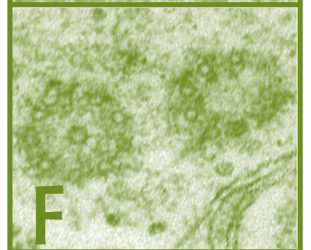
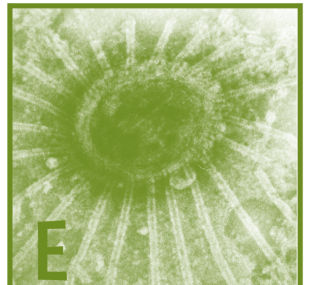
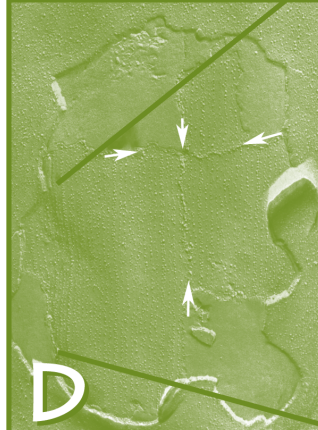
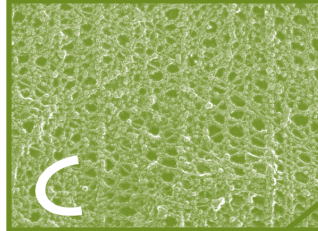
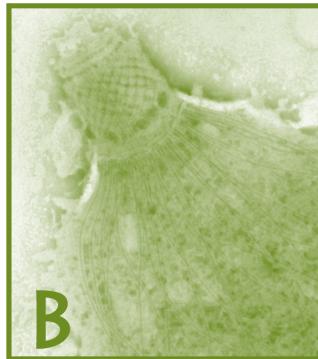


# *TOXOPLASMA*

MOLECULAR AND CELLULAR BIOLOGY

JAMES W. AJIOKA

DOMINIQUE SOLDATI



# ***Toxoplasma***

## Molecular and Cellular Biology

Edited by James W. Ajioka and Dominique Soldati

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# Contents

	List of Contributors	vii
	Preface	xii
Part I	Overview	1
1	The Life Cycle of <i>Toxoplasma gondii</i> J.P. Dubey	3
2	Ultrastructure J.-F. Dubremetz and D.J.P. Ferguson	17
Part II	Pathogenesis, Immunology, and Virulence	35
3	Epidemiology, Diagnostics, and Chemotherapy Eskild Petersen	37
4	Ocular Toxoplasmosis: Clinical Features, Pathology, Pathogenesis, Animal Models, and Immune Responses Fiona Roberts, Annie Kuo, Leigh Jones, Rima McLeod, and Craig W. Roberts	59
5	Congenital Toxoplasmosis A.W. Pfaff, O. Liesenfeld, and E. Candolfi	93
6	Innate Recognition and the Regulation of Protective Immunity to <i>Toxoplasma gondii</i> Marion Pepper and Christopher A. Hunter	111
7	Mechanisms of Immune Evasion Eric Y. Denkers, Chiang W. Lee, Leesun Kim, and Barbara A. Butcher	127
8	Apoptosis and Its Impact on the Parasite–Host Interaction Carsten G.K. Lüder	143



9	<b>Pathogenicity and Virulence in <i>Toxoplasma gondii</i></b>	159
	Sonya Taylor, Asis Khan, Chunlei Su, and L. David Sibley	
10	<b>Cellular Response to Infection</b>	171
	Ashley Fouts and John C. Boothroyd	
<b>Part III</b>	<b>Genome and Genetics</b>	191
11	<b>Genetics and Genome Organization of <i>Toxoplasma gondii</i></b>	193
	Asis Khan, Sonya Taylor, Chunlei Su, L. David Sibley, Ian Paulsen, and James W. Ajioka	
12	<b>Evolution and Comparative Genomics of <i>Toxoplasma gondii</i></b>	209
	Jessica Kissinger and Chih-Horng Kuo	
13	<b>Population Genetics, Sex, and the Emergence of Clonal Lines of <i>Toxoplasma gondii</i></b>	227
	Michael E. Grigg	
14	<b>Manipulating the <i>Toxoplasma</i> Genome</b>	241
	Marc-Jan Gubbels, Jolly Mazumdar, Giel van Dooren, and Boris Striepen	
<b>Part IV</b>	<b>Growth, Development, and Metabolism</b>	263
15	<b>Cell Cycle Control/Parasite Division</b>	265
	Michael W. White, Jay R. Radke, Magnolia Conde de Felipe, and Margaret Lehmann	
16	<b><i>Toxoplasma</i> Gene Regulation and Bradyzoite Development</b>	285
	Stanislas Tomavo and Louis M. Weiss	
17	<b>Biology of Bradyzoites</b>	303
	Florence Dzierszinski and Laura J. Knoll	
18	<b>Chromatin Remodeling</b>	321
	William J. Sullivan, Jr.	
19	<b>Lipid Synthesis and Uptake</b>	345
	Isabelle Coppens	
20	<b>Nucleotides and Amino Acids</b>	365
	Barbara A. Fox, Kshitiz Chaudhary, and David J. Bzik	
<b>Part V</b>	<b>Organelles: Sorting, Content, and Function</b>	389
21	<b>Protein Targeting to the Apicoplast</b>	391
	Marilyn Parsons, Amy DeRocher, and Jean E. Feagin	

22	<b>The Metabolic Functions of the Mitochondrion and the Apicoplast</b>	409
	Frank Seeber and Dominique Soldati	
23	<b>Microneme Protein Repertoire and Function</b>	437
	M.-H. Huynh and V.B. Carruthers	
24	<b>Proteomic Analysis of the Rhoptry Organelles of <i>Toxoplasma gondii</i></b>	455
	J.M. Wastling and P.J. Bradley	
25	<b>Dense Granules of the Infectious Stages of <i>Toxoplasma gondii</i>: Their Central Role in the Host–Parasite Relationship</b>	475
	Corinne Mercier, Marie-France Cesbron-Delauw, and David J.P. Ferguson	
26	<b>Calcium Homeostasis and Acidocalcisomes in <i>Toxoplasma gondii</i></b>	493
	Silvia N.J. Moreno, Kildare Miranda, and Wanderley de Souza	
Part VI	<b>Cytoskeleton, Signaling, Motility, and Invasion</b>	507
27	<b>Tubulin, Microtubules, and Microtubule-associated Structures in <i>Toxoplasma gondii</i></b>	509
	Naomi S. Morrisette	
28	<b>Molecular Motors</b>	525
	Bernardo J. Foth and Dominique Soldati	
29	<b>Actin Dynamics and Motility in Apicomplexans</b>	555
	Jennifer L. Gordon, Nivedita Sahoo, Simren Mehta, and L. David Sibley	
30	<b>Proteases of <i>Toxoplasma gondii</i></b>	571
	Artemio Jongco and Kami Kim	
31	<b>The <i>Toxoplasma gondii</i> Parasitophorous Vacuole Membrane: Transactions at the Parasite–Host Interface</b>	591
	Angela M. Martin and Anthony P. Sinai	
	<b>Index</b>	611
	<b>Color Plate</b>	



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# Preface

The direction and progress of any research discipline is determined by the community's collective ability to navigate, through observation and experimentation, in the pursuit of illumination. From the outset, the route to understanding *Toxoplasma gondii* as a parasitic protozoan was convoluted. In hindsight, this can be largely attributed to it possessing a comparatively complex, elusive life cycle, remaining a parasite of "doubtful nature" decades after its discovery (Wenyon, 1926; Lapage, 1968). More recently, a migration of researchers working with a core of established *Toxoplasma* investigators has led to something of a renaissance in the field. At this point, our collective knowledge is sufficiently great, so, as one of the contributors to this volume amusingly suggested, it is time to "demystify" *Toxoplasma*.

*T. gondii* was a relative latecomer in its discovery as a parasitic disease agent, and even the current name was not established until a couple of years after its initial description by Charles Nicolle at the Institut Pasteur, Tunis, in 1907 (Nicolle, 1907). Although Nicolle was better known for his work on typhus, for which he was awarded the Nobel prize, he was a scientist with the "Pastorian mission" to eradicate infectious disease (Pelis, 2006). Thus, he retained a broad interest in infectious disease agents, including enigmatic intracellular parasites of rodents. Based on similarities to the pattern of replication of piroplasms, the parasite taken from the desert rodent *Ctenodactylus gundi* (or *gondi* as referred to by Nicolle) was tentatively classified as *Piroplasma quadrigeminum* (Nicolle, 1907). Nicolle, wishing to share his finding, kindly lent a blood smear from the infected rodent to the then world's expert on piroplasms, the Cambridge parasitologist George Nuttall. Nuttall, a native Californian and head of the Molteno Institute for Parasitology, concluded that it did not belong to the *Piroplasma* and suggested a new genus, classifying the parasite as *Nicollia quadrigemina* (Nuttall, 1913). This name and information was presumably offered to Nicolle in a personal communication, but was never formally published by Nuttall<sup>1</sup>. After further observation, in 1908, Nicolle and his colleague L. Manceaux made another proposal based on "grandes analogies entre lui et les *Leishmania*" and suggested a new name *Leishmania gondii* (Nicolle and Manceaux, 1908). This speculation was based on several criteria, but two are notable. First, in liver preparations:

---

<sup>1</sup>This is inferred from a section describing Nicolle's parasite in Nuttall's 1913 review of Piroplasms.

*Les éléments parasités sont toujours des mononucléaires; on ne rencontre jamais de protozoaires dans les globules rouges, dans les polynucléaires ni dans les cellules du foie.*

Second:

*...la présence fréquente de deux corps chromatiques: grand et petit karyosomes.*

The first observation that the parasite resides in monocytes and not red blood cells argued against the parasite being a piroplasm, and the apparent absence in liver cells reinforced the leishmania paradigm of monocyte occupation. The second and frequent observation of a small chromatin body in addition to the nucleus is certainly reminiscent of a kinetoplast, and it is ironic that its true nature as the apicoplast would not be definitively revealed for another 90 years. Concurrent with this analysis in 1908, A. Splendore in Brazil published the first in a series of three papers that described effectively the same parasite isolated from a rabbit. He also tentatively identified it as a leishmania (Splendore, 1908). Finally in 1909, after extensive microscopic analysis of several tissues and experimental infection studies, Nicolle and Manceaux endowed the parasite with its current name (Nicolle and Manceaux, 1909):

*...et nous proposons, pour désigner ce genre nouveau, le nom de Toxoplasma (de τοξον, arc). Le nom du parasite du Gondi sera donc T. gondii.*

Notably, they finish this seminal publication by reporting on a friendly collaboration with A. Splendore, concluding that the parasite he isolated from the rabbit was “morphologiquement identique” to their newly named *T. gondii*. Splendore, apparently in agreement with this assessment, classified his parasite as *T. cuniculi* and published these results in the third paper of his series the following year (Splendore, 1910). This was the first of many such papers describing the isolation of *Toxoplasma* from a variety of host species.

Over the next 50 years, *Toxoplasma* (including species now known to be *gondii*) was isolated from a wide variety of animal species including humans. In this time, the parasite's current status as a model organism came to the fore as the acute disease form could be easily cultured and lent itself to microscopic study. EM studies began in the early 1950s and the ultrastructure of *T. gondii*, descriptions of the apical complex, and secretory organelles such as the rhoptries became a paradigm for the invasive form of apicomplexan parasites (see, for example, Gustafson *et al.*, 1954; Scholtyseck and Melhorn, 1970). Ironically, the morphology of the parasite was well described, but the nature and extent of *T. gondii* infection remained a mystery because only the acute disease form of the parasite was readily observed as the parasite was primarily seen post-mortem. This situation changed dramatically in 1948 with the advent of the serologically based dye test developed by Albert Sabin and Harry Feldman (Sabin and Feldman, 1948). The test not only allowed diagnosis of human toxoplasmosis but also provided a way to gather epidemiological data from which the close relationship between *T. gondii* isolates and the solidification of the single species concept emerged. Although *T. gondii* had been isolated from many mammalian and bird species, the natural route of transmission remained unknown. Since the large majority



Charles Nicolle with pet cat on the steps of the Institut Pasteur, Tunis. Reprinted with permission from the Director General, Institut Pasteur, Tunis.

cases of human toxoplasmosis were via congenital transmission, the rarity of the condition could not explain the widespread infection in both man and animals.

Like many infectious agents, epidemiological data provided the first clues. In the 1950s and early 60s, evidence began to accrue that the consumption of undercooked meat may be an important route of *T. gondii* infection (Weinman and Chandler 1954; Jacobs *et al.*, 1960). Through observation of children in a Paris sanatorium in the mid-1960s, Desmonts and co-workers followed children before and after admission to the sanatorium and assessed the rate of seroconversion as a measure of infection (Desmonts *et al.*, 1965). From a baseline 10% seroconversion per year, the figure rose to 50% with the daily consumption of undercooked beef or horse meat and to nearly 100% with rare lamb. A study of the adult population in Paris at this time showed that over 80% were positive for antibodies against *T. gondii*, consistent with the rate of acquired infection observed in the sanatorium. Curiously, a survey of strict vegetarians in India showed that the prevalence of infection was similar to non-vegetarians and herbivorous animals (Rawal, 1959). This and similar investigations prompted the search for other routes of infection.

As current *Toxoplasma* researchers, the coccidian nature of the parasite is such an integral part of our thinking that we tend to forget the difficulties in understanding the

parasite in the absence of this knowledge (D. Ferguson, personal communication). In the mid-1950s, L. Jacobs and co-workers first tested fecal transmission of *T. gondii* in dogs, albeit with negative results (Jacobs *et al.*, 1955). Unfortunately between their lack of success and the similarity to other known coccidian parasites of cats and dogs, *T. gondii* transmission remained enigmatic for another 10 years. William Hutchison, working at the University of Strathclyde in the mid-1960s, revisited the fecal transmission route hypothesis. Although he wanted to follow Jacobs and use dogs for his experiments, these could not be maintained at his facility so he used a cat instead (J.P. Dubey, personal communication). Hutchison showed that it was possible to isolate from cat feces, 2 weeks after infection with tissue cysts, an unknown form of the parasite that could transmit infection to mice (Hutchison, 1965). Importantly, this form was very stable and could survive for up to 12 months stored in water. However, since the feces-derived inoculum potentially contained pathogens other than *T. cati* ova and an unknown form of *T. gondii* (specified pathogen-free cats were not available at the time), the only way Hutchison could control the infection was by a microscopic examination of the inoculum, and he noted:

*The only living material observed, apart from bacteria, were the oocysts of Isospora and the ova of T. cati.*

In retrospect, it probable that the *Isospora*-type oocysts were in fact the unrecognized form of *T. gondii*, highlighting how unthinkable it was to consider *Toxoplasma* as a coccidian at the time (D. Ferguson, personal communication). Thus, the prime hypothesis was that the unknown infectious form could be protected within the nematode eggs, but Hutchison cautiously concluded:

*At present it is impossible to say whether the infection induced by this material was transmitted by helminth ova or by some other means.*

This finding motivated Hutchison and other researchers, notably J. Frenkel, J.P. Dubey, H.G. Sheffield and M.L. Melton to address this question. Despite some data to the contrary, the collective results clearly showed that *T. gondii* transmission was not dependent on nematode ova (Frenkel *et al.*, 1969; Sheffield and Melton, 1969). Finally, with the discovery of the sexual cycle in the cat intestine, these researchers and their colleagues revealed the true coccidian nature of *T. gondii* and better defined the unique asexual transmission between secondary hosts, culminating in the elucidation of the complete life cycle (Frenkel *et al.*, 1970; Hutchison *et al.*, 1970, 1971). Following this painstaking microscopy by Dubey, D. Ferguson and co-workers described gametogenesis and the sexual cycle in great detail within the cat intestine (Dubey and Frenkel, 1972; Ferguson *et al.*, 1974, 1975, 1979a, 1979b; Speer and Dubey, 2005). For asexual reproduction, Frenkel coined the now commonly used terms “tachyzoite” to describe the rapidly dividing form seen in the acute infection and “bradyzoite” to describe the slowly dividing form seen in the chronic infection (Frenkel, 1973). Through characterizations of the bradyzoite infection and infective fecal forms, Dubey and co-workers clarified a confusing situation in the literature by defining the bradyzoite containing mass as “tissue cysts” (Dubey and Beattie 1988) compared to the fecal shed “oocysts” (Dubey and Frenkel 1976).

With knowledge of the life cycle in hand, in the late 1970s and early 80s, Elmer Pfefferkorn and co-workers at Dartmouth initiated a series of studies to establish the transmission genetics of *T. gondii*. In an elegant set of crosses using drug resistance and auxotrophic markers they had previously developed (Pfefferkorn *et al.*, 1977, 1978), Pfefferkorn confirmed Mendelian inheritance and the haploid nature of the parasite (Pfefferkorn and Pfefferkorn, 1980). Moreover, they and others showed that a single cloned parasite could give rise to the complete sexual cycle in the cat, demonstrating that the sexual cycle did not involve fixed mating types (Pfefferkorn *et al.*, 1977; Cornelissen and Overdulve, 1985). These results were consistent with microscopic/cellular observations of gametogenesis in the cat intestine, where both macro- and microgametocytes appeared to be produced from a single organism. Thus, in mixed infections, both selfing and outcrossing occur in about the same frequency (Pfefferkorn and Pfefferkorn, 1980). These studies laid the foundation for what would become the molecular genetics and genomics of *T. gondii*.

Compared to other model organisms, the early path to understanding *T. gondii* ran a circuitous route, but from the beginning, the community of investigators has been intellectually incisive and generous with sharing information, techniques and authorship on publications. The current community is a testament to this tradition.

We would like to thank J.P. Dubey and D. Ferguson for historical insights and, most importantly, thanks to all of the contributors to this book and publisher who made it possible.

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July, 2007

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# Part I

## Overview

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# The Life Cycle of *Toxoplasma gondii*

1

J.P. Dubey

## Abstract

Infections by the protozoan parasite *Toxoplasma gondii* are widely prevalent worldwide in animals and human beings. Cats are the only definitive hosts for *T. gondii* and all other warm-blooded animals are intermediate hosts. Cats excrete the environmentally resistant oocysts after ingesting any of the three infectious stages of *T. gondii*, tachyzoites, bradyzoites, and oocysts. Humans and other hosts become infected with *T. gondii* by ingesting uncooked infected meat, by ingesting food and water contaminated with oocysts, or transplacentally. Cats can excrete millions of oocysts after ingesting tissues of infected animals and oocysts can survive in the harsh environment for months. Contamination of sea water can occur from the oocysts washed off the land and marine mammals can acquire *T. gondii* infection and some die from toxoplasmosis.

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## Etiologic agent

*Toxoplasma gondii* is a coccidian parasite with cats as the definitive host, and warm-blooded animals as intermediate hosts. It belongs to:

Phylum: Apicomplexa; Levine, 1970

Class: Sporozoasida; Leukart, 1879

Subclass: Coccidiasina; Leukart, 1879

Order: Eimeriorina; Leger, 1911

Family: Toxoplasmatidae, Biocca, 1956

There is only one species of *Toxoplasma*, *T. gondii*.

## Basic structure and life cycle

Coccidia are among the most important parasites of animals and they were the first protozoa discovered. The oocyst is the key stage of all coccidians and their classification was based on the structure of the oocyst. Oocysts with four sporocysts, each with two sporozoites (total eight sporozoites), are classified as *Eimeria*. Oocysts containing two sporocysts, each with four sporozoites historically were classified as *Isospora*. Coccidiosis due to *Eimeria* species is one of the most economically important diseases of poultry, cattle, sheep, goats and many other herbivores and it is difficult to raise livestock coccidia free.

Before the discovery of the life cycle of *T. gondii* coccidians were considered to be host-specific with a simple one-host life cycle, with infection confined to intestines and to mostly enterocytes. With few exceptions eimerians still follow this life cycle. The host becomes infected by ingesting sporulated oocysts of *Eimeria*. After excystation, the sporozoites penetrate intestinal epithelial cells and multiply asexually before forming male and female gamonts. Oocysts are produced after fertilization, and are passed in feces in unsporulated stage. Sporulation occurs outside the host. Unlike *Eimeria*, the life cycle of which has been known for many years, little was known of the complete life cycle of most *Isospora* species until 1970 when the life cycle of *T. gondii* was discovered. Until then, *Isospora* species were considered parasites of carnivores (dogs, cats) and birds and were thought to be not host specific. In 1970, *T. gondii*, a parasite previously known to parasitize extraintestinal tissues of virtually all warm-blooded hosts, was found to be an intestinal coccidium of cats and to have in its life cycle an isosporan-like oocysts. This finding was a major breakthrough in medical and veterinary sciences and eventually led to the discovery of life cycles and recognition of several new taxa of economically important *Toxoplasma*-like parasites (e.g. *Neospora*, *Sarcocystis*).

The universal distribution of *T. gondii* is probably related to its adapted transmission by fecal–oral cycle, by carnivory, and transplacentally (Frenkel *et al.*, 1970; Dubey and Beattie, 1988).

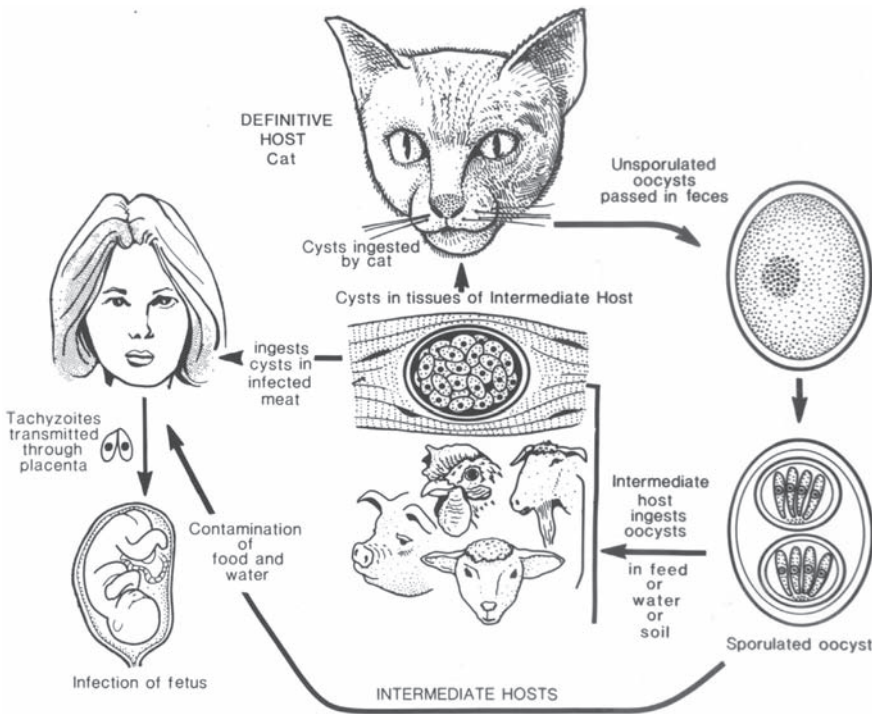
There are three infectious stages of *T. gondii*: the tachyzoites (in groups), the bradyzoites (in tissue cysts), and the sporozoites (in oocysts). These stages are linked in a complex life cycle (Figure 1.1).

### *Tachyzoite*

The term “tachyzoite” (tachos = speed in Greek) was proposed by Frenkel (1973) to describe the stage that rapidly multiplied in any cell of the intermediate host and in non-intestinal epithelial cells of the definitive host. The term tachyzoite replaces the previously used term trophozoite (trophicos = feeding in Greek). Tachyzoites have also been termed endodyozoites or endozoites. Aggregates of numerous tachyzoites are called clones, terminal colonies or groups. This stage of the parasite is directly associated with acute phase of the disease (see Chapter 6). Tachyzoites can multiply and kill virtually all host cells of warm-blooded animals. Congenital infection can occur when tachyzoites from maternal blood are carried to fetal tissues (see Chapter 5).

The tachyzoite is often crescent-shaped, approximately  $2 \times 6 \mu\text{m}$  (Figure 1.2A), with a pointed anterior (conoidal) end and a rounded posterior end. In histological sections tachyzoites are often round with a central nucleus (Figure 1.2B). Ultrastructurally, the tachyzoite consists of various organelles and inclusion bodies including a pellicle, apical rings, polar rings, conoid, rhoptries, micronemes, micropore, mitochondrion, subpellicular microtubules, endoplasmic reticulum, Golgi complex, ribosomes, rough and smooth endoplasmic reticula, micropore, nucleus, dense granules, and apicoplast (see Chapter 2). The nucleus is usually situated toward the central area of the cell.

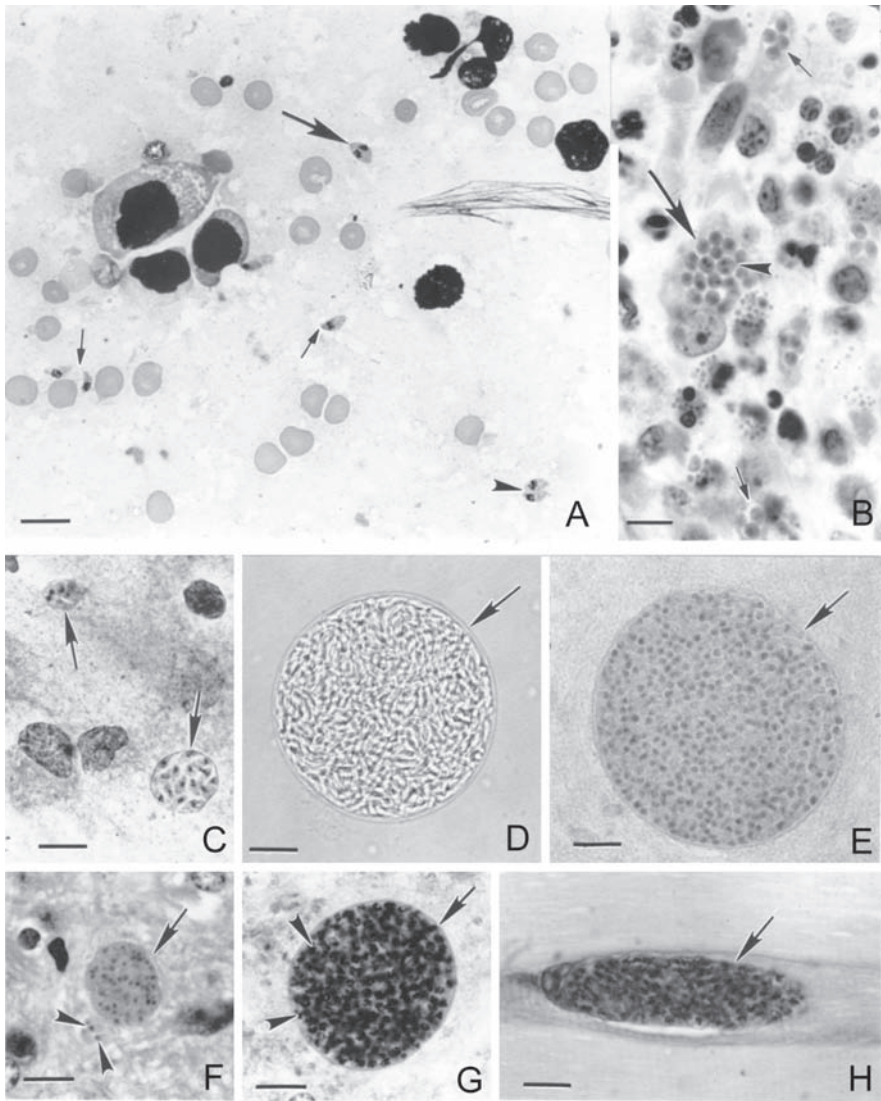
Although tachyzoites can move by gliding, flexing, undulating and rotating, they have no visible means of locomotion such as cilia, flagella or pseudopodia (see Chapter 28). No differences were found in locomotion of tachyzoites, bradyzoites, and sporozoites (Wetzel *et al.*, 2005).



**Figure 1.1** Life cycle of *Toxoplasma gondii*.

Tachyzoites enter host cells by actively penetrating through the host cell plasmalemma or by phagocytosis (see Chapter 29). After entering the host cell, the tachyzoite becomes ovoid and is surrounded by a parasitophorous vacuole (PV), which appears to be derived from both the parasite and the host cell (see Chapter 31). Tachyzoites multiply asexually within the host cell by repeated endodyogeny, a specialized form of reproduction in which two progeny form within the parent parasite, consuming it (Sheffield and Melton, 1968; see Chapter 15). Tachyzoites continue to divide by endodyogeny. *In vivo*, most groups of tachyzoites are arranged randomly due to asynchronous cycles of endodyogeny. However, rosettes are occasionally formed due to synchronous division. In rapidly dividing tissue culture-adapted strains, *T. gondii* within a vacuole may divide synchronously (Bommer, 1969; Roos *et al.*, 1994) but this is not the norm. Rarely, tachyzoites of certain strains divide by binary fission (Ferguson and Hutchison, 1981; Rondanelli *et al.*, 1974). The host cell ruptures when it can no longer support the growth of tachyzoites.

The rates of invasion and growth vary depending on the strain of *T. gondii* and the type of host cells (Appleford and Smith, 1997; Kaufman and Maloney, 1962). After entry of tachyzoites into a host cell, there is a variable lag period phase before the parasite divides and this lag phase is partly parasite dependent (Appleford and Smith 1997). Mouse virulent strains of *T. gondii* grow faster in cell culture than do “avirulent” strains and some strains of *T. gondii* form more rosettes than others (Appleford and Smith, 1997). Although *T. gondii* isolates have been classified genetically into type I, II and III (Howe and Sibley, 1995) there are no appreciable structural differences among different isolates of *T. gondii*.



**Figure 1.2** Tachyzoites (A, B) and tissue cysts (C–H) of *T. gondii*. Bar=10μm. (A) Individual (small arrows), binucleate (large arrow), and divided (arrowhead) tachyzoites. Impression smear of lung. Compare size with red blood cells and leukocytes. Giemsa stain. (B) Tachyzoites in a group (large arrow) and in pairs (small arrows) in section of a mesenteric lymph node. Note organisms are located in parasitophorous vacuoles and some are dividing (arrowhead). H&E stain. (C) Two tissue cysts (arrows). Note thin tissue cyst wall enclosing bradyzoites. Impression smear of mouse brain. Silver impregnation and Giemsa stain. (D) A tissue cyst freed from mouse brain by homogenization in saline. Note thin cyst wall (arrow) enclosing many bradyzoites. Unstained. (E) A large tissue cyst in section of rat brain 14 months post infection. Note thin cyst wall (arrow). H&E stain. (F) A small tissue cyst (arrow) with intact tissue cyst wall (arrow) and 4 bradyzoites (arrowheads) with terminal nuclei adjacent to it. Section of mouse brain 8 months post infection. H&E stain. (G) A tissue cyst in section of mouse brain. Note PAS-negative tissue cyst wall (arrow) enclosing many PAS-positive bradyzoites (arrowheads). The

### *Bradyzoites and tissue cysts*

The term “bradyzoite” (brady = slow in Greek) was also coined by Frenkel (1973) to describe the encysted stage of the parasite in tissues. Bradyzoite are also called cystozoites. To avoid confusion between the terms cysts (also called pseudocysts) in tissues versus oocysts in feces Dubey and Beattie (1988) proposed the term tissue cyst for the encysted stage of the parasite.

Bradyzoites and tissue cysts are usually associated with chronic infection but bradyzoites are formed as early as 3 days post infection (Dubey and Frenkel, 1976; Dubey *et al.*, 1998; see Chapter 16). The conversion of bradyzoites to tachyzoites is of great clinical significance because it can lead to reactivation of a latent infection (e.g. in AIDS patients).

Tissue cysts (Figure 1.2) grow and remain intracellular as the bradyzoites divide by endodyogeny (Ferguson and Hutchison 1987a,b). Tissue cysts vary in size; young tissue cysts may be as small as 5  $\mu\text{m}$  in diameter and contain only two bradyzoites (Dubey *et al.*, 1998), while older ones may contain hundreds of organisms (Figure 1.2). Tissue cysts in the brain are often spheroidal and rarely reach a diameter of 70  $\mu\text{m}$ , whereas intramuscular cysts are elongated and may be 100  $\mu\text{m}$  long. Although tissue cysts may develop in visceral organs, including the lungs, liver and kidneys, they are more prevalent in the neural and muscular tissues, including the brain, eyes and skeletal and cardiac muscles (Dubey *et al.*, 1998). Intact tissue cysts probably do not cause any harm and can persist for the life of the host without causing host inflammatory response.

The tissue cyst wall is elastic and thin ( $< 0.5 \mu\text{m}$  thick) (Figure 1.2C), and it encloses hundreds of crescent-shaped bradyzoites,  $5\text{--}8.5 \times 1\text{--}3 \mu\text{m}$  in size (Mehlhorn and Frenkel, 1980; Speer *et al.*, 1999; Dubey and Sreekumar, 2003). The tissue cyst develops within the host cell cytoplasm. The tissue cyst wall is argyrophilic but results vary depending on the silver impregnation method used (Figure 1.2C). The cyst wall is composed of host cell and parasite materials (Ferguson and Hutchison, 1987a,b; Sims *et al.*, 1988). It is ultimately lined with granular material which also fills the space between the bradyzoites. Some bradyzoites degenerate, especially in older tissue cysts (Pavesio *et al.*, 1992). The cyst wall is only faintly periodic acid–Schiff (PAS) positive (Figure 1.2G).

Bradyzoites differ structurally only slightly from tachyzoites. They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located. The contents of rhoptries in bradyzoites are usually electron dense, whereas those in tachyzoites are labyrinthine. However, the contents of rhoptries in bradyzoites vary with the age of the tissue cyst. Bradyzoites in younger tissue cysts may have labyrinthine rhoptries, whereas those in older tissue cysts are electron dense. Bradyzoites contain several amylopectin granules which stain red with PAS reagent; such material is either in discrete particles or absent in tachyzoites. Bradyzoites are more slender than are tachyzoites. Bradyzoites are less susceptible to destruction by proteolytic enzymes than are tachyzoites (Jacobs *et al.*, 1960).

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bradyzoites stain bright red with PAS but they appear black in this photograph. Periodic acid Schiff hematoxylin (PASH). (H) An elongated tissue cyst (arrow) in section of skeletal muscle of a mouse. PASH.



### Oocysts

Unsporulated oocysts are subspherical to spherical and are  $10 \times 12 \mu\text{m}$  in diameter (Figure 1.3A). Under light microscopy, the oocyst wall consists of two colorless layers (Dubey *et al.*, 1970). Polar granules are absent and the sporont almost fills the oocyst. A micropyle can be seen ultrastructurally (Speer *et al.*, 1998). Sporulation occurs outside the cat within 1 to 5 days of excretion depending upon aeration and temperature.

Sporulated oocysts are subspherical to ellipsoidal and are  $11 \times 13 \mu\text{m}$  in diameter (Figure 1.3E). Each sporocyst contains two ellipsoidal sporocysts without Stieda bodies. Sporocysts measure  $6 \times 8 \mu\text{m}$ . A sporocyst residuum is present; there is no oocyst residuum. Each sporocyst contains four sporozoites (Figure 1.3E).

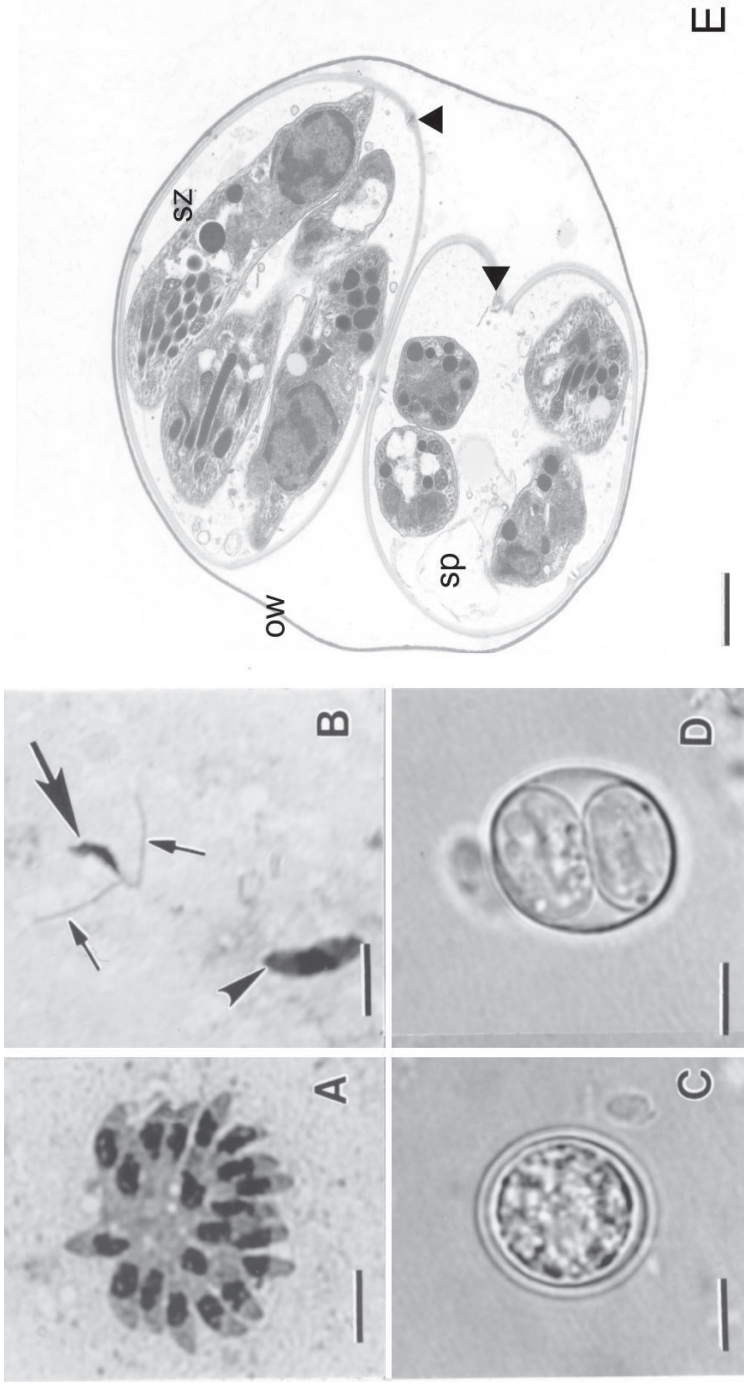
### Life cycle in the definitive host, cat

Cats, not only domestic (*Felis domesticus*) but nearly all species of felines, including bob cats (*Lynx rufus*), mountain lion (*Felis concolor*), Asian leopard (*Felis bengalensis*), ocelot (*Felis pardalis*), jaguarondi (*Felis yagouaroundi*), Pallas cat (*Felis manual*), Iromote cat (*Felis iriomotensis*), Amur leopard cat (*Felis euphilurus*), Geoffroy's cat (*Oncifelis geoffroyi*), wild cat (*Felis sylvestris*), Pampas cat (*Oncifelis colocolo*), Siberian tiger (*Panthera tigris altaica*), lion (*Panthera leo*), cougars (*Felis concolor*), African wild cat (*Felis lybica*), and cheetah (*Acinonyx jubatus*), are the definitive hosts for *T. gondii* (Jewell *et al.*, 1972; Miller *et al.*, 1972; Janitschke and Warner, 1972; Marchiondo *et al.*, 1976; Pizzi *et al.*, 1978; Polomoshnov, 1979; Akuzawa *et al.*, 1987; Dubey *et al.*, 1988; Ochoi *et al.*, 1989; Dorny and Franken, 1989; Aramini *et al.*, 1998; Lukešová and Literák, 1998; Basso *et al.*, 2005).

Cats, like other hosts of *T. gondii*, can become infected by ingesting food or water contaminated with oocysts, by ingesting infected tissues containing tachyzoites or bradyzoites, or transplacentally. *T. gondii* infection is common in many animals used for food (Dubey and Beattie, 1988; Tenter *et al.*, 2000). Sheep, goats, pigs, and rabbits are more commonly infected than horses and infection is very low in battery-raised chickens and non-existent in cattle (Dubey *et al.*, 2005b). Tissue cysts are present in most edible portions of infected pork and uncooked scraps of meat can cause shedding of oocysts in cats.

Parasitemia during pregnancy can cause placentitis followed by spread of tachyzoites to the fetus. In people, sheep, and other higher mammals, congenital infection usually occurs only once when the mother becomes infected during pregnancy. Unlike humans, repeated congenital infection can occur in some strains of mice, guinea pigs, and rabbits. Several litters maybe born infected from an infected mouse without reinfection from outside sources. Congenitally infected mice can themselves produce congenitally infected mice for ten generations (Beverley, 1959). Congenitally infected mice and rats maybe born infected but not develop antibodies to *T. gondii* (Dubey *et al.*, 1995; Dubey 1997b).

*T. gondii* has also adapted to an oocyst–oral cycle in herbivores (intermediate hosts) and tissue cyst–oral cycle in carnivores, especially in the cat. *T. gondii* oocysts are less infective and less pathogenic for the cat than for other hosts (Dubey, 1996). For example, 1 live oocyst is orally infective to mice and pigs (Dubey *et al.*, 1996) whereas 100 or more oocysts may be required to establish infection in a cat (Dubey, 1996). The reverse may be true for bradyzoites. By mouth, bradyzoites are less infective to mice than cats (Dubey, 2001). Cats can shed millions of oocysts after ingesting as few as obne bradyzoite whereas 100 brady-



**Figure 1.3** Enteropithei stages of *T. gondii*. Bar in A–D = 5  $\mu$ m, in E = 6  $\mu$ m. (A) Schizont from intestinal epithelium of a cat. Impression smear. Giemsa stain. (B) A microgamete (large arrow) with 2 flagella (small arrows), and a merozoite (arrowhead). Impression smear of cat intestine. Giemsa stain. (C) Unsporulated oocyst in cat feces. Unstained. (D) Sporulated oocyst from cat feces. Unstained. (E) Transmission electron micrograph of a sporulated oocyst. Note thin oocyst wall (ow), 2 sporozoites (sp) and sporocysts (sz), one of which is cut longitudinally. Each sporocyst has 4 sutures (arrowheads) that collapse during excystation of sporozoites.

zoites may not be infective to mice by the oral route (Dubey, 2001). Although *T. gondii* can be transmitted orally by ingesting tissue cysts, epidemiologic evidence indicates that cats are essential in perpetuation of the life cycle as *T. gondii* infection is rare or absent in areas devoid of cats (Wallace, 1969; Munday, 1972; Dubey *et al.*, 1997c) and prevalence of *T. gondii* was reduced drastically in rodent population on farms where cats were vaccinated with *T. gondii* to prevent oocysts shedding (Mateus-Pinilla *et al.*, 1999).

Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*, i.e. tachyzoites, bradyzoites, and sporozoites. Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage of *T. gondii* ingested. Prepatent periods are 3–10 days after ingesting tissue cysts, 3–18 days after ingesting oocysts, irrespective of the dose (Dubey and Frenkel, 1972, 1976; Dubey, 1996, 2001). The prepatent period after ingesting tachyzoites may vary (Dubey, 2005). Fewer than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts (Dubey and Frenkel, 1972, 1976; Dubey, 1996, 2001, 2002, 2005).

*Bradyzoite-induced cycle in the cat:* Only the bradyzoite-induced cycle is known in cats (Dubey and Frenkel, 1972). After the ingestion of tissue cysts by cats, the tissue cyst wall is dissolved by proteolytic enzymes in the stomach and small intestine. The released bradyzoites penetrate the epithelial cells of the small intestine and initiate the development of numerous generations of *T. gondii*. Five morphologically distinct types of *T. gondii* develop in intestinal epithelial cells before gametogony begins. These stages are designated types A to E instead of generations because there are several generations within each *T. gondii* type. These asexual stages in the feline intestine are structurally distinct from tachyzoites that also develop in the lamina propria. Types C, D, and E multiply by a schizogony. In schizogony the nucleus divides two or more times without cytoplasmic division. Whether daughter organisms (merozoite) formation begins after 4 or more nuclei have been formed is uncertain. Before or simultaneous with the last nuclear division, merozoite formation is initiated near the center of the schizont. The merozoites eventually move towards the periphery of the schizont and the schizont plasmalemma invaginates around each merozoite forming the plasmalemma of the merozoite. The merozoites separate from the schizont at their posterior ends; sometimes leaving a residual body (Dubey and Frenkel, 1972; Speer and Dubey, 2005).

After the asexual development (types A–E), the sexual cycle starts 2 days after tissue cysts were ingested by the cat. The origin of gamonts has not been determined, but the merozoites released from schizont types D and E probably initiate gamete formation. Gamonts are found throughout the small intestine but most commonly in the ileum, 3–15 days after inoculation. They are found above the nucleus of the host epithelial cell near the tips of the villi of the small intestine. Female gamonts are subspherical and each contains a single centrally located nucleus and several PAS-positive granules.

Mature male gamonts (microgamonts) are ovoid to ellipsoidal in shape. During microgametogenesis, the nucleus of the microgamont divides to produce 10–21 nuclei. The nuclei move toward the periphery of the parasite and enter protuberances formed in the pellicle of the microgamont. One or two residual bodies remain in the microgamont after division into microgametes. Microgametes are elongated and consist mainly of nuclear

material and 2 long free flagella. Microgamonts have up to 21 microgametes (Dubey and Frenkel, 1972). Microgametes use their flagella to swim to and penetrate and fertilize mature macrogametes to form zygotes. After fertilization, an oocyst wall is formed around the parasite. Infected epithelial cells rupture and discharge oocysts into the intestinal lumen.

*Oocyst-induced cycle in cats* As stated earlier cats shed oocysts with a minimum prepatent period of 18 days after feeding sporulated oocysts, irrespective of the dose (Freyre et al., 1989, Dubey, 1996) and not all cats that become infected shed oocysts. Enterioepithelial stages preceding the formation of gamonts have not been found. It has been hypothesized that sporozoites first convert to tachyzoites, and tachyzoites convert to bradyzoites, and when tissue cysts rupture a few bradyzoites return to the intestinal epithelium to initiate the enterioepithelial cycle and this event is unpredictable.

*Tachyzoite-induced cycle in the cat* Cats can shed oocysts after feeding tachyzoites with a prepatent period of 19 days or more, similar to the oocysts-induced cycle. However, in some cats fed tachyzoites the prepatent period was 11–17 days (Dubey, 2005), probably related to the ingestion of *T. gondii* transitional stages between tachyzoites and bradyzoites. The transition of bradyzoite to tachyzoite and tachyzoite to bradyzoite is not an all or none phenomenon and the transitional stage has not been morphologically and biologically characterized. For example, tachyzoites lack PAS-positive granules that are numerous in bradyzoites and their synthesis and accumulation is gradual (Dubey and Frenkel, 1976). Although tachyzoites are not as resistant to acid as bradyzoites, it is likely that some *T. gondii* might penetrate pharyngeal-buccal mucosa when *T. gondii* was poured in the mouth of the cat (Dubey, 2002, 2005). However, 17 of 31 cats administered *T. gondii* by a stomach tube also became infected indicating that they survived acid-pepsin digestion in the stomach (Dubey, 1998, 2005). From a practical point of view, bradyzoites are likely to be present in most infected animals, even during acute infection because bradyzoites were found in mice infected for only 3 days (Dubey and Frenkel, 1976). These results indicate that humans can become infected if they accidentally ingest *T. gondii* tachyzoites in the laboratory.

#### *Oocyst shedding and survival in the environment*

Cats can shed millions of oocysts in a short period of a few days (Dubey and Frenkel, 1972; Dubey, 2001). Sporulated oocysts survive for long periods under moderate environmental conditions (Dubey 1998b; Dubey, 2004). For example, they can survive in moist soil for months to years (Dubey and Beattie, 1988). Oocysts in soil can be spread mechanically by flies, cockroaches, dung beetles, and earthworms. Oocysts are known to survive on fruits and vegetables for long periods (Kniel et al., 2002). Humans may acquire toxoplasmosis by petting dogs that have rolled over in infected cat feces (Frenkel et al., 1995; Lindsay et al., 1997) and oocysts have been found in dog feces (Schaes et al., 2005). While only a few cats (< 1%) may be shedding *T. gondii* oocysts at any given time the enormous numbers produced and their resistance to destruction assure widespread contamination (Dubey, 2004). Latently infected cats can shed oocysts after challenge infection (Dubey, 1976,

1995; Ruiz and Frenkel, 1980b). Congenitally infected kittens can also excrete oocysts (Dubey and Johnstone, 1982; Dubey and Carpenter, 1993).

Infection rates in cats are largely determined by the rate of infection in the local avian and rodent populations which serve as a food source. For example, *T. gondii* oocysts were found in 23.2% of 237 cats in Costa Rica, where infection in local rodents and birds was high (Ruiz and Frenkel, 1980a). For epidemiologic surveys, seroprevalence data for cats are more useful than results of fecal examination because cats with antibodies have probably already shed oocysts and are an indicator of environmental contamination (Dubey and Frenkel, 1972). *T. gondii* can injure the neural tissues of infected mice and rats, resulting in impaired learning and memory and behavioral abnormalities. *T. gondii*-infected rodents become less neophobic, leading to decreased aversion to the odor of cats. This behavior might help ensure that rodents would be eaten by cats and the life cycle would be preserved.

#### *Life cycle in the intermediate hosts, including humans and cats*

The life cycle of oocyst-transmitted infection has been studied in mice (Dubey *et al.*, 1997a; Speer and Dubey, 1998). After ingestion of sporulated oocysts, sporozoites excyst, penetrate enterocytes and goblet cells of the intestinal epithelium, and are carried to the lamina propria via an unknown mechanism. Sporozoites were found in parasitophorous vacuoles (PV) in enterocytes and goblet cells as early as 2 hours post feeding. Some sporozoites can be found circulating in peripheral blood as early as 4 hours after ingestion. However, most remain in the lamina propria where they multiply in a variety of cells including vascular endothelium, fibroblasts, mononuclear cells and segmented leukocytes, but not in erythrocytes. It is likely that sporozoites break out of the PV before reaching the lamina propria. At 12 hours post infection (PI), sporozoites had divided into 2 tachyzoites (Dubey *et al.*, 1997a). By 6 days PI, bradyzoites and tissue cysts had formed as demonstrated immunohistochemical staining with bradyzoite-specific antibodies and by bioassays in cats. Tissue cysts were persisted in many organs of mice even during chronic infection.

The bradyzoite-induced cycle in the intermediate host is similar to that of oocyst-induced cycle but bradyzoites are less infective to mice than sporozoites (Dubey, 1997b). After feeding bradyzoites to mice, bradyzoites were found in enterocytes and in the lamina propria at 1 hour PI. By 2 days PI, tachyzoites were formed and tissue cysts were formed by 6 days PI. Tissue cysts persisted in several organs.

#### *T. gondii infection in marine mammals*

Contamination of marine environment with *T. gondii* is of epidemiological, veterinary, and public health concern. There are numerous reports of *T. gondii* infections in marine mammals including sea otters, dolphins, seals, whales (Miller *et al.*, 2001; Dubey *et al.*, 2003; Conrad *et al.*, 2005) and toxoplasmosis has been considered a cause of death in sea otters (Thomas and Cole, 1996; Cole *et al.*, 2000; Dubey *et al.*, 2003; Lindsay *et al.*, 2001b; Miller *et al.*, 2002; Kreuder *et al.*, 2003) yet how marine mammals become infected is unknown. Ingesting oocysts directly from sea water or ingesting tissues of animals that have ingested oocysts are most likely. Miller *et al.* (2002) presented evidence that coastal



fresh water surface runoff presented a risk of infection to sea otters, so it is possible that *T. gondii* oocysts could be washed into the sea via runoff contaminated by cat excrement. Grey seals developed patent *T. gondii* infection after they were fed oocysts (Gajadhar *et al.*, 2004). *T. gondii* oocysts are extremely resistant to environmental influences and oocysts can sporulate and survive in seawater for months (Lindsay *et al.*, 2003). Even unsporulated oocysts can remain viable at 4°C for months (Lindsay *et al.*, 2002).

The role of marine invertebrates in the life cycle of *T. gondii* is unknown. Although *T. gondii* does not parasitize cold-blooded animals, mollusks are filter feeders of water and may thus concentrate oocysts from the water. Experimentally, *T. gondii* oocysts were removed by oysters from water tanks seeded with oocysts (Lindsay *et al.*, 2001a). Viable *T. gondii* was recovered from tissues of oysters for at least 21 day post exposure with oocysts using bioassay in mice (Lindsay *et al.*, 2004). Similarly, Arkush *et al.* (2003) found viable *T. gondii* in tissues of mussels (*Mytilus galloprovincialis*) 3 days post exposure. Finding of antibodies to *T. gondii* in nearly 100% of free-ranging bottlenose dolphins (*Tursiops truncatus*) is even more intriguing because they drink very little water (Dubey *et al.*, 2003, 2005a).

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## Abstract

*Toxoplasma gondii* belongs to the phylum Apicomplexa and as such shares a number of specific organelles with other members of the phylum. These organelles, predominantly located in the apical cytoplasm, were characterized ultrastructurally over 35 years ago (Scholtyseck and Mehlhorn, 1970; Vivier *et al.*, 1970). The tachyzoite of *T. gondii* can actually be considered the paradigm of Apicomplexa invasive stages, as most of the structural, biological and molecular data concerning these “zoites” have been obtained using this organism as a model, even before its life cycle and phylogenetic links were discovered. Other stages, and especially those occurring in the definitive host have been less well studied in many respects, but their ultrastructure is nevertheless very well known. The present chapter will summarize the main ultrastructural characteristics of the different stages of *T. gondii*, in both the intermediate and definitive hosts, including the tachyzoite and the other stages of asexual reproduction, and the sexual stages, that are typical of enteric Coccidia.

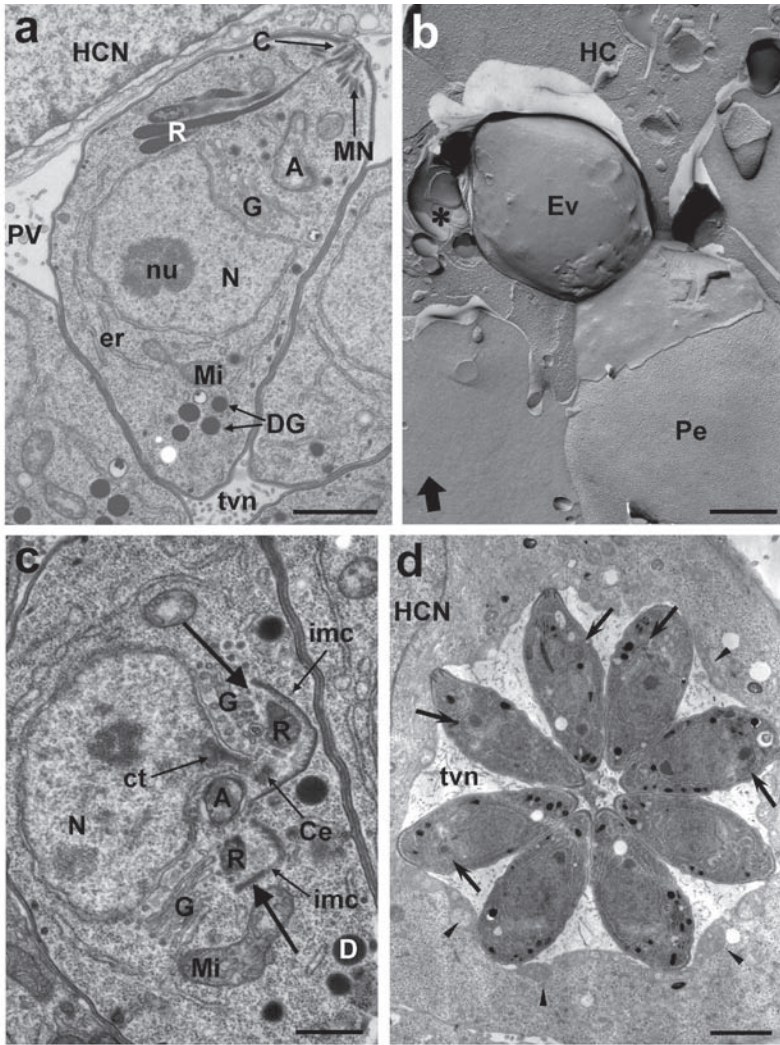
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## Invasive stage ultrastructure and genesis

### Basic ultrastructural morphology

There are four invasive forms of *T. gondii*; the tachyzoite, bradyzoite, merozoite and sporozoite. The tachyzoites and bradyzoites are associated with the intermediate host and the merozoites and sporozoites associated with the definitive host. The tachyzoites and merozoites are responsible for expansion of the population within a host while the bradyzoites and sporozoites are capable of transmission to new hosts.

The tachyzoite is the most extensively studied stage in the *T. gondii* life cycle because of the ease with which large numbers can be obtained both *in vivo* and *in vitro*. The invasive stages are crescent shaped cells ( $2 \times 7 \mu\text{m}$  approx) with a slightly more pointed anterior end (the anterior being defined by the direction of motility) (Figure 2.1a and see Figures 2.2a and 2.3b). They are comprised of a unique cytoskeleton (subpellicular microtubules, conoid, inner membrane complex), secretory organelles (rhoptries, micronemes, dense granules), endosymbiotic derived organelles (mitochondrion, apicoplast), eukaryotic universal organelles (nucleus, endoplasmic reticulum, Golgi apparatus, ribosomes), specific



**Figure 2.1** *Toxoplasma gondii* tachyzoites developing in Human foreskin fibroblasts *in vitro*. (a) Sagittal section of an intravacuolar tachyzoite. A—apicoplast, C—conoid, DG—dense granule, er—endoplasmic reticulum, G—Golgi body, HCN—host cell nucleus, MN—micronemes, Mi—mitochondria, N—nucleus, nu—nucleolus, PV—parasitophorous vacuole, R—rhoptry, tvn—tubulo-vesicular network. Bar is 1  $\mu$ m. (b) Freeze fracture of an invading tachyzoite showing the parasitophorous vacuole membrane (Ev), a clump of membrane whorls that may correspond to material exocytosed from the rhoptries (asterisk), and the plasmalemma of the tachyzoite (Pe). Arrow (bottom left) shows angle of shadowing. Bar is 0.5  $\mu$ m. (c) Early stage of endodyogeny showing two developing daughters (arrows), with early rhoptries (R). The Golgi body (G) has divided. Only one nuclear pole (ct—centrocone, Ce—centriole) is in the section plane. A—apicoplast, D—dense granule, imc—inner membrane complex, Mi—mitochondrion, N—nucleus. Bar is 0.5  $\mu$ m. (d) Intracellular rosette of tachyzoites: typical figure of intracellular tachyzoite multiplication in adherent cells grown *in vitro*, where divisions occur in one single plane. The vacuole and tubulo vesicular network (tvn) surround the parasites, all of which are in an early stage of endodyogeny (arrows). Host cell mitochondria (arrowheads) surround the parasitophorous vacuole membrane. Bar is 2  $\mu$ m.

structures (acidocalcisomes,) all enclosed by a complex membranous structure termed the pellicle.

The cytoskeleton comprises:

- Two apical rings located beneath the plasma membrane at the apical tip of the parasite. They are made of a thin ring of electron dense material, the upper one is 160 nm, the posterior one 200 nm in diameter.
- The conoid is a hollow truncated cone consisting of fibers wound into a spiral, like a compressed spring, 400 nm in diameter at the base and 250 nm high, made of tubulin organized in a unique fashion, consisting of asymmetrical filaments of about 9 protofilaments (Hu *et al.*, 2002b).
- Two polar rings encircle the top of the resting conoid. The outer ring consists of dense material covering the anterior rim of the inner membrane complex (IMC, see below). The inner ring anchors the 22 subpellicular microtubules that extend underneath the IMC for approximately two-thirds of the body length (Nichols and Chiappino, 1987). These microtubules are classical 22 nm in diameter hollow tubes, comprising 13 protofilaments made of alpha and beta tubulin (Hu *et al.*, 2002b).
- A pair of adjacent intraconoidal microtubules is also found, extending for a short distance (less than 1  $\mu\text{m}$ ) into the apical cytoplasm (Hu *et al.*, 2002b).

The pellicle is a distinctive membrane complex that surrounds the infectious stages. It consists of an outer unit membrane (plasmalemma) that completely encloses the organism and an inner layer of two closely applied unit membranes found at a fixed distance (approx 15 nm) from the plasmalemma. The inner membrane complex consists of fused plates formed from flattened vesicles derived from the ER–Golgi system (Vivier and Petitprez, 1969). The inner layer is interrupted by circular apertures at the anterior end (outer polar ring), where the conoid protrudes, and at the posterior end. The organization of the IMC has been essentially unraveled by EM freeze fracture (Morrisette *et al.*, 1997; Porchet and Torpier, 1977). It is made of an apical plate which is a single truncated cone approximately 1  $\mu\text{m}$  high, to which six longitudinal rows of rectangular plates are attached. The rows end at the posterior of the tachyzoite in triangular plates. The rows can extend straight or be twisted helically. The protoplasmic faces on both sides of the IMC are covered with lines of intramembranous particles (IMPs), with 22 lines of higher density corresponding to the underlying subpellicular microtubules (Porchet and Torpier, 1977). An additional organized structure associated with the inner side of the IMC has been described by negative staining after detergent extraction, as a network of 8–10 nm filaments (Mann and Beckers, 2001). The precise correlation between this network and the IMP alignments has not been defined.

The pellicle has an additional adaptation termed the micropore, which is located in the apical half of the cell normally just anterior to the nucleus. The single micropore consists of a circular (approximately 150 nm diameter) invagination of the plasmalemma through a break in the inner membrane complex. The latter infolds to form an electron dense collar around the invagination (see Figure 2.2a). These structures are present throughout development and increase in number during endopolygony and gametogony. They are thought to act as a cytostome-like structure (Nichols *et al.*, 1994).

Three distinct secretory organelles have been identified, which can vary in numbers and shape between the invasive stages (see below) (Figure 2.1a and see Figures 2.2a and 2.3b). First are small rod shaped micronemes ( $250 \times 50 \text{ nm}$ ), located in the most apical area of the parasite, behind the conoid. They are homogeneously electron dense. Second are the rhoptries, organized as a group of elongated, club shaped organelles that extend from within the conoid toward the nucleus. They show a long narrow neck up to  $2.5 \mu\text{m}$  long, and a sac like body about  $0.25 \times 1 \mu\text{m}$  in the posterior area. The contents are electron dense, except in the widened part, where the structure can be either labyrinthine or electron dense in appearance depending of the specific stage. Different proteins are found in the bulbous region (the ROP proteins) and the neck region (the RON proteins) (Bradley *et al.*, 2005). A third type of organelle, found throughout the cell but mostly in the posterior part of the parasite, are spherical shaped ( $0.3 \mu\text{m}$  diameter) structures with electron dense contents, which have been termed the dense granules.

The nucleus occupies a central or basal location depending on the invasive stage (see below). It is often flattened on the upper side, where the Golgi apparatus is located. It contains a central nucleolus and small clumps of electron dense heterochromatin scattered throughout the nucleoplasm. The nuclear envelope has numerous nuclear pores, and is covered on its external side with ribosomes, except on the upper face, where the Golgi apparatus is located (Figure 2.1a). The nuclear envelope is in continuity with sheets of rough endoplasmic reticulum that extend into the cytoplasm of the tachyzoite.

Using certain preparative techniques, one or two vesicles of c.a.  $200 \text{ nm}$  containing one or several electron dense droplets or crystals of various sizes in a clear background are found near the nucleus or in the posterior part of tachyzoites (Figure 2.1a). These have been termed the acidocalcisomes, and the dark contents are believed to be calcium bound to pyrophosphate and polyphosphates (Luo *et al.*, 2005).

Several mitochondrial profiles of  $0.5 \mu\text{m}$  width and various lengths can usually be observed at various locations above and below the nucleus (Figure 2.1a). These represent sections through a single branched and elongated mitochondrion. They show the typical apicomplexan structure, with bulbous cristae.

Above the Golgi is the apicoplast (Figure 2.1a). This Apicomplexa specific organelle, limited by multiple membranes, has been identified morphologically since the early 1960s (Ogino and Yoneda, 1966; Sheffield and Melton, 1968; Vivier and Petitprez, 1969) but was only recently shown to be a typical plastid (Kohler *et al.*, 1997). In the infectious stage it is relatively uniform in shape, up to  $500 \text{ nm}$  in diameter, bounded by possibly 4 membranes, and filled with granular and filamentous content, in which ribosomes can be observed. The origin of the four membranes is still a matter of debate but could result from a secondary phagocytosis of an alga already containing an endosymbiont (Kohler *et al.*, 1997).

## Comparison of the invasive stages

The infectious stages consisting of the tachyzoite, bradyzoite, merozoite and sporozoite differ from each other in the number of the apical organelles, the shape and electron density of the rhoptries, the location of the nucleus and the presence or absence of polysaccharide granules. The polysaccharide granules are ovoid structures ( $250\text{--}180 \text{ nm}$ ) of variable electron density located in both the apical and basal cytoplasm. They contain an



unusual form of carbohydrate which is biochemically more similar to plant amylopectin than animal glycogen (Coppin *et al.*, 2005). These granules are rarely found in tachyzoites or merozoites but are present in large numbers in bradyzoites and sporozoites (see Figure 2.3b) The most obvious difference between stages relates to variations in the number and structure of the apical organelles (see review by Dubey *et al.*, 1998). These stage differences are summarized in Table 2.1.

### Host cell invasion

Invasion is operated by a moving junction, which has the same morphological features as the one described for *Plasmodium knowlesi* (Aikawa *et al.*, 1981), both in thin section, and in freeze fracture. Interestingly, *T. gondii* makes the same junction with nucleated cells and red blood cells (Michel *et al.*, 1979; Porchet-Hennere and Torpier, 1983). It is a very close apposition of the parasite and host plasma membranes, with thickening of the host side, and an accumulation of rhomboidally organized intramembranous particles on the protoplasmic face (lipid layer adjacent to the cytoplasm) of the host plasma membrane. This forms a very tight junction which excludes small electron dense tracers such as ruthenium red. The molecular organization of the moving junction is still unclear, but recent data have shown that it involves proteins derived from the rhoptry neck in association with the microneme protein AMA1 (Alexander *et al.*, 2005; Lebrun *et al.*, 2005).

Microneme exocytosis has never been clearly visualized, although it is thought to occur both during gliding motility and invasion. The docking site for microneme exocytosis is not known in *T. gondii*. Rhoptry exocytosis is easily documented upon invasion, as an apical opening in continuity with the parasite plasma membrane, facing the developing parasitophorous vacuole (PVM) (Nichols *et al.*, 1983). At very early stages of invasion, when the moving junction forms, small vesicles can be seen budding from the developing vacuole or lying in the host cell cytoplasm. At this stage, empty rhoptries are already observed. Therefore these vesicles correspond to the physiological counterpart of the evacuoles, which are the product of frustrated rhoptry exocytosis in the host cell cytoplasm when invasion is blocked by cytochalasin D (Hakansson *et al.*, 2001).

The membrane of the developing vacuole is completely devoid of intramembranous particles (Figure 2.1b) (Dubremetz *et al.*, 1993), reflecting the selective exclusion of the transmembrane host cell proteins at the moving junction. However it will acquire IMPs during the first hour of development (Porchet-Hennere and Torpier, 1983), probably of parasite origin resulting from protein secretion, especially the contents of the rhoptries and dense granules that are released into the PV (Dubremetz *et al.*, 1993).

### Parasitophorous vacuole, intracellular development

Within minutes after closure of the parasitophorous vacuole, the posterior part of the parasite invaginates and the tubulo-vesicular network (TVN) starts developing in this invagination (Sibley *et al.*, 1995). The origin of the TVN is not fully understood: it contains dense granule proteins that are exocytosed from the anterior end of the parasite, this exocytosis beginning before the completion of the invasion process (Dubremetz *et al.*, 1993). These tubules are in direct continuity with the PVM, although these two structures contain distinct dense granule derived proteins (Cesbron-Delauw, 1994).

**Table 2.1** Summary of the morphological differences between stages of *T. gondii*

Life cycle stage	Nucleus	Micronemes	Rhoptries			Polysaccharide granules
			Number	Appearance	Dense granules	
Tachyzoite	Central	Few	5–12	Labyrinthine	Numerous	Few
Bradyzoite	Basal	Numerous	5–10	Solid	Numerous	Numerous
Merozoite	Central	Few	3–5	Solid	Few	Absent
Sporozoite	Basal	Numerous	5–10	Labyrinthine	Numerous	Numerous



Immediately after invasion, host cell mitochondria and endoplasmic reticulum surround the PV and persist throughout the intracellular development (Sinai *et al.*, 1997). The rhoptry protein ROP2, exocytosed during invasion, has been shown to anchor the host mitochondria to the PVM (Sinai and Joiner, 2001). The host ER is devoid of ribosomes on the side facing the vacuole.

The parasitophorous vacuole described above is only formed by actively invading parasites. It is characterized by its ability to prevent the fusion of host cell lysosomes thus protecting the parasite during intracellular development (Jones and Hirsch, 1972; Jones *et al.*, 1972).

## Endodyogeny

The tachyzoite is unique in its ability to undergo indefinite proliferation by a distinctive process termed endodyogeny, which involves parasite growth and division to form two daughters. Despite grossly resembling binary fission, endodyogeny is a highly complex event, related to the structural complexity of the formation of polarized daughters. In contrast with the canonical asexual division mode of most Apicomplexa and even the coccidian stages of *T. gondii*, the tachyzoite retains the apical complex until the end of the endodyogeny process. Endodyogeny is the exclusive form of asexual division undergone within the intermediate host (during tachyzoite and bradyzoite formation). The processes of mitosis and daughter formation occur simultaneously during endodyogeny

## Mitosis

There have been few descriptions of *T. gondii* mitosis at the EM level, and what has been observed can be interpreted by comparison with more detailed studies in related Apicomplexa, especially *Eimeria* *sp.* (Dubremetz, 1973). One characteristic feature of apicomplexan mitosis is the retention of an intact nuclear membrane throughout the process of division. Coccidian type centrioles (150 nm D) consist of 9 short tubules (100 nm long) centered on a central tubule. Centrosomes or spindle pole bodies, are made of 2 centrioles oriented in parallel always found associated with mitotic spindle poles, usually on the apical side of the nucleus. The earliest stage of mitosis is a trans-nuclear funnel, corresponding to an invagination of the nuclear envelope opened on both sides towards the cytoplasm. The mitotic spindle most likely polymerizes in this funnel, which then opens in the nucleoplasm in its middle part, whereas the poles give rise to the centrocones. The centrocones are at first subspherical invaginations of the nuclear envelope opened towards the centrosomes and through which the spindle microtubules extend. The intranuclear spindle is usually very short and transient and has rarely been described. What occurs most likely is that the kinetochores are separated immediately after the funnel opening and assemble on the nucleoplasmic side of the centrocones. This association between centrosomes, centrocones and kinetochores ensures genetic material partition between daughter tachyzoites (Figure 2.1c).

## Zoite biogenesis

Soon after the centrosomes separate and centrocones are formed, the future apical complex of each daughter tachyzoites starts to develop adjacent to each centrosome. The details of

this biogenesis have not been studied as thoroughly as in *Eimeria sp.* (Dubremetz, 1975), but follow the same scheme (Hu *et al.*, 2002a; Vivier, 1970; Vivier and Petitprez, 1969). At an early stage of development the conoid forms near each centrosome (Figure 2.1c). The inner membrane complex and underlying subpellicular microtubules array appear to form around the conoid and, in a coordinated manner, start to grow posteriorly (Figure 2.1c). This occurs within the mother cell cytoplasm rather than in association with the mother cell plasmalemma that is characteristic of daughter formation in classical schizogony undergone by most apicomplexans. Early stages are short flattened cones above the centrocones (Figure 2.1c), which later elongate into the grossly cylindrical shape that will eventually surround the mature organism. The Golgi apparatus divides, concomitantly with spindle formation, with each newly formed Golgi body being found adjacent to the upper nuclear envelope, near each centrocone (Figure 2.1c) (Pelletier *et al.*, 2002). The apicoplast elongates and appears to divide during daughter formation with a portion entering each daughter. Rhoptry precursors are observed at this time as heterogeneous irregularly shaped vesicles of about 0.3  $\mu\text{m}$  located near the Golgi bodies, within the inner membrane complex (Figure 2.1c). As development proceeds, the nucleus becomes U-shaped and the developing inner complex elongates and engulfs the daughter nuclei while additional organelles (rhoptry precursors and then micronemes) are formed anterior to the Golgi body. The rhoptry contents condense while the rhoptry ducts appear and elongate towards the conoid. As the daughters grow, the inner membrane complex of the mother cell breaks down along with the anterior organelles. The fully formed daughters fill much of the mother cell cytoplasm and their inner membrane complex comes in contact with the mother cell plasmalemma to form the daughter pellicle. This is initiated at the anterior end and results in the daughters remaining connected via a small portion of residual cytoplasm before finally separating. In certain cases, each of the daughters, while remaining attached by their posterior ends, can undergo a new cycle of endodyogeny. There is evidence for the repeated synchronized divisions of the daughters leading to the accumulation of tachyzoites within the vacuole (Figure 2.1d).

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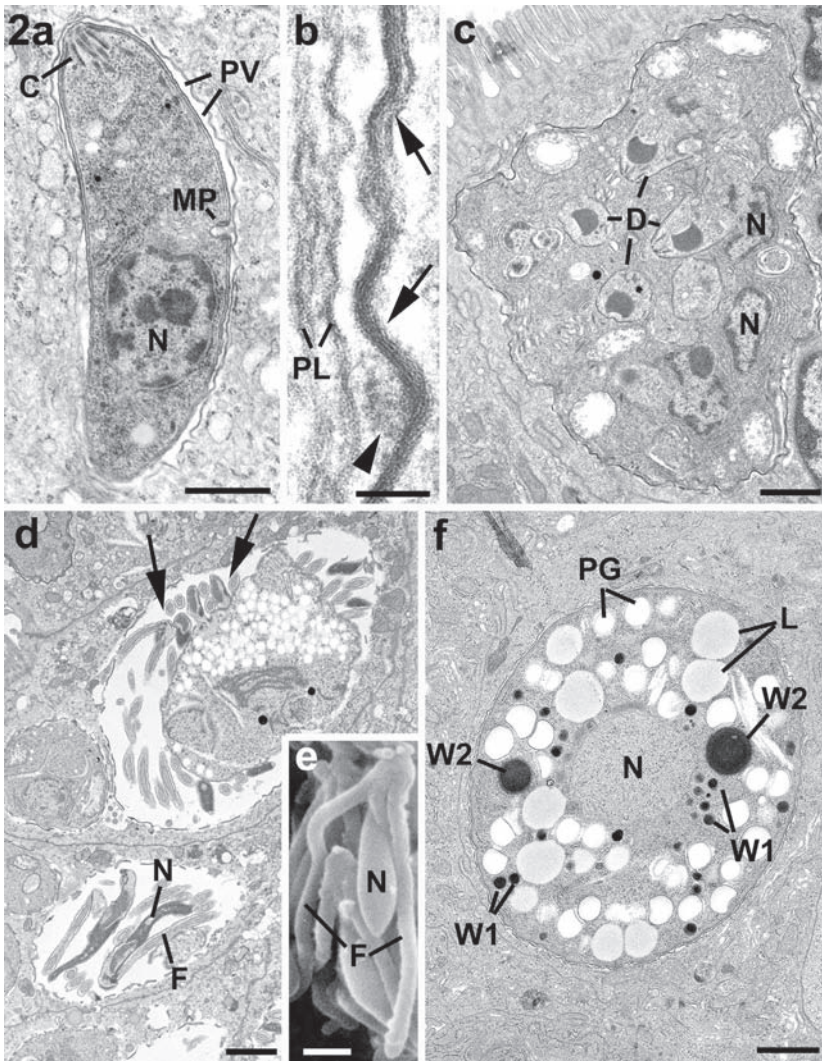
## Coccidian development in the definitive host

### Host–parasite relationship

Coccidian development is limited to the epithelial cells of the small intestine of the cat (the definitive host). The coccidian stages develop within a tight fitting, thick walled parasitophorous vacuole consisting of three closely applied unit membranes with a number of conical-shaped dense structures (Figure 2.2a,b) (Ferguson, 2004; Ferguson *et al.*, 1974). There is no evidence of formation of the tubular network within the PV or the congregation of the host cell mitochondrion or strands of rER around the periphery of the PV (Figure 2.2a). These structural differences correlated with the lack of expression of the majority of dense granule proteins (see Chapter 25, this volume, Mercier *et al.*).

### Asexual development

During coccidian development, asexual multiplication involves a process with unique structural features, which has been termed endopolygeny (Piekarski *et al.*, 1971). The



**Figure 2.2** *Toxoplasma gondii* developing in enterocytes of the cat intestine. (a) Early intracellular stage located in a thick walled tight fitting parasitophorous vacuole (PV). The merozoite has lost the apical organelles but still retains the conoid (C) and a micropore (MP) located close to the nucleus (N). Bar is 1  $\mu$ m. (b) Enlargement showing the laminated structure of the electron dense membrane limiting (arrows) the parasitophorous vacuole. Note a conical structure protruding into the membrane (arrowhead). PL—parasite pellicle. Bar is 100 nm. (c) Low power of a schizont with a number of nuclei (N) showing the formation of a larger number of daughters (D) by endopolygony. Bar is 1  $\mu$ m. (d) Low power showing two stages of microgametogony. In the upper vacuole microgametes can be seen budding from the surface (arrows). In the lower vacuole the elongated nucleus (N) and flagella (F) of the fully formed microgamete can be seen. Bar is 1  $\mu$ m. (e) Scanning electron micrograph of mature microgamete showing the nucleus (N) and two long flagella (F). Bar is 1  $\mu$ m. (f) Mature macrogamete showing the central nucleus (N). The cytoplasm containing a number of wall forming bodies type 1 (W1) and a few wall forming bodies type 2 (W2) plus numerous polysaccharide granules (PG) and lipid droplets (L). Bar is 1  $\mu$ m. (Images supplied by David Ferguson, Oxford University; copyright retained.)

process involved growth of the parasite and repeated nuclear divisions employing an eccentric intra-nuclear spindle. There is also a marked increase in the size of the mitochondria and apicoplast (Ferguson *et al.*, 2005). This proliferative phase differentiates the process from endodyogeny. After a variable number of nuclear divisions, the parasite enters the differentiation phase (daughter formation), which coincides with the apicoplast division (Ferguson *et al.*, 2005). Daughter formation can occur at any time between the 4 and about 20 nuclei stage and is initiated during or just after the final nuclear division (Figure 2.2c). The first evidence of daughter formation is the appearance of a conical structure formed by a number of flattened vesicles each with underlying longitudinally running microtubules and with the conoid in the apex (Figure 2.2c). The initiation of daughter formation is synchronized with all daughters forming at the same time (Figure 2.2c). The mechanism of daughter formation is similar to that observed for the two daughters formed during endodyogeny. The simultaneous formation of a large number of daughters requires an extremely well coordinated process to ensure that all daughters receive a full complement of organelles and are therefore viable. As daughter formation progresses by the posterior growth of the inner membrane complex it encloses a nucleus, apicoplast and mitochondrion and the apical organelles develop. At this point the daughters fill the mother cell cytoplasm but are still enclosed in the schizont plasmalemma. The final stage is the invagination of the mother cell plasmalemma starting at the anterior of the daughters and progressing posteriorly to form the outer membrane of the pellicle of each daughter. A single micropore is formed in the pellicle just anterior to the nucleus. The merozoites often remain attached to a small amount of residual cytoplasm at the posterior end. These banana shaped daughters can be seen forming (a) fan-like structures. This process differs from classical schizogony in that the daughters are formed within the cytoplasm rather than budding from the surface.

### Sexual development

After an unknown number of asexual cycles, certain merozoites on entering a new enterocyte can develop into either male (microgametocyte) or female (macrogametocyte) gametocytes. In the case of microgametogony, this results in the formation of multiple (15–30) male (microgametes) while during macrogametogony, a single female (macrogamete) gamete forms. The trigger for the conversion from asexual to sexual development is unknown. Nor is it known what is responsible for deciding whether an invading merozoite develops into either a microgametocyte or a macrogametocyte.

#### *Microgametogony and the microgamete*

Initially it is impossible to differentiate between endopolygony and microgametogony with both processes involving continued growth and repeated nuclear divisions. However, characteristic chromatin condensation within the nuclei allows the identification of microgametocytes (Ferguson *et al.*, 1974). The nuclei then move to the periphery of the cell with two centrioles and a dense plaque (perforatorium) located between the nuclei and the plasmalemma. The centrioles become the basal bodies for the developing flagella. Microgamete development continues with flagellar growth and protrusion of a portion of cytoplasm containing the basal bodies, the electron dense portion of the nucleus and a



mitochondrion into the lumen of the PV (Figure 2.2d). As this occurs there is division of the nucleus with the electron dense portion separating from the electron lucent portion. The electron dense portion enters the developing microgamete and the lucent portion remains within the mother cell as a residual nucleus. The microgametocyte of *T. gondii* produces relatively few (15–30) microgametes (Figure 2.2d). Maturation continues with each microgamete becoming elongated in appearance and consisting of an electron dense nucleus with a mitochondrion located between the nucleus and the basal bodies from which the two very long flagella run toward the posterior (Figure 2.2e). Once fully formed, the microgametes detach from the microgametocyte leaving a large residual cytoplasmic body (Dubey *et al.*, 1998; Ferguson *et al.*, 1974).

### *Macrogametogony and the macrogamete*

The development of the macrogametocyte is associated with growth of the trophozoite and the appearance of a large nucleus with dispersed chromatin and a large nucleolus but no nuclear division. As the macrogametocyte grows there is a marked increase in the size of the peripherally located mitochondrion and the centrally located apicoplast. In addition, a number of Golgi bodies are distributed throughout the cytoplasm. The first distinctive feature of macrogametogony is the appearance of flocculent material condensed within dilations of the rER which represents the formation of the wall forming body type 2 (WFB2); so called because of their role in the formation of the oocyst wall (see below). As maturation continues, there is an increase in size and number of the WFB2 and a number of electron dense membrane bound granules appear to form from vesicles produced by the Golgi bodies, which can be differentiated by immuno-electron microscopy into two populations. One which appears to be involved in the formation of the outer veil and is termed the veil forming bodies (VFB) and the other the wall forming bodies type 1 (WFB1) (Ferguson *et al.*, 2000; Ferguson *et al.*, 2003). As the veil and wall forming bodies are being synthesized there is also the synthesis of numerous polysaccharide granules and lipid droplets and an expansion of the apicoplast (Figure 2.2f). When fully developed, the macrogametocyte can be considered to be a mature macrogamete (Ferguson *et al.*, 1975).

### *Oocyst wall formation*

The oocyst wall is extremely resistant to physical and chemical insults. As such it is fundamental to the survival of the parasite. The wall is a complex structure consisting of a number of distinct layers and is synthesized while the macrogamete is still within the host cell (Ferguson *et al.*, 1975; Speer *et al.*, 1998). Formation of this multi-layered structure requires the control and sequential secretion of the various components from the macrogamete. During the maturation of the macrogamete the contents of the VFB are released to form a loose outer veil. This is followed by the triggered secretion of the WFB1, which occurs simultaneously in the mature macrogamete to form the outer layer of the oocyst wall. Finally, the contents of the WFB2 are released and coalesce to form the electron lucent inner layer of the oocyst wall (Ferguson *et al.*, 1975). This results in a cytoplasmic mass characterized by a central electron lucent nucleus and cytoplasm packed with polysaccharide granules and lipid droplets. The formation and polymerization of the inner layer has a dramatic effect on the ability to process the oocyst for ultrastructural examination. To

date no technique has been developed that will allow the oocysts of *T. gondii* or any other coccidian oocyst to be examined by electron microscopy. The outer layer contains mostly proteins and carbohydrate and appears to provide structural strength. In contrast the inner layer has a high lipid content and appears to provide the protection from chemical insult by its impervious nature (Belli *et al.*, 2006).

### Fertilization

It would appear logical that if sexual development takes place, there will be fusion between a microgamete and a macrogamete to form a fertilized zygote. However, this process has never been visualized.

### Oocyst and extracellular sporulation

Due to the technical difficulties, ultrastructural studies of sporulation have been limited to one series of papers (Ferguson *et al.*, 1979a; Ferguson *et al.*, 1979b; Ferguson *et al.*, 1979c). The oocyst is the only stage of *T. gondii* that is capable of undergoing extra-cellular development. The oocysts are excreted with a single undifferentiated cytoplasmic mass; the primary sporoblast. In the external environment, asexual development (sporulation) occurred with two rounds of nuclear division giving rise to four nuclei. The cytoplasmic mass then underwent division to form two spherical secondary sporoblasts each with two nuclei. Each secondary sporoblast developed into a sporocyst, which is characterized by the formation of the sporocyst wall consisting of four plates joined by sutures. Within the sporocyst, a nucleus moved to either end of the sporocyst. The formation of daughters is initiated adjacent to the plasmalemma above each nucleus and progressed by posterior growth. This resulted in the formation of four daughters (two from each end) within each sporocyst.

### Excystation

Excystation is stimulated by incubation in a mixture of trypsin and bile salts (sodium taurocholate), which appears to act on the sporocyst wall causing increased tension. This results in an infolding of the edges of the plates, which eventually rupture along the suture line thus releasing the sporozoites (Christie *et al.*, 1978; Ferguson *et al.*, 1979d; Speer *et al.*, 1998).

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## Development in the intermediate host

### Tachyzoite development.

When an intermediate host is infected by ingestion of tissue cysts or oocysts, the bradyzoites and sporozoites are released into the lumen of the small intestine. The sporozoites and bradyzoites invade the enterocyte or intra epithelial lymphocytes of the small intestine or pass through into the lamina propria and invade cells there. In either case the parasite (bradyzoite or sporozoite) defaults to tachyzoite development with formation of the characteristic parasitophorous vacuole and undergoes multiplication by endodyogeny (Dubey *et al.*, 1998; Speer and Dubey, 1998). Thereafter the tachyzoites disseminated systemically via the vascular system to all organs of the body and proliferation by endodyogeny in many

cell types. However, in immuno-competent animals the number of lesion and tachyzoites peaked during the acute phase, about 12 days, and by 21 days it is difficult to identify tachyzoites in any organ, including the brain, even using immuno-cytochemistry.

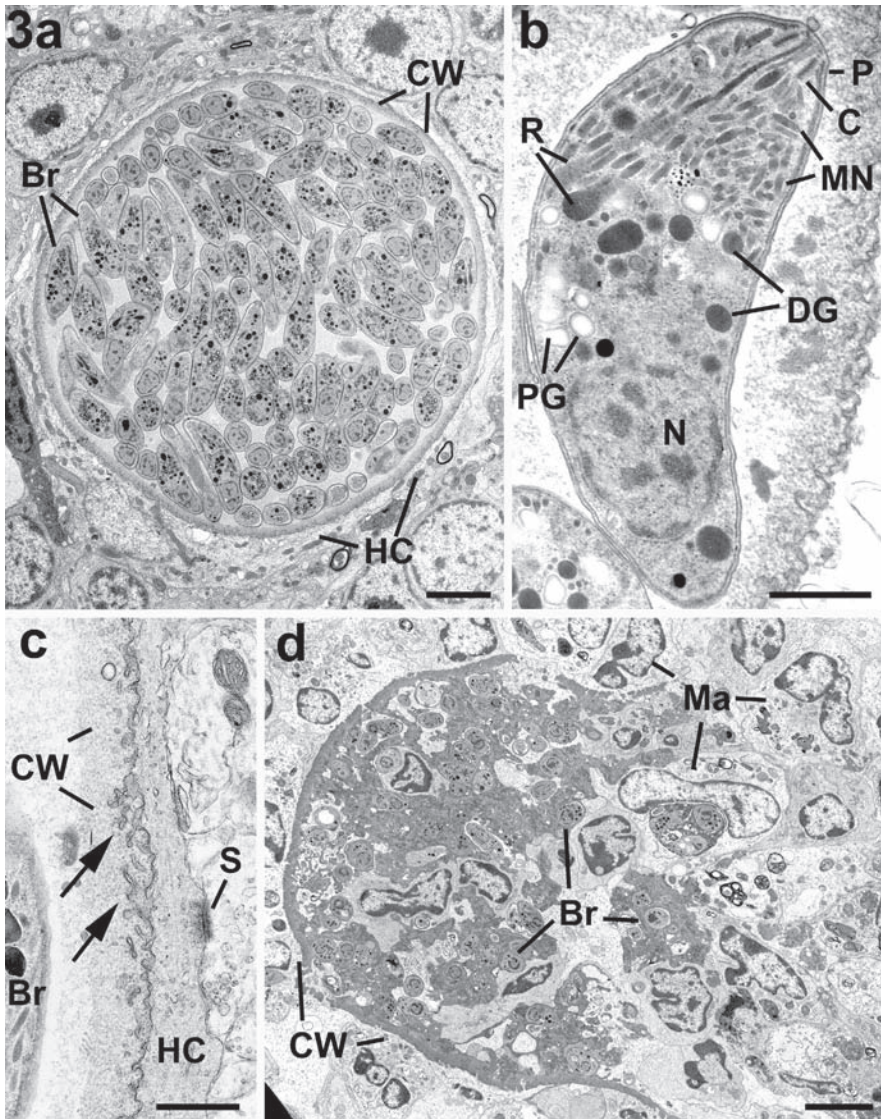
### Stage conversion: tachyzoite to bradyzoite

Stage conversion can be examined in detail in mice at 12–15 days post-oral infection. In mice, the lesions showing stage conversion are limited to the brain and consist of numbers of parasites undergoing tachyzoite development admixed with parasites forming early tissue cysts. A number of cysts can be seen within the lesion and indeed it is possible to observe two cysts forming within the one host cell. The early cysts could be differentiated from tachyzoite-like vacuoles by the distinctive structure of the PV consisting of a tight fitting undulating PVM with no associated host cell mitochondrion or rER. These vacuoles lacked the tubular network but possessed a thin layer of amorphous material. As the tissue cyst develops, the depth of the invaginations of the PVM increases, as does the thickness of the layer of homogenous material underlying the membrane (Figure 2.3a). These tissue cysts continue to enlarge over the next 3 weeks with a concomitant decrease in the proportion of dividing bradyzoites (Ferguson and Hutchison, 1987a). Within the early tissue cyst the bradyzoites still have similar ultrastructural features to the tachyzoites; particularly the rhoptries which retain a labyrinthine appearance (Ferguson and Hutchison, 1987b). It is often 21–28 days before typical bradyzoites could be identified.

### Structure of the tissue cyst and bradyzoite

The structure of the mature tissue cyst observed from 3 to 24 months post-infection (approx. the life span of the mouse) remained relatively unchanged (Figure 2.3a). The first important observation was that throughout this period the tissue cysts were retained within a viable host cell (Figure 2.3a,b). It was originally thought that the mature cysts were extracellular. However on ultrastructural examination, a thin rim of host cell cytoplasm could be observed enclosing the tissue cysts (Ferguson and Hutchison, 1987b). This may explain the lack of an immune response to the tissue cysts; they are masked from the immune system by the host cell. With the limited host cytoplasm available it is difficult to identify the cell type. However, in the majority of cases, the host cells could be identified as neurons because of the defining presence of synapses (Figure 2.3c). There are variations in the thickness of the cyst wall between tissue cysts with some showing deep invaginations of the limiting membrane forming a complex of interconnecting channels all embedded in the homogeneous granular material (Ferguson and Hutchison, 1987a). In the mature cysts, the bradyzoites appeared more elongated than the tachyzoites with a posteriorly located nucleus (Figure 2.3b). There are numerous micronemes and few dense granules although this can be variable. The rhoptries had more bulbous ends and are uniformly electron dense. The major difference is the presence of numerous polysaccharide granules (Figure 2.3a,c). It is possible to find small groups of tissues cysts of different sizes but there is no evidence to support the idea that this represents cysts arising from “escaped” bradyzoites forming daughter cysts.





**Figure 2.3** Ultrastructural features of tissue cysts of *Toxoplasma gondii* in mouse brain. (a) Low power of a mature tissue cyst containing a large number of bradyzoites (Br) enclosed by a cyst wall (CW). Note the thin rim of host cell cytoplasm (HC) enclosing the cyst. Bar is 5  $\mu$ m. (b) Longitudinally sectioned bradyzoite showing the posteriorly located nucleus, numerous polysaccharide granules (PG) plus dense granules (DG), rhoptries (R), micronemes (MN), conoid (C) and anterior polar rings (P). Bar is 1  $\mu$ m. (c) Detail of the periphery of a tissue cyst showing the cyst wall (CW) with deep invaginations of the limiting membrane (arrows) into the underlying granular material. Note that the host cell (HC) can be definitively identified as a neuron because of the presence of synapses (S). Br—bradyzoite. Bar is 100 nm. (d) Section through a ruptured tissue cyst in an immuno-competent host showing the fractured cyst wall (CW) partially enclosing the bradyzoites (Br). Note the numerous macrophages (M) surrounding and invading into the tissue cyst and phagocytosing the bradyzoites. Bar is 5  $\mu$ m. (Images supplied by David Ferguson, Oxford University; copyright retained.)

## Cyst rupture in immunocompetent hosts

It has been shown that, a very small percentage of tissue cysts are rupturing at any given time during chronic infections (Ferguson *et al.*, 1989). The initial change appeared to be death of the host cell. With the exposure of the parasite antigens within the cyst wall there is evidence for a rapid and massive cell mediated immune response involving numerous inflammatory cells (monocytes and even neutrophils). These can be observed around the still apparently intact cyst. With the rupture of the cyst wall there was further influx of macrophages into the cyst (Figure 2.3d). The macrophages phagocytosed the bradyzoites where there is fusion with lysosomes to form phagolysosomes within which the parasites are destroyed. This resulted in the formation of small inflammatory lesion (microglial nodule) with limited host tissue damage. In immuno-competent animals, bradyzoites appeared to be destroyed before they could undergo replication or stage conversion to tachyzoites.

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# Part II

## **Pathogenesis, Immunology, and Virulence**

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Eskild Petersen

## Abstract

*Toxoplasma gondii* occur world-wide, but the incidence is higher in tropical areas and decreases with increasing latitude. Seroprevalence in Europe is high, up to 54% in Southern European countries and decrease with increasing latitude to 5% to 10%. The *T. gondii* seroprevalence of 15.8% in the age group 12–49 years in the United States. *T. gondii* is a common infection in South America and seroprevalence is high in people from poor socio-economic conditions probably due to water borne transmission and infection often takes place during childhood.

Risk factors for infection with *T. gondii* is mainly through meat products and poor kitchen hygiene in Europe, and surface water in Brazil. *T. gondii* can be divided into three major genotypes of which type II dominate in Europe, and type I have been found over-represented in patient with eye disease.

The finding of *Toxoplasma*-specific IgM-antibodies do not necessarily mean an acute infection, and the major problem of diagnosis of *T. gondii* infection in pregnant women is the finding of *Toxoplasma*-specific IgM- and IgG-antibodies. Two-test strategies with IgM-capture assays and direct IgG assays followed by assay of the *Toxoplasma*-specific IgG-avidity ratio is presently the best option.

*T. gondii* is treated with sulfadiazine and pyrimethamine, but there are only one randomized controlled trials and treatment efficacy is based on animal models and studies with historic controls. A randomized controlled trial found good effect on *T. gondii* eye disease of trimethoprim and sulfamethoxazol. In animal models the best results for a new drug for treatment is atovaquone.

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## Introduction

*Toxoplasma gondii* occur world-wide, but the incidence is higher in tropical areas and decreases with increasing latitude. Seroprevalence in Europe is high, up to 54% in southern European countries (Table 3.1) and decrease with increasing latitude to 5% to 10% in northern Sweden and Norway (Evengard *et al.*, 2001; Jenum *et al.*, 1998). The age-specific prevalence has been decreasing in Europe over the past three to four decades (Forsgren *et al.*, 1991; Welton and Aedes, 2005).

**Table 3.1** Prevalence and incidence of *T. gondii* infections in pregnant women and newborn children based on data from studies performing population wide screening

Country	Year	Protocol	N	Sero-prevalence	Infected pregnant women	CT infected children	Incidence per 1000 pregnant women	Incidence per 10,000 liveborns
Netherlands <sup>a</sup>	1986 - 87	PN	28,049	45%	55	12	1.96	4.27
Norway <sup>b</sup>	1992 - 94	PN	35,940	10.9%	47	11	1.31	3.06
Finland <sup>c</sup>	1988 - 89	PN	16,733	20.3%	13	4	0.77	2.3
New England <sup>d</sup>	1986 - 92	NN	635,000	10%	-	52	-	0.82
Denmark <sup>e</sup>	1992 - 96	NN	89,873	25.3%	141	27	1.56	3.00
Sweden <sup>f</sup>	1997 - 98	NN	35,000	10.1%	-	3	-	0.85
France <sup>g</sup>	1995	PN	13,459	54.3%	32	8-10	2.3	5.9-7.4
Poland <sup>h</sup>	1998 - 00	NN	17,653	45.6%	-	19	-	10.8

NN = neonatal screening; PN = Prenatal screening

a Gilbert *et al.*, 2001.

b Jenum *et al.*, 1998.

c Lappalainen *et al.*, 1995.

d Guerina *et al.*, 1994.

e Lebech *et al.*, 1999.

f Evengard *et al.*, 2001.

g Baril *et al.*, 1999.

h Paul *et al.*, 2001.

In the United States data are collected regularly through the NHANES study (National Health and Nutrition Examination Study) and the NHANES III 1999–2000 found a *T. gondii* seroprevalence of 15.8% in the age group 12–49 years. *T. gondii* seroprevalence was higher among non-Hispanic black persons than among non-Hispanic white persons (age-adjusted prevalence 19.2% vs. 12.1%). No statistically significant differences were found between *T. gondii* antibody prevalence in NHANES 1999–2000, and NHANES III (1988–94) (Jones *et al.*, 2003; McQuillan *et al.*, 2004).

*T. gondii* is a common infection in South America, and a study from Brazil found that seroprevalence was high in people from poor socio-economic conditions probably due to water borne transmission (Bahia-Oliveira *et al.*, 2003). Another study found a seroprevalence of 73% in slaughterhouse workers and suggested that fresh meat is an important source of infection in Brazil (Dias *et al.*, 2005). A study of children from Guatemala found that infection with *T. gondii* often took place before the age of 5 years, at which age 43% were seropositive (Jones *et al.*, 2005).

The seroprevalence is low in most Asian countries, and a study from Korea found an IgG prevalence in pregnant women of 0.8% (Song *et al.*, 2005), and a recent study of HIV-positive patients from Taiwan found a seroprevalence of 10.2% (Hung *et al.*, 2005).

A recent study from India found a seroprevalence of *Toxoplasma*-specific IgG antibodies of 45% (Singh and Pandit, 2004), and a study of HIV-infected patients from Japan found an overall seroprevalence of 44.8%, and the majority of these patients were in the age of 25 to 34 years (Nissapatorn *et al.*, 2004). A study of 327 adult cat owners in Thailand found a seroprevalence of 6.4% (Sukthana *et al.*, 2003), and a study from Malaysia found high seroprevalence in Malays of 55.7% and people of belonging to the Indian ethnic group of 55.3%, but low in ethnic Chinese of 19.4% (Nissapatorn *et al.*, 2003).

A study from Sao Tomé found a prevalence of 21.5% in children below 5 years of age (Fan *et al.*, 2005) and a study from Sudan found a seroprevalence in pregnant women from Khartoum of 34.1% (Elnahas *et al.*, 2005). Of 1828 HIV positive patients from Bobo-Dioulasso, Burkina Faso, 25.4% had positive *T. gondii* serology (Millogo *et al.*, 2000). Immigrants to the United Kingdom born in West Africa had a 100-fold higher incidence of symptomatic eye disease due to *T. gondii* compared with white people born in Britain (Gilbert *et al.*, 1999).

### Risk factors for infection with *Toxoplasma gondii*

There is no biological test which can distinguish infections from oocysts ingestion transmitted by felines, from tissue cysts ingestion from infected meat (Dubey 1996; Dubey 2000; Hill and Dubey 2002). Therefore, epidemiological surveys examining risk factors in infected and non-infected persons remain the most useful way of assessing the relative importance of different sources of *T. gondii* infection in humans. Soil contact through gardening allows contact with infective oocysts deposited by any recently infected cat. Oocysts take one to five days to become infective, but they can remain infective in soil and probably water for up to one year depending on ambient temperature and humidity.

A systematic sample of 1157 women of reproductive age, aged 15–49 years in Belgrade, Serbia, found a seroprevalence of 77% (Bobic *et al.*, 1998). Consumption of undercooked meat and exposure to soil (farming, gardening) were found to be associated with *T. gondii* infection, but cat ownership was not. A prospective case–control study from Norway 1992–94 found that eating raw or undercooked meat and meat products, poor kitchen hygiene; cleaning the cat litter box and eating unwashed raw vegetables or fruits were associated with increased risk of *T. gondii* infection (Kapperud *et al.*, 1996). From 1991 through to 1994 a prospective risk factor study in pregnant women infected during pregnancy and controls was performed in Italy. Eating cured pork or raw meat at least once a month increased the risk of *T. gondii* infection threefold (OR: 3.1; 95% CI: 1.6–6.0) (Buffolano *et al.*, 1996). The OR, odds ratio, is the risk of infection in a group sharing a common risk factor compared to a population without the risk factor. An OR of two indicate a doubling of the risk. The 95% confidence interval, show the limits within which the true OR is found with 95% probability. A case–control study from France found the following risk factors: poor hand hygiene (OR = 9.9; 95% CI 0.8–125), consumption of undercooked beef (OR = 5.5; 95% CI: 1.1–27), having a pet cat (OR = 4.5; 95% CI 1.0–19.9), frequent consumption of raw vegetables outside the home (OR = 3.1; 95% CI 1.2–7.7) and consumption of undercooked lamb (OR = 3.1; 95% CI 0.85–14) (Baril *et al.*, 1999).

A European multicentre case–control study in six centers in Belgium, Denmark, Italy, Norway, and Switzerland included 252 cases and 708 controls (Cook *et al.*, 2000). The study found that contact with raw or undercooked beef, lamb, or other sources of meat, as well as with soil, were independent risk factors for *T. gondii* seroconversion during pregnancy. In addition, travel outside of Europe, the United States, and Canada was a risk factor for seroconversion. The population attributable fraction showed that 30% to 63% of seroconversions were due to consumption of undercooked or cured meat products and 6% to 17% were a result of soil contact, but ownership of a cat was not a risk factor (Cook *et al.*, 2000). Information about how to avoid toxoplasmosis in pregnancy could be a cost effective approach to preventing congenital toxoplasmosis (Conyn-van Spaendonck and van Knappen, 1992; Lopez and Dietz, 2000). Based on the knowledge of these identified risk factors for primary toxoplasmosis, pregnant women should be appropriately advised by their obstetricians and primary care providers on how to lower the risk of congenital toxoplasmosis by avoiding risk factor exposure. Recommendations to prevent congenital *Toxoplasma* infection in pregnant women are shown in Table 3.2.

*Toxoplasma gondii* genotypes

*Toxoplasma gondii* can be divided into three main genotypes (Sibley and Boothroyd 1992; Grigg *et al.*, 2001; Khan *et al.*, 2005; see Chapters 11 and 13). It has been proposed that the different genotypes may be partly responsible for the different pathogenicity observed in the infection. In mice, one *T. gondii* parasite of type I is lethal to outbred mice whereas the lethal dose of types II and III is about a three to four orders of magnitude greater (Boothroyd and Grigg 2002; see Chapter 9). One study has reported an unusual abundance of type I and recombinant strains in patients with retinochoroiditis (Grigg *et al.*, 2001) and a recent study from Brazil of ocular disease caused by *T. gondii*, analysis of lesions found only type I and III strains and no type II strains (Vallochi *et al.*, 2005). Recent work, however, suggest a more complicated picture in Brazil with both pathogenic and apathogenic isolates overall belonging type I (Ferreita *et al.*, 2006; Khan *et al.*, 2006). A study of 86 pregnant women from France found predominantly type II (Ajzenberg *et al.*, 2002), which confirm previous studies which has also primarily found type II (Howe *et al.*, 1997).Recently, methods have been developed which allow *T. gondii* in patients to be at least partially typed using genotype specific markers (Kong *et al.*, 2003), and this will be a valuable tool for assessing the geographical distribution of genotypes as well as the

**Table 3.2** Advice for how to avoid infection with *T. gondii*

Washing hands before handling food
Cooking meat to a safe temperature (i.e. one sufficient to kill <i>Toxoplasma</i> )
Cleaning cooking surfaces and utensils after they have contacted raw meat, poultry, seafood, or unwashed fruits or vegetables
Peeling or thoroughly washing fruits and vegetables before eating
Avoiding cat feces in soil or changing cat litter or, if no one else is available to change the cat litter, using gloves, then washing hands thoroughly
Wearing gloves and thoroughly washing hands after gardening or handling soil
Avoid untreated surface water for drinking

importance of genotype for pathogenicity. The phylogentic development over time of the different genotypes suggest that the “atypical” or “exotic” genotypes may be the ancestral types and types I, II and III more recent (Su *et al.*, 2003).

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### Diagnosis of *Toxoplasma gondii* infection in pregnant women

In countries where pre-natal screening programs are in place a test of the first blood sample from the pregnant women for *Toxoplasma*-specific IgM- and IgG-antibodies is performed. Approximately 5% of seropositive women in the first trimester have *Toxoplasma*-specific IgM-antibodies (Gilbert *et al.*, 2007), but only approximately 4% of these give birth to a child with congenital *Toxoplasma* infection. It is therefore a considerable problem to diagnose whether women with specific IgM antibodies are infected before or after conception. This is particularly a problem in countries where testing of pregnant women in the beginning of pregnancy are common. This problem has been partly solved by obtaining two samples from pregnant women to see if there is any development of the immune response. It is generally agreed that there is a development of the *Toxoplasma*-specific IgG-antibody response within the first 8 weeks after infection after which the IgG levels are maintained at a high level, with or without declining IgM antibodies (Jenum *et al.*, 1997; Jenum *et al.*, 1998).

#### Combined, two-test strategies

A study showed that the best strategy for diagnosing acute and recent infection with *T. gondii* was a two-test strategy with a sensitive IgM test first followed by an IgG-avidity test (Robert *et al.*, 2001). This confirmed the need of the *Toxoplasma*-specific IgG-avidity index assay in the diagnosis of acute and recent infection. However, the increased use of the *T. gondii* IgG-avidity test has highlighted an inherent problem with the test that many pregnant women have long-lasting low IgG-avidity antibodies (Petersen *et al.*, 2005) and the IgG-avidity assay needs further development which could be by the use of recombinant antigens (Beghetto *et al.*, 2003).

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### EIA tests for *Toxoplasma*-specific IgG and IgM antibodies

The problems with IgM-based diagnostics in *T. gondii* infections have resulted in attempts to improve the tests. The accepted reference test is the ISAGA (Immuno-Sorbent-Agglutination-Assay), but most analyses are performed with an Enzyme-Immuno-Assay, EIA  $\mu$ -capture test. The assays use whole-cell, lysed *T. gondii* as antigen, and attempts have been made to improve the test by using recombinant antigens (Ferrandiz *et al.*, 2004). The ISAGA IgM and EIA IgM and IgM immunofluorescence were evaluated in a prospective, European cohort study, EMSCOT, European Multicentre Study on Congenital Toxoplasmosis, of women diagnosed with primary *T. gondii* infection during pregnancy and newborns identified through neonatal screening (Gilbert *et al.*, 2007).

The EMSCOT study provided data on sensitivities for diagnosing congenital infection in the newborn of four *Toxoplasma*-specific IgM-antibody assays and three *Toxoplasma*-specific IgA antibody assays. The study also provided data on the sensitivity of neonatal testing related to the estimated gestational age of infection with *T. gondii*, showing that the IgM seropositivity at birth only detects infections in the second half of pregnancy (Gilbert

*et al.*, 2007). The study included 5223 samples from 996 children of which 3742 were tested with an EIA system, 2011 with an ISAGA IgM and 316 with the IgM immunofluorescence assay (Gilbert *et al.*, 2007). The children were followed for one year to ascertain the diagnosis by demonstrating the presence of *Toxoplasma*-specific IgG antibodies at 12 months of age, which is the gold standard for confirming congenital infection with *T. gondii*. With this as the reference the sensitivity and specificity of the different blood tests taken at birth is shown in Table 3.3 below. The sensitivity for EIA tests is low, 29.3%, clearly demonstrating the need for better tests. Table 3.4 shows the same data according to the gestational age of infection. This was the first study to provide data on the sensitivity of *Toxoplasma*-specific IgM antibodies related to gestational age at infection. It is clearly seen that a sensitivity of 50% is not reached until after the 30th gestational week.

Recombinant IgG-assays, adults

Conventional assays have so far used whole-cell, lysed, *T. gondii* antigens, which have batch variations. With increasing emphasis on need for reproducibility the use of recombinant antigens in diagnostics assays provide a theoretical advantage. Previous studies have shown that the GRA1, GRA7 and SAG1 molecules are immunodominant (Aubert *et al.*, 2000; Harning *et al.*, 1996; Jacobs *et al.*, 1999; Johnson *et al.*, 1992; Li *et al.*, 2000). Our study showed that recombinant antigens including a mixture of GRA1, GRA7 and SAG1 were not as sensitive as whole-cell, lysed, antigen if sera had an IgG titer of less than 1:1600 using an EIA test and less than 1:512 in an IgG-immunofluorescence test (Pietkiewicz *et al.*, 2004). The test did, however, have 100% sensitivity in a panel of sera from individuals who had *Toxoplasma*-specific IgM and/or IgA antibodies, indicating that the infection was recent (Pietkiewicz *et al.*, 2004). Future assays for *Toxoplasma*-specific IgG antibodies

**Table 3.3** Sensitivity and specificity of IgM tests at birth (Gilbert *et al.*, 2007)

	Sensitivity (95% CI)	Specificity (95% CI)
ISAGA IgM	0.536 (0.45, 0.62)	0.964 (0.95, 0.98)
ELISA IgM	0.293 (0.17, 0.46)	0.963 (0.93, 0.98)
IFAT IgM	0.100 (0.01, 0.46)	1.000 (0.92, 1.0)

**Table 3.4** Sensitivity and specificity of IgM tests according to gestational age at infection (Gilbert *et al.*, 2007)

IgM	Sensitivity	Specificity	Post test probability for CT given a pos. test	Post test probability for CT given a neg. test
Trimester				
1: 0–14 weeks + 6 days	0.36	0.96	0.23	0.02
2 :15w to 27w+ 6 days	0.33	0.97	0.81	0.20
3 28+IgM	0.71	0.89	0.90	0.32

CT= congenital toxoplasmosis.



relying on recombinant antigens need to include a panel of antigens and the test with recombinant antigens has not yet been optimized to the same sensitivity as the whole-cell, lysed antigen assay.

### Recombinant IgM and IgG assays, newborns

Diagnostic assays based on recombinant antigens for measuring the *Toxoplasma*-specific IgM-antibodies were evaluated in infants with or without congenital toxoplasmosis born to mothers with toxoplasmosis acquired during pregnancy (Buffolano *et al.*, 2005). Antigen fragments from the MIC2, MIC3, M2AP, and SAG1 protein were tested in an EIA test on 104 serum samples from newborns born to mothers infected with *T. gondii* during pregnancy (Buffolano *et al.*, 2005). The presence of *T. gondii*-specific, IgM antibodies against recombinant MIC2, MIC3, M2AP, and SAG1 antigens may be used for the early postnatal diagnosis of congenital toxoplasmosis. It was also found that the newborn, *Toxoplasma*-infected child primarily produces IgG<sub>2</sub> and IgG<sub>3</sub> against recombinant *Toxoplasma*-antigens, whereas the maternally transferred antibodies primarily were IgG<sub>1</sub> (Buffolano *et al.*, 2005). Thus subclass analysis of serum samples from mother and child against defined recombinant antigens may further improve diagnosis of congenital *Toxoplasma*-infection in newborns.

### The *Toxoplasma*-specific IgG avidity index

The maturation of the IgG-response varies considerably between individuals. In the study of Lappalainen *et al.* (1993) two seroconverting mothers already had an IgG-avidity index above 20% at the time of diagnosis, but most patients had developed an IgG-avidity index above 15% after 180 days (Lappalainen *et al.*, 1993). A study from France found an average IgG-avidity index of 0.2 in pregnant women infected within 5 months (Leolier and Pucheu 1993).

The original method developed by Hedman *et al.* (1989) used serial dilutions tested in EIA with and without 6M urea, but automated assays today calculate the IgG-avidity index from two single measurements of the sample with and without urea. This introduces an uncertainty, although experiments with only two serum sample dilutions showed an excellent agreement with IgG-avidity measurements using four serial serum sample dilutions (Korhonen *et al.*, 1999). Prince and Wilson (2001) evaluated the IgG-avidity assay and found that using single dilution assays with and without urea and showed that because the signal obtained in an EIA system is not linear, it makes a difference whether the *Toxoplasma* IgG-avidity index is calculated from the OD values or the activity measured in International Units of *Toxoplasma*-specific IgG antibodies per ml (Prince and Wilson, 2001). Up to half of the patients with acute infections may show a low or borderline IgG-avidity index 6 months after the infection (Montoya *et al.*, 2002; Rossi 1998) which is in concordance with the results reported in our study (Petersen *et al.*, 2005). The cut-off value defining a low IgG-avidity index differs markedly between different studies and one study found that patients infected within the past 3 months had IgG-avidity index below 0.45 (Holliman *et al.*, 1994). A comparison between the VIDAS and the Labsystems IgG-avidity index showed a correlation coefficient of 0.6 in pregnant women but 0.88 in other patients (Alvarado-Esquivel *et al.*, 2002), but the difference was not further discussed.



The IgG response matures rapidly in some individuals (Pelloux *et al.*, 1998), who showed that at least in pregnant women, a low IgG-avidity index persisted up to 9 months post-infection and all women were treated. In a study of *T. gondii*-infected pregnant women identified through prenatal screening, Jenum *et al.* (1997) found that only 2 out of 73 women had an IgG-avidity index above 0.2 before 20 weeks of gestation, but many continued to have a low IgG-avidity index even a year after infection. It is assumed that all women were treated during pregnancy. This long lasting low IgG-avidity appears to be common in pregnant women (Petersen *et al.*, 2005). The comparison of IgG-avidity maturation in treated, pregnant women with samples from patients with acute infection with *T. gondii* who were not pregnant and were not treated, revealed a significantly more rapid IgG maturation during the first four months after infection in subjects who were not treated and not pregnant (Petersen *et al.*, 2005). This finding indicates that treatment may influence the IgG-avidity maturation and underlines the need for further studies to better clarify the avidity maturation process in pregnant women under therapy in comparison with the untreated individuals. If confirmed, different cut-off values will have to be defined for treated and untreated and/or pregnant and non-pregnant individuals.

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### Diagnosis of *Toxoplasma gondii* infection in live-born neonates

Diagnosis of congenital infection with *T. gondii* is difficult at birth if *Toxoplasma*-specific IgM- and/or IgA-antibodies are not present, because current diagnostic methods can only distinguish between maternal and fetal IgG with difficulty. The traditional method of diagnosing congenital toxoplasmosis in IgM and IgA negative newborns is to wait up to 12 months and observe if the maternal *Toxoplasma*-specific IgG antibodies disappear. If the child has been treated continuously with sulfadiazine and pyrimethamine the synthesis of *Toxoplasma*-specific IgG-antibodies can be suppressed and the serological confirmation of the infection can sometimes not be made with certainty before the second year of life (Wallon *et al.*, 2001). IgM and IgA antibodies do not cross the placenta, and neonatal screening programs for congenital toxoplasmosis are based on detection of *Toxoplasma*-specific IgM-antibodies eluted from blood spots from PKU-filter papers (Guthrie cards) (Guerina *et al.*, 1994; Lebech *et al.*, 1999; Neto *et al.*, 2004; Sorensen *et al.*, 2002). Different cut-offs for maternal and newborn *Toxoplasma*-specific IgM antibodies has been proposed (Candolfi *et al.*, 1993). It has been hypothesized that treatment of acute toxoplasmosis during pregnancy reduced the duration of the *Toxoplasma*-specific IgM response, but two studies did not find such an effect (Gras *et al.*, 2004; Gilbert *et al.*, 2007).

Transferred maternal and neo-synthesized *T. gondii*-specific IgG-antibodies can be differentiated by immunoblot or immunocomplexing (Chumpitazi *et al.*, 1995; Gross *et al.*, 2000; Remington *et al.*, 2004; Robert-Gagneaux *et al.*, 1999; Pinon *et al.*, 1996; Pinon *et al.*, 2001; Robert-Gagneux *et al.*, 1999). The Western blot technique and immunocomplexing were compared in a double-blind study and found to be equally sensitive (Pinon *et al.*, 2001). The Immunoblot technique identifies newborns with congenital toxoplasmosis with a sensitivity of approximately 70% (Rilling *et al.*, 2003; Tissot Dupont *et al.*, 2003) increasing to 85% within the first 3 months of life (Gross *et al.*, 2000; Rilling *et al.*, 2003; Tissot Dupont *et al.*, 2003). A two-dimensional immunoblot, 2DIB, assay improve sensi-

tivity of distinguishing between maternal and neonate *Toxoplasma*-specific IgG (Nielsen *et al.*, 2005).

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### Diagnosis of *Toxoplasma gondii* in the fetus

This review will not include ultrasound examinations of the fetus. Cordocentesis has been abandoned because a high risk of fetal loss, and fetal diagnosis today is focused on detection of *T. gondii* in amniotic fluid. There is no barrier between the amniotic fluid and the fetus, and detection of *T. gondii* DNA in amniotic fluid is therefore regarded as proof that the fetus is infected. The first study of PCR for *T. gondii* nucleic acid in amniotic fluid found a high sensitivity and specificity (Hohlfeld *et al.*, 1994), and the methods soon replaced the previously used mouse inoculation method. It soon became clear that the results varied between different centers (Guy *et al.*, 1996; Pelloux *et al.*, 1998) so there is a huge need to for standardization and quality control. Later studies (Romand *et al.*, 2001, 2004; Thalib *et al.*, 2005) could not confirm the results of the first study, and it is now generally agreed that the diagnostic sensitivity in the best centers is around 70% and the diagnostic specificity around 90%.

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### Immunocompromised patients

*T. gondii* infections in cardiac transplants can be managed by careful prevention with treatment of the recipient. Monitoring bone marrow transplant patients by PCR on peripheral blood, BAL fluid and CSF (according to local symptoms), treatment with pyrimethamine has reduced mortality to the same levels as for *T. gondii* negative BMT patients. The same strategy can be applied to other immunosuppressed patients in risk of developing *T. gondii* infection including HIV patients with low CD4<sup>+</sup> T cell count and also pulmonary toxoplasmosis is well known in HIV-infected patients with low CD4<sup>+</sup> T cell counts (Rabaud *et al.*, 1996). An early study showed that trimethoprim/sulfamethoxazole prevented *T. gondii* infection in most but not all immunosuppressed patients (Norrby *et al.*, 1975), results which are supported by our data showing that *T. gondii* infection in individuals despite trimethoprim/sulfamethoxazole treatment. Thus, *T. gondii* infection should be suspected in immunosuppressed patients with unspecific symptoms of infection irrespective of trimethoprim/sulfamethoxazole prophylaxis.

The most important factor in the management of the immunosuppressed patient is to consider *T. gondii* as a potential causative agent in patients presenting with unspecific symptoms including focal symptoms from CNS, heart, lungs and liver. Here, diagnosis by serology has been replaced by PCR analysis of *T. gondii*-specific nucleic acid.

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### Chemotherapy

#### Development of drugs against *Toxoplasma gondii*

The effect of sulfonamides were shown in 1942 by Sabin and Warren and confirmed in later studies (Eyles 1953). Pyrimethamine was found effective against *T. gondii* (Eyles and Coleman 1952) and the synergy between sulfadiazine and pyrimethamine demonstrated soon after (Eyles and Coleman 1953). Spiramycine was shown to be effective against *T. gondii* in 1958 (Beverly 1958; Garin and Eyles 1958). These three drugs are still the main

treatment for *T. gondii* infections in pregnancy, infants with congenital toxoplasmosis and ocular toxoplasmosis.

In 1976, it was shown that sulfadoxine combined with pyrimethamine was effective against *T. gondii* (Garin *et al.*, 1976) and some centers advocate postnatal treatment with sulfadoxine/pyrimethamine for up to two years in infants with congenital toxoplasmosis (Villena *et al.*, 1998). However, there are no prospective, placebo controlled studies of treatment of *T. gondii* infections in pregnant women or newborns and only a few studies of treatment of adults with retinochoroiditis. A recent follow-up study of 120 children with severe, congenital *T. gondii* infection found an improved outcome in children receiving one year treatment with sulfadiazine and pyrimethamine compared with historical control (McLeod *et al.*, 2006).

### Treatment of *Toxoplasma gondii* eye disease

Perkins *et al.* (1956) included 164 cases of acute uveitis randomized to treatment for four weeks with pyrimethamine or placebo and found a significant improvement of the uveitis lesions. A randomized open-labeled clinical trial comparing the recurrence of retinochoroiditis in 61 patients treated by sulfamethoxazole and trimetoprim (co-trimoxazole) every three days for up to twenty months (duration of the study) and 63 patients without treatment, found a significantly lower rate of recurrence in the treatment group ( $p = 0.054$ ; 6/61 vs. 15/63) (Silveira *et al.*, 2002). In a prospective multicenter study of 149 consecutive patients with active toxoplasmic retinochoroiditis who were randomly assigned to a treatment with pyrimethamine and sulfadiazine, clindamycin plus sulfadiazine or co-trimoxazole found no difference in resolving of the eye lesion or recurrence over two years follow up between the treated groups and the untreated group (Rothova *et al.*, 1993). A Double blind clinical trial included 94 children with congenital toxoplasmosis receiving either pyrimethamine and sulfafadiazine in different doses compared to a group of untreated children (Mets *et al.*, 1996). Thirteen percent (7/54) of treated patients have developed active retinal disease or new lesions during 189 patients-years of follow-up compared to 8/18 (44%) of untreated patients developed new lesions during 160 patients-years of follow-up (Mets *et al.*, 1996). A descriptive study of the effect of an additional course of pyrimethamine and sulfadiazine compared to historical controls did not report a reduced rate of recurrence after additional treatment (Wallon *et al.*, 2001). A study comparing pyrimethamine and sulfadiazine with pyrimethamine and azithromycine in adult patients with choroiretinitis found no difference in the clinical outcome (Bosch-Driessen *et al.*, 2002).

### Treatment of *Toxoplasma gondii* in pregnant women and newborns with congenital toxoplasmosis

Table 3.5 show prenatal treatment regimens in Europe and Table 3.6 summarize different postnatal treatment protocols currently used in different European centers. Postnatal treatment of congenital toxoplasmosis has recently been reviewed (Petersen and Schmidt 2003). There are no randomized, controlled trials of treatment effect of either pre- or postnatal treatment.

### Immunosuppressed patients

In patients receiving a heart transplant, it has been shown that prophylaxis with pyrimethamine has been very effective in preventing clinical toxoplasmosis (Wulff *et al.*, 2005) and trimethoprim/sulfamethoxazole prophylaxis for *P. jirovecii* was equally effective (Orr *et al.*, 1994). An early study found *T. gondii* in 4 of 7 recipients with mismatched *T. gondii* (either donor or recipient being infected with *T. gondii* the other not) status who did not receive pyrimethamine compared to 1 of 37 receiving pyrimethamine (Wreghitt *et al.*, 1992). Trimethoprim-sulfamethoxazole prophylaxis for *P. jirovecii* also reduced the risk of clinical *T. gondii* infection in HIV patients (Luft and Remington 1992; Gallant *et al.*, 1994; Richards *et al.*, 1995). A recent review of *T. gondii* infection in 2193 autologous transplants and 381 allogenic transplants found an overall rate 1.9 per 1000 patients but 0.9 per 1000 receiving autologous transplant, and 8.0 per 1000 receiving allogenic transplant (Pagano *et al.*, 2004). Bone marrow transplant patients should be regularly monitored to *T. gondii* infections for instance with monthly PCR to detect circulating parasites so early treatment can be started (Martino *et al.*, 2005).

### New drugs

The most promising new drug for the treatment of *T. gondii* is atovaquone, and studies in mice suggest that it may be partially effective against the tissue cyst (Huskinson-Mark *et al.*, 1991) and azithromycin has also been found to have a partial effect on *T. gondii* tissue cysts (Derouin *et al.*, 1992).

Artemisinins have been tested in mice models and one study found it reduced brain cyst load (Sarciron 2000). A study in the hamster model of *T. gondii* eye infections found no effect of atovaquone on the eye lesions but a 90% reduction in brain cyst numbers (Gormley *et al.*, 1998). Other studies of atovaquone have also reported significant increased survival and reduction in brain cyst burden (Araujo *et al.*, 1998; Djurkovic-Djakovic 2002; Alves 2005). Atovaquone resistance has been generated in the laboratory by inducing mutations in the *T. gondii* cytochrome b gene (McFadden *et al.*, 2000), but no wild type *T. gondii* isolate resistant to atovaquone has been found (McFadden *et al.*, 2001), and it is uncertain whether prolonged treatment of *T. gondii* cysts carriers pose a risk of inducing resistance.

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## Summary and future directions

The risk of infection with *T. gondii* has been decreasing in Europe over the past 50 years, and at the same time population based studies show that the prevalence and severity of congenital toxoplasmosis in Europe is less than compared to fifty years ago. Nevertheless, the finding that the burden of acquired *T. gondii* infection is higher than previously thought show that more data on this aspect of *T. gondii* in the population is needed. Recent European risk factor studies show that up to two thirds of infections come through food, and provision of *T. gondii* free food could thereby prevent some infections.

The present screening programs in Europe were started based on data from the 1950s and 60s to identify infected pregnant women or newborn, but new studies show that treatment does not prevent transmission and has only a modest effect on symptoms in the newborn. The long-term effect of treatment on the prevention of late-onset eye disease need to be confirmed and alternatives to the present pre- and neonatal screening

**Table 3.5** Prenatal treatment regimens in Europe (European Multicenter Study on Congenital Toxoplasmosis, 2003). PN = postnatal testing of seronegative women; RT = rising IgG titers; Avidity = log IgG avidity; HT = high IgG titre. All enrollments were consistent with the definite and probable criteria reported by Lebech *et al.* (1996).

Centre	Method for detecting maternal infection	Tests of recent infection IgM and IgG positive plus tests shown	Prenatal treatment		After diagnosis of fetal infection
	Seroconversion: recommended testing		After diagnosis of maternal infection for infection acquired in:		
	Schedule for IgG negative women		First and second trimesters	Third trimester	
France					
Lyon	Monthly + postnatal <sup>a</sup>	Avidity <35%, RT	Spiramycin <sup>b</sup>	P-S <sup>c,d</sup>	P-S <sup>c,d</sup>
Paris	Monthly + postnatal <sup>a</sup>	None enrolled <sup>e</sup>	Spiramycin <sup>b</sup>	Spiramycin <sup>b</sup>	P-S <sup>c,d</sup>
Marseille	Monthly + postnatal <sup>a</sup>	RT	Spiramycin <sup>b</sup>	Fansidar <sup>e,f</sup>	Fansidar <sup>e,f</sup>
Grenoble	Monthly	RT	Spiramycin <sup>b</sup>	Fansidar <sup>e,f</sup>	Fansidar <sup>e,f</sup>
Nice	Monthly + postnatal <sup>a</sup>	RT	Spiramycin <sup>b</sup>	P-S <sup>c,d</sup>	P-S <sup>c,d</sup>
Toulouse	Monthly + postnatal <sup>a</sup>	RT, IgA	Spiramycin <sup>b</sup>	Spiramycin <sup>b</sup>	Fansidar <sup>e,f</sup>
Reims	Monthly + postnatal <sup>a</sup>	RT, IgA, IgE	Spiramycin <sup>b</sup>	Spiramycin <sup>b</sup>	Fansidar <sup>e,f</sup>

Austria					
Austria	10, 20, 32 weeks	RT, HT, low avidity	P-S <sup>c,g</sup>	P-S <sup>c,g</sup>	P-S <sup>c,g</sup>
Italy					
Nales	12, 20, 36 weeks	IgA, RT	Spiramycin <sup>b</sup>	Spiramycin <sup>b</sup>	P-S <sup>c,d</sup>
Milan	Monthly	IgA, RT	Spiramycin <sup>b</sup>	Spiramycin <sup>b</sup>	P-S <sup>c,d</sup>
Sweden					
Stockholm	10 weeks + postnatal <sup>h</sup>	avidity < 15%	Nil	Nil	Nil

<sup>a</sup> Routine postnatal testing of cord blood and/or postnatal sample in seronegative women.

<sup>b</sup> Daily dose in all centres = 3 g/day until delivery or until regimen changed after prenatal diagnosis.

<sup>c</sup> Pyrimethamine-sulphonamide combination with folic acid alternates with spiramycin in three or four weekly cycles until delivery except in Paris, Reims, Marseille, Toulouse and Grenoble where continuous pyrimethamine-sulphonamide is given.

<sup>d</sup> Pyrimethamine-sulphadiazine: Daily dose in Lyon, Paris and Nice = 50 mg/day pyrimethamine, 3 g/day sulphadiazine; in Naples = 25 mg/day pyrimethamine, 2 g/day sulphadiazine; in Milan 50 mg/day pyrimethamine, 2 g/day sulphadiazine.

<sup>e</sup> Enrollment was restricted to women who seroconverted.

<sup>f</sup> Fansidar. Dose in Marseille, Toulouse and Grenoble = 50 mg/week pyrimethamine, 1 g/week sulphadoxine; Reims = 75 mg/2 weeks pyrimethamine and 1.5 g/2 weeks sulphadoxine.

<sup>g</sup> Daily dose in Austria = 25 mg/day pyrimethamine (50 mg for the first dose), 0.75 g/day sulphadiazine (1.5 g for first dose), prescribed from 16 weeks of gestation onwards and continued until delivery. Spiramycin given before 16 weeks gestation.

<sup>h</sup> Neonatal screening for specific IgG antibodies in Guthrie card filter paper bloodspots followed by retrospective testing of stored prenatal samples.

**Table 3.6** Different protocols for postnatal treatment of congenital toxoplasmosis in European centers (Gras *et al.*, 2005)

	Prenatal treatment		Postnatal treatment
	Trimester		
	1 <sup>st</sup> and 2 <sup>nd</sup>	3 <sup>d</sup>	
France			
Lyon	Spira	P-S	P-S for 3w <sup>1</sup> , Spira till >5kg <sup>2</sup> , Fansidar for 12m <sup>3</sup>
Paris	Spira	Spira	P-S for 12m <sup>4,5</sup>
Marseille	Spira	Fansidar	No manifestations: Fansidar for 12 m <sup>3</sup> Manifestations: Fansidar for 24 m <sup>3</sup>
Grenoble	Spira	Fansidar	No manifestations: Fansidar for 12 m <sup>3</sup> Manifestations: Fansidar for 24 m <sup>3</sup>
Nice	Spira	P-S	P-S for 3w <sup>4</sup> , Fansidar for 24m <sup>3</sup>
Toulouse	Spira	Spira	Fansidar/spira for 12m <sup>3</sup>
Reims	Spira	Spira	Fansidar for 24m <sup>6</sup>
Austria			
Austria	P-S <sup>10</sup>	P-S	No manifestations: P-S/spira for 12 m <sup>4</sup> Manifestations: P-S for 6m, P-S/spira for 6m <sup>4</sup>



Italy					
Naples	Spira	Spira	P-S	No manifestations: P-S/spira for 12 <sup>4,7,8</sup> Manifestations: P-S for 6m, P-S/spira for 6m <sup>4,7,8</sup> P-S for 12m Spiramycin <sup>4</sup>	
Milan	Spira	Spira	P-S		
Sweden					
Stockholm	Nil	Nil	Nil	No manifestations-S/spira for 12 m <sup>4,7,8</sup> Manifestations: P-S for 6m, P-S/spira for 6m <sup>4,7,8</sup>	
Poland					
vPoznan	Nil	Nil	Nil	No manifestations-S/spira for 12 m <sup>2,4</sup> Manifestations: P-S for 6m, P-S/spira for 6m <sup>2,4</sup>	
Denmark					
Copenhagen	Nil	Nil	Nil	P-S for 3m <sup>4,8,9</sup>	

P-S = pyrimethamine-sulphonamide; Spira=spiramycin; P-S/spiramycin indicates 4–6 cycles alternating with Spiramycin; m = months; all prenatal treatment was continued until delivery. Folic acid was prescribed to all infants receiving P-S or Fansidar.

<sup>1</sup>Pyrimethamine (3 mg/kg every 3 days), sulfadiazine (75 mg/kg/day).

<sup>2</sup>Spiramycin (125 mg/kg/day).

<sup>3</sup>Fansidar consists of pyrimethamine (1.25 mg/kg every 10 days) and sulphadoxine (25 mg/kg every 10 days).

<sup>4</sup>Pyrimethamine (1 mg/kg/day), sulfadiazine (75–100 mg/kg/day).

<sup>5</sup>Pyrimethamine dose reduced to (1 mg/kg/3 days) after 3 weeks treatment.

<sup>6</sup>Fansidar consists of pyrimethamine (1.25 mg/kg every 15 days) and sulphadoxine (25 mg/kg every 15 days).

<sup>7</sup>Spiramycin (100 mg/kg/day).

<sup>8</sup>Pyrimethamine 2 mg/kg/day for 1–3 days then reduce to 1 mg/kg/day.

<sup>9</sup>Prednisolone given if intracranial manifestations or active retinochoroiditis.

<sup>10</sup>P-S given after 15 weeks of gestation, otherwise spiramycin used.

are badly needed. Infection with *T. gondii* is more prevalent in South America and whether infections with oocysts through contaminated drinking water results in a more virulent clinical picture, needs further study. Data on the distribution of *T. gondii* types indicate a difference between Europe, South and North America and the importance of this should shed light on emerging virulence patterns.

The *T. gondii* IgG-avidity assay is now widely used for diagnosis of recent infection in pregnant women. A high proportion of pregnant women show persistent, low-avidity IgG antibodies to *T. gondii*, and it needs to be studied whether treatment with anti-*T. gondii* antibiotics results in a sustained low-avidity IgG response. The IgG-avidity assay also urgently need to be standardized between different manufacturers.

The drugs used for treatment of *T. gondii* has not changed for fifty years and trials with new drugs like atovaquone for both treatment of clinical disease and prevention of later relapse should become a priority for the development of new chemotherapeutic agents.

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# Ocular Toxoplasmosis: Clinical Features, Pathology, Pathogenesis, Animal Models, and Immune Responses

4

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## Abstract

Ocular toxoplasmosis occurs during the acute acquired infection, during active congenital infection *in utero* and the newborn period and as a recrudescent sequela of both types of infection. It also occurs in immune compromised persons usually as reactivation disease. Destruction of the eye is secondary to parasite replication and the immune response to parasite growth may augment this destruction. Clinical features, pathogenesis, immunology and protective and pathogenic mechanisms, pathology, animal models for study of the disease, and methods of treatment are summarized and discussed.

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## Introduction and history

In 1908, Nicolle and Manceaux found *Toxoplasma gondii* in tissues of *Ctenodactylus gundi*, a North African rodent (Nicolle and Manceaux, 1908), but it was not until 1923 when Janku, a Czech ophthalmologist, gave an early description of ocular manifestations of *T. gondii* infection (Janku, 1923). In the 1930s, toxoplasmosis was recognized as a serious disease in humans (Wolf *et al.*, 1936), and various serologic tests to diagnose *T. gondii* infection became available in the 1940s. Wilder, in a breakthrough study in 1952, found *T. gondii* parasites in enucleated eyes with severe intraocular inflammation (Wilder, 1952), and this changed the established view that tuberculosis was the primary cause of posterior uveitis (Guyton and Woods, 1941; Woods and Guyton, 1944).

Early studies by Perkins led him to conclude that recrudescent lesions occurring in the second and third decades of life were consequences of congenitally acquired *Toxoplasma* infection (Friedmann *et al.*, 1969; Perkins, 1973; See Chapter 5). However, studies in Brazil by Glasner and associates (Glasner *et al.*, 1992) and in other areas of the world by other investigators (Couvreur *et al.*, 1996; Montoya *et al.*, 1996; Burnett *et al.*, 1998; Gilbert *et al.*, 2000) demonstrated that postnatally acquired ocular toxoplasmosis (OT) might be more prevalent than previously believed. One of the largest reported series of acute acquired OT was described by Burnett and associates in the Greater Victoria area of British Columbia between 1994 and 1995 (Burnett *et al.*, 1998). Table 4.1a contains the characteristics of 20 patients with initial ocular findings, later identified as acquired *Toxoplasma* infection in the Victoria epidemic. Most of the patients had improvement in their active inflammatory ocular process when they received trimethoprim, sulfamethoxazole and prednisone orally. This particular epidemic was

**Table 4.1a** Patient clinical data and outcomes in Victoria epidemic

Case no.	Age (years)	Sex	Eye involved	Follow-up (weeks)	Initial acuity	Latest acuity	Lesion activity	Toxoplasmosis pathology	Other existing pathology	Treatment	Treatment duration (weeks)
1	30	M	R	121.3	20/30	20/25	Active			T/S + P	3
2	48	F	R	129.6	20/20	20/20	Active			T/S	3
3	26	M	R	47.0	20/60	20/20	Active			T/S + P	3
4a	79	F	R	129.7	10/100	10/400	Inactive		MH	T/S + P	3
4b	79	F	L	129.7	20/200	20/60	Active		Cat	T/S + P	3
5	44	M	L	133.0	20/50	20/25	Active			T/S + P + C	4
6	83	M	L	64.7	HM	20/40	Active			T/S + P	3
7	83	F	R	170.4	20/200	NLP	Active	Vit, phs		S/P + P	4
8	40	M	L	135.0	20/50	20/20	Active			T/S + P	4
9	77	F	R	132.6	20/70	20/50	Active	Vit		C + P + M	6
10	67	F	R	140.3	20/30	20/30	Inactive		ERM	N	

11	32	M	L	105.9	20/20	20/20	Inactive		N
12	49	M	R	86.1	20/20	20/50	Active	Cat	T/S + P 3
13	16	F	L	105.6	20/20	20/20	Inactive		N
14	15	M	L	158.3	20/30	20/40	Active	Mt	T/S + P 4
15	75	F	R	86.7	5/400	5/400	Inactive	Fib	N
16	45	F	R	121.4	20/400	20/40	Active		T/S + P 3
17	74	M	L	38.1	5/400	20/60	Active	Fib	T/S + P 3
18	56	M	L	152.4	20/50	20/200	Active	Vit, rec	T/S + P 4
19	83	F	R	137.1	HM	20/40	Active		T/S + P 3
20	66	F	L	79.9	20/50	20/20	Inactive		N

HM = hand motion; NLP = no light perception; vit = vitritis; phs = phthisis; Mt = macular traction; Fib = macular fibrosis; rec = recurrent lesion in macula; cat = cataract; ERM = epiretinal membrane; MH = macular hole; N = none; T/S = trimethoprim/sulfamethoxazole; P = prednisone; C = clindamycin; M = minocycline; S/P = sulfadiazine + pyrimethamine (with leucovorin).

Adapted with permission from Burnett A.J., Shortt S.G., Isaac-Renton J., King A., Werker D. (1998). Multiple cases of acquired toxoplasmosis retinitis presenting in an outbreak. *Ophthalmology* 105(6), 1032–1037.

**Table 4.1b** Clonal types assigned after analysis at 5 independent loci of amplified *T. gondii* DNA from patients with ocular disease

Clonal type, patient	Immunosuppression before ocular toxoplasmosis	Clinical finding	Allele at indicated locus				
			SAG1	SAG2	SAG3	SAG4	B1
I; RVG 06	No	Chronic, dense vitritis	I	I	I	I or II	ND
I; RVG 08	No	Chronic retinitis	I	I	I	I or II	I
I; JSR 14	Yes	HIV positive, chorioretinitis	NA	NA	NA	I or II	I
II; 1039	Yes	Lymphoma	II or III	II	II	I or II	II or III
II; 2021	Yes	AIDS, bilateral ocular disease	II or III	II	II	I or II	II or III
II; V92	Yes	AIDS	II or III	II	NA	NA	ND
III; G23	Yes	Severe vitritis	II or III	III	III	III	II or III
IV; JSR 02	No	Chronic vitritis; elderly adult	I	III	III	III	I
IV; JSR 05	Yes	HIV, atypical chorioretinitis	I	III	III	III	I
IV; 179	No	Chronic and severe retinitis	I	III	III	III	I
IV; 2033	No	Chronic and severe retinitis	I	III	III	NA	ND
New; 2035	No	Atypical, persistent anti-toxoplasma IgM, elderly adult	I	I	III	I or II	II or III

Adapted with permission from Grigg M.E., Ganatra J., Boothroyd J.C., Margolis T.P. (2001). Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *J. Infect. Dis.* 184, 633–639.

**Table 4.1c** Ocular characteristics of 14 patients with postnatally acquired ocular toxoplasmosis

	Iris atrophy no. (%)	Cataract no. (%)	Persistent vitreous opacities no. (%)	Size of primary retinal lesion <3 ODD no. (%)	Persistent cystoid macular edema no. (%)	Retinal detachment no. (%)	Ischemic retinal areas no. (%)	Phthisis no. (%)	Final visual acuity <20/200 no. (%)
Recurrences (n = 8)	2 (25)	5 (63)	5 (63)	5 (63)	2 (25)	3 (38)	3 (38)	2 (25)	5 (63)
No recurrences (n = 6)	0 (0)	1 (17)	1 (17)	1 (17)	0 (0)	0 (0)	2 (33)	0 (0)	1 (17)
Total (n = 14)	2 (14)	6 (35)	6 (35)	6 (43)	2 (14)	3 (21)	5 (36)	2 (14)	6 (43)

ODD= optic disk diameters.

Adapted with permission from Bosch-Driessen E.H., Rothova, A. (1999). Recurrent Ocular Disease in postnatally acquired toxoplasmosis. Am. J. Ophthalmol. 128, 421–425.

**Table 4.1d** Effect of therapy on the duration of inflammatory activity

Duration of inflammatory activity	No. of patients (%)			
	Group 1	Group 2	Group 3	Group 4
Less than 3 wks	6 (17%)	4 (9%)	4 (15%)	7 (17%)
Between 3 and 6 wks	20 (57%)	23 (50%)	16 (59%)	19 (46%)
More than 6 wks	9 (26%)	19 (41%)	7 (26%)	15 (37%)
				Total
				21 (14%)
				78 (52%)
				50 (34%)

**Table 4.1e** Decrease in size of retinal lesion during therapy

Decrease in size of retinal lesion	No. of patients (%)			
	Group 1	Group 2	Group 3	Group 4
> 0.5 disk diameter in diameter	17 (49%)	13 (28%)	3 (11%)	8 (20%)
				Total
				41 (27.5%)

Group 1 = pyrimethamine, sulfadiazine, corticosteroid.

Group 2 = clindamycin, sulfadiazine, corticosteroid.

Group 3 = trimethoprim, sulfamethoxazole, corticosteroid.

Group 4 = no treatment.

Adapted with permission from Rothova A., Meenken C., Buitenhuis H.J., et al. (1993). Therapy for ocular toxoplasmosis. Am. J. Ophthalmol. 115, 517-523.



attributed to one reservoir contaminated by oocysts from infected cats (Bowie *et al.*, 1997) and similar outbreaks with oocyst infected water supplies have also been reported. Other outbreaks of toxoplasmosis involved ingestion of infected meat (Masur *et al.*, 1978; Choi *et al.*, 1997; Ross *et al.*, 2001).

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### Clonal types of parasites and ocular disease

Different clonal types of *T. gondii* may contribute to varying severity of ocular disease. Three distinct genotypes and atypical organisms have been identified (Howe *et al.*, 1995; Saeij *et al.*, 2006; see Chapters 11 and 13). Clonal type I parasites cause high mortality in mice, whereas clonal type II parasites form more cysts and are less virulent in mice (see Chapter 9). Clonal type III parasites are intermediate in virulence. Genetic analysis of parasites isolated from infected eyes from Southern Brazil has shown presence of type I and I/III recombinants or atypical organisms (Sibley *et al.*, 1992; Vallochi *et al.*, 2005). Recombinant parasites or atypical organisms may be unique in their virulence phenotype, patterns of dissemination, or immunologic responses (M. Grigg, International Toxoplasmosis Meeting, France 2005). An analysis of parasites isolated from a child in the Victoria epidemic has shown atypical parasites (Burnett *et al.*, 1998; Lehmann *et al.*, 2000, Personal communication from D. Sibley to R. McLeod, 2006; Boyer *et al.*, in press 2006). In a small cohort of immunocompetent humans with severe OT, clonal type I or recombinant genotypes were detected (Grigg *et al.*, 2001) (Table 4.2), rather than clonal type II, which are the most commonly isolated clonal types from infected humans in France (Darde, 1996; Sibley *et al.*, 1996). Early reports have shown that there is also a high predominance of type II parasites in patients with AIDS in the U.S. (Boothroyd *et al.*, 2002), France (Howe *et al.*, 1997; Honore *et al.*, 2000), and Spain (Fuentes *et al.*, 2001).

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### Clinical manifestations

Clinical manifestations of OT include complaints of floaters and decrease in visual acuity. Additional complaints of red eye, photophobia, and pain indicate involvement of the anterior segment. The active retinal lesion of acute acquired OT is unilateral and creamy in appearance (Figure 4.1, upper left). The initial retinitis may later involve all layers of the retina and choroid (Figure 4.1, upper right) (Neussenblatt *et al.*, 1994). Anterior uveitis, cataracts, glaucoma, and changes in the iris may occur secondary to posterior uveitis (Westfall *et al.*, 2005). In the immunocompetent patient, resolution of retinal inflammation is reported to take at least two months, depending on the size of the retinal lesion (Rothova *et al.*, 1993). Over time, the lesion may become whiter in appearance surrounded by areas of hyperpigmentation from migration of retinal pigment epithelial cells.

Visual prognosis depends on many factors, including location and size of lesion, duration of infection, and baseline visual status. Visual sequelae such as retinal detachment or visual impairment (visual acuity < 20/200) have been reported in patients with OT (Friedmann *et al.*, 1969; Bosch-Driessen *et al.*, 2000; Bosch-Driessen *et al.*, 2002; Atmaca *et al.*, 2004). Recurrences of OT more frequently occur during the period immediately following the first episode and then diminish over time. The cause of the recurrent episodes is thought to be due to release of parasites from latent cysts in the retinal tissue, though specific factors that precipitate recurrences remained to be studied. In a cohort of 14 patients with acute acquired OT, recurrence occurred in 57% of patients during second year of follow up (Bosch-Driessen

**Table 4.2a** Ophthalmologic manifestations of congenital toxoplasmosis in children in the US (Chicago) national collaborative treatment trial

Manifestation	Treatment group ( <i>n</i> = 76)	Historical group ( <i>n</i> = 18)	Total ( <i>n</i> = 94)
Strabismus	26 (34%)	5 (28%)	31(33%)
Microphthalmia	10(13%)	2 (11%)	12 (13%)
Phthisis	4 (5%)	0 (0%)	4 (4%)
Microcornea	15 (20%)	3 (17%)	18 (19%)
Cataract	7 (9%)	2 (11%)	9 (10%)
Vitritis (Active)	3 (4%)	2 (11%)	5 (5%)
Retinitis (Active)	6 (8%)	4 (22%)	10 (11%)
Chorioretinal scars	56 (74%)	18 (100%)	74 (79%)
Retinal detachment	7 (9%)	2 (11%)	9 (10%)
Optic atrophy	14 (18%)	5 (28%)	19 (20%)

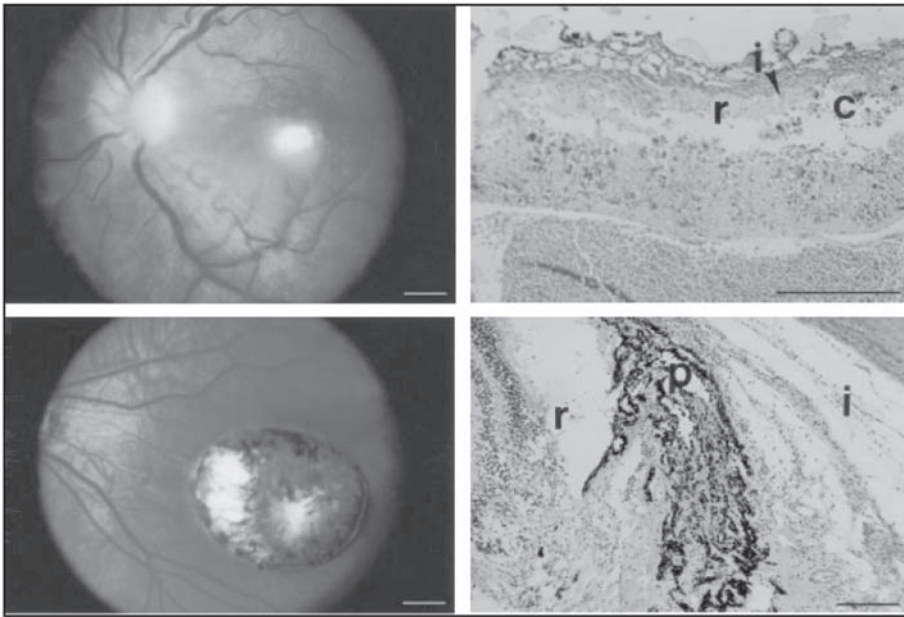
**Table 4.2b** Visual impact of congenital toxoplasmosis in children in the US (Chicago) national collaborative treatment trial

Eye status (most recent examination)	Treated patients ( <i>n</i> = 76)	Historical patients ( <i>n</i> = 18)	Total ( <i>n</i> = 94)
No disease	18 (24%)	0 (0%)	18 (19%)
In those with retinochoroidal lesions:			
Normal visual acuity	19 (25%)	4 (22%)	23 (24%)
Unilateral visual impairment	17 (22%)	9 (50%)	26 (28%)
Bilateral visual impairment	22 (29%)	5 (28%)	27 (29%)
New Lesion	7 (13%)	8 (44%)	15 (21%)

Adapted with permission from Mets M.B., Holfels E., Boyer K.M., Swisher C.N., Roizen N., *et al.* (1997). Eye manifestations of congenital toxoplasmosis. *Am. J. Ophthalmol.* 123(1), 1–16.

*et al.*, 1999). This study is remarkable in the many adverse consequences and frequent recurrences of ocular *T. gondii* infection (Table 4.1c). In another study comparing different treatment regimens (Table 4.1d and e), the mean recurrence rate during a three-year follow-up period between the treated and untreated cohort was 49% (Rothova *et al.*, 1993).

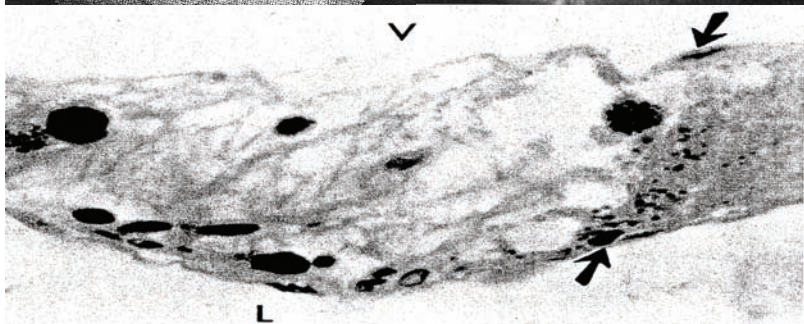
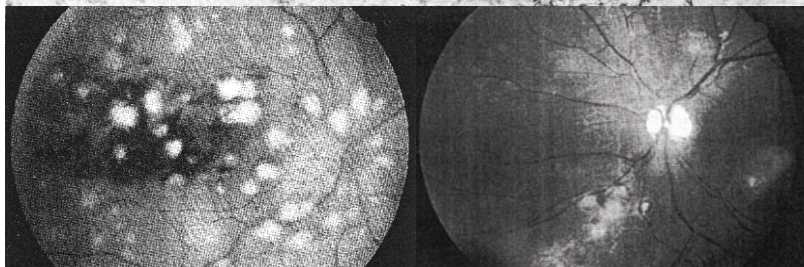
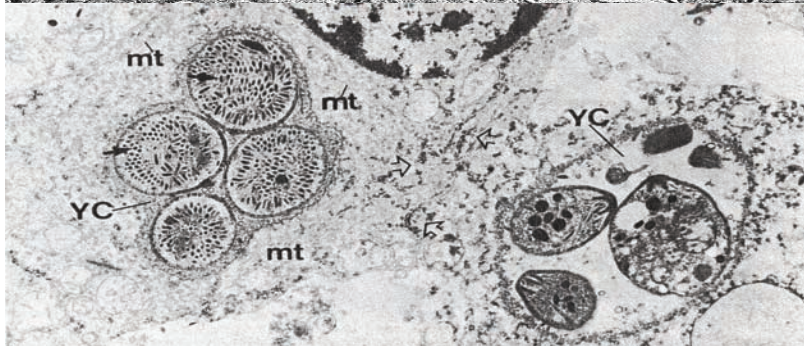
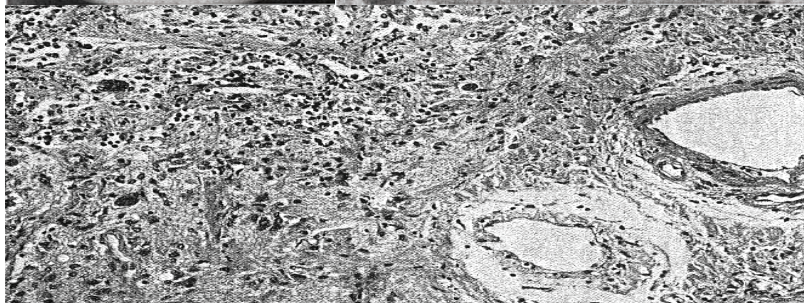
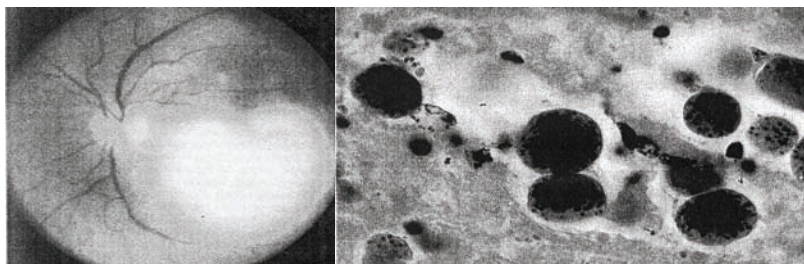
OT lesions in immunocompromised patients are more varied, usually perivascular in location (Holland, 1989), and may present as either unilateral or bilateral retinochoroiditis, multifocal lesions, or retinal necrosis (Holland *et al.*, 1988). Since cytomegalovirus (CMV) and *T. gondii* infection can both cause necrotizing retinopathy and similar clinical abnormalities, some distinguishing features of OT may include: smooth, non-granular lesion border, presence



**Figure 4.1** Toxoplasmic retinochoroiditis in immunocompetent patients. *Upper left.* Active toxoplasmic retinochoroiditis in a patient with acute acquired infection (provided by Dr. Jack S. Remington, Stanford University). Scale bar = 1.5  $\mu$ m. *Upper right.* Acute lesion in a five-day-old infant born prematurely. There is complete necrosis in all layers of the retina (r) with numerous inflammatory cells (i) and focal calcification (c). Scale bar = 100  $\mu$ m. *Lower left.* Quiescent toxoplasmic retinochoroidal scar in a congenitally infected patient. Scale bar = 1.5  $\mu$ m. *Lower right.* Retinochoroidal scar in a two-year-old child. The hyperpigmentation at the edge of a retinochoroidal scar seen with ophthalmoscopy as in *lower left* is the result of disruption and proliferation of the retinal pigment epithelium (p). The retina adjacent to this also shows disruption of the normal architecture (r). Scattered chronic inflammatory cells persist in the lesion (i). Scale bar = 100  $\mu$ m. (Adapted with permission from Roberts F. and McLeod R. (1999). Pathogenesis of Toxoplasmic Retinochoroiditis. *Parasitol. Today.* 15(2), 51–57.)

intraocular inflammation, and absence of retinal hemorrhage (Elkin *et al.*, 1994). In patients with AIDS, initial presentation of diffuse toxoplasmic retinochoroiditis may also mimic acute retinal necrosis syndrome (Parke *et al.*, 1986), herpetic-associated acute retinal necrosis, and syphilis (Gagliuso *et al.*, 1990). Other clinical manifestations of OT in patient with AIDS have included toxoplasmic papillitis (Falcone *et al.*, 1993), and bilateral military retinitis (Berger *et al.*, 1993) (Figure 4.2, lower left). In patients with toxoplasmic retinochoroiditis after cardiac transplantation, ocular symptoms usually appear within 3 to 6 months after transplantation (Conrath *et al.*, 2003). The source of infection may have been from reactivation secondary to immunosuppression or the transplanted heart. Similarly, in patients after bone marrow transplantation, reactivation of ocular disease usually occurs within 6 months after transplantation. In addition, recipients who already have antibody to *T. gondii* or previous OT lesions prior to transplantation have a higher risk for reactivation (Peacock *et al.*, 1995).





Animal studies suggest that tissue destruction in toxoplasmic retinochoroiditis is caused by proliferation of parasites, rather than from secondary hypersensitivity reaction to toxoplasmic antigens. Postmortem examination with light and electron microscopy (Figure 4.2, upper left, upper right, upper center, lower center) of the retinal tissue from a patient receiving prolonged chemotherapy showed presence of *T. gondii* in encysted bradyzoites within the necrotic retina (Yeo *et al.*, 1983). Histologic study of another patient receiving chronic systemic corticosteroid therapy demonstrated focal zones of retinal necrosis adjacent to vessels (Figure 4.2, bottom), with numerous *T. gondii* at the interface between necrotic and healthy retina (Nicholson *et al.*, 1976). In another study, development of ocular lesion ranged from 20 days to 1 year after initiation of corticosteroid therapy (Morhun *et al.*, 1996) (Figure 4.2, lower right).

*T. gondii* is transmitted congenitally from mothers who have primary *Toxoplasma* infection during pregnancy (Remington *et al.*, 1976), or those who are chronically infected and immunocompromised. The rate of transplacental transmission of *T. gondii* parasites increases from the first to the third trimester of pregnancy, but the severity of fetal infection usually decreases with increasing gestation (Desmont *et al.*, 1974). The most common clinical manifestation of congenital toxoplasmosis is retinochoroidal scarring (Figure 4.1, lower left and lower right). The occurrence of the retinal lesions tends to be bilateral and macular in location (Remington *et al.*, 1976; Mets *et al.*, 1997). In addition, even though these retinal lesions may not develop for years after birth, 90% will develop retinochoroidal lesions by adolescence if infected infants are left untreated (Koppe *et al.*, 1974; Wilson *et al.*, 1980; Koppe *et al.*, 1986). Ocular manifestations of congenital toxoplasmosis in the US are listed in Table 4.2a (Mets *et al.*, 1997). Large, prospective studies of congenital *T. gondii* infections conducted in Amsterdam (Koppe

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**Figure 4.2** Toxoplasmic retinochoroiditis in immunocompromised patients. *Upper left.* Fundus photograph with red-free light showing irregular margins and presence of multiple satellite lesions. *Upper right.* Multiple cysts of *T. gondii* in necrotic retina (hematoxylin-eosin,  $\times 440$ ). *Upper Center.* Mononuclear inflammation within the optic nerve, with several *T. gondii* cysts on the left. *Lower Center.* Two young cysts (YC) within a host retinal cell. The cell on the left has many microtubules (mt) and Nissl body-like structures (open arrows), suggesting a neuroretinal cell. (Adapted with permission from Yeo J.H., Jakobiec F.A., Iwamoto T., Richard G., Kreissig I. (1983). Opportunistic toxoplasmic retinochoroiditis following chemotherapy for systemic lymphoma. A light and electron microscopic study. *Ophthalmol.* 90(8), 885–98.). *Lower Left.* Fundus appearance of right eye in an AIDS patient with bilateral miliary toxoplasmic retinitis, showing multiple small, round, white, inflammatory retinal lesions, as well as hemorrhage and edema in the macula. (Adapted with permission from Berger B.B., Egwuagu C.E., Freeman W.R., Wiley C.A. (1993). Miliary toxoplasmic retinitis in acquired immunodeficiency syndrome. *Arch. Ophthalmol.* 111(3), 373–6.) *Lower Right.* Fundus appearance of left eye from an 8-year-old child treated with systemic corticosteroids, showing active retinal inflammatory lesion adjacent to an old retinochoroidal scar along the inferonasal vascular arcade. (Adapted with permission from Morhun P.J., Weisz J.M., Elias S.J., Holland G.N. (1996). Recurrent ocular toxoplasmosis in patients treated with systemic corticosteroids. *Retina.* 16(5), 383–7.) *Bottom.* Histologic section from an adult receiving long-term corticosteroid therapy, showing focal zones of inner retinal necrosis adjacent to the vessels. Arrows showing cysts and released organisms lie at the interface of intact and necrotic retina. V= vitreous cavity, L= blood vessel lumen (hematoxylin-eosin,  $\times 700$ ). (Adapted with permission from Nicholson D.H., Wolchok E.B. (1976). Ocular toxoplasmosis in an adult receiving long-term corticosteroid therapy. *Arch. Ophthalmol.* 94(2), 248–54.)

*et al.*, 1974; Koppe *et al.*, 1982; Koppe *et al.*, 1986; Koppe *et al.*, 1989), France (Daffos *et al.*, 1988; Hohlfield *et al.*, 1989; Binquet 2003; Brezin *et al.*, 2003; Wallon *et al.*, 2004), and in the U.S. (Fair, 1958; Wilson *et al.*, 1980; McAuley *et al.*, 1994; Mets *et al.*, 1997) have all shown that congenital *T. gondii* infection can lead to serious long-term sequelae and adverse visual outcomes in both treated and untreated children (Table 4.2b).

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## Treatment

Treatment of OT is always warranted for severe or persistent lesions involving the macula or the optic nerve, in large retinal lesions with severe inflammation, and in immunocompromised patients. There is some controversy about whether active lesions confined to the peripheral retina warrant treatment. We believe that all active disease should be treated, because these lesions are caused by replicating *T. gondii* parasites that destroy retinal tissue (Table 4.3b). In immunocompromised patients, prophylaxis with trimethoprim-sulfamethoxazole is indicated, although sulfonamide allergies occur in a substantial number of patients with AIDS who receive these medicines (Peters *et al.*, 1994; Bayard *et al.*, 1992), and pyrimethamine can have an antagonistic effect when used concurrently with zidovudine (Israelski *et al.*, 1989). Substitution of clindamycin for sulfonamides (Leport *et al.*, 1989) or the use of trimethoprim and sulfamethoxazole have been described as acceptable alternatives (Haverkos, 1987). In the case of sulfadiazine or clindamycin toxicity, clarithromycin and atovaquone can be used as second line therapy. The use of corticosteroids may increase the risk of further suppression of immune response, without benefit since little ocular inflammation is usually associated with OT in immunocompromised patients (Holland *et al.*, 1988).

In immunocompetent patients, the combination of pyrimethamine and sulfadiazine with corticosteroids has been the major therapy for active disease caused by either congenital or acquired OT (Table 4.3b). Pyrimethamine is associated with bone marrow suppression, and sulfadiazine with renal damage and hypersensitivity reactions. In addition, corticosteroid should always be used concurrently with antimicrobial agents, otherwise severe tissue destruction may result (O'Connor *et al.*, 1976; Sabates *et al.*, 1981). In pregnant women with acute acquired infection, spiramycin may reduce transmission to the fetus. Before 17 weeks of gestation, sulfadiazine is used alone or with clindamycin to treat the fetus due to concerns about teratogenicity. After that time, the combination of pyrimethamine, sulfadiazine with leucovorin is used (Table 4.3a). The treatment protocol for treating active lesions in infants and older children is listed in Table 4.3b.

There is currently no effective therapy against bradyzoites that prevents recurrent ocular disease in humans. Medical therapies that would eradicate bradyzoites in tissue cysts would be the ideal way to prevent recrudescence.

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## Pathogenesis

*T. gondii* is considered to reach the eye via a hematogenous route. Tachyzoites are arrested within the capillary bed of the retina where the parasites can invade (Roberts and McLeod, 1999). In a minority of cases *T. gondii* may be transmitted to the eye, from a severely infected brain, via the optic nerve. Once within the retina the organisms multiply, cause cell lysis and spread to adjacent cells. The outcome of infection is modulated by the intraocular immune response.



**Table 4.3a** Guidelines for treatment of *T. gondii* infection in the pregnant woman infected during gestation

Infection	Medication	Dosage	Duration of therapy
First 18 wk of gestation or until term if fetus found not to be infected by amniocentesis at 18 wks	Spiramycin <sup>a</sup>	1 g every 8 hours without food	Until fetal infection is documented or until it is excluded at 18 wk of gestation
Fetal infection confirmed after 18 wks of gestation and in all women infected after 24 wks	Pyrimethamine <sup>b</sup>	Loading dose: 50 mg each 12 hours for 2 days; then beginning on day 3, 50 mg per day	Until term <sup>c</sup>
	Sulfadiazine	Loading dose: 75 mg/kg; then beginning 50 mg/kg each 12 hours (maximum 4 g per day)	Until term <sup>c</sup>
	Leucovorin (folinic acid) <sup>b</sup>	10–20 mg daily <sup>d</sup>	During and for 1 wk after pyrimethamine therapy

<sup>a</sup>Available only on request from the U.S. Food and Drug Administration (telephone number 301–443–5680), and then with this approval by physician's request to Aventis.

<sup>b</sup>Adjusted for megaloblastic anemia, granulocytopenia, or thrombocytopenia; blood cell counts, including platelets, should be monitored.

<sup>c</sup>Subsequent treatment of the infant is the same as that described under treatment of congenital infection. When the diagnosis of infection in the fetus is established earlier, we suggest that sulfadiazine be used alone until after the first trimester, at which time pyrimethamine should be added to the regimen. After 24 wks of gestation, we recommend that amniocentesis be performed and that pyrimethamine/leucovorin/sulfadiazine be used instead of spiramycin.

<sup>d</sup>Optimal dosage, feasibility, and toxicity currently are being evaluated in the ongoing Chicago-based National Collaborative Treatment Trial (NCTT) (telephone number 773–834–4152).

Adapted with permission from Remington J.S., McLeod R., Thulliez P., Desmonts G. (2005). Toxoplasmosis. In *Infectious Diseases of the Fetus and the Newborn Infant*, J.S. Remington, J.O. Klein, C. Baker, C.B. Wilson, eds. (Philadelphia, U.S.A.: WB Saunders), chapter 31.



**Table 4.3b** Guidelines for treatment of *T. gondii* infection in the infant and older children and adults

Infection	Medication	Dosage	Duration of therapy
Congenital <i>T. gondii</i> infection in the infant <sup>a</sup>	Pyrimethamine <sup>a, b</sup>	Loading dose: 1 mg/kg each 12 hours for 2 days; then beginning on day 3, 1 mg/kg per day for 2 or 6 mo; then this dose every Monday, Wednesday, Friday	1 yr <sup>c</sup>
	Sulfadiazine <sup>c</sup>	50 mg/kg each 12 hours	1 yr <sup>c</sup>
	Leucovorin <sup>b</sup>	10 mg three times weekly	During and for 1 wk after pyrimethamine therapy
	Corticosteroids <sup>d</sup> (prednisone) have been used when CSF protein is > 1 g/dL and when active chorioretinitis threatens vision	0.5 mg/kg each 12 hours	Corticosteroids are continued until resolution of elevated (> 1 g/dL) CSF protein level or active chorioretinitis that threatens vision
Active chorioretinitis in older children and adults	Pyrimethamine <sup>b</sup>	Loading dose: 1 mg/kg each 12 hours (maximum 50 mg per dose) for 2 days; then beginning on day 3, maintenance, 1 mg/kg per day (maximum 50 mg). Doses up to 75 mg per day have been used in adult patients with AIDS.	Usually 1–2 week beyond the time that signs and symptoms have resolved in immunocompetent persons, see text and other texts for discussions of suppression in persons with irreversible immunocompromise

Sulfadiazine <sup>c</sup>	Loading dose: 75 mg/kg; then beginning 12 hours later, maintenance, 50 mg/kg every 12 hours	Usually 1–2 wk beyond the time that signs and symptoms have resolved, as above
Leucovorin <sup>b</sup>	10–20 mg 3 times weekly	During and for 1 wk after pyrimethamine therapy
Corticosteroids <sup>d</sup> (prednisone)	1 mg/kg/day, divided bid; maximum 40 mg per day followed by rapid taper	Steroids are continued until inflammation subsides (usually 1–2 wk) and then tapered rapidly. Pyrimethamine and sulfadiazine are always continued at least 1 week beyond discontinuation of prednisone.

<sup>a</sup>Optimal dosage, feasibility, and toxicity currently are being evaluated in the ongoing Chicago-based National Collaborative Treatment Trial (NCTT) (telephone number 773–834–4152).

<sup>b</sup>Adjusted for megaloblastic anemia, granulocytopenia, or thrombocytopenia; blood cell counts, including platelets, should be monitored as described in text.

<sup>c</sup>Alternative medicines for patients with atopy or severe intolerance of sulfonamides have included pyrimethamine and leucovorin with clindamycin, or azithromycin, or atovaquone, with standard dosages as recommended according to weight.

<sup>d</sup>Corticosteroids should be used only in conjunction with pyrimethamine, sulfadiazine, and leucovorin treatment and should be continued until signs of inflammation (high CSF protein, > 1 g/dL) or active chorioretinitis that threatens vision have subsided; dosage can then be tapered and the steroids discontinued.

Adapted with modifications and permission from Remington J.S., McLeod R., Thulliez P., Desmonts G. (2005). Toxoplasmosis. In *Infectious Diseases of the Fetus and the Newborn Infant*, J.S. Remington, J.O. Klein, C. Baker, C.B. Wilson, eds. (Philadelphia, U.S.A.: WB Saunders), chapter 31.

## Histopathology of ocular toxoplasmosis

The histopathological features of OT depend on the stage of the disease and the immune status of the patient. The pathological features of human OT have been described in a number of reports (Hogan, 1951; Wilder, 1952; Zimmerman, 1961; Rao and Font, 1977). The acute lesions of OT consist of focal retinal necrosis in which tachyzoites are occasionally identified. Tissue cysts may be found adjacent or at some distance from these lesions. Distant from the active lesion the retina may show perivascular inflammation, edema and gliosis. Usually there is associated choroidal chronic inflammation, which is occasionally granulomatous. Rarely acute toxoplasmic retinochoroiditis may simulate bilateral acute retinal necrosis syndrome.

Active lesion heals to leave a scar. Secondary changes include retinal detachment, iridocyclitis, and cataracts. Papillitis, papilledema and later optic atrophy are recognized manifestations of toxoplasmic encephalitis (Perkins, 1973; Mets *et al.*, 1996).

In congenital OT irreversible damage occurs *in utero* (Brezin *et al.*, 1994; Roberts *et al.*, 2001). The features include zones of retinochoroiditis with retinal necrosis, edema and inflammatory infiltration of the retina (Figures 4.3 and 4.4). Tissue cysts have not been identified in fetal eyes although extracellular organisms have been identified in lesions by immunohistochemistry (Brezin *et al.*, 1994; Roberts *et al.*, 2001). Optic neuritis has also been identified in eyes from second trimester fetuses but is most likely a secondary manifestation of toxoplasmic encephalitis (Roberts *et al.*, 2001) (Figure 4.3, left panel). Established retinochoroidal scars have also been identified in infants at birth and in a third trimester fetus confirming that resolution of infection can also occur *in utero* (Guerina *et al.*, 1994; Mets *et al.*, 1996; Roberts *et al.*, 2001) (Figure 4.3, right panel). Severe congenital infection may result in microphthalmos or microcornea.

In AIDS related OT the lesions differ both clinically and histologically from those of immunocompetent patients. There is usually no evidence that the disease originated in pre-existing retinochoroidal scars. Lesions are frequently bilateral and multifocal with extensive retinal necrosis, minimal inflammation and large numbers of parasites. OT is relatively uncommon in AIDS compared with cerebral toxoplasmosis. Immunosuppression from other causes may cause a similar pathological picture.

## Ocular toxoplasmosis in animals

### Naturally occurring disease

Naturally occurring OT has been described in many species including dogs, cats, pigs, sheep, cattle and wallabies (Piper *et al.*, 1970; Vainisi and Campbell, 1969; Petrak and Carpenter, 1965; Ashton, 1979). All animals have several features in common including retinal necrosis, focal retinochoroiditis and organisms within the retina. The constituents of the inflammatory response are similar between species although granulomatous choroidal lesions are more common in sheep whereas in cattle the inflammatory infiltrate is predominantly plasma cells. In contrast to the histopathological features in humans organisms have been identified in the uveal tract.

## Experimentally induced disease

### *General considerations*

Ocular disease is not common in animals compared with *T. gondii* infection rates. An animal model should ideally be infected by a conventional route and reliably develop disease. Some animal models require intraocular inoculation of parasites to effectively induce disease. This trauma may itself produce significant inflammation and carries the theoretical risk of sympathetic ophthalmitis.

### *Guinea pig*

Early models of toxoplasmosis produced disease in guinea pigs by intravitreal inoculation of trophozoites (Hogan *et al.*, 1956; Hogan *et al.*, 1957; Hogan *et al.*, 1958). Live organisms were identified in the eye 10 months later but the course of the disease was not altered by treatment with sulfadiazine and pyrimethamine.

### *Hamsters*

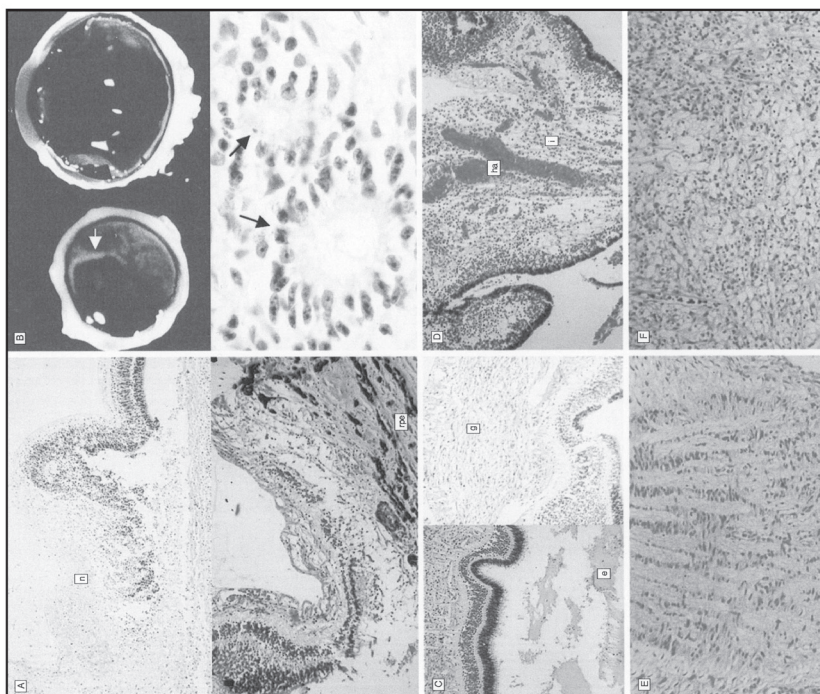
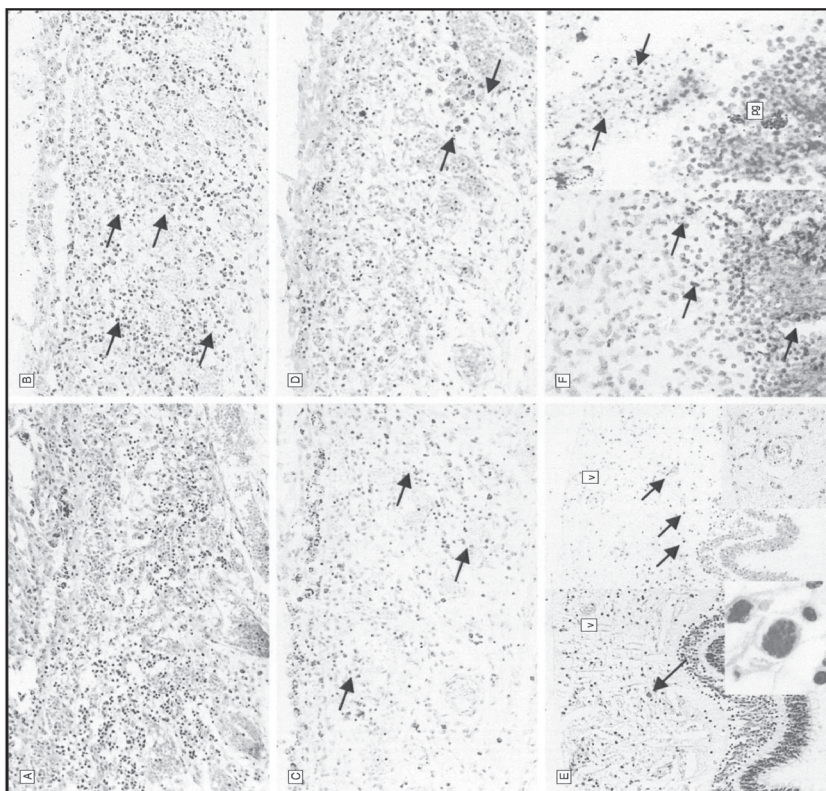
Systemic infection of hamsters with the RH strain of *T. gondii* can produce widespread retinal necrosis in the majority of infected eyes with focal retinochoroiditis (Frenkel, 1955). Intraperitoneal or oral infection with the less virulent ME49 strain can also produce retinochoroiditis within 3 weeks of inoculation that resolves by 12 weeks (Pavesio *et al.*, 1995; Gormley *et al.*, 1999). The course of this acute disease is not altered by chemotherapy with sulfadiazine and pyrimethamine (Gormley *et al.*, 1998).

### *Rabbit models*

Intravitreal injection with the RH strain of *T. gondii* can produce acute retinitis. (Nozik and O'Connor, 1970). However disease recurrence cannot be stimulated with *T. gondii* antigen, local trauma, systemic adrenaline and corticosteroids (Nozik and O'Connor, 1970). Similarly immunosuppression with cyclosporin A does not alter the course of disease (Friedrich *et al.*, 1992). In this model both naive and primed rabbits developed toxoplasmic retinochoroiditis but the severity and progression of the disease was more severe in the naive group some of which died of generalized infection (Garweg *et al.*, 1998). This model was adapted to assess the affect of chemotherapeutic agents on the retinitis produced (Chowchuvech *et al.*, 1972; Tabbara *et al.*, 1974). Yoshizumi described the light and electron microscopic features of retinochoroiditis in this model (Yoshizumi, 1976).

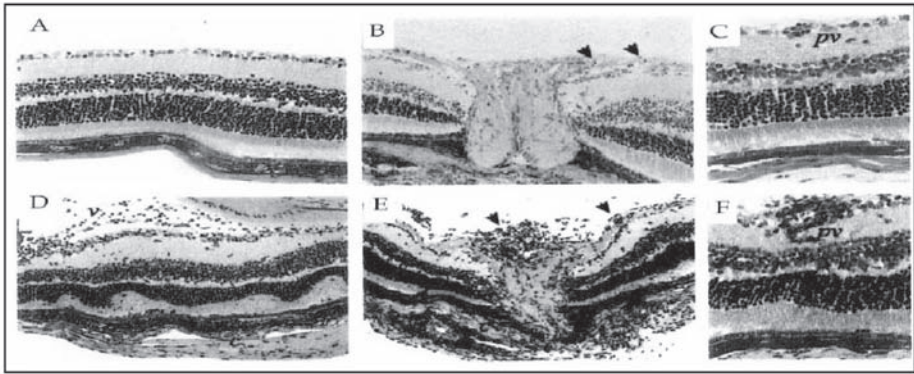
### *Primate models*

The first primate model was achieved by inoculation of live parasites into the inner retinal layers (Culbertson *et al.*, 1982). All eyes developed a necrotizing retinochoroiditis with lesions healing by scarring. Subsequent intraocular challenge with *T. gondii* antigen produced iridocyclitis, vitritis and retinal edema but not necrotizing retinochoroiditis supporting a role for hypersensitivity in recurrent disease (Newman *et al.*, 1982).





**Figure 4.3** Left Panel: Ocular histopathology in congenital toxoplasmosis. A, top, A well-demarcated area of retinal necrosis (n) at the posterior pole in the eye of a 22-week-gestation fetus (hematoxylin-eosin, original magnification  $\times 250$ ). A, bottom, The edge of a large retinohoroidal scar from the eye of the 2-year-old child. The scar is well demarcated with tubuloacinar proliferation of the retinal pigment epithelium (rpe) at the edge of the scar. The center of the scar is devoid of retina (hematoxylin-eosin, original magnification  $\times 250$ ). B, top, Eye from the 32-week-gestation fetus showing a large hyperpigmented scar, with a white rim, in the superotemporal region of the eye (arrow). B, bottom, The retina from the edge of the scar shows disorganization with formation of Flexner-Wintersteiner rosettes (arrows) (hematoxylin-eosin, original magnification  $\times 400$ ). C, left, Retina from the 5-day-old infant eye showing retinal detachment with an exudate (e) between retina and choroids. The inner retinal layer is edematous and inflamed (hematoxylin-eosin, original magnification  $\times 100$ ). C, right, Retina from the eye of a 22-233k gestation fetus showing gliosis (g) of the inner retinal layers (hematoxylin-eosin, original magnification  $\times 250$ ). D, Eye from a 23-233k gestation fetus showing a moderate inflammatory infiltrate (i) within the primary vitreous and surrounding the hyaloid artery (ha) (hematoxylin-eosin, original magnification  $\times 20$ ). E, Optic nerve from the eye of a 23-week-gestation fetus with congenital toxoplasmosis. The nerve architecture is disrupted with an inflammatory cell infiltrate (hematoxylin-eosin, original magnification  $\times 100$ ). Right Panel: Inflammatory cells and *Toxoplasma gondii* organisms present in ocular toxoplasmosis. A through D represent the same discrete ocular lesion from a 21-week-gestation fetus with a peripapillary lesion. A, Disruption of the retinal pigment epithelium (RPE) with choroidal congestion and inflammation (hematoxylin-eosin, original magnification  $\times 400$ ). Immunohistochemical staining for T cells, CD3 (B) and T-cell subset, CD4 (C), shows numerous positive lymphocytes within the choroids (arrows). D, In this case CD68-positive macrophages are numerous within the choroids underlying the area of RPE disruption (arrows). No staining was identified in the negative control or in sections stained with anti-CD8 (not shown). E and F demonstrate staining for *T. gondii*. E, left, Retina from the 5-day-old infant showing a collection of intracellular *T. gondii* within the retina (arrow) (hematoxylin-eosin, original magnification  $\times 100$ ). Note the small blood vessel (v). The inset shows a high-power view of these organisms (hematoxylin-eosin, original magnification  $\times 400$ ). Right, Retina from the 5-day-old infant eye showing immunohistochemical staining for *T. gondii* antigen. Note the same small blood vessel (v) also identified in E, left. Many extracellular *T. gondii* organisms are identified (arrows). In addition, the inset shows the presence of organisms in a perivascular location (L43 stain, original magnification  $\times 100$  and  $\times 250$ ). F, left, Gliotic retina in an eye from a 22-week-gestation fetus showing extracellular organisms scattered throughout the retinal layers (arrows) (polyclonal antibody, original magnification  $\times 250$ ). Right, Disrupted retina and necrotic debris in an eye from a 23-week-gestation fetus. Numerous extracellular *T. gondii* organisms (arrows) are present within the necrotic debris (L43 stain, original magnification  $\times 400$ ). The red staining product allows distinction from melanin pigment granules (pg) of disrupted RPE. Adapted with permission from Roberts, F., Mets, B., Ferguson, D.J. et al. (2001). Histopathological features of ocular toxoplasmosis in the fetus and infant. Arch. Ophthalmol. 119, 51-58.



**Figure 4.4** C57BL/6 mice develop toxoplasmic retinochoroiditis that can be exacerbated by inhibition of NO production by L-NAME treatment. In infected mice not treated with L-NAME, the inflammatory infiltrate is focal and in areas where the retina appears normal (A). In other areas, there is a mild inflammatory cell infiltrate (arrows), confined to the inner retinal layers and optic nerve head (B). In these mice there is only mild perivascular cuffing (pv) (C). In L-NAME treated mice, inflammatory infiltrate is severe and diffuse (D and E). There are numerous cells in the vitreous (v) and expanding choroid as well as within the inner retinal layers and optic nerve head (arrows). Perivascular cuffing by inflammatory cells is more severe in L-NAME treated mice (F) than in untreated mice. Adapted with permission from Roberts, F., Mets, B., Ferguson, D.J. *et al.* (2001). Histopathological features of ocular toxoplasmosis in the fetus and infant. *Arch. Ophthalmol.* 119, 51–58.

### Cat models

The cat is an unusual choice for an animal model since there may be a different response to parasitemia. In the first feline model, pathogen-free cats were inoculated in the right carotid artery with ME49 tachyzoites and developed bilateral retinochoroiditis up to 8 days later which resolved by 70 days post infection with scarring (Davidson *et al.*, 1993).

### Mouse models

The immunology of OT has been most extensively studied in murine models, but is complicated due to different routes of infection, different mouse strains and different parasite lineages employed in each study. These studies are summarized below and in Table 4.4.

#### *Murine congenitally acquired ocular toxoplasmosis*

Congenital OT has been studied in a murine model (Hay *et al.*, 1981; Hutchison *et al.*, 1982; Hay *et al.*, 1984). In these studies, mice infected *in utero* developed vasculitis, choroiditis, vitritis, retinitis and necrosis in affected eyes. The composition of inflammatory infiltrates included lymphocytes and macrophages which contained degraded photoreceptors in the phagosomes (Hay *et al.*, 1981; Dutton *et al.*, 1986; McMenamin *et al.*, 1986). The low rate of ocular disease in this model (approximately 2.5% of pups), make it less than ideal (Hay *et al.*, 1981).

#### *Murine adult acquired ocular toxoplasmosis*

C57BL/6 mice infected intraperitoneally with *T. gondii* (ME49 strain) develop OT 4–6 weeks post infection (Gazzinelli *et al.*, 1994; Roberts *et al.*, 2000), but similar infection of other



**Table 4.4** The effect of host genetic or immunological manipulation on the outcome of ocular toxoplasmosis in murine models.

Manipulation	Mouse strain	Parasite strain	Clonal lineage	Route of infection	Effect on parasite burden	Effect on pathology	Effect on immune parameters	Reference
Aminoguanidine treatment	C57BL/6	ME49 cysts	Type II	Intraperitoneal	Nd <sup>~</sup>	Increase in inflammation	Nd <sup>-</sup>	Hayashi <i>et al.</i> , 1996
B cell gene deficiency	C57BL/6	RH tachyzoites	Type I	Intraocular	Increased parasite burden	Increased necrosis and inflammation	Same increase in IFN- $\gamma$ and TNF- $\alpha$ as seen in WT	Lu <i>et al.</i> , 2004
CD4 gene deficiency	C57BL/6	RH tachyzoites	Type I	Intraocular	Increased parasite burden	Decreased necrosis and inflammation	No increase in IFN- $\gamma$ or TNF- $\alpha$ as seen in WT	Lu <i>et al.</i> , 2004
CD4 neutralizing antibody	C57BL/6	ME49 cysts	Type II	Intraperitoneal	Increased cyst burden	Increase in number and severity of lesions	Nd <sup>-</sup>	Gazzinelli <i>et al.</i> , 1994
CD8 gene deficiency	C57BL/6	RH tachyzoites	Type I	Intraocular	Increased parasite burden	Increased necrosis and inflammation	Same increase in IFN- $\gamma$ and TNF- $\alpha$ as seen in WT	Lu <i>et al.</i> , 2004
CD8+ neutralizing antibody	C57BL/6	ME49 cysts	Type II	Intraperitoneal	Increased cyst burden	Increase in number and severity of lesions	Nd <sup>-</sup>	Gazzinelli <i>et al.</i> , 1994
FAS non functional mutant ( <i>pr</i> ) <sup>*</sup>	C57BL/6	ME49 cysts	Type II	Intraperitoneal	No difference	No difference	Early increase in IFN- $\gamma$ and increased nitrite levels	Shen <i>et al.</i> , 2001

Manipulation	Mouse strain	Parasite strain	Clonal lineage	Route of infection	Effect on parasite burden	Effect on pathology	Effect on immune parameters	Reference
FAS non functional mutant ( <i>pr</i> )*	C57BL/6	ME49 tachyzoites	Type II	Intraocular	Nd <sup>-</sup>	Increase in inflammation and pathology	Decreased apoptosis	Hu <i>et al.</i> , 1999
FASL non functional mutant ( <i>gl</i> Δ) <sup>§</sup>	C57BL/6	ME49 cysts	Type II	Intraperitoneal	No difference	No difference	Early increase in IFN-γ and increased nitrite levels	Shen <i>et al.</i> , 2001
FASL non functional mutant ( <i>gl</i> Δ) <sup>§</sup>	C57BL/6	ME49 tachyzoites	Type II	Intraocular	Nd <sup>-</sup>	Increase in inflammation and pathology	Decreased apoptosis	Hu <i>et al.</i> , 1999
IFN-γ gene deficiency	BALB/c	Fukaya strain cysts	Undescribed	Peroral	Increased dissemination of parasites in multiple ocular tissues Decreased tachyzoite to bradyzoite conversion	Increased inflammation including sludging of blood in vessels	Nd <sup>-</sup>	Norose <i>et al.</i> , 2003
IFN-γ gene deficiency	C57BL/6	Fukaya strain cysts	Undescribed	Peroral	Increased dissemination of parasites in multiple ocular tissues Decreased tachyzoite to bradyzoite conversion	Increased inflammation including sludging of blood in vessels	Nd <sup>-</sup>	Norose <i>et al.</i> , 2003

IFN- $\gamma$ neutralizing antibody	C57BL/6	ME49 cysts	Type II	Intraperitoneal	Increased tachyzoite proliferation	Increase in inflammation and pathology	Increased expression of macrophage and T cell markers	Gazzinelli <i>et al.</i> , 1994
IFN- $\gamma$ neutralizing antibody	Swiss-webster	Beverley cysts	Type II	Intraperitoneal	High parasitic loads maintained after day 45 when control group loads drop Higher cyst burden	Increase in inflammation and pathology	Nd <sup>-</sup>	Olle <i>et al.</i> , 1996
IL-6 gene deficiency	129/SVJ	Beverley cysts	Type II	Intraperitoneal	Increased bradyzoite number Increased cyst burden	Increased inflammation in retina and vitreous humor	Decreased IL-1 transcription Increased TNF- $\alpha$ transcription No difference in transcription of $\beta 2$ -microglobulin, TGF- $\beta$ iNOS and IL-6 (transcripts in KO mice are non-functional)	Lyons <i>et al.</i> , 2001
IL-10 gene deficiency	BALB/c	RH tachyzoites	Type I	Intraocular	Increased tachyzoite proliferation	Increased necrosis and severe inflammation	No difference in IFN- $\gamma$ or TNF- $\alpha$ production	Lu <i>et al.</i> , 2003

Manipulation	Mouse strain	Parasite strain	Clonal lineage	Route of infection	Effect on parasite burden	Effect on pathology	Effect on immune parameters	Reference
IL-10 gene deficiency	C57BL/6	RH tachyzoites	Type I	Intraocular	Increased tachyzoite proliferation	Increased necrosis and severe inflammation	No difference in IFN- $\gamma$ or TNF- $\alpha$ production	Lu <i>et al.</i> , 2003
IL-10 transgenic	C57BL/6	RH tachyzoites	Type I	Intraocular	Increased tachyzoite proliferation	Less severe inflammation and no evidence of necrosis	No increase in IFN- $\gamma$ and TNF- $\alpha$ as seen in WT or IL-10 KO	Lu <i>et al.</i> , 2003
L $\omega$ -nitro-L-arginine methyl ester (L-NAME) treatment	C57BL/6	ME49 cysts	Type II	Intraperitoneal	Nd <sup>-</sup>	Increase in inflammation	Nd <sup>-</sup>	Roberts <i>et al.</i> , 2000
Prednisolone treatment (with sulfadiazine)	Swiss-Webster	Beverley cysts	Type II	Intraperitoneal	Increased parasite encystment and cyst number	Decreased inflammation and pathology	Nd <sup>-</sup>	Olle <i>et al.</i> , 1996
TNF- $\alpha$ neutralizing antibody	C57BL/6	ME49 cysts	Type II	Intraperitoneal	Increased tachyzoite proliferation	Increase in inflammation and pathology	Increased expression of macrophage and T cell markers	Gazzinelli <i>et al.</i> , 1994

\*lymphoproliferation

§general lymphoproliferative disease

~not determined

strains such as BALB/c mice fails to induce OT. Direct inoculation tachyzoites into the anterior chamber (intracameral infection) has been successfully used to initiate BALB/c (Lu *et al.*, 2005). Instillation with *T. gondii* (ME49 strain) can also induce OT in mice (Tedescos *et al.*, 2005).

## Immune response

IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-10, and nitric oxide

IFN- $\gamma$  and TNF- $\alpha$  have been demonstrated to play a crucial protective role during systemic *T. gondii* infection by inducing NO production and restricting *T. gondii* replication (Suzuki *et al.*, 1989; Suzuki, 1988; Blackwell *et al.*, 1994; Gazzinelli *et al.*, 1993; Langermans *et al.*, 1992; see Chapter 6). In keeping with this, inhibition of NO has been demonstrated to increase mortality in C57/BL6 mice infected orally and exacerbate chronic toxoplasmic encephalitis in C57BL/6 mice (Schluter *et al.*, 1999; Nickdel *et al.*, 2001). However the role of these mediator in eye disease is complex and although most literature point to a detrimental role there are contradictory reports (Goureau *et al.*, 1995; Wang and Hakanson, 1995; Smith, 1998; Fukushima, 2005; Rosenbaum *et al.*, 1998; Smith *et al.*, 1998; Brito *et al.*, 1999).

C57BL/6 mice infected intraperitoneally with *T. gondii* (ME49:clonal lineage II) develop ocular disease which is exacerbated by neutralization of TNF- $\alpha$ , IFN- $\gamma$  or inhibition of NO production (Gazzinelli *et al.*, 1994; Olle *et al.*, 1994; Roberts *et al.*, 2000; Hayashi *et al.*, 1996) (Figure 4.4). IFN- $\gamma$  deficient mice have increased parasite proliferation ocular pathology and reduced visual function following intraocular *T. gondii* infection compared with wild-type control mice (Norose *et al.*, 2003; Norose *et al.*, 2005).

IL-6 is important in controlling parasite number in the eye as IL-6 deficient mice develop increased retinitis, vitritis and have increased bradyzoite numbers in their eyes compared with infected wild type mice. (Lyons *et al.*, 2001).

IL-10 has been demonstrated to inhibit *T. gondii* killing and to exacerbate encephalitis (Gazzinelli *et al.*, 1993); however, IL-10 deficient mice ultimately die due to uncontrolled production of IL-12 and IFN- $\gamma$  following *T. gondii* infection (Gazzinelli *et al.*, 1996). Transgenic mice constitutively expressing IL-10 have a reduced ability to control *T. gondii* proliferation following intraocular infection with RH tachyzoites, but paradoxically develop less severe inflammation (Lu *et al.*, 2003). IL-10 gene deficient mice develop increased pathology in their eyes compared with wild type mice (Lu *et al.*, 2003).

## Lymphocytes

B cells and antibodies have been demonstrated in a number of systems to contribute to the protective immune response against *T. gondii* (Frenkel and Taylor, 1982; Grimwood and Smith, 1992; Hauser and Remington, 1981; Couper *et al.*, 2005). Consistent with this B cell deficient ( $\mu$ MT) mice infected intraocularly with *T. gondii* develop increased ocular inflammation, necrosis, and parasite burdens compared with wild type control mice (Kang *et al.*, 2000).

Long term immunity to *T. gondii* infection is dependent on CD4+ and CD8+ T cells (Brown and McLeod, 1990; Gazzinelli *et al.*, 1991; Parker *et al.*, 1991; Gazzinelli *et al.*, 1992). Depletion of either CD4+ or CD8+ T cells was found to increase ocular parasite burdens and inflammation in C57/BL6 mice chronically infected with *T. gondii* (ME49) (Gazzinelli

*et al.*, 1994). Similarly, CD4 gene deficient C57BL/6 mice have a reduced ability to control intraocularly inoculated *T. gondii* (RH strain) compared with wild type mice. However, CD4+ T cells contribute to tissue destruction as CD4 gene deficient mice had reduced inflammation (Lu *et al.*, 2004). CD8 gene deficient C57BL/6 mice also have a reduced ability to control parasite multiplication in their eyes compared but develop increased inflammation compared with wild type mice (Lu *et al.*, 2004).

### Ocular toxoplasmosis and immune privilege

MHC class I which is normally expressed at reduced levels in immune privileged tissues including the eye is upregulated in the ganglion and retinal epithelial cells of eyes from *T. gondii* infected mice (Lyons *et al.*, 2001). This would provide the means for CD8+ T cells to recognize and kill infected cells.

The constitutive expression of FasL (CD95 ligand) in eyes serves to induce apoptosis on infiltrating inflammatory cells through interaction with Fas (CD95) on their surface. However, the same interaction provides a mechanism by which cytotoxic lymphocytes induce apoptosis in *T. gondii* infected target cells (Gavrilescu and Denkers, 2003). The study of B6MRL/*lpr* and B6MRL/*gld* mice that have non-functional mutations in Fas (CD95) and FasL (CD95 ligand) genes, respectively has yielded conflicting data in the role of these molecules during OT (Hu *et al.*, 1999; Shen *et al.*, 2001). In one study, B6MRL/*lpr* and B6MRL/*gld* mice developed similar ocular disease following intraperitoneal infection with the ME49 strain of *T. gondii* (Hu *et al.*, 1999). However, following intraocular infection with the PLK strain of *T. gondii*, B6MRL/*lpr* and B6MRL/*gld* mice had a decreased ability to control parasite proliferation and developed more severe inflammation (Shen *et al.*, 2001). The apparent differences in the findings of these studies may reflect the different routes of inoculation.

TGF- $\beta$ , a molecule that is normally constitutively expressed in eyes as part of immune privilege, has been demonstrated to be raised during OT and may act to limit the potentially damaging inflammation in the eye (Lyons *et al.*, 2001).

### The conflict of anti-parasitic mechanisms with immune privilege

The eye is an immune privileged site and as such has a number of mechanisms in place to limit inflammation and prevent damage. During OT there is a need to control parasite proliferation, but limit damage to eye. A number of mechanisms have evolved in the eye to prevent potentially damaging inflammation that are often in conflict with the mechanisms that are known to control *T. gondii* infection at other anatomical sites. During OT a number of these mechanisms are ablated which facilitates parasite killing. Notably, mediators such as IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and NO that are often associated with potentially damaging inflammatory responses, but also parasite killing are produced within the eye. Furthermore, MHC class I is upregulated to allow CD8+ T cell recognition of infected cells. IL-10 would appear to play an important role in limiting immune mediated necrosis in the eye. In addition, TGF- $\beta$  is upregulated and may also play a role in limiting the potential damaging effects of the immune response as noted in other ocular disease models (Peng *et al.*, 1997).

## Summary and conclusions

OT may be a consequence of congenital or acute acquired infection and recrudescent disease occurs in each of these clinical settings. Current treatments described herein are not ideal as they only treat active disease, but do not eliminate chronic infection. From animal models it is evident that disease and retinal destruction can be due to replicating organisms or the inflammatory response they elicit. Consequently the balance of inflammation with parasite killing is critical in limiting pathology and destruction of the eye.

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## Abstract

*Toxoplasma gondii* is one of the few pathogens that regularly cross the placenta. The consequences of maternal infection during pregnancy depend largely on the timing. The risk of fetal infection continually increases with the duration of pregnancy. Conversely, early infection bears the greatest risk of severe sequelae for the unborn child. Brain and eye lesions are the most common consequences of *in utero* infection. Importantly, babies born without overt clinical symptoms are at risk of developing the disease during childhood or adolescence. These features explain why congenital toxoplasmosis continues to be a major health problem throughout the world. This chapter gives an overview of clinical disease, principles of diagnosis of maternal and fetal infection and treatment of congenital toxoplasmosis. The management and legal situation in different countries is discussed. Finally, the actual knowledge of immunologic control of materno-fetal transmission is presented.

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## Introduction

Primary infection in pregnant women can result in transplacental transmission of *Toxoplasma gondii* and cause congenital toxoplasmosis (CT). CT can lead to a wide array of clinical manifestations ranging from no symptoms at all, abortion, fetal death, hydrocephalus, microcephaly, seizures and mental retardation and retinochoroiditis, which can manifest itself many years after birth in spite of treatment (Kodjikian *et al.*, 2006). Pregnant women should be informed about the prevalence of CT, the risk factors and the preventive measures to lower the risk of congenital infection. Obstetricians should diagnose a primary infection as early as possible and set up an amniocentesis if necessary. Medical biologists should offer the adequate methods for the prenatal and postnatal diagnosis of CT and pediatricians should ensure a perfect follow-up of infants suspected to be infected and treat correctly CT as soon as it is diagnosed (Jones *et al.*, 2001; Montoya and Liesenfeld, 2004; Kravetz and Federman, 2005).

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## Epidemiology

*T. gondii* is ubiquitous in nature and among the most common infections of humans. The seroprevalence in humans increases with age indicating past exposure. There is no significant difference in the prevalence between men and women. There are marked geographic

differences in the rate of seroprevalence that can be explained by differences in the exposure to either tissue cysts in meat or oocysts in cat feces. Whereas a high seroprevalence in France is most likely caused by consumption of undercooked meat, similar high rates in other geographic regions may be explained by high numbers of stray cats. The incidence of maternal infection estimated from population based cohort studies ranges from 1 to 8/1000 susceptible pregnancies, with the highest reported rates in France.

During the 1940s, there was improved understanding of the cause of maternal infection for congenital toxoplasmosis in newborns, and in 1953, Feldman reported a series of 103 children of which 99% had eye lesions, 63% had intracranial calcifications and 56% had psychomotor retardation (Feldman, 1953). This initiated interest in congenital infection among scientists in Europe (Couvreur, 1955). In Gothenburg, Sweden, 50% of mothers had had previous infection with *T. gondii* and two out of 23 260 children had clinical toxoplasmosis during a 1948–51 study period (Holmdahl and Holmdahl, 1955). A study from Austria reported frequent symptoms in children with congenital toxoplasmosis (Eichenwald, 1957). A French study concluded that treatment prevented transmission from mother to child and reduced the clinical symptoms in children (Couvreur and Desmonts, 1962), and another study from France showed that the seroprevalence in pregnant women in Paris was 85% with a high risk of *Toxoplasma* infection in sero-negatives (Desmonts *et al.*, 1965). This was followed by a larger study from France of 374 pregnancies (Desmonts and Couvreur, 1974).

The proportion of pregnant women who are susceptible to *Toxoplasma* infection ranges between 80% to 90% in northern Europe, 50% in France, and 10 and 20% in parts of Brazil (Guimaraes *et al.*, 1993). The prevalence of IgG antibodies in the childbearing age group indicating previous *Toxoplasma* infection, has fallen over the past 3 decades in many European countries, leaving more women susceptible to infection during pregnancy (Ades and Nokes, 1993). The overall rate of transmission of *T. gondii* from the mother to the fetus is approximately 30%. Almost all infections acquired shortly before or around the time of conception do not result in transmission to the fetus. Infections acquired in the first trimester will result in congenital infection in 10% to 25% of fetuses. The rate of transmission rises to 30% to 50% and 60% to 70% in women infected in the second and third trimester, respectively (Dunn *et al.*, 1999). The reason for this remains unknown. It might be simply due to the gradual increase in the size of the materno-fetal interface, which statistically facilitates transmission. Alternatively, there may be an augmented exchange activity of trophoblast cells in the course of pregnancy, which would render the fetus more susceptible to infection, e.g. by enhanced expression of adherence receptors.

The incidence of congenital transmission has previously been estimated either indirectly based on incidence rates of maternal infection and the overall transmission rate or directly based on clinical observations. However, these numbers do not allow an accurate determination of the incidence of congenital infection. More recently, serologic screening of newborns has been used to determine the incidence of congenital infection. These studies estimated the incidence of congenital infection to be approximately 0.1 to 1 per 1000 births (Remington *et al.*, 2000). However, screening of newborns is known to diagnose only between 70 and 80% of infected newborns. Systematic screening programs in pregnant women have allowed the determination of the incidence of congenital infection

in Europe. The birth prevalence of congenital toxoplasmosis ranges from  $< 1/10,000$  live births in Sweden and Massachusetts,  $3/10,000$  in Brazil, to an estimated  $10/10,000$  live births in France (Gilbert, 2000; Neto *et al.*, 2000; Evengard *et al.*, 2001).

## Physiopathology

Understanding the conditions which favor congenital infection is an elementary prerequisite for developing preventive measures, such as developing vaccines. However, while there is substantial knowledge regarding the control of systemic infection, there are only few data on the regulation of the transplacental passage of *T. gondii*. This is partly due to the relatively low number of human cases. On the other hand, human pregnancy is difficult to model in animal studies. In particular, the short duration of murine gestation of about three weeks restricts the value of such models. Nevertheless, the use of mouse and other small mammal models, gave valuable answers to specific questions. The existence of numerous transgenic and knockout strains allows selecting and investigating single mechanisms. For this reason, insights gained from such work in mouse models occupy a substantial part of this chapter.

## Control of materno-fetal infection

It is important to note that primary maternal toxoplasmosis does not necessarily result in fetal infection, as the transmission rate depends on the time point of infection during pregnancy. While only 9 percent of infections during the first trimester result in fetal infection, this number climbs to 27 percent and 59 percent for infections during the second and third trimester, respectively (Desmonts and Couvreur, 1979). This clearly implicates the existence of critical steps of transmission, which “decide” whether the parasite infects the fetus or not. Another striking characteristic of congenital toxoplasmosis is the protection of the unborn child when the mother had already been infected before pregnancy (Remington *et al.*, 2000). Several mechanisms have been implicated in this protection. Most of the studies focused on cell mediated mechanisms, but recent work show that antibodies also play a protective role. Clearly, this is difficult to investigate in humans. The importance of cell-mediated protective mechanisms can be deduced from a study which showed a limited, but visible risk of HIV infected women to pass on their *T. gondii* infection to their offspring (Minkoff *et al.*, 1997). Despite the obvious discrepancies, animal studies gave some insights in the mechanisms at play. The mouse strain BALB/c shares central features with the human case of congenital toxoplasmosis. First, primo-infection of the mother during pregnancy also results in about 50 per cent of transmission. Second, this infection confers resistance to materno-fetal infection during subsequent infections (Roberts and Alexander, 1992). Vaccination with soluble *T. gondii* antigen conferred a certain degree of protection for the fetus (Roberts *et al.*, 1994). This was associated with an enhanced Th1-type immune response. The importance of such a Th1-type immune response, especially mediated by CD8+ cells and IFN- $\gamma$  production, has been extensively proven (Denkers and Gazzinelli, 1998). However, data obtained in non pregnant mice have to be extrapolated carefully to congenital models, since pregnancy modifies the balance between Th1 and Th2 immune responses by generating a Th2 environment essential to maintain pregnancy (Ng *et al.*, 2002). The interaction between genetic background, pregnancy and stimulation of

the immune system was evident in a vaccination study using two different mouse strains (Letscher-Bru *et al.*, 2003). Vaccination with the major surface protein, SAG1, in BALB/c mice resulted in a mixed Th1–Th2 response and conferred partial protection to congenital infection. In contrast, the same vaccination protocol induced in another mouse strain, CBA/J, a biased Th2 response to SAG1, and resulted in an even increased materno-fetal transmission of *T. gondii*. Consequently, a finely tuned balance between the anti-parasitic Th1 response and the pregnancy induced Th2 response has to be achieved when developing vaccines.

### Cellular immune response and transmission

When looking for the mechanisms which control parasite transmission, the role of the cellular arm of the immune system becomes evident (see Chapter 6). Protection against materno-fetal transmission is diminished when CD8+ cells are depleted or IFN- $\gamma$  is neutralized (Abou-Bacar *et al.*, 2004b). This underlines the importance of CD8+ cells as IFN- $\gamma$  producing cells for protection of congenital toxoplasmosis, which has been shown before in vaccination studies of non-pregnant mice (Denkers, 1999). CD4+ cells, while not completely redundant, seem to play a minor role in such recall responses.

However, while CD8+ cells play a central role for a secondary response, as shown by the vaccination and reinfection studies, they might not be as important in the case of a primary infection. In the absence of immunologic memory to *T. gondii* infection, mechanisms of the innate immune response occupy the central place. Importantly, knock-out BALB/c mice (RAG-2<sup>-/-</sup>), which are unable to produce T- and B-cells, showed an enhanced splenocyte production of IFN- $\gamma$  in response to *T. gondii* infection thought to be due to greater NK cell production relative to the BALB/c control mice (Abou-Bacar *et al.*, 2004a). Cell enumeration revealed considerably enhanced numbers of circulating NK cells, and other studies have shown that this cell type is very important for a quick reaction to *T. gondii* infection through IFN- $\gamma$  production (Sher *et al.*, 2003) and by inference from *in vitro* studies, cytotoxic activity (Hauser and Tsai, 1986). Notably, congenital *T. gondii* transmission rates in RAG-2<sup>-/-</sup> mice were significantly lower than in the BALB/c controls. Curiously, whereas depletion of NK cells enhanced parasite transmission in the RAG-2<sup>-/-</sup> compared to the BALB/c controls, neutralization of IFN- $\gamma$  appears to decrease the risk of transmission despite an increased maternal parasite burden in both cases. This apparent conundrum highlights the need for further investigation into the effects of cytokine secretion and cytotoxic activity by cells in the innate and adaptive immune responses during *T. gondii* infection/congenital transmission.

### Structure of the placenta

Before discussing the role of the placenta in the transmission of *T. gondii*, it is useful to get an idea of the placental structure, with regards to control of pathogen transmission. The placenta is a unique organ that is formed both by maternal and by fetal cells. The main function of this complex structure is to ensure exchange of nutrients and waste products between mother and fetus while avoiding adverse reactions of the mother's immune system towards the fetus. Given the transient nature of the placenta, this has to be a dynamic process. Following implantation of the conceptus in the uterus, trophoblast progenitor

cells start to invade the uterine tissue and the maternal blood vessels. There, they replace the maternal endothelial cells, which allow the fetus to control the blood flow in the uterine wall. For a more detailed insight of the placentation process, numerous reviews describe the actual knowledge (Staun-Ram and Shalev, 2005). For our purpose, it is important to state that a syncytium of trophoblast cells ultimately form an impermeable barrier between maternal blood and fetal tissues. Only very few pathogens are able to cross this border, mostly viruses and bacteria, but, outstanding among protozoan parasites, also *T. gondii*. To explore the mechanisms at play, mouse models were established for different pathogens. The placentae of mice and humans have in common their hemochorial structure with the fetal trophoblasts bathing directly in maternal blood (Georgiades *et al.*, 2002). The resulting direct exposure of placental cells to cells of the maternal immune system poses a problem because they should naturally be treated as immunologically “non-self” and thus being rejected. The entire process is far from being elucidated, but the present knowledge reveals a highly complex construction designed to suppress anti-fetal immunity within the placenta (Moffett and Loke, 2004). A central feature is the absence of classical MHC molecules and the expression of non-polymorphic MHC types, like HLA-G by trophoblast cells (Hunt *et al.*, 2005).

#### The role of the placenta in *T. gondii* transmission

The above results generally show that regulation of materno-fetal transmission is correlated with parasite density in the maternal peripheral blood. However, it is always important to bear in mind the above mentioned differences of infection in pregnant and non-pregnant mice. It is evident that the placenta, owing to its capacity to secrete hormones, cytokines and chemokines, not only assures maternal tolerance towards the fetus, but indeed actively participates in the regulation of the systemic immune response (Szekeres-Bartho, 2002). Additionally, some data directly show the existence of a placental barrier function which is independent from parasite control in the periphery. A study using DNA vaccination with SAG1 found a protective effect for the mother, but no reduction in *T. gondii* transmission to their offspring (Couper *et al.*, 2003). Furthermore, the above mentioned study using knock-out BALB/c mice (Abou-Bacar *et al.*, 2004a), revealed an unexpected finding. When IFN- $\gamma$  was completely neutralized by the use of a monoclonal antibody, a considerable increase of parasite numbers in the mothers' peripheral blood was observed, whereas the materno-fetal transmission rate was diminished. *In vitro* studies on the human trophoblast cell line BeWo suggest that IFN- $\gamma$  is necessary for adhesion of *T. gondii* infected monocytes, thereby facilitating materno-fetal transmission (Pfaff *et al.*, 2005a). Combining *in vivo* and *in vitro* results, the parasite therefore depends on the immune system and its production of IFN- $\gamma$  to facilitate its transmission to the fetus. Following infection, trophoblast cells are not able to limit *T. gondii* multiplication when stimulated by IFN- $\gamma$ , in contrast to most other cell types (Pfaff *et al.*, 2005b). This shows, once again, the delicate balance between infection control and pregnancy maintenance.

In a mouse model of congenital toxoplasmosis, infection of the placenta precedes fetal infection. Furthermore, immunohistochemical studies in a rat model suggest that the parasite first infects placental trophoblast cells, which form a barrier between maternal blood and fetal tissue. The fetal part of the placenta, and ultimately the fetus itself, are

then infected (Ferro *et al.*, 2002). Consequently, trophoblast cells play a major role for materno-fetal infection. However, the very small numbers of parasites actually seen in such immunohistochemical studies makes it very difficult to draw conclusions. Trophoblast cells have been proven to support productive *T. gondii* infection (Abbasi *et al.*, 2003), but so do virtually all cell types. Other ways of transmission, for example through breaches in the trophoblast layer, are not excluded.

Human studies also gave some insights. When placental samples of mothers who had acquired *T. gondii* infection during pregnancy were checked at birth, some placentae proved to be positive in the absence of fetal infection (Fricker-Hidalgo *et al.*, 1998; Ajzenberg *et al.*, 2002). This proves the barrier function of the placenta. However, it is often difficult to draw conclusions from findings in term placentae, when the actual infection may date several months back. In fact, the same studies found a considerable number of *T. gondii* negative placentae despite proven transmission to the fetus. *T. gondii* cysts were detected in sheep placentae (Dubey, 1987), and it is probable that the parasite is also able to form cysts in human placentae. However, nothing is known about the frequency or relevance of such parasite persistence in the placenta.

In summary, the available means of investigation and model systems revealed some details of how materno-fetal *T. gondii* transmission is controlled. Although many questions remain unresolved, current evidence suggests that the placenta is actively involved in transmission control and that transmission occurs rapidly following infection.

### Physiopathological characteristics of congenital toxoplasmosis

The immune system of the fetus and the newborn is, of course, far from being perfect. Therefore, any infection contracted during this critical time, even those which are harmless for adults, consist a serious threat to health and life of the infected individual. Toxoplasmosis is a remarkable example for such opportunistic diseases. When contracted in the mother's womb, *T. gondii* disseminates quickly throughout the defenseless body, causing severe lesions in multiple organs, brain and eye being the most affected organs. However, even when the symptoms develop only after birth, or even during adolescence, patients having acquired the parasite *in utero* react immunologically different to the infection, compared to those infected after birth. Population-based studies suggest that patients with ocular disease due to congenital infection have diminished anti *T. gondii* responses (Yamamoto *et al.*, 2000; Vallochi *et al.*, 2002). This shows that the parasite, when present at such early time of development, is able to condition the host's immune system to its own advantage, i.e. to render it tolerant towards its antigens.

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### Clinical disease and clinical diagnosis of congenital *Toxoplasmosis*

Congenital infection may manifest itself either as subclinical or symptomatic disease. Infection may remain subclinically or affect the previously undiagnosed individual during infancy, childhood, or adolescence. In contrast, severe disease may occur in the neonatal period or during the first months of life. These different forms and the clinical manifestations vary depending on the trimester when the infection was acquired. There is an inverse relationship between the rate of transmission and the severity of the infection (Hohlfeld *et*



*al.*, 1994; Dunn *et al.*, 1999). Clinical manifestations of congenital toxoplasmosis are most severe before week 26 of gestation (Dunn *et al.*, 1999); the central nervous system is commonly affected, non-specific signs include retinochoroiditis, strabismus, blindness, epilepsy, psychomotor or mental retardation, encephalitis, microcephaly, intracranial calcification, hydrocephalus, anemia, jaundice, rash, and petechiae due to thrombocytopenia (Remington *et al.*, 2000). Those newborns infected in the third trimester may be asymptomatic at birth, but sequelae may develop later in life. A recent European multicenter study found that out of 244 newborns with congenital toxoplasmosis, 19 had cerebral calcifications and that treatment within four weeks of infection reduced the risk (Adjusted Odds Ratio 0.28; CI 0.08–0.75) (Gras *et al.*, 2005). The same study found retinochoroiditis in 12% (30/255) of newborns with congenital toxoplasmosis.

Most infants with subclinical infection at birth subsequently develop signs or symptoms of congenital toxoplasmosis. Retinochoroiditis is commonly the initial manifestation, appearing in most cases between 3 and 4 years of age. However, similar signs and symptoms of congenital toxoplasmosis may be observed in patients infected with viral (rubella virus, CMV, *Herpes simplex virus*), bacterial (including *Treponema pallidum*, *Listeria monocytogenes*), and other infections affecting the brain. Chorioretinitis is frequently caused by *Herpes simplex virus*, CMV, and rubella virus. A markedly elevated CSF protein concentration is a hallmark of congenital toxoplasmosis.

The diagnostic work-up of all cases of proven or suspected congenital infection in newborns should include physical and neurological examination, pediatric ophthalmologist examination of the retinae, ultrasound examination of the head, and a variety of microbiological tests (see below). Ultrasound examination of the head may reveal uni- or bilateral ventricular dilatation and calcification.

During pregnancy, sonographic detection of fetal abnormalities can assist in the diagnosis of fetal infection (Hohlfeld *et al.*, 1989). However, the sensitivity is relatively low (Pratlong *et al.*, 1994). In rare cases, ventricular dilatation can be detected by fetal ultrasound in patients in whom other direct diagnostic tests, including PCR on amniotic fluid, see below, gave negative results (Gay-Andrieu *et al.*, 2003). Since fetal abnormalities may be detected by ultrasound examination, repeated ultrasound examinations are indicated during the course of pregnancy. The decision to terminate the pregnancy may be based on fetal abnormalities detected in ultrasound examinations; necropsy should then be performed to confirm the etiology of congenital toxoplasmosis.

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### **Biological diagnosis of congenital *Toxoplasmosis***

Clinical and therapeutical consequences of the detection of a seroconversion must be carefully considered because the majority of the children are not infected at birth. Tools for an efficient prenatal and postnatal diagnosis of congenital infection are now affordable and ensure an early diagnosis and treatment, reducing the sequelae of the disease in newborns (Foulon *et al.*, 1999).

## Prenatal diagnosis

The prenatal diagnosis is based on ultrasound examination follow-up, and amniocentesis. Ultrasound examination allows to observe potential defects (see clinical part of the chapter).

Empirical data suggest that amniocentesis should be done one month after the putative maternal infection, to allow for the delay of transplacental passage, but in any case not before week 18 of pregnancy. Of course, the mothers are treated during that period, as treatment probably decreases the rate of clinical signs in the fetus (Foulon *et al.*, 1999), that point being, however, still controversial (Thiebaut *et al.*, 2006; SYROCOT *et al.*, 2007). PCR amplification is now the gold standard for prenatal diagnosis. Two targets are used: the 35-fold repetitive B1 gene and the 300-fold repetitive AF146527 (Filisetti *et al.*, 2003). The sensitivity of the AF-PCR is estimated to be 64% with a negative predictive value of 87.8% and specificity and positive predictive value of 100% (Romand *et al.*, 2001). The use of AF-PCR quantification of *T. gondii* in maternal blood may improve prognosis of fetal infection. Parasite loads greater than 100 per mL are associated with an elevated risk of severe fetal outcome (Romand *et al.*, 2004). Mouse inoculation, used as a back-up method, slightly enhances the sensitivity, compared to PCR alone, and may allow the isolation of the strain for genotyping (Ajzenberg *et al.*, 2002; Filisetti *et al.*, 2003). Therefore, a negative result does not exclude congenital toxoplasmosis, especially if contamination has occurred during the first or the third trimester. In contrast, positive PCR and/or mouse inoculation proves fetal infection, and treatment based on pyrimethamin/sulfadiazine should be established.

## Neonatal diagnosis

### Introduction

Specific postnatal diagnosis of CT is crucial for the newborns from mothers infected or supposed to be infected during pregnancy, but for whom the antenatal diagnosis was negative or not done. Parasitological and immunological diagnosis is easier postnatally than *in utero*, owing to the better accessibility of appropriate samples (cord blood, placenta, serum, etc.). However, CT is mainly subclinical, especially in countries with effective screening programs (France, Austria), where treatment of the fetus reduces the risk of major complications. Early postnatal diagnosis is of paramount importance to identify infants qualifying for aggressive treatment, in order to reduce the incidence of ocular sequelae later in life (Kodjikian *et al.*, 2006).

### Parasite detection

At birth, the sensitivity of parasite detection is poor, ranking between 25% and 61% (Fricker-Hidalgo *et al.*, 1998). Detection of the parasite is always done in multiple samples: placenta, cord blood, amniotic fluid (if available), having in mind that an isolated detection of *Toxoplasma* in placenta does not confirm a congenital toxoplasmosis. Other samples, such as cerebrospinal fluid (CSF) and necropsy samples are anecdotic; however, detection of the parasite in such samples is always the proof of CT.

### *Immunological diagnosis*

The use of immunological methods is of paramount importance for definitive postnatal diagnosis. The diagnosis is based on the detection of neosynthesized antibodies. Infant's serum contains maternal antibodies transmitted through the placenta during gestation. Only IgG crosses the placenta, but IgM and IgA may leak through the placenta during labor and can be found in the newborn's serum. Therefore, it is crucial to monitor the disappearance of passively acquired Ig of maternal origin. Mean antibody half-lives are 23 days for IgG, 5 days for IgM and 6 days for IgA. Therefore the detection of IgM and IgA in the infant's blood from 15 days to 3 months after birth is a proof of CT. The date of final clearance of maternal IgG antibodies depends on the initial level at birth. Maternal IgG usually disappears after 5 to 10 months of life. Given the IgG half-life, titers should fall by approximately one-half every month. Recently, the identification of neosynthesized antibodies has been highly improved by the use of comparative mother–infant methods which allows distinguishing neonate antibodies (IgG, IgM and IgA) from passively or accidentally transmitted maternal Ig. These new methods have allowed to exceed 90% of sensitivity in the detection of CT (Pinon *et al.*, 2001).

The first sample should be cord blood for both parasite detection (see above chapter on prenatal diagnosis) and antibody detection. Sequential infant blood sampling should be done 15 days later and one month after birth. Thereafter, monthly testing is sufficient and allows monitoring the decay of passively transferred maternal antibodies for serologic rebound due to neosynthesis by the infant.

There is a wide spectrum of methods for antibody detection; however only a few are suitable for diagnosis of CT. Schematically, we can distinguish non comparative and comparative antibody detection methods.

A non-comparative method is a method which measures total levels of neonatal anti-*Toxoplasma* antibodies, IgG, IgM, IgA and IgE, independently from the maternal sample (Montoya, 2002; Montoya and Liesenfeld, 2004). These methods require quite often modifications, such as lesser serum dilution or lower cut off (Pinon *et al.*, 2001). For detecting IgG, ELISA is the method of choice for precise and reproducible quantification in international units (IU/mL). For IgM detection, ISAGA and ELISA are mostly used with modifications with a reported sensitivity of 81.1% and 64.8%, respectively. ELISA may be also a useful method for detection of IgA or IgE, a marker of poor adherence or inadequate treatment. The simultaneous measurement of the three isotypes, IgM, IgA and IgE, may improve the diagnosis at birth (Foudrinier *et al.*, 2003). On the other hand, IgM Immunoblotting (IB) gave better results than the other two methods used for IgM detection (Pinon *et al.*, 2001).

Owing to the fact that the placenta allows the passage of maternal IgG (and sometimes IgM and IgA during labor, making new-born blood non contributive for the first ten days), methods based on comparative mother-infant and then infant-infant immunological profiles are of paramount importance during the first three months of life. This passage of maternal Ig considerably hinders and delays the immunological diagnosis of CT. Fortunately, with current methods, we are able to identify neosynthesized antibodies by comparing an infant sample with the corresponding maternal sample at birth, and subsequently by following consecutive infant samples. There are three methods: calculation of

the specific antibody load, immunoblotting (IB) and Enzyme Linked Immuno Filtration Assay (ELIFA).

Historically, levels of specific IgG production were compared between mother and newborn. This approach compares toxoplasmic IgG levels expressed in IU/mL to total non specific IgG levels and allows to calculate the “specific antibody load” (Wong and Remington, 1994), which may be used to detect increased antibody production in infants due the neosynthetized antibodies. This quantitative method should be employed if methods for comparing mother-infant profiles are not available.

IB combines electrophoresis of toxoplasmic antigens under denaturing conditions, electrotransfer to a nitrocellulose membrane and a specific antibody assay. The result is a recognition band pattern of *T. gondii* antigens by the infant sera. The presence of bands in the infant's pattern which are absent in the mother's pattern points to antibody neosynthesis. IgG IB and IgM IB reach sensitivities of 65.4% and 68.5% and specificities of 96% and 98%, respectively. However, false positive results due to natural IgM limit the use of this method after three months of age. Combined detection of IgG and IgM by IB gives an enhanced sensitivity of 78%, with a specificity of 96%. Classical IB using one-dimensional electrophoresis (1DIB) may be improved by using a two-dimensional electrophoresis (2DIB). 2DIB allows to characterize more than 1000 *Toxoplasma* epitopes, compared to the 50 bands of the 1DIB. 2DIB clearly enhances the sensitivity of CT diagnosis (Nielsen *et al.*, 2005). On the other hand, specificity as well as sensitivity may be improved by the use of recombinant antigens, such as MIC2 and SAG1 (Buffolano *et al.*, 2005).

ELIFA is an elegant method that employs a microporous cellulose acetate membrane in a co-immunoelectrodiffusion procedure to simultaneously study antibody specificity by immunoprecipitation and antibody isotypes by immunofiltration through the membrane of an enzyme labeled antibody. The profile allows to investigate four parameters in mother–infant or infant–infant serum pairs: (i) the number of precipitating arcs, (ii) the isotypes (IgG or IgM), (iii) the comparative specificity by continuity (coalescence) of the arcs present in the two serums samples revealing the same antigen involved in the precipitate and (iv) the relative amounts of antibodies of the same specificity in each sample. Neosynthesized antibodies are revealed by the presence of specific antibodies present in neonatal serum and absent in the maternal blood, or by higher concentration of neonatal antibodies with a specificity identical to that of the maternal antibodies. By combining IgG ELIFA and IgM ELIFA, sensitivity reaches an excellent 88.8% with a specificity of 100%.

### *Interpretation of the results*

Detection of the parasite in the placenta does not unequivocally demonstrate transmission of *Toxoplasma* to the fetus. Only detection of the parasite in cord blood, CSF, newborn blood or other tissues within the first 6 months of life can prove CT. In case of positive antenatal diagnosis, confirmation of diagnosis at birth is not always obtained. Indeed, treatment with pyrimethamine and sulfadoxine may have eradicated the placental infection, decreased parasitemia and attenuated the immunological responses. Given the possibility of false positive results of antenatal diagnosis, post-natal follow-up is still crucial to confirm antenatal diagnosis.

If antenatal screening and postnatal detection of parasite are negative, apart from clinical signs the following parameters unequivocally confirm CT during an immunological follow-up:

- Detection of neosynthesized IgG by comparative mother-infant or infant-infant immunological profiles within the first 6 months of life.
- A rise in antitoxoplasmic IgG titers within the first 12 months of life.
- A lack of decrease in specific IgG titers.
- Detection of antitoxoplasmic IgM and/or IgA within the first 6 months of life.

Most often, during postnatal follow-up, the naturally transmitted maternal antibodies disappear after 5 to 10 months of life. This first negative antibody test does, however, not prove the absence of parasite transmission to the fetus, the negative result should be absolutely reconfirmed three months later, without treatment during the interval.

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## Treatment

Therapy of congenital toxoplasmosis is always a combination therapy. Most drugs against *T. gondii* only interact with the tachyzoite form and do not eradicate cysts. Pyrimethamine as an antagonist of folinic acid is considered the most effective anti-*Toxoplasma* agent. Suppression of the bone marrow is the main side effect. All patients treated with pyrimethamine should have a peripheral blood cell and platelet count, initially twice weekly. Folinic acid is therefore always associated. Pyrimethamine is potentially teratogenic and should not be used during the first trimester of pregnancy. Sulfadiazine is in most cases used as the second drug and acts synergistically with pyrimethamine. The most common side effects associated with sulfadiazine are skin rashes with electromyographic abnormalities and elevated hepatic enzyme serum levels.

### Congenital infection

A variety of treatment schedules have been described to treat newborns with congenital toxoplasmosis. The continuous combination of pyrimethamine (2 mg/kg/day for 2 days, then 1 mg/kg/day for 2 to 6 months, then 1 mg/kg/day three times a week), sulfadiazine (50 mg/kg twice daily), and folinic acid (10 mg three times weekly) is most widely used (McAuley *et al.*, 1994). The optimal duration of therapy is not known. Most investigators recommend a minimum of 12 months. The administration of pyrimethamine-sulfadiazine-folinic acid, alternated with spiramycin (100 mg/kg/day) has also been described. Improvements include gains in intellectual function, regression of retinal lesions, reduction in anticonvulsant drug requirements, and prevention of auditory sequelae. The efficacy of spiramycin is unknown. Therefore, spiramycin is only used in the setting of acute acquired infection in pregnant women.

### Acute acquired *Toxoplasma* infection in pregnant women

Treatment of an acutely infected pregnant woman does not eliminate but does seem to decrease the incidence of fetal infection. In the past, spiramycin has been accepted by most investigators as being effective in reducing the frequency of maternal transmission of *T.*

*gondii* to the fetus by approximately 60% (Forestier *et al.*, 1991). Recent data from Europe, however, indicate that spiramycin may not reduce the rate of transmission of the parasite from the mother to the fetus. If fetal infection is documented or acute infection of the mother is highly suspected, the combination of pyrimethamine (25 mg/day), sulfadiazine (1 g four times a day), and folinic acid (5 to 15 mg/day) is administered. Pyrimethamine must not be used in the first 12 to 14 weeks of pregnancy because of its teratogenic effects. In recent studies, prenatal treatment did not appear to have reduced the risk of mother-to-child transmission (Foulon *et al.*, 1999; Gilbert *et al.*, 2001a; Gilbert *et al.*, 2001b; Gilbert and Gras, 2003; SYROCOT *et al.*, 2007). However, since these studies included only few untreated women, and untreated women were mostly infected during the third trimester, a definitive conclusion regarding the efficacy of prenatal treatment is impossible at present.

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### Risk factors and preventive measures

Infection in humans most commonly occurs through the ingestion of raw or undercooked meat that contains tissue cysts, through the ingestion of water or food contaminated with oocysts, or congenitally through transplacental transmission from a mother who acquired the infection during gestation. Less common is transmission by transplantation of an infected organ or transfusion of contaminated blood cells. Transmission has also occurred through laboratory accidents.

Although raw or undercooked meat containing tissue cysts appears to be the main source of infection in Europe, the relative contribution of this route of infection compared to the risk by ingestion of oocysts is unclear. A French study found that only 17% of women who knew that they were susceptible and had received health information reported any action to avoid infection (Wallon *et al.*, 1994). A case-control study in six European centers identified undercooked meat and cured meat products as the principal factor contributing to between 30% and 63% of infections in pregnant women. Contact with soil contributed to a substantial minority of infections (6% to 17%) (Cook *et al.*, 2000), and soil contamination of unwashed fruit and vegetables has been reported as a risk factor in other studies (Kapperud *et al.*, 1996). Game animals and sheep are showing high rates of infection, rates of infection in pigs and cattle are lower. Recently, water has been described as a potential source for *T. gondii* infection in humans and animals (Bowie *et al.*, 1997; Miller *et al.*, 2002; Bahia-Oliveira *et al.*, 2003).

Since the routes of infection described above indicate that infection with *T. gondii* is a preventable disease, education of pregnant women on how to prevent infection is of utmost importance. Contact with all materials potentially contaminated with cat feces (i.e. handling of cat litter and gardening) should be avoided, alternatively, gloves may be used. Contact of mucous membranes with raw meat should be avoided and hands, kitchen surfaces, and utensils should be washed after contact with raw meat. Meat should be cooked "well done" and fruits and vegetables should be washed before consumption.

Systematic, prenatal serologic screening is performed in Austria, France, Greece, and Slovenia. When seroconversion is detected in the mother, treatment is started. Neonatal screening for congenital toxoplasmosis is performed in New England, Denmark and parts of Brazil by analyzing the blood samples obtained on filter paper (Guthrie cards) (Guerina



*et al.*, 1994; Lebech *et al.*, 1999; Neto *et al.*, 2004). Positive titers in IgM antibody tests result in further confirmatory diagnostic tests in mother and infant.

## Conclusion

A strict education of *Toxoplasma* seronegative pregnant women should be promoted for primary prevention of CT (SYROCOT *et al.*, 2007). In parallel, the use of serological tools for an early diagnosis of maternal *Toxoplasma* infection is mandatory. In case of maternal *Toxoplasma* infection, prenatal and postnatal diagnosis of CT should be started with the optimal set of methods as soon as possible. Consequently, more than 90% of CT cases will be diagnosed during the first three months of life (Pinon *et al.*, 2001; Rilling *et al.*, 2003). Early pre and postnatal diagnosis of CT will allow early pyrimethamine–sulfadoxine treatment which will reduce the clinical complications (McAuley *et al.*, 1994; Roizen *et al.*, 1995; Foulon *et al.*, 1999; Gras *et al.*, 2005). Finally, more relevant studies, both fundamental and clinical, should be promoted for the understanding of transplacental passage of *Toxoplasma*, on physiopathology of CT, especially ocular pathogenesis, and on more accurate epidemiological data in order to evaluate the efficacy of preventive and therapeutic measures.

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# Innate Recognition and the Regulation of Protective Immunity to *Toxoplasma gondii*

6

Marion Pepper and Christopher A. Hunter

## Abstract

Most infections with *T. gondii* are relatively asymptomatic as a consequence of the development of protective immunity that allows long-term control of this persistent organism. However, this pathogen can cause severe disease in immune competent individuals and is a major opportunistic infection in patients with primary or acquired defects in T cell function. The study of these events has led to the identification of the innate and adaptive components required to control *T. gondii* and highlighted the importance of cell-mediated immunity in resistance to intracellular pathogens. This infection has also provided a model to study general immuno-regulation and the balance between protective and pathological T cell responses. This chapter reviews our current understanding of immunity to *T. gondii* and outlines some of the prominent questions in this area of research.

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## Introduction

Since the initial description of *T. gondii* as a pathogen of the fetus and then as an opportunistic infection in the immunocompromised host there has been an interest in understanding the events that lead to the development of clinical disease. The ability of the tachyzoite stage of *T. gondii* to invade and replicate within any nucleated cell, eventually leading to lysis, is an important element of the pathogenesis of this infection. Unrestricted parasite replication has the capacity to cause damage to any tissue type and can lead to acute virulence (Chapter 9). Moreover, because this organism has a specialized developmental stage, the tissue cyst, that allows parasite persistence and which can lead to recrudescence of infection there is a need for the long-term control of this infection. Nevertheless, as a consequence of the immune response, individuals infected with *T. gondii* rarely develop clinical toxoplasmosis. The study of these events has led to the identification of the cell types and effector functions required to control toxoplasmosis and highlighted the critical role of IL-12-mediated production of IFN- $\gamma$  in resistance to intracellular pathogens. More recently this infection has provided a system to model general immuno-regulation and the balance between protective and pathological T cell responses. This chapter will review the studies that have led to our current appreciation of the factors involved in innate and adaptive immunity to *T. gondii*.

## Innate resistance to *Toxoplasma gondii*

### Establishment of infection

The ingestion of oocysts or tissue cysts of *T. gondii* results in the release of the parasite in the small intestine where it crosses the gut and replicates within the lamina propria in enterocytes and lymphocytes (Speer and Dubey, 2005). Within several days, tachyzoites can be found in multiple tissues, a consequence of parasite dissemination via the blood and lymphatics. Indeed, although *T. gondii* is an obligate intracellular pathogen, the tachyzoite is motile and virulent strains of *T. gondii* can transmigrate through the gut for longer distances than avirulent strains (Barragan *et al.*, 2005; Barragan and Sibley, 2002). Recently, it has been suggested that this organism can use the host immune system to facilitate its spread. It has been reported that this parasite has chemokine-like activities that recruit immature dendritic cells (DC) (Diana *et al.*, 2005) and there is evidence that enterocytes contribute to infection-induced production of chemokines at the site of infection within the small intestine (Luangsay *et al.*, 2003). These soluble factors recruit inflammatory cells to the site of infection which in turn amplify chemokine production within hours of infection (Bennouna *et al.*, 2003; Robben *et al.*, 2005). Thus, early events promote the influx of monocytes and DC into the lamina propria and there is evidence that these cells act as Trojan horses to disseminate *T. gondii* to distant sites including the brain (Courret *et al.*, 2006).

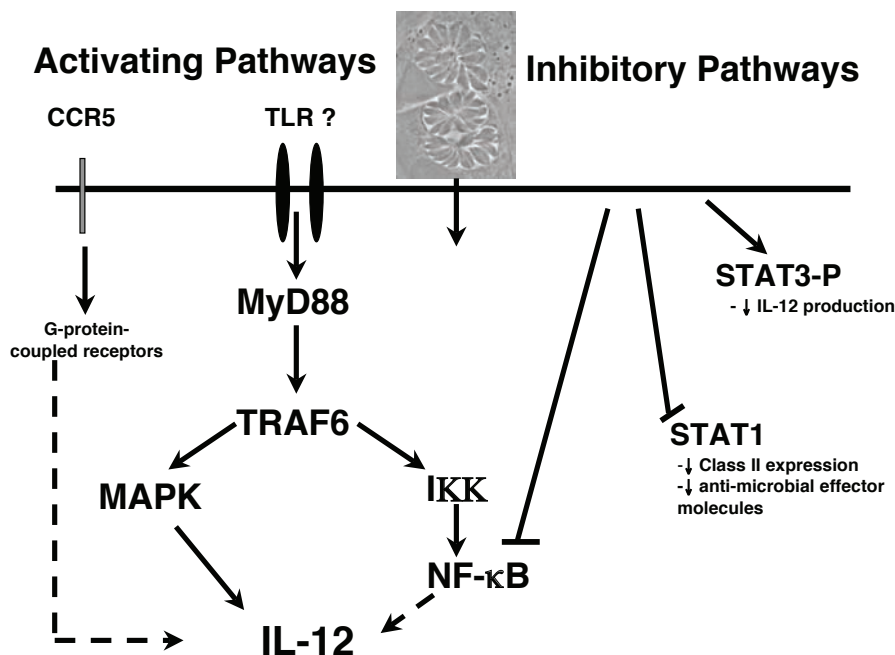
### The role of IL-12 in the activation of NK cell-mediated resistance to *T. gondii*

The production of IFN- $\gamma$ , by NK and T cells, has a major role in mediating resistance to *T. gondii* and accessory cell production of IL-12 is a critical step that promotes these responses. Thus, the absence of IL-12 results in reduced IFN- $\gamma$  levels and failure to control parasite replication (Gazzinelli *et al.*, 1993b; Hunter *et al.*, 1994; Khan *et al.*, 1994). IL-12 deficient animals therefore succumb to infection with similar kinetics to IFN- $\gamma$  deficient animals. Initial reports demonstrated that macrophages were the major source of IL-12 during toxoplasmosis (Gazzinelli *et al.*, 1993b), however injection of soluble *Toxoplasma* antigens (STAg) established that *in vivo*, DCs make IL-12 (Aliberti *et al.*, 2000; Reis e Sousa *et al.*, 1997). Further work indicated that neutrophils, a prominent cell type recruited within hours to the site of infection, contained pre-stored IL-12 that is released in response to *T. gondii* (Bliss *et al.*, 1999). Consistent with this observation, depletion of neutrophils in infected mice using an anti-GR-1 antibody antagonized IFN- $\gamma$  responses, had a profound effect on infection-induced activation of DC and resulted in susceptibility to acute infection (Bennouna *et al.*, 2003; Bliss *et al.*, 2001; Sayles and Johnson, 1996). While GR-1 is widely used as a marker for neutrophils, these studies are complicated by the expression of this molecule on monocytes, CD8 $\alpha^+$  T cells and certain DC populations (Asselin-Paturel *et al.*, 2003). While it is unclear what the exact temporal sequence of IL-12 production may be *in vivo*, these studies indicate that multiple cell types are involved in the production of IL-12 following challenge with *T. gondii*.

## Proximal signaling events leading to IL-12 production

Given the importance of IL-12 for resistance to *T. gondii* there has been a concerted effort to understand how cells of the innate immune system recognize this organism. Figure 6.1 outlines some of the interactions of *T. gondii* with host cell signaling pathways. Initial studies identified a parasite-derived cyclophilin that bound to the chemokine receptor CCR5 and stimulated DC production of IL-12 and reported that in the absence of CCR5 there is decreased IL-12 and increased susceptibility to *T. gondii* during chronic infection. Interpretation of these latter results is complicated by the requirement for CCR5 in the migration of CD8<sup>+</sup> T cells to sites of *Toxoplasma*-induced inflammation (Luangsay *et al.*, 2003). In addition, there are emerging data that suggest that G-protein coupled signaling inhibits IL-12 production.

The other major pathway involved in recognition of *T. gondii* involves Toll Like Receptors (TLRs), a family of evolutionarily conserved pattern recognition receptors that recognize different pathogens (Medzhitov, 2001). Following infection, mice lacking MyD88, an essential adapter protein in the TLR signaling pathway, have a major defect in their ability to make IL-12 and are highly susceptible to acute toxoplasmosis (Hitziger *et al.*, 2005; Scanga *et al.*, 2002). Furthermore, mice lacking TRAF-6, another molecular scaffold downstream of MyD88, are unable to produce IL-12 in response to *T. gondii* antigens (Mason *et al.*, 2004b). In contrast, several studies have established that mice that lack either TLR1, 2, 4, 6, 9 or 11 are not acutely susceptible to toxoplasmosis (Chen *et al.*,



**Figure 6.1** Activation and inhibition of pro-inflammatory signals by *T. gondii*. Infection activates CCR5 and/or TLR to signal the MAPK-dependent production of IL-12. The ability of this parasite to interfere with NF-κB and STAT1 activation and induce STAT3 activity are associated with inhibition of pro-inflammatory events (Chapter 7).

2002; Hitziger *et al.*, 2005; Mun *et al.*, 2003; Yarovinsky *et al.*, 2005), but the identification of a parasite profilin as a ligand for murine TLR 11 and increased susceptibility of TLR11 deficient mice to *T. gondii* indicates that this molecule represents a major receptor involved in these events (Yarovinsky *et al.*, 2005). Although the absence of CCR5 or any single TLR does not recapitulate the phenotype of the MyD88–/– mice it could be argued that a combination of these receptors are required for the high levels of IL-12 produced during this infection.

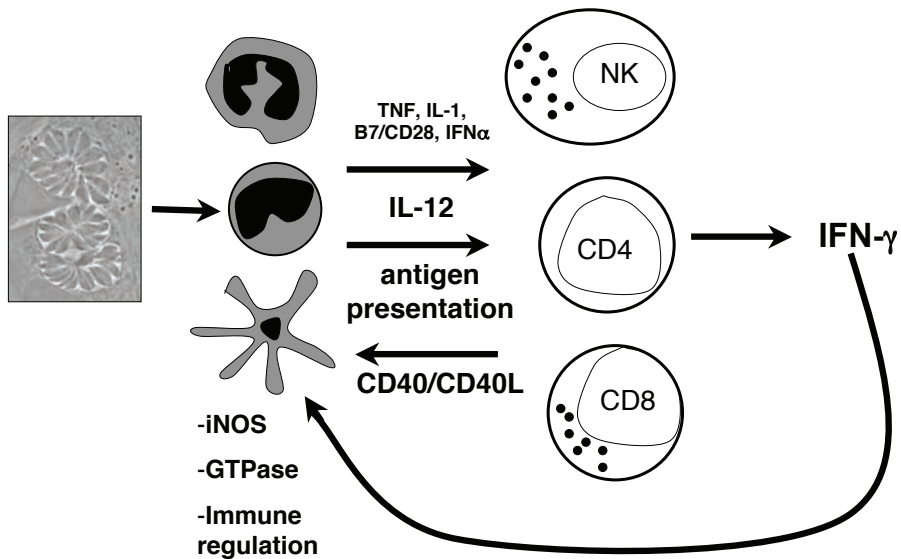
While the most proximal events leading to the production of IL-12 in innate cells are still being studied, some of the distal pathways are better understood. Downstream of MyD88 and TRAF6, signaling branches towards the NF- $\kappa$ B or MAP kinase pathways. Both of these cascades have important roles in innate immunity and in the regulation of IL-12 production and although virulent strains of *T. gondii* or parasite antigens fail to activate NF- $\kappa$ B (Butcher *et al.*, 2001; Shapira *et al.*, 2005; Shapira *et al.*, 2002, Chapter 7) there is evidence that avirulent strains of *T. gondii* can directly activate low levels of NF- $\kappa$ B (Robben *et al.*, 2004). However, infection-induced production of IL-12 by either type of parasite is independent of multiple NF- $\kappa$ B family members (Mason *et al.*, 2004a). In contrast, MAP kinases represent the most dominant pathway for *Toxoplasma*-induced synthesis of IL-12 since stimulation of macrophages with STAg or live organisms results in the transient activation of MAP kinases and inhibitors of p38MAPK prevent the subsequent production of IL-12 (Butcher *et al.*, 2001; Kim *et al.*, 2005; Mason *et al.*, 2004b; Valere *et al.*, 2003).

### NK cell mediated resistance to *T. gondii*

NK cells are a population of lymphocytes that lacks antigen specific receptors but which have the innate ability to lyse certain types of class I deficient targets (tumor cells or virally infected targets) and to produce IFN- $\gamma$  (Cerwenka and Lanier, 2001). Early studies established that challenge with *T. gondii* or parasite antigens leads to the activation of this population (Hauser *et al.*, 1982; Sharma *et al.*, 1986) but the significance of this response to infection was unclear. The availability of SCID mice that lack T and B cells but have normal NK cells revealed the presence of a T cell independent mechanism of resistance to *T. gondii* (see Figure 6.2) that was driven by the ability of IL-12 to stimulate NK cell production of IFN- $\gamma$  (Gazzinelli *et al.*, 1993b; Hunter *et al.*, 1993; Johnson, 1992). Other co-factors such as IL-1, IL-15, IL18, TNF- $\alpha$  and CD28 synergize with IL-12 to enhance the NK production of IFN- $\gamma$  both *in vitro* and *in vivo* during infection (Hunter, 1996). There is also evidence that human NK cells can lyse infected cells (Subauste *et al.*, 1992) indicating that these effectors can distinguish uninfected from infected cells but the basis for this recognition is unclear.

### Anti-microbial mechanisms involved in the control of parasite replication

It has long been appreciated that IFN- $\gamma$ , derived from NK or T cells, plays a critical role in activating macrophages to control the replication of *T. gondii* (Black *et al.*, 1987; Nathan *et al.*, 1983). In humans, there is evidence that IFN $\gamma$  primes phagocytes to produce reactive oxygen intermediates and leukotrienes that lead to parasite killing (Murray, 1985; Yong



**Figure 6.2** Events involved in innate and adaptive immunity to *T. gondii*. Macrophages, neutrophils and dendritic cells produce IL-12 in response to infection and, in combination with other immune molecules, drives the production of IFN- $\gamma$  by NK cells as well as CD4<sup>+</sup> and CD8<sup>+</sup> T cells. IFN- $\gamma$  is then able to activate anti-microbial effectors that lead to the control of parasite replication, including the production of iNOS and the upregulation of the p47 GTPase family.

*et al.*, 1994). In mice, the absence of IFN- $\gamma$  or IFN- $\gamma$ -mediated signaling during acute or chronic infection results in uncontrolled parasite burden and death of the host (Gavrilescu *et al.*, 2004; Gazzinelli *et al.*, 1993a; Lieberman *et al.*, 2004a; Scharon-Kersten *et al.*, 1996; Suzuki *et al.*, 1989; Suzuki *et al.*, 1988; Yap and Sher, 1999). However, IFN- $\gamma$  alone is not efficient at activating the anti-microbial activities of macrophages and the best characterized second signal is provided by TNF- $\alpha$  (Sibley *et al.*, 1991). The synergistic protection mediated by these two cytokines is attributed to the upregulation of the enzyme inducible nitric oxide synthase (iNOS) which promotes the production of nitric oxide (Adams *et al.*, 1990) but iNOS deficient mice challenged with *T. gondii* only succumb to the chronic phase of this infection (Scharon-Kersten *et al.*, 1997). These findings indicated that there was an IFN- $\gamma$ -dependent iNOS-independent mechanism of resistance to *T. gondii* and subsequent studies implicated a family of 47 kDa IFN- $\gamma$ -induced GTPases (IGTP, LRG47, TGTP, IRG-47) in these events. Thus, mice deficient in IGTP or LRG-47 die during the acute phase and IRG-47 knockout mice die in the chronic phase of infection (Collazo *et al.*, 2001; Taylor *et al.*, 2000). The underlying mechanism whereby these enzymes promote anti-*Toxoplasma* activity is uncertain but there is evidence that these GTPases are recruited to the parasitophorous vacuole where they disrupt the vacuolar membrane (Martens *et al.*, 2005). Other studies have also identified a role for LRG47 in resistance to *Mycobacterium tuberculosis* and suggested that it is involved in lysosomal fusion (MacMicking *et al.*, 2003) and/or autophagy (Gutierrez *et al.*, 2004), two distinct

mechanism that can lead to control of intracellular pathogens. Searches of the human genome indicate that human orthologs to the murine p47 GTPase family exist, but none have been cloned or characterized (Taylor *et al.*, 2004). There are also studies based on the identification of a *Toxoplasma* seropositive patient with a primary genetic deficiency in the ability of IFN- $\gamma$  to activate STAT1 that has led to the proposal that TNF- $\alpha$ , not IFN- $\gamma$ , has the dominant role in activating human macrophages (Janssen *et al.*, 2002).

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### **The role of adaptive immunity in resistance to *T. gondii***

The previous sections have focused on the innate responses to *T. gondii* but long-term resistance to this pathogen is dependent on the development of parasite specific adaptive immunity. The hallmark of these responses is the selection and expansion of B and T cells that express non-germline encoded clonal receptors (TCR, BCR) that permit specific recognition of parasite antigens and which promote anti-parasite effector responses.

#### **B cells and antibody**

During toxoplasmosis there is a prominent expansion of the B cell compartment and these cells produce high levels of *T. gondii*-specific IgM and IgG which can be used to distinguish acute and chronic infections (Montoya and Liesenfeld, 2004). Several functions have been attributed to these antibodies, including opsonization of extra-cellular parasites and subsequent phagocytosis and killing by macrophages (Sibley *et al.*, 1985) as well as complement activation and parasite lysis. Moreover, mice which lack B cells ( $\mu$ MT), survive the acute stage of infection yet succumb to fatal toxoplasmic encephalitis 3–4 weeks post-infection (Kang *et al.*, 2000). Interestingly, immunized  $\mu$ MT mice are not protected against challenge with a virulent strain of parasite and the transfer of serum from immunized wild types to immunized  $\mu$ MT mice enhances survival but does not save these mice (Sayles *et al.*, 2000). Together, these findings suggest an additional role for B cells in resistance to *T. gondii* possibly through the production of IFN- $\gamma$ , TNF and IL-12 (Harris *et al.*, 2000) or in antigen presentation.

#### **T cell mediated immunity**

The importance of T cells for resistance to *T. gondii* is demonstrated by the loss of T cells that precedes the reactivation of this infection in individuals with AIDS (Israelski *et al.*, 1993; Israelski and Remington, 1992). For example, while PBMC from asymptomatic patients co-infected with HIV and *Toxoplasma* show reduced levels of parasite-specific IL-12 and IFN- $\gamma$  in recall responses this is completely absent in patients with clinically apparent TE (Gazzinelli *et al.*, 1995). Moreover, patients undergoing immunosuppressive therapy or affected by cancers that lead to defects in T cell function are also susceptible to toxoplasmosis (Britt *et al.*, 1981; Gaines *et al.*, 1973; Israelski and Remington, 1993; Slavin *et al.*, 1994). Consistent with these observations, studies with mouse models have shown that long term protection is dependent on parasite specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells that produce high levels of IFN- $\gamma$  (Beaman *et al.*, 1994; Gazzinelli *et al.*, 1992; Gazzinelli *et al.*, 1991; Johnson, 1992; Parker *et al.*, 1991; Suzuki and Remington, 1988). In this section we will review some of the major factors involved in the generation and maintenance of these T cell responses.



### Initiation of the adaptive immune response

The adaptive immune response depends upon the innate ability of accessory cells to present parasite derived peptides in the context of MHC molecules. Almost all cells can express MHC class I which is involved in the presentation of peptides derived from the host cytosol to CD8<sup>+</sup> T cells. Presentation of parasite-derived Class I peptides occurs primarily in infected cells and depends upon the ER transporter protein TAP, consistent with a role for canonical pathway of class I presentation (Gubbels *et al.*, 2005). However, this observation is not consistent with evidence that the parasitophorous vacuole in which *Toxoplasma* resides is non-fusogenic and that leakage of parasite products into the cytoplasm of the host cell does not occur (Schwab *et al.*, 1994). This concept has been challenged by the report of the escape of a 69 kDa protein from the parasitophorous vacuole into the cytosol in a small percentage of infected cells (Gubbels *et al.*, 2005).

The expression of class II molecules required for activation of CD4<sup>+</sup> T cells is largely restricted to professional antigen presenting cells, which includes macrophages, B cells and DC. For many years the macrophage was regarded as the major antigen presenting cell population during toxoplasmosis. This was based on the observations that following challenge these cells are prominent at sites of infection, control parasite growth, express increased levels of class II and can stimulate parasite specific CD4<sup>+</sup> T cells. However, DC represent a specialized class of APC that express much higher levels of class II molecules than macrophages or B cells (Banchereau and Steinman, 1998; Cella *et al.*, 1997). Moreover, their ability to sample antigens at sites of inflammation and then traffic to lymphoid tissues, undergo maturation and prime naive T cells contrasts with macrophages which remain at sites of inflammation and are less efficient at priming T cells. Indeed infection or parasite antigens lead to the rapid activation and migration of DC to the T cell area of the lymph node (Reis e Sousa *et al.*, 1997; Straw *et al.*, 2003). Furthermore *T. gondii*-infected human monocyte-derived dendritic cells directly upregulate genes involved in MHC Class I antigen processing and presentation (Chaussabel *et al.*, 2003). Additionally, DC infected with transgenic *T. gondii* which express the model antigen OVA are able to activate naive OVA-specific T cells (Pepper *et al.*, 2004) whereas activated macrophages are unable to prime naive CD4<sup>+</sup> T cells (unpublished data). A similar approach was used to show that DC from infected mice were able to activate an OVA-specific CD8<sup>+</sup> T cell hybridoma (Gubbels *et al.*, 2005).

Once naive T cells are primed, they then acquire the ability to mediate effector functions and there is considerable evidence from human and murine systems that these cells can be cytolytic for infected cells (Chardes *et al.*, 1994; Hakim, 1991; Kasper, 1992; Khan *et al.*, 1990; Montoya *et al.*, 1996; Subauste *et al.*, 1992; Subauste, 1991; Yano *et al.*, 1989). Nevertheless, experimental evidence suggests that the major function of CD4<sup>+</sup> and CD8<sup>+</sup> T cells during toxoplasmosis is to produce IFN- $\gamma$  required to control parasite replication. The expansion of these effector cells is not just dependent on MHC/TCR interactions but co-stimulatory molecules like CD28 and ICOS are important for providing the second signals necessary for T cell production of IL-2 required for proliferation (Villegas *et al.*, 1999; Villegas *et al.*, 2002b). While early studies revealed that treatment with the autocrine T cell growth factor IL-2 enhances resistance to *T. gondii* (Sharma *et al.*, 1985), subsequent work identified the ability of CD4<sup>+</sup> T cells to make IL-2 as important in parasite-specific

production of IFN- $\gamma$  (Gazzinelli *et al.*, 1991) and that IL-2 deficient mice are susceptible to acute toxoplasmosis associated with a defect in their ability to make IFN- $\gamma$  (Villegas *et al.*, 2002a). There is also evidence that other cytokines, including IL-7 and IL-15 that are involved in T cell growth and homeostasis can also play a role in immunity to *T. gondii* (Kasper *et al.*, 1996; Khan and Kasper, 1996; Khan *et al.*, 2002; Lieberman *et al.*, 2004b).

### Pathological T cell responses

The acute stage of this infection, even in immune competent individuals, is frequently characterized by flu-like symptoms. In mice, this manifests as high systemic levels of pro-inflammatory cytokines and the development of severe inflammation in multiple tissues including the gut and liver (Liesenfeld *et al.*, 1996). While some of this pathology can be attributed to the presence of replicating parasites there is also immune-mediated bystander damage (Mordue *et al.*, 2001). It is now recognized that the CD4<sup>+</sup> T cells induced by this infection are major contributors to these events and depletion of this subset prevents the infection-induced ileitis that can occur in certain strains of mice (Liesenfeld *et al.*, 1996). The high levels of IL-12 and IFN- $\gamma$  produced during this effector phase of infection are counteracted by several different anti-inflammatory mechanisms including TGF- $\beta$  (Buzoni-Gatel *et al.*, 2001), IL-10 (Gazzinelli *et al.*, 1996), IL-27 (Villarino *et al.*, 2003) and lipoxins (Aliberti *et al.*, 2002a; Aliberti *et al.*, 2002b) which are important for preventing hyper-inflammation. Moreover, the absence of IL-4 or IL-5 can lead to exacerbated pathology in the gut and/or liver (Nickdel *et al.*, 2004; Nickdel *et al.*, 2001) and the absence of fibrin leads to severe immune-pathology (Johnson *et al.*, 2003). More recently the class I like molecule CD1d and  $\gamma\delta$  T cells have also been implicated in the resolution of *Toxoplasma*-induced inflammation (Egan *et al.*, 2005; Smiley *et al.*, 2005). Together, these studies indicate that there are multiple checks in the immune system to balance the consequences of a strong protective response.

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### Toxoplasmic encephalitis

The emergence of the adaptive response is associated with the transformation of the parasite from the tachyzoite into the encysted bradyzoite which persists for the life of the host, mostly localized in brain and muscle. These cysts periodically release bradyzoites that are able to infect new cells and become tachyzoites and immune-competent individuals are normally able to control this reactivation. For patients with acquired defects in T cell function, this recrudescence leads to clinical disease that can involve multiple sites but primarily the CNS where it causes toxoplasmic encephalitis (TE). Since the brain is the site most commonly affected in clinical disease a major challenge has been to understand how anti-parasite responses are mediated in this immune privileged organ. Thus, despite the presence of the blood brain barrier that restricts the access of the immune system, the lack of a lymphatic system and low constitutive levels of class I and II expression, the immune system is still able to control parasites in this site. In chronically infected mice, the disease in the brain is characterized by a prominent influx of macrophages, DC as well as large numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells accompanied by the local production of pro-inflammatory cytokines (Fischer *et al.*, 2000; Hunter and Remington, 1994). In certain strains of mice (Balb/c, 129) inflammation associated with this phase of the infection is resolved as

parasite replication is controlled and this resistance is associated with the Ld class I locus (Brown, 1995). In contrast, in some strains (B6, CBA/ca), despite the development of a protective response, and no obvious underlying immune deficiencies these animals develop a progressive encephalitis (Brown, 1995).

Given the immune privileged status of the CNS it has been unclear what events lead to T cell recruitment and activation to this site. During the early phase of TE there is an early astrocyte activation that precedes the infiltration of protective T cells (Hunter *et al.*, 1992), and which correlates with the production of chemokines that are likely to have a role in the induction of inflammation (Brenier-Pinchart *et al.*, 2004; Strack *et al.*, 2002a; Strack *et al.*, 2002b). Additionally, *in vitro* studies have reported that infection of astrocyte cultures with bradyzoites or tachyzoites results in the production of the pro-inflammatory cytokines IL-1, IL-6 and TNF- $\alpha$  (Fischer *et al.*, 1997) which are known to have protective effects during TE and astrocytes are also sources of other cytokines (IL-10 and 12) that have a role in resistance to TE (Wilson *et al.*, 2005). Moreover astrocyte expression of class I and II has been noted in mice with TE (Schluter *et al.*, 1993) although there are also reports that astrocytes infected with *T. gondii* downregulate the levels of these molecules (Luder *et al.*, 2003). Nevertheless, the role for astrocytes in the regulation of T cell mediated resistance to TE remains an open question.

### Anti-parasite effectors in the CNS

Experimental models have highlighted the importance of T cell production of IFN- $\gamma$  required for the control of *T. gondii* in the CNS (Suzuki *et al.*, 1989; Suzuki *et al.*, 1990). One of the major anti-microbial functions of IFN- $\gamma$  is (in combination with TNF- $\alpha$ ) to activate infiltrating macrophages and microglia (the CNS resident macrophages) to produce nitric oxide which controls the replication of *T. gondii* (Chao *et al.*, 1993). Thus, mice that lack iNOS or TNF- $\alpha$  are highly susceptible to TE (Deckert-Schluter *et al.*, 1998; Gazzinelli *et al.*, 1993a; Schariton-Kersten *et al.*, 1997; Yap *et al.*, 1998). These latter findings have been recapitulated in a recent report in which an arthritic patient treated with anti-TNF developed TE (Young and McGwire, 2005). Moreover, T cells do not only produce IFN- $\gamma$  but can also express CD40L which stimulates the ability of macrophages to kill *T. gondii* (Andrade *et al.*, 2003; Reichmann *et al.*, 2000). Consistent with this idea, CD40LKO mice have increased susceptibility to TE suggesting an important role for these interactions in resistance to chronic infection consistent with the observation that patients deficient in this pathway develop TE (Subauste *et al.*, 1999). Similarly, while the ability of CD8<sup>+</sup> T cells to produce IFN- $\gamma$  is essential for resistance to TE, the finding that perforin-deficient mice develop higher parasite burdens than WT controls indicates that optimal resistance to TE is dependent on the ability of cytotoxic T cells to recognize and lyse cells infected with *T. gondii* (Denkers *et al.*, 1997). There are also reports that non-hematopoietic cells (which don't express iNOS) in the CNS need to be able to respond to IFN- $\gamma$  in order to control infection (Yap and Sher, 1999). Astrocytes represent the most numerous non-hematopoietic cell type in the brain and IFN- $\gamma$  can stimulate these somatic cells to inhibit parasite replication (Wilson and Hunter, 2004) independently of reactive nitrogen or oxygen intermediates but dependent on IGTP (Halonen *et al.*, 2001).

## Conclusion

Technical advances in the ability to experimentally manipulate the immune response have contributed to the progress in understanding many aspects of the immune response to *T. gondii*. For example the use of monoclonal antibodies to deplete cytokines or lymphocyte subsets and the availability of transgenic and knock-out mice have allowed the dissection of the roles of cell subsets, cytokines or even individual anti-microbial effector molecules in resistance to this parasite. Many of these studies have focused on the host response to the parasite but there is a growing appreciation that many aspects of the biology of *T. gondii* impact the development of protective immunity. This includes the parasite's ability to compromise pro-inflammatory signaling, prevent apoptosis (Chapter 8), interfere with antigen presentation or mobilize monocytes for its dissemination. With the completion of the *Toxoplasma* genome, and the relative ease with which forward and reverse genetics can be performed on this haploid organism, new tools to examine the parasites' effect upon the immune response have been generated and there is now an unprecedented opportunity to understand how the biology of *T. gondii* affects the development of protective immunity (Fox and Bzik, 2002; Gubbels *et al.*, 2005; Kim and Boothroyd, 2005; Kwok *et al.*, 2003; Pepper *et al.*, 2004).

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# Mechanisms of Immune Evasion

7

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## Abstract

*Toxoplasma gondii* employs multiple strategies to avoid, deflect or subvert host defense mechanisms. The parasite avoids immunity through cyst formation and sequestration in immunoprivileged tissues as well as in the parasitophorous vacuole within the host cell. It deflects immunity through stage-specific antigen variation. *Toxoplasma*-induced suppression of immunity occurs through induction of anti-inflammatory mediators such as IL-10 and lipoxin A<sub>4</sub>. In infected cells, it is now evident that *T. gondii* directly subverts proinflammatory signaling cascades. Suppression of NFκB and MAPK signal transduction, and activation of anti-inflammatory transcription factor STAT3 have each recently emerged as targets of manipulation by the parasite. As a result, the parasite blocks many activities associated with activation in innate immune cells, including macrophages, dendritic cells and neutrophils. The many ways that *Toxoplasma* downmodulates immune responses reflects the sophistication of this parasite in its interactions with the host.

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## Introduction

Encounter between *Toxoplasma* and the immune system is a major determinant in the outcome of this host–parasite interaction. Without an intact host defense system, as in HIV AIDS, cysts harbored by chronically infected individuals can reactivate leading to devastating disease or death (Luft *et al.*, 1993). Similarly, congenital infection may result in severe consequences for the fetus in the absence of functionally mature immunity (Remington *et al.*, 2001). Nevertheless, viewed from the parasite's perspective it is a benefit to be sensed by the host immune system. If not, *T. gondii* is likely to cause lethal infection—an outcome minimizing the probability of host-to-host transmission. The ability of *Toxoplasma* to establish asymptomatic, long-term infection in 1 billion or more humans is a dramatic testament to the success of this parasite in dealing with the host immune system.

As reviewed in this volume and elsewhere, *Toxoplasma* is highly effective at inducing protective host immunity (Alexander and Hunter, 1998; Denkers and Gazzinelli, 1998). This property resides largely in the ability of the parasite to induce IL-12 from cells of the innate immune system (Sher *et al.*, 2003). In turn, IL-12 release leads to early natural killer cell IFN-γ production and generation of protective IFN-γ secreting Th1 T lymphocytes



and CD8<sup>+</sup> cytolytic T cells (Denkers *et al.*, 1997; Gazzinelli *et al.*, 1991; Schar-ton-Kersten *et al.*, 1996; Suzuki *et al.*, 1988). IFN- $\gamma$ -inducible molecules such as the 47 kDa GTPases and inducible nitric oxide synthase that catalyzes nitric oxide production are important players in resistance during acute infection, as well as in preventing cyst reactivation during chronic infection (Butcher *et al.*, 2005a; Collazo *et al.*, 2001; Khan *et al.*, 1997; Schar-ton-Kersten *et al.*, 1995; Taylor *et al.*, 2004). Each of these mediators can be highly effective at protecting the host from the effects of uncontrolled infection.

*Toxoplasma* plays a dangerous game. It has found ways to stimulate host microbicidal effector activity, as it must to ensure host and parasite survival. Nevertheless, host protective responses cannot be so strong that they eliminate the parasite before establishing latency. In this regard, *T. gondii* must evade the immune response. This review focuses on newly emerging cellular and molecular evidence that highlights the sophisticated ways *T. gondii* avoids immune elimination.

**Parasite niche within the host**

Characteristics of the parasite life-cycle favor evasion of immunity at several key points (Table 7.1). The intracellular residence of tachyzoites and the localization of bradyzoites to immunoprivileged tissues of the central nervous system act to shelter parasites from host immunity. Bradyzoites, themselves, are contained within cysts that do not elicit inflammation, acting further to sequester *T. gondii* from the immune system.

*Toxoplasma* enters cells and establishes an intracellular niche using mechanisms that favor evasion of immunity. Cell invasion involves parasite actin-based motility, establishment of a moving junction, and sequential discharge of micronemes, rhoptries and dense granules (Dobrowolski and Sibley, 1996). Together, these processes lead to creation of a specialized parasitophorous vacuole in which tachyzoites reside and multiply (Carruthers, 2002; Carruthers and Sibley, 1997; Sibley, 2003).

The parasitophorous vacuole is devoid of host transmembrane proteins, though glycosylphosphatidylinositol (GPI)-anchored proteins and some G-protein-linked seven-transmembrane receptor proteins appear to be selected for inclusion (Charron and Sibley,

**Table 7.1** *T. gondii* strategies of avoiding host immunity during infection

Immune-evasion strategy	Mechanism	Representative references
Nature of the niche	Cyst formation, location in immunoprivileged anatomical site (CNS) Antigen switching during stage differentiation Intracellular sequestration	(Hill and Dubey, 2002) (Kim and Boothroyd, 2005) (Mordue and Sibley, 1997)
Stimulation of anti-inflammatory host molecules	IL-10 Lipoxin A <sub>4</sub>	(Gazzinelli <i>et al.</i> , 1996) (Aliberti <i>et al.</i> , 2002a)
Interference with proinflammatory signaling cascades	Block NF $\kappa$ B translocation Defective LPS-induced p38 MAPK phosphorylation STAT3 activation	(Butcher <i>et al.</i> , 2001; Shapira <i>et al.</i> , 2005) (Kim <i>et al.</i> , 2004) (Butcher <i>et al.</i> , 2005b)



2004; Mordue *et al.*, 1999). As a result of excluding proteins such as Rab5 and Rab7 that target vacuoles to endocytic pathways, *T. gondii* evades endocytic fusion (Mordue and Sibley, 1997). It was long ago recognized that invasion fails to trigger an oxidative burst that normally accompanies phagocytosis in macrophages (Sibley *et al.*, 1985; Wilson *et al.*, 1980). Other studies using antibody-coated parasites and Fc-receptor-bearing CHO cells demonstrate rapid degradation of tachyzoites entering cells through receptor-mediated endocytosis (Joiner *et al.*, 1990; Mordue and Sibley, 1997).

To all intents and purposes, the parasitophorous vacuole sequesters the parasite from the cell cytoplasm and host pathways of endocytosis. Nevertheless, *T. gondii* elicits robust CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses that depend upon cytoplasmic and endocytic pathways of MHC class I and II antigen presentation (Luder and Seeber, 2001). How can they occur? One possibility is that these responses are driven by phagocytic uptake of inactivated or opsonized tachyzoites, resulting in classical MHC class II presentation to CD4<sup>+</sup> T cells, or cross-presentation to CD8<sup>+</sup> T cells (Ackerman and Cresswell, 2004).

More recent evidence indicates that *T. gondii* possesses the ability to direct proteins across the parasitophorous vacuole membrane into the host cell cytoplasm, and that this can result in MHC class I-restricted antigen presentation to CD8<sup>+</sup> T cells (Gubbels *et al.*, 2005). Robey and colleagues recently engineered parasites to express a secreted form of the model antigen ovalbumin. In infected dendritic cells, parasite-secreted ovalbumin was subjected to proteolytic degradation and transporter associated with antigen processing (TAP)-dependent peptide loading onto MHC class I complexes for presentation to ovalbumin-specific CD8<sup>+</sup> T cells (Gubbels *et al.*, 2005). This finding appears to provide an example of immunostimulation and immuno-evasion occurring concurrently. Invasion and creation of the parasitophorous vacuole occur without entry into phagolysosomal pathways of degradation. Yet, at the same time, infected antigen-presenting cells are capable of presenting endogenously synthesized antigenic peptide to T cells. An important proviso to this study is that it is not yet clear if results using transgenic parasites expressing secreted ovalbumin are applicable to native *Toxoplasma* proteins expressed under normal conditions.

Stage differentiation of *Toxoplasma* from rapidly dividing tachyzoites to cyst-forming bradyzoites is accompanied by a major shift in the antigenic expression profile of the parasites (Lyons *et al.*, 2002). This occurs concurrently with the rise in the adaptive immune response. Antigenic shifting is a commonly used strategy for persistence of microbial pathogens (Kyes *et al.*, 2001; van der Woude and Baumler, 2004). For *Toxoplasma*, stage differentiation is associated with a change in expression pattern of members of the surface antigen (SAG)1 superfamily (He *et al.*, 2002; Jung *et al.*, 2004). The tachyzoite-specific surface proteins SAG1 and SAG2A are major targets of T and B cell immunity (Bessieres *et al.*, 1992; Khan *et al.*, 1988; Partanen *et al.*, 1984; Prigione *et al.*, 2000). Indeed, SAG1 activates IFN- $\gamma$ -producing T cells, and SAG1-specific CD8<sup>+</sup> T lymphocytes transfer protection against *T. gondii* challenge infection (Khan *et al.*, 1994). It is tempting to speculate that termination of SAG1 and SAG2A expression during tachyzoite-to-bradyzoite stage differentiation is a parasite mechanism to escape host immunity. Thus, as vigorous T and B lymphocyte responses develop to these molecules, their expression is switched off and

stage differentiation initiates. In this manner, such immunodominant tachyzoite-specific antigens may serve as decoys for the acquired immune response.

The consequences of interfering with stage-specific expression of *Toxoplasma* antigens were recently investigated by generating parasites that constitutively express proteins normally restricted to tachyzoites or bradyzoites (Kim and Boothroyd, 2005). Enforced expression of SAG1 into the bradyzoite stage results in reduced cyst counts early during chronic infection, but later leads to hyperproduction of IFN- $\gamma$ , TNF- $\alpha$  and IL-10, at the same time causing severe encephalitis. Conversely, premature tachyzoite expression of SRS9, a normally bradyzoite-specific antigen, elicits an SRS9-specific IFN- $\gamma$  response and cysts formed by these transgenic parasites fail to persist *in vivo* (Kim and Boothroyd, 2005).

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### Induction of host anti-inflammatory mediators as an immuno-evasion strategy

*T. gondii* infection results in robust production of pro-inflammatory cytokines, and the parasite is regarded as a prototypic inducer of Th1 responses (see Chapter 6). *Toxoplasma* also activates pathways leading to anti-inflammatory mediators, such as IL-10, TGF- $\beta$  and lipoxin A<sub>4</sub> (Aliberti and Bafica, 2005). A major function of anti-inflammatory mediators during infection with *T. gondii* and other microbial pathogens is to dampen down proinflammatory cytokine responses, thereby avoiding immunopathological effects of overproducing these potentially lethal mediators. Such responses are most often viewed as host self-protective responses. However, while direct evidence is lacking (and would actually be difficult to obtain), anti-inflammatory responses may also be driven by the parasite's need to keep the host alive through infection. Induction of anti-inflammatory networks may also represent a microbial strategy to avoid elimination that would otherwise result from host proinflammatory anti-microbial effector mechanisms.

#### Interleukin-10

The cytokine IL-10 is well known for its potent ability to downregulate production of pro-inflammatory cytokines such as IL-12 and TNF- $\alpha$ . During macrophage infection with *T. gondii*, IL-10 increases parasite replication through downregulatory effects on nitric oxide production (Gazzinelli *et al.*, 1992), and *Toxoplasma* itself stimulates IL-10 during infection (Hunter *et al.*, 1994). Nevertheless, absence of IL-10 results in lethal inflammatory pathology during acute infection that is at least in part dependent upon IFN- $\gamma$ -producing CD4<sup>+</sup> T lymphocytes (Gazzinelli *et al.*, 1996; Neyer *et al.*, 1997). C57BL/6 strain mice undergoing oral infection with type II strain ME49 parasites develop lethal ileitis associated with proinflammatory cytokine overproduction, whereas BALB/c strain animals do not (Liesenfeld, 1999; Liesenfeld *et al.*, 1999; Liesenfeld *et al.*, 1996). Lack of pathology in BALB/c mice is dependent upon the parasite's ability to trigger IL-10 production. Thus, like C57BL/6 animals, BALB/c background IL-10<sup>-/-</sup> mice also develop lethal gut pathology (Suzuki *et al.*, 2000).

IL-10 is found in the central nervous system of hosts latently infected with *T. gondii* (Hunter and Remington, 1994; Schluter *et al.*, 1997). Similar to macrophages, IFN- $\gamma$ -activated astrocytes can block parasite replication, and this response is antagonized by IL-10

(Halonen *et al.*, 2001; Wilson *et al.*, 2005). Some studies suggest that absence of IL-10 leads to diminished cyst numbers in the central nervous system (Deckert-Schluter *et al.*, 1997). However, others suggest no effect on parasite burden (Wilson *et al.*, 2005). The major effect of IL-10 in this situation may be to downregulate inflammatory pathology that contributes to toxoplasmic encephalitis. Thus, induction of IL-10 for the parasite may reflect the need to maintain stable infection in the host without causing detrimental host pathology.

### Lipoxin A<sub>4</sub>

During systemic triggering with tachyzoite extracts, splenic dendritic cells become unresponsive to *in vitro* stimulation, a process termed “paralysis” (Reis e Sousa *et al.*, 1999). Following injection of soluble parasite extracts, splenic dendritic cells become refractory to a second injection for several days, as measured by release of IL-12 *in vitro*. The physiological relevance of this phenomenon is suggested by the observation that IL-10<sup>-/-</sup> mice can be rescued from *Toxoplasma*-induced hyperinflammatory pathology by induction of dendritic cell paralysis prior to infection.

The mechanism underlying induction of dendritic cell unresponsiveness appears to be a parasite-induced downregulation of chemokine receptor CCR5 (Aliberti *et al.*, 2002a). The latter is a seven-transmembrane G-protein coupled receptor contributing to *T. gondii*-induced dendritic cell IL-12 responses (Aliberti *et al.*, 2000). Parasite-triggered lipoxin A<sub>4</sub>, an arachidonate-derived inhibitor of inflammation, seems to be involved in maintaining dendritic cell paralysis and CCR5 downregulation, since in the absence of this enzyme, CCR5 expression is recovered within a few days. Mice lacking 5-lipoxygenase, an enzyme involved in lipoxin A<sub>4</sub> biosynthesis, succumb from toxoplasmic encephalitis associated with elevated levels of IL-12 and IFN- $\gamma$ , but decreased numbers of cysts in the central nervous system (Aliberti *et al.*, 2002b). Interestingly, it was recently found using a proteomics approach that tachyzoite extracts contain a 15-lipoxygenase activity that can contribute to host lipoxin A<sub>4</sub> synthesis (Bannenberg *et al.*, 2004). This suggests that the parasite itself may use the lipoxin anti-inflammatory pathway to prevent pathology and promote parasite persistence (Aliberti, 2005). Lipoxins play an anti-inflammatory role in other infections, including *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (Bafica *et al.*, 2005; Vance *et al.*, 2004). Indeed, *P. aeruginosa* also expresses an enzyme with 15-lipoxygenase activity (Vance *et al.*, 2004), although it is not known if *M. tuberculosis* has the same features. These findings suggest that lipoxin pathways are common targets of microbial pathogens.

### TGF- $\beta$

The cytokine TGF- $\beta$  also plays a role in protection against murine ileitis following oral *Toxoplasma* infection. Intraepithelial CD8<sup>+</sup> T lymphocytes producing TGF- $\beta$  transfer protection against gut inflammation (Buzoni-Gatel *et al.*, 2001; Buzoni-Gatel *et al.*, 1997). This effect appears to be mediated by TGF- $\beta$ -mediated downregulation of inflammatory cytokine production by lamina propria CD4<sup>+</sup> T cells (Mennechet *et al.*, 2004).

## Interference with proinflammatory signaling cascades

Several intracellular protozoans are capable of manipulating to their own advantage host cell signaling pathways. For example, *Leishmania* blocks IL-12 production and interferes with protein kinase C and JAK/STAT signaling (Denkens and Butcher, 2005). The transforming apicomplexan, *Theileria parva*, hijacks I $\kappa$ B kinase signalsomes to constitutively activate NF $\kappa$ B signaling and induce resistance to apoptosis (Dobbelaere and Kuenzi, 2004). Increasing evidence indicates that *Toxoplasma* delivers immunosuppressive messages from within the host cell, or possibly during the invasion process itself (Denkens *et al.*, 2004). Elsewhere in this volume, the ability of the parasite to block apoptosis is discussed. Here, we focus on suppression of proinflammatory mediators. Tachyzoite-infected mouse macrophages are resistant to LPS activation through Toll-like receptor (TLR)4, as measured by suppressed production of IL-12p40, TNF- $\alpha$ , nitric oxide and many other proinflammatory cytokines and chemokines (Butcher *et al.*, 2001; Lee *et al.*, 2006; Luder *et al.*, 2003). Recent work from our laboratory indicates that signaling through other TLR is also blocked. Suppression of nitric oxide (through decreased expression of inducible nitric oxide synthase) is associated with increased tachyzoite replication *in vitro*, suggesting this as a possible mechanism for parasite dissemination during infection (Luder *et al.*, 2003). Furthermore, *Toxoplasma* downregulates MHC class II expression, resulting in reduced antigen presentation to T cells (Luder *et al.*, 2001).

Recent studies from our laboratory employing pathway-specific gene arrays indicate that infection with *T. gondii* blocks most LPS-triggered cytokine and chemokine responses (Lee *et al.*, 2006). Nevertheless, a small subset of LPS responsive genes escape suppression by the parasite. Most prominently, LPS induction of IL-10 is not prevented by *T. gondii* infection. This finding reinforces the idea that IL-10 induction itself may play an important role in immune evasion by the parasite.

Importantly, the suppressive properties of *T. gondii* in macrophages depend upon the ability of the parasite to actively invade cells (Butcher and Denkers, 2002). Thus, neither heat inactivated tachyzoites nor soluble parasite extracts display inhibitory effects on LPS-triggered macrophages. In addition, treatment with cytochalasin D, a drug that allows parasite attachment but prevents cell entry, interferes with the ability of *T. gondii* to block LPS-induced TNF- $\alpha$  production.

Recent evidence indicates that the immunosuppressive effects of *Toxoplasma* extend beyond macrophages. Thus, in populations of mature and immature dendritic cells, tachyzoites preferentially infect the latter (McKee *et al.*, 2004). Immature dendritic cells then become functionally inactivated as a result of infection, as measured by resistance to activation by TLR or CD40 signaling. The effect of dendritic cell inactivation is that, like macrophages, cells are unable to secrete TNF- $\alpha$  or IL-12 upon LPS stimulation, or to activate naive CD4<sup>+</sup> T lymphocytes (McKee *et al.*, 2004). It has been suggested that *T. gondii* exploits dendritic cells as vehicles for dissemination during early infection (Courret *et al.*, 2006). By inactivating these cells, the parasite may promote its establishment within the host.

For suppression of LPS-induced IL-12, it is important to realize that the parasite itself triggers low amounts of this cytokine (Kim *et al.*, 2005). Production of IL-12 in response to RH tachyzoite infection in macrophages occurs with kinetics delayed relative

to LPS. Interestingly, *Toxoplasma* appears to trigger a signaling pathway leading to IL-12 that is different from the TLR4 transduction cascade, in that it does not require NF $\kappa$ B family member c-Rel, and it involves a newly recognized autophosphorylation activity of p38 MAPK (Kim *et al.*, 2005; Mason *et al.*, 2002). Thus, the parasite appears to have found its own ways to induce IL-12 in macrophages, and it is tempting to speculate that this is driven by the need to keep the host alive during infection.

Neutrophils are also rendered dysfunctional by *Toxoplasma* infection. Triggering of polymorphonuclear leukocytes through TLR induces upregulation of membrane-bound TNF- $\alpha$ , but in parasite-infected cells this response is defective (Bennouna *et al.*, 2006). Neutrophils contain preformed pools of TNF- $\alpha$  (Bennouna and Denkers, 2005), and *T. gondii* appears to interfere with TNF- $\alpha$  mobilization to the cell surface, rather than with upregulation of gene transcription. This differs from macrophages where parasite infection results in decreased transcriptional responses (Lee *et al.*, 2006).

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### Intracellular mechanisms of immune evasion

Discovering how *T. gondii* subverts signaling pathways in infected cells remains an area to be fully explored. Two major signaling pathways play important roles in induction of TNF- $\alpha$ , IL-12 and many other mediators suppressed by *Toxoplasma* infection. These are the NF $\kappa$ B and mitogen-activated protein kinase (MAPK) transduction cascades. Both Rel/NF $\kappa$ B and MAPK pathways are evolutionarily ancient pathways, and they play important roles in many physiological processes, including host immunity. Both pathways are emerging as targets for dismantling by *T. gondii*.

#### Rel/NF $\kappa$ B

The Rel/NF $\kappa$ B proteins are a family of heterodimeric molecules made up of RelA/p65, RelB, c-Rel, NF $\kappa$ B1 (p50 and its precursor p105), and NF $\kappa$ B2 (p52 and its precursor p100) (Gosh and Karin, 2002; Karin and Ben-Neriah, 2000). Each possesses a Rel homology region (RHR) that promotes dimerization, DNA binding and interaction with I $\kappa$ B molecules that inhibit NF $\kappa$ B function. Only RelA and c-Rel contain strong transcriptional activating domains, and of these c-Rel is predominantly used for LPS-triggered IL-12p40 induction (Sanjabi *et al.*, 2000).

In the classical activation pathway, I $\kappa$ B inhibitor proteins bind to NF $\kappa$ B, masking nuclear import sequences. This results in predominantly cytoplasmic localization of NF $\kappa$ B under resting conditions. When I $\kappa$ B molecules undergo phosphorylative activation, mediated by an upstream I $\kappa$ B kinase complex, they are targeted for polyubiquitination followed by proteasomal degradation. The latter event reveals NF $\kappa$ B nuclear localization sequences, enabling rapid import into the nucleus. Here, NF $\kappa$ B dimers bind to  $\kappa$ B sites on target promoters, activating transcription of NF $\kappa$ B-responsive genes. Expression of the I $\kappa$ B protein itself is controlled by NF $\kappa$ B, and in this manner NF $\kappa$ B regulates its own signaling activity (Chiao *et al.*, 1994). Activation of NF $\kappa$ B signaling is triggered through several receptors in innate immune cells, including TLR, TNF receptors and CD40.

Infection with the type 1 RH *Toxoplasma* strain induces rapid phosphorylation and degradation of I $\kappa$ B activation in macrophages (Butcher *et al.*, 2001). Nevertheless, we, and others, found that there is no subsequent nuclear translocation of RelA/p65, c-Rel or p50



in infected cells (Butcher *et al.*, 2001; Shapira *et al.*, 2002). In addition, when macrophages harboring parasites are subjected to LPS triggering, NF $\kappa$ B translocation is prevented. However, this block is a short-term phenomenon because after approximately 6 hours of infection, the ability of NF $\kappa$ B to translocate in response to LPS is restored (Butcher and Denkers, 2002; Kim *et al.*, 2004). Even at this time point or later, macrophages infected with RH tachyzoites alone do not exhibit nuclear localization of NF $\kappa$ B.

Others have reported that RH infection triggers RelA/p65 and p50 translocation (Molestina *et al.*, 2003). Whether the discrepancy results from use of different host cell types or possibly divergent parasite substrains is presently unclear. Another study of interest found that while RH strain tachyzoites do not induce RelA/p65 nuclear localization, low virulence PTG tachyzoites trigger low level nuclear translocation of this NF $\kappa$ B family transcription family member, and this was tied to increased amounts of IL-12 production relative to infection with RH (Robben *et al.*, 2004).

The lack of NF $\kappa$ B translocation in RH infected macrophages despite I $\kappa$ B degradation led us to investigate the molecular mechanisms underlying this defect. At the same time that defects in NF $\kappa$ B nuclear translocation were reported (Butcher *et al.*, 2001), an independent investigation found that the function of transcription factor IFN- $\gamma$ -stimulated signal transducer and activator of transcription (STAT)1 was also blocked by *T. gondii* (Luder *et al.*, 2001). Because STAT1 underwent normal IFN- $\gamma$ -driven phosphorylation in infected macrophages, yet failed to translocate to the nucleus, we hypothesized that *Toxoplasma* might induce a generalized block in nuclear import in host cells (Denkens *et al.*, 2003).

Recent evidence argues against a global shut-down in nuclear import pathways in infected cells. Thus, constitutive shuttling of NF $\kappa$ B between the cytoplasm and nucleus continues to occur in parasite-infected cells (Shapira *et al.*, 2005). Instead, termination of NF $\kappa$ B signaling was found to be associated with reduced RelA/p65 phosphorylation. Regulation of NF $\kappa$ B activity through phosphorylation and acetylation is emerging as an important factor in the transactivational capacity and nuclear retention of this transcription factor family (Chen and Greene, 2004). Whether *Toxoplasma*-induced RelA/p65 modifications occur by inactivation of an upstream kinase or activation of a downstream phosphatase of either host or parasite origin is not yet known.

## MAPK

Like the NF $\kappa$ B signaling cascade, MAPK pathways take part in both immune and non-immune cellular functions (Dong *et al.*, 2002). There are three major mammalian MAPK. These are p38, ERK1/2 (p42/p44), and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK). Activation of MAPK results in phosphorylation of transcription factors such as Elk-1, NF-IL-6, and c-Jun, as well as phosphorylation of other kinases such as MAPK-activating protein (MAPKAP) kinase-2. The MAPK are activated through dual phosphorylation of Thr-X-Tyr motifs that is mediated by upstream MAPK kinases (MKK). In turn, MKK are activated through phosphorylation mediated by a large family of MKK kinases (M3K). Deactivation of MAPK is mediated by a family of MAPK phosphatases (Camps *et al.*, 2000; Chen *et al.*, 2002; Ropert *et al.*, 2003). The M3K-MKK-MAPK signaling cascades are often triggered in response to infection. In particular, cell



activation through TLR-MyD88 leads to rapid MAPK phosphorylation. MAPK are also activated by proinflammatory cytokines such as IL-1 and TNF.

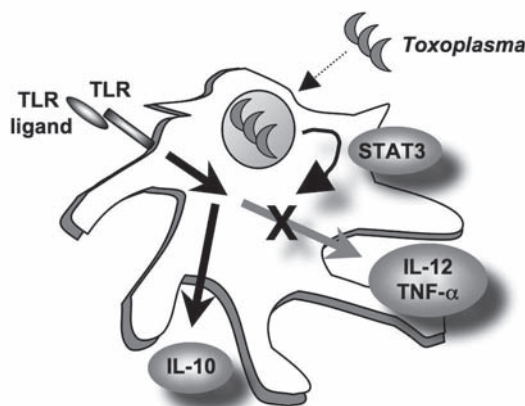
Infection of macrophages with RH strain *Toxoplasma* leads to activation of p38 and ERK1/2 MAPK, and low levels of SAPK/JNK (Kim *et al.*, 2004; Valere *et al.*, 2003). This occurs within 10–20 min of infection, and is followed by dephosphorylation such that MAPK background phosphorylation levels are re-attained within approximately 2 hours. Production of IL-12 mediated by live RH parasites or RH parasite extract is dependent upon p38 MAPK activation (Kim *et al.*, 2005; Mason *et al.*, 2004).

Despite early activation of MAPK by the parasite, when infected macrophages are subjected to LPS stimulation reactivation of this kinase cascade is defective (Kim *et al.*, 2004). Inasmuch as p38 MAPK is required for LPS-induced IL-12p40 and TNF- $\alpha$  induction (Lu *et al.*, 1999; Wysk *et al.*, 1999), inability to reactivate this MAPK family member is likely to contribute to the suppressive effects of *Toxoplasma* on LPS-triggered cytokine production. This could result from defects in upstream kinase activity, although reactivation of MKK3, a major MAPK kinase involved in p38 activation is unaffected (Kim *et al.*, 2004). Alternatively, inactivation might be the result of MKK phosphate activity, either induced in the host by *T. gondii* infection, or possibly produced by the parasite itself. There is evidence that two other protozoan parasites, *Trypanosoma cruzi* and *Leishmania major*, influence host signaling through induction of host phosphatases during infection (Blanchette *et al.*, 1999; Forget *et al.*, 2001; Ropert *et al.*, 2003).

## STAT3

Many aspects of *T. gondii* induced suppression of macrophage activation resemble the anti-inflammatory effects of IL-10 (Donnelly *et al.*, 1999). In particular, both IL-10 and the parasite downregulate LPS-induced TNF- $\alpha$  and IL-12. Nevertheless, experiments employing IL-10<sup>-/-</sup> macrophages ruled out parasite-induced IL-10 as the mechanism of suppression (Butcher *et al.*, 2001). Over 15 years after the discovery of IL-10, how this cytokine mediates its suppressive effects remains unclear. Nevertheless, there is agreement that activation of STAT3, a molecule whose phosphorylation is driven by JAK-mediated association with the IL-10 receptor, is required for IL-10-mediated proinflammatory cytokine suppression (Williams *et al.*, 2004).

We recently found that *Toxoplasma* invasion in macrophages triggers rapid STAT3 activation independently of IL-10 (Butcher *et al.*, 2005b). This response is rapid, occurring within minutes of infection. Parasite-induced STAT3 phosphorylation is also sustained for up to 22 hours of infection. Prolonged parasite-driven STAT3 activation is reminiscent of constitutive I $\kappa$ B $\alpha$  phosphorylation and NF $\kappa$ B activation that occurs in *Theileria*-transformed leukocytes (Heussler *et al.*, 2002). Importantly, in STAT3 KO macrophages, *Toxoplasma* is unable to suppress LPS-induced TNF- $\alpha$  or IL-12p40 production (Butcher *et al.*, 2005b). Therefore, *T. gondii* bypasses IL-10 and directly activates STAT3 to mediate suppression of LPS-induced activation. Because *Toxoplasma* does not block TLR4-triggered IL-10, the parasite presumably targets a point in the TLR signaling cascade that lies downstream of a bifurcation point leading to IL-10 on the one hand, and TNF- $\alpha$ /IL-12 on the other (Figure 7.1). Whether STAT3 contributes to inactivation of NF $\kappa$ B and MAPK signaling, or whether it operates as a parallel suppression mechanism is not presently known.



**Figure 7.1** STAT3 is a mediator of *T. gondii*-mediated IL-12 and TNF- $\alpha$  suppression. LPS triggers macrophages through TLR4 to produce IL-10, IL-12 and TNF- $\alpha$ . Infection with *Toxoplasma* attenuates IL-12 and TNF- $\alpha$  release but does not suppress production of IL-10. During infection, STAT3 is rapidly activated and as a result translocates to the nuclei of infected cells. Phosphorylation of STAT3 mediates suppression of IL-12 and TNF- $\alpha$ . While STAT3 is not activated by molecules spontaneously released by extracellular tachyzoites, it may be activated during invasion (dashed arrow). The ability of *Toxoplasma* to sustain STAT3 phosphorylation over several hours suggests that the parasite maintains activation of this host signaling molecule from within the parasitophorous vacuole.

## Conclusions and questions

*Toxoplasma* is a potent immune response inducer. Nevertheless, the parasite also attenuates immunity at several points in its life-cycle. The ability of *T. gondii* to directly manipulate signaling pathways in infected cells is an emerging aspect of the parasite's biology that is increasingly attracting the interest of toxoplasmodologists. Inhibition of proinflammatory cytokines (reviewed here) and induction of resistance to apoptosis (reviewed by Luder in this volume) are two important outcomes of the manipulative aspects of *Toxoplasma*.

Why is it necessary for *T. gondii* to block responses induced by TLR ligands such as bacterial endotoxin? One possibility is that the parasite possesses its own molecules that can signal through TLR. In this case, it may be important to attenuate host responses in order to avoid induction of TLR driven anti-microbial activity or proinflammatory cytokine pathology. In this regard, a *Toxoplasma* profilin with specificity for mouse TLR11 was recently isolated from soluble extracts of the parasite (Yarovinsky *et al.*, 2005). Another possibility is that down modulation of TLR signaling is a means to prevent harmful host responses to gut microflora that could occur as *Toxoplasma* crosses the intestinal epithelium and enters the host.

Definitive identification of *Toxoplasma* molecules that interact with host signaling pathways and the molecular mechanisms of how this is achieved are areas awaiting elucidation. In addition to STAT3 activation by an as-yet-unidentified host or parasite kinase, there is evidence for a parasite I $\kappa$ B kinase activity (Molestina and Sinai, 2005). A recent study identified several potential phosphatase and kinase molecules contained within the set of *Toxoplasma* rhoptry proteins released during invasion (Bradley *et al.*, 2005). Thus, it is possible that the parasite itself introduces kinases or phosphatases into the cell to di-

rectly manipulate host signal transduction cascades. In addition, several rhoptry and dense granule proteins span the parasitophorous vacuole membrane such that they are partially immersed in the host cell cytoplasm. Proteins with this property would be candidate molecules for involvement in recruiting and activating host cell signaling components. There is evidence that dense granule protein GRA7 is expressed at the surface of infected host cells, presumably after escaping the confines of the parasitophorous vacuole (Neudeck *et al.*, 2002). In addition, recombinant antigens released by intracellular tachyzoites can escape into the cytoplasm and even target to the host cell nucleus if expressing the appropriate nuclear localization sequence (Gubbels *et al.*, 2005). Seizing control of host signaling pathways by directing *Toxoplasma* effector molecules into the host cell cytoplasm is a tantalizing idea that awaits firm experimental verification.

The view of *Toxoplasma* solely as a trigger of cell-mediated immunity requires modification. In addition to long recognized aspects of immune evasion such as formation of cysts and induction of host anti-inflammatory mediators, the parasite directly exploits intracellular signaling cascades that attenuate proinflammatory responses. The sophisticated means by which this elegant intracellular parasite deals with host defense responses provides a prime example of a highly elegant host–parasite relationship.

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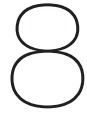


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# Apoptosis and Its Impact on the Parasite–Host Interaction



Carsten G.K. Lüder

## Abstract

Apoptosis is a tightly regulated form of programmed cell death in multicellular organisms. With the unexpected observation of an apoptosis-like cell death in protozoans including *Toxoplasma gondii* it is now clear, however, that it is not confined to metazoans. In vertebrates, apoptosis fulfils essential roles in development, tissue turnover, regulation of immune responses and in innate as well as adaptive defense mechanisms against intracellular pathogens. Importantly, infection with *T. gondii* considerably modulates apoptosis exerting both pro- and anti-apoptotic activities within its host. During acute infection, apoptosis is triggered in leukocytes including activated T cells and this diminishes the anti-parasitic immune response but also avoids overwhelming immunopathology. The induction of exaggerated leukocyte apoptosis by *T. gondii*, on the other hand, may completely abrogate anti-parasitic immunity leading to life-threatening disease. *T. gondii* also inhibits apoptosis and this either affects uninfected bystander cells or parasite-infected host cells. In order to develop within its intracellular niche, *T. gondii* may indeed rely on direct countermeasures to shut down apoptotic cascades that would normally be triggered as a cellular suicide program in parasite-infected cells. Apoptosis is, however, also indirectly inhibited in uninfected bystander cells, e.g. in macrophages and granulocytes thereby sustaining the inflammatory response during acute toxoplasmosis. Apoptosis and its complex modulation during infection with *T. gondii*, thus, have a crucial impact on the parasite–host interaction and the pathogenesis of disease.

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## Introduction

Apoptosis is an evolutionary conserved form of programmed cell death that—paradoxically—constitutes an integral part of the survival of multicellular organisms. In vertebrates, apoptosis fulfils essential roles during development, tissue homeostasis, regulation of the immune system and as effector mechanism of the innate and adaptive immune response (for reviews see Meier *et al.*, 2000; Opferman *et al.*, 2003). Not surprisingly, apoptosis also has a major impact on infections with *Toxoplasma gondii*, both as potential anti-parasitic immune mechanisms and as a pathogenicity factor during disease. However, *T. gondii* would not have been able to become one of the most successful intracellular parasites unless mechanisms have been evolved which counterbalance harmful apoptosis-mediated

effector mechanisms during infection. There is now convincing evidence that *T. gondii* exhibits different activities to inhibit apoptosis of certain cells of the host and this may facilitate intracellular survival or regulate the inflammatory response to the parasite. Going a step further, infection with *Toxoplasma* also triggers apoptosis of T lymphocytes and this may help to dampen the adaptive immunity during acute toxoplasmosis. This indicates that the parasite “hijacks” the regulatory capacities of apoptosis during immune responses. It should, however, be stressed that parasite-triggered T cell death probably represents a “normal” mechanism to turn lymphocytes off and to avoid immunopathology during prolonged infection (Opferman and Korsmeyer, 2003). It is the dual activity of the parasite which makes apoptosis and its modulation by *T. gondii* a fascinating aspect of the parasite-host interaction. Furthermore, due to the major impact on the course of toxoplasmosis, apoptosis and its parasite-mediated modulation might also represent a putative novel target for anti-parasitic therapies. Manipulation of apoptosis for the treatment of diseases which involve deficient or exaggerated levels of cell death, i.e. certain types of cancer and neurodegenerative diseases, respectively, may be exploited in the near future (Nicholson, 2000; Cummings *et al.*, 2004). Therapeutic intervention with the modulation of apoptosis exerted by *T. gondii* as a strategy to facilitate its survival within the host might thus also be feasible for the treatment of toxoplasmosis. The scenario of apoptosis as a target for chemotherapy may even become more realistic with the recent unexpected observation of apoptosis or apoptosis-like cell death in *T. gondii* (Peng *et al.*, 2003). Triggers of apoptosis or apoptosis-like cell death have already been shown to kill other protozoan parasites (Picot *et al.*, 1997; Sen *et al.*, 2004) and are, thus, potentially also useful for the treatment of toxoplasmosis.

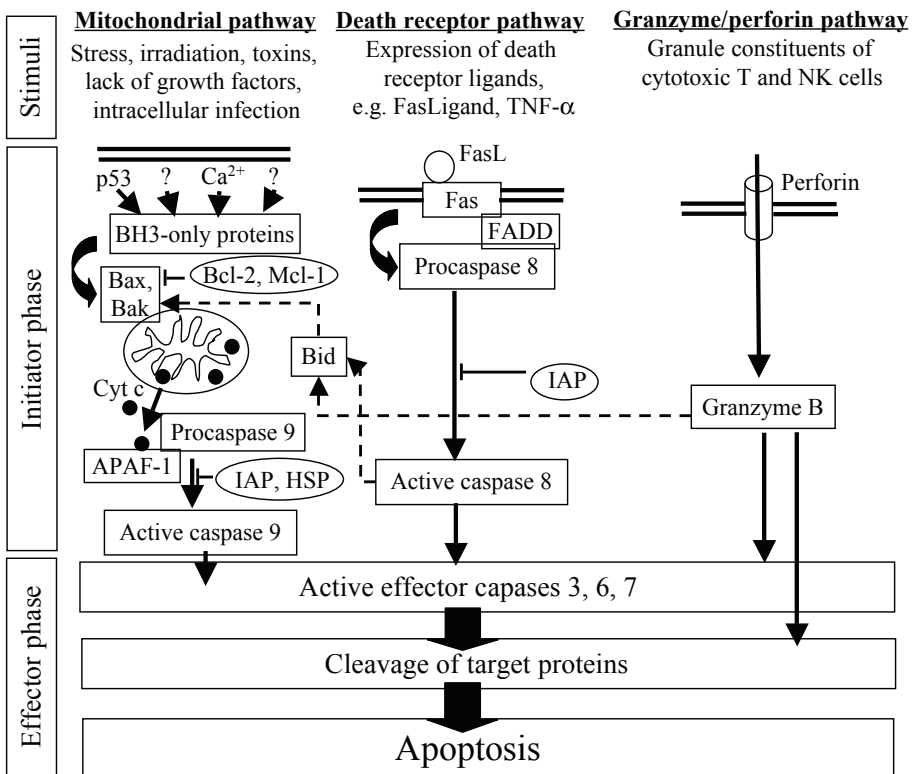
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### Apoptosis and its regulation in mammalian cells

Apoptosis in multicellular organisms has evolved as a mechanism to remove “unwanted” cells without inducing tissue damage due to inflammation. Taking into consideration that, for example,  $3.5 \times 10^{10}$  granulocytes ( $5 \times 10^6$  per ml blood) of a human adult have to be replaced every 2 to 3 days, i.e. 160 000 granulocytes have to die and be silently removed every second in each of us, it may become clear that this is a tremendous task. It is obvious that this has to be tightly regulated and it indeed involves a variety of control mechanisms both at the transcriptional and posttranscriptional level. During the execution phase of apoptosis, cells undergo disintegration into membrane-bound apoptotic vesicles which display “eat-me” signals including phosphatidylserine (PS) or distinct sugar moieties on their outer membrane leaflet and which are readily engulfed by phagocytes (Savill *et al.*, 2000). Obviously, host cells which undergo apoptosis may not sustain the development of *T. gondii*. A disturbed nutrient and growth factor supply would hamper or prevent parasite growth and replication. Furthermore, the final phagocytosis of apoptotic vesicles derived from infected cells which eventually contain viable *T. gondii* may lead to the parasites death since they would end up in a phagolysosome which is an unsuitable compartment for parasite survival (see A. Sinai, Chapter 31, this book).

Apoptosis can be initiated via three major pathways which transduce different proapoptotic stimuli into a common effector phase of cell death (Figure 8.1). The mitochondrial pathway is activated when cells encounter stress signals including DNA damage,

growth factor withdrawal, nutrient starvation or toxins. Since infection by intracellular parasites may also trigger the mitochondrial pathway unless anti-apoptotic activities of the parasite counterbalance this “natural” cell response, this pathway is supposed to be of particular relevance also for the interaction of *T. gondii* with apoptosis of the host cell. The diverse stress-related stimuli converge via largely unknown pathways at the level of several active BH3-only proteins which inactivate anti-apoptotic members of the Bcl-2 family and activate proapoptotic Bax/Bak-like Bcl-2 proteins (Figure 8.1; Bouillet *et al.*, 2002). Bax-like proteins then disrupt the outer mitochondrial membrane leading to the release of cytochrome c into the cytoplasm which in turn catalyzes the formation of a multimeric complex, called the apoptosome (Adams *et al.*, 2002). Within the apoptosome, caspase 9 is then autocatalytically activated and consequently activates the effector caspase 3. Caspases constitute the central component of apoptotic pathways (Riedl *et al.*, 2004) and lead—due



**Figure 8.1** Three major pathways lead to apoptosis in vertebrate cells. Various proapoptotic stimuli including internal and external signals are transduced via different initiator phases into a common effector phase during which apoptosis is executed. The death receptor pathway and the granzyme/perforin pathway eventually also activate the mitochondrial pathway by cleavage of Bid, which induces cytochrome c (Cyt c) release from mitochondria. Apoptosis is counterbalanced by several anti-apoptotic proteins including certain Bcl-2 family members, inhibitors of apoptosis (IAPs), or heat shock proteins (HSPs). See text for further details.

to their proteolytic activity—to those cellular and biochemical changes observed during apoptosis, e.g. cleavage of regulatory and structural proteins, DNA fragmentation and disintegration of the cell. They are synthesized as inactive procaspases and are activated by cleavage into active subunits although other mechanisms may also participate (Hengartner, 2000).

The second major apoptosis pathway is initiated by ligation of death receptors including Fas/CD95 and tumor necrosis factor-receptor 1 (TNF-R1) and is hence called the death-receptor pathway (Figure 8.1; Ashkenazi *et al.*, 1999). It plays crucial roles in removal of autoreactive T and B cells as well as during the course of an immune response. It is, therefore, also of particular importance for the course of parasitic infections (Dockrell, 2003). Ligation of these receptors leads to the recruitment of FADD (Fas-associated death domain protein) and procaspase 8 leading to a heteromultimeric complex, i.e. the death inducing signaling complex (DISC). After activation of caspase 8, this in turn activates caspase 3 which finally executes those events observed during apoptosis (see above). In addition, caspase 8 can also indirectly activate caspase 3 via the cleavage of Bid and consecutive cytochrome *c* release (Kuwana *et al.*, 1998; Scaffidi *et al.*, 1999). The interconnection between the death-receptor and the mitochondrial pathway may be relevant in order to amplify the death receptor signal in certain cells or under certain conditions.

Cytotoxic lymphocytes, i.e. T lymphocytes (CTL) and Natural Killer (NK) cells eventually employ a third apoptotic pathway in order to eliminate malignant or pathogen-infected cells (Lieberman, 2003). Recently, this granzyme/perforin pathway has also been implicated in immunological homeostasis (Trambas *et al.*, 2003). After cell-cell interaction, granzymes, i.e. serine proteases are exocytosed and enter—with the help of perforins—the target cell (Figure 8.1). They then induce apoptosis by caspase-dependent and -independent mechanisms including direct proteolytic activation of caspases, cleavage of Bid and consecutive activation of the mitochondrial pathway as well as induction of DNA fragmentation (Lieberman, 2003).

Apoptotic cascades are counterbalanced by a variety of prosurvival signals (Figure 8.1). Such molecules not only suppress physiological apoptotic cell death, but may also be employed by *T. gondii* to inhibit host cell apoptosis (see below). Cellular survival-promoting proteins include members of the Bcl-2 and the death effector domain (DED) families, inhibitors of apoptosis (IAPs), TNF-R-associated factors (TRAFs) and heat shock proteins (HSPs) (Ekert *et al.*, 1999; Beere *et al.*, 2001; Tibbetts *et al.*, 2003; Cory *et al.*, 2003).

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### Apoptosis-like cell death in *T. gondii*

It has long been assumed, that cellular suicide only makes sense for metazoans. However, a variety of protozoans including *T. gondii* (Peng *et al.*, 2003) and *Plasmodium spec.* (Picot *et al.*, 1997; Al-Olayan *et al.*, 2002) have now been shown to undergo a form of programmed cell death which shares several features of apoptosis as observed in higher eukaryotes. Apoptosis-like cell death in *T. gondii* is characterized by chromatin condensation, formation of apoptotic bodies, DNA fragmentation and hypoploid nuclei (Peng *et al.*, 2003). It is induced in extracellular tachyzoites by nitric oxide (NO) and thus resembles NO-induced apoptosis as described for different mammalian cells *in vitro* and *in vivo* (Dimmeler



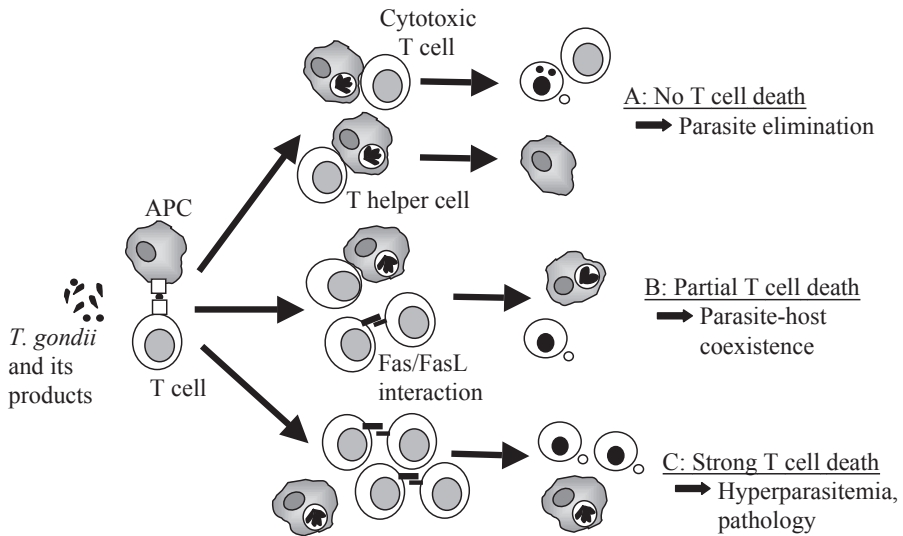
*et al.*, 1997). It should be stressed, however, that high concentrations of the NO donor sodium nitroprusside were required to induce apoptosis-like cell death in *T. gondii* and the relevance of these observations for the viability of the parasite under more physiological conditions remains unknown. After prolonged incubation of *T. gondii* without host cells, we also observed an increase in apoptotic tachyzoites as determined by terminal transferase dUTP-mediated nick end labeling and DNA fragmentation into 180bp multimers (C. Weller and C.G.K. Lüder, unpublished). This indicates that apoptosis may also occur when tachyzoites are deprived of suitable growth factors which are only provided within the parasite intracellular niche. Together, these results provide evidence that tachyzoites may die an apoptosis-like cell death under certain conditions although the impact on infection remains unknown. The driving force for *T. gondii* to die by a cellular suicide program may be found in the genetic relationships between individual cells. Since co-infections with different *T. gondii* strains are rare and, therefore, the population structure within a host mostly clonal, the self-destruction of individual cells may help genetically closely related parasites to survive. One might, thus, speculate that apoptotic-like cell death in *T. gondii* assists—beside other factors—in the control of parasite numbers and in the avoidance of host death due to hyperparasitemia as recently hypothesized for *Plasmodium spec.* in the mosquito vector (Al-Olayan *et al.*, 2002) and the mammalian host (Deponete *et al.*, 2004). Alternatively, clearance of apoptotic parasites could also render phagocytes anti-inflammatory (Savill *et al.*, 2000), thereby, diminishing the immune response against *T. gondii*.

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### **Leukocyte apoptosis triggered after infection with *T. gondii***

Acute infection of both humans and mice with *T. gondii* induces a state of transient immunosuppression as determined by decreased antibody and T lymphocyte responses to homologous and heterologous antigens (Strickland *et al.*, 1977; Wing *et al.*, 1983; Luft *et al.*, 1984). Among other factors, apoptosis of T lymphocytes triggered by *T. gondii* may restrict the immune response to the parasite, thus, allowing parasite survival and establishment of persistent infection (Figure 8.2; Khan *et al.*, 1996; Wei *et al.*, 2002). Under conditions of exaggerated apoptosis in splenocytes, parasites multiply unrestrictedly leading to high parasite burdens in various tissues of the host (Figure 8.2; Mordue *et al.*, 2001; Gavrilescu *et al.*, 2001). Under such conditions, cell death within the spleen is not restricted to certain cell populations, but occurs in CD4+ and CD8+ T lymphocytes, B lymphocytes, NK cells, granulocytes (Gavrilescu *et al.*, 2003) and macrophages (Hisaeda *et al.*, 1997). It has to be stressed, however, that the level of splenocyte apoptosis was markedly determined by the genotype and the virulence of the parasite and thus represents a characteristic determinant for the course of toxoplasmosis (see below). Importantly, lymphocyte apoptosis may also influence the local immune response after natural parasite transmission via the gut, since oral infection with *T. gondii* leads to apoptosis in Peyer's patch T cells (Liesenfeld *et al.*, 1997). Such a cell death again appears to depend on the course of infection, being only observed in inbred mouse strains susceptible to severe disease.

Apoptosis of CD4+ T cells during acute infection of mice with *T. gondii* had been preceded by a state of unresponsiveness to antigenic or mitogenic stimulation (Khan *et al.*, 1996). T cell maturation markers could be readily detected whereas costimulatory molecules CD28 and CTLA-4 were not expressed. This suggests that the T cells die as a



**Figure 8.2** T cell apoptosis is triggered after infection with *T. gondii* and diminishes anti-parasitic immunity. A: In a hypothetical scenario, infection with *T. gondii* that does not trigger T cell apoptosis would lead to appropriate priming of T helper cells and cytotoxic T cells which in turn activate infected target cells for parasitocidal activity or kill infected target cells including the intracellular parasites, respectively. B+C: During infection with *T. gondii*, however, T cell apoptosis is induced to various extents and this may lead to parasite-host coexistence (B) or exaggerated parasite replication and eventually host death (C). Parasite-triggered T cell death involves different mechanisms including Fas/FasL interaction.

consequence of absent costimulation, i.e. a mechanism called cell death “by neglect.” This hypothesis is supported by the fact that exogenous IL-2 partially restores T cell proliferation (Khan *et al.*, 1996). Recently, human dendritic cells infected with viable parasites have been shown to induce T lymphocyte apoptosis in a contact-dependent fashion (Wei *et al.*, 2002). T cells again failed to upregulate CD28 in response to parasite-infected dendritic cells, thus, resembling cell death “by neglect.”

Another important mechanism of T cell death, i.e. activation-induced cell death (AICD) also participates in the apoptosis of T lymphocytes during acute toxoplasmosis. This form of apoptosis is initiated by the interaction of Fas and Fas ligand (FasL), augmented by IL-2, and is counteracted by Bcl-2 or Bcl-X<sub>L</sub> (Hildeman *et al.*, 2002). Infection with *T. gondii* indeed leads to the upregulation of Fas expression in Peyer’s patch T cells (Liesenfeld *et al.*, 1997) as well as in splenocytes and ocular tissue (Hu *et al.*, 1999). Furthermore, induction of apoptosis by *T. gondii* is abolished in mutant mice lacking a functional Fas/FasL system (Liesenfeld *et al.*, 1997; Gavrilescu *et al.*, 2003). This suggests a crucial role for the interaction of Fas and its ligand in *T. gondii*-triggered apoptosis of T cells. Expression of Fas as well as Fas/FasL-mediated apoptosis in *T. gondii*-infected mice is regulated by the secretion of the proinflammatory cytokines IFN- $\gamma$  and IL-12 (Liesenfeld *et al.*, 1997; Gavrilescu *et al.*, 2003), and may be counterbalanced by activation of NF- $\kappa$ B<sub>2</sub> (Caamano *et al.*, 2000).

Proinflammatory cytokines may, however, also directly participate in splenocyte apoptosis during acute toxoplasmosis. Apoptosis can be initiated by ligation of death receptors belonging to the TNF-receptor (TNF-R) superfamily which also includes the TNF-R1 (p55; Ashkenazi *et al.*, 1999). High level apoptosis of splenocytes during acute toxoplasmosis is indeed accompanied by increased mRNA levels of TNF-R1 (Gavrilescu *et al.*, 2001) and high TNF- $\alpha$  serum levels (Mordue *et al.*, 2001). More importantly, deficiencies in TNF-R and TNF-R1 expression at least partially abolish splenocyte apoptosis following *T. gondii* infection (Mordue *et al.*, 2001, Gavrilescu *et al.*, 2003). This indicates that increased expression of TNF and TNF-R1 may facilitate the ligation of the TNF-R1 thereby inducing leukocyte apoptosis. Taken together, the above results indicate that both Fas-dependent and Fas-independent cell death deplete T cells during acute toxoplasmosis. Further experiments have to unravel the relative contribution of these different forms of cell death for the course of infection. Furthermore, its overall impact on the transient immunosuppression during toxoplasmosis also awaits clarification.

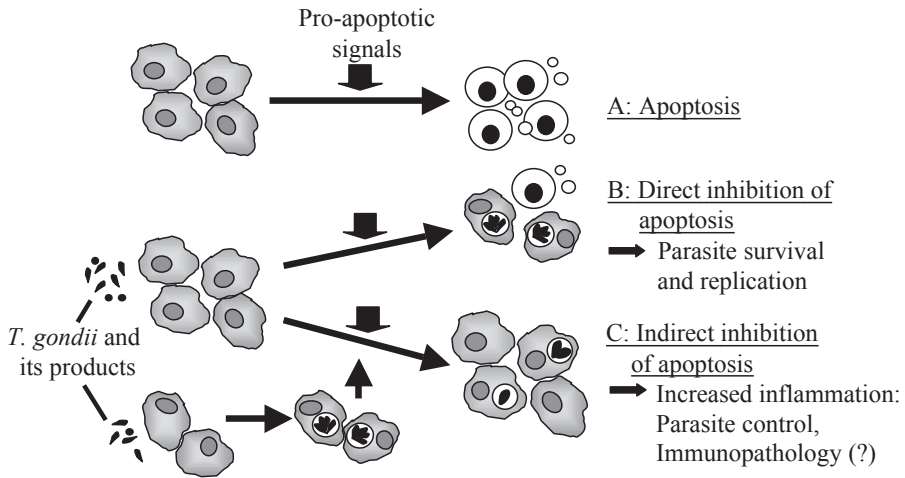
### **Anti-apoptotic activities conferred by infection with *T. gondii***

Besides increased apoptosis of distinct cell populations after infection, *T. gondii* has been clearly shown to also inhibit host cell death (Nash *et al.*, 1998; Goebel *et al.*, 1999, 2001; Channon *et al.*, 2002; Payne *et al.*, 2003). Parasite-mediated resistance against apoptosis was observed in murine and human cell lines treated with diverse inducers of apoptosis including CTL-mediated cytotoxicity, irradiation, growth factor withdrawal, TNF- $\alpha$  and/or several toxic agents (Nash *et al.*, 1998; Goebel *et al.*, 2001; Payne *et al.*, 2003). Furthermore, *T. gondii* also led to decreased apoptosis in primary cells cultured *ex vivo* (Hisaeda *et al.*, 1997; Channon *et al.*, 2002) although this appears to depend on the parasite strain used for infection (Hisaeda *et al.*, 1997). Importantly, inhibition of apoptosis has recently been shown to occur *in vivo* after intraperitoneal infection of mice with *T. gondii* (Orlofsky *et al.*, 1999, 2002). This suggests that interference of *T. gondii* with the suicide program of host cells may modify the course of toxoplasmosis.

Since *T. gondii* targets apoptosis that is transduced via the mitochondrial pathway, the death-receptor pathway and the granzyme/perforin pathway, it might be hypothesized that this is achieved by parasite interference with a component common to all pathways. To date, however, experimental evidence rather suggests that *T. gondii* inhibits apoptosis of host cells by different mechanisms. While direct inhibition of host cell apoptosis by *T. gondii* is restricted to parasite-positive host cells, indirect mechanisms protect both infected and uninfected host cells against apoptosis (Figure 8.3).

#### **Indirect mechanisms to inhibit apoptosis**

Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF) secreted by *T. gondii*-infected human fibroblasts increases expression of the anti-apoptotic Bcl-2 family member Mcl-1 and abolishes apoptosis in neutrophils *in vitro* (Figure 8.3C; Channon *et al.*, 2002). A similar mechanism may also operate *in vivo*, since the inflammatory response to *T. gondii* after intraperitoneal infection of mice was shown to be accompanied by increased levels of A1/Bfl-1, an anti-apoptotic protein similar to Mcl-1 (Orlofsky *et al.*, 1999). Importantly, parasite-induced expression of A1/Bfl-1 leads



**Figure 8.3** Different mechanisms of *T. gondii* contribute to inhibition of host cell apoptosis during infection. A: In the absence of the apoptosis-blocking activities of the parasite, pro-apoptotic signals either related or unrelated to infection induce apoptosis. B: In the presence of the parasite and its products, proapoptotic signals induce apoptosis in parasite-negative cells only, while direct interference of the parasite with signaling in parasite-positive host cells blocks apoptosis and enables parasite survival. C: Apoptosis induced by proapoptotic signals is also inhibited by indirect mechanisms, i.e. production of host-derived anti-apoptotic molecules by *Toxoplasma*-infected cells. These molecules in turn block apoptosis in parasite-infected cells and uninfected bystander cells, thereby, leading to an inflammatory response with reduced parasite replication but potentially also immunopathology.

to increased numbers of peritoneal macrophages and neutrophils possibly by inhibiting apoptosis in these cells. Although it has not been directly addressed, increased expression of A1/B $\beta$ -1 may result from parasite-driven secretion of inflammatory cytokines such as GM-CSF (Orlofsky *et al.*, 1999).

Expression of the heat shock protein (HSP) 65 in inflammatory macrophages following infection of mice with low-virulent *T. gondii* strains was also shown to prevent apoptosis of these cells (Hisaeda *et al.*, 1997). Furthermore, depletion of  $\gamma\delta$  T cells abolishes parasite-driven HSP65 expression and induces apoptosis in macrophages from *Toxoplasma*-infected mice (Hisaeda *et al.*, 1997). This indicates that under certain conditions, priming of  $\gamma\delta$  T lymphocytes by *Toxoplasma* or its products can counterbalance apoptosis in inflammatory macrophages in an indirect fashion. HSPs including HSP27, HSP70 and HSP90 are highly conserved chaperones which promote cell survival in response to various stress stimuli and this is at least partially achieved by interference with the cytochrome *c*-triggered caspase activation (Figure 8.1; Beere *et al.*, 2001). Whether HSP65 fulfils similar functions in the inhibition of macrophage apoptosis after infection with *T. gondii* remains, however, to be elucidated. Furthermore, whether the increase in HSP65 expression is mediated by the secretion of inflammatory cytokines *in vivo* also awaits further clarification.

## Direct inhibition of apoptosis

In addition to indirect mechanisms, *T. gondii* has evolved strategies to directly inhibit host cell apoptosis (Nash *et al.*, 1998; Goebel *et al.*, 1999, 2001; Payne *et al.*, 2003). Such inhibition requires the presence of intracellular parasites which either induce the expression of anti-apoptotic molecules by the host cell or directly interfere with apoptotic signaling cascades of the host cell (Figure 8.3B). It is, therefore, restricted to parasite-positive host cells and is not observed in parasite-negative bystander cells (Goebel *et al.*, 1999). Direct inhibition of host cell apoptosis was predominantly investigated *in vitro* after treatment of host cells with proapoptotic stimuli in order to obtain high level apoptosis. However, it was recently also observed in peritoneal exudate macrophages from *Toxoplasma*-infected mice indicating that it operates *in vivo* as well (Orlofsky *et al.*, 2002).

Efforts have been undertaken to unravel the underlying mechanisms of this parasite-host cell interaction. The results clearly show that *Toxoplasma* interferes with activation of the caspase cascade, thereby, abolishing cleavage of nuclear target proteins (Goebel *et al.*, 2001; Payne *et al.*, 2003). While activation of caspase 9 and caspase 3 via the mitochondrial pathway was unequivocally inhibited by *T. gondii*, clear evidence for the theory that the death receptor pathway involving caspase 8 activation is also targeted by the parasite has not yet been found. Inhibition of caspase 8 activity by *T. gondii* was demonstrated in murine fibroblasts by Payne *et al.* (2003) but other workers did not find it occurring in human histiocytic cells (Goebel *et al.*, 2001) possibly indicating species- or cell type-specific differences. Furthermore, since the death receptor pathway is interconnected with the mitochondrial pathway via the cleavage of Bid at least in certain cell types (Figure 8.1; Kuwana *et al.*, 1998; Scaffidi *et al.*, 1999), inhibition of caspase 8 activity by *T. gondii* as described (Payne *et al.*, 2003) might be due to an anti-apoptotic effect on the mitochondrial pathway. Whether *T. gondii* is able to directly inhibit the death receptor signaling pathway, thus, remains to be elucidated.

Several mechanisms have been described that accompany decreased caspase activation in the presence of intracellular *T. gondii*. Inhibition of caspase 9 and caspase 3 activity clearly correlated with decreased cytochrome c release from mitochondria into the cytosol of *T. gondii*-infected human-derived tumor cells (Goebel *et al.*, 2001). Such interference may be at least partially mediated by increased levels of anti-apoptotic proteins of the Bcl-2 family since expression of Mcl-1 and Bfl-1/A1, but not other anti-apoptotic Bcl-2 proteins was increased after parasitic infection (Goebel *et al.*, 2001; Molestina *et al.*, 2003). Increased levels of these proteins might reduce the activity of Bax/Bak-like proapoptotic Bcl-2 proteins, thereby, decreasing the release of cytochrome c from mitochondria of infected cells (Adams *et al.*, 2002). In addition to increased levels of anti-apoptotic Bcl-2 family members, inhibition of apoptosis by *T. gondii* may also involve parasite-driven expression of inhibitors of apoptosis (IAP) proteins, namely neuronal apoptosis inhibitory protein (NAIP) 1 and IAP2 (Molestina *et al.*, 2003). Since IAPs are known to block apoptosis by direct inhibition of distinct caspases, such mechanism would operate downstream of cytochrome c release and activation of caspase 8 (Deveraux *et al.*, 1998). Degradation of the poly(ADP-ribose) polymerase (PARP) in the presence of intracellular parasites as described by Goebel *et al.* (2001) is also possibly involved in the inhibition of apoptosis. PARP is well known as a target of activated caspases, but also promotes cell death under

certain conditions (Tanaka *et al.*, 1995; Jacobson *et al.*, 1999). Although direct evidence is lacking, it still appears plausible that diminished PARP levels in *Toxoplasma*-infected cells may inhibit apoptosis in a caspase-independent fashion (Alano *et al.*, 2004). In conclusion, three different mechanisms have already been described that are possibly involved in the blockade of host cell apoptosis by intracellular *T. gondii*. Further analyses are clearly required to determine whether they represent redundant mechanisms which are of functional significance for inhibition of apoptosis in *T. gondii* infected cells.

The transcription factor NF- $\kappa$ B regulates proinflammatory responses during microbial infection and also functions as a promoter for cellular survival (Wang *et al.*, 1998; Van Antwerp *et al.*, 1998). This is mediated by activating the transcription of genes encoding anti-apoptotic molecules including Bcl-2 and IAP proteins. Upregulation of distinct anti-apoptotic Bcl-2 proteins and IAPs after infection of murine fibroblasts with *T. gondii* indeed requires a functional NF- $\kappa$ B pathway (Molestina *et al.*, 2003). Whether, however, the anti-apoptotic effect exerted by *T. gondii* also relies on a functional NF- $\kappa$ B pathway has not been consistently answered. Blockade of apoptosis in murine fibroblasts by *T. gondii* was abolished in fibroblasts from NF- $\kappa$ B p65 knockout mice (Payne *et al.*, 2001). However, in human-derived promyelocytic HL-60 cells, *T. gondii* inhibited actinomycin D-induced apoptosis in the absence of DNA-binding activity of NF- $\kappa$ B (S. Goebel and C.G.K. Lüder, unpublished). Therefore, NF- $\kappa$ B activation seems not to represent a general trait of intracellular *T. gondii* (see also E. Denkers, Chapter 7, this book). Further studies are, thus, urgently required to unambiguously clarify the role of NF- $\kappa$ B in the inhibition of apoptosis by *T. gondii*.

Knowledge of the parasite molecule(s) that interferes with apoptosis-regulating signaling cascades of the host cell is still limited. Inhibition of apoptosis requires the presence of intracellular parasites while intracellular replication of *T. gondii* is dispensable (Goebel *et al.*, 1999). Notably, infection by a single viable parasite is thus sufficient to block apoptosis of the host cell. In addition, inhibition of apoptosis by *T. gondii* is reversible since it is abolished after killing the parasite (Nash *et al.*, 1998). This indicates that the continuous production and/or secretion of a *T. gondii* molecule is required to block apoptosis. *T. gondii* resides intracellularly in a membrane-bound parasitophorous vacuole. It may be hypothesized that the parasite molecule is either small enough to diffuse through pores within this membrane (Schwab *et al.*, 1994) or is inserted into or even translocated across the membrane by still unknown transport pathways (Cesbron-Delauw, 1994; Beckers *et al.*, 1994). Interestingly, it was recently described that the inhibitor of NF- $\kappa$ B activation, I $\kappa$ B, accumulates on the vacuolar membrane surrounding intracellular *T. gondii* and appears to be phosphorylated by a novel *T. gondii* kinase activity (Molestina *et al.*, 2003, 2005). This might then lead to the ubiquitinylation and proteasomal degradation of I $\kappa$ B and activation of NF- $\kappa$ B. It supports the hypothesis that parasite molecules with access to the host cell cytoplasm may indeed interfere with signaling cascades of the host cell thereby abolishing apoptosis.

### Significance of decreased host cell apoptosis for parasite survival

In order to grow and replicate, *T. gondii* relies on the sustained viability of its host cell. The cellular suicide of a parasite-infected cell is, therefore, believed to serve as an innate



response to counteract the intracellular development of the parasite (Williams, 1994). To date, however, the consequences of potential host cell apoptosis on the development of *T. gondii* have not been directly addressed in detail. This question is not trivial since host cell apoptosis not only disturbs the intracellular development of the parasite but can also facilitate dissemination of certain pathogens (Gao *et al.*, 2000; Van Zandbergen *et al.*, 2004). Yamashita *et al.* (1998) reported that induction of apoptosis in *T. gondii*-infected target cells by CTL-mediated cytotoxicity does not lead to parasite death *in vitro*. *In vivo*, however, engulfment of apoptotic bodies containing viable parasites by phagocytic cells and subsequent elimination of the parasite may considerably contribute to parasite death due to apoptosis. It thus appears likely that interference of *T. gondii* with apoptosis of its host cell facilitates the intracellular development of the parasite and increases parasitemia (Figure 8.3B). This view is sustained by the strength of experimental data from Orlofsky *et al.* (2002) who reported a decreased number of parasites in apoptotic macrophage population. However, further investigations are required to determine whether the development of *T. gondii* indeed relies on the inhibition of host cell apoptosis.

As discussed above, infection of *T. gondii* also triggers mechanisms that diminish apoptosis in uninfected bystander cells under certain conditions (Figure 8.3C). Importantly, such reduction of apoptosis in distinct cell populations of the host may lead to an enhanced inflammatory response to the parasite (Hisaeda *et al.*, 1997; Orlofsky *et al.*, 1999, 2002). Inflammatory leukocytes limit parasite replication by T cell-independent effector mechanisms but can also induce host mortality due to overwhelming immunopathology (see also C. Hunter, Chapter 6, this book). Therefore, enhanced survival of inflammatory cells probably fulfils a dual role during toxoplasmosis depending on the host and parasite strain as well as the dose and route of infection. Indeed, depletion of  $\gamma\delta$  T cells in mice infected with a low-virulent strain of *T. gondii* abolished parasite-triggered inhibition of apoptosis in peritoneal macrophages and reduced host survival (Hisaeda *et al.*, 1997). This suggests that indirect inhibition of apoptosis after infection with *T. gondii* contributes to efficient parasite control. In contrast, reduced cell death possibly also increases the inflammatory response to highly virulent *T. gondii* strains thereby leading to increased immunopathology and host mortality (Orlofsky *et al.*, 2002). From these experimental studies in mice, it can be concluded that *T. gondii*-mediated inhibition of apoptosis by indirect mechanisms fulfils a crucial role in the regulation of the immune response and the outcome of infection.

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## Roles of apoptosis during infection

As outlined above, apoptosis may potentially fulfill a variety of innate and adaptive effector as well as regulator functions in the response to *T. gondii*. However, on the basis of the fact that parasite infection exerts multiple effects on apoptosis of host-derived cells, what are the actual roles of apoptosis during toxoplasmosis?

### Apoptosis as an effector mechanism against *T. gondii*

After infection with *T. gondii*, CD8+ and CD4+ T lymphocytes with cytotoxic activity against parasite-infected target cells have been isolated from seropositive humans (Montoya *et al.*, 1996) whereas in mice, T cell-mediated cytotoxicity appears to be restricted to

the CD8+ subset (Hakim *et al.*, 1991). Such cytotoxicity may be achieved by the induction of apoptosis via the granzyme/perforin pathway (Figure 8.1; Lieberman, 2003). The view on cytotoxicity as an important effector mechanism to control parasite replication was supported by experimental findings in mice showing that CD8+ T cells represent the more relevant effector cell type for effective control of *T. gondii* as compared to CD4+ T cells (Suzuki *et al.*, 1988; Gazzinelli *et al.*, 1992). However, the role of CTL activity during toxoplasmosis is obscured by the fact that these T cells simultaneously exert cytotoxicity and secrete the protective cytokine IFN- $\gamma$  (Suzuki *et al.*, 1988). Using perforin knockout mice, granule-mediated cytotoxicity of T lymphocytes and NK cells have indeed been shown to be dispensable in the control of parasite replication during the acute stage of infection (Denkers *et al.*, 1997). In contrast, perforin-mediated target cell lysis partially restricts tissue cyst development within the brain and decreases susceptibility of mice during chronic *Toxoplasma* encephalitis (Denkers *et al.*, 1997). This suggests that cells harboring latent bradyzoite-containing tissue cysts are more susceptible to apoptosis than tachyzoite-containing host cells. It leads to the interesting hypothesis that bradyzoites and tachyzoites differ in their ability to interfere with signaling cascades of their host cells, only the latter being able to considerably block apoptosis. Alternatively, distinct conditions within the brains of infected mice may lead to the higher susceptibility of tissue cyst-containing host cells to CTL-mediated cytotoxicity.

Beside granule-mediated cytotoxicity, CTL also induce apoptosis via the death-receptor pathway (Tibbetts *et al.*, 2003). Its impact on the control of *T. gondii* has not been thoroughly investigated. However, CTL-mediated apoptosis via this pathway rather represents a regulator of the immune response than an effector function against intracellular pathogens (Lieberman, 2003). It may, therefore, play only a minor role in the parasite control during toxoplasmosis.

Apoptosis as a suicide program of the cell in response to intracellular infection with *T. gondii* does not seem to play a significant role as an innate effector mechanism against the parasite. This may be largely due to the broad anti-apoptotic effects of *T. gondii* (see above). However, since the effect of the latent bradyzoite stage of *T. gondii* on host cell apoptosis is unknown, it cannot be excluded that apoptosis restricts parasite development during chronic toxoplasmosis.

In conclusion, apoptosis plays only a minor role in the innate and adaptive defense against acute *T. gondii* infection, but may contribute to parasite control during chronic toxoplasmic encephalitis.

## Apoptosis in the pathogenesis of toxoplasmosis

In contrast to the limited role in combating the parasite, apoptosis plays a crucial role in the pathogenesis of toxoplasmosis. Induction of high levels of apoptosis in splenocytes (Mordue *et al.*, 2001; Gavrilescu *et al.*, 2001), Peyer's patch T cells (Liesenfeld *et al.*, 1997), and peritoneal macrophages (Hisaeda *et al.*, 1997) after infection of mice with *T. gondii* leads to defective immune responses to the parasite (Figure 8.2). Importantly, extensive apoptosis was associated with high level parasitemia and an increased number of deaths after infection (Mordue *et al.*, 2001; Gavrilescu *et al.*, 2001; Liesenfeld *et al.*, 1997; Hisaeda *et al.*, 1997). Splenocyte apoptosis was clearly less evident after infection of mice

with *T. gondii* strains of lower virulence leading to reduced parasite burdens (Lee *et al.*, 1999). The level of apoptosis in T lymphocytes and possibly other leukocytes, thus, appears to correlate with the induction of pathology during toxoplasmosis. Such apoptosis may dysregulate the immune response, thereby, leading to unrestricted parasitemia and damage of host tissues, at least under certain conditions. In contrast, low or intermediate levels of T cell death rather contribute to the parasite's ability to establish persistent infections. Thus, a tight regulation of T cell death may be critical for a stable parasite–host interaction during toxoplasmosis. This view is supported by the finding that apoptosis is able to restrict the intraocular inflammation in response to *T. gondii* (Hu *et al.*, 1999). Furthermore, low levels of apoptosis gradually decrease the number of intracerebral T cells during chronic toxoplasmic encephalitis and may favor persistence of *T. gondii* within neuronal cells without the induction of immunopathological effects (Schlüter *et al.*, 2002). Apoptosis of T cells, therefore, not only restricts the parasite-specific immune response but also immunopathological changes of host tissue at least in mice. It will be of major interest to determine whether apoptosis fulfils similar roles in the pathogenesis of human toxoplasmosis.

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## Conclusions

Apoptosis and its modulation during toxoplasmosis represent a fascinating example of the complex interaction of *T. gondii* with the mammalian host. More importantly, however, it has a major impact on the host's immune response to the parasite and determines the outcome of infection and the pathogenesis of disease. Considering the general concept that infection of cells normally triggers a cellular suicide program in order to restrict or even avoid parasite development, the broad anti-apoptotic activities of *T. gondii* may even represent an essential feature of its parasitic life style. Thus, the interaction of *T. gondii* with apoptosis of the host's cells has certainly become an important topic in *Toxoplasma* research. We are now beginning to understand the underlying principles of how the parasite triggers apoptosis in certain immune cells or blocks apoptosis in its own host cell. Interestingly, multiple mechanisms appear to be involved in either case and it will be important to unravel whether they represent redundant mechanisms or rather operate under different physiological conditions. In order to further our knowledge of the underlying molecular and cellular mechanisms it is also crucial to identify and characterize those parasite molecules which mediate the pro- and anti-apoptotic activities of *T. gondii*. In addition, we still have very limited information on the effect of bradyzoites of *T. gondii* on host cell apoptosis and its impact on the chronic stage of toxoplasmosis. Unraveling these topics will further our understanding of a crucial determinant of the parasite–host interaction and the outcome of infection. It might also help to develop novel therapeutic strategies for the treatment of toxoplasmosis.

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# Pathogenicity and Virulence in *Toxoplasma gondii*

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## Abstract

*Toxoplasma gondii* is a widespread protozoan parasite that causes opportunistic disease in humans. *T. gondii* has a complex life cycle that alternates between haploid asexually dividing forms that infect a variety of hosts, and a sexual phase, which occurs exclusively in the intestinal epithelial cells of the cat. Despite the potential for sexual recombination, *T. gondii* maintains a highly clonal population structure consisting of three clonal lineages that predominate in North America and Europe. This unusual population structure may result from the recent adaptation for asexual oral infectivity, which bypasses the cat and allows direct transmission between intermediate hosts. Notably, type I strains are distinguished by their high mortality in the mouse model. Forward genetic approaches have been developed to allow mapping of complex phenotypes based on linkage analysis. Acute virulence in the type I lineage is tightly linked to a major locus on chromosome VIIa. Identification of genes that regulate virulence and pathogenicity will be aided by the recently completed genome and the assembly of a genome map based on genetic linkage analysis.

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## ***Toxoplasma gondii*: genetics and population biology**

*Toxoplasma gondii* has a heteroxenous life cycle that consists of a sexual phase in the intestine of the cat and asexual replication in a wide range of warm-blooded vertebrates (Dubey, 1998). Despite the existence of a sexual phase, the population structure is remarkably clonal, consisting of three major lineages that are predominant in North America and Europe (Ajzenberg *et al.*, 2002; Dardé *et al.*, 1992; Howe and Sibley, 1995; Sibley and Boothroyd, 1992) (see also other contributions in this volume). The three archetypal lineages are derived from two closely related parental strains and following their recent origin in the past 10 000 yrs, they have undergone rapid expansion (Grigg *et al.*, 2001; Su *et al.*, 2003). Toxoplasmosis can be studied in a variety of small animal models that mimic the acute and chronic phases of infection (Denkers *et al.*, 1997). Production of mutants is facilitated by its haploid genome and ease of propagation in a variety of cell lines (Pfefferkorn, 1988). *Toxoplasma gondii* is equipped with excellent methods for classical (Khan *et al.*, 2005) and molecular genetics (Roos *et al.*, 1994), making it a model system for exploring the genetic basis of virulence in protozoan parasites.

## Phenotypic variation among clonal *T. gondii* lineages

The predominantly clonal population structure has several implications for understanding the biology of *T. gondii* and its potential to cause disease (see Chapters 11 and 13). First, members of separate clonal lineages may differ dramatically in biological traits including growth, infectivity, pathogenesis, and virulence (Saeij *et al.*, 2005; Sibley and Howe, 1996). Second, isolates from the same clonal lineage will share common phenotypes. Finally, such phenotypic differences are likely to be mediated by the small number of genetic differences that exist between the lineages.

Type II strains are the most abundant in animal infections and human toxoplasmosis cases in North America and Europe (Ajzenberg *et al.*, 2002; Howe *et al.*, 1997; Howe and Sibley, 1995; Mondragon *et al.*, 1998). While type III strains are also abundant in animals, they are rarely seen in humans (Howe and Sibley, 1995; Howe *et al.*, 1997; Ajzenberg *et al.*, 2002). The majority of infections in AIDS patients (Honoré *et al.*, 2000; Howe *et al.*, 1997), and congenital cases of toxoplasmosis in France (Ajzenberg *et al.*, 2002) are caused by type II parasites. Hence, while these strains are classified as being relatively non-virulent in the mouse model (see below), they are certainly capable of causing serious human disease. This difference also highlights the concept that “virulence” may be due to host specific factors that influence the host–parasite interaction. The reasons for the prevalence of type II strains in human infection are unclear but could relate to immune status, the burden of exposure, or particular adaptations that make these strains successful in causing human infection. Additionally, while type I strains are relatively rare in animals and humans, they may be more prone to reactivation in immunocompromised patients as shown an increased prevalence in several independent studies of toxoplasmosis in AIDS patients (Howe and Sibley, 1995; Khan *et al.*, 2005).

The type I lineage has been shown to exclusively contain those strains of *T. gondii* that are acutely virulent in mice (Sibley and Boothroyd, 1992). Acute virulence is normally monitored by establishing a dose-response curve relating infection to mortality. The development of chronic infections in mice that survive acute infection is easily determined by seroconversion, which occurs within the first several weeks of infection (Howe *et al.*, 1996). Virulent strains are defined by those which never give rise to seropositive survivors even at doses of 10 or fewer tachyzoites (Howe *et al.*, 1996; Sibley and Boothroyd, 1992). This definition provides a reliable means of classifying strains as virulent (a single viable organism leads to mortality;  $LD_{100} = 1$ ) vs. non-virulent (readily give rise to chronic infections characterized by seropositive survivors). Importantly, acute virulence typical of type I strains is independent of the genetic background of the mouse and is evident in inbred and outbred lines. Because types II and III readily give rise to chronic infections (leading to seropositive survivors) it is possible to determine a  $LD_{50}$  for these strains using standard titration methods (Reed and Muench, 1938). Types II and III typically have  $LD_{50}$  values of  $\geq 10^3$  tachyzoites based on survival after i.p. challenge in outbred mice (Howe *et al.*, 1996; Sibley and Boothroyd, 1992).

It has been suggested that the virulence of the commonly used RH type I strain may be due to extensive laboratory passage since it was isolated in 1941 (Sabin, 1941). However, more than 20 recently isolated type I strains also share this highly virulent phenotype in mice, indicating that acute virulence is correlated with the genotype of the parasite and not

with passage history (Sibley and Boothroyd, 1992; Su *et al.*, 2002). Type I strains have been isolated from a variety of domestic animals including, pigs, goats and cows, and from humans in North America, Europe other parts of the world, thus indicating this lineage and the acute virulence phenotype is widespread.

An added complication of monitoring the virulence in normally non-virulent strains is that the LD<sub>50</sub> can decrease following repeated passage (requiring fewer tachyzoites to cause lethal infection in outbred mice). Such a spontaneous change has been recorded in a clone of the type III strain VEG; this clone replicates at faster rates typical of virulent strains (Radke *et al.*, 2001). However, type II and III strains never reach the extreme lethality of type I strains (unpublished results) but instead they invariably give rise to chronic infections at low doses, making it possible to classify these strains as non-virulent. Importantly, inbred mice show very different susceptibilities to non-virulent strains of *T. gondii* (Araujo *et al.*, 1976; Brown and McLeod, 1990; Johnson, 1984; McLeod *et al.*, 1989). In particular, C57BL/6 mice challenged with the type II strain ME49 are susceptible to parasite induced intestinal necrosis (Liesenfeld, 2002) and the development of spontaneous CNS disease that resembles reactivation (Hunter and Remington, 1994). While inbred mice have been useful for defining the contribution of host genetics to resistance, estimation of parasite virulence in these various genetic backgrounds complicates comparisons between different parasite lineages. As such, the estimation of acute virulence in outbred mice provides the most unbiased method for direct comparison of parasite strains.

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### Host response and pathology during acute *Toxoplasmosis*

*Toxoplasma gondii* induces a potent Th1 response in mice that is characterized by production of IL-12 and IFN- $\gamma$ , both critical factors for control of infection (Denkers *et al.*, 1997; Hunter and Reichmann, 2001; See Chapter 7). During acute infection with *T. gondii* the parasite initially replicates rapidly but it is eventually contained by innate and adaptive cell-mediated immune responses. A variety of effector cells including macrophages, dendritic cells, and neutrophils produce IL-12, a critical mediator for inducing IFN- $\gamma$  production (Hunter and Reichmann, 2001). High levels of IFN- $\gamma$  are required for the control of both chronic and acute stages of infection in the murine host (Yap and Sher, 1999). Recent studies indicate that recruitment of inflammatory monocytes to the site of primary infection is essential to control toxoplasmosis in the murine system (Robben *et al.*, 2005). These cells produce IL-12 and are also activated by IFN- $\gamma$  to kill and inhibit parasites and thus they play several important roles in combating infection (Mordue and Sibley, 2003; Robben *et al.*, 2005).

While infection with type I strains is uniformly lethal in mice, the specific causes of pathology during the acute infection are poorly understood. There are several possible explanations for the observation that acutely virulent strains are not adequately controlled by the immune system including: (1) inadequate stimulation of the protective immune response, (2) refractoriness on the part of the parasite to killing mechanisms of the host, (3) increased growth potential of the parasite resulting in greater tissue damage. Studies in the mouse model indicate that all three lineages induce a vigorous immune response typified by induction of Th1 cytokines (Gavrilescu and Denkers, 2001; Mordue *et al.*, 2001). Moreover, different parasite strains are equally sensitive to killing by activated

macrophages, indicating they may not have inherent differences in susceptibility (Mordue and Sibley, 1997; Mordue and Sibley, 2003). The pathology of acute infection is associated with excess production of acute inflammatory mediators, signifying that disease is due to excessive inflammatory immune responses (Gavrilescu and Denkers, 2001; Mordue *et al.*, 2001).

A variety of studies, primarily using type I strains, indicate that infection of macrophages by *T. gondii* leads to down regulation of inflammatory mediators by inhibition of cellular signaling pathways (Denkers *et al.*, 2003). Thus, parasite “virulence” may result in part from inhibition of host cellular responses designed to mediate protection. Additionally, there is evidence that type II strains may prime the immune system more effectively, thus leading to an earlier response that is important for control of infection. Type II strains induce a much stronger IL-12 response from macrophages *in vitro* (Robben *et al.*, 2004) and this stimulus may be important *in vivo* for priming early production of IFN- $\gamma$ . Type I strains are refractory in this capacity (Robben *et al.*, 2004) and hence may delay activation of the Th1 immune response *in vivo*.

Understanding the basis acute virulence is complicated by the high degree of similarity between parasite lineages. All lineages infect a variety of cell types equally well, including naive macrophages, and significant differences in cell invasion *in vitro* have not been reproducibly demonstrated. In contrast, differences in growth rate have been observed *in vitro*, with type I strains exhibiting a faster doubling time (5–6 hours) versus types II and III (7–9 hours) (Radke *et al.*, 2001). Extrapolating these differences over time could account for the different tissue burdens achieved *in vivo* when low doses of parasites are administered by i.p. injection (Mordue *et al.*, 2001). High tissue burdens are associated with lethality and when type II strains are inoculated at high initial doses, they reproduce the lethality seen in type I infections (Gavrilescu and Denkers, 2001; Mordue *et al.*, 2001). Additionally, the capacity for strains to differentiate into slower-growing bradyzoites may also affect expansion during acute infections. Type I strains appear to be less likely to undergo differentiation in response to stress induced by the immune system, and thus less likely to curtail growth kinetics *in vitro*. Differentiation is also likely to have a strong epigenetic component based on recent studies indicating that chromatin remodeling contributes substantially to stage-specific gene expression (Saksouk *et al.*, 2005).

Comparison of different strains of *T. gondii* *in vitro* reveals dramatic differences between lineages in the ability to migrate under soft agarose or to cross biological barriers such as polarized epithelial cells (Barragan and Sibley, 2002). Type I strains are adept at both of these two processes and presumably this leads to faster spread *in vivo*, and possible escape from local immune responses. Traditional measurements of parasite dissemination such as plaque formation assays support this conclusion (Mordue *et al.*, 2001) as do more recent studies using bioluminescence (Hitziger *et al.*, 2005). While active migration across biological barriers may facilitate spread, the ability to infect trafficking leukocytes may also lead to dissemination. Infected leukocytes offer a prime means of dissemination of parasites *in vivo*, and recent studies in the murine model indicate such a role for dendritic cells and also some populations of monocytes (Courret *et al.*, 2005). Infection of murine dendritic cells by *T. gondii* suppresses differentiation, thus compromising their ability to prime the immune system (McKee *et al.*, 2004). Studies with human dendritic cells also

indicate a capacity for the parasite to prevent maturation and to enhance cellular migration (Diana *et al.*, 2004). Consequently, successful dissemination *in vivo* depends on a combination of active migration, hitchhiking within leukocytes, and modulating the responsiveness of immune effector cells.

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## Defining virulence genes

Defining virulence at the molecular level has been hampered in parasites by their complex biology and until recently, limited availability of genetic tools. In contrast, bacterial pathogens provide a paradigm for how genetic approaches can be used to identify virulence genes. One strict definition of virulence genes is provided by an extension of the Henle-Koch's postulates for identifying the etiological cause of infection (Falkow, 1988). These molecular Koch's postulates define virulence genes based on the following criteria: (1) the gene should be found in pathogens and not in non-pathogens, (2) deletion of the gene reduces virulence and this can be restored by complementation, (3) transfer of the gene of interest to a non-pathogen background leads to gain-of-function. These postulates require the availability of a genetic system and consequently cannot be fulfilled in all situations. They also limit the definition of virulence genes to those that directly cause or contribute to disease in a pathogen-specific fashion.

An alternative definition for virulence is based on large-scale insertional mutagenesis studies that have attempted to define the question more broadly as: *what genes are required for infection?* (Groisman and Ochman, 1994). Not surprisingly, the list of genes identified by this approach is much longer and includes a large number of basal metabolic functions that are essential *in vivo*. Notably, many of these are not restricted to pathogens and may not contribute directly to disease, yet they are required for survival *in vivo*. Many similar genes are also found in non-pathogens that face hostile, nutrient-poor conditions in the environment and thus may require a similar complement of metabolic pathways for survival. Regardless of the definition used, genetic approaches provide a powerful standard for establishing the role of specific genes in virulence.

While genetic systems have been developed for a number of parasites, they have not been extensively applied to classifying virulence. The majority of studies reported thus far have employed reverse genetic approaches to ask the question: *is this gene required for infection?* Such studies have revealed a requirement for several surface proteins, secretory adhesins, and motor proteins in infection by *T. gondii* in the mouse model (i.e. MIC1-MIC3 (Cerede *et al.*, 2005), GRA2 (Mercier *et al.*, 1998), M2AP (Huynh *et al.*, 2003), SAG3 (Dzierszinski *et al.*, 2000), MyoA (Meissner *et al.*, 2002), SAG3 (Dzierszinski *et al.*, 2000), and AMA1 (Mital *et al.*, 2005)). While less well investigated, a number of metabolic pathways are also essential for parasite survival *in vivo* (Fox and Bzik, 2002; See Chapter 20). Such reverse genetic approaches are useful for evaluating the role of specific genes; however, these studies do not systematically identify genes that are responsible for disease progression or define the molecular basis for differences in pathogenicity between naturally occurring strains of *T. gondii*.

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## Genetic approaches for defining virulence genes

The availability of classical genetic crosses and linkage analysis in *T. gondii* provides a forward genetic system for identifying genes involved in virulence (Khan *et al.*, 2005; Su

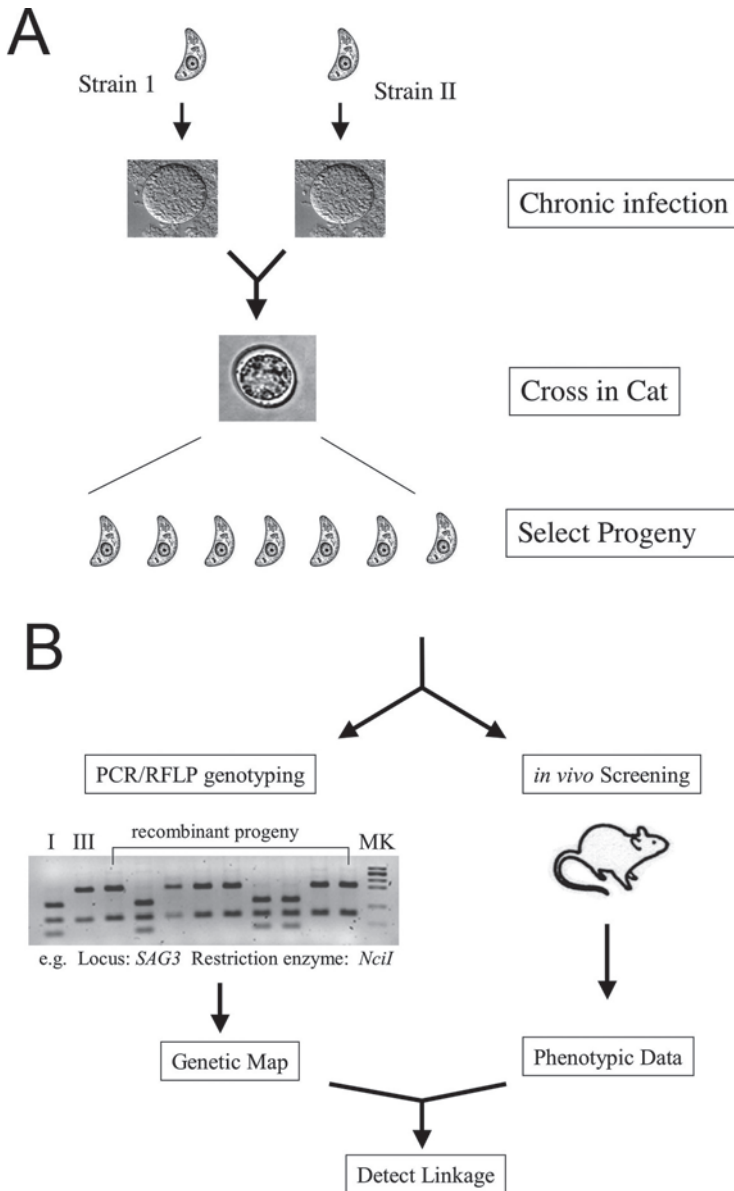
*et al.*, 2002; see Chapter 11) (Figure 9.1). To explore the genetic basis of acute virulence, a highly virulent type I strain called GT-1 (Dubey, 1980), was crossed with a non-virulent type III strain called CTG (Pfefferkorn *et al.*, 1977). GT-1 has a virulent phenotype like RH, and a single viable parasite is uniformly lethal in the mouse when injected i.p. (Su *et al.*, 2002). In contrast, CTG has an LD<sub>50</sub> of  $\sim 10^5$  in outbred mice (Su *et al.*, 2002). Recombinant clones were genotyped using restriction fragment length polymorphism markers distributed across the genome. Separately, each clone was tested to establish the virulence phenotype in mice following i.p. inoculation with  $10^1$ ,  $10^2$  or  $10^3$  tachyzoites. Phenotypic analysis of recombinant progeny revealed a range from fully virulent to non-virulent, including clones that showed intermediate levels of virulence (Su *et al.*, 2002). The definition of virulence used in this cross is that fully virulent clones do not give rise to chronic infection (all infected animals succumb, even at low dose of parasites), while clones with intermediate virulence were categorized as high (70–95% mortality, but with chronically infected survivors), intermediate (30–70% mortality) and low (< 30% mortality). These results indicate that virulence is largely heritable rather than epigenetic and that it is likely multigenic. Importantly, when acute mortality is considered as single gene model, it maps strongly to the central region of chromosome VII (Su *et al.*, 2002) (since renamed VIIa) (Khan *et al.*, 2005).

Many genetic traits are not all-or-none but rather vary along a continuum. Such traits are typically not governed by a single locus but rather by the combination of genes at different loci. Phenotypes such as growth and virulence fit this profile. Additionally, other regions of the genome can modulate traits that are largely mediated by a single locus. For example, while chloroquine resistance is primarily due to the PfCRT gene, drug sensitivity in field isolates is also influenced by other loci (Ferdig *et al.*, 2004). Very likely, biological traits like virulence will prove to have a similar complex genetic underpinning.

Studies by Lander and Botstein (Lander and Kruglyak, 1995; Lander and Botstein, 1989; Lander *et al.*, 1987) stimulated enormous interest in the genetic analysis of complex traits. In particular, these studies drew attention to the idea that interval mapping based on genetic markers could be used to genetically localize quantitative trait loci (QTLs). The development of new QTL mapping strategies and the successful identification of genes responsible for many complex traits in a wide variety of organisms has kindled interest in the use of QTL mapping in parasites (Su and Wootton, 2004). Linkage analysis can be performed using a variety of software tools and the use of statistical analyses such as maximum likelihood ratio or Log odds ratio (LOD scores) takes into account the potential false-positive associations that may occur with large datasets (Lynch and Walsh, 1998).

Analysis of the progeny of the *T. gondii* genetic cross for virulence revealed a contribution for multiple genetic loci (Su *et al.*, 2002). When cumulative mortality is modeled based on the assumption that it may be due to several genes, the major contribution is still located on chromosome VIIa. QTL analysis revealed that virulence was strongly linked to a single locus on chromosome VIIa flanked by the two markers M95 to cS10-A6 with minor contributions from other regions of the genome (Su *et al.*, 2002). The implication of this finding is that a single genetic locus is largely responsible for the virulence phenotype of type I strains of *T. gondii* in mice. This region on chromosome VIIa contains more than 100 genes and interestingly none of them correspond to genes that have previously





**Figure 9.1** Genetic crosses between *T. gondii* strains for mapping complex phenotypes. (A) Schematic for producing genetic crosses in *T. gondii*. Tachyzoites of two separate strains are grown *in vitro* and then used to establish chronic infections in mice or rats. Strains are crossed by co-feeding cysts to cats and oocysts are collected and sporulated. Haploid F1 progeny are isolated following *in vitro* culture. (B) Schematic of analysis of progeny from a cross. Progeny are genotyped using RFLP markers (left) and phenotyped for virulence in mice (right). Linkage analysis can then be performed (Khan *et al.*, 2005; Su *et al.*, 2002), to identify candidate genetic regions for the phenotype of interest.

been shown to be essential *in vivo*. To determine whether the polymorphisms associated with the QTLs identified in the progeny from the I×III cross are also found in other virulent strains, 45 naturally occurring isolates from humans and animals were analyzed. Included in this group were 19 isolates that displayed the type I phenotype, all of which were uniformly lethal in mice with  $LD_{100} = 1$  (Su *et al.*, 2002). Analysis of the markers on chromosome VIIa showed that the type I genotype was conserved in all virulent strains (Su *et al.*, 2002). Collectively, these data indicate that the gene(s) associated with the QTL for virulence on chromosome VIIa is likely conserved in all strains that exhibit acute virulence characteristic of the type I lineage.

The differences seen in virulence between the three archetypal strain types are likely due to the different assortment of alleles they acquired following the relatively few genetic crosses since their common origin (Grigg *et al.*, 2001, Su *et al.*, 2003). Two models could explain the much higher level of virulence in the type I lineage. First, the type I lineage may have inherited a pre-existing virulence gene from one of the two parental strains. The parental strains that gave rise to the archetypal lineages are not directly known, so direct assessment of this model is not possible. Alternatively, neither parental strain may have expressed this trait and only through the combination of alleles present in the progeny did virulence fully manifest.

To determine if virulence can come about by recombination in a cross, recombinant progeny derived from a cross between non-virulent type II and type III parental strains (Sibley *et al.*, 1992) were assessed for their virulence phenotype in mice (Grigg *et al.*, 2001). When inbred CBA/CaJ mice were used in this study, high mortality was observed for two of the progeny S23 and CL11 (Grigg *et al.*, 2001). While these results illustrate the importance of recombination in generating complex phenotypes like virulence, it is difficult to directly compare these results to other studies due to the use of inbred mice, which are highly susceptible to toxoplasmosis. Retesting of the clone CL11 in outbred mice revealed it had an  $LD_{50}$  of  $> 10^3$ , which is not significantly different from the parental types (Su C, and Sibley LD, unpublished). While the clone S23 exhibits higher virulence in both inbred (Grigg *et al.*, 2001) and outbred mice (Su C, and Sibley LD, unpublished), notably it leads to chronic infections at low doses in both types of mice. By definition this is an intermediate phenotype albeit exhibiting a much lower  $LD_{50}$  than the parental strains. In this regard S23 is similar to the clones with intermediate virulence derived from the I×III cross (Su *et al.*, 2002). Backcross studies using the S23 clone may be useful in defining what genes contribute to this intermediate virulence phenotype.

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## Future directions

Genetic linkage mapping provides a powerful first step for identifying the genetic basis of complex traits. The success of mapping QTLs for acute virulence demonstrates the usefulness of this approach (Su *et al.*, 2002). Additionally, there are numerous other quantitative traits that likely contribute to pathogenesis and which could also be studied by genetic linkage analysis including: migration capacity, transmission, growth rate, differentiation, induction or suppression of host signaling pathways. One limitation to genetic mapping is the relative infrequency of crossovers in *T. gondii* (Khan *et al.*, 2005). Nonetheless, by comparing the segregation of markers that are located at known map positions to the physical

map of the genome it is possible to identify candidate genes for complex traits such as acute virulence. The integration of a combined physical genome map with genetic linkage markers in the *T. gondii* genome database (<http://ToxoDB.org>) facilitates such analyses. Additionally, the availability of tools for reverse genetics (Roos *et al.*, 1994) provides a means to identify the role of specific genes involved in conferring complex traits such as virulence. *Toxoplasma* is among the few parasitic systems that offer a robust combination of *in vitro* and *in vivo* models, forward, and reverse genetics. As such, it provides a model system for investigating the genetic basis of complex traits such as virulence and pathogenicity.

#### Note added in proof

Recent genetic mapping studies have revealed that several specific genes encoding rhoptry proteins are important for virulence in *T. gondii* (Saeij *et al.*, 2006; Taylor *et al.*, 2006).

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## Abstract

The high prevalence, worldwide distribution, and ability to persist in the face of a competent immune system all indicate that *Toxoplasma gondii* is very well adapted to life within a host. As an obligate intracellular parasite, these adaptations necessarily include mechanisms to co-opt the host cell for its own purposes. Much of the phenomenology associated with the intracellular niche has been known for decades. In recent years, however, some of the molecules that mediate a sustained interaction between host and parasite have begun to be identified and the way in which they promote intracellular survival have likewise begun to be uncovered. In this chapter, we will review the recent findings in the area of host response. In addition, some of the methods that are just beginning to be developed and that seem most likely to give further detail on these processes will also be discussed. We will focus on the best-studied stage, the tachyzoite, but what information exists on the other stages will also be briefly summarized.

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## Introduction

The high prevalence, worldwide distribution and ability to persist in the face of a competent immune system all indicate that *Toxoplasma gondii* is very well adapted to life within a host. This chapter will review what is known about how the infected cell responds to *Toxoplasma*. While including those host responses that are presumed to be pro-host (aiding host survival), we will focus primarily on those that may be parasite-driven and pro-parasite. By studying the nature of the host cell's response to the parasite, we can potentially learn not only about the basic biology of this human pathogen, but also more about our own immune defenses, novel methods that pathogens use to avoid our immune systems, and possible targets for controlling the infection.

This chapter will be broken down into five parts. The first four parts comprise discussions on the environment set up by *Toxoplasma* inside the host cell, nutrients acquired from the host, immune evasion strategies, and experiments using microarray technology to profile the global effects of infection on the host cell. Largely because tachyzoites are easily studied *in vitro*, the majority of this article will pertain to the tachyzoite stage of the parasite. We will conclude, however, with a brief discussion of what is known for the bradyzoite, merozoite, and sexual stages of the parasite.

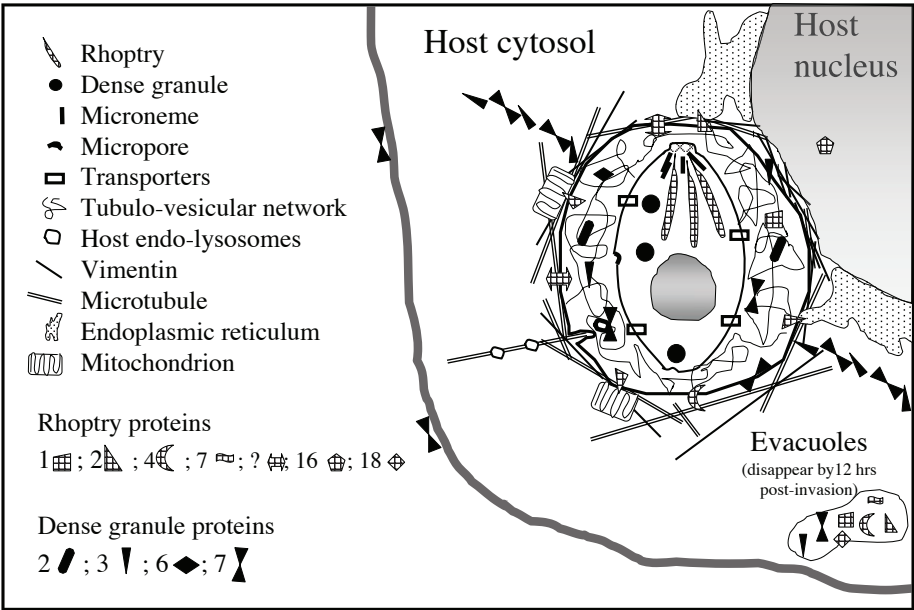
Specialized environment inside the host cell

To examine the interactions between *Toxoplasma* and its host cell, it is important first to understand the conditions in which *Toxoplasma* survives in the host cell. The parasite most likely begins to manipulate the host cell processes as it enters the cell and forms a parasitophorous vacuole (PV). Proteins likely to interact with host processes include those located in the parasitophorous vacuolar membrane (PVM) as well as proteins that may be secreted into the host cytoplasm. Morphological changes to the host cell that can be detected by light or electron microscopy are likely important clues to how *Toxoplasma* modifies the host cell. A diagram of on tachyzoite inside the parasitophorous vacuole will clarify the text (Figure 10.1).

Invasion and parasitophorous vacuole formation

*Toxoplasma* is able to invade virtually any mammalian or avian cell and create a specialized environment within that cell. Vacuole formation will be reviewed in detail in Chapters 29 and 31, therefore, only some aspects of invasion will be discussed here.

Parasite entry into the host cell involves invagination of the host cell plasma membrane to form the PVM around the parasite. This unique membrane is composed of both host and parasite constituents. The removal of certain host proteins results in a vacuole that does not fuse with other host cell membranes including lysosomes (Mordue and Sibley, 1997). The addition of parasite proteins to the PVM may be an essential part of the inva-



**Figure 10.1** A schematic view of the parasitophorous vacuole is shown containing a single tachyzoite with the localization of various secreted components indicated, where known. Not all components are seen at the same time but this represents a general view early in the lytic cycle. See text for details.

sion process and may enable contact with the host cytoplasm including essential nutrients, key proteins, organelles and other critical cell machinery.

*Toxoplasma* invasion is an active process in which the parasite secretes proteins and drives the creation of a parasite-containing vacuole inside the host cell. Three distinct types of secretory organelles, micronemes, rhoptries, and dense granules, aid invasion and intracellular survival. Microneme and rhoptry neck contents are secreted in the earliest stages of invasion and generally mediate adhesion and entry into a host cell (Chapter 22). Rhoptry bulb proteins are secreted into the nascent vacuole and vacuolar membrane, as well as the host cell, during invasion (Chapter 23). Dense granules can be secreted throughout the lifecycle of the parasite but appear key to modifying the parasitophorous vacuole and infected host cell (Chapter 24).

Because many rhoptry proteins are located within the PVM and even inside the host cell (discussed below) they are good candidates for aiding parasite nutrient acquisition. A recent effort to identify the protein constituents of rhoptries has led to the discovery of 38 proteins, many of which have potentially interesting functions (Bradley *et al.*, 2005). For example, the PVM appears to have pores made of proteins presumably of parasite origin. These pores allow polar molecules (including amino acids and nucleotides) up to 1300 daltons access to the vacuolar space (Schwab *et al.*, 1994). The identities of the molecules comprising this pore are unknown but ROP14 is a good candidate, being a predicted multipass transmembrane protein (Bradley *et al.*, 2005).

Between the PVM and the parasite membrane is a specialized environment made up of a tubulo-vesicular matrix that is established by secretion of dense granules within the PV (Sibley *et al.*, 1995). Electron micrographs show this matrix between parasites and possibly linking parasites to each other and also to the PVM (Magno *et al.*, 2005). In particular, GRA2 is thought to be instrumental in forming the tubular network, as vacuoles that contain GRA2-knockout parasites lacked the nanotubule conformation (Mercier *et al.*, 2002). The purpose of this structure is not known but it has been postulated to aid in various processes including nutrient transfer or structural integrity of the vacuole (Sibley *et al.*, 1995; Magno *et al.*, 2005).

### Parasite proteins that enter the host cell

Secretion of *Toxoplasma* proteins into the host cell is from both rhoptries and dense granules. There is a massive secretion at initial penetration of the host cell, then there appears to be additional secretion from dense granules during replication of the parasite. During tachyzoite invasion, proteins and membranous material are released into the host cell. This material, termed “evacuoles” (short for “empty vacuoles” since they contain no parasites), has been shown by indirect immunofluorescence to contain ROP1, ROP2, ROP4, ROP7, GRA3, and GRA7 (Hakansson *et al.*, 2001; Carey *et al.*, 2004; Hajj, *et al.*, 2005; Dubremetz *et al.*, 1993; Coppens *et al.*, in press). After release into the cell, the evacuoles traffic away from the true PV in a host microtubule-dependent manner. Introduction of evacuoles occurs at the time of invasion after which they appear to rapidly disappear (or at least the markers by which they are detected do). The function of evacuoles is unknown but they could serve as delivery vehicles for parasite proteins that must interface with host cell components directly. Examples of such molecules could include many of the recently

identified rhoptry proteins that include several predicted protein kinases, phosphatases (PP2C-type), and proteases (serine and insulinasinase-type metalloprotease). Some of these proteins are secreted into the host cell (Gilbert *et al.*, 2007; Taylor *et al.*, 2006; Saeij *et al.*, 2006; Saeij *et al.*, 2007) where they may be virulence factors capable of modulating host functions, like signal transduction, that require a very fast response and are regulated at the level of protein phosphorylation. For example, STAT proteins are phosphorylated in response to cytokines or growth factors and then are able to translocate to the nucleus and activate transcription. Research has shown that a *Toxoplasma* kinase is able to sustain the phosphorylation and activity of STAT3/6 in infected cells (Saeij *et al.*, 2007). These proteins are discussed in more detail under “Strain differences” in this chapter. A parasite kinase may play a role in modulating the NF $\kappa$ B pathway to promote anti-apoptotic and other responses (Molestina and Sinai, 2005a; Molestina and Sinai, 2005b). *Toxoplasma*’s anti-apoptotic effects will be discussed later in this chapter and also Chapter 8. Much more work will need to be done to elucidate the specific functions of rhoptry proteins that are targeted to the host cell.

Dense granule proteins also enter the host cell, but with kinetics that are generally different from those of rhoptry proteins. As mentioned above, introduction of some dense granule proteins occurs with evacuoles in a pattern that is partially overlapping that of rhoptry proteins. In addition to this initial event, however, some dense granule proteins continue to be secreted as the parasitophorous vacuole is formed. Specifically, GRA3 and GRA7 are detectable by indirect immunofluorescence assay in strands sometimes emanating from the PVM and their fluorescence intensity persists during replication (Dubremetz *et al.*, 1993; Coppens *et al.*, in press). These strands can be seen to connect vacuoles within the same host cell (Dubremetz *et al.*, 1993). In addition to presence within the host cell, GRA7 is seen on the surface of infected cells (Neudeck *et al.*, 2002). It is not yet clear what function the structures connecting vacuoles, or cell surface localization may perform.

One clue to the possible function of some of these secreted proteins, or at least the means by which they are regulated, could come from the fact that several of them are phosphorylated. Evidence suggests that ROP2, ROP4, and GRA7 are all phosphorylated when *Toxoplasma* is inside a host cell, but not in extracellular parasites (Carey *et al.*, 2004; Neudeck *et al.*, 2002; J.D. Dunn and J.C. Boothroyd, unpublished results). It is, however, unknown whether the kinase(s) responsible are of host or parasite origin. Regardless, protein kinases are well-studied drug targets in mammalian systems and so make excellent drug targets. Note that an inhibitor of a host kinase has the potential to affect the parasite in a way that also leaves less chance of creating a parasite line that is resistant to the drug. While resistance in *Toxoplasma* is unlikely to be a significant problem (only humans are treated and there is no human-to-human transmission), drug resistance in the closely related *Plasmodium* species that causes malaria is a very large problem and many of these pathways could be related in the two systems. Further work on the parasite proteins just described, as well as the 17 additional proteins recently identified as being secreted from dense granules and micronemes (Zhou *et al.*, 2005), will undoubtedly be key to understanding how these parasites co-opt and reproduce within the infected host cell.

### Ultrastructural changes

Clues to the role of the secreted proteins discussed above have come from careful analysis of ultrastructural changes in the infected host cell. These include redistribution of vimentin, microtubules, and host organelles. Vimentin is an intermediate filament, known to be involved in intracellular transport between the nucleus and plasma membrane of a cell. In an infected cell, the vimentin distribution appears normal throughout the cell except near the parasite where many filaments originate at the host cell nucleus and terminate at the PVM (Halonen and Weidner, 1994). This association may facilitate tethering of the PV to the host nucleus and/or transfer of nutrients from the host cell cytosol into the parasite although nothing has yet been identified that traffics via vimentin to the *Toxoplasma* PVM. Disruption of the vimentin filaments does result in loss of apposition between the PVM and host nucleus (Halonen and Weidner, 1994) but growth of the parasite in cells lacking vimentin appears otherwise normal (Sehgal *et al.*, 2005). As with all such analyses, it is possible that the importance of the vimentin distribution is necessary only *in vivo* or in different cell types or parasite life stages not yet investigated.

Similar to vimentin, host microtubules form an actively remodeled ring around the parasite (Melo *et al.*, 2001; Coppens *et al.*, in press). Microtubules appear to be important for both the trafficking of parasite proteins into the host as well as scavenging host cholesterol. Disruption of host microtubules decreases strand formation by evacuoles as parasites enter the host cell (Hakansson, 2001). Additionally, disruption of normal microtubule polymerization and depolymerization by the drugs nocodazole or taxol reduces cholesterol delivery to the parasite by 40% (Sehgal *et al.*, 2005). Despite these changes, taxol treatment for 12 hours does not inhibit *Toxoplasma* growth, raising a question as to the necessity for cholesterol acquisition and dense granule secretion into the host. However, 12 hours may not have been enough to see detrimental effects of transport reduction.

Another quite dramatic ultrastructural modification of the host involves rearrangement of mitochondria and ER. The PVM and evacuoles associate with host cell endoplasmic reticulum (ER) and mitochondria (Hakansson *et al.*, 2001). ER and mitochondria are vital host cellular machinery and are involved in synthesis of energy and lipid biosynthesis. Additionally, extracellular nutrients obtained from the surface of the cell can be transferred from the cytoplasm to the ER for use, storage, or recycling back to the surface. It seems logical, then, that the apposition of PVM and host organelles would aid nutrient transfer into the parasite. Interestingly, cholesterol, a nutrient that follows this cycle and is scavenged by *Toxoplasma* is not reliant on ER for transfer into the parasite (see below) but there may be other nutrients for which organellar apposition to the PVM does aid transfer.

The apposition of mitochondria could play quite a different role from that of aiding nutrient transfer. Mitochondria are key players in cellular apoptosis. *Toxoplasma* infection renders cells resistant to multiple inducers of apoptosis partly by targeting the proteins downstream of the convergence of the different apoptosis-inducing pathways (Nash *et al.*, 1998). Whatever the reason, the association between PVM and host is quite remarkable with reports that as much as 50% of the PVM can be associated with host organelles (Nakaar *et al.*, 2003) and, from the host's perspective, over 90% of its mitochondria can flank the PVM.

ROP2 is a good candidate for a parasite protein that mediates mitochondrial association to PVM because, in addition to being present on the PVM, it is processed to reveal what may be a functional mitochondrial import sequence exposed to the host cytosol. Indeed, recombinant ROP2 with this sequence exposed has been shown to associate with purified mitochondria and ER membranes (Sinai and Joiner, 2001). While efforts to knock out ROP2 were unsuccessful, an antisense mediated suppression of ROP2 revealed pleiotropic effects such as misshapen rhoptries, aborted vacuoles, and termination of cytokinesis (Nakaar *et al.*, 2003). Importantly, however, suppression did significantly decrease the association of host cell mitochondria and PVM. Unfortunately, the other effects of ROP2 depletion were so great that it is impossible to know how abolition of ER and mitochondrial apposition affects the parasite.

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## Necessary nutrients and their acquisition

Many parasites have either lost or not developed the ability to synthesize, *de novo*, certain compounds in favor of obtaining them from their host. Some of those deficiencies in *Toxoplasma* appear to have been exploited by the host. For example *Toxoplasma* is unable to manufacture tryptophan and the host has developed methods to limit this key amino acid in infected cells. *Toxoplasma* also lacks *de novo* synthesis machinery for cholesterol. As these nutrients represent critical points of cross-talk between the host and parasite, they will be briefly discussed here. More detailed discussion of these and other aspects of nutrient acquisition are discussed elsewhere in this volume (Chapter 19 and Chapter 20).

### Tryptophan

Similar to many intracellular microbes, *Toxoplasma* is a tryptophan auxotroph, and thus it must scavenge this essential amino acid from the host. Host defenses capitalize on this deficiency through a pathway dependent on interferon-gamma (IFN- $\gamma$ ). Fibroblasts and lymphocytes decrease available tryptophan by IFN- $\gamma$ - and JAK/STAT-dependent up-regulation of indoleamine 2,3-dioxygenase (IDO). IDO catabolizes cellular tryptophan, thus reducing intracellular tryptophan levels and starving intracellular pathogens unable to synthesize this essential amino acid. IFN- $\gamma$  reduces replication of *Toxoplasma* in various cell types (Ceravolo *et al.*, 1999; Aline *et al.*, 2002; Brunton *et al.*, 2001). The growth reduction in fibroblasts of 54% appears to be due primarily to tryptophan starvation because addition of tryptophan to the medium or transfection of bacterial tryptophan synthase into the parasite ameliorated the effect of IFN- $\gamma$  pretreatment of the fibroblasts (Pfefferkorn, 1984; Ceravolo *et al.*, 1999). Tryptophan starvation is only one effect of IFN- $\gamma$ ; other JAK/STAT effects, induction of nitric oxide synthetase, or p47 GTPase induction have effects on growth of *Toxoplasma* in other cell types (Luder *et al.*, 2003; Butcher *et al.*, 2005; Fujigaki *et al.*, 2003; Martens *et al.*, 2005).

### Cholesterol

The doubling of parasites within the vacuole as well as growth of the PVM requires rapid addition of lipid membrane. To meet this demand, *Toxoplasma* is able to synthesize as well as scavenge fatty acids, but cholesterol must be obtained from the host. Since *Toxoplasma* lacks *de novo* cholesterol synthesis, the parasite must gain this nutrient from the



host cell. To understand this process, it is useful first to appreciate how a mammalian cell obtains and uses cholesterol. Although mammalian cells are able to make cholesterol through the mevalonate synthesis pathway, the majority of cholesterol is synthesized in the liver or obtained from the diet. Both processes for obtaining cholesterol are regulated at the transcriptional level in accordance with sterol levels. Low-density lipoprotein (LDL) is a particle with protein as well as lipid components that facilitates solubilization of cholesterol as well as uptake into cells. Cells obtain most of their cholesterol through receptor-mediated endocytosis of LDL from the bloodstream. LDL enters host endocytic compartments that later fuse with lysosomes. Degradation ensues and egress of cholesterol in non-vesicular (free) form or vesicular forms takes place. This cholesterol can transit to the plasma membrane and then be incorporated into other organelle membranes or be transported in a vimentin dependent manner for re-esterification in the ER and storage inside the cell (Sarria *et al.*, 1992).

There are a variety of steps in the host cholesterol synthesis and acquisition pathways where *Toxoplasma* may interfere. There is some evidence that enzymes of the mevalonate synthesis pathway are increased in *Toxoplasma*-infected cells (Blader *et al.*, 2001). This pathway is transcriptionally controlled in response to low cholesterol and the increases seen in *Toxoplasma* infection could result from host sensing of decreased cholesterol or from a parasite-driven mechanism. The activity of the rate-limiting enzyme HMG-CoA reductase, however, was not increased in infected cells (Coppens *et al.*, 2000). Therefore, it seems most likely that if there is an increase in mevalonate synthesis it is triggered by parasite mediated host cell sterol depletion.

Receptor-mediated uptake of LDL into endosomes is significantly increased in infected cells and appears to be the main source of cholesterol in *Toxoplasma*. It is not thought that endocytic vesicles fuse with the *Toxoplasma* PVM because three endocytic markers (transferrin receptor, mannose phosphate receptor, and LAMP1) do not colocalize with the PVM (Mordue *et al.*, 1999), but abolition of lysosomal function reduces cholesterol acquisition (Coppens *et al.*, 2000). Further research has shown that cholesterol acquisition by *Toxoplasma* is dependent on energy and dynamic host microtubules but not on host golgi, ER, or vimentin (Sehgal *et al.*, 2005; Coppens *et al.*, 2000; Charron and Sibley, 2002). These data indicate that the delivery of cholesterol may depend on vesicular-like transport.

Consistent with all of the above data, electron micrographs show LDL adsorbed to colloidal gold within membrane bound structures in the vacuole. There are also host microtubules visibly invaginating the PVM in these micrographs (Coppens *et al.*, 2006). The data suggest that microtubules somehow create invaginations in the PVM that can route LDL-laden endo-lysosomes into the PV. A parasite protein (GRA7) within the vacuole may aid in the pinching off of these invaginations.

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## Parasite subversion of host defenses

Vertebrates have many ways to minimize the growth and spread of intracellular pathogens. Classic cellular host response includes induction of apoptosis, upregulation of MHC presentation, and secretion of immunomodulatory factors. Its obligate intracellular existence has undoubtedly required the evolution of a battery of methods for *Toxoplasma* to subvert

these responses. Apoptosis and immune evasion are detailed in Chapters 8 and 7, and so this chapter will briefly discuss these in the context of specific sub-cellular responses.

## Apoptosis

Apoptosis is a method of programmed cell death that can be instigated by several different pathways. Exogenous activation of apoptosis can be from cytolytic T cells, TNF- $\alpha$ , or withdrawal of growth factor, whereas internal inducers include reactive oxygen species, irradiation, or mitochondrial cytochrome c release. All of these pathways converge to cause cell death by activating a specific protease known as caspase 3. *Toxoplasma*-infected cells were found to be resistant to both extrinsic and intrinsic inducers of apoptosis (Nash *et al.*, 1998) and activation of caspase 3 as well as additional upstream caspases is known to be inhibited by *Toxoplasma*-infection (Goebel *et al.*, 2001). The mechanism for this inhibition, however, is unknown but it could well be mediated by one of the secreted parasite proteins discussed above.

An additional anti-apoptotic effect of *Toxoplasma* may be through the NF $\kappa$ B signaling pathway. Inactive NF $\kappa$ B is bound to I $\kappa$ B $\alpha$  in the host cytosol. The phosphorylation of I $\kappa$ B $\alpha$  causes its degradation and exposes the nuclear localization signal on NF $\kappa$ B. Nuclear translocation and subsequent promoter binding by NF $\kappa$ B upregulate numerous pro-inflammatory cytokines and anti-apoptotic genes. I $\kappa$ B $\alpha$  degradation has been demonstrated in infected fibroblasts and macrophages, and recent evidence points to both parasite and host kinase activities that phosphorylate I $\kappa$ B $\alpha$  (Molestina and Sinai, 2005a).

The subsequent extent of NF $\kappa$ B activation in infected host cells is a matter of controversy and will need further work to resolve. To act as a transcriptional activator, NF $\kappa$ B must be in the nucleus. One group has seen nuclear accumulation and activation of NF $\kappa$ B (Molestina, 2003; Kim *et al.*, 2001). But, nuclear accumulation of NF $\kappa$ B appears to be reduced in infected macrophages (Butcher *et al.*, 2001). In this case, blocking nuclear export increased NF $\kappa$ B accumulation. This evidence implicates export as a key factor in regulating NF $\kappa$ B accumulation (Shapira *et al.*, 2005).

## MHC

Major histocompatibility complex (MHC) expression plays a critical role in mammalian immune responses. MHC class I molecules are at the surface of all nucleated cells and present peptides derived from endogenous proteins synthesized within these cells (self proteins as well as viral or intracellular bacterial or parasitic proteins). Cytotoxic T lymphocytes are able to identify foreign antigens presented in MHC class I proteins and kill the target cell. The expression of MHC class I and many genes involved in antigen processing and presentation can all be up regulated upon exposure to IFN- $\gamma$ .

MHC class II molecules are constitutively expressed by dendritic cells and macrophages, the so-called professional antigen-presenting cells, but can be induced in most other cell types upon exposure to IFN- $\gamma$ . MHC class II molecules present peptides derived from phagocytosed or endocytosed foreign antigens to helper T lymphocytes which when they recognize a foreign antigen become activated and exert their protective effects.

NK and T cells secreting IFN- $\gamma$  play a critical role in defending against *Toxoplasma* and the parasite, perhaps not surprisingly, is capable of blocking the IFN- $\gamma$ -induced up-

regulation of MHC class I and II expression (Luder *et al.*, 1998). Induction of MHC class II depends on a transcription factor (STAT1) activating a class II trans-activator (CIITA). In the case of MHC class II, the transcriptional activity of CIITA is impaired in *Toxoplasma*-infected cells (Luder *et al.*, 2001; Luder *et al.*, 2003). Furthermore, STAT1 activity appears to be altered in murine macrophages and, thus, the expression of many IFN- $\gamma$ -inducible genes (in addition to MHC) is impaired in infected cells.

### Immunomodulatory factors

Microarray studies (described below), in addition to other work, show that *Toxoplasma* invasion is not silent; rather, it causes secretion of many proinflammatory cytokines (Gail *et al.*, 2001; Blader *et al.*, 2001; Chaussabel *et al.*, 2003; Brenier-Princhart *et al.*, 2005; Denney *et al.*, 1999). Some of these, e.g. IFN- $\gamma$  and IL-12, are critical for host survival (Sharton-Kerston *et al.*, 1996; Hunter *et al.*, 1995). Although IFN- $\gamma$  is protective to the host, *Toxoplasma* has mechanisms to ameliorate its effects in certain cell types. For example, macrophages and microglia (among other cells) produce inducible nitric oxide synthase (iNOS) and thus the potent antimicrobial nitric oxide in response to IFN- $\gamma$ ; in both of these cell types, *Toxoplasma* infection appears to inhibit iNOS upregulation (Luder *et al.*, 2003; Rozenfeld *et al.*, 2005).

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### Microarrays investigate global host changes

Recent research has used DNA microarray technology to ask in an unbiased and global way what are some of the major changes in transcript abundance that occur upon infection. These data can be used to identify induced genes or pathways that are likely involved with parasite survival. Four reports have been published using this technology and they all show overarching similarities despite use of different platforms, cell types and strains of parasite.

### Comparison with other pathogens

In 2001, Gail *et al.* (2001) used BD Atlas arrays containing roughly 600 genes including immunomodulatory factors, receptors, signal transduction components, and cell-cycle regulators. mRNA from uninfected human foreskin fibroblasts (HFFs) and HFFs infected by *Toxoplasma* RH strain, *Salmonella typhimurium* or *Chlamydia trachomatis* were compared. In these experiments, 40 genes were upregulated more than 3-fold with infection by *Toxoplasma*. Those genes most induced (10-fold or greater) encode primarily immunomodulatory factors and were induced by all three intracellular organisms. Interestingly, there was only one noted decrease brought about by infection with *Toxoplasma*. The PCR verification showed that the false positive rate on these arrays was roughly 25% and so conclusions concerning unverified genes must be considered tentative (as for most array work). Nevertheless, certain trends were clear (e.g. effect on immunomodulatory genes) and results for key genes were confirmed by other means.

The comparison between different intracellular pathogens allowed for identification of *Toxoplasma*-specific and likely pro-pathogen changes in the host. Of the genes induced more than 3-fold on these arrays, only two were *Toxoplasma*-specific: MacMARKS and

Transferrin receptor (TfR). The transferrin receptor increase is particularly interesting because iron is a necessary nutrient for *Toxoplasma* to gain from its host.

Additional research has been performed to determine whether the transferrin receptor mRNA increase is specifically modulated by the parasite. The RNA levels for this gene are known to be regulated post transcriptionally by an iron response protein (IRP) that binds a *cis* iron regulatory element (IRE). In low iron conditions, IRP binds the IRE and stabilizes the transferrin receptor mRNA resulting in production of more receptor protein. Interestingly, *Toxoplasma* infection appears to increase TfR levels directly because addition of available iron does not ameliorate the TfR increase (Gail *et al.*, 2004). Therefore, in infected cells, the IRP stabilizes the receptor mRNA even when transferrin is abundant. It is unclear whether a parasite or host protein secreted during infection mediates the stability change. Host transferrin receptor levels may indeed be manipulated directly by the parasite, but further research will be necessary to distinguish these two possibilities.

A subsequent report (Chaussabel *et al.*, 2003) also compared *Toxoplasma* infection with that of other pathogens, but this time in two different immune cell populations. Intracellular (*Leishmania major*, *Leishmania donovani*, *Toxoplasma*) and extracellular parasites (*Brugia malayi*) were each grown in human dendritic cells (DCs) or macrophages (MPs) for 16 hours before mRNA was extracted, labeled, and hybridized to Agilent arrays with oligonucleotides for 12 000 genes. Profiles of gene induction were compared between the different parasites as well as between the different cell types. Although there were gene changes common to both cell types (720), there were roughly twice as many unique changes in DCs (799) than in MPs (340). Both of these cells are important members of the host immune system and it is not surprising that they would have different transcript profiles upon exposure to pathogens.

Only those genes whose expression increased were grouped and studied further. The data were categorized, first according to the expression patterns for different infectious agents, then by putative function of the gene paying special attention to those involved in immune response and signaling. Strikingly, nearly every gene that was induced by any of the other pathogens was induced by *Toxoplasma* in at least one cell type. *Toxoplasma* is known to elicit a very strong Th1 immune response and these data are in good agreement with this fact.

One interesting category of gene from these arrays encompasses those where infection with the *Leishmania* parasites increased transcript levels of a gene but *Toxoplasma* did not. Upon inspection of this category, 2 genes stand out, IL10R and WARS; these were the only genes that showed an upregulation with the other parasites but not *Toxoplasma* in both DCs and MPs. Acute *Toxoplasma* infection stimulates interferon signaling and IL-10 is a potent suppressor of this signaling, so it was somewhat expected to see that its receptor is not increased. WARS is a tryptophanyl t-RNA synthetase that is upregulated by IFN- $\gamma$  (like IDO, see nutrient acquisition section above). Its lack of upregulation in *Toxoplasma* infection is curious and could be a hint to a previously unknown mechanism used by the parasite to enhance its own survival. Additional work will be needed to determine if this lack of upregulation is parasite driven and how it impacts the host–pathogen interaction.

Certain genes identified as upregulated in fibroblasts are also increased in either DCs or MPs. For example, IL-6 and RANTES were both increased in HFFs and DCs but not

in MPs. A number of type I interferon-inducible genes also follow this same pattern. There are a number of possible explanations for phenomena like this. It is possible that MPs lack mechanisms to upregulate these mRNAs in response to infection, but the data on increases due to infection with other intracellular pathogens disproves this hypothesis. Various host cells have differing responses to the parasite, and these differences can be exploited to learn more about mammalian immune systems and how it combats *Toxoplasma*. It is also possible that the parasite has a cell-specific method of reducing these mRNA levels in that cell type alone. This is an interesting possibility and the data from these microarrays will be key to developing hypotheses to discriminate between the alternatives of how *Toxoplasma* specifically modulates these immune cells.

### Time course and other factors

Blader *et al.* (2001) used cDNA microarrays with roughly 22000 sequenced genes. To examine the kinetics of gene induction, arrays were performed at 1, 2, 4, 6, and 24 hours post-infection. Although some changes in RNA transcript level took place immediately, the majority of the host transcript level changes were not visible before 24 hours in this time course.

The earliest responding genes were mostly involved with immune response. Experiments using “conditioned” media (from previously infected cell cultures) as well as transwells indicated that parasite invasion was not required for many of these changes. Additionally, cycloheximide, a chemical that inhibits protein synthesis, was used to determine that 82% of the early response genes (2 hours post infection) did not require protein synthesis for their upregulation. This is not surprising because the necessity of a fast response may demand that regulation be effected through phosphorylation and/or protein degradation.

The majority of the response observed by Blader *et al.*, at 24 hours post-infection did require parasite invasion. Interestingly, two pathways that were seen to be upregulated at this time-point were the glycolytic and mevalonate synthesis pathways. This is particularly noteworthy because *Toxoplasma* scavenges the products of these pathways from the host. As mentioned previously for mevalonate synthesis enzymes, it is likely that as the parasite uses up nutrients from the host, the levels drop and the HFF cell senses this depletion and responds with a synthesis increase. Similarly, it is likely that glycolytic enzymes are increased in response to host cell energy depletion. It is possible, however, that the parasite specifically directs this increase by somehow targeting the genes, RNA and/or enzymes involved in these two synthesis pathways.

### HIF1

Examination of the array data led to identification of a pro-parasite induced transcription factor HIF1 $\alpha$ . A number of genes that were increased, those encoding glycolytic enzymes, glucose transporters, the transferrin receptor, and growth factor VEGF, are all known to be regulated by the same transcription factor, HIF1 $\alpha$ . Further research has indicated that HIF1 $\alpha$  is induced by *Toxoplasma* infection and thus likely to be at least a part of the mechanism by which these genes are modulated (Spear *et al.*, 2006). Interestingly, this increase in protein levels of HIF1 does not require invasion, or physical juxtaposition of live parasites and host cells: parasites in a transwell chamber are also able to induce HIF1 $\alpha$

production in mouse embryonic fibroblasts. Additionally it does not appear to be induced by a factor in parasite lysates. Two alternative mechanisms have been put forth: consumption of oxygen by the parasite causes a hypoxic environment thus increasing HIF1 $\alpha$ , or short-lived, diffusible factors released by the parasite signal the host to increase HIF1 $\alpha$ . Protozoan oxygen consumption does not appear to cause the HIF1 $\alpha$  induction because infection by another protozoan, *Trypanosoma cruzi*, does not cause the induction (Vaena de Avalos *et al.*, 2002). It seems likely that parasite factors induce HIF1 $\alpha$  but the mechanism is currently unknown.

Regardless of the mechanism of induction of HIF1 $\alpha$ , it is clearly necessary for intracellular growth of *Toxoplasma*: examination of parasites by transmission electron microscopy showed gross defects in parasites grown in HIF1 $\alpha$  knockout cells at 3% (hypoxic) but not at 21% (normoxic) oxygen concentrations where HIF1 function is normally not required. There is evidence that acute *Toxoplasma* infection may actually cause hypoxic conditions by inhibiting angiogenesis: *Toxoplasma*-infected mice show reduced melanoma growth and specifically, reduced vascularization of implanted material (Hunter *et al.*, 2001). Hence, the HIF1 $\alpha$  induction may be a parasite-driven adaptation to combat the low oxygen environment caused by parasite. Interestingly, there are many inhibitors known for mammalian HIF1 $\alpha$  and so it will be interesting to see whether these inhibitors also exhibit anti-*Toxoplasma* activity.

The most recent array experiments utilized BD Atlas arrays containing 4000 genes to examine the responses of retinal vascular endothelial cells to infection at 2 and 24 hours post-infection (Knight *et al.*, 2005). The results at 2 hours generally agree with those from Blader *et al.*, and indicate that chemokine and inflammatory molecule transcript levels are upregulated by *Toxoplasma* infection. The results at 24 hours however differ from those of the other three groups. In contrast with the much greater number of genes induced at 24 hours in fibroblasts (Blader *et al.*, 2001), these retinal endothelial cells have many fewer transcript changes at this time point. Transcripts that were increased at 2 hours post infection, but decreased at 24 hours post infection include chemokines GRO1, FKN, MCP1, and RANTES (Knight *et al.*, 2005). This decrease is likely cell type specific because increases in GRO1, MCP1, and RANTES were noted in fibroblasts at 24 hours post infection (Gail *et al.*, 2001, Blader *et al.*, 2001). Interestingly, transcript levels of GRO1 and RANTES at 16 hours post infection were quite different in dendritic cells and macrophages (Chaussabel *et al.*, 2003); these results add evidence to the conclusion that there are cell specific differences not only in induction, but also in subsequent downregulation of gene transcripts. Rampant immune activation will kill an animal, therefore mammals have mechanisms to shut down the immune response once it has begun. Perhaps these retinal endothelial cells are suppressing the immune response and are thus a good cell type in which to study the mechanism behind this important phenomenon.



## Difference in host-response between parasite strains and stages

### Strain differences

There are three dominant strains of *Toxoplasma* in Europe and the Americas (see Chapter 12) and each is characterized by different virulence in mice. For type I strains, infection with only one parasite causes mortality. For type III strains,  $10^5$  parasites rarely cause lethality, while type II strains have an intermediate virulence. In humans, the data are less clear although there is some indication that here too there are strain-specific differences in disease outcome (Grigg *et al.*, 2001; Ajzenberg *et al.*, 2002).

The molecular basis for these differences has recently been partially elucidated using *Toxoplasma* genetic maps (Kahn *et al.*, 2005) in combination with microarray analysis. Two specific parasite factors and their interaction with host proteins are instrumental to the different virulence phenotypes seen in the strains. Through genetic mapping of virulence loci, two *Toxoplasma* kinases were identified, Rop16 and Rop18 (Saeij *et al.*, 2007; Saiej *et al.*, 2006; Taylor *et al.*, 2006). Both of these proteins are particularly polymorphic between different strains and both are secreted into the host open cell upon invasion. Rop16, in type I and III strains, mediates the sustained phosphorylation of STAT3 and STAT6 transcription factors, while the type II Rop16 shows very minimal STAT3/6 phosphorylation at 18 hours (Saeij *et al.*, 2007). Rop18 was found to be a virulence factor and the type I or type II Rop18, when transfected into the avirulent type III parasites, was found to be sufficient to drastically increase virulence (Taylor *et al.*, 2006; Saeij *et al.*, 2006). Increasing levels of Rop18 in a parasite tends to correlate with virulence as well, but the mechanism for the virulence increase is unknown at this time.

Virulence is also linked to dissemination through the host and this can be facilitated by parasite motility or by hijacking of circulating host cells such as macrophages. Dissemination was examined using parasites engineered to express luciferase so that when the substrate luciferin is injected into infected animals the parasites emit light that can be quantitated. Two F1 progeny from a cross between type II and III parents—one, S23, is highly virulent ( $LD_{50} < 10$ ) while the other, S22, is not ( $LD_{50} > 100\,000$ )—were imaged every other day for about 2 weeks post-infection. The images show distinctly higher levels of dissemination and parasite number when the infection was with the more virulent strain (Saeij *et al.*, 2005) even though the *in vitro* growth rate was not significantly different from S22. The basis for this difference in dissemination is not known, but there is evidence that the virulent type I strain exhibits enhanced migration through tissues (Barragan and Sibley, 2002). There are also noted differences between strains in attraction of mobile immune cells to sites of infection (Diana *et al.*, 2005; Robben *et al.*, 2004). Research has shown that macrophages infected with type I strains exhibit reduced adhesion to extracellular matrix and thus may travel more freely through the host than uninfected macrophages (Da Gama *et al.*, 2004). As yet, the molecules and mechanisms involved in all of these phenomena are unknown, but all are almost certain to involve specific interactions with the host cell.

## Stage differences

The vast majority of research to elucidate host response has been done with tachyzoites, but this form of the parasite is relatively transient during the course of an infection. Bradyzoites on the other hand, are capable of surviving within host cells for extended periods of time (Remington *et al.*, 1963; Dzierszinski *et al.*, 2004). Bradyzoites are critical to the persistence of the parasite for the lifetime of the host, and also to the asexual transmission of *Toxoplasma* between subsequent hosts. The tachyzoite and bradyzoite stages of the parasite can be found in nearly all mammals, but there are additional, sexual stages of the parasite that survive only in a cat. Very little data exist on how the bradyzoite stage manipulates its host cell and even less concerning the feline stages. This section, therefore, will discuss primarily what is known about bradyzoites but data concerning the sexual stages will be included when known.

Bradyzoites live inside of a cyst wall made of proteins and carbohydrates secreted by the parasite. This is generated inside the limits of the PVM within an infected host cell and appears to be derived largely from the secreted contents of dense granules although few components are known definitively. The lectin dolichos biflorus that is known to bind the sugar GalNAc recognizes the bradyzoite cyst wall suggesting it may have a chitin-like composition although this has not been directly demonstrated (Boothroyd, *et al.*, 1997). The wall could play a number of different roles in host manipulation and bradyzoite survival but it is almost certain to represent a significant barrier to exchange between the parasite and host cell. This could impact uptake of nutrients as well as export of parasite constituents into the host cell. This latter aspect has long been considered as a key function of the cyst wall because of its presumed impact on antigen presentation and consequent recognition of the parasites by immune surveillance mechanisms. As yet, however, there are no experimental data to substantiate this supposition.

As with tachyzoites, clues to how the bradyzoite interacts with the host cell, before or after the cyst wall is formed, are likely to come from an inventory of the proteins it secretes. Although less well characterized, it is already clear that there are major differences between the bradyzoite and tachyzoite with the three types of secretory organelles, micronemes, rhoptries, and dense granules, all showing stage-specific proteins based on immunofluorescence and/or RNA data (Alexander *et al.*, 2005; Schwarz *et al.*, 2005; Ferguson *et al.*, 1999; Cleary *et al.*, 2002). One function of these differences could be to minimize the immune response to bradyzoites if they do not produce the antigens to which the immune system was primed during the acute (tachyzoite) stages of the infection. This has been seen to be the case for surface antigens belonging to the SRS (SAG1-related sequence) family (Kim and Boothroyd, 2005) and could easily be imagined for the secreted antigens, as well.

Rhoptries are secreted at the time of invasion and they, too, contain a distinct complement of proteins in bradyzoites. ROP1 and ROP9 are not present in bradyzoites (unpublished results; Reichmann *et al.*, 2002) and there is evidence that others are similarly stage specific. These include proteins that are secreted into the host cell and may be interacting with the host. There is only one rhoptry protein, BRP1, which is known to be present in bradyzoites and not tachyzoites. BRP1 is visible by IFA in the bradyzoite rhoptries as early as 12 hours after bradyzoite induction *in vitro*. This protein, however has no homology to known proteins and gives little insight into the function of bradyzoite rhoptries. Parasites

in which BRP1 is replaced with HXGPRT grow, invade, and form cysts similarly to wild type. Therefore, BRP1 is not essential for any of these functions. There was some evidence that this protein was secreted into the vacuole during *in vitro* bradyzoite growth, but no secretion was seen *in vivo* in human brain slices stained with antibodies to BRP1 (Schwartz *et al.*, 2005) and so the *in vitro* result seems likely to be an artifact of culture conditions. Interestingly, this protein is also seen in merozoite rhoptries in infected cat epithelium. This suggests this protein might play a role in invasion of mucosal intestine cells since both merozoites and bradyzoites but not tachyzoites must invade this tissue.

Dense granules are good candidates for proteins that interact with and manipulate host cells. GRA1–8 are expressed in bradyzoites and all except GRA4 can be seen by indirect fluorescence microscopy to be present in the cyst wall (Ferguson *et al.*, 1999; Ferguson, 2004; Fisher *et al.*, 1998). The only known parasite protein, however, that has been seen to enter the bradyzoite-infected host cell is GRA7. In cultures containing murine brain cells infected with bradyzoites, this protein was seen by indirect immunofluorescence to be secreted into the host cell cytoplasm and maybe even cell culture supernatant (Fisher 1998). The pattern, however, was distinct from that of the other GRA proteins seen in tachyzoite-infected host cells post invasion; GRA7 was distributed through the cytoplasm of the host cell and did not form long strands between vacuoles. It is also interesting to note that only GRA7 is seen in the feline stages and the parasitophorous vacuole containing these parasites looks quite different (by EM) from that containing tachyzoites (Ferguson, 2004).

The nutrient requirements of bradyzoites are presumed to be much less than that of tachyzoites since bradyzoites divide much more slowly. Consistent with this, drugs such as purine analogs that are targeted to DNA replication kill tachyzoites much more efficiently than bradyzoites. Whether these differences in nutrient requirements are reflected in a decrease in the machinery for their synthesis and/or salvage is currently unknown.

One way to learn more about the host manipulation by bradyzoites would be to use microarrays to examine any changes in host gene expression patterns in infected cells. *In vitro*, it is possible to cause the conversion of tachyzoites to bradyzoites with the application at 4 hours post infection of pH stress and serum starvation and we have recently used this approach to examine differences in host gene expression (Fouts and Boothroyd, 2007). Using human cDNA microarrays, mRNA from cultures of bradyzoite-infected cells was compared with that of tachyzoite-infected and uninfected controls 2 days post invasion. Although the magnitude of changes caused by infection was less with bradyzoites than for tachyzoites, the trends were very similar with few if any host genes specifically upregulated by the bradyzoites. This may be due to the fact that the invasion necessarily began identically in the two infected cultures (both started with tachyzoite invasion and then one culture was switched to bradyzoite conditions 4 hours later) and we looked only 44 hours after that. Bradyzoite differentiation appears to be a prolonged affair *in vitro* and *in vivo* and major differences might not manifest for many days by which time the *in vitro* bradyzoite cultures appear extremely unhealthy and the tachyzoites have lysed out, making comparisons impossible.

Resolution of this issue, therefore, will likely await direct *in vivo* comparisons of the two stages but these are technically far more difficult. One hurdle that will need to be

overcome is identifying the rare infected cell *in vivo* and isolating its RNA away from the vast majority of (uninfected) cells. A technique that could aid in this might be developed by exploiting another recently developed method involving the *Toxoplasma* enzyme, uracil phosphoribosyltransferase (TgUPRT). This enzyme allows incorporation of thiolated uracil into *Toxoplasma* RNA (Iltzsch and Tankersley, 1994; Cleary *et al.*, 2005). If parasites could be engineered to secrete TgUPRT into the infected host cell then infected cells would specifically incorporate thiouracil into their RNA; the RNA from uninfected cells would not be affected. The resulting thiolated RNA could then be biotinylated and isolated on streptavidin columns, giving the purification necessary for microarray studies and eliminating the problem of a huge background from the RNA of uninfected cells. The possibility of doing this has recently been partially demonstrated when parasite RNA was isolated away from host RNA using tissue from an infected mouse into which thiouracil had been introduced a few hours earlier (Cleary *et al.*, 2005). Expression of a secreted form of TgUPRT that functions in the PV or cytosol of the infected host should be possible and experiments to achieve this are in progress.

## Conclusion

Past research by many groups has shed tremendous light on *Toxoplasma* biology and the many, complex interactions between this parasite and the infected host cell. We are, however, just beginning to gain insight into the molecular differences underlying these interactions. Recent advances in genetic, genomic, biochemical, cell biological and immunological methods will surely accelerate the pace of discovery and we should soon be examining specific molecular interactions with the ultimate hope of developing highly effective interventions. As a by-product, we can also look forward to a much more detailed understanding of how the parasite has evolved to co-opt the host for its own purposes and how the host has evolved to defend against pathogens. Looking at these interactions is also likely to reveal much about normal host cell processes since, in one respect, the parasite is like a mechanic with a box of tools, each designed to interfere with a particular host function in a highly specific, and informative, way.

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# Part III

## **Genome and Genetics**

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# Genetics and Genome Organization of *Toxoplasma gondii*

1 1

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## Abstract

*Toxoplasma gondii* is a member of the phylum Apicomplexa, a diverse group of early branching eukaryotes related to dinoflagellates and ciliates (see Chapter 12; Baldauf, 2003). The definitive host is the cat where the sexual cycle takes place in the intestinal epithelia and oocysts are shed in the feces where meiosis occurs to produce environmentally resistant sporozoites (see Chapter 1). The life cycle is unusual compared to closely related coccidians in that the parasite can transmit directly between secondary hosts. This has allowed clonal growth and expansion in the population where the vast majority of isolates in North America and Europe are dominated by three clonal lineages (Howe and Sibley, 1995). Moreover, the predominance of the three clonal lineages appears to have emerged from a population bottleneck about 10 000 years ago (Su *et al.*, 2003) where the lineages appear to be derived from just a few related ancestral strains (Grigg *et al.*, 2001a). *T. gondii* in South America appear to be more divergent but a greater analysis of isolates is required. Genetic mapping and sequence analysis reveals an ~65 Mb genome distributed across 14 chromosomes which is over twice the size of *Plasmodium falciparum* (Khan *et al.*, 2005). This difference is due to higher predicted gene content, lower gene density and more introns per gene. The genome annotation shows that compared to *P. falciparum*, *T. gondii* retains many more enzymes in carbohydrate, lipid, amino acid and nucleic acid metabolic pathways (see Chapters 19 and 20).

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## Biology and life cycle

*Toxoplasma* belongs to the tissue-cyst forming coccidia, a group that also contains *Neospora*, *Sarcocystis*, and numerous other genera (Levine, 1988). Tissue-cyst forming coccidia typically have a two-host life cycle (referred to as heteroxenous), alternating between a definitive host carnivore and various intermediate hosts that are herbivores (Dubey, 1977). Molecular phylogenetic reconstructions suggest that *Toxoplasma* diverged from *Neospora* at ~10 million years ago (mya) (Su *et al.*, 2003), closely matching the speciation of cats and dogs, their respective definitive hosts. Heteroxenous life cycles are thought to have arisen several independent times in the coccidia (Barta, 1989), attesting to the advantages of this life style for transmission.

During the acute phase of infection, *Toxoplasma* grows rapidly as a haploid form called the tachyzoite that invades and replicates within virtually any nucleated cell from warm-blooded animals (see Chapter 15). During the chronic phase of infection, the parasites convert to a slow growing form called bradyzoites that are encased in a thick-walled cyst. Ingestion of these cysts by cats leads to sexual differentiation within intestinal epithelia cells and the resulting micro and macro gametocytes fuse to form a diploid zygote (Dubey and Frenkel, 1972). Following the development of an impervious wall, oocyst stages are shed in the feces and they undergo meiosis in the environment to yield eight haploid sporozoite progeny (Cornelissen *et al.*, 1984). Oocysts are long-lived and resistant to environmental conditions and are responsible for disseminating infection by contamination of food or water (Mead *et al.*, 1999).

Although the sexual cycle is usually initiated by bradyzoites, ingestion of tachyzoites and sporozoites by the cat can also lead to the sexual cycle (Dubey, 2005; Dubey and Frenkel, 1972). Each form has different prepatent periods prior to shedding of oocysts: 3–10 days for bradyzoites, > 19 days for tachyzoites and sporozoites (Dubey and Frenkel, 1972; Dubey and Frenkel, 1976; Dubey *et al.*, 1970; Freyre *et al.*, 1989). Moreover, these studies show that cats fed with bradyzoites rapidly shed oocysts with little or no dependence on strain or dose, whereas the prepatent period for both the sporozoite and tachyzoite forms is 2 to 3 times longer. These studies suggest that the sexual cycle is initiated by the bradyzoite form, so ingested sporozoites and tachyzoites must first initiate and sustain an acute infection in the cat intestinal tract such that bradyzoites are allowed to develop. If this range of prepatent periods occurs in natural infections, there would be a greater opportunity to establish a productive co-infection for recombination between different strains.

Early studies on the experimental transmission of *T. gondii* revealed that a single cloned organism can give rise to the complete sexual cycle in the cat (Cornelissen and Overdulve, 1985; Pfefferkorn *et al.*, 1977), precluding the existence of a fixed mating type. This is consistent with cellular observations during gametogenesis and establishment of the sexual cycle. *T. gondii* appears to have as many as five morphologically distinct schizont types prior to gamont formation and gamete production (Dubey and Frenkel, 1972; Speer and Dubey 2005). Both micro- and macrogametocytes develop exclusively within the epithelial enterocytes and are generally seen in close proximity to what is thought to be the final schizont-type prior to gamont differentiation. Although it is not clear at what point the differentiation is fixed, the observation of a what appears to be a shared developmental pathway supports the notion that a single organism gives rise to both macro and microgametes. Definitive confirmation of this model of gametogenesis was provided by co-feeding bradyzoites from two different drug-resistant lines. Analysis of progeny showed that recombination in the cat occurs both by self-mating and outcrossing with approximately equal frequency (Pfefferkorn and Pfefferkorn, 1980). These early studies on genetic recombination established that meiosis in *T. gondii* follows conventional Mendelian rules, and paved the way for further genetic mapping analysis described below.



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## Clonal population structure

The presence of a sexual phase in the life cycle in *T. gondii* strains predicts that the genetic diversity among isolates should be high. However, multilocus genotyping based on either restriction fragment length polymorphisms (RFLP) or isoenzyme markers revealed a much lower genetic variation than would be expected for a panmictic population (Dardé *et al.*, 1992; Sibley and Boothroyd, 1992). Clonality among isolates of *T. gondii* is evident by the repeated isolation of strains with identical genotypes from different geographical regions and hosts and by the absence of many possible recombinant genotypes (Sibley and Boothroyd, 1992). Further sampling revealed that *T. gondii* has a highly clonal population structure consisting of three predominant lineages (Howe and Sibley, 1995). The vast majority of *T. gondii* strains isolated from humans and animals in North America and Europe can be readily grouped into one of these clonal lineages. This clonal population structure indicates that propagation in nature occurs predominantly by asexual replication. In addition to the original three genotypes, a large number of isolates from sea otters fall into a genotype called type X, which is highly similar to type II strains at most loci (Miller *et al.*, 2004). This isolate has been associated with encephalitis in otters, although it is not clear if this is due to genetic differences in the parasite or extreme susceptibility of otters to toxoplasmosis.

There are likely several explanations for the maintenance of clonality in *T. gondii*. Genetic exchange in the wild may be limited by the restricted meiotic phase of the life cycle: sexual development only occurs within cat intestinal epithelial cells during a short period after initial infection and the chance of two or more strains infecting a single cat simultaneously may be low. Second, the current widespread occurrence of these three clonal lineages limits the opportunity for crossing to unique genotypes even when double infections do occur. However, clonality does not imply that no differences exist between individual members of a clonal lineage; small differences are expected to arise by mutation or genetic changes as a result of mitotic propagation. Microsatellite markers are particularly adept at picking up such differences and therefore well suited to defining minor variants within a population (Ajzenberg *et al.*, 2002; Lehmann *et al.*, 2000; Lehmann *et al.*, 2004). However, interpretation of microsatellite patterns for inferring molecular phylogeny is complicated due the multiple ways that such repeats can expand and contract. This can result in different lineages that look alike despite not sharing a common ancestry (referred to as homoplasy), thus leading to inaccurate phylogenies.

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## Biallelic population structure and recent origin

Sequencing of several unlinked polymorphic antigens from the three archetypes identified only two alleles at all loci examined, and these alleles are assorted randomly between the three lineages (Grigg *et al.*, 2001a). Therefore, at any given locus two strains will always be identical (with the exception of minor mutations) while the third is unique. This biallelic gene pool indicates that the three dominant lineages share a common ancestry from two closely related parental strains (Grigg *et al.*, 2001a). The two allelic types have been designated A (Adam) and E (Eve) and analysis of the frequency of single nucleotide polymorphism (SNPs) indicates about 1/100 base pairs (bp) differ between the lineages (Su *et*

*al.*, 2003). The expansion and predominance of the three lineages is likely due to a selective advantage of alleles that confer a strong selective advantage.

One possible explanation for the success of the clonal lineages is the acquisition of a new mode of transmission that has been termed direct oral infectivity between intermediate hosts (Su *et al.*, 2003). Endowed with potential to transmit directly between intermediate hosts, a small group of strains evidently expanded rapidly at the expense of more ancestral strains that were restricted to the conventional, obligatory two-host life cycle. Asexual oral transmission is absent in related genera such as *Neospora*, *Sarcocystis*, and consistent with it being a more effective means of transmission, *T. gondii* has a much broader host range than any of its close relatives (Dubey, 1977). One prediction of these studies is that ancestral strains of *T. gondii*, which predate the common origin of the clonal strains, should lack efficient asexual oral transmission. Evidence in support of this model was provided by the finding that several atypical strains of *T. gondii*, which have more diverse genotypes and hence predate the common origin of the clonal lineages, also have decreased asexual oral transmission (Su *et al.*, 2003). Testing this model will require examining a wider collection of exotic strains and it is also possible that additional adaptive traits are responsible for the recent expansion of the clonal lineages.

Despite the success of the clonal lineages, this population structure may not predominate in all settings. A number of strains isolated from distant geographical sites such as French Guyana were found to have atypical genotypes (Dardé *et al.*, 1998). These more divergent strains have unique multi-locus genotypes and contain alleles that were not observed in strains from other areas (Ajzenberg *et al.*, 2002). Other atypical strains have been associated with severe clinical presentation such as recurrent ocular toxoplasmosis (Grigg *et al.*, 2001b). Recent studies of *T. gondii* isolates from Brazil, where ocular toxoplasmosis is common, also indicate a different genetic makeup than seen in North America and Europe (Khan *et al.*, 2006). Other studies using animal isolates of *T. gondii* from South America have emphasized the much greater genetic diversity in strains found there (Ferreira *et al.*, 2004; Ferreira *et al.*, 2006; Lehmann *et al.*, 2006). This pattern contrast sharply with that seen in North America and Europe. However, large regions of the world have not been adequately sampled, and others examined using only a single genetic marker (Dubey *et al.*, 2004), which under estimates the true genetic diversity in regions where clonality does not predominate. Hence, comprehensive population genetic studies of *T. gondii* remains a highly important area for future research.

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## Development of genetic linkage maps

Application of genetics to defining virulence and pathogenicity requires a robust forward genetic system. Classical genetic linkage analysis provides one such approach for identifying the contributions of genetic loci to complex biological traits. Genetic mapping approaches have been developed in several apicomplexan parasites including *Eimeria tenella* (Shirley and Harvey, 2000), *Plasmodium falciparum* (Su *et al.*, 1999) and *T. gondii* (Khan *et al.*, 2005). All three of these organisms contain 14 haploid chromosomes, although the relative size of the genomes differs with *T. gondii* being the largest at ~65 million bp. The *P. falciparum* genetic map is comprised of more than 900 microsatellite markers spaced across the ~25–30 million bp genome (Su *et al.*, 1999). Recombination is relatively frequent in

malaria and hence the map unit (defined as the average distance for 1% recombination or centiMorgan (cM) is equal to ~17 kilobases (Su *et al.*, 1999). Genetic linkage studies were instrumental in positional cloning of the chloroquine resistance locus in *P. falciparum* (Fidock *et al.*, 2000). Genetic linkage maps have also been useful in mapping other loci that contribute quantitatively to drug resistance in malaria (Ferdig *et al.*, 2004) and for analyzing complex phenotypes such as development in *Eimeria* (Shirley and Harvey, 2000).

Microsatellites tend to be rare in *T. gondii*, hence RFLPs have been used to define genetic markers (Sibley *et al.*, 1992). Large scale sequencing of expressed sequence tags (ESTs) in *T. gondii* has allowed for gene discovery and the identification of SNPs between the three lineages (Ajioka *et al.*, 1998; Li *et al.*, 2003). Genetic crosses in *T. gondii* are conducted by: (1) inducing chronic infections in mice; (2) co-feeding tissue cysts from two strains to cats; (3) isolating oocysts and reinitiating cultures *in vitro*; (4) identifying recombinant progeny by drug resistance or RFLP markers. More than 250 strain-specific markers were used to construct a genetic map of *T. gondii* based on the analysis of 71 distinct progeny from several genetic crosses (Khan *et al.*, 2005). Included in this analysis are progeny from genetic crosses between the type II (Me49 clone B7) and III (CTG) lineages as well as crosses between the type I (GT-1) and III (CTG) lineages. The average map unit in *T. gondii* is ~104 kb/cM, although this can vary by as much as 3-fold across the genome (Khan *et al.*, 2005). The resolution of the current *T. gondii* map allows mapping of a particular single-gene trait to a region of 200–500 kb based on the analysis of a relatively small number of progeny (i.e. 20–30). Genetic linkage maps of the 14 *T. gondii* chromosomes are shown in Figure 11.1.

The recent completion of a 10X whole genome shotgun sequence for *T. gondii* has allowed the assembly of a combined physical and genetic linkage map that assigns the majority of large contigs-scaffolds to particular chromosomes (Khan *et al.*, 2005). Approximately 95% of the genome was assembled using this approach, revealing that it contains 14 chromosomes ranging in size from ~2 million bp to more than 7 million bp (Khan *et al.*, 2005). Previous karyotype analysis revealed that only the smallest 5–6 of these can be resolved on conventional pulsed-field gels; however, the karyotypes are stable with repeated passage *in vitro* (Sibley and Boothroyd, 1992). Collectively, the parameters of genetic linkage analysis in *T. gondii* are well suited to mapping specific phenotypes to particular chromosomes and for defining regions of the genome that underlie both simple and complex traits. Further information on the genetic crosses, markers, and genetic linkage maps for the *T. gondii* genome map can be found at <http://toxomap.wustl.edu/>.

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## Mapping drug resistance

The *T. gondii* genome map has been utilized for mapping specific drug resistance traits in *T. gondii*. In the process of conducting genetic crosses, drug-resistance markers were typically introduced into the parental lines. For example, the type III CTG strain carried resistance markers to sinefungin (SNF) (Pfefferkorn and Pfefferkorn, 1980) and adenine arabinoside (AraA) (Pfefferkorn and Pfefferkorn, 1978). Similarly, the type II strain Me49 was tagged with resistance to 5-fluorouridine deoxyribose (FUDR), as described previously (Pfefferkorn and Pfefferkorn, 1977). Consequently, the association between resistance in the progeny and specific genetic markers can be determined using linkage



analysis. Resistance to each of these compounds is primarily determined by a single genetic locus. However, their association with specific genetic markers was evaluated using quantitative trait locus (QTL) mapping (Lander and Kruglyak, 1995), which is capable of analyzing both single-locus effects and secondary linkages that influence resistance. QTL linkage analysis showed a single strong association for each of these compounds with log likelihood ratios of  $> 6$  with no secondary QTLs (Figure 11.2).

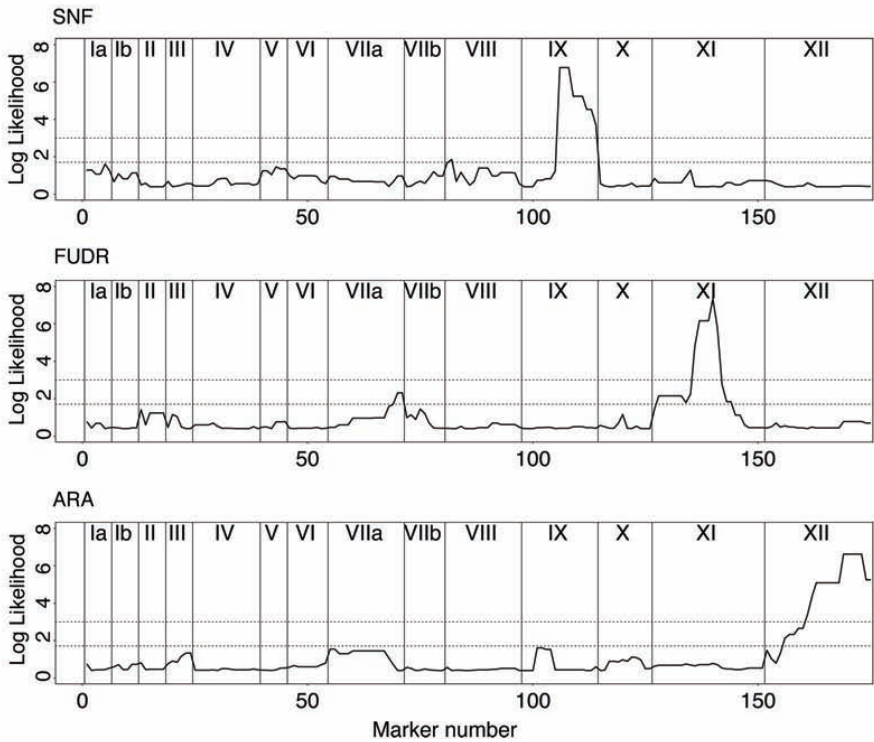
The target for two of the above compounds, FUDR and ARA-A, was already known before linkage analysis was undertaken and hence they serve as an estimate of the precision of mapping by linkage analysis. The target for FUDR is the uracil phosphoribosyl transferase (UPRT) gene. The UPRT gene is located in the center of chromosome XI and mapping of the resistance phenotype showed perfect correspondence to this locus (Figure 11.2 and <http://www.toxodb.org>). The target for ARA-A is adenosine kinase, which is located at the end of chromosome XII and once again there was perfect correspondence with this locus between markers AK165 and AK163 (Figure 11.2 and <http://www.toxodb.org>). Although, the target for SNF in *T. gondii* is presently unknown the resistance phenotype mapped to a region on chromosome IX at marker AK123. This marker lies on the same node as three other markers and these markers span a 540 kb region (Khan *et al.*, 2005). SNF is known to inhibit methylation reactions (Martin and McMillan, 2002), although preliminary examination of this region of the genome does not identify any obvious target that fits this profile (<http://www.toxodb.org>).

The relatively large intervals defined by these mapping studies illustrates one of the primary limitations of the linkage map at present. The relatively low rate of recombination limits resolution for mapping. Positional cloning will thus require higher density maps in regions where candidate loci have been identified. This can be accomplished by isolating new progeny and examining them for recombination within these nodes using additional polymorphic markers. Linkage mapping has also been used to map genes related to complex traits such as virulence (Su *et al.*, 2002) (see Chapters 9 and 12).

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## Basic features of the genome

Together, the genetic map and the DNA sequencing contig assemblies show that the genome of *T. gondii* consists of 14 chromosomes ranging from just under 2 Mb to nearly 7.5 Mb (<http://www.toxodb.org/>; I. Paulsen, D. Roos, unpublished data). Perhaps the most striking difference between the genome of *T. gondii* and the genomes of the other apicomplexans currently available for comparison, is the overall size. At nearly 65 Mb, it is nearly three times larger than *Plasmodium falciparum* and seven times larger than *Cryptosporidium parvum* and *Theileria parva* (see Table 11.1) (Abrahamsen *et al.*, 2004; Gardner *et al.*, 2002; Pain *et al.*, 2005). This difference is primarily due to a lower gene density, more predicted genes and more introns per gene. Compared to *P. falciparum*, *T. gondii* has half the gene density, 50% more predicted genes, four times as many introns per gene and an average intron size three times larger. The reason for these differences is not clear, but it has been suggested that the very wide secondary host range, the ability to form spores and be passed between secondary hosts may necessitate more genes and complex gene structures. The other striking difference is the GC content, where *T. gondii* is GC rich at 52% GC compared to the AT rich genome of *P. falciparum* at 19% GC. The genomes of *C. parvum*



**Figure 11.2** Mapping of drug resistance to SNF, FUDR and ARA-A across the genome of *T. gondii*. For each drug a single locus was found to be statistically associated with resistance, any secondary peaks were non-significant. Resistance to SNF localized to chromosome IX, FUDR localized to chromosome XI and ARA-A mapped to chromosome XII. Significance levels are given by dotted lines, the lower line is significant log likelihood 1.7, while the upper line is highly significant with a log likelihood of 3. Reproduced with permission from (Khan *et al.*, 2005).

and *T. parva* also have low GC contents at 30 and 34% respectively (Abrahamsen *et al.*, 2004; Pain *et al.*, 2005). In contrast, the coccidian *Eimeria tenella* shows a GC content of about 53% (K.L. Wan, unpublished data), in agreement with the closer phylogenetic relationship to *T. gondii*.

### Chromosomes and evolution

The population structure of *T. gondii* is clearly clonal, where the vast majority of strains isolated in Europe and North America appear to belong to one of three clonal lineages (see Chapter 13). The three clonal lineages have recent ancestry and analysis of type-specific polymorphism across the genome revealed a couple of surprising results. First, the three clonal lineages appear to be related through a small number of crosses, perhaps as few as two (Boyle *et al.*, 2006). The analysis showed that a type II-like strain is likely to be the common parent for both type I and type III strains where another known strain P89 is



**Table 11.1** Comparison of *T. gondii* and *P. falciparum* genomes

	<i>Toxoplasma gondii</i>	<i>Plasmodium falciparum</i>
<b>Chromosomes</b>	14	14
Total genome size (bp)	63 495 144	22 906 652
GC content (%)	52.2	19.4
Number of Genes	7793	5297
Mean gene length (bp)	2264.8	2274.9
Gene Density	9121.6	4324.5
Percent Coding	30.4	52.6
Percent Genes with Introns	74.3	53.7
<b>Exons</b>		
Number	39 843	12 700
Mean number per gene	5.1	2.4
GC content (%)	57.2	23.7
Mean Length (bp)	484.7	948.8
Total Length (bp)	19 310 050	12 050 244
<b>Introns</b>		
Number	32 050	7403
GC content (%)	49.2	13.5
Mean Length (bp)	613.3	178.6
Total Length (bp)	18 323 201	1 322 331

thought to be the other type III parent. Second, chromosome Ia is completely conserved between clonal lineages whereas every other chromosome shows evidence for crossing over (Khan *et al.*, 2006). Given the very rapid clonal expansion of these strains after the crossing events (Su *et al.*, 2003), it is speculated that the success of these strains is somehow conferred by genes located on chromosome Ia. Together, these studies suggest that sexual recombination and natural selection for clonal growth have dramatically altered the population structure of *T. gondii* and events such as these will continue to produce new strains that punctuate the evolution of this parasite.

### Gene function and classification

In order to classify genes into functional GO Slim categories (<http://www.geneontology.org/GO.slims.shtml>; GO=Gene Ontology; GO Slim is a cut down version of the complete GO), the annotated genes were assigned by a combination of two methods: (1) transferring the GO assignments from *P. falciparum*, where they share clear orthologues and (2) using Pfam (Protein family) (Finn *et al.*, 2006) hits for automated assignments (I. Paulsen, D Roos, unpublished data).

The cellular component may be roughly divided into a few GO categories: (1) the cell (GO:0005623), which is effectively the cell membrane and associated structures, (2) intracellular (GO:0005622), which includes the nuclear and cytoplasmic components but

excludes vacuolar and secretory products and (3) the vacuole/lysosome (GO:0005773; GO:0005764), mitochondrion (GO:0005739) and unknown (GO:0008372). About one third of the gene products have unknown cellular locations. Of the intracellular components identified, about a third show nuclear localization (GO:0005634), a quarter with the mitochondrion, a quarter with the ribosome (GO:0005840) and the remainder associated with other organelles like the Golgi apparatus (GO:0005794), endoplasmic reticulum (GO:0005783) etc. The apparent over representation in particular localizations is in part, due to the initial filter which will select for conserved genes/gene products (e.g. ribosomal proteins). The GO classification for biological process is dominated by the generation of precursor metabolites and energy (GO:0006091), transport (GO:0006810) and unknown processes (GO:0000004).

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## Metabolic pathways

One of the underlying reasons for the increased genome size of *T. gondii* compared to other apicomplexans becomes readily apparent when comparing metabolic pathways. Using *P. falciparum* and humans for comparison, pathways for the metabolism of carbohydrates, lipids, nucleic acids and amino acids all show significant differences (<http://apidb.org/apidb/>; I. Paulsen, D. Roos, unpublished data).

### Carbohydrate metabolism

*T. gondii* appears to share all of the major carbohydrate metabolic pathways seen in humans whereas *P. falciparum* does not retain obvious orthologues for enzymes required for gluconeogenesis, polysaccharide metabolism and O-glycosylation.

Because there are some essentially irreversible steps in glycolysis, gluconeogenesis requires bypass reactions in three places in the pathway. *T. gondii* and humans appear to share the key first bypass step to form oxaloacetate from pyruvate via pyruvate carboxylase (EC 6.4.1.1) whereas *P. falciparum* does not appear to retain this activity, but both parasites appear to be able to convert the oxaloacetate to phosphoenolpyruvate via through the reversible activity of PEP carboxykinase (EC 4.1.1.49). In *T. gondii* and humans but not *P. falciparum*, the second bypass step, conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, is carried out by fructose-1,6-bisphosphatase (EC 3.1.3.11). Since there is no evidence for the third bypass reaction catalyzed by glucose-6-phosphatase, *T. gondii* either does not require alpha-D-glucose and/or regenerates and uses beta-D-glucose via the reversible reactions of glucose-6-phosphate isomerase (EC 5.3.1.9) and hexokinase type IV (EC 2.7.1.1). The cell's ability to regenerate glucose is thought to be important in times of abundant energy and more importantly for *T. gondii*, for the recovery of energy stored as fatty acids or protein (see below). It is likely that the sporozoites and perhaps the relatively quiescent bradyzoites, store energy in these forms to facilitate transformation and re-activation into rapidly dividing tachyzoites seen in the acute infection. *T. gondii* also appears to use complex carbohydrates like glycogen for energy storage, as the genome annotation provides evidence for several proteins important for glycogen metabolism, including a 1,4-alpha-glucan branching enzyme (EC 2.4.1.18) for building glycogen linkages and a 1,6 glucosidase (EC 3.2.1.33) for the hydrolysis of branch linkages.

Earlier studies provided good evidence that *T. gondii* uses N-glycosylation to modify proteins (Odenthal-Schnittler *et al.*, 1993). The recent genome annotation shows the presence of a previously characterized N-acetyl-galactosaminyltransferase (Wojczyk *et al.*, 2003) and a putative mannosyltransferase (EC 2.4.1.83). Together this suggests that unlike *P. falciparum*, *T. gondii* also uses O-glycosylation to modify proteins such as SAG1 (Letourneur *et al.*, 2001). These cell surface protein modifications may promote the parasite's ability to bind the host's extracellular matrix and assist subsequent invasion.

### Lipid metabolism

The genome annotation of *T. gondii* for enzymes involved with lipid metabolism shows some unique features compared to *P. falciparum* and humans. *T. gondii* appears to use both type I and type II fatty acid biosynthetic pathways whereas *P. falciparum* and humans are limited to type II and type I respectively. In lipid energy production, oxidation of odd number carbon chain fatty acids requires propionate (propanoate) metabolism. Since excess propionate inhibits growth of many organisms, the metabolite requires conversion to succinyl-CoA for entry into the TCA cycle. *T. gondii*, *P. falciparum* and humans are able to convert propionate to propionyl-CoA. At this point humans use propionyl-CoA carboxylase to form methymalonyl-CoA whereas *T. gondii* appear to employ a methylcitrate synthase (EC 2.3.3.5) to form 2-methylcitrate. *P. falciparum* does not appear to have either option. The enzymes catalyzing other key steps to succinyl-CoA (EC 4.2.1.79, EC 4.1.3.30 and EC 6.2.1.1) also appear to be present in *T. gondii*. Propionate metabolism appears to be important for *T. gondii*, as part of odd-chain fatty acid metabolism and/or directly as a carbon source in ways not required by *P. falciparum*. Short chain fatty acids such as propionate are abundant in vertebrate intestinal tracts. Propionate may be used directly as an energy source by ingested bradyzoites, sporozoites and newly converted tachyzoites in the intestinal epithelia. Another possibility is that propionate may be converted to glucose for energy storage in the developing oocyst.

### Nucleic acid and amino acid metabolism

Both *P. falciparum* and *T. gondii* retain the ability to synthesize pyrimidines *de novo*, but *T. gondii* can salvage both purines and pyrimidines whereas *P. falciparum* can only salvage purines. Although *T. gondii* mouse virulence *in vivo* is compromised if *de novo* pyrimidine synthesis is knocked out (Fox and Bzik, 2002), pyrimidine salvage can support limited parasite growth and must confer a selective advantage as an addition to *de novo* synthesis. Overall, the profile of amino acid biosynthesis and degradation for *T. gondii* is strikingly similar to humans. The genome annotation suggests that alanine, cysteine, proline, serine and tyrosine can all be synthesized by *T. gondii* but not by *P. falciparum*. Moreover, like humans and unlike *P. falciparum*, *T. gondii* appears to have the ability to degrade isoleucine, leucine, and valine. In general the annotation revealed an essentially complete set of orthologues for the relevant enzymes in each pathway. However it is useful to note a couple of exceptions as a *caveat emptor* in using these data. The tyrosine transaminase (EC 2.6.1.5) appears to reversibly catalyze: L-tyrosine + 2-oxoglutarate = 4-hydroxyphenylpyruvate + L-glutamate. It is probably reasonable to assume that glutamate is present, but there isn't any evidence for orthologous enzymes that synthesize 4-hydroxyphenylpyruvate.

Similarly, unless serine is produced indirectly from cystathione, the more direct synthesis is through the serine-glyoxylate transaminase reversible reaction between glycine and serine (EC 2.6.1.45): L-serine + glyoxylate = 3-hydroxypyruvate + glycine. Again, although it is reasonable to assume glycine is present, there is lack of obvious orthologues for the enzymes used in the synthesis of hydroxypyruvate. These examples suggest that substrate scavenging may be an important part of the synthesis pathways. Despite these “missing” enzymes, on balance the data suggest that *T. gondii* has a much greater capacity to synthesize and utilize amino acids compared to *P. falciparum*. This may be due to the difference in host cell types and therefore the available strategies for nutrient acquisition. Although *P. falciparum* scavenges a large amount of hemoglobin, only about 16% of the resulting amino acids are incorporated into parasite protein (Krugliak *et al.*, 2002). The bulk of the amino acids used are probably acquired by the increased permeation and uptake by the infected erythrocyte (Ginsberg, 1994; Kirk, 2001). These adaptations are unlikely to be possible across the nucleated host cell range used by *T. gondii*, hence the necessity for maintaining *de novo* biosynthesis and degradation of amino acids. This principle may be the underlying explanation for the comparatively greater retention of metabolic pathways by *T. gondii* compared to *P. falciparum*. The advent of new genomic data from more closely related species, e.g. coccidians like *Eimeria tenella* and *Neospora caninum*, will test this generalization.

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# Evolution and Comparative Genomics of *Toxoplasma gondii* 12

Jessica C. Kissinger and Chih-Horng Kuo

## Abstract

Molecular data have shed considerable light on the evolution of many organisms, especially unicellular organisms, like protists and bacteria, many of which have no fossil record. Sequence data have shown that *Toxoplasma gondii* groups with other apicomplexan parasites and not surprisingly is most closely related to other coccidia. Analyses of currently available sequence data have revealed an evolutionary history for the Apicomplexa that includes the endosymbiosis of an alga, giving rise to the apicoplast organelle found in *T. gondii* and many other (but not all) apicomplexan parasites. Comparative sequence analyses have also revealed the presence of horizontal gene transfers from diverse sources into the *T. gondii* nuclear genome. Analyses of *T. gondii* expressed sequence tags (ESTs) and preliminary nuclear gene content have revealed the largest and most diverse gene content of any apicomplexan examined thus far.

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## Introduction

*Toxoplasma gondii* is classified as a coccidian and a member of the phylum Apicomplexa (Levine, 1982; Perkins *et al.*, 2000). This classification tells us much about the physical properties and lifestyle of this organism, but it does not tell us where this organism came from. In the absence of a fossil record, the evolutionary biologist must turn to the presence of shared derived physical characters (synapomorphies) and molecular data to ascertain relationships and evolutionary histories for this group of organisms. This chapter will take a historical perspective to examine the data we have concerning the evolution of *Toxoplasma*. The morphological and developmental characteristics that have been used to greatly facilitate classification are not always informative with respect to the determination of evolutionary history and relatedness. For example, the character, “infects humans” is highly useful for classification but less meaningful with respect to evolution since many distantly related pathogenic organisms have independently evolved the capacity to infect humans.

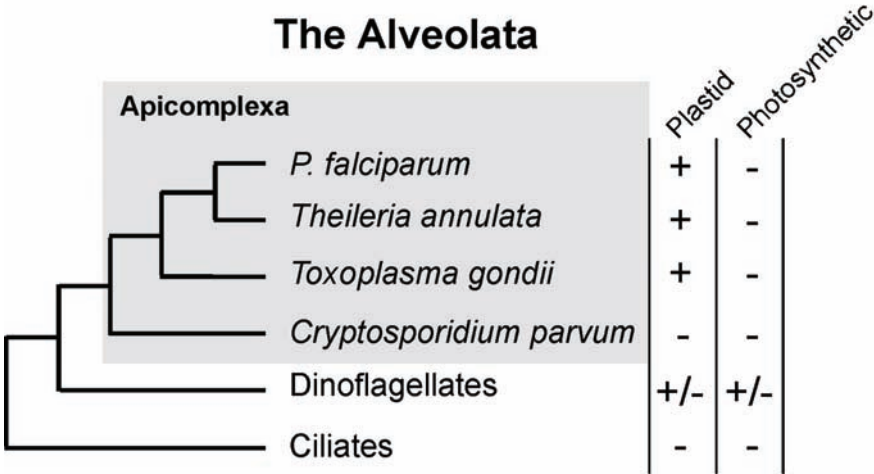
Our current views of protist evolution have been dramatically influenced by the influx of molecular data and increased taxonomic sampling representative of the diversity of life. Whittaker’s five kingdom schema (Whittaker, 1969) in which the monera (bacteria) gave rise to the protista (unicellular eukaryotes) which in turn gave rise to the multicellular

plants, animals and fungi has been replaced with a complex multi-domain classification of life in which the sheer diversity of protists has become apparent (Baldauf, 2003). Protists do not form a single clade on the tree of life. They are found on nearly every branch of the eukaryotic tree and can be more diverse from one another than are the plants from the animals (Adl *et al.*, 2005; Baldauf, 2003). *Toxoplasma gondii* resides within a eukaryotic super-group called the Alveolata. This group contains the ciliates and dinoflagellates, which are free-living, and the parasitic phylum Apicomplexa (Figure 12.1).

**Classical taxonomy**

The microscope, and in particular the electron microscope, has greatly facilitated and enhanced the number of characters that are available for classification and evolutionary analyses. Organisms belonging to the phylum Apicomplexa are so named because of the apical complex located at the apical end of the protist during particular life cycle stages. The apical complex consists of one or more polar rings, a conoid, several (usually 2–8) rhoptries, and micronemes (Chapter 2). Prior to the coining of the term “Apicomplexa” (Levine, 1970) to describe protozoa that contain an apical complex, the term, “Sporozoa,” which is still used, was the more popular term. However, since many organisms that are not closely related have evolved the capacity to form spores (e.g. microsporidians and haplosporidians) and because plants form a completely unrelated type of spore and hence could be confusing, this term is less used by some protozoologists.

There are an estimated 300 genera of apicomplexans representing nearly 4800 named species. All are parasitic (Perkins *et al.*, 2000). The Apicomplexa are officially divided into three taxonomic classes, the Perkinsasida, Conoidasida and the Aconoidsida (Levine, 1988) but most species fall into the last two classes and are commonly, but not officially, referred to in terms of five “principal groups,” the Eugregarine, Haemogregarine, Eimeriorine,



**Figure 12.1** Relationships of the organisms within the Alveolata. “+,” present; “-,” absent; The four species shaded by the grey box belong to the phylum Apicomplexa. Together, the Apicomplexa, Dinoflagellata and the Ciliates comprise the eukaryotic supergroup, Alveolata.

Haemosporine and the Piroplasmid (Perkins *et al.*, 2000). *Toxoplasma gondii* is taxonomically classified as Apicomplexa: Conoidasida: Eucoccidiorida: Eimeriorina: Sarcocystidae: *Toxoplasma gondii* (Perkins *et al.*, 2000). The NCBI taxonomic classification is quite similar, Apicomplexa: Coccidia: Eimeriida: Sarcocystidae: *Toxoplasma gondii*. Within the family Sarcocystidae, there are six recognized genera, *Frenkelia*, *Sarcosystis*, *Besnoitia*, *Hammondia*, *Neospora* and *Toxoplasma*. All genera are heteroxenous (utilize more than one host species) although *Toxoplasma* can directly develop in its definitive host, felids (Chapter 1). All genera in this family share several synapomorphies. They produce oocysts that contain two sporocysts and each sporocyst contains 4 sporozoites (Hammond and Long, 1973; Long, 1982; Perkins *et al.*, 2000).

## Molecular phylogenies

One of the first molecules to be examined in apicomplexan parasites was the Small Subunit 18S rRNA molecule (SSU-rRNA). The utility of this molecule for ascertaining evolutionary relationships among distantly related organisms in the tree of life had been well established (Pace *et al.*, 1986; Sogin, 1991; Sogin *et al.*, 1986; Sogin *et al.*, 1993). Several apicomplexan SSU-rRNA gene sequences were determined and a number of observations were made. The Apicomplexa are monophyletic (have a single origin) and they represent a distinct clade on the eukaryotic tree of life. Additionally, apicomplexan SSU-rRNA sequences, especially those from *Plasmodium* have very long branch lengths (Johnson *et al.*, 1987; McCutchan *et al.*, 1988), and this can, under some circumstances, cause a problem for phylogenetic analysis known as long branch attraction which causes sequences with long branches on the phylogenetic tree to group together rather than in their correct locations (Lyons-Weiler and Hoelzer, 1997).

Despite the long-branch issue, a number of informative studies on the evolution of *T. gondii*, other coccidia and their heteroxenous lifestyle emerged. Analyses of SSU-rRNA confirmed the family Sarcocystidae and identified *Neospora caninum* as the sister group to *T. gondii* (Holmdahl *et al.*, 1994). The genera *Isospora* and *Hammondia* were placed as sister taxa to the *Toxoplasma-Neospora* group (Carreno *et al.*, 1998; Ellis *et al.*, 1999). As more sequences became available, the structure of molecular relationships began to emerge for species within the order Eimeriorina. One group contains the tissue-cyst-forming genera *Isospora*, *Neospora*, *Toxoplasma* and *Hammondia*. Another group contains all members of the genus *Sarcocystis* and a third the members of the family Eimeriidae (*Eimeria* and *Cyclospora*) (Franzen *et al.*, 2000; Su *et al.*, 2003). An analysis of many diverse apicomplexan SSU-rRNA sequences including adelids, coccidia, haemosporidia and piroplasms revealed that the heteroxenous life styles characteristic of many genera within this phylum appear to have evolved independently and probably reflect evolutionary changes in the feeding behavior of the definitive hosts (Barta, 1989).

SSU-rRNA molecules can be ideal candidates for phylogenetic studies of distant relationships because they are slow evolving and the high copy numbers present in most genomes are usually homogenous and thus PCR-generated sequences should be uniform. However, as more apicomplexan SSU-rRNA sequences were obtained from within single species and as more species were examined, a complex picture began to emerge. Multiple, distinct SSU-rRNA genes were discovered in *Plasmodium* (Gunderson *et al.*, 1987) as well

as gene conversion among the different types (Enea and Corredor, 1991). These observations meant that SSU-rRNA was not a good evolutionary indicator in these species and perhaps the entire phylum. Later, it was also discovered that the apicomplexan SSU-rRNA gene families were very small (Abrahamsen *et al.*, 2004; Gardner *et al.*, 2005; Gardner *et al.*, 2002; Pain *et al.*, 2005; Xu *et al.*, 2004) and could, potentially be under selection, especially since stage-specific expression was observed in *Plasmodium* (Li *et al.*, 1997; McCutchan *et al.*, 1995). Initially the long apicomplexan SSU-rRNA branch lengths were interpreted as being indicative of ancient early branching species rather than the result of selection. Estimates of the origin of the Apicomplexa based on SSU-rRNA analyses have been made. The origin of the Alveolata was placed at > 1 billion years ago and *Plasmodium* at ~120 million years ago (Escalante and Ayala, 1995), but these estimates were based on a uniform molecular clock and if selection has been acting, these estimates will be over-estimates.

One of the first attempts to circumvent the emerging SSU-rRNA issues for apicomplexan phylogenies was to examine a slowly evolving mitochondrial gene, cytochrome B (Escalante *et al.*, 1998), but large numbers of species outside of the haemosporinids were never examined. Current phylogenetic studies have focused on the use of multiple protein encoding genes to create phylogenies for eukaryotes (Baldauf, 2003) the alveolates (Harper *et al.*, 2005) and the Apicomplexa (Zhu *et al.*, 2000).

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## EST comparisons

The first large-scale analysis of gene content in an apicomplexan parasite was an examination of Expressed Sequence Tags (ESTs) in *T. gondii* (Ajioka *et al.*, 1998). Comparisons of these EST data to other available sequence data revealed that more than half of the sequences had no significant similarity to sequences then present in public databases. This finding was quite significant and underscored the highly unusual nature of *T. gondii* relative to data (mainly model organisms) available at the time. Nine years later, the situation has not improved for *T. gondii*. Currently, 4521 of the predicted 7793 protein encoding genes are hypothetical but significantly, 752 of these are conserved hypothetical proteins indicating that while they are unknown, they are also found in other species, often apicomplexans (ToxoDB). Other apicomplexan species have better percentages of hypothetical genes ~30, but they also have many fewer genes in their genomes (Abrahamsen *et al.*, 2004; Gardner *et al.*, 2005; Gardner *et al.*, 2002; Xu *et al.*, 2004).

Since the aforementioned *T. gondii* EST sequence publication, data sets have become available for additional apicomplexan species (Table 12.1). Within the Sarcocystidae, EST data are available for *T. gondii*, *N. caninum*, and *Sarcocystis neurona* (Howe, 2001). Comparative analyses at multiple taxonomic levels have proven to be of considerable value for understanding the biology, evolution and adaptation of apicomplexan parasites to their hosts. One comparative study of 55K apicomplexan ESTs (~20 000 assembled clusters) from *T. gondii*, *N. caninum*, *S. neurona*, *E. tenella* and *P. falciparum* demonstrated that ~20% of the clustered sequences had homologs to plants, fungi, bacteria and other eukaryotes that could be identified in the public databases (Li *et al.*, 2003a). Interestingly, 62 genes were identified that appear to be apicomplexan-specific. Similar findings were observed in a recent analysis of *T. gondii* SAGE tags. In this study, comparative approaches revealed phylum-specific genes specifically involved in invasion and transmission (Radke *et al.*, 2005).



**Table 12.1** Status of apicomplexan sequence resources available for comparative analyses. Genome statistics were obtained from relevant publications, sequence center websites or genome databases, whichever was most current. EST counts were obtained from NCBI GenBank, or sequencing center if not available in GenBank. “†”—Taxonomic status is uncertain; *Perkinsus* is an alveolate but its affiliation with the Apicomplexa and/or Dinoflagellata remains controversial. “^”—Additional ESTs from *Eimeria tenella*, *E. acervulina* and *E. maxima* are located at <http://www.lbm.fmvz.usp.br/eimeria/>. “#”—EST sequences are located at Sanger, [http://www.sanger.ac.uk/Projects/B\\_bovis/](http://www.sanger.ac.uk/Projects/B_bovis/). “@”—ESTs in the collection represent a mixture of ESTs obtained from *C. hominis* and *C. parvum*. ND—not determined; NA—not applicable; TIGR—<http://www.tigr.org>; WTSI—<http://www.sanger.ac.uk>; VCU—<http://www.hominis.mic.vcu.edu/>; UMN—<http://cryptogenome.umn.edu/>; WU—<http://www.genome.wustl.edu/est/>; Stanford—<http://sequence-www.stanford.edu/group/malaria/index.html>.

Organism	Mb	Coverage	Genes	ESTs	Source
<i>Perkinsus marinus</i> <sup>†</sup>	~130	WGS	ND	0	TIGR
<i>Babesia bigemina</i>	10	WGS	ND		WTSI
<i>Babesia bovis</i>	9.4	WGS	ND	12 565 <sup>#</sup>	TIGR WTSI
<i>Cryptosporidium hominis</i>	9.16	WGS 12X	3994	0	VCU
<i>Cryptosporidium parvum</i>	9.11	Complete	3952	567 <sup>@</sup>	UMN
<i>Eimeria tenella</i>	55	PGS	~8000	34 035 33 585 <sup>^</sup>	WTSI
<i>Gregarina niphandrodes</i>	ND	NA	NA	1919	WU
<i>Neospora caninum</i>	~55	WGS	NA	25, 064	WU WTSI
<i>Plasmodium berghei</i>	23	WGS 3X	12 235	13, 095	WTSI
<i>Plasmodium chabaudi</i>	23	WGS 3X	ND	0	WTSI
<i>Plasmodium falciparum</i> 3D7*	23	Complete	5444	21, 176	WTSI Stanford TIGR
<i>Plasmodium gallinaceum</i>	23	WGS 3X	ND	0	WTSI
<i>Plasmodium knowlesi</i>	23	WGS 8X	5052	0	WTSI
<i>Plasmodium reichenowi</i>	23	WGS 3X	ND	0	WTSI
<i>Plasmodium vivax</i>	~25	Complete	5402	22 238	TIGR
<i>Plasmodium yoelii yoelii</i>	23	WGS 5X	7861	13, 925	TIGR
<i>Sarcocystis neurona</i>	ND	NA	NA	15 382	WU
<i>Theileria annulata</i>	8.5	WGS 8X	3792	17 031	WTSI
<i>Theileria parva</i>	8.5	Complete	4035	4380	TIGR
<i>Toxoplasma gondii</i> *	63	WGS 12X	7793	129, 421	Sanger TIGR

\*Note: other strains being sequenced.

Currently, the most limiting factors for comparative analyses are a lack of taxonomic sampling and bioinformatics resources for storing and analyzing the data. Within the Apicomplexa, prominent pathogens of human and agricultural importance have been

initially fairly well characterized. However, compared to the estimated 4800 species we have barely scratched the surface. Our taxonomic coverage is also highly biased towards members of the coccidia and haemosporidia. Only one EST project is available for gregarines, *Gregarina niphandrodes* (Omoto *et al.*, 2004). In the absence of greater taxonomic diversity, our ability to infer the direction of evolutionary change and identify adaptations e.g. host specificity is significantly hampered.

Apicomplexan EST sequence data for comparative analyses are available from a variety of sources (Table 12.1). Most data are available in the NCBI GenBank (Benson *et al.*, 2003), from the sequence source, or in specialized databases like ToxoDB (Kissinger *et al.*, 2003) GeneDB (Hertz-Fowler *et al.*, 2004) or the Apicomplexan Bioinformatics Resource Center, ApiDB (<http://ApiDB.org>) (Aurrecochea *et al.*, 2007).

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## The apicoplast and its evolutionary implications

As discussed in detail in Chapter 20, the evolutionary history of *T. gondii* includes the acquisition and maintenance of an apicoplast. The exact origin of the apicoplast is still debated but all evidence points to its acquisition via a secondary endosymbiotic event (Figure 12.2) in which one unicellular eukaryote endosymbiosed an alga, probably a red alga (Coppin *et al.*, 2005; Kohler *et al.*, 1997; Lang-Unnasch *et al.*, 1998; Roos *et al.*, 1999; Williamson *et al.*, 1994; Wilson *et al.*, 1996; Wilson *et al.*, 1991). Exactly when in evolutionary history this endosymbiotic event occurred is also debated, but increasing evidence suggests that it may have occurred prior to the formation of the phylum Apicomplexa and likely before the formation of the super group Alveolata (Fast *et al.*, 2001; Harper and Keeling, 2003). Under this scenario the ancestor of both the Heterokonta (sea kelp and diatoms) and the Alveolata acquired the endosymbiont and was photosynthetic. During the course of evolution, several lineages subsequently lost the endosymbiont/plastid e.g. the ciliates and *Cryptosporidium* (Abrahamsen *et al.*, 2004; Xu *et al.*, 2004) or the capacity to perform photosynthesis while others retained it (Figure 12.1). There is increasing evidence that the endosymbiotic event happened so long ago it might provide evidence for an even larger and still-debated super group of eukaryotes termed the “Chromalveolata” (Cavalier-Smith, 1999; Harper and Keeling, 2003; Harper *et al.*, 2005). This group would include the Alveolata, and the Chromista (Heterokonta, Cryptophyta (unicellular flagellated photosynthetic organisms) and Haptophyta (a branch of algae including phytoplankton)).

Of significance to the evolution of *T. gondii* is the profound metabolic impact provided by the apicoplast organelle (Roos *et al.*, 1999) and its potential to serve as a drug target in *T. gondii* (Fichera and Roos, 1997), *Plasmodium* (Foth and McFadden, 2003; McFadden and Roos, 1999), and other plastid-containing apicomplexan parasites. A second evolutionary implication of plastid acquisition is the transfer of genetic information from the organellar genome to the nuclear genome (Figure 12.2). The extent of intracellular gene transfer differs by lineage but includes hundreds of genes in *T. gondii* (Harb *et al.*, 2004) and *P. falciparum* (Foth *et al.*, 2003) (Chapter 20).

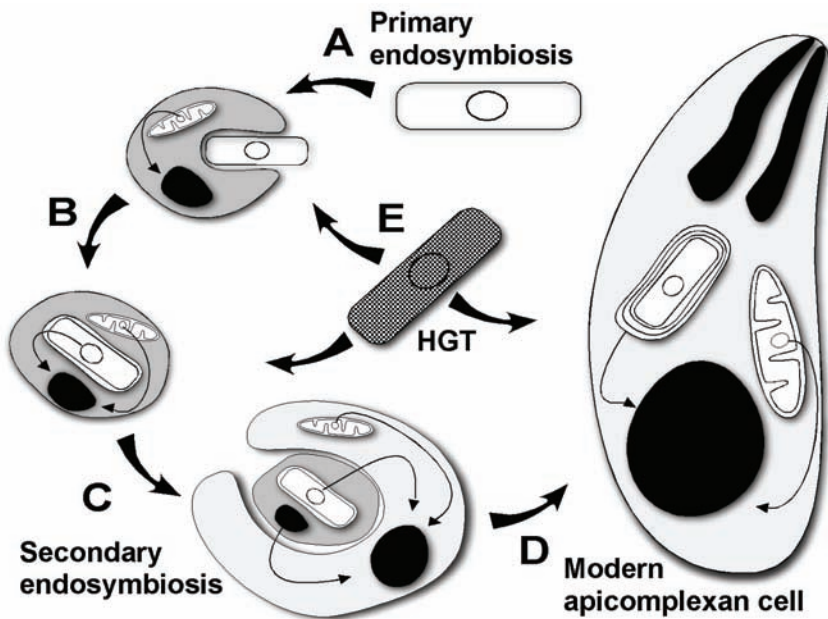
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## Gene transfer, evolution, and adaptation

As EST and genome sequence became available comparative analyses against public databases, and phylogenetic analyses revealed that *T. gondii* (and other apicomplexans)

contained genes of “plant-like” origin or similarity. The first reported case for *T. gondii* was the discovery of a “plant-like” enolase gene (Dzierszinski *et al.*, 1999). Additional putative homologs of plant-like and bacterial-like genes were identified in comparisons of ESTs (Li *et al.*, 2003a). At the time, due to poor taxonomic sampling, it was not clear if these genes represented shared ancestral genes or if they represented acquired genes. Genes can be acquired in nuclear genomes via two basic mechanisms. They can be acquired by intracellular gene transfer (IGT) from organellar genomes (mechanism unknown) or, they can be acquired from sources exterior to the cell, horizontal gene transfer (HGT) (Figure 12.2).

Given the evolutionary history of the apicoplast and its acquisition from an alga, it would not be surprising to detect genes of both algal (from the algal nuclear genome) and plastid (from the chloroplast) origin in the host nuclear genome. While *T. gondii* and other Apicomplexa no longer harbor the algal nuclear or mitochondrial genomes, they



**Figure 12.2** Apicomplexan evolution has been affected by endosymbiosis and gene transfer. Solid black circles are eukaryotic nuclei. Cristate, solid and spotted white ovals are mitochondria, cyanobacteria and other prokaryotes respectively. (A) Primary endosymbiotic event in which a cyanobacterium is endosymbiosed by a eukaryotic cell to give rise to a photosynthetic algal cell. (B) Intracellular gene transfer occurs from the organelles (chloroplast and mitochondrion) to the nuclear genome and indicated by the fine black arrows. Organellar gene loss also occurs. (C) An algal cell is endosymbiosed by another eukaryotic cell. Gene transfer to the new host nuclear genome begins. The algal mitochondrion is lost. The vestigial algal nucleus becomes a nucleomorph. (D) The nucleomorph is lost giving rise to a modern apicomplexan cell in which the “apicoplast” is surrounded by 4 membranes. (E) Throughout the evolution of the Apicomplexa, genes have also been acquired horizontally from additional, diverse, prokaryotic (and potentially eukaryotic) sources via Horizontal gene transfer, HGT.

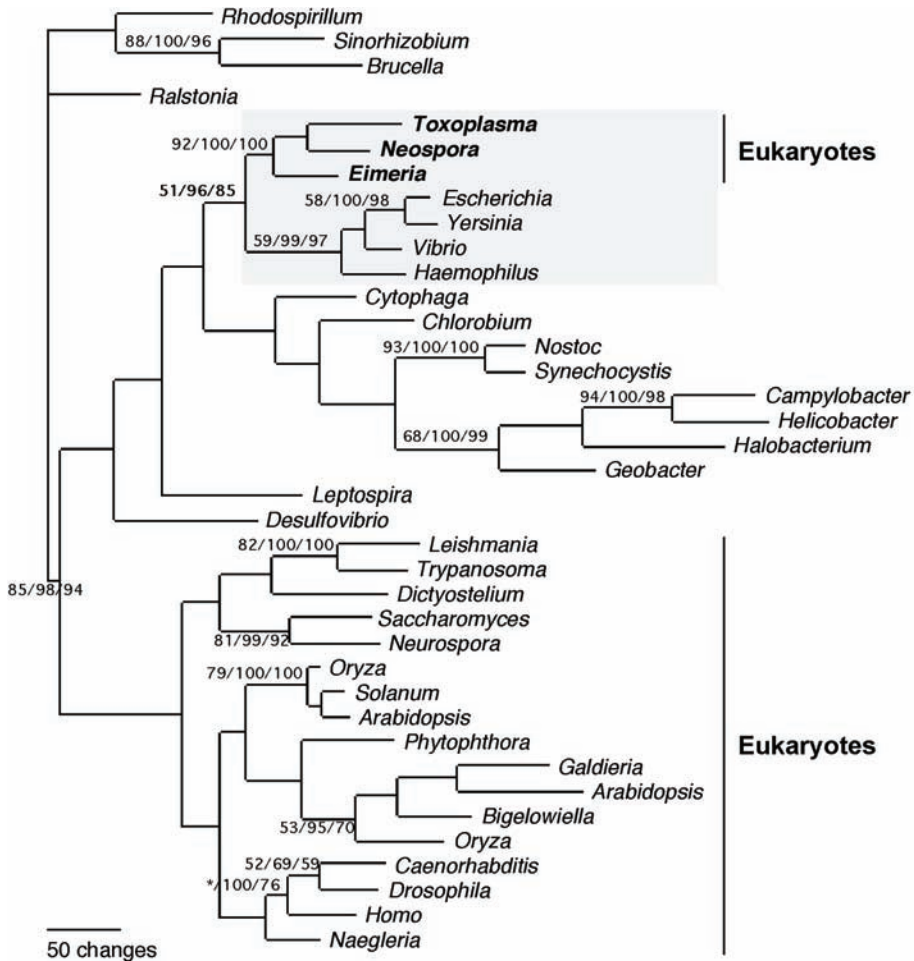
were undoubtedly present in the pre-alveolate ancestor. The *T. gondii* apicoplast does still retain a genome. It is circular, indicative of its cyanobacterial origin, but highly reduced at only 35 kb (<http://www.sas.upenn.edu/~jkissing/toxomap.html>). Most of the reduction appears to be the result of gene loss, for example the genes involved in photosynthesis, but several hundred genes have been transferred to the *T. gondii* nuclear genome (Harb *et al.*, 2004; Roos *et al.*, 1999).

A systematic survey of draft apicomplexan genome sequences to identify putatively acquired genes from any donor source identified many of the expected “plant-like” genes but surprisingly also identified many sequences of bacterial and archaeal ancestry (Huang and Kissinger, 2006; Huang *et al.*, 2004). One gene, fructose-1,6-bisphosphatase is most closely related to  $\gamma$ -proteobacterial sequences and thus far has only been detected in members of the coccidia, *E. tenella*, *N. caninum* and *T. gondii* (Figure 12.3). This enzyme is involved in a pathway, the mannitol cycle that has only been detected in coccidia. The function of the fructose-1,6-bisphosphatase enzyme is to shunt fructose-1,6-bisphosphate from the glycolytic pathway and convert it to mannitol (Schmatz, 1997). Mannitol forms the major energy reserve in the oocyst life cycle stage of *Eimeria*.

Parasites that have an oocyst life cycle stage require an energy source while they exist in an extracellular environment. However, the particular energy source appears to vary by evolutionary lineage. While *Eimeria* uses mannitol (Schmatz, 1997), *Cryptosporidium* uses trehalose (Abrahamsen *et al.*, 2004; Xu *et al.*, 2004). The extent to which these different energy sources represent adaptations of the parasite versus stochastic evolutionary events is currently unknown, but they represent intriguing avenues to explore.

A strong case for functional benefit that can be attributed to gene transfer in *T. gondii* is the acquisition of plant-like calcium protein kinases, CDPKs (Huang *et al.*, 2004; Nagamune and Sibley, 2006) and calcium ATPases (Nagamune and Sibley, 2006). Calcium levels regulate a number of processes in *T. gondii* including motility (Wetzel *et al.*, 2004), differentiation (Billker *et al.*, 2004) and secretion (Carruthers *et al.*, 1999; Carruthers and Sibley, 1999). The presence of these plant-like calcium-dependent enzymes is conserved across many apicomplexan species and the CDPKs are amplified in *T. gondii* relative to other apicomplexans suggesting expanded roles (Nagamune and Sibley, 2006).

*Toxoplasma gondii*, unlike any other examined apicomplexan thus far, exhibits an additional type of intracellular gene transfer. Thus far, transfers from the algal and plastid genomes have been discussed, but all eukaryotes contain genes acquired from mitochondria in their genomes, even those organisms that no longer possess a mitochondrion (Hashimoto *et al.*, 1998). Of interest to this discussion is the extent to which fragments of the mitochondrial genome are observed in the *T. gondii* nuclear genome. Analysis of *T. gondii* cosmid clones revealed the presence of cytochrome oxidase 1 and cytochrome B fragments that were flanked by repeats. Estimates of the copy number of the gene in the *T. gondii* genome by hybridization intensity revealed > 700 copies in the nuclear genome (Ossorio *et al.*, 1991). Analyses of the draft *T. gondii* genome sequence reveal 1549, 709, and 1097 copies respectively of fragments of the cytochrome B, cytochrome oxidase 1 and cytochrome oxidase 3 genes respectively in the nuclear genome (Hall and Kissinger, unpublished observation). It is not yet known if the mitochondrial fragments in the nuclear genome represent the result of independent transfer events or post-transfer amplification



**Figure 12.3** Identification of a bacterial gene in *T. gondii* and other Coccidia. Phylogenetic analysis of fructose-1,6-bisphosphatase. Note, this gene is not detected in *P. falciparum* and *C. parvum*. The tree shown is a phylogram generated via parsimony analysis. Bootstrap and quartet puzzling frequency values above 50% are indicated on branches (maximum likelihood using puzzle frequencies/distance with neighbor-joining/parsimony). “\*” indicates that a value below 50% was observed for this particular method. Grey=region of tree uniting genes from coccidia and proteobacterial organisms. The scale is as indicated. Accession numbers for each sequence are as in (Huang *et al.*, 2004).

and fragmentation. The level of sequence divergence observed in the fragments suggests that the process has been ongoing since sequences can vary by a continuum ranging up to at least 40% in their nucleotide divergence. Unlike the earlier observation of transferred fragments being flanked by repeats, the majority of fragments identified from the *T. gondii* ME49 genome sequence to not appear to be directly flanked by repetitive sequences (Hall and Kissinger, unpublished observation).

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## Insights into recent *Toxoplasma* evolution and population structure

As if the early evolutionary history of *T. gondii* were not exciting enough, the recent evolutionary history has been no less intriguing. Examination of the population structure of *T. gondii* revealed that it was basically clonal and that sexual reproduction was highly reduced (Howe and Sibley, 1995) (Chapter 12). Follow-up of this observation has revealed that the majority of the world's clonal *T. gondii* population is derived from a single genetic cross (Chapter 11) and that this cross produced offspring that were capable of being spread via an orally infective route as opposed to the normal ingestion of oocysts. (Boyle *et al.*, 2006; Khan *et al.*, 2005; Su *et al.*, 2003). The evolution of oral infectivity marks a major evolutionary event in the history of *T. gondii* that has had profound effects on the distribution and population structure of this organism by facilitating direct organism to organism transfer of the haploid parasite in the absence of sexual recombination. The longer-term evolutionary consequences of this major shift in propagation strategy are unknown, but they can be modeled. As a general rule, shifts to entirely or almost entirely sex-free modes of reproduction leads to the accumulation of deleterious mutations (Muller, 1932).

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## Comparative genomic insights and whole genome phylogenies

Genome sequences are now available for many organisms within the Alveolata (Table 12.1) and throughout the tree of life. These resources permit new levels and types of comparisons. For example, it is now possible to determine the core set of genes or metabolic pathways that are shared by a given group of organisms and to determine those genes, pathways or processes that are unique to an organism, a group of organisms, or even an entire phylum. When complete genome sequences are available we can ask questions about gene loss in a meaningful way and look for differences in metabolic processes between the host and pathogen and potentially utilize them in the development of therapeutics (Chaudhary and Roos, 2005). On a wider scale, it becomes possible to ask questions about the evolution of adaptive strategies (Roos, 2005). Are similar genetic components, pathways or processes conserved in pathogens that affect the same host or utilize a particular life history strategy? Comparative genomics has been likened to “a recapitulation of the grand era of natural history during the 1800s when the detailed cataloging of nature gave way to the hunger to understand why there is such diversity and structure to the natural world” (Clark, 2006).

To address the questions posed above we first need an “outgroup.” An outgroup is a related species from outside the taxonomic group of interest that will represent the “ancestral state” and polarize the direction of change within the group under study. Among the alveolates, there are two completed ciliate genome sequences, *Tetrahymena thermophila* (Eisen *et al.*, 2006) and *Paramecium tetraurelia* (<http://paramecium.cgm.cnrs-gif.fr/>) that can serve this purpose. Unfortunately, these species are quite distant from the Apicomplexa. A dinoflagellate would be more appropriate for comparative purposes. A genome project is under way for the oyster parasite, *Perkinsus marinus* (TIGR) and this organism is believed to be a dinoflagellate or an early branching apicomplexan (Goggin and Barker, 1993; Reece *et al.*, 1997; Saldarriaga *et al.*, 2003; Siddall *et al.*, 1997).

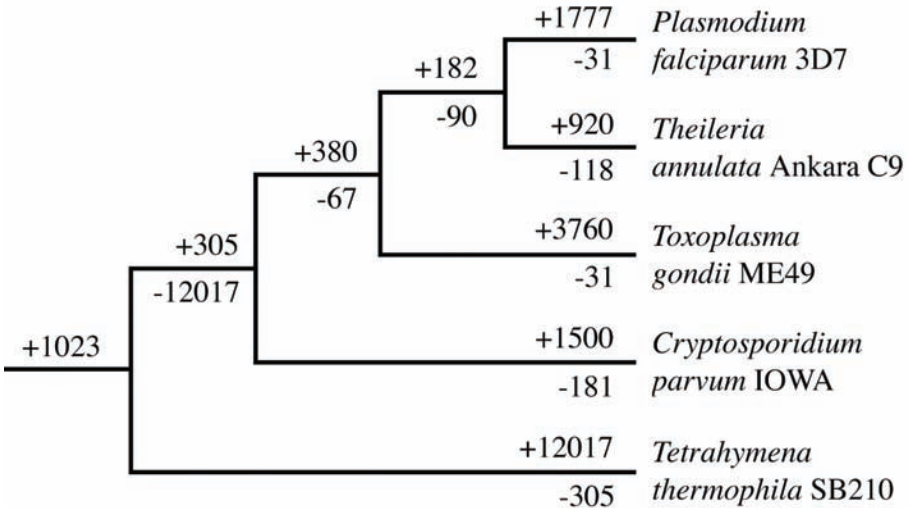


To determine the sets of orthologous genes shared by *T. thermophila*, *C. parvum*, *T. annulata*, *P. falciparum* and *T. gondii*, published, or predicted (ToxoDB; (Kissinger *et al.*, 2003)), protein-encoding gene sets were compared (Kuo and Kissinger, unpublished data). Our analyses reveal that the apicomplexans share more genes with each other than with the free-living ciliate, *T. thermophila* (Table 12.2). *Toxoplasma gondii* ME49 has the largest number of annotated protein-coding genes of the apicomplexans examined thus far (Chapter 11); but considerably fewer than *T. thermophila* (Table 12.2). Interestingly, *P. falciparum* shares a greater number of orthologous gene clusters with *T. gondii* than with the more closely related *T. annulata* (Table 12.2). One possible explanation is that the *T. annulata* lineage has an extremely reduced genome and has lost many orthologous genes following divergence from *T. gondii*. Under this hypothesis, lineages that are more closely related do not necessarily share a higher portion of their gene content compared to lineages of greater evolutionary distance. Both *C. parvum* and *T. annulata* have extremely reduced genomes (Abrahamsen *et al.*, 2004; Pain *et al.*, 2005) and thus may appear less related to nearest neighbors if analyzed solely on the basis of shared gene content. Phylogenetic analysis will not be biased by this difference beyond the unavoidable lack of orthologous sequences due to gene loss.

If the set of orthologous genes are analyzed to determine which species share which sets of genes, the results can be mapped onto a cladogram (tree of species relationships) and the patterns of gene gain or loss mapped. Figure 12.4 shows that 1023 gene clusters are shared by all five alveolate species, *T. thermophila*, *C. parvum*, *T. gondii*, *T. annulata* and *P. falciparum*. This “core” alveolate gene set represents highly conserved genes, most of which were easily identified since 70% have similarity to a Pfam domain (Kuo and Kissinger, unpublished observation). *Tetrahymena thermophila* contains 12017 gene clusters that are not shared with any of the apicomplexan genomes examined (Figure 12.4).

**Table 12.2** Clusters of orthologous genes as determined by OrthoMCL (Li *et al.*, 2003b). Values indicate the number of orthologous gene clusters shared by each species-pair. These clusters may, or may not, contain genes from other species. Numbers on the diagonal are the number of gene clusters found in each species (orthologs and paralogs). The versions of the genome annotations analyzed are as indicated by the release date and number of annotated proteins, nSeq. OrthoMCL parameters were E = 1e-9 cutoff for BLASTP and Inflation = 1.5 (Kuo and Kissinger unpublished observations).

Species	Release	nSeq	<i>C.p.</i>	<i>P.f.</i>	<i>T.a.</i>	<i>T.g.</i>	<i>T.t.</i>
<i>Cryptosporidium parvum</i>	4/2/06	3806	3598	1706	1493	1865	1386
<i>Plasmodium falciparum</i> 3D7	12/7/05	5411		4756	2156	2587	1601
<i>Theileria annulata</i>	7/15/05	3795			3251	2012	1321
<i>Toxoplasma gondii</i> ME49	1/04/06	7793				6912	1828
<i>Tetrahymena thermophila</i>	4/14/06	27424					14058



**Figure 12.4** Cladogram illustrating orthologous gene cluster distribution among five alveolate species. Numbers shown on the internal branches indicate the counts of orthologous genes that are uniquely present (preceded by a “+” sign) or uniquely absent (preceded by a “-” sign) in all daughter lineages. For example, 182 orthologous gene clusters are found in both *P. falciparum* and *T. annulata* but none of the other species; 90 orthologous gene clusters are shared by all lineages (including the out-group) but not found in either *P. falciparum* or *T. annulata*.

Three hundred and five orthologous gene clusters are shared by the four apicomplexan lineages examined, but absent in the free-living ciliate (Figure 12.4). Of this conserved apicomplexan core, ~60% of the identified sequences have similarity to Pfam domains. Most of the remaining genes encode conserved hypothetical proteins. Based on the available predicted *T. gondii* ME49 protein-encoding sequences (ToxoDB, Chapter 11) 3760 genes appear to be *T. gondii*-specific. This number is presumed to be artificially high since no other coccidia were included in the analysis. When the genome data become available for *Eimeria* and *Neospora*, it is hypothesized that this number will be reduced significantly since many genes are expected to be shared among the coccidia, especially among species as closely related as *N. caninum* and *T. gondii*. Given the overall similarity of *N. caninum* and but their use of distinct definitive hosts, canid and felid, respectively, comparative genomics will an important role in the initial identification of candidate genes that may play a role in adaptation to host.

A close look at Figure 12.4 also reveals 31 genes that are shared by the other three apicomplexans examined that are not observed in the predicted *T. gondii* ME49 gene set (Figure 12.4). While it is possible that these genes represent true losses, it is also possible given the preliminary nature of the available annotation that these genes were simply missed in the first-pass annotation or represent genes located within physical gaps in existing genome sequence. When the genome sequence is fully complete it will be possible to definitively determine the fate of these genes.

## Prospects for positioning *T. gondii* within the Apicomplexa

One of the largest phylogenetic issues facing *T. gondii* is a determination of its exact relationship to other members of the Sarcocystidae. As was mentioned above, once this framework of relationships is determined it becomes possible to assess the directionality of evolutionary and adaptive change. To address this question, sequences will need to be obtained from many more species within this group so that the phylogeny can be created. But what molecular sequences will be appropriate to resolve these relationships? We have developed an approach that allows us to use available genome sequences from the group of interest (in this case, we will have *Eimeria*, *Neospora* and *Toxoplasma*) and determine, via phylogenetic analysis and selective constraints, the set of genes that would be useful to determine the phylogeny. We used this approach to determine the relationships depicted in the cladogram in Figure 12.4 (Kuo *et al.*, submitted). The value of this approach is that it removes the guesswork out of determining the best genes to use for the analysis and since the approach is based on the analysis of amino acid sequences, it will permit the use of sequences that can be found in existing EST resources (Table 12.1). Once candidate genes are identified, these can be selectively cloned from the species under study and the phylogeny can be created.

This is an exciting time in both evolutionary biology and parasitology. “Omics” technologies are facilitating questions on a scale that was here-to-fore unimaginable. This phenomenon is no less true for comparative genomics. In a window of 4 years we have gone from the publication of the first apicomplexan genome, *P. falciparum* in 2002, to more than 15 apicomplexan genomes completed or in progress (Table 12.1). These data combined with strategic taxonomic sampling of the non-model apicomplexan organisms will create the framework to facilitate analyses designed to provide insight into the biology of *T. gondii*.

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# Population Genetics, Sex, and the Emergence of Clonal Lines of *Toxoplasma gondii*

13

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## Abstract

The population biology of the cosmopolitan parasite *Toxoplasma gondii* is highly dependent on the pathogen's ability to propagate both clonally and sexually. This highly prevalent zoonosis utilizes its sexual cycle to reassort and produce new lines capable of emerging from wild niches to expand its host range and cause outbreaks. Highly successful clones then rapidly spread clonally to endemic levels worldwide.

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## Introduction

*Toxoplasma* is a protozoan parasite in the phylum Apicomplexa, a monophyletic group of > 5000 species comprising almost entirely parasitic protists (Levine, 1988). It is related to other important animal and human pathogens including *Plasmodium*, *Cryptosporidium*, and *Eimeria*, the etiologic agents of malaria, cryptosporidiosis and chicken coccidiosis respectively. *Toxoplasma gondii* exists as a single species and is most closely related to the tissue-cyst forming coccidia *Neospora*, *Sarcocystis*, *Besnoitia*, and *Hammondia*. These protozoa are prevalent zoonoses that all reproduce sexually in nature, with *Toxoplasma gondii* distinguishing itself as the most "successful" pathogen in the group capable of infecting essentially any warm-blooded vertebrate at often high prevalence. Indeed, *Toxoplasma* is a cosmopolitan pathogen that chronically infects approximately one-third of the world's human population as well as a substantial number of other intermediate hosts including most birds and mammals.

Toxoplasmosis and associated sequelae (including loss of motor and/or cognitive function, sight, hearing, fatal encephalitis) is a substantial burden to congenital and immunosuppressed patients who are infected or experience reactivation of infection each year. In healthy adults, disease is of variable severity, ranging from largely asymptomatic to mild influenza-like symptoms before resolving into a relatively benign, life-long infection. However, some strains of *Toxoplasma* have been linked with causing a recurrent and often quite severe ocular disease in healthy adults (Grigg *et al.*, 2001b). *Toxoplasma* is also a leading cause of death due to food-borne illness in the United States (Mead *et al.*, 1999), and the incidence of toxoplasmic encephalitis in patients with AIDS, although diminished in recent years since the introduction of more effective treatment modalities for HIV infection, still remains a significant problem for patients with AIDS and those who are

immunocompromised (Montoya and Liesenfeld, 2004). In these patients, prognosis can be poor because no sterilizing prophylactic drug currently exists.

Despite the fact that the parasite was discovered in 1908 infecting tissues of the African rodent *Ctenodactylus gundi*, its complete life cycle was only determined in 1970 (see for example Dubey *et al.*, 1970). Wild and domestic felids are the only known animals capable of serving as the definitive or egg-shedding hosts. *Toxoplasma* is so ubiquitous in nature, no ecological niche capable of sustaining warm-blooded vertebrates has been spared. Recently, it has even been discovered as a common pathogen infecting virtually any marine mammal including mustellids (i.e. sea otters), cetaceans (i.e. whales, dolphins, porpoises), pinnipeds (i.e. seals, sea lions, walruses) and manatees (Dubey *et al.*, 2003). How *Toxoplasma* gains access to, for example, deep-sea dolphins is truly enigmatic and raises the question as to whether additional non-feline hosts can serve as definitive hosts, at least in the marine environment.

Its global prevalence is largely the result of its incredibly flexible life cycle. Two major routes of transmission are known to exist in nature and each is dependent on ingestion of infectious bradyzoites (the end-stage of the asexual cycle) or sporozoites (the end-stage of the sexual cycle). In effect, the parasite can be propagated either asexually or sexually. The asexual cycle occurs in most warm-blooded animals and consists of a dynamic equilibrium between two haploid forms that can be propagated indefinitely: a lytic tachyzoite that disseminates infection and causes acute disease, and an infectious, cyst-forming, bradyzoite that establishes a life-long, chronic infection. Transmission can occur by ingestion of undercooked meat harboring *Toxoplasma* tissue cysts, and scavenging allows the parasite to cycle through the food chain. The ability of the parasite to readily interconvert between tachyzoites and bradyzoites in intermediate hosts is thus critical for the success of its asexual phase as this serves to regulate the parasite's pathogenesis and establish its long-term persistence. It is also a unique feature exclusive to *Toxoplasma*; other tissue-dwelling coccidia do not form infectious tissue-cysts capable of oral transmission through intermediate hosts and so, are rarely transmitted asexually by carnivorous (Sibley, 2003; Su *et al.*, 2003) (see Chapter 11). The sexual cycle occurs exclusively in the gut of feline species (the definitive host) and involves schizogony, gametogenesis and mating within the intestinal epithelium. The sexual cycle culminates with the production of oocysts that are excreted in the feces of cats. In the environment, oocysts sporulate to produce two sporocysts, each containing four highly infectious sporozoites that can persist in the environment for months to years. Propagation through a single cat can yield in excess of 10 million highly infectious oocysts shed in its feces, and here, transmission can occur by ingestion of food and water contaminated with sporulated oocysts, e.g. in drinking water or on fresh vegetables (Dubey *et al.*, 1970; Frenkel *et al.*, 1970). Several oocyst-derived infections have been linked to epidemiologic outbreaks of the parasite in Canada, Panama and Brazil (Benenson *et al.*, 1982; Bowie *et al.*, 1997; Burnett *et al.*, 1998; de Moura *et al.*, 2006). The extent to which either cycle contributes to the successful propagation of the parasite life cycle is currently unclear. This certainly represents a central question toward understanding the population biology of this parasitic protozoan and this chapter reviews the current literature and comments on the role of sexual versus clonal reproduction in shaping *Toxoplasma*'s current population genetic structure.

## Population biology

*Toxoplasma* is endemic worldwide. For such a widespread pathogen having apparently few geographic or host boundaries and a well-described sexual cycle in cats, it was anticipated that genetic diversity among isolates would be substantial and that the population structure be effectively panmictic. However, early studies using multi-locus isoenzyme analyses on a large number of isolates of mostly European origin revealed remarkably little variation across different genetic loci (Darde *et al.*, 1988; Darde *et al.*, 1992). When the first PCR-Restriction Fragment Length Polymorphism (RFLP) analyses were applied against a larger set of isolates of principally North American and European origin, strains that were acutely virulent in mice comprised a single, clonal "type" that bifurcated from the non-virulent strains (Sibley and Boothroyd, 1992). The virulent type I strains possessed extremely limited genetic diversity and grouped together as one lineage using highly polymorphic microsatellite markers. A subsequent and more exhaustive multilocus RFLP study applied against 106 strains derived largely from symptomatic humans and domestic animals across three continents revealed that the majority of non-virulent strains grouped into just two discrete clonal lineages that were referred to as types II and III (Howe and Sibley, 1995). The two molecular studies showed that the vast majority of strains (> 94%) possess only a limited number of alleles for any given genetic locus and cluster into just three biologically discrete, clonal lineages (or archetypes, hereafter referred to as types I, II, and III) (Darde, 1996; Sibley and Howe, 1996). The limited number of clonally propagated lines coupled with the extensive linkage disequilibrium among strains strongly suggested that *Toxoplasma* exists in nature as a striking example of a eukaryotic pathogen with a capacity for Mendelian genetics that instead propagates itself largely asexually (Tibayrenc *et al.*, 1991). However, it is important to note, that the majority of parasite isolates came from a limited host range and were largely from symptomatic infections, which might have biased the results. Nevertheless, for a pathogen capable of genetic sex with no obvious geographic or host range restrictions, to suggest that meiotic recombination is not a major force driving strain diversity in natural populations of *Toxoplasma* was entirely not expected.

## Genetic diversity

### Clonal propagation theory

The vast majority of natural isolates from other parasitic protozoa such as *Trypanosoma cruzi*, *Leishmania* spp. and perhaps *T. brucei*, propagate asexually and all can be classified into just a few major phylogenetic lineages based on isoenzyme and genetic analyses (Tibayrenc *et al.*, 1990; Tibayrenc *et al.*, 1986). That is, they too possess a "clonal" population genetic structure consisting of independently propagating, genetically quite divergent clonal lines. Although hybrid or recombinant genotypes have also been identified in nature, these lines are typically ancient in origin (as measured by their accumulation of point mutations at loci under no obvious positive selection) indicating that genetic sex has contributed little recently, if at all, to the extensive variation found between these clonally propagated lines in their natural population structure (Ayala, 1998; Tibayrenc and Ayala, 2002; Tibayrenc and Ayala, 1999; Tibayrenc *et al.*, 1991; Tibayrenc *et al.*, 1990; Tibayrenc *et al.*, 1986). Evidence for this comes from the persistence of genetic variation

among alleles in stable lines over vast geographic areas, all of which suggest an ancient origin for these meiotic events (Oliveira *et al.*, 1998). Clonality is not an unusual feature among prokaryotic pathogens and serves the basis for the “Clonal Propagation Theory” reported previously for these protozoan parasites. In essence, the clonal theory states that sexual reproduction is too infrequent to be an effective force in the emergence of new lines with a capacity to alter the population genetic structure. Whether or not genetic sex is an effective force driving the emergence of new, clonal “types” in the population biology of the sexual protozoa (e.g. *Toxoplasma* and *Plasmodium*) has been the subject of intensive study and debate over the past decade.

### Inbreeding and clonality

For *Toxoplasma*, a haploid organism capable of sexual reproduction in its definitive feline host, the sheer predominance of three dominant, clonal lines has been difficult to fathom. This point is however easily reconcilable if sexual recombination contributes little, if at all, to the reproduction and dissemination of successful lines of the parasite. This is an entirely feasible explanation for two reasons. First, *Toxoplasma* can be clonally propagated asexually because tissue cysts are orally infectious (Su *et al.*, 2003). Here, carnivorous and scavenging through intermediate hosts can effectively maintain its life cycle indefinitely. Second, as a haploid organism, reproduction in feline species can proceed by self-fertilization in the absence of prey chronically infected with two or more distinct genotypes. Hence, inbreeding in the cat gut can yield progeny genetically identical to the infecting parent strain. Thus, for any given geography that is dominated by a limited number of different genotypes, or where there are few prey superinfected with more than one strain of parasite, inbreeding would be favored. This would limit the opportunity for sexual recombination to play a role in diversifying the parasite’s population genetic structure. These two scenarios would thus support the clonal propagation theory for a sexually reproducing organism. Inbreeding in *Plasmodium falciparum*, which has an obligate sexual phase in its insect vector, has already been suggested to explain the lack of genetic polymorphism found in most genes and sequences not subjected to immune pressure in *P. falciparum* (Hartl *et al.*, 2002; Volkman *et al.*, 2001). In fact, the paucity of mixed genotype infections found in individuals from South and Central America coupled with data consistent with a recent population genetic bottleneck in *P. falciparum* has been interpreted to support the clonal propagation theory (Rich *et al.*, 1998). More importantly perhaps is the recent data in high-infectivity regions, where no barriers for mixed-strain infections in the insect vector exist (which should promote genetic sex), that shows inbreeding is actually favored in these high-transmission endemic sites, the genetic basis for this is unclear (Razakandrainibe *et al.*, 2005).

### Meiotic recombination and clonal propagation

Because *Toxoplasma* is such a prevalent pathogen in nature, it is hard to envisage that a small, effective population size or bottleneck has conspired to limit meiotic recombination in the species. Mixed genotype infections in prey have not only been identified in nature (Ajzenberg *et al.*, 2002; Aspinall *et al.*, 2003; Su *et al.*, 2006), they can readily be induced experimentally (Araujo *et al.*, 1997; Dao *et al.*, 2001; Reikvam and Lorentzen-Styr, 1976). Experimental laboratory crosses likewise indicate that, unlike *Plasmodium*, inbreeding is



not favored and no obvious barriers exist for divergent strains to mate and undergo productive meioses (Pfefferkorn and Kasper, 1983; Sibley *et al.*, 1992; Su *et al.*, 2002). Moreover, the existence of naturally occurring “recombinant” genotypes in the *Toxoplasma* population genetic structure clearly demonstrates that sex has occurred in the species (Ferreira Ade *et al.*, 2006; Grigg *et al.*, 2001b; Howe and Sibley, 1995; Howe *et al.*, 1996).

An alternative, conciliatory explanation to the clonal propagation theory might be that meiotic recombination is actually quite an effective force driving strain diversity and produces new lines that can emerge as the dominant “clonotypes” in *Toxoplasma*. This is not blasphemous logic given the high fecundity of its sexual cycle; this parasite is capable of producing  $\sim 10^8$  recombinant progeny from just one mating. In effect, a limited population structure of just three predominant genotypes could represent a recent clonal outbreak of biologically successful lines derived from a mating between genotypically distinct strains. This type of epidemic epidemiology is found in bacteria such as *Neisseria meningitidis*, which is considered a sexual organism because it is easily transformable and undergoes frequent recombination but that, because of periodic epidemics of highly successful clones, is superficially clonal (Smith *et al.*, 1993). To distinguish between such “epidemic clonality,” which pre-supposes a diverse population structure that is diluted by the rapid expansion of a few (or just one) genotypes, from that of clonal propagation, in which lines show a more ancient origin through the accumulation of within-lineage mutations acquired through genetic drift, polymorphism among distinct, largely unlinked loci need only be assessed among *Toxoplasma* lineages. When DNA sequencing was applied against archetypal strains isolated from multiple host species collected over three continents at 18 mostly unlinked polymorphic antigens, a total of just two allelic classes were identified at all loci examined. These two alleles segregated randomly between the three lineages (Grigg *et al.*, 2001a). Validation of this limited, essentially dimorphic, gene pool came with the release of the *Toxoplasma* Expressed Sequence Tag (EST) database derived of sequences from representatives of the three archetypes. This analysis confirmed that the major lineages share a common ancestry and have emerged as a result of meiotic recombination. Thus, for some loci, type I and II strains shared essentially the same allele and type III the other, whereas for other loci, type I and III shared the same allele with type II being different, and so on. In over  $\sim 100$  kb of sequence, only two unique within-lineage polymorphisms have been so far detected for the three clonal types (Grigg *et al.*, 2001a; Su *et al.*, 2003). This remarkable and near total absence of genetic variation suggests that the major types emerged as the dominant strains relatively recently. Su *et al.* (2003) postulated, based on neutral mutation rates calculated for other pathogens, that the three predominant types emerged sometime within the past 10 000 years. The genetic drift calculations obviously support the recent expansion explanation and thereby question the validity of applying the clonal propagation theory to the population biology of *Toxoplasma*. Even highly polymorphic microsatellite loci show remarkably little variation and thus support the recent expansion explanation (Ajzenberg *et al.*, 2002; Blackston *et al.*, 2001; Lehmann *et al.*, 2006).

To emphasize the fact that sexual recombination between two distinct founders is apparently responsible for this emergence, the two inferred ancestries that each possess discrete allelic types have been referred to as A (Adam) and E (Eve). The true origin of these two extant lines and the timing of this expansion will likely require, however, a *Toxoplasma* fossil.

Rarely, atypical genotypes have been isolated that, based on very limited RFLP analyses, appear to possess new, shuffled combinations of the same A and E alleles typically found in the three major lineages (Darde, 1996; Grigg *et al.*, 2001b; Howe *et al.*, 1996). Multi-locus DNA sequencing of these less abundant reassortants has confirmed that many of these genotypes possess A and E alleles, but that the alleles have segregated differently among the loci analyzed (Boyle *et al.*, 2006; Grigg *et al.*, 2001a; Grigg and Suzuki, 2003). These data are consistent with the less abundant and often called “recombinant” genotypes being the less prevalent or less “successful” sibling progeny or cousins of the three major lineages. The fact that the three archetypal lines predominate against a background of other recombinants supports the idea that genetic sex has produced three lines that possess a selective advantage or more potent assortment of the A and E alleles, that confers on these three lineages an altered biologic potential. Su and colleagues (2003) produced evidence to show that the three dominant lineages are more successful because they have acquired an increased capacity for asexual transmission. Furthermore, the inheritance of a particular combination of alleles along chromosome Ia appears to correlate strongly with this orally infectious acquired phenotype (Khan *et al.*, 2006a). Hence, these three “successful” lineages, with their innate ability to be readily transmitted among prey asexually, have rapidly expanded without the need for sexual propagation in the definitive feline host. These results thus provide a striking example of epidemic clonality for a eukaryotic pathogen.

To prove this, the genealogy and number of matings involved in the evolution of the three highly successful lines need only be assessed. A genome-wide SNP analysis recently applied against archetypal strains showed that just two crosses gave rise to the three extant lineages (Boyle *et al.*, 2006). In effect, type I and III strains are offspring from two separate crosses between one ancestry or parent, comprising the type II lineage (or “A” type), with one of two strains derived from the otherwise distinct (or “E” type), ancestral gene pool (Boyle *et al.*, 2006). As just one example of how two ancestral lines might have diverged and then reunited, there could have been Old World and New World strains which drifted since the last ice age and then were given the opportunity to cross after European colonization of the New World (via the massive importation/exportation of animals chronically infected with divergent strains of *Toxoplasma* derived from the two “worlds”). These data are clearly important and suggest that only a few matings are sufficient to dramatically alter the population genetic structure of *Toxoplasma*.

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## Panmixia

To explain the apparent “epidemic” clonality of just three predominant lines, there must exist for any given geography or time isolates that have recombinant genotypes as well as rare strains that possess atypical alleles. Recent analyses of strains isolated from both domestic and remote geographic sites and exotic species (i.e. wild animals from North and South America) have identified a rich abundance of diverse, recombinant, and “exotic” strains in the underlying population genetic structure of *Toxoplasma* (Blackston *et al.*, 2001; Darde, 1996; Dardé *et al.*, 1998; Grigg *et al.*, 2001b; Lehmann *et al.*, 2000). Atypical strains have been identified infecting humans, chickens and wild cats in South America (Ajzenberg *et al.*, 2004; Dubey *et al.*, 2006; Ferreira Ade *et al.*, 2006; Khan *et al.*, 2006b; Lehmann *et al.*, 2006; Su *et al.*, 2006), wild animals throughout North America

(Dubey *et al.*, 2004; Lehmann *et al.*, 2000), as well as a unique set of strains, collectively called type X, infecting marine mammals (Cole *et al.*, 2000; Conrad *et al.*, 2005; Miller *et al.*, 2004). Multi-locus DNA sequencing of these “exotic” or atypical strains infecting humans and wild animals in tropical or remote geographic niches has identified truly novel alleles that are highly polymorphic. Importantly, many of these atypical strains also possessed A and E alleles among the loci examined, except in different, shuffled combinations relative to the archetypes (Ajzenberg *et al.*, 2004; Boyle *et al.*, 2006; Grigg *et al.*, 2001a; Grigg *et al.*, 2001b; Grigg and Suzuki, 2003; Su *et al.*, 2006). The fact that these “exotic” strains possess A and E alleles in addition to unique alleles demonstrates that significant introgression has occurred in the species, such that the underlying population structure for *Toxoplasma* is effectively panmictic. Hence, the relationship of alleles among exotic isolates supports a role for frequent genetic sex and possibly even intragenic recombination in the diversification of these alleles (Grigg *et al.*, 2001a). Collectively, these data support the notion that *Toxoplasma gondii* is a global pathogen that possesses both a complex and simple population genetic structure that is punctuated by the clonal expansion of “successful” lines that emerge as a result of frequent genetic sex in wild cycles or environments where the prevalence of its definitive feline host is high, e.g. in South America.

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## Serotyping and disease

*Toxoplasma* causes a wide spectrum of species-specific diseases ranging from chronic asymptomatic infection to lymphadenopathy, chorioretinitis, ileitis, encephalitis, congenital disease as well as behavioral modifications in a variety of infected hosts. To begin to address whether parasite strain “type” is associated with a particular disease, a serologic strain-typing assay has recently been developed that takes advantage of the fact that polymorphism within the three dominant archetypes (representing > 94% of strains infecting people in North America and Europe) is generally limited to just two alleles that are only ~1–2% polymorphic at the amino acid level for the majority of *Toxoplasma* strains worldwide (Ajioka *et al.*, 1998; Grigg *et al.*, 2001a; Kong *et al.*, 2003). This methodology obviates the necessity to isolate sufficient parasite DNA or actual organisms (which are generally only obtained from symptomatic patients) and instead relies on detecting strain-specific antibodies present in infected animal and human sera. This is possible because every infection with *Toxoplasma* produces a strong anti-*Toxoplasma* antibody response in all otherwise healthy hosts. Although the humoral response is known to cross-react among all strains of *Toxoplasma* and is largely restricted to the surface antigens and secretory proteins, a significant proportion of natural antibodies circulating in infected individuals are, however, restricted to polymorphic epitopes present in the major immunogens. Hence, many antibodies to *Toxoplasma* are strain-specific and clear serotypes exist (Kong *et al.*, 2003). This is borne out by the fact that 5/5 monoclonal antibodies to the immunodominant SAG2A surface antigen isolated after a natural infection react to just one allele-specific epitope (Kong *et al.*, 2003; Parmley *et al.*, 1994). Thus, polymorphic epitopes appear to be highly immunogenic and possibly even immunodominant. This conservation or focus of strong immunity to polymorphic epitopes, coupled with the limited number of alleles possessed by different *T. gondii* strains (in North America and Europe at least), has allowed for the development of diagnostic strain-typing methodologies using polymorphic

peptides to pull out strain-specific antibodies circulating in infected patients to effectively “serotype” the major strains of *T. gondii* known to cause disease in humans and animals globally (Kong *et al.*, 2003; Nowakowska *et al.*, 2006).

The serotyping assay is both highly specific and has been validated using > 40 sera from human patients known to be infected by either type I, II, III or X strains (Kong *et al.*, 2003; Nowakowska *et al.*, 2006; James, Shobab, and Grigg, unpublished results). Using this technique, it is now possible to discriminate infections caused by type I/III from II from X strain infections using just a few microliters of human or animal sera (Figure 13.1). It is also my laboratory’s experience that people infected with unique strains that possess different alleles at the diagnostic GRA6 and GRA7 genes from the type I/III, II or X epitopes generally induce antibodies that either cross-react to produce serologic “signatures” different from standard profiles or don’t react at all. This is presumably the result of infection by a new genotype that induces a different humoral response characterized by no reactivity (e.g. the polymorphic peptides are not recognized in these infected hosts) or because the underlying epitope sequence is different so that a cross-reactive serologic response is detected (e.g. no antibodies exist in infection sera that recognize the type I/III or type II allele-specific polymorphisms in the epitope). These “signatures” are thus diagnostic in their own right and point to the likelihood of infection with an atypical parasite genotype. This assay should provide new tools to address the extent of strain heterogeneity infecting humans and animals in different geographic locations and assess whether particular serotypes are associated with different clinical disease. Ultimately this should provide important information for the establishment of health care guidelines to reduce communities and/or susceptible population groups that are more at risk of exposure to particular diseases caused by *Toxoplasma*.

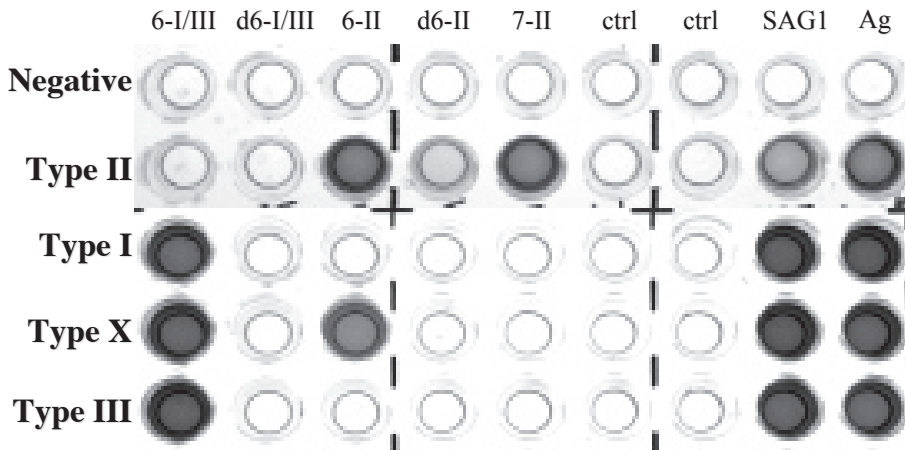
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## Sex and the evolution of virulence

From an evolutionary genetics vantage, sex in eukaryotic pathogens is advantageous by promoting (1) recombination (shuffling alleles into new and different combinations along a chromosome) and (2) segregation (reassortment of hybrid chromosomes during meiosis) to yield progeny harboring variation. In effect, sex profoundly alters genetic associations (Agrawal, 2006). It is this variation that can confer on progeny new biologic potentials for survival, expansion into new ecological niches or to cause new disease. Successful progeny are thus measured by their transmissibility. Put another way, by their ability to colonize a vast array of organisms, to survive host immunity and to remain infectious in order to propagate their life cycle. However, genetic sex is not without its limitations. It can rapidly break down favorable allele combinations brought about through millennia of genetic drift and selection. This can prove disadvantageous for offspring if they are less “fit” than their parents.

The recent pandemic clonality of just three dominant strains in the global population structure of *Toxoplasma* has generated significant debate surrounding the origin of these three lines. To establish whether sexual recombination is, in and of itself, capable of rapidly generating progeny with an increased biological potential (e.g. virulence or oral infectivity), progeny from a single meiotic cross were analyzed to see if “sex” could yield new biologic traits among recombinant progeny of the type that could explain the current day “epidemic

6-I/III: CLHPERVNVFDY  
 6-II: CLHPGSVNEFDF  
 6-X: CLHPERVNEFDF



**Figure 13.1** Detection of strain-specific serologic profiles using polymorphic allele-specific peptides at GRA6 (6-I/III, d6-I/III, 6-II, d6-II) and GRA7 (7-II) to discriminate infection caused by type II from X from I/III strains. Type II strain infections produce allele-specific antibodies restricted to the “E” amino acid in the 6-II peptide epitope and react with the 7-II peptide epitope. Type I/III strain infections produce allele-specific antibodies restricted to the “ER” amino acids in the 6-I/III peptide epitope but do not react with the 7-II peptide epitope. Type X infections produce allele-specific antibodies that react to both the “E” and “ER” residues contained in the type 6-I/III and the 6-II peptide epitopes but do not react with the 7-II peptide epitope. Reactivity at SAG1 and parasite lysate (Ag) indicate the presence of anti-*Toxoplasma* antibody reactivity. Ctrl are irrelevant peptides derived from randomizing the amino acids present in the GRA6 peptide and serve as specificity negative controls.

clonality” of *Toxoplasma*. Taking advantage of a genetic cross performed by Sibley and Pfefferkorn between avirulent type II and type III parents (Sibley *et al.*, 1992), recombinant progeny were assessed for an altered virulence phenotype. A pathogen’s virulence is often an accurate gauge by which to measure the likely success of a given organism. Of the 16 progeny analyzed, 14 were avirulent like their parents, but remarkably, two progeny possessed altered virulence, one of which was three orders of magnitude more virulent (Grigg *et al.*, 2001a). Because these organisms are haploid and neither parent exhibits a virulent phenotype, the increased virulence is not likely the result of an acquisition of some intrinsic virulence gene or “pathogenicity island” as seen for example with bacteria. Instead, the most compelling argument is that virulence behaves as a quantitative trait and the species is drawing on its underlying genetic diversity, in this case two distinct ancestral gene

pools, to reshuffle genetic associations among alleles to promote the punctuated evolution of new biologic potentials. Recent quantitative trait loci (QTL) analysis of this cross supports this view, as several loci appear to contribute to the virulence phenotype (Saeij *et al.*, 2006). However, in contrast to this type II  $\times$  type III cross, QTL analysis of a type I  $\times$  type III cross showed virulence to map effectively as a single major locus with a couple of minor modifying loci (Su *et al.*, 2001). Notably, the mapping data from both crosses identified a major contribution from the same region of chromosome VIIa. Using a candidate gene approach, both the type I  $\times$  type III and type II  $\times$  type III studies identified the same highly polymorphic locus encoding the secreted protein ROP18 (Taylor *et al.*, 2006; Saeij *et al.*, 2006). Transgene studies with either the type I allele or the type II allele incorporated into a type III strain dramatically increased virulence compared to the parental type III strain. Despite the fact that the type I allele transformants increased virulence by 4–5 logs compared to the parental type III, they did not reach the typical type I level of extreme virulence, suggesting other loci influence the phenotype. The convergence of these two independent QTL analyses on a major polymorphic locus demonstrates that the virulence phenotype has a common genetic basis. Perhaps the most important principle illustrated here is that the contribution of a given locus to virulence as a quantitative trait will vary, depending upon the specific combination of alleles at contributing loci that reassort in a cross. Hence the resulting virulence can range across several orders of magnitude. Although the specific function of the proteins encoded by this and other contributing loci remain to be determined, the range of phenotypes observed from allele-specific interactions raise the possibility that secondary hosts other than mice may be the evolutionarily relevant secondary hosts. Other abundant small mammals and birds may be where natural selection produced the ancestral diversity present in the current type strains.

Such punctuated evolution of virulence by recombination or “misis of genes” is commonly exploited by many viruses to rapidly evolve new pathogenic “types,” particularly for the influenza virus and HIV-1 (Robertson *et al.*, 1995; Scholtissek *et al.*, 1979). The fact that reassortment via a sexual cross in a cat can so rapidly evolve new pathogenic parasite strains has striking biological ramifications for the sexual protozoa. Establishing whether protozoal parasites utilize genetic sex to evolve to occupy new niches, or cause more severe disease will no doubt be an important series of experiments to perform given this first example of a virulence “shift” occurring in a non-viral, eukaryotic pathogen.

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## Conclusions

The biological success of the three naturally derived and highly successful, archetypal lineages of *Toxoplasma* shows that, while outbreeding can be infrequent, it has benefited the species tremendously since particularly successful progeny from genetic sex between distinct genotypes have recently come to dominate (Grigg *et al.*, 2001a; Su *et al.*, 2003). Furthermore, the fact that punctuated evolution of new biologic traits such as virulence can be readily mimicked in a cross in the lab suggests that sexual eukaryotes do not necessarily have to evolve by the gradual acquisition of virulence traits or the rapid acquisition of “pathogenicity islands” as seen for instance in bacteria. Instead, reassortment of existing alleles is sufficient to yield progeny with dramatically different properties. What this suggests is that several genes will contribute to quantitative traits like virulence. Genetic sex can



create a new “mix,” where some loci will contribute to the trait more than others, but when alleles are reshuffled, the new genetic associations formed can confer a new phenotypic potential among progeny. This sends an important public health message: understanding genetic variation in a sexual species is tantamount for those species targeted for widespread vaccination. The genetic impact of these types of selection pressures should be an important consideration in the design of future vaccine and therapeutic control strategies to curtail any possibility of selecting for new recombinants that are more virulent or that cause new disease.

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# Manipulating the *Toxoplasma* Genome

14

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## Abstract

The parasitic protozoan *Toxoplasma gondii* has many hallmarks of a robust genetic model system: straightforward continuous culture, a fast generation time, standard codon usage, efficient transient (30%) and stable (1–5%) transfection, and a haploid genome carrying mostly single copy genes which now has been fully sequenced. The parasite's microscopically well-defined subcellular structure also makes it highly suitable for cell biological studies. It is therefore no surprise that *Toxoplasma* has attracted a continuously growing research community. *Toxoplasma* serves as a model system for medically important yet experimentally less or not tractable apicomplexan parasites like *Plasmodium* and *Cryptosporidium*, respectively. Research on *T. gondii* has produced exciting progress in our understanding of how apicomplexan parasites invade and modify cells to suit their needs, how they grow and replicate within these cells, and how they subvert the host's counter measures. Work using the *Toxoplasma* model has also contributed significantly to more general problems in cell biology especially in the areas of motility and organelle evolution and biogenesis. This chapter reviews the genetic approaches and techniques that drive this progress

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## Introduction

Genetic studies on *Toxoplasma* were pioneered in the 1970s by the Pfefferkorn laboratory (Pfefferkorn and Pfefferkorn, 1976b). This work included the development of protocols to reproducibly culture, clone, mutagenize and select parasites in a tissue culture model. Based on these protocols a series of chemically induced mutants were used to map out the parasite's nucleotide biosynthetic pathways (Pfefferkorn and Pfefferkorn, 1976a; Pfefferkorn and Pfefferkorn, 1977a; Pfefferkorn and Pfefferkorn, 1977b; Pfefferkorn and Pfefferkorn, 1978). An excellent review of these early studies is given in (Pfefferkorn, 1988). The genes characterized in this work form the basis of many of the selectable markers used today. These studies were also critical for the establishment of protocols for genetic crosses in the cat (Pfefferkorn and Pfefferkorn, 1980), which have since been used to map virulence factors and to analyze *Toxoplasma* population structure and evolution (see Chapter 13).

*Toxoplasma* cell and molecular biology has experienced a gold rush through the development of reverse genetics. This approach is based on the ability to introduce foreign DNA into the parasites through transfection allowing the genome to be directly manipu-

lated through the introduction of reporter genes, allelic replacements or gene knock-outs. The aim of this chapter is to provide an overview of these transgenic techniques and to indicate how they can mesh with forward genetic approaches to provide a powerful system to identify parasite genes and to unravel their biological function.

### ***Toxoplasma* transfection—getting genes into parasites**

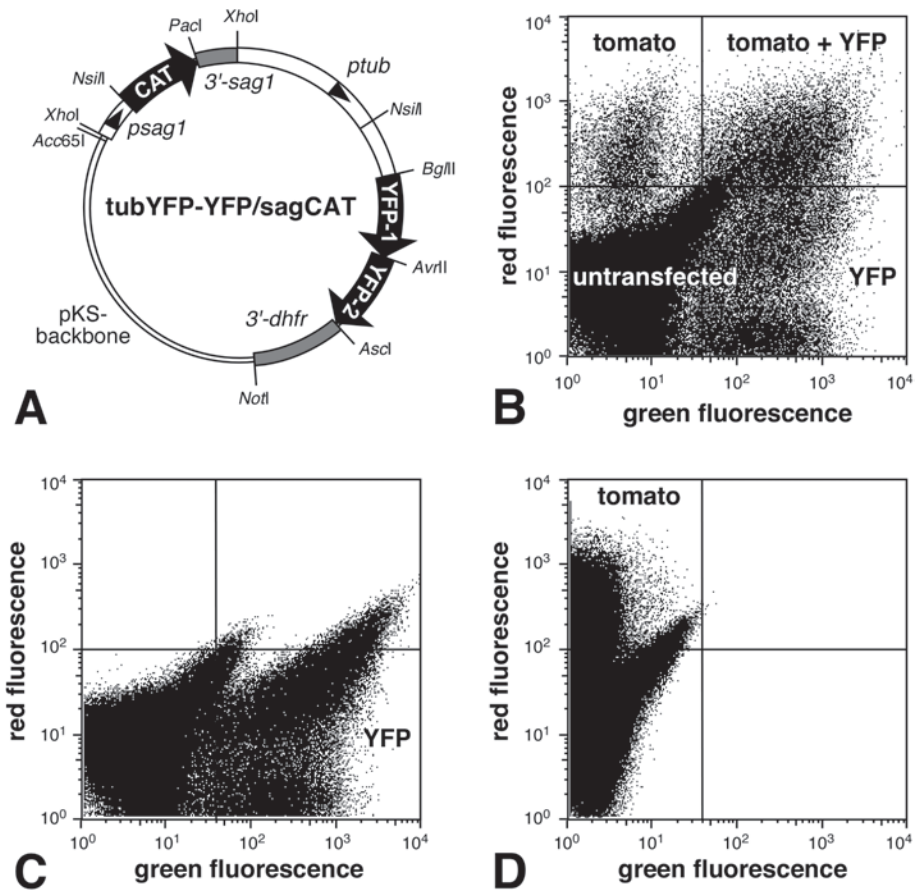
*T. gondii* transfection was established through the combined effort of several laboratories (Donald and Roos, 1993; Kim *et al.*, 1993; Sibley *et al.*, 1994; Soldati and Boothroyd, 1993). The key observations were that plasmid DNA can be introduced into tachyzoites through electroporation and that flanking a marker gene with sequences derived from the 5' and 3' end of a *T. gondii* open reading frame (ORF) will result in expression of this marker (Soldati and Boothroyd, 1993) (Figure 14.1A highlights the features of a *T. gondii* transfection vector). Several selection strategies have been developed to establish continuous and genetically stable transgene expression. In general, stable transformation in *T. gondii* is linked to physical integration of the plasmid DNA into the chromosome. Detailed mapping of numerous insertion sites indicated that plasmid insertion predominantly occurs through non-homologous recombination with no apparent site preference. While episomes can persist for at least a week after transfection they are unstable and, if not integrated, eventually lost. The efficiency of transient (30%) and stable transfection (up to 5%) is remarkably high and has permitted the development of a powerful set of genetic tools.

Transcription in *T. gondii* is monocistronic and depends on promoter elements found in close proximity to the transcription start site. The choice of an appropriate promoter sequence is an important consideration in the construction of transfection vectors and different promoters are discussed in detail below. Sequence features within the mRNA and the translated protein also influence the success of transfection experiments. The translation machinery will not always use the first initiation codon present in the mRNA (e.g.  $\alpha$ -tubulin (Nagel and Boothroyd, 1988)). The majority of *T. gondii* initiation codons is preceded by several A residues with the -3 residue being the most conserved (this rule is not absolute). Protein stability is another major determinant of the level of expression. It has been noticed that lack of expression for certain “problem” transgenes can be overcome by translational fusion to the C-terminus of a well expressed protein like chloramphenicol acetyl transferase (CAT (Darling *et al.*, 1999; Radke and White, 1999; Striepen *et al.*, 1998)). In addition, there is evidence for an N-end rule in *T. gondii* (Matrajt *et al.*, 2002b). According to this model, protein stability is influenced by the amino acid following the initiation methionine. Ala, Glu and Asp were found to be most conducive to a high level of expression.

### **Promoters**

The exact sequence elements controlling gene expression in *T. gondii* have been studied relatively sparsely, although this area is quickly expanding as the genome sequence and transcriptome data become available (Radke *et al.*, 2005). The choice of a specific promoter element will influence both timing and level of transgene expression. In general, promoter activity is contained in a DNA segment of a few hundred base pairs (bp) upstream of





**Figure 14.1** Construction of *T. gondii* transfection vectors. (A) Schematic representation of the modular assembly of the *T. gondii* transfection plasmid *tubYFP-YFP/sagCAT*. The plasmid is built upon the pKS+ backbone (Stratagene). *T. gondii* promoters (*psag1* from SAG1, *ptub* from  $\alpha$ -tubulin) are represented as white bars with a black arrowhead indicating the direction of transcription. Large black arrows indicate open reading frames. Exchanging YFP-1 or YFP-2 with the gene of interest will generate a C- or N-terminal fusion protein, respectively. (B–D) Flow cytometry dot profile of parasites transiently transfected with tandem YFP (C), tandem Tomato (D) or both (B). Single and double expressors can be identified and sorted individually.

the initiation start site and the 5' untranslated region (UTR). Several studies have been undertaken to identify sequence elements controlling basal and/or enhanced expression (Kibe *et al.*, 2005; Nakaar *et al.*, 1998; Soldati and Boothroyd, 1993). A classical TATA box seems absent in the majority of promoters while the genome harbors the genes for a TATA binding protein, as well as other basal eukaryotic transcription factors (Callebaut *et al.*, 2005). Few homologs of proteins known to be involved in enhanced transcription have been identified in sequence similarity searches in different apicomplexans (Meissner and Soldati, 2005; Templeton *et al.*, 2004). This either means apicomplexan transcription

factors are unusual and remain to be discovered or gene regulation is controlled at another level (e.g. through histone modification (Saksouk *et al.*, 2005), post-transcriptionally or a limited set of transcription factors is used in a combinatorial fashion (van Noort and Huynen, 2006).

Constitutive promoters commonly used in *T. gondii* transfection vectors are derived from the TUB1 ( $\alpha$ -tubulin), DHFR (dihydrofolate reductase), ROP1 (rhoptry protein 1) and HXGPRT (hypoxanthine-guanine phosphoribosyl transferase) genes (Donald *et al.*, 1996b; Soldati and Boothroyd, 1993). The activity of promoters from SAG1 (surface antigen 1), ENO1 (enolase 1) and LDH1 (lactate dehydrogenase 1), are restricted to the tachyzoite stage (Ferguson, 2004). Examples of promoters specific for the bradyzoite stage are elements from the BAG1 (bradyzoite antigen 1), HSP70 (heat shock protein 70) and ENO2 (enolase 2) genes. Some of these promoters have been mapped through transfection experiments to identify sequence elements associated with stage specificity (Bohne *et al.*, 1997; Kibe *et al.*, 2005; Ma *et al.*, 2004). In contrast to other protozoan parasite systems, the 3'-UTR of *T. gondii* genes studied to date seems not to play a role in stage dependent gene expression (Bohne *et al.*, 1997). Promoters specific for gametes or sporozoites have not been experimentally characterized so far, although genes specific for these stages have been identified (Radke *et al.*, 2004) or can be deduced from sequence comparisons with *Plasmodium falciparum* genes expressed in these stages.

It has not been formally established whether *Toxoplasma* promoters are differentially regulated throughout the parasite's cell cycle. The lack of synchronous development in culture and the relatively short cell cycle (6–8 hours for the RH strain) compared with the long half-life of most reporters have posed technical obstacles. However, the correlation of developmental timing and gene expression in other apicomplexans make a cell-cycle dependent regulation in *T. gondii* highly likely (Baldi *et al.*, 2000; Bozdech *et al.*, 2003; Brown *et al.*, 2000; Hoane *et al.*, 2003; Vaishnav *et al.*, 2005). This view is further supported by the observation that correct targeting or function of certain genes seems to require the use of the native promoter (e.g. several microneme proteins do not target correctly when not driven by their native promoter (Reiss *et al.*, 2001; Soldati *et al.*, 2001)). Functional expression of the MORN1 protein, which plays a critical role in apicomplexan cell division equally depends on its native promoter (Gubbels *et al.*, 2006), but it has not been established if this is a timing or expression level dependent phenomenon. In any case, cloning the native promoter should be considered if transgenic gene expression results in toxicity or mistargeting.

## Regulatable promoters

A regulatable promoter offers the attractive option of inducing or repressing the expression of a gene at will. This can be especially useful when combined with elimination of the native locus through gene targeting (see below). Currently the most effective approach to regulate expression in *T. gondii* is based on the tetracycline repressor system, which is widely used in a variety of organisms. Derived from the tetracycline resistance operon of *E. coli* (Hillen and Wissmann, 1989), this regulation system has three main components: the Tet repressor protein (TetR), a DNA sequence to which TetR will bind specifically (the Tet operator, TetO) and tetracycline (Tet) as inducer. In the absence of tetracycline, TetR

binds to TetO and sterically interferes with transcription. When added to the medium, tetracycline binds TetR triggering a conformational change resulting in dissociation from the TetO element allowing transcription. This so called “tet on” model has been adapted for *T. gondii* but only yields modest transgene expression (Meissner *et al.*, 2001). Recent modifications to both the TetR (fusion to YFP) and the promoter (four TetOs flanking the initiation site of the RPS13 gene) have resulted in robust expression and relatively tight (88 fold) induction (van Poppel, 2006a; van Poppel, 2006b). This system is responsive to a low inducer concentration permitting the use of the less expensive, yet toxic, tetracycline instead of anhydrotetracycline (ATc).

The “tet off” model is based on the observation that fusion of TetR with a viral transactivator can result in regulatable transactivation (Gossen and Bujard, 1992), in this case presence of Tet will inhibit expression. Meissner and colleagues identified a short peptide sequence which functions as a tet-dependent transactivator (TATi-1) when fused to TetR in a genetic screen (Meissner *et al.*, 2002). A cell line stably expressing TATi-1 has been established and transgenes under the transcriptional control of a minimal Sag1 or Sag4 promoter containing seven TetO elements are robustly expressed and can be effectively downregulated with ATc (Meissner *et al.*, 2002).

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## Heterologous reporter genes as tools for tracing *Toxoplasma* biology

A large and growing number of heterologous reporter transgenes has been adapted for use in *T. gondii*. These genes provide powerful tools to study gene expression, parasite growth and development, protein trafficking, organelle biogenesis and, more recently, host–parasite interactions in mice.

### Enzymatic reporters

Enzymes form the core of heterologous transgenic markers. Their ability to convert numerous substrate molecules per expressed protein molecule enables sensitive detection. This sensitivity can be further boosted through the use of colorimetric, radioisotope or luminescence substrates. Some enzymes can also confer drug resistance or complement auxotrophy and hence be used for selection (see below). Enzymatic markers that have been successfully expressed in *T. gondii* include chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase (LacZ), firefly and *Renilla* luciferase,  $\beta$ -lactamase and alkaline phosphatase. The last two enzymes lack activity within the parasite (likely due to the presence of inhibitors) but can be useful to assay parasite protein secretion (Chaturvedi *et al.*, 1999; Karsten *et al.*, 1997; Karsten *et al.*, 1998).

Enzymatic activity is typically measured in a cell free parasite protein extract. CAT activity is measured by radiolabeled chloramphenicol conversion and can be quantified by thin layer chromatography (Kim *et al.*, 1993; Soldati and Boothroyd, 1993), scintillation counting or using colorimetric assays.  $\beta$ -galactosidase is measured using a colorimetric assay that transforms yellow chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) substrate into a red product using an absorbance spectrophotometer at 570 nm (Seeber and Boothroyd, 1996). Notably this assay can be adapted to multiwell plates for high throughput drug screening (McFadden *et al.*, 1997). CPRG is very potent and stable, and can be used in

live parasites by adding it directly to the well at the outset of the experiment. In this case culture medium without phenol red should be used. Luciferase can be detected with exquisite sensitivity and two luciferase genes (*Renilla* and firefly) have been shown to express in *T. gondii* when fused to CAT (Matrajt *et al.*, 2002b). Since these enzymes use different substrates they can be measured in subsequent assays within the same sample (Michael White, personal communication). This assay can be adapted to multiwell plates (Saeij *et al.*, 2005).

### Fluorescent reporters: glowing and flowing

The expression of autofluorescent proteins can be detected *in vivo* without the addition of substrates. This provides a unique tool to follow biological processes over time and has been particularly useful for microscopy based assays (see (Gubbels and Striepen, 2004) for a review). The first fluorescent protein expressed in *Toxoplasma* was green (GFP) (Kim *et al.*, 2001; Striepen *et al.*, 1998) but now a range of colors is available making the simultaneous use of multiple markers feasible. Cyan (CFP) and yellow fluorescent protein (YFP) are a suitable pair for microscopic analysis and have been used to study *Toxoplasma* organelle biogenesis (Joiner and Roos, 2002; Pelletier *et al.*, 2002; Striepen *et al.*, 2000). A tandem repeat of the YFP gene yields highly fluorescent transgenics which are now widely used to track parasites in tissue culture and in infected animals (Gubbels *et al.*, 2003; Gubbels *et al.*, 2005; Gubbels *et al.*, 2004). Red fluorescent proteins (RFP) further extends the options for labeling. DsRed produces brightly fluorescent parasites (Striepen *et al.*, 2001), however, this protein has to assemble into a tetramer to become fluorescent which can be problematic if the tagged protein is part of a complex or structure. Monomeric mutants of RFP (e.g. mRFP (Campbell *et al.*, 2002)) can help overcome these problems but suffer from considerably weaker fluorescence. The new “cherry” and “tomato” variants (Shaner *et al.*, 2004) provide a reasonable compromise and the tandem tomato marker produces exceptionally bright fluorescence when expressed in *T. gondii* (see Figure 14.1B, GvD and BS, unpublished; Hu *et al.*, 2006).

Parasites expressing fluorescent proteins can also be analyzed and sorted by flow cytometry. Like drug markers, fluorescent proteins can be used as positive or negative selectable markers. To obtain clonal parasite lines stably expressing fluorescent protein, we routinely use two rounds of fluorescence activated cell sorting (FACS) and expansion of sorted parasites in culture (Gubbels *et al.*, 2003). Multiple fluorescent proteins can be used simultaneously however the laser and filter configuration and the need of compensation impose some restrictions (see Figure 14.1B). Finally, fluorescent protein expression can also be detected using a plate reader. This approach can be used to conveniently measure growth of parasites expressing bright YFP-YFP, dsRed or dTomato transgenes. Special plates with black wells and a thin optical bottom and culture medium without phenol red should be used for optimal results (Gubbels *et al.*, 2003).

### Epitope tagging

Epitope tags can be placed at the N- and C-terminus as well as inserted internally. Due to their short length, epitope tags cause less steric hindrance than bulky fluorescent proteins. Epitope tags require fixation and staining with a specific antibody before visualization and

as such cannot be used to study live parasites. However, epitope tags are highly suitable for subcellular and ultrastructural localization, immunoprecipitation experiments or to monitor protein processing during targeting or maturation. A variety of epitope tags have been used successfully in *Toxoplasma* (e.g. cMyc (Delbac *et al.*, 2001), HA (Karsten *et al.*, 1997), FLAG (Sullivan *et al.*, 2005) and Ty-1 (Herm-Gotz *et al.*, 2002)).

### Reporters for tracking parasites in animal models

Several groups have begun to explore the use of reporter parasites to follow the course of infection and toxoplasmosis in the mouse.  $\beta$ -galactosidase and YFP-YFP expressing parasites are detected in tissue sections with excellent sensitivity and specificity (Dao *et al.*, 2002; Egan *et al.*, 2005; Seeber and Boothroyd, 1996). Cells can also be retrieved from infected animals (e.g. from the spleen or lymph nodes) at different stages of the infection for flow cytometric analysis (Gubbels *et al.*, 2005). Alternatively, parasites can be observed in real time in the animal. At the organ level YFP-YFP or tandem Tomato expressing parasites can be followed *in vivo* within the lymph nodes of mice by two-photon microscopy (Chtanova, MJG, GvD, BS and Robey, unpublished). On a slightly larger scale, the dissemination of parasites within an animal has been studied using luciferase-expressing parasites and bioluminescent imaging (Hitziger *et al.*, 2005; Saeij *et al.*, 2005).

Transgenic parasites have also enabled immunological studies, which were previously hampered by the lack of well-defined epitopes for *T. gondii* (Luder and Seeber, 2001). Parasites were engineered to express model antigens like ovalbumin (Gubbels *et al.*, 2005; Pepper *et al.*, 2004) or  $\beta$ -galactosidase (Kwok *et al.*, 2003) for which defined T-cell receptor transgenic mouse strains and reporter assays are available. Interestingly, these studies found that efficient MHC class I and II presentation requires tachyzoite infection of the presenting cell and secretion of the antigen into the parasitophorous vacuole.

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### Selectable markers: generating stable transgenic parasite lines

The isolation of parasite clones carrying a stable transgene or genomic modification requires the ability to select or sort for these relatively rare events against a background of wild type parasites. A straightforward way to achieve this goal is to use a transfection plasmid that harbors two expression cassettes; one with the gene of interest, and a second one with a selectable marker (see Figure 14.1A). The selectable marker is usually a drug resistance gene, but can also be a fluorescent protein or an enzyme which overcomes auxotrophy. Transformation with both circular and linearized plasmid results in plasmid integration and stable transformation. If a high efficiency of transformation is required the DHFR-TS marker can be used as an enhancer (see below). Restriction enzyme mediated integration (REMI) can also significantly enhance transformation efficiency (Black *et al.*, 1995). However, REMI frequently results in multiple plasmids integration, which can complicate subsequent analysis of the transgenics (Gubbels *et al.*, 2004).

#### Chloramphenicol acetyl transferase (CAT)

Selection for parasites expressing the CAT gene requires growth in the presence of 10–20  $\mu$ M chloramphenicol (Kim *et al.*, 1993). Chloramphenicol targets the prokaryotic

translation machinery in the apicoplast resulting in a so-called “slow-death” phenotype (Camps *et al.*, 2002; Fichera and Roos, 1997). Parasites under selection show no significant growth delay in the first and second passage under drug and requires prolonged selection. When expressed from a relatively weak promoter like *sag1*, the use of the CAT marker often selects for multiple transgene insertions which can be exploited to achieve overexpression of a second transgene (Striepen *et al.*, 1998).

#### Hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT)

The *T. gondii* HXGPRT gene can be employed for both positive and negative selection. This strategy requires the use of a parasite mutant in which the native HXGPRT locus has been deleted (Donald *et al.*, 1996a). Such mutants are now available for the RH, Prugnault and PLK strains. HXGPRT deficient mutants depend on the interconversion of AMP into GMP and the rate-limiting enzyme in this pathway is IMPDH (inosine monophosphate dehydrogenase) (Chaudhary *et al.*, 2004). IMPDH can be inhibited by mycophenolic acid treatment. Mycophenolic acid (25 µg/ml) combined with xanthine supplementation (50 µg/ml) will result in mostly single insertions of plasmids carrying a HXGPRT minigene (Donald *et al.*, 1996a).

The pro-drug 6-thioxanthine is converted into a lethal compound by HXGPRT and can be used for selection against this marker. This has been used for marker “recycling” (Brecht *et al.*, 1999), hit-and-run gene targeting (Donald and Roos, 1998) and genetic analysis of stage specific gene expression (Bohne and Roos, 1997; Knoll and Boothroyd, 1998; Matrajt *et al.*, 2002a).

#### Pyrimethamine-resistant dihydrofolate reductase–thymidylate synthase (DHFR-TS)

The drug pyrimethamine interferes with folate synthesis and is used in combination with sulfonamide drugs for the treatment of toxoplasmosis and malaria. Potent pyrimethamine resistance has emerged in *Plasmodium falciparum* through mutations in its target, the DHFR-TS gene (Peterson *et al.*, 1988). The point mutations resulting in drug resistance have been engineered into a *T. gondii* DHFR-TS mini-gene cassette (Donald and Roos, 1993; Reynolds and Roos, 1998). Stable transgenics of mostly single copy insertions can be selected in the presence of 1 µM pyrimethamine. Plasmids carrying a DHFR-TS cassette integrate at a very high frequency (up to 5% stable transfectants). This enhancement does not require expression of the marker but seems to depend on sequence elements in the 5′ flanking region of the gene (Donald and Roos, personal communication). Pyrimethamine resistant parasites should be handled with care as pyrimethamine is used for the treatment of humans.

#### Other drug selectable markers

Additional selectable markers are available which are used less frequently for various technical reasons. The bacterial BLE gene confers phleomycin resistance to *T. gondii* but involves elaborate extracellular parasite treatment for selection (Messina *et al.*, 1995; Soldati *et al.*, 1995). *T. gondii* is a tryptophan auxotroph and is highly sensitive to interferon gamma induced tryptophan starvation (Pfefferkorn, 1984). An elegant complementation



strategy is based on the *E. coli* tryptophan synthase B gene and indole supplementation (Sibley *et al.*, 1994). Like CAT, TrpB selection results in multi-copy integration. UPRT or uridine phosphoribosyltransferase is a gene not encountered in the mammalian host and has been exploited to specifically incorporate radioactive or affinity tagged uracil into the parasite's RNA (Cleary *et al.*, 2005; Pfefferkorn and Pfefferkorn, 1977a). Although this gene cannot be positively selected for, it can be used as a negative selectable marker using the pro-drug 5'-fluoro-2'-deoxyuridine (FUDR). Negative selection requires a UPRT knock-out line (Donald and Roos, 1995). Two other negative selectable markers are found in herpes simplex virus thymidilate kinase (TK: conferring sensitivity to ganciclovir (Fox *et al.*, 2001; Radke and White, 1998)) and bacterial cytosine deaminase (CD: conferring sensitivity to 5-fluorocytosine (Fox *et al.*, 1999)). Fusion of these transgenes with CAT or DHFR-TS generated additional positive-negative selectable markers (Fox *et al.*, 1999; Fox *et al.*, 2001; Radke and White, 1998).

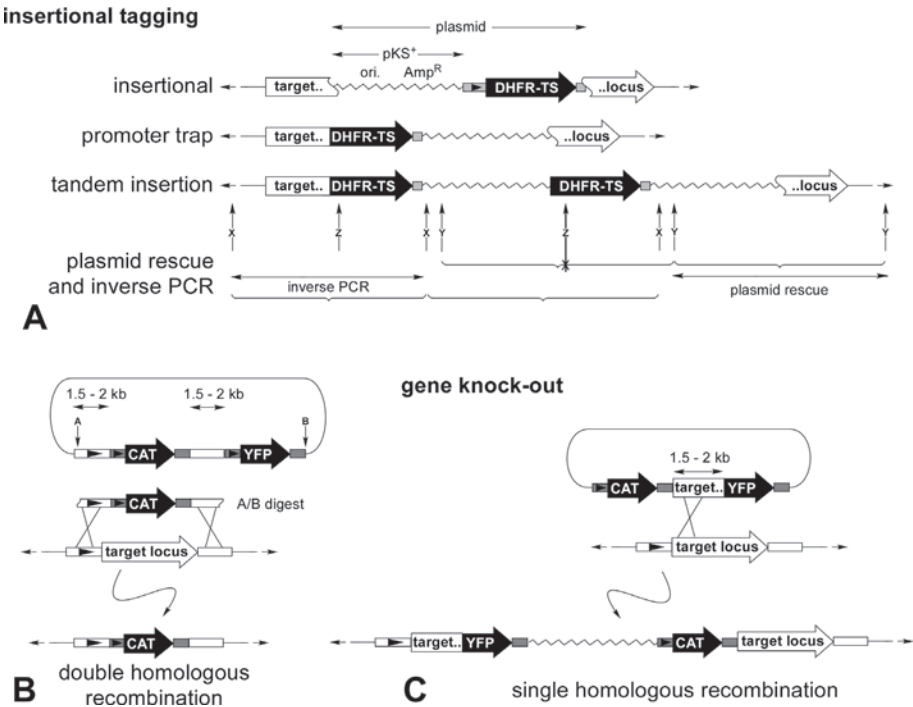
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### **Taking genes out—gene silencing through gene targeting and RNA mediated approaches**

#### Allelic replacement or knock-out through gene targeting

Transfection technology not only enables the introduction of genes but can also be used to remove or alter endogenous genes. Gene targeting or “knock-out” experiments serve as rigorous tests for the involvement of a gene and its product in a given biological process. *T. gondii* is haploid and most proteins are encoded by a single copy gene. Therefore, targeting a single locus is usually sufficient to produce a null mutant. Gene targeting is based on homologous recombination between the altered locus provided by transfection of a plasmid and target locus in the genome. The most widely used procedure aims for a double cross-over event to replace the target. Linearized knock-out constructs usually harbor genomic sequences from the 5' and 3' end of the target gene flanking a selectable marker (see Figure 14.2). Several markers have been used successfully in knock-out constructs. If phenotypic selection against the target gene is available gene targeting is straightforward. However, in the absence of selection, random integration can produce considerable background. The ratio between random integration versus homologous recombination varies widely between different loci and targeting plasmids. While there is anecdotal evidence that the length of the flanking regions can influence the frequency of homologous recombination this has not been firmly established; successful gene replacements were reported with homologous regions ranging from 2–16 kb (Donald and Roos, 1994; Kim *et al.*, 1993; Luo *et al.*, 2005). Introduction of a second negative selectable marker outside of the homologous flanking sequence can be used to counter-select against random integration (e.g. UPRT or YFP (Figure 14.2B) (Mazumdar *et al.*, 2006) or GFP (Gilbert *et al.*, 2007)).

When circular plasmids are used, homologous recombination can occur through single cross-over (Donald and Roos, 1994). A single DNA segment homologous to the genomic region to be targeted is sufficient using this strategy. This will produce a pseudo-diploid target gene locus. Using a positive-negative marker like HXGPRT, one can select for a second recombination event, which “resolves” the pseudo-diploid by excising the marker along with either 5' or 3' sequence. This can be exploited to perform hit-and-run mutagenesis



**Figure 14.2** Exploiting non-homologous insertion and homologous recombination to manipulate the *T. gondii* genome. (A) Schematic representation of insertional genomic tagging using a DHFR-TS plasmid (based on (Roos *et al.*, 1997). Plasmid DNA is indicated on top, genomic insertions below. For insertional mutagenesis expression of the DHFR-TS pyrimethamine resistance gene is driven by its own promoter, the insertion therefore is not necessarily within the open reading frame but might also act through inactivating a regulatory region (e.g. promoter). In case of promoter trapping DHFR-TS does not carry its own promoter, and expression of the resistance gene depends on insertion close to an active promoter, or as an in frame fusion into an expressed gene. Tandem insertions can complicate the identification of the tagged locus by plasmid rescue (using restriction enzyme X) and/or inverse PCR (using restriction enzyme X or Y). However, simultaneously applying restriction enzyme Z, cuts the tandem into two fragments incompatible with plasmid rescue or inverse PCR (Roos *et al.*, 1997; Sullivan *et al.*, 1999). (B) Schematic representation of gene knock-out through double homologous recombination. The homologous regions destined for homologous recombination are represented by white boxes. Restriction enzymes A and B are used to generate fully homologous ends. In this case YFP is used as a negative selectable marker to enrich for homologous recombination (YFP is lost and parasites are FACS negative). (C) Schematic representation of allelic replacement through single homologous recombination. In this strategy a circular plasmid inserts and tags the locus with a YFP fusion (which can be omitted, or replaced by a shortened ORF to create a functional knock-out). The gene-locus 3' of the plasmid backbone is functionally inactivated by the lack of a promoter.

which results in “clean” mutants leaving no plasmid or marker sequences behind (Donald and Roos, 1998).

### Targeting essential genes in a haploid organism

While gene targeting is a powerful tool to analyze gene function it has an obvious limitation in haploid organisms like *T. gondii*—a knock-out of an essential gene will produce a non-viable mutant that cannot be studied. It is not uncommon for *T. gondii* gene targeting experiments to “fail,” i.e. does not result in the isolation of a knock-out line after multiple attempts. This can indicate that the locus is either essential or that the ratio of homologous versus non-homologous recombination was unfavorable for this specific locus and/or targeting plasmid. This question can be resolved by demonstrating that the locus can be targeted successfully if an ectopic copy of the gene is provided as a transgene (e.g. Wichroski and Ward, 2003). However this does not indicate why it is essential. Combining gene targeting with the tetracycline regulatable expression system can now be used to create conditional knock-outs which allow the observation of loss of function mutants for essential genes (Meissner *et al.*, 2002). In this approach, the gene of interest is stably ectopically expressed under control of a tet-off promoter. To differentiate between endogenous and ectopic genes the latter is usually epitope tagged. The endogenous genomic copy is subsequently disrupted using one of the approaches discussed above. In this background, a null phenotype can now be induced by treatment with ATc. Addition of ATc to the drinking water can be used to study the importance of the target gene for virulence in a mouse model. While elaborate and not without pitfalls (e.g. strength or timing of the tet promoter might not be appropriate for the target gene, insufficient regulation can result in leaky expression etc.), this strategy has been successful for a growing number of essential genes including MyosinA (Meissner *et al.*, 2002), AMA1 (Mital *et al.*, 2005) MIC2 (Huynh and Carruthers, 2006) and acyl carrier protein (Mazumdar *et al.*, 2006).

### RNA mediated knock-down

RNA mediated gene silencing through anti-sense and more recently double stranded (ds) RNA has emerged as a powerful alternative to gene targeting in many organisms. These approaches have been explored in *T. gondii* and success has been reported using hammerhead ribozymes and dsRNA (Al-Anouti and Ananvoranich, 2002; Nakaar *et al.*, 2000). Specific suppression of gene expression has been observed after electroporation of *in vitro* transcribed dsRNA and using plasmids in which the target gene was flanked by opposing sag1 promoters for dsRNA production in the parasite. In these experiments the level of target mRNA was reduced to 20% within 3 hours over multiple days (Al-Anouti *et al.*, 2003). The hammerhead ribozyme strategy is based on anti-sense RNA fused to a hammerhead ribozyme to retain the transcript in the nucleus and enhance stability. Nuclear hybridization with the target mRNA prevents translocation into the cytoplasm for translation. So far most experiments have been performed with genes that provide strong negative selection like UPRT and HXGPRT (Al-Anouti *et al.*, 2003; Nakaar *et al.*, 2000; Sheng *et al.*, 2004) but there are also reports on the knock-down of rhoptyr protein ROP2 (Nakaar *et al.*, 2003) and lactate dehydrogenase (Al-Anouti *et al.*, 2004) along with the biological effects of these knock downs. It appears that several laboratories failed in their efforts to establish dsRNA mediated knock-down. The reasons for this are unclear but could be due to technical issues or differences in the susceptibility of individual genes. In contrast to *P. falciparum*, the *T. gondii* genome seems to encode a full set of proteins

with similarity to the RNAi machinery in other organisms (Ullu *et al.*, 2004). Additional work is needed to resolve current difficulties and develop this approach into a robust and generally applicable tool for *T. gondii*.

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## Generating mutants to dissect parasite specific pathways by forward genetics

### Insertional mutagenesis and promoter trapping

Random high frequency integration of a genetic element into the parasite genome can be used to disrupt loci and produce pools of insertional mutants. The main advantage of this approach is that the integrated sequence can be exploited to identify the targeted gene with modest effort. The high frequency of non-homologous insertion of transgenes in *T. gondii* allows the use of simple plasmid constructs the way transposons are used in other systems (Donald *et al.*, 1996b). Several non-essential genes have been identified using random insertion of a DHFR-TS or HXGPRT element (Arrizabalaga *et al.*, 2004; Chiang *et al.*, 1999; Donald and Roos, 1995; Sullivan *et al.*, 1999). The tagged locus in the genome can be identified by plasmid rescue or inverse PCR strategies (see Figure 14.2; Donald *et al.*, 1996b). The insertional strategy is not limited to gene disruption but can also be used to trap promoters and genes. Several bradyzoite specific genes (Bohne and Roos, 1997; Knoll and Boothroyd, 1998) as well as genes controlling differentiation (Matrajt *et al.*, 2002a; Vanchinathan *et al.*, 2005) have been identified using differential HXGPRT selection under culture conditions that favor differentiation to bradyzoites followed by counter-selection under “tachyzoite” conditions.

### Signature-tagged mutagenesis

Signature-tagged mutagenesis is a genetic strategy that has been used to identify genes associated with pathogenesis and persistence in bacterial and fungal systems (see (Autret and Charbit, 2005; Lorenz, 2002) for recent reviews) and has been adapted for *Toxoplasma* (Knoll *et al.*, 2001). Wild-type parasite clones are first tagged with unique oligonucleotide insertions (the signature-tag) to enable differential identification of the clones. These clones are then mutagenized (chemical or insertional) followed by another cloning step. Pools of mutants, which are distinguishable by their tag, are subsequently exposed to a selective condition e.g. infection into a mouse. Mutations/insertions in genes critical in this condition will result in loss of the respective mutant. Such “missing” mutants can be identified by comparing the tags present in pools before and after selection. Several candidate genes playing critical roles in establishing persistent mouse infections genes have been identified using this approach (Knoll, personal communication).

### Conditional mutants to identify genes with essential functions

Genetic analysis of pathways essential for growth in culture requires conditional mutants. Temperature sensitivity (ts) due to chemically induced point mutations can be exploited to obtain strains that are viable at the permissive temperature and display a mutant phenotype at the restrictive temperature. For *Toxoplasma* there are both heat-sensitive (Pfefferkorn and Pfefferkorn, 1976b; Radke *et al.*, 2000) MJG and BS, unpublished) and cold-sensitive

(Uyetake *et al.*, 2001) examples. ENU (N-ethyl-N-nitrosourea) induces random point mutations and has been the mutagen of choice in most *T. gondii* studies. Chemical mutagenesis has been successfully used in *T. gondii* to produce mutants with defects in stage differentiation (Singh *et al.*, 2002), invasion and egress (Black *et al.*, 2000; Uyetake *et al.*, 2001) and cell division and cell-cycle progression (Radke *et al.*, 2000; White *et al.*, 2005).

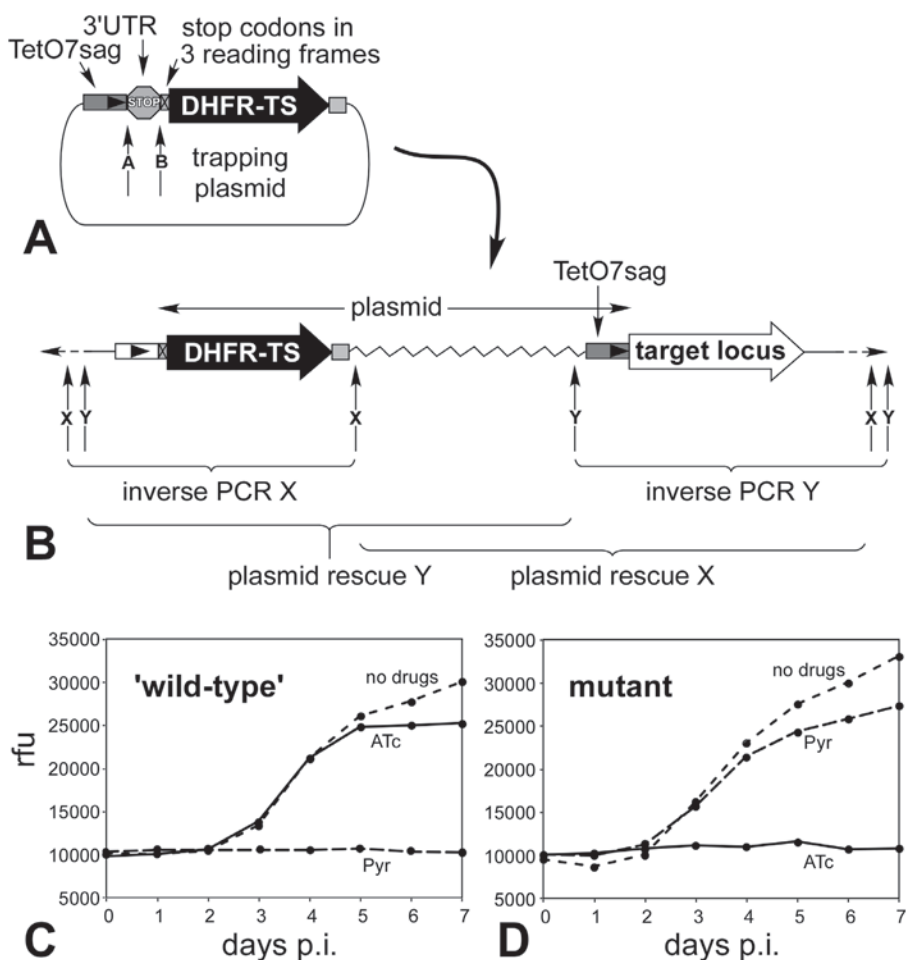
As *T. gondii* is haploid and almost all genes are single copy, insertional mutagenesis strategies to isolate conditional mutants should be feasible. We have explored the possibility to replace endogenous promoters with the tetracycline regulatable promoters discussed above. In the absence of tet the “new” promoter should drive expression of the targeted gene enabling recovery of viable mutants. In a second step a phenotypic screen can be applied in the presence (or absence) of tet to identify defects. This approach is outlined in Figure 14.3 (MJG and BS, unpublished).

### Genetic complementation

While generating chemical mutants is straightforward, identifying the mutated gene responsible for the phenotype is not. The two avenues most widely used to accomplish this goal are physical mapping through crosses, and phenotypic complementation by transfection with a wild type DNA library. While crosses are feasible in *T. gondii*, their limited throughput makes them less practical as a general tool for mutant analysis (also the RH strain used as the molecular biology work horse for *T. gondii* is unable to complete the sexual life cycle). Robust complementation faces two challenges: full representation of the genome (or transcriptome) and efficient recovery of the complementing sequence. Black and colleagues identified a genetic element that maintains stable episomes in *T. gondii* (Black and Boothroyd, 1998) allowing convenient rescue by host lysis and transformation of bacteria. A library harboring an episomal maintenance sequence on the backbone successfully complemented the HXGPRT locus in the knock-out mutant under mycophenolic acid selection. Analysis of the recovered plasmids suggested that they might undergo recombination, potentially decreasing their stability (Black and Boothroyd, 1998).

The second effort to generate a complementation system was built on high frequency integration of library plasmids (Striepen *et al.*, 2002). Genomic inserts are rescued into plasmid by an *in vitro* recombination protocol (Invitrogen Gateway system (Hartley *et al.*, 2000)). Rescued library insert can be shuttled back into a parasite expression plasmid through a second recombination step to confirm complementation capacity. A cDNA library build on this model successfully complemented the *Toxoplasma* HXGPRT locus at high efficiency. Transformation of this library into a *T. gondii* ts cell cycle mutant (Radke *et al.*, 2000) identified a suppressor of this mutant (White *et al.*, 2005). An analogous library carrying *Cryptosporidium parvum* genomic DNA resulted in identification of the *Cryptosporidium* IMPDH gene (Striepen *et al.*, 2002; Umejiego *et al.*, 2004).

Several ts mutants could not be complemented using the cDNA libraries described above (MJG, White and BS unpublished). Genes encoding large mRNAs and/or transcribed at low levels are typically under-represented in cDNA libraries. To overcome these problems we have constructed a large insert (40–50 kb) genomic cosmid library build on a DHFR-TS containing super-cos vector. This library provides sufficient coverage and transformation efficiency to complement the lack of HXGPRT in every transfection reac-



**Figure 14.3** A prototype conditional insertional tagging system (A) Schematic representation of the trapping plasmid used to replace endogenous promoters with Tet conditional promoters (Tet7sag1 or Tet7sag4). Before transfection into TATI parasites, the plasmid is digested with restriction enzymes A and B and gel purified to remove the HXGPRT 3'UTR. The background of DHFR-TS expression from contaminating undigested plasmid is low since the 3'UTR terminates transcription (preliminary experiments identified this as a major source of background). Furthermore, the insertion of DHFR inside an ORF rather than between promoter and startcodon is prevented by the insertion of stop codons in all three frames right before the start codon. The expected insertion site is shown in (B), where the endogenous promoter of a trapped gene drives DHFR-TS expression, and the Tet dependent promoter drives expression of the trapped gene. Fluorescence growth curves are shown for YFP expressing lines of wild-type TATI (C) and a trapped mutant (D) isolated through a replica plating screen. Wild-type growth in the presence of ATc is undisturbed but highly sensitive to pyrimethamine (Pyr). The mutant is pyrimethamine resistant, but sensitive to ATc, suggesting that the tag now controls the expression of a gene essential for growth.



tion attempted. In addition, we recently complemented a mutant with a *ts* cell division defect. A 7 kb complementing region could be identified from overlapping recovered cosmids (MJG and BS, unpublished; cosmid complementation was observed in several additional *ts* mutants, MJG and BS, unpublished; White, personal communication).

Expression cloning to identify the function, developmental regulation, or subcellular localization of gene products

Expression cloning using libraries is not limited to mutant complementation and can be applied to the identification of any gene for which a positive selection protocol can be established. Exploiting the availability of antibody reagents specific for sporozoites, Radke and colleagues recently cloned the gene of *sporoSAG1* by expression cloning (Radke *et al.*, 2004). In similar approaches, fluorescent protein tagging has been used to identify novel genes based on the subcellular localization of their products. Bradley *et al.* employed an insertional element harboring a GFP marker (Bradley *et al.*, 2004) whereas Gubbels *et al.*, constructed a genomic DNA-YFP fusion library (Gubbels *et al.*, 2004). In both approaches cell sorting was used to isolate fluorescent clones. Several interesting new proteins were identified that targeted to the rhoptries (Bradley *et al.*, 2004) and the inner membrane complex (Gubbels and Striepen, 2004; Gubbels *et al.*, 2006).

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## Outlook: digging deeper into the gold mine

While the last decade has seen tremendous progress, many aspects of apicomplexan biology remain poorly understood. *T. gondii* has proven itself as an excellent reverse genetic model and this approach will undoubtedly be critical to further progress. From the standpoint of an experimental biologist the field currently faces two main challenges. The first is to exploit the now completed genome sequence of the parasite most effectively, and the second is to define functions for the many genes and processes unique to Apicomplexa. The genome is obviously a tremendous resource, but the number of "candidate" genes produced by computational screens easily overwhelms the throughput of many of the functional genetic assays currently available. While feasible, gene knock-outs remain time consuming. Protocols that would increase the frequency of homologous over non-homologous recombination could be very helpful to streamline the process. While the use of site-directed recombinases holds some promise they do not solve the problem of targeting the locus in the first place (Brecht *et al.*, 1999). A better understanding of DNA repair mechanisms in *T. gondii* could be helpful (Dendouga *et al.*, 2002). A strategy which has met with some success in other systems is to disrupt the genes encoding the non-homologous recombination pathway (Nayak *et al.*, 2005), or conversely to overexpress (heterologous) elements of the homologous recombination pathway (Shaked *et al.*, 2005). RNA mediated knock-down could serve as an alternative to improved gene targeting, allowing large numbers of genes to be analyzed with minimal effort, making this approach well suited for functional genomics. While promising, RNAi in *T. gondii* currently seems not sufficiently robust for large scale screens (Al-Anouti and Ananvoranich, 2002; Al-Anouti *et al.*, 2003; Sheng *et al.*, 2004).

Forward genetic analysis has equally seen great progress. The reliance on cat infection experiments currently limits the power of genetic crosses. A model for completion

of the entire life cycle in tissue culture would be highly desirable. An avenue to this goal could be improved culture conditions (the use of primary feline cells and/or co-culture with bacterial flora) to “naturally” induce the developmental steps to gametes and oocysts. Alternatively, the machinery that regulates the induction of sexual differentiation could be genetically targeted (in a directed or random fashion) to produce a mutant that can proceed into gametogenesis even in the absence of the correct environmental cues.

While the tools to complement mutants have improved and may now be at a level to permit robust analysis, the ways to generate and select such mutants still lag behind. Screens that select a specific set of mutants in a particular aspect of apicomplexan biology would be powerful tools. Recent “chemical genetics” screens (Carey *et al.*, 2004) show that by exploiting transgenic markers and high throughput robotics this should be an achievable goal. Since the seminal studies on *Toxoplasma* genetics in the 1970s, there has been considerable progress in the development of *T. gondii* as a tractable model system for apicomplexan biology. Nevertheless, digging deeper into the veritable gold mine of *Toxoplasma* biology will require the development of a new generation of tools with sufficient throughput to take full advantage of the genome sequence.

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# Part IV

## **Growth, Development, and Metabolism**

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Michael W. White, Jay R. Radke, Magnolia Conde de Felipe, and Margaret Lehmann

## Abstract

The virulence and developmental competency of *Toxoplasma* vary significantly between strain types (Grigg *et al.*, 2001; Howe and Sibley, 1995; Sibley and Boothroyd, 1992; Sibley and Howe, 1996). The molecular basis for these differences is mostly unknown, but control of the replication cycle, the ability to form tissue cysts, as well as distinct differences in migration or the ability to stimulate the immune response have all been implicated (Saeij *et al.*, 2005). Parasite burden plays a primary role in pathogenesis and may result from fast growth, or indirectly, when parasites fail to switch to the slow growing (or growth arrested) bradyzoite form. Tachyzoites from type I strains proliferate unchecked in mice (until the death of the animal) (Radke and White, 1999; Sibley *et al.*, 2002) and cell culture (5–6 hours doubling) (Jerome *et al.*, 1998) and do not readily form bradyzoites. The growth of type II and III parasites is less vigorous, but these strains typically demonstrate some degree of cell cycle control after infection that always leads to bradyzoite development and cyst formation. These observations link parasite replication to the pathogenesis of disease (Jerome *et al.*, 1998), yet we know few of the molecular details that define this relationship. In this chapter, we will initially summarize the available “tool-kit” (*Development of a “tool-kit” for the study of the Toxoplasma cell cycle*) and reagents developed over the last decade to investigate the *Toxoplasma* cell cycle. We will then discuss the major features of the known tachyzoite cell cycle (*Major phases of the tachyzoite cell cycle*) and describe the order and timing of organelle replication and daughter formation (*Organelle replication and daughter budding*). We then outline the evidence for checkpoint control of chromosomal replication (*Regulation of the parasite cell cycle*), and finally, discuss observations that link cell cycle control to parasite development (*Changes in parasite growth are linked to development*).

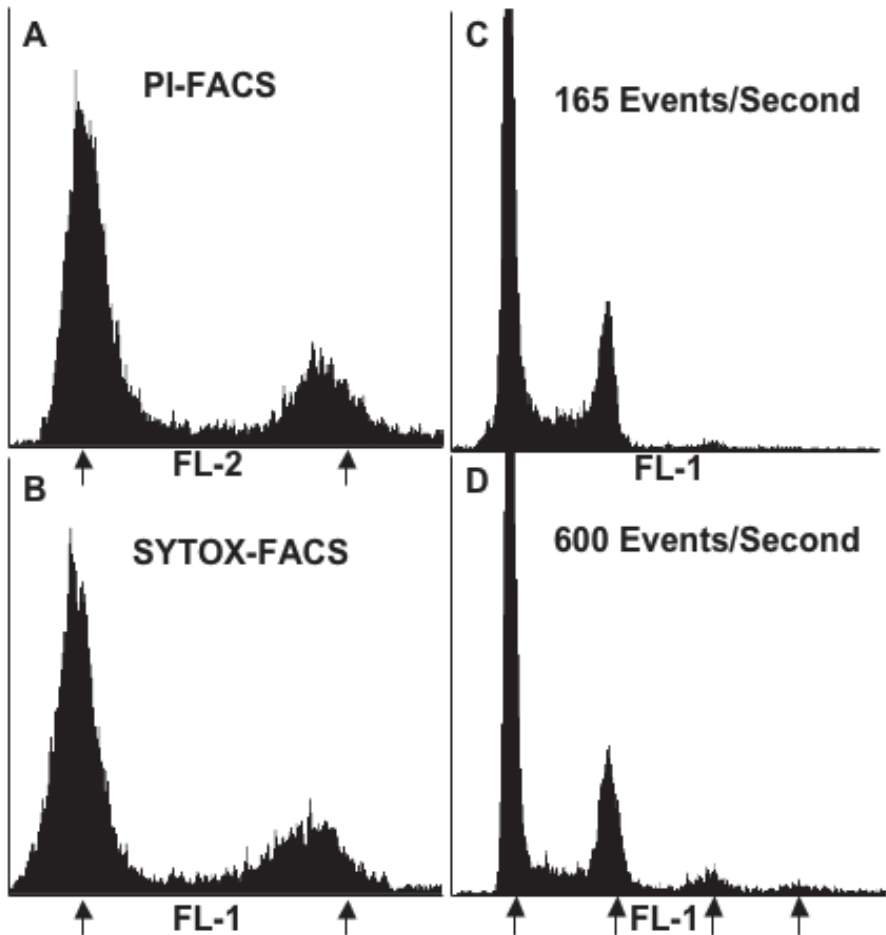
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## Development of a “tool-kit” for the study of the *Toxoplasma* cell cycle

### Assessment of genomic DNA content

Estimates of genome size and DNA content of various *Toxoplasma* developmental stages reported more than two decades ago (Cornelissen *et al.*, 1984) established the essential haploidy of mature asexual stages and the bimodal profiles of proliferating stages. While

these *in situ* methods have now been largely replaced by flow cytometric (FACS) protocols, the results of these early studies have proven remarkably accurate. Standard protocols now in routine use to evaluate nuclear DNA in relation to cell cycle distribution are based on chromosome staining with propidium iodide (PI, 5-bromo-2'-deoxyuridine; basic protocol in Darzynkiewicz *et al.*, 1987). Quantitative analysis of *Toxoplasma* populations using PI-FACS methods have been optimized for appropriate fixation and PI-staining and standard FACS operating protocols originally developed for animal cells have been adjusted to accommodate for the relative small size and reduced nuclear fluorescence associated with parasite preparations (see Figure 15.1 for complete protocols; Radke and White, 1998). Recently, PI has been replaced in yeast protocols with SYTOX-green, a newer generation of DNA stain (Figure 15.1). SYTOX-green exhibits greater sensitivity (up to 1000-fold over PI staining), improved linearity between fluorescence and nuclear DNA content; and has proven less sensitive to dye dilution (higher affinity for DNA) or changes in cell number, size or morphology that limit precise cell cycle phase assignments in yeast ex-





periments that employ PI-fluorescence (Haase and Reed, 2002). A comparison of nuclear DNA fluorescence from asynchronously growing tachyzoite populations stained with PI versus SYTOX-green reveals identical profiles indicating that parasite preparations stained with either dye give equivalent results (Figure 15.1). As noted in the yeast model, we found significant improvements in sensitivity and fluorescence signal stability when using SYTOX-green. The membrane permeant, DNA specific dye, Hoechst 33342 is also an alternative to the PI-stain, and its use in *Toxoplasma* has been recently reported (Hu *et al.*, 2004). Unfortunately, excitation of Hoechst 33342 requires a UV-laser not commonly found on many flow cytometers. Chromosomal DNA content can be correlated to the level of chromatin proteins such as histones (Chabanas *et al.*, 1983) and this approach has been demonstrated in a *Toxoplasma* transgenic clone that was engineered to express the fusion protein histone 2B-YFP (Hu *et al.*, 2004). This method offers the opportunity to sort live parasites based on relative DNA content that is not possible with fixed parasite

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**Figure 15.1** Comparison of PI and SYTOX-green staining of asynchronously growing tachyzoites. (A) Standard analysis for DNA content of ethanol fixed parasite material using PI-staining. Tachyzoite populations are initially identified using forward and side scatter analysis. DNA profiles for PI staining are then analyzed using FL-2 fluorescence and data collected in linear mode in order to accurately define DNA contents. Calibration of the 1N DNA content of the asynchronous populations to average 400 mode fluorescent (first arrow) was used for all sample collections in this analysis. Note the second broad peak in the tachyzoite bimodal DNA distribution ranges from near-diploid to diploid (second arrow indicates 800 mode fluorescence) suggesting that this population is not pure 2N. (B) DNA profile of SYTOX-green stained tachyzoites is virtually identical to PI. DNA analysis followed the protocol in (A) with data collection in FL-1. (C, D) Effect of parasite density on DNA profiles of asynchronously growing tachyzoites. Due to the relative large size of the typical flow chamber (FACS Calibur chamber 20  $\mu\text{m}$   $\times$  64  $\mu\text{m}$  oval) in contrast to the average tachyzoite ( $\sim$ 2  $\mu\text{m}$   $\times$  8  $\mu\text{m}$ ), analysis of samples with increased parasite density leads to spurious populations with apparent incremental ploidy. Examples shown here were analyzed in sequence after calibration of the 1N DNA peak to 200 average mode fluorescence and the histograms shown represent equal collections of 10000 events. (C) The profile in the plot demonstrates that at  $\leq$ 165 (in practice  $<$ 250 events) parasite events per second there are no significant populations containing  $>$ 2N DNA contents, however, (D) as parasite density in the sample increases new populations appear with greater DNA fluorescence that are multiples of 1N and 2N mode fluorescence (four populations  $\sim$ 1–4N are indicated by the arrows). Populations  $>$ 2N have higher forward scatter indicative of a larger particle or multiple parasites (not shown). Methods: The DNA content of purified *Toxoplasma* tachyzoites fixed in 70% (v/v) ethanol/30% 1XPBS (and stored at least overnight but up to a month at  $-20^{\circ}\text{C}$ ). Following fixation, parasites are pelleted by centrifugation to remove ethanol (6500 rpm, 5 min microfuge) and resuspended in freshly made 50  $\mu\text{M}$  Tris pH 7.5 solutions containing either propidium iodide (PI, 0.1 mg/ml final concentration, Sigma-Aldrich #P4864) or SYTOX-green (1  $\mu\text{M}$  SYTOX-green final concentration; Molecular Probes #S7020, Invitrogen Corp., Carlsbad, CA). RNase cocktail (RNase A, RNase T1, Ambion Corp., Austin TX) is added to the staining solutions (250U or 5  $\mu\text{l}$  of the stock to 500  $\mu\text{l}$  of staining solution) and the samples incubated for 30 min at room temperature in the dark. Standard FACS analysis is performed at equal parasite densities corresponding to  $<$ 250 events per second and employ a variety of common flow cytometers such as a FACS-Scan or FACScalibur (Becton-Dickinson, San Jose, CA) equipped with a 488nm argon laser. Fluorescence results (excitation for FL-1 at 515–545nm or FL-2 at 564–606nm) collected in linear mode (10000 events and best resolution at 400 mode calibration for 1N) is quantified using CELLQuest™ v3.0 software and the percentages of G1, S and G2+M parasites are calculated using defined gates for major subpopulations containing haploid (1N), intermediate, and diploid (2N) genome equivalents.

material. It is unclear whether histone 2B-YFP marker fluorescence is precise enough to distinguish DNA contents intermediate between 1 and 2N (Hu *et al.*, 2004) and a lack of correlation in histone levels to DNA content has been noted to occur under some growth conditions in animal cells (Chabanas *et al.*, 1983). Thus, the lack of precision and potential for uncoupling reduces the value of this method for defining the major phases of the *Toxoplasma* cell cycle and the requirement to introduce a stable copy of the histone-YFP marker limits its use as a routine method of genomic DNA assessment.

### Synchronization of parasite replication

Methods to establish synchronous growth in eukaryotic models have employed selective methods that exploit cell cycle differences in cell adhesion to plastic surfaces (mitotic shake-off) or changes in cell density, shape, and size (centrifugal or membrane-based elutriation or sedimentation gradient) that accompany progressive transit through the cell cycle. Metabolic depletion (serum or growth factor starvation) or treatment with growth-inhibitors (e.g. high thymidine, hydroxyurea, aphidicoline, colchicines) have also been utilized in attempts to reversibly block cell growth at specific points in the cell cycle (Hung *et al.*, 1996). The precise synchronization of cells varies significantly among these methods with membrane-based elutriation considered to be the new gold standard for growth synchronization of animal cells (recently reviewed in Cooper and Shedden, 2003). Attempts to synchronize the growth of apicomplexa parasites present unique challenges but also opportunities as cell cycle progression is intertwined with parasite development. For example, initial attempts to synchronize *Plasmodium falciparum* cultures exploited the relative resistance of erythrocytes infected with the ring stage to lysis in D-sorbitol or mannitol solutions (Lambros and Vanderberg, 1979), while other protocols have taken advantage of febrile disruption of parasite development (Haynes and Moch, 2002; Kwiatkowski, 1989). Each of these methods has recently been employed in whole cell microarray analysis of *Plasmodium* growth and development within the animal red cell (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003). Developmental synchrony in *Toxoplasma* has been achieved *in vitro* using native sources of sporozoite and bradyzoite inocula (Jerome *et al.*, 1998; Radke *et al.*, 2003) where differentiation of sporozoite(or bradyzoite)-to-tachyzoite-to-bradyzoite follows a series of synchronous transitions in growth that parallel parasite development in the animal (Jerome *et al.*, 1998; Radke *et al.*, 2003). There has been little success employing growth-inhibitors to synchronize *Toxoplasma* growth as many traditional compounds used to synchronize animal cells are cytotoxic in this parasite (e.g. aphidicoline, hydroxyurea, and colchicines are all cytotoxic). One exception has been the use of exogenous thymidine to reversibly block the endodyogenic growth of tachyzoites engineered to express the herpes simplex virus thymidine kinase (HSTK)—an enzyme these parasites normally lack. Human fibroblast cultures infected with transgenic strain RH<sup>TK+</sup>, carrying multiple copies of the HSTK gene, are arrested at the G1-S boundary following a short treatment with 10  $\mu$ M thymidine (Radke and White, 1998)(synchronization of animal cells typically requires > 1 mM thymidine). Based on changes in genomic DNA content, parasites released from the thymidine block immediately enter S phase, and then transit into G2+M starting at 3 hours post-thymidine release and returning to G1 within 4–6 hours of thymidine removal (Radke and White, 1998). Synchronous progression through the

RH<sup>TK+</sup> cell cycle following thymidine-release was independently confirmed by following the centriole duplication/migration and concerted daughter budding that concludes with a stepwise parasite division in the synchronized population (Radke *et al.*, 2001; White *et al.*, 2005).

### Markers to visualize parasite replication

Development of new fluorescent molecular markers and protocols, including real-time methods, have permitted a greater understanding of the order and timing of organelle and nuclear replication during progression through the tachyzoite cell cycle. Visualization of the newly formed daughter inner membrane complex (Hu *et al.*, 2002) and subpellicular microtubules of the parasite cytoskeleton (Striepen *et al.*, 2000) within viable parasites is now possible as well as real-time microscopic viewing of plastid replication (Striepen *et al.*, 2000), Golgi (Pelletier *et al.*, 2002) and other parasite secretory organelles (Striepen *et al.*, 1998). The *Toxoplasma* cell cycle “tool-kit” includes markers that illuminate key features of the parasite spindle (centrin) (Hartmann *et al.*, 2006) and nucleus (chromatin content, histone 2B; replication foci, TgPCNA1; nucleolar division, TgXPMC2) (Hu *et al.*, 2004; Radke *et al.*, 2001; White *et al.*, 2005). It can be anticipated new reagents will be developed in the coming years that will provide higher resolution of the structural changes occurring during parasite replication.

### Generation of conditional cell cycle mutants

Temperature-sensitive growth mutants have been vital to the functional identification of cell cycle-related molecules in yeast (Hartwell, 1991; Hartwell *et al.*, 1973) and animal cells (Simchen, 1978) and represents the most forward and far-reaching approach to characterize essential regulatory molecules. Temperature sensitive phenotypes that arrest parasite growth at G1, S or M likely result from mutations in molecules that regulate transit to a subsequent phase of the cycle. It is these proteins that impact parasite growth and their regulatory roles are critical to understanding the tachyzoite replicative cycle. The Pfefferkorn laboratory first demonstrated conditional mutants could be generated by chemical mutagenesis in *Toxoplasma* (Pfefferkorn and Pfefferkorn, 1976). The specific defect in these mutants was not determined nor were changes to the parasite cell cycle assessed, although virulence was found to be fully attenuated in two mutants inoculated into albino mice. Following the principles of this early success, three separate mutagenesis panels were screened using criteria established in yeast to isolate cell cycle mutants (Hartwell *et al.*, 1973) where clones were initially characterized based on their time-of-arrest (those arresting within 1–2 divisions selected) and then further classified by morphological criteria (e.g. cell size, percentage of abnormal mitotic and/or cytokinetic forms). Collectively, 40 clones displayed conditional growth at 40°C and 8 of these mutants (20%) were classified as carrying a cell cycle defect (Radke *et al.*, 2000). Several of these mutants had a unimodal 1N DNA distribution (G1) at the non-permissive temperature while one arrested equally into 1N and 2N subpopulations (Radke *et al.*, 2000; White *et al.*, 2005). The number of *ts* cell cycle mutants obtained in this pilot screen was remarkably close to the frequency at which they occur in mutagenized yeast and animal cells (Hartwell *et al.*, 1973; Hirschberg and Marcus, 1982). In collaboration with the Striepen laboratory (U. Georgia), a semi-

automated production technique was applied to the generation of *ts*-mutants (Gubbels *et al.*, 2003) in order to expand the number and potential cell cycle phenotypes represented. Over 40 000 chemical mutant clones have now been screened by replica plate assay with the number of conditional growth and cell cycle mutants isolated exceeding 100 clones (Gubbels, Lehmann, Striepen, and White, unpublished results). Phenotypic characterization of this large panel of mutants and genetic complementation of selected clones is now under way.

### Genetic complementation in *Toxoplasma*

Forward genetics is a classical strategy to identify genes associated with a variety of biochemical events (Eki *et al.*, 1990; Hartwell *et al.*, 1970; Talavera and Basilico, 1977), and thus, devising methods for genetic complementation in *Toxoplasma* was a priority for the field. Efforts in this area have resulted in the development of an episomal-based strategy (Black and Boothroyd, 1998) and a second approach that couples phage recombination (Hartley *et al.*, 2000) with the high frequency of integration in the *Toxoplasma* genome (Donald and Roos, 1993) to mobilize cDNA and genomic fragments integrated into parasite transformants. The later method provides for recovery of inserts directly from the parasite genome into vector *in vitro*, thus streamlining the process of propagation, enrichment, and re-testing of complementing sequences (Striepen *et al.*, 2002). This recombination-based strategy has been used to identify genes conferring drug resistance (Striepen *et al.*, 2002) and genes encoding stage-specific surface antigens (Radke *et al.*, 2004); and complementation of a mitotic cell cycle mutant has been achieved using progressive cycles of temperature selection followed by insert recovery and re-complementation (White *et al.*, 2005). Genetic complementation of *ts11C9* (Radke *et al.*, 2000) identified the *T. gondii* homolog of eukaryotic XPMC2, which is a known suppressor of a mitotic catastrophe (Su and Maller, 1994; Su and Maller, 1995) suggesting that the defect contained in *ts11C9* is associated with mitotic cyclin function. More recently, a genomic-based approach to complementation has been developed that takes advantage of the large insert size afforded by cosmid vectors (see Chapter 14 in this book for details of this model). An RH strain cosmid library constructed with ~40 kbp genomic inserts has shown promise in complementing temperature sensitive mutants that had previously failed rescue attempts using cDNA libraries (Gubbels and Striepen, unpublished data). With the successful production of *ts*-mutants on a large scale in *Toxoplasma* and the development of a variety of genetic methods to identify effected genes in individual mutants, the next few years should see significant strides in deciphering the molecular mechanisms regulating the *Toxoplasma* division cycle.

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### The major features of the *Toxoplasma* cell cycle

Morphological studies that span several decades have identified two major types of asexual division utilized by apicomplexan parasites—a multi-nuclear process called schizogony and modified binary division termed endodyogeny, exemplified by the replication of *Toxoplasma* tachyzoites. Multiple nuclei in schizogony result from repeated rounds of mitosis (e.g. merozoites of *Eimeria callospermophilli*, *T. gondii*, and *Plasmodium falciparum*) (Read *et al.*, 1993; Roberts *et al.*, 1970; Speer and Dubey, 2005) or arise as the result of endopolygeny

(e.g. *Sarcocystis tenella*, *P. berghei*, and *P. lophurae*) (Beaudoin and Strome, 1973; Canning and Sinden, 1973; Speer and Dubey, 1981) wherein a single nucleus, that is presumably polyploid, undergoes mitosis followed by the budding of parasites. *T. gondii* endodyogeny has been examined in detail by electron microscopy (Ogino and Yoneda, 1966; Sheffield and Melton, 1968) to establish the basic framework upon which the order and timing of subcellular events during the tachyzoite cell cycle have now been placed.

### Major phases of the tachyzoite cell cycle

Depending on the *Toxoplasma* development stage, parasites have a population distribution that is either unimodal or bimodal based on genomic DNA content. Unimodal DNA patterns consistent with a haploid genome are characteristic of the asexual end-point stages of the *Toxoplasma* life cycle i.e. sporozoites produced in oocysts shed by the feline definitive host and bradyzoites contained within murine brain tissue cysts (Cornelissen *et al.*, 1984; Radke *et al.*, 2003; Radke and White, 1998). Bimodal patterns that encompass haploid-to-diploid DNA contents are a feature of asynchronous tachyzoite populations undergoing active endodyogeny (Cornelissen *et al.*, 1984; Radke *et al.*, 2003; Radke and White, 1998) and reflect ongoing chromosome replication and mitosis (Radke *et al.*, 2003). Ploidy  $> 2N$  is rarely observed in healthy extracellular tachyzoite populations (Hu *et al.*, 2004) analyzed at appropriate parasite densities by flow cytometry (see section on FACS protocols) indicating that tachyzoite replication is governed by mechanisms that limit division to single trips around the complete cell cycle. Tachyzoites containing more than two nuclei are infrequently observed *in situ*, but importantly, give rise to progeny that undergo the normal binary division (Hu *et al.*, 2004) indicating there is significant regulatory pressure coordinating the parasite cell cycle.

The comparative simplicity of tachyzoite division makes this developmental stage a suitable model for defining the major phases of the cell cycle and where the basic order and timing of nuclear and organelle division may be established. Static and time-lapse microscopic observations of asynchronous tachyzoites, combined with evaluations of synchronous RH<sup>TK+</sup> parasites, has led to the following summary of the tachyzoite cell cycle (see Figure 15.2). *Toxoplasma* tachyzoites divide using a three-phase cycle comprised of a G1 (60%), S (30%) and G2+mitosis (10%) (Cornelissen *et al.*, 1984; Radke and White, 1998). In RH parasites, which double on average every ~6 hours, transit through G1 consumes ~3 hours, S phase ~2 hours, and mitosis/cytokinesis ~1 hour of the division cycle. The length of G1 and S phases are longer in slower growing strains whereas mitosis/cytokinesis appears to be relatively invariant. The length of the S phase period was independently confirmed by [<sup>3</sup>H]-radiolabeling of asynchronously growing RH<sup>TK+</sup> parasites (Radke *et al.*, 2001) and in transgenic parasites expressing TgPCNA1-YFP where the subpopulation containing replication foci and the duration of foci estimated by time-lapse microscopy were found to match the FACS estimates of the length of S (Radke *et al.*, 2001). It is not yet possible to monitor the start of mitosis, although replication foci containing TgPCNA-YFP dissolve only minutes before nuclear division suggesting there is little pause between S and mitosis (Radke *et al.*, 2001)—a finding that parallels similar observations in *Theileria* and other protozoa (Fujishima, 1983; Irvin *et al.*, 1982). Time-lapse microscopy of TgPCNA1-GFP transgenic parasites (Hu *et al.*, 2004; Radke



*et al.*, 2001) reveals that the nuclear division begins within 20 minutes of the dissolution of replication foci; and within ~30 minutes nuclei have resolved into each daughter. A similar timeframe has now been reported in transgenic parasites expressing the histone 2B-YFP marker (Hu *et al.*, 2004).

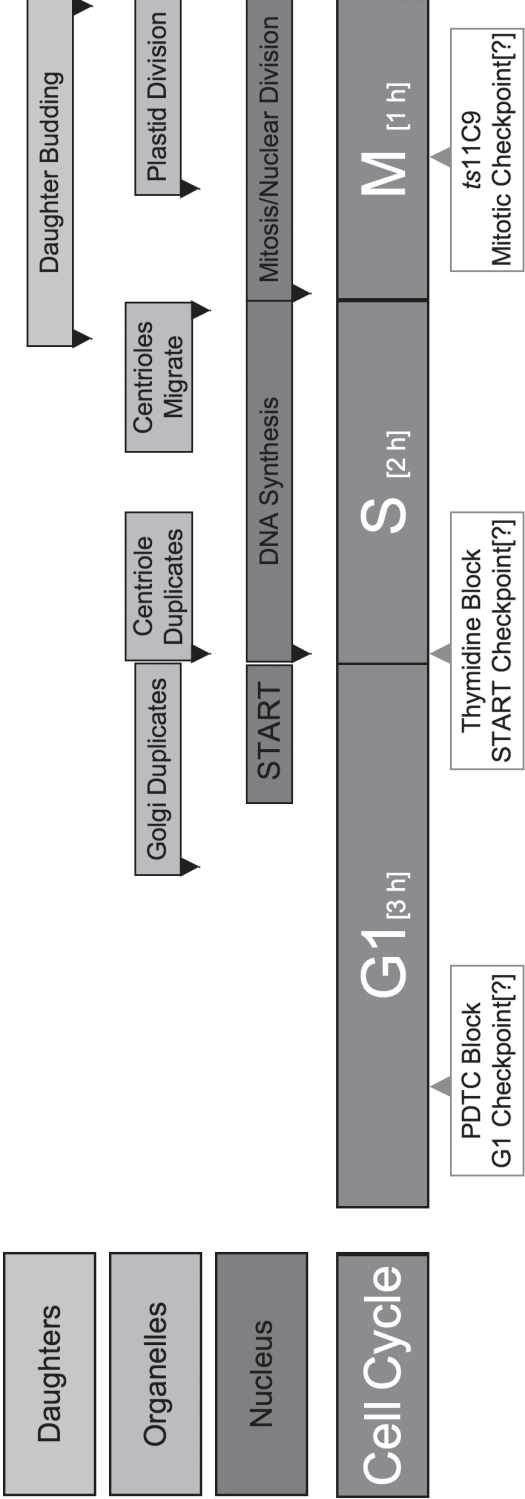
Nuclear fluorescence and FACS to measure S phase length as a function of the total cell cycle demonstrates that the broad second peak observed in a growing tachyzoite population is comprised of both near-diploid late-S and 2N mitotic parasites, and suggests DNA replication may be unequal across S phase. Although these observations remain to be biochemically confirmed, it should be noted that mature gametocytes of *Toxoplasma* and *Plasmodium* do not possess a haploid DNA complement, as expected, but are instead near-diploid (1.7N) (Cornelissen *et al.*, 1984; Janse *et al.*, 1986a; Janse *et al.*, 1986b). *Plasmodium* gametocytes carry the partially duplicated genome into the insect host where it is converted to 2N and then duplicated to 8N prior to becoming a gamete. Similarly, *Toxoplasma* M3 merozoites also exceed a 1N complement of nuclear DNA and are presumed to become diploid before differentiating into gametes in the next host cell (Cornelissen *et al.*, 1984). It is significant that distinct subpopulations containing a 1N-plus DNA content were detected in mature *Toxoplasma* tissue cysts (Cornelissen *et al.*, 1984), and potentially related to this, we have encountered a premitotic, near-diploid population that arises in conjunction with pH-stress or spontaneous bradyzoite differentiation (Radke *et al.*, 2003). Finally, a shift in pattern from 2N to a near-diploid distinguished the DNA profiles of mutant *ts11C9* parasites arrested at 40°C from genetic complements able to grow at this temperature (White *et al.*, 2005) demonstrating that the methods used to measure DNA content in these parasites are capable of resolving near-diploid from true 2N.

### Organelle replication and daughter budding

It has become increasingly clear that the traditional cell cycle viewed from the perspective of G1, S, G2 and M boundaries is overly simplistic (Nasmyth, 1996). The mechanism of chromosome duplication is not strictly an S phase process as replication origins are occupied throughout the cycle and active replication complexes assemble either as cells exit mitosis or in early G1 (Nasmyth, 1996). Commitment to replication occurs at late-G1/S (Murray and Hunt, 1993) but may continue beyond S into G2 or early mitosis (Hansen *et al.*, 1993; Widrow *et al.*, 1998). Chromosome segregation begins with the duplication of the spindle pole body, which in yeast occurs at the start of S (Murray and Hunt, 1993), and it has been reported recently that sister chromatids also form during S (Toth *et al.*, 1999)—long before classical mitosis takes place. Finally, cytokinesis does not always follow the conclusion of mitosis and, in fact, may also begin in S phase (Kron and Gow, 1995). Figure 15.2 summarizes our current knowledge of these processes within the context of the major cell cycle divisions defined for the *Toxoplasma* tachyzoite stage.

The onset of *T. gondii* tachyzoite division is visually manifest by changes in the Golgi and the formation of daughter apical complexes in the mother cell cytoplasm (Gavin *et al.*, 1962; Ogino and Yoneda, 1966; Sheffield and Melton, 1968). In this process, the Golgi is first elongated and then divided, ultimately between each daughter cell. In G1 of the tachyzoite cell cycle, the diameter of the mother cell Golgi ultimately doubles due to lateral cisternal growth (Pelletier *et al.*, 2002), which is coordinate with growth of the ER export





**Figure 15.2** Summary of the major divisions in the tachyzoite cell cycle and the order and timing of organelle replication. Cell cycle timing presented here is based on the RH strain with relative positions of PDTC and thymidine blocks indicated based on DNA profile and time to S phase of synchronous populations. IMC=inner-membrane-complex

site on the nuclear envelope. However, there is no evidence that the ER itself gives rise to growth of the Golgi (Pelletier *et al.*, 2002). Complete division yields two Golgi per daughter cell, which then coalesce to form a single structure (Pelletier *et al.*, 2002).

Studies demonstrate plastid division occurs before or simultaneously with nuclear division (Striepen *et al.*, 2000; White *et al.*, 2005) and is intimately coupled with the mitotic machinery (Striepen *et al.*, 2000). During nuclear division and segregation, the apicoplast is closely associated with the centriole in the apical juxta-nuclear region of parasite cells (Matsuzaki *et al.*, 2001; Striepen *et al.*, 2000) and becomes elongated as the two daughter centrioles migrate to opposite sides of the nucleus. This division is characterized by elongated and then U- and dumb-bell-shaped plastid forms just prior to the appearance of lobulated parasite nuclei (He *et al.*, 2001; Matsuzaki *et al.*, 2001; Striepen *et al.*, 2000). Apicoplast division appears to depend on the force generated by the mitotic spindle and the pellicle of the forming daughter cells. The ends of dividing plastids are inserted into the forming daughters and appear to be attached to one side of the spindle.

Daughter cell budding (cytokinesis) itself appears to initiate in late S phase prior to or in concert with mitotic onset and is closely coordinated with mitotic progression throughout (Hu *et al.*, 2002; Sheffield and Melton, 1968; Striepen *et al.*, 2000). The apical polar ring microtubule-organizing center (MTCO) (Russell and Burns, 1984) directs daughter cell budding, but the spindle pole structure appears to administrate nuclear division (Morrisette and Sibley, 2002a). During endodyogeny, the tachyzoites subpellicular microtubules are not reorganized prior to the division of the nucleus (Sheffield and Melton, 1968). This replication process preserves the tachyzoite infectivity by maintaining a highly polarized organization throughout the cell cycle (Morrisette and Sibley, 2002a). The apical polar ring also contains a centriole-based MTOC, or centrosome (Sheffield and Melton, 1968) that is adjacent during the initial stages of daughter cell formation, or associated with the spindle pole plaques and embedded in the nuclear envelope. The centrosomes also play a role in the assembly of intra-nuclear spindle microtubules (Morrisette and Sibley, 2002a), in the division of membrane-bounded organelles, including the apicoplast (Foth and McFadden, 2003) and in the biogenesis of the Golgi apparatus, as the highly conserved centrosome component centrin localizes near the Golgi during mitosis (Stedman *et al.*, 2003). Each centriole gives rise to one new centriole per cell cycle (Marshall, 2001) that typically forms adjacent to the original. During G1, a single centrosome is located in association with one end of the Golgi stack (Hartmann *et al.*, 2006) and duplication then occurs at the G1/S phase boundary, following division of the Golgi. Duplicated centrosomes are observed throughout S phase and mitosis (G2 is short or non-existent; Radke *et al.*, 2001). Following duplication, centrosomes migrate to the basal end of the nucleus, are separated, and then migrate back to the apical end, adjacent (but opposite) the dividing Golgi (Hartmann *et al.*, 2006). The centrosome migration appears to coordinate and mark mitotic entry and the initiation of daughter cell formation (budding) (White *et al.*, 2005).

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## Regulation of the parasite cell cycle

Models for regulation of the eukaryotic cell cycle are based on a succession of active cyclin/CDK (cyclin dependent kinase) complexes, where accumulation of each successive cyclin leads to activation of its associated kinase. Specifically, each cyclin/CDK represses

its predecessor and stimulates expression of its successor until mitosis and then cytokinesis are concluded (Hunt *et al.*, 1992). Thus, the changes in cellular morphology that once characterized the original cell cycle description(s), now give way to the action of factors on multimeric protein complexes associated with chromosomes and have specific functions in DNA replication or segregation (see reviews, Dalton, 1998; Stillman, 1996; Zachariae, 1999).

### Apicomplexa cyclins/cyclin-dependent kinases

The life cycle of *Toxoplasma* is complex involving two hosts and at least four asexual and two sexual end-point stages that are haploid (sporozoite, tachyzoite, bradyzoite, merozoite and micro- and macro-gametes) and the diploid zygote formed in the feline definitive host. Progression through each developmental stage is likely controlled, in part, by cyclin-dependent kinases (CDK). In human cells, nine CDK proteins are uniquely paired with 11 cyclins to regulate a different cell cycle phase transition (Knockaert *et al.*, 2000). In unicellular eukaryotes, including the protozoa, the number of cyclin/CDK combinations appear more limited but not necessarily less complex (Zhang *et al.*, 1999). For example, a single CDK interacting with different cyclin partners is sufficient to regulate the onset of both S and mitotic phases in the fungi *Schizosaccharomyces pombe* (Nurse and Bissett, 1981). Similar to *S. pombe*, expanded roles for each cyclin/CDK complex may account for relatively few cyclin and CDK protein combinations regulating the complex developmental pathways in Apicomplexa parasites and may shed light on the unusual bimodal cell cycle-arrest of some conditional *Toxoplasma* *ts*-mutants (White *et al.*, 2005). Given the recent progress made in characterizing the cyclin/CDK repertoire in these parasites, we should gain in the coming few years a better understanding of how cyclin/CDKs complexes function in Apicomplexa growth and development.

Complete genome sequence and extensive ESTs generated for a number of Apicomplexa have permitted the identification of parasite homologs of cyclin-dependent kinases (CDKs) and a few cyclins. In comparison to cyclins, CDKs are moderately conserved, and therefore identification of multiple CDKs in genome sequence from *Plasmodium* (reviewed in Anamika *et al.*, 2005; Doerig *et al.*, 2002; Hammarton *et al.*, 2003), *Cryptosporidium* (White and Kvaal, unpublished), *Eimeria* (Kinnaird *et al.*, 2004), *Theileria* (Kinnaird *et al.*, 2001; Kinnaird *et al.*, 1996), and *Toxoplasma* (Wastling and Kinnaird, 1998) has been reasonably straightforward. Eight distinct CDKs and CDK-related genes are now recognized in *Plasmodium* with the parasite expression of six of these confirmed at the mRNA level. By comparison, the *Toxoplasma* genome appears to encode fewer CDK-related genes, yet two *Toxoplasma* CDK-related sequences (CDC2-related AF042172 and CAK AJ534295) have already been identified and one CDK-related protein has been partially characterized (Khan *et al.*, 2002). So far, relatively few cyclin partners have been identified or characterized in Apicomplexan parasites. Three of four *Plasmodium* cyclins with homology to cyclin hours, mitotic cyclins and ania-type cyclins (Pfcyc-1, -3, -4) are able to activate the CDK-PfPK5 *in vitro* (Merckx *et al.*, 2003) and Pfcyc1 shows flexibility in activating a second CDK (Pfmrk). This lack of specificity is incongruent with the exclusive cyclin/CDK pairings that govern cell cycle checkpoints in other eukaryotic cells and suggests that conjecture of cyclin/CDK function in these parasites based on sequence

similarity will likely be unproductive. Because cyclins are weakly conserved, we recently adopted a yeast two-hybrid strategy to isolate these proteins in *Toxoplasma* (Kvaal *et al.*, 2002). From several independent yeast screens, we have identified two divergent cyclins based on their interaction with the *Toxoplasma* CDK-related protein (Khan *et al.*, 2002). TgCYC1 is one of the largest proteins within the cyclin family and contains an unusual repetitive N-terminal domain (Kvaal *et al.*, 2002), whereas TgCYC2 is similar to the A/B family of cyclins (Kvaal and White, unpublished results). Experiments in yeast confirmed cyclin function of TgCYC1 *in vivo*, although the replacement of yeast G1 cyclin (CLN2) in these studies was surprising in view of the similarity of TgCYC1 to cyclin H.

### Evidence for checkpoints in the tachyzoite cell cycle

Gene Ontology defines a cell cycle checkpoint (GO:0000075) as “a point in the eukaryotic cell cycle where progress through the cycle can be halted until conditions are suitable for the cell to proceed to the next stage.” The presence of cell cycle factors in Apicomplexa genomes (e.g. cyclins, CDKs, MAPKs) suggest checkpoints exist in these parasites. However, the unusual replication of these parasites presents unique challenges to our thinking about the cell cycle machinery as the activities of three (or more) microtubule-organizing-complexes (mother and daughter inner-membrane-complexes and mitotic spindle) must be coordinated with chromosome replication and segregation (Morrisette and Sibley, 2002a). Thus, it is commonly assumed that molecular mechanisms regulating cell cycle and developmental transitions in these parasites are novel, yet tachyzoites appear to have mechanisms that act similar to other eukaryotes in regulating G1 progression, S phase entry (START), and chromosome segregation (cyclin B-anaphase promoting complex) as discussed below.

1. G1 checkpoints. The DNA content of end-point development stages in *Toxoplasma* are uniformly haploid, demonstrating that parasites naturally arrest in a G1/G0 state as part of normal developmental progression. G1 arrest is also observed in conditional mutants containing mutations in cell cycle mechanisms suggesting also that G1 checkpoints are active in the proliferating tachyzoite (Radke *et al.*, 2000). This conclusion is supported by recent experiments showing that the G1 cyclin inhibitor (Moon *et al.*, 2004), pyrrolidine dithiocarbamate (PDTC), reversibly blocks the growth of intracellular tachyzoites in G1 (based on a near uniform haploid genome content). Tachyzoites released from a short PDTC block were found to synchronously enter S phase within 3 hours post-drug release demonstrating that PDTC arrests parasites early in G1 (Conde de Felipe, Lehmann, and White, unpublished results).
2. The START checkpoint. The existence of cyclin (Kvaal *et al.*, 2002) and CDC28/cdc2 (Wastling and Kinnaird, 1998) homologs in *Toxoplasma* suggests START checkpoints are active in these parasites. The START checkpoint regulates entry into S phase, and in *Saccharomyces*, START also controls the initiation of spindle formation and budding (cytokinesis) (Hartwell *et al.*, 1974). Arrest at START, which can be caused when signals for DNA synthesis are blocked, prevents all three branches of the cell cycle cascade in yeast (Hartwell *et al.*, 1974; Reed, 1980). The tachyzoite cell cycle also appears to have START, and passage through this checkpoint activates a cascade

that is akin to that in yeast. As discussed above tachyzoites, duplicate their centriole as they enter S phase (Hu *et al.*, 2002; Morrisette and Sibley, 2002b), and before they exit S, and enter mitosis, daughter budding initiates and spindles migrate into position for chromosome segregation (Hu *et al.*, 2002). Treatment of RH<sup>TK+</sup> tachyzoites with excess thymidine (Radke *et al.*, 2001; Radke and White, 1998), which is known to cause dNTP depletion (Terasima and Tolmach, 1963), causes parasites to arrest at the G1/S boundary and in addition blocks centriole duplication (the sentinel event of START activation in yeast) and daughter formation (White *et al.*, 2005). The fact that RH<sup>TK+</sup> parasites synchronously divide upon release from the thymidine block (Radke and White, 1998) provides clear evidence that thymidine in these parasites is acting via a checkpoint control.

Inhibition of parasite growth by the DNA polymerase inhibitor, aphidicoline (Shaw *et al.*, 2001) or hydroxyurea (de Melo *et al.*, 2000), which act similarly to high thymidine in animal cells, are toxic in *Toxoplasma*. Parasites exposed to aphidicoline pass START (as evidenced by multiple centriole duplications (Shaw *et al.*, 2001) and form spindles and initiate daughter budding, but fail to undergo nuclear division causing a fatal outcome. It is unclear whether these results provide evidence for a lack of S phase or DNA damage checkpoints as the authors suggest. It should be noted that we and others have not observed a pause or lengthening of S phase when tachyzoites (and other Apicomplexa parasites) are exposed to conditions that damage chromosomes (Behnke and White, unpublished; Doerig *et al.*, 2000). However, like *Saccharomyces*, *Toxoplasma* tachyzoites may check for damaged DNA when they attach chromosomes to the spindle (DNA damage in yeast results in arrest in mitosis; Rhind and Russell, 1998), owing to the fact that spindles and daughters are regulated by START (Hu *et al.*, 2002; Radke *et al.*, 2001); and these parasites lack a G2 period (Radke *et al.*, 2001) where DNA damage checkpoints would otherwise operate in animal cells (Smits and Medema, 2001). Thus, the phenotype seen with aphidicoline, and other DNA synthesis inhibitors, could be consistent with a DNA damage checkpoint that acts in mitosis. It is not surprising then, that synchronization of tachyzoite growth with these inhibitors has not been successful (de Melo *et al.*, 2000; Shaw *et al.*, 2001).

3. Mitotic checkpoints. Spindle and anaphase checkpoints are conserved widely in lower (Huberman, 1996), as well as higher eukaryotes (Smits and Medema, 2001), and one or both of these checkpoints are likely active in *Toxoplasma* as well as other Apicomplexa. The presence of CDC28/cdc2 and cyclin and putative homologs for some spindle and many anaphase checkpoint proteins in *Toxoplasma* supports this conclusion. *Toxoplasma* homologs to known cell cycle factors have been assembled within Gene Ontology and may be accessed through the following web site; <http://vmbmod10.msu.montana.edu/vmb/white-lab/informatics.htm>. Experimental evidence for mitotic checkpoints is also emerging from studies of a tachyzoite mitotic mutant (*ts11C9*; Radke *et al.*, 2000) and this is likely to expand as additional mutants are characterized. A single mutation in *ts11C9* results in the coordinate arrest of mitosis and cytokinesis (Radke *et al.*, 2000) and this defect is alleviated by factors previously shown to suppress mutations in the mitotic cyclin pathway of yeast and *Xenopus*. At the non-permissive temperature, *ts11C9* parasites maintain an intact

spindle and are capable of organelle segregation but do not continue budding in the absence of nuclear division (White *et al.*, 2005) as has been observed in parasites exposed to microtubule agonists (Morrisette and Sibley, 2002b). Altogether, these data suggest mitotic checkpoints in *Toxoplasma* actively communicate with the developing daughters and mother through a mechanism that requires an intact cytoskeleton. Proof of this model will come from further genetic analysis of mitosis in these parasites, however, there is precedence in higher eukaryotes that factors controlling mitotic checkpoints may act locally (Rieder *et al.*, 1997) and are tethered to the centrosome and spindle microtubules during mitosis (Clute and Pines, 1999).

Classical cell fusion experiments have established a hierarchal order in cell cycle controls with mitosis commonly delayed when one of two nuclei in the heterokaryon cell had not completed DNA replication (Murray and Hunt, 1993). Recent studies that demonstrate multi-nuclear division in the tachyzoite provide a glimpse that cell controls follow a hierarchy in *Toxoplasma* (Hu *et al.*, 2004). Although rare, tachyzoites containing odd nuclear and daughter numbers suggest that unequal re-initiation of DNA replication and nuclear division can occur in tachyzoites that have not completed cytokinesis (resolving the mother) (Hu *et al.*, 2004). Re-initiation appears strictly limited to one additional cycle as parasites containing > 4 nuclei are not observed (Hu *et al.*, 2004). Remarkably, there is equal stoichiometry of daughters and nuclei in these parasites suggesting S phase re-initiation and daughter formation is tightly coupled and daughter maturation is precisely synchronized indicating that the S phase nucleus undergoing re-initiation must prevent the second daughter-nuclear pair from advancing until the second round of DNA replication and mitosis has finished.

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### Changes in parasite growth are linked to development

Mechanisms that control replication are commonly linked with parasite development (Wong, 1996) and control of DNA synthesis and its relation to the developmental program has been described across genera. Differentiation of the *Typanosoma brucei* bloodstream form into procyclic forms (Mutomba and Wang, 1996), *Plasmodium* merozoites into gametocytes (Ono *et al.*, 1993), *Theileria annulata* schizonts into merozoites (Shiels *et al.*, 1997), *Leishmania* amastigotes into promastigotes (Sacks and Perkins, 1984), and *T. gondii* tachyzoites into bradyzoites (Bohne *et al.*, 1994) are all induced to varying degrees by inhibitors of DNA synthesis. In Apicomplexa development, cell cycle control also dictates the size of schizonts across distinct species. *Eimeria bovis* sporozoites invariably produce very large schizonts (> 100 000 first-generation merozoites) (Reduker and Speer, 1987), while sporozoites of *Cryptosporidium parvum* form small schizonts that produce 6–8 merozoites (Fayer, 1997). Importantly, studies of sporozoite-initiated development indicate control of tachyzoite growth is linked to the molecular switch that triggers bradyzoite differentiation (Jerome *et al.*, 1998). Tachyzoites emerging from a sporozoite infection undergo roughly 20 division cycles (7 hours doubling time), but then spontaneously slow (15 hours doubling time) and within 24 hours of this growth shift commence bradyzoite development. Similarly, bradyzoites from mature cysts, like sporozoites, are uniformly 1N (Radke *et al.*, 2003), and without evidence for active mitosis, cytokinesis or DNA synthesis (Ferguson and Hutchison, 1987). These parasites also can differentiate into tachyzoites (recrudesce)



and initiate a tightly controlled succession of growth transitions leading to reformation of the tissue cyst (Dubey, 1998; Jerome *et al.*, 1998; Radke *et al.*, 2003), and thus tachyzoites emerge similarly from sporozoite or bradyzoite infections suggesting that common cell cycle mechanisms may control both branches (oocyst and tissue cyst) of the intermediate life cycle in *Toxoplasma*. In every case described above, changes in the replication cycle appear critical for continued development, and the associated remodeling of nucleoprotein complexes during chromosomal replication may provide the basis for requisite changes in transcriptional patterns that ultimately administrate development (Edgar and McGhee, 1988; Saksouk *et al.*, 2005; Wolffe and Hayes, 1999).

Characterizing cell cycle changes during bradyzoite development demonstrate distinct changes in G2 that correlate with differentiation. In rapidly growing tachyzoite populations, parasites with more than a 1N DNA content are either in S (30%) or mitosis/cytokinesis (10%) indicating little G2 (Radke *et al.*, 2001). In differentiating populations, parasites contain 1.8–2N nuclear DNA content but do not actively synthesize DNA, or engage in mitosis or cytokinesis (< 1%)(Radke *et al.*, 2003). This late-S/G2 population can represent 25% of the total, and co-analysis of tachyzoite (SAG1) and bradyzoite (BAG1) markers with respect to DNA content demonstrate 85% of these parasites express both SAG1 and BAG1. Parasites from *in vivo* tissue cysts do not express SAG1 and are uniformly haploid (Radke *et al.*, 2003), thus expression of both SAG1 and BAG1 in the late-S/G2 suggests an intermediate stage in the developmental pathway that leads to the tissue cyst. This late S/G2 mechanism is not without precedent given that both *Dictyostelium* and *Acanthamoeba* differentiate from late-S/G2 following inhibition of the cell cycle (Jantzen *et al.*, 1988; weeks and Weijer, 1994).

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## Conclusions and prospects for future studies

The importance of growth to *Toxoplasma* pathogenesis is underscored by the obligate intracellular parasitic life style that leads to host cell destruction upon lysis and spread of the organism into new tissues; in the midst of this destruction parasite development unfolds ensuring parasite escape and transmission of the infection. This life style complicates current treatments of this infection in animals that are generally efficacious in blocking the pressing parasite expansion, but are unable to prevent chronic disease due to the unfortunate linkage between anti-proliferation measures that promote development. In order to develop therapeutic strategies that avoid this “catch 22” and approach a true cure, it is imperative that we understand the molecular controls of parasite proliferation and their links to development. In the last decade of Apicomplexa research there has been extraordinary progress in the development of model systems, genomics, and new reagents that are contributing to rapid advances in our understanding of the parasite cell cycle. This progress is likely to continue as long as basic research into these mechanisms continues to receive support. The discovery of important cell cycle factors in the next few years is expected to accelerate studies into the mechanisms that control growth and development of these important parasites. Given the unusual nature of their cell cycles, these factors will provide a new direction for drug development in the Apicomplexa, and cell cycle factors, like cyclin-dependent kinases, already are subject to intensive studies directed at identifying new compounds that target these essential mechanisms of parasite growth (Bhattacharjee *et al.*, 2004; Keenan *et al.*, 2005; Woodard *et al.*, 2003).

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# *Toxoplasma* Gene Regulation and Bradyzoite Development

16

Stanislas Tomavo and Louis M. Weiss

## Abstract

Due to its central importance in disease pathogenesis, the biology of stage differentiation has been an active area of research and this topic is reviewed in the current chapter. Tachyzoites can transform into bradyzoites and visa versa depending on the environmental (i.e. host) conditions. Investigations into bradyzoite biology and this differentiation event have been accelerated by the development of *in vitro* techniques to study and produce bradyzoites as well as by the genetic tools that exist for the manipulation of *T. gondii*. Bradyzoite differentiation is coupled with a slowing of the cell cycle. Differentiation is a programmed response as it proceeds in a reproducible fashion when host cells are infected with sporozoites leading to the formation of tachyzoites and then terminating with the formation of bradyzoites. This programmed response can be altered by changes in the environment. Unfortunately, the genetic triggers and sensors for this differentiation response have yet to be identified. The development of a bradyzoite is a stress mediated differentiation response that leads to metabolic adaptations. Transcription of a whole set of bradyzoite specific genes occurs during differentiation in a coordinated fashion. These gene products include metabolic enzymes, surface antigens, secretory antigens (including rhoptry proteins) and cyst wall components. Many of the features of this differentiation event also have elements reminiscent of epigenetic phenomena described in other systems.

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## Introduction

*Toxoplasma gondii* has three infectious stages: tachyzoites (an asexual reproductive stage), bradyzoites (in tissue cysts, an asexual reproductive stage) and sporozoites (in oocysts, produced by sexual reproduction) (McLeod *et al.*, 1991; Wong and Remington, 1993). Natural infection occurs from either the ingestion of tissue cysts or oocysts. Upon ingestion either bradyzoites or sporozoites can invade the intestinal epithelium and then differentiate into tachyzoites which disseminate throughout the body. While oocysts are produced only in the intestine of definitive hosts (i.e. cats), bradyzoites can develop in both definitive and intermediate hosts. The number of tissue cysts formed in mouse brain appears to be regulated by the class I gene *L<sup>d</sup>* (Brown *et al.*, 1995), with larger numbers of tissue cysts being produced in mice that become mildly ill from infection than in those that become highly symptomatic.

It is hypothesized that in chronic toxoplasmosis, bradyzoites regularly transform to tachyzoites which are either controlled by the immune system or transform back into bradyzoites. Such a dynamic equilibrium between encysted and replicating forms would lead to recurrent antigenic stimulation, possibly accounting for the life-long persistence of antibody titers found in chronically infected hosts (Ferguson *et al.*, 1989; Frenkel and Escajadillo, 1987). New tissue cysts have been demonstrated to be formed in murine models during chronic infection (Ferguson *et al.*, 1994; Gross *et al.*, 1992; McLeod *et al.*, 1991; van der Waaij, 1959) and cyst rupture has been demonstrated in the primate *Aotus lemurinus* (Frenkel and Escajadillo, 1987). The development of *Toxoplasma* encephalitis as well as relapsing chorioretinitis in congenital infection is thought to be a consequence of the transition of bradyzoites in tissue cysts into the active and rapidly replicating tachyzoite stage which is not cleared by the immune deficient or immune tolerant host. Due to its central importance in disease pathogenesis, the biology of stage differentiation has been an active area of research. While progress has been made, bradyzoite to tachyzoite interconversion is not well understood. Many initial seminal observations have been reviewed elsewhere (Weiss and Kim, 2000).

Both tachyzoites and bradyzoites replicate by endodyogeny although bradyzoites often display asynchronous division resulting in an odd number of parasites within a parasitophorous vacuole. The parasitophorous vacuole is modified by stage specific proteins into a stage specific vacuole with its limiting membrane forming the cyst wall of the *T. gondii* tissue cyst. Bradyzoites differ ultrastructurally from tachyzoites in that they have a posteriorly located nucleus, solid rhoptries which are often looped back on themselves, numerous micronemes and polysaccharide (amylopectin) granules (Dubey, 1997; Ferguson and Hutchison, 1987b). Mature tissue cysts in the brain are spherical and 50 to 70  $\mu\text{m}$  in diameter containing approximately 1000 crescent-shaped 7 by 1.5  $\mu\text{m}$  bradyzoites (Dubey *et al.*, 1998). The tissue cyst wall or bradyzoite parasitophorous vacuole membrane is elastic, thin ( $< 0.5 \mu\text{m}$  thick), faintly PAS positive, argyrophilic, phase lucent by phase-contrast microscopy and autofluorescent at 330–385 nm (Dzierszinski *et al.*, 2004; Lei *et al.*, 2005; Sims *et al.*, 1988; Weiss *et al.*, 1995). The cyst wall is lined by granular material and matrix material fills up the space between the bradyzoites particularly in mature cysts (Ferguson and Hutchison, 1987a, b; Sims *et al.*, 1988).

In mature tissue cysts bradyzoites may enter a  $G_0$  stage of the cell cycle becoming non-replicative with a DNA content of 1N (Radke *et al.*, 2003). In mature cysts occasional degenerating bradyzoites are seen (Pavesio *et al.*, 1992) and *in vitro* missegregation and loss of apicoplasts in mature bradyzoites has been demonstrated (Dzierszinski *et al.*, 2004). Tissue cysts can develop in any visceral organ (e.g. lungs, liver and kidneys), but are more common in neural (brain and eyes) or muscle (skeletal and cardiac) tissue (Dubey *et al.*, 1998). In neural tissue, cysts can develop in neurons, astrocytes and microglia. It is not known, however, which cell is the preferred or predominant cell in which cysts form and whether host cells influence cyst formation (Fagard *et al.*, 1999; Ferguson and Hutchison, 1987a,b; Ferguson *et al.*, 1989; Halonen *et al.*, 1996; Halonen *et al.*, 1998a). In astrocytes, the bradyzoite parasitophorous vacuole is surrounded by a layer of host cell intermediate filaments (glial fibrillary acidic protein) that limits the contact of the vacuole with host ER and mitochondria (Halonen *et al.*, 1998b).

## Bradyzoite-specific genes

Cloning of genes encoding stage-specific antigens is of importance for understanding the mechanism of stage conversion between tachyzoites and bradyzoites. Several bradyzoite specific genes related to antigens identified by monoclonal antibodies have been cloned and characterized including: BAG1, MAG1, BSR4 (p36), LDH2 and SAG4A (p18) (Bohne *et al.*, 1995; Knoll and Boothroyd, 1998a; Knoll and Boothroyd, 1998b; Odberg-Ferragut *et al.*, 1996; Parmley *et al.*, 1994; Parmley *et al.*, 1995; Yang and Parmley, 1995, 1997). Random sequencing of cDNA libraries (EST projects) from bradyzoites and tachyzoites has resulted in the identification of about 50 stage specific cDNA sequences that are available at <http://www.cbil.upenn.edu/apidots/> and <http://cmgm.stanford.edu/micro/boothroyd/boothroydlabhome.html>. Genes induced on bradyzoite differentiation or unique to the bradyzoite libraries have also been identified, but the majority of these encode proteins of unknown function (Ajioka, 1998; Ajioka *et al.*, 1998; Manger *et al.*, 1998a). At least 21 bradyzoite-specific genes have also been identified by using a subtractive cDNA library approach (Yahiaoui *et al.*, 1999). Reverse transcriptase PCR confirmed differences in gene expression between bradyzoites and tachyzoites (Roos unpublished, Knoll and Boothroyd, 1998b). A bradyzoite specific rhoptry protein, BRP1, was described which is secreted into the parasitophorous vacuole upon invasion (Schwarz *et al.*, 2005).

Many *T. gondii* surface antigens are members of a gene family with similar structure to SAG1 (i.e. a cysteine rich fold) and a glycolipid (GPI) anchor (Boothroyd *et al.*, 1998; Lekutis *et al.*, 2001; Manger *et al.*, 1998b). Several of these surface antigens appear to be stage specific, i.e. SAG1 SRS1-SRS3, SAG2A and SAG2B in tachyzoites and SAG2C, SAG2D, SAG4A, SAG5A (SAG 5.1) and SRS9 in bradyzoites (Jung *et al.*, 2004; Kim and Boothroyd, 2005). The reason for stage-specific expression of these surface proteins is not known. SAG2C and SAG2D are only detected on *in vivo* bradyzoites not *in vitro* bradyzoites, suggesting they may be expressed later in bradyzoite differentiation and could be markers of cyst maturation (Lekutis *et al.*, 2001).

A defining characteristic of the *T. gondii* tissue cyst, which contains bradyzoites, is the presence of a cyst wall. *Dolichos biflorus* (DBA) bind to the cyst wall and is a useful marker of cyst wall formation *in vitro*. CST1 is a 116 kDa glycoprotein recognized by DBA as well as the monoclonal antibody 73.18 (Weiss *et al.*, 1992; Zhang *et al.*, 2001). It appears to be identical to an antigen recognized by the serum of animals with chronic infection (Smith, 1993; Smith *et al.*, 1996; Zhang and Smith, 1995) and by a rat monoclonal antibody CC2 (Gross *et al.*, 1995). MAG1 was originally identified as a 65 kDa protein expressed in the cyst matrix (Parmley *et al.*, 1994), but it has been demonstrated that MAG1 is also expressed in tachyzoites and secreted into the parasitophorous vacuole, albeit less abundantly than in bradyzoites (Ferguson and Parmley, 2002).

It is worth noting that genes encoding several enzymes involved in glucose metabolism such as glycolysis and polysaccharide storage or amylopectin are also stage-specifically regulated (Yang and Parmley, 1997; Dzierszinski *et al.*, 1999; Coppin *et al.*, 2005). Lactate dehydrogenase (Yang and Parmley, 1997), glucose 6-phosphate isomerase (Dzierszinski *et al.*, 1999) and enolase (Dzierszinski *et al.*, 1999; Dzierszinski *et al.*, 2001) have been shown to be present as two distinct stage-specific isoenzymes, suggesting that these enzymes may have different biochemical properties that are important to parasite growth

in the two distinct stage conditions (Tomavo, 2001). For example, the significance of *T. gondii* lactate dehydrogenases, LDH1 and LDH2 in the control of a metabolic flux during parasite differentiation has been investigated. The expression of the two LDH isoenzymes was knocked down in a stage-specific manner (Al-Anouti *et al.*, 2004). These LDH knock down parasites exhibited variable growth rates in either the tachyzoite or the bradyzoite stage when compared with the wild type parasites, were less virulent in mice and were unable to form tissue cysts in a murine model system (Al-Anouti *et al.*, 2004). Genes involved in the expression of enzymes required for amylopectin degradation such as  $\alpha$ -glucosidase,  $\alpha$ -glucan phosphorylase, R1 protein and  $\alpha$ -amylase are found to be bradyzoite specific while the genes for amylopectin synthesis (iso-amylase, amylopectin synthase and branching enzymes) are expressed in both tachyzoites and bradyzoites (Coppin *et al.*, 2005). This gene expression pattern is consistent with the production of amylopectin during differentiation of tachyzoites into bradyzoites and with the mobilization of the glucose stores during bradyzoite-to-tachyzoite interconversion.

### Gene regulation in *Toxoplasma gondii*

In *Plasmodium falciparum* microarray studies have found that developmental gene expression is tightly regulated so that about 80% of the identified genes have a peak expression occurring within a narrow timeframe that corresponds to one of the various developmental life stages (Bozdech *et al.*, 2003; Hall *et al.*, 2005; Llinas and DeRisi, 2004). Such stage specific coordinate regulation of genes accounts for a large fraction of the *Plasmodium* transcripts and this has been confirmed by proteomic studies (Hall *et al.*, 2005). This has led to the hypothesis of "just in time" mechanisms of mRNA and protein expression for *Plasmodium* (Llinas and DeRisi, 2004). Such extensive data do not yet exist for *T. gondii*, however, cDNA microarrays based on EST data do suggest that similar regulatory mechanisms may be operating in *T. gondii* differentiation. Microarray studies have demonstrated that in *T. gondii*, transcriptional mechanisms with timed expression of clusters of genes is seen in bradyzoite development and that for most genes changes in transcription is tied to changes in protein expression (Ellis *et al.*, 2004; Matrajt *et al.*, 2002; Singh *et al.*, 2002). Further confirmatory data is provided by the Serial Analysis Gene Expression (SAGE) project done with VEG strain *T. gondii* differentiating over a 15 day period *in vitro* from sporozoites to bradyzoites (Jerome *et al.*, 1998). This data is accessible at Toxodb ([www.Toxodb.org](http://www.Toxodb.org)) and at TgSAGEDb (<http://vmbmod.10.msu.edu/vmb/white-lab/newesage.htm>), which supports that transcriptional mechanisms play a key role in regulating the developmental program of this organism.

RNA transcription in most eukaryotes is carried out by RNA polymerase II and its general transcription factors (GTF), which are often conserved throughout evolution (Ranish and Hahn, 1996). In addition, activating transcription factors (ATF) bind to *cis*-acting promoter elements recruiting chromatin remodeling enzymes allowing recruitment of the RNA polymerase II complex (Blazek *et al.*, 2005). It should, however, be appreciated that in other protozoa RNA polymerase I rather than RNA polymerase II has been demonstrated to be involved in transcription, i.e. VSG in *T. brucei* (Belli, 2000). In the Apicomplexa, it appears that gene transcription is similar to other eukaryotes with the genome of *T. gondii* demonstrating the presence of all three RNA polymerases (for a review see Meissner and Soldati,

2005). Homologs for various GTFs (TFIID, TFIIE, TFIIF and TFIIH) and a subunit of Mediator (*mdt6*) have been identified in the *T. gondii* genome (Meissner and Soldati, 2005) as have genes similar to GTFs described in *Plasmodium* (Callebaut *et al.*, 2005). Studies have also demonstrated the presence of chromatin remodeling enzymes in *T. gondii* (Sullivan and Smith, 2000; Sullivan *et al.*, 2003).

Regulatory elements are present upstream of *T. gondii* ORFs as demonstrated by the ability of *SAG1* (tachyzoite specific expression), *ROP1*, and *TUB1* regions to serve as promoters in vector constructs (Kim *et al.*, 1993; Soldati and Boothroyd, 1993) and the presence of bradyzoite specific regulatory regions (Bohne and Roos, 1997; Eaton *et al.*, 2005; Ma *et al.*, 2004). It appears that the 3' UTR of *T. gondii* genes does not play a significant role in controlling developmental gene expression. Functional studies of promoter elements from both *T. gondii* and *P. falciparum* support the view that, similar to other eukaryotes, they have a bipartite organization of basal and *cis*-elements. There have been attempts to map these promoter regions (reviewed in (Meissner and Soldati, 2005) in *GRA* genes, *NTPase*, *DHFR-TS*, *SAG1*, *Enolase 1*, *Enolase 2*, *BAG1*, *hsp70* and *LDH2* (Bohne *et al.*, 1997; Kibe *et al.*, 2005; Ma *et al.*, 2004; Matrajt *et al.*, 2004; Mercier *et al.*, 1996; Nakaar *et al.*, 1998; Roos *et al.*, 1997; Soldati and Boothroyd, 1995). It is evident that the sequence A/TGAGACG is present in both *sag1* and *gra* promoter regions (Mercier *et al.*, 1996) and that the sequence GAGACG in the *NTP1/II* and *DHFR-TS* promoters (Matrajt *et al.*, 2004; Nakaar *et al.*, 1998). In a genome wide analysis of upstream regions of ORFs using MEME the motif GAGACG has been commonly found suggesting this might be similar to the TATA or CAAT box seen in other eukaryotes (Mullapudi N. and Kissinger J., Eighth International Congress on Toxoplasmosis, Porticcio-Corsica, France, abstract 114; 2005).

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## The cell cycle and bradyzoite development

It is probable that bradyzoite differentiation from tachyzoites is a programmed response related to a slowing of replication and lengthening of the cell cycle (Jerome *et al.*, 1998). When sporozoites infect human fibroblasts *in vitro* they transform to rapidly dividing tachyzoites having a doubling time of 6 hours. After 20 divisions, approximately 5 days in culture, these tachyzoites shift to a slower growth rate with a doubling time of 15 hours (Khan *et al.*, 2002; Radke *et al.*, 2001). Bradyzoite differentiation, as defined by the expression of bradyzoite specific antigens, occurs spontaneously when the population shifts to a slower growth rate, but is not seen in the rapidly dividing organisms. This is consistent with observations that spontaneous bradyzoite differentiation occurs less readily in rapidly dividing strains of *T. gondii* such as RH and that stress conditions that slow growth induce bradyzoite differentiation (Bohne *et al.*, 1994; Jerome *et al.*, 1998; Weiss and Kim, 2000). In tachyzoites, the cell cycle is characterized by prominent G1 and S phases with a short G2+M. In tachyzoites, a small proportion of replicating parasites (< 10%) have 1.8 to 2N DNA content (i.e. are in a G2 premitotic state), whereas parasites that express bradyzoite antigens (*BAG1*) are much more likely (~ 50%) to have a G2 premitotic DNA content (Radke *et al.*, 2003)). As *T. gondii* replication slows and bradyzoite development begins there is also an increase in duration of the G1 phase of the cell cycle and bradyzoites from mature cysts *in vivo* have a 1N DNA content suggesting they are in a quiescent G<sub>0</sub> (Radke *et al.*, 2003; Khan *et al.*, 2002; Radke *et al.*, 2001).



Conditions that slow, but do not arrest the cell cycle, result in bradyzoite differentiation, whereas conditions that block cell cycle progression do not result in appreciable differentiation (Fox and Bzik, 2002; Khan *et al.*, 2002). Bradyzoite differentiation cannot be uncoupled from slowing of the cell cycle and may be a stochastic event that occurs at a specific point in the cell cycle when replication has slowed sufficiently. Overall, commitment to bradyzoite differentiation probably occurs at a particular point in the cell cycle and that transit through the cell cycle is required for differentiation. These early “pre-bradyzoites” can continue to replicate, but at some point in the development and maturation of the tissue cyst, the fully mature bradyzoites enter a quiescent G<sub>0</sub> phase.

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### The development of tissue cysts and bradyzoites *in vitro*

Bradyzoite development *in vitro* was reported in the 1960s (Hogan *et al.*, 1960; Matsukayashi and Akao, 1963) and biologic evidence of oocyte production with a bradyzoite prepatent period from cats fed tissue cultures was reported in 1977 (Hoff *et al.*, 1977). Using electron microscopy, cyst-like structures can be seen in tissue culture as early as 72 hours after infection, however by bioassay mature cysts are not present until 6d post infection (Dubey, 1997). Bradyzoite-specific monoclonal antibodies have greatly facilitated studies of bradyzoite development *in vitro* and the recognition of techniques for the induction of differentiation. Recently, parasite lines have also been developed that use GFP under the control of various stage specific promoters (Eaton *et al.*, 2005; Ma *et al.*, 2004). In tissue culture studies, it is evident that bradyzoites spontaneously convert to tachyzoites and that tachyzoites spontaneously convert to bradyzoites (Bohne *et al.*, 1993, 1994; De Champs *et al.*, 1997; Jones *et al.*, 1986; Lane *et al.*, 1996; Lindsay *et al.*, 1991; Lindsay *et al.*, 1993a; Lindsay *et al.*, 1993b; McHugh *et al.*, 1993; Parmley *et al.*, 1995; Popiel *et al.*, 1994; Popiel *et al.*, 1996; Soete *et al.*, 1993; Soete *et al.*, 1994; Soete and Dubremetz, 1996; Weiss *et al.*, 1994).

Stress conditions are associated with an induction of bradyzoite development including: temperature stress (43°C (Soete *et al.*, 1994)), pH stress (pH 6.6–6.8 or 8.0–8.2 (Soete *et al.*, 1994; Soete and Dubremetz, 1996; Weiss *et al.*, 1995)), chemical stress (sodium arsenite (Soete *et al.*, 1994), mitochondrial inhibitors (Bohne *et al.*, 1994; Tomavo and Boothroyd, 1995)), and nutrient stress (arginine starvation (Fox *et al.*, 2004)). Nitric oxide produced by inflammatory cells in response to IFN- $\gamma$  or sodium nitroprusside (SNP, an exogenous NO donor) is an important stimulus for bradyzoite antigen expression (Bohne *et al.*, 1994). Exposure of extracellular tachyzoites to stress conditions (pH 8.1) will result in an increase in bradyzoite differentiation, consistent with a direct effect of stress on the parasite (Weiss *et al.*, 1998; Yahiaoui *et al.*, 1999). The contribution of the host cell to stage conversion remains to be evaluated.

Conversion between tachyzoites and bradyzoites is a rapid event and the commitment to differentiation may be occurring at the time of or shortly after invasion and formation of the parasitophorous vacuole. When bradyzoite differentiation occurs in cell culture following infection with tachyzoites, all of the currently available markers for bradyzoite formation, with the exception of p21 (mAb T84G10), can be detected within 24 hours of infection (Gross *et al.*, 1996; Lane *et al.*, 1996). By bioassay in cats, mature/functional cysts are not formed until at least six days in culture; currently there are no antigenic markers for mature cysts. When cells are infected by bradyzoites from tissue cysts differentiation to tachyzoites



and the appearance of tachyzoite specific antigens (i.e. SAG1) occurs within 15 hours and before cell division had occurred (Soete and Dubremetz, 1996).

### The stress response and bradyzoites

There is a significant body of evidence relating heat shock proteins with differentiation in various phyla (Heikkila, 1993a,b). Bradyzoite differentiation probably shares features common to other stress induced differentiation systems such as glucose starvation and hyphae formation in fungi or spore formation in *Dicyostelium* (Soderbom and Loomis, 1998; Thomason *et al.*, 1999). BAG1 (also known as BAG5 or hsp30) mRNA and protein are upregulated within 24 hours during bradyzoite formation suggesting transcriptional regulation of its expression (Bohne *et al.*, 1995; Parmley *et al.*, 1995). The carboxyl-terminal region of BAG1 has a small heat shock motif most similar to the small heat shock proteins of plants and the near the N-terminus is a synapsin Ia like domain that may be involved in the association of this small heat shock protein with cytoskeletal proteins (actin, tubulin and intermediate filaments) during development (Baines *et al.*, 1995). Four other small heat shock proteins are present in *T. gondii* hsp20, hsp21, and hsp29 are seen in both tachyzoites and bradyzoites and hsp 28 is specific for tachyzoites (de Miguel, 2005); however, none of these other small heat shock proteins is associated with bradyzoite differentiation. A homolog of heat shock protein 70 (hsp70) has been demonstrated by several authors to be induced during both the transition from tachyzoite to bradyzoite and from bradyzoite to tachyzoite (Lyons and Johnson, 1995, 1998; Miller *et al.*, 1999; Silva *et al.*, 1998; Weiss *et al.*, 1998). Hsp90 mRNA and protein levels increase during bradyzoite differentiation (Echeverria *et al.*, 2005) and hsp90 is found in the cytosol of tachyzoites, but in both the nucleus and cytosol of mature bradyzoites. In *T. gondii* mutants that were unable to differentiate, hsp90 was only found in the cytoplasm (Echeverria *et al.*, 2005). A mitogen activated protein kinase (TgMAPK-1) related to human p38, which is a MAPK involved in the stress response, is also increased during bradyzoite formation (Brumlik *et al.*, 2004). It is probable that other components of the traditional stress response pathway(s) are involved in differentiation in *T. gondii*.

The stress response in many eukaryotes is associated with phosphorylation of the alpha subunit of eukaryotic initiation factor-2 (eIF2) which enhances the translational expression of bZIP (basic leucine zipper proteins) proteins such as GCN4 in yeast (Hinnebusch and Natarajan, 2002). The phosphorylation of eIF2 is increased by conditions that induce bradyzoite formation (Sullivan *et al.*, 2004). This phosphorylation is dependent on a novel eIF2 protein kinase (TgIF2K-A) (Sullivan *et al.*, 2004). In *T. gondii*, homologs of other genes involved in this stress response pathway, GCN1 and GCN20, have been identified (Sullivan *et al.*, 2004) as well as the histone acetyltransferase GCN5 (Hettmann and Soldati, 1999; Sullivan and Smith, 2000) and a SWI/SNF homolog (Sullivan *et al.*, 2003). Histone modification is probably a key factor in the differentiation process resulting in bradyzoite formation. In yeast, GCN5 and SWI/SNF are recruited by GCN4 resulting in changes in gene expression as a consequence of stress conditions (Rodrigues-Pousada *et al.*, 2004). In *Dictyostelium*, a GCN2 ortholog is involved in the development of the fruiting body in response to nutrient stress (Fang *et al.*, 2003).

TgSRCAP (*Toxoplasma gondii* Snf2-related CBP activator protein) is a SWI2/SNF2 family chromatin remodeler whose expression increases during cyst development (Nallani and Sullivan, 2005). Using yeast two hybrid analysis TgSRCAP was found to interact with several proteins that could be transcription regulators including TgLZTR (Nallani and Sullivan, 2005). SRCAP in other eukaryotes is also involved in the regulation of CREB (Sullivan *et al.*, 2003). Refer to Chapter 18 on chromatin remodeling for more details.

Analysis of reporter genes driven by the *hsp70* promoter demonstrates that promoter responds to conditions that induce bradyzoite formation (Ma *et al.*, 2004). The pH regulated cis-element of the *hsp70* promoter was mapped to the region –420 through –340 from the initial ATG of the *hsp70* gene (Ma *et al.*, 2004). At –650 bp from the initial ATG the sequence AGAGACG is present which has been described as a *cis*-acting element that acts as an enhancer in the transcription of several *T. gondii* genes (Mercier *et al.*, 1996). There are a series of nGAAn repeats –385 from the initial ATG, which have similarity to the heat shock element (HSE) described in other eukaryotes (Morimoto *et al.*, 1994) and a CCGGGG motif located right next to this HSE is similar to the sp1-*hsp70* site in the human *hsp70* promoter (Morgan, 1989). In addition, the *hsp70* promoter contains several AGGGG or CCCCT regions which are similar to the core region of the STRE (stress response element) described in many eukaryotic genes (Estruch, 2000). Similar STRE and HSE elements are seen in the promoter region of *enolase 1*, a bradyzoite specific isoform of enolase (Kibe *et al.*, 2005). In yeast, enolase is also known as *hsp48*, as it is a stress related heat shock protein (Iida and Yahara, 1985). The STRE-binding activity detected in nuclear extract from stress-induced bradyzoites was significantly higher than that from non-stressed tachyzoites (Kibe *et al.*, 2005). Transcription factors responsible for regulation of *hsp70* and *enolase 1* have not yet been identified although electrophoretic mobility shift assays (EMSA) suggest that there are specific proteins that recognize the STRE and HSE elements of these genes (Kibe *et al.*, 2005; Ma *et al.*, 2004). Although there is an area of similarity between the *BAG1* promoter region and that of *hsp70* promoter region, oligonucleotides from this *BAG1* upstream region do not compete in EMSA (Ma *et al.*, 2004). Stress responses result not only in the induction, but also in the down regulation of many genes. The development of bradyzoites may prove to be the default differentiation pathway for *T. gondii* (Khan *et al.*, 2002; Radke *et al.*, 2001) and turning off of genes that maintain the tachyzoite stage could be the critical early event in differentiation that permits bradyzoite development to go forward.

It is interesting to note that both of the enolases (ENO1 and ENO2) of *T. gondii* are detected in the nucleus of dividing parasites (Ferguson *et al.*, 2002). The nuclear signal of ENO1 in the brain of 12-day-infected mice is detected at an early time point during bradyzoite differentiation before the expression of the bradyzoite surface marker, P36 or SAG4. In addition, the tachyzoite specific ENO2 is exclusively expressed in the dividing sexual forms of *T. gondii* examined in infected cells derived from the cat. It is probable that this novel sub-cellular localization of the enolases is due to their having nuclear functions. In addition, based on the studies of enolase in other organisms, it is likely that ENO1 and ENO2 also have heat shock activity. Similar functions have been described for the enolase of *S. cerevisiae* (Iida and Yahara, 1985). Alternatively, the nuclear functions of *T. gondii* ENO1 and ENO2 may also be similar to that of human cells where the enzyme binds to

the c-Myc promoter and acts as transcriptional repressor in cancer cells (Feo *et al.*, 2000). The nuclear factor isolated from cold-resistant mutants of *Arabidopsis thaliana* using genetic approaches was also identified as an enolase which binds to a DNA motif similar to that described in human cells (Lee *et al.*, 2002). It is, therefore, tempting to postulate that the *T. gondii* enolases might display similar transcriptional functions which are involved in the intracellular growth of this parasite.

Studies of other microorganisms including the fungi and other protozoa suggest that differentiation involves conserved signaling pathways, such as cyclic nucleotides, which are also involved in the response to stress or nutrient starvation. The effect of cyclic nucleotides on bradyzoite differentiation has been assessed using non-metabolized analogues of cAMP and cGMP as well as forskolin (to stimulate a short pulse of cAMP) and demonstrated that cGMP or forskolin induced bradyzoite formation (Eaton *et al.*, 2005; Kirkman *et al.*, 2001). Several kinases potentially involved in differentiation have been cloned including: *T. gondii* PKA1 and PKA2 [Eaton, Tang and Kim, unpublished data], a glycogen synthase kinase (GSK-3) homolog (Qin *et al.*, 1998) and a unique apicomplexan PKG (Donald *et al.*, 2002; Donald and Liberator, 2002; Gurnett *et al.*, 2002; Nare *et al.*, 2002)). It should be appreciated, that the Kinome of the Apicomplexa demonstrates significant divergence between these parasites and their mammalian hosts (Doerig *et al.*, 2005). Protein phosphorylation has proven to be a major mechanism of regulation of gene expression and integration and amplification of extracellular signals, the presence of highly conserved signaling molecules suggests that many of the pathways identified in other eukaryotes are preserved in *T. gondii*.

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## Genetic studies on bradyzoite

Bradyzoite and tachyzoite specific promoter regions have been utilized to create reagents for the study of differentiation *in vitro* (Gross, 1996). Reporter genes such as chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase and luciferase are useful for mapping promoter activity and the definition of minimal promoter sequences (Bohne *et al.*, 1997; Yang and Parmley, 1997; Eaton *et al.*, 2005; Ma *et al.*, 2004). Another reporter gene that has proven useful for the identification of bradyzoite differentiation mutants by FACS is green fluorescent protein (Matrajt *et al.*, 2002; Singh *et al.*, 2002).

Knockout of bradyzoite-specific genes should not be essential as growth could occur in the tachyzoite stage. A *bag1* knockout was generated using HGXPRT as a selectable marker in an HGXPRT<sup>neg</sup> PLK strain of *T. gondii* and another *bag1* knockout was performed using CAT as a selectable marker in a clone of PLK strain that had been passaged through mice to ensure it made cysts at the start of the study (Bohne *et al.*, 1998; Zhang *et al.*, 1999). Cyst formation both *in vitro* and *in vivo* occurred in both knockouts, indicating that BAG1 is not essential for cyst formation. Zhang *et al.*, however, found that the number of cysts formed *in vivo* in CD1 mice was reduced in the *bag1* knockouts and that complementation resulted in the production of similar numbers of cysts *in vivo* as the wild type PLK strain (Zhang *et al.*, 1999). The decrease in cyst formation is a relatively subtle phenotype, which was not observed when BAG1 was disrupted in the HGXPRT<sup>neg</sup> PLK strain background and cyst formation was tested in highly susceptible C57BL/6 mice (Bohne *et al.*, 1998). When parasites were grown in SNP the *bag1* knockout grew faster than PLK suggesting that *bag1*

knockout may affect the efficiency of differentiation from rapidly growing tachyzoites to the slowly growing bradyzoite stages (Zhang *et al.*, 1999). No phenotype was seen when the gene for a bradyzoite specific rho-try protein, BRP1, was knocked out (Schwarz *et al.*, 2005). The capacity to convert from tachyzoite to bradyzoite is a key feature for *T. gondii* persistence in the host, and it is likely that multiple genes with redundant functions are involved in this process.

Promoter trapping has been an effective technique for the identification of genes induced during bradyzoite differentiation. A promoterless hypoxanthine-xanthine-guanine phosphoribosyltransferase (HGXPRT) gene with 6-thioxanthine (6-TX) or 8-azaguanine (8-AzaH) as negative selection and mycophenolic acid with xanthine (MPA-X) as a positive selection has been utilized as a promoter trap by several groups (Bohne *et al.*, 1997; Knoll and Boothroyd, 1998a; Knoll and Boothroyd, 1998b). For this technique, the promoterless HXGRPT construct was randomly integrated into an HGXPRT<sup>neg</sup> PLK strain of *T. gondii*. Recombinants with a tachyzoite-specific or constitutive promoter are killed at pH 7.0 growth conditions in the presence of 6-TX or 8-AzaH (i.e. incorporation of 6-TX or 8-AzaH only occurs in the presence of active HGXPRT) and only recombinants with a bradyzoite-specific HGXPRT can grow at pH 8.0 in the presence of MPA-X (i.e. active HGXPRT in the presence of xanthine bypasses the MPA induced block of IMP dehydrogenase). By repeating these 6-TX and MPA-X selections several times a population of organisms enriched for HGXPRT under the control of bradyzoite specific gene promoters emerges. Using this technique several bradyzoite specific promoters (Donald and Roos, unpublished; cited in (Matrajt *et al.*, 2002)) and eight bradyzoite specific recombinant (BSR) *T. gondii* strains have been obtained (Knoll and Boothroyd, 1998b).

Two additional genetic strategies have been developed to identify mutants which are unable to undergo bradyzoite differentiation (Matrajt *et al.*, 2002; Singh *et al.*, 2002). Singh *et al.* generated point mutants by chemical mutagenesis in a LDH2-GFP Prugnaud (type II) background to obtain mutants with altered ability to transform into bradyzoites (Singh *et al.*, 2002). Parasites unable to differentiate were identified by FACS enrichment of GFP-negative parasites exposed to bradyzoite inducing conditions. Subsequently, this group used an insertional mutagenesis strategy rather than chemical mutagenesis to generate differentiation mutants that were identified by FACS enrichment (Vanchinathan *et al.*, 2005). Matrajt *et al.* also utilized insertional mutagenesis of an engineered stable line expressing a bradyzoite-specific pT7-HGXPRT cassette in UPRT-deficient RH (type I) parasites (Matrajt *et al.*, 2002). Insertional mutagenesis was performed with the DHFR cassettes that earlier were shown to have a high frequency of random integration (Donald and Roos, 1993). Earlier studies had demonstrated that when cultured in a low CO<sub>2</sub> environment this UPRT<sup>neg</sup> strain differentiates into bradyzoites (Bohne and Roos, 1997). The pT7 stable line was obtained using several rounds of negative and positive selection (6-TX for tachyzoite conditions and MPA-X for bradyzoite conditions) resulting in a *T. gondii* strain where HGXPRT was tightly regulated by differentiation conditions.

Both groups demonstrated that the differentiation mutants obtained were unable to make bradyzoites at the same efficiency as the parental strains and had global defects in their expression of previously characterized bradyzoite markers. Due to technical difficulties the exact mutations were not identified. The insertional mutants identified by

Matrajt *et al.* similar to the *bag1* knockout described by Zhang *et al.* (Zhang *et al.*, 1999) had a more rapid growth under bradyzoite inducing conditions than seen in wild-type parasites (Matrajt *et al.*, 2002). Microarray analysis of these mutants identified classes of genes, including a 14-3-3 homolog and a probable vacuolar ATPase, whose expression was decreased in the differentiation mutants (Matrajt *et al.*, 2002; Singh *et al.*, 2002). Vanchinathan *et al.* using an insertional mutagenesis FACS strategy identified two differentiation mutants TBD-5 and TBD-6 that switch to bradyzoites at 10 and 50% of wild-type levels respectively (Vanchinathan *et al.*, 2005). TBD-5 had multiple insertions and was not characterized further, but TBD-6 had a single insertion 164bp upstream of the transcription start site of a gene encoding a zinc finger protein (ZFP1). ZFP1 is targeted to the parasite nucleolus by CCHC motifs and significantly altered expression levels appear to be toxic to *T. gondii*. The phenotype of decreased bradyzoite development could be replicated by directed knockout of the same upstream locus (Vanchinathan *et al.*, 2005).

## Summary

Despite the identification of many bradyzoite specific genes as well as *T. gondii* mutants that are compromised in their ability to undergo differentiation, a unified model for bradyzoite differentiation has not yet been developed. Several themes, however, have emerged from the available data: (1) tachyzoites and bradyzoites express related genes encoding structural homologs in a mutually exclusive way; (2) metabolic genes that are stage specific exist suggesting these stages are metabolically distinct; (3) stress related differentiation pathways and stress proteins are associated with these stage transitions; and (4) it is likely that coordinate regulation of a developmental program exists and some of the control elements of this program involve chromatin remodeling. Investigations into bradyzoite biology and the differentiation of tachyzoites into bradyzoites have been accelerated by the development of *in vitro* techniques to study and produce bradyzoites as well as by the genetic tools that exist for the manipulation of *T. gondii*. Unfortunately, the genetic triggers and sensors for the differentiation response have yet to be identified. Bradyzoite differentiation may be the default pathway and that any situation that perturbs positive signals necessary for tachyzoite replication results in the formation of bradyzoites.

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## Abstract

Formation and maintenance of the bradyzoite cyst is essential for the lifecycle of *Toxoplasma gondii*. To understand this critical stage, many aspects of bradyzoite biology must be examined. In this chapter, we consider how the bradyzoite adapts to an encysted lifestyle by discussing its morphology and metabolism. We also explore bradyzoite tissue cyst development *in vivo* by analyzing different parasite strains, mouse models, the influence of host genetic background, and bradyzoite cyst rupture with parasite reactivation. Finally, we consider the long-term survival of cysts and the relationship between bradyzoite biology and host immunity. This discussion includes tissue tropism, the SRS family of surface antigens, and immune recognition or diversion. While the bradyzoite stage is challenging to study, a wealth of fascinating information will be learned as we dissect out its secrets.

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## Introduction

In order to guarantee parasite transmission, many parasites differentiate into resistant forms to carry on from one life cycle to another, or between hosts (examples include *Giardia* and *Entamoeba* cysts, *Schistosoma* and *Ascaris* eggs, *Cryptosporidium* and *Cyclospora* oocysts, *Trypanosoma cruzi* trypomastigotes). This chapter discusses the biology of the encysted form of *T. gondii* asexual cycle (described in Chapter 1), called the bradyzoite. The triggering of tachyzoite to bradyzoite differentiation, as well as the regulation of gene expression, are described in Chapter 16. The bradyzoite (i) characterizes the chronic phase of the disease, (ii) insures persistence of the infection (apparently without inducing an inflammatory response), and (iii) is the most efficient precursor to sexual stages in the cat (Frenkel, 1973; Dubey and Frenkel, 1976). Importantly, the requirement for bradyzoites re-differentiation into tachyzoites to initiate an acute infection not only occurs during infection of the definitive feline host, but has been recently co-opted by *T. gondii* to initiate a distinct asexual cycle for transmission between intermediate hosts (Su *et al.*, 2003).

Providing a complete biological definition of a bradyzoite is not trivial for several reasons: (1) while mature tissue cysts have been studied for a long time, and their cytological and morphological description is precise (Dubey *et al.*, 1998), biochemical studies are challenged by the limited amount of biological material that can be harvested, as well as contamination by host molecules; (2) studies of early bradyzoite stages are simply not

possible *in vivo*, because of the difficulties in accessing these parasite stages, and because bradyzoite development is a stochastic, asynchronous and multi-step process with many intermediates; (3) some laboratories use the greater resistance to pepsin acid of bradyzoites as a marker, but since the technique is not always consistent, it is difficult to use this as a definition; (4) the criterion of oral infectivity in mice is equally problematic, since extracellular tachyzoites can be infective orally (Dubey *et al.*, 1998). A better molecular characterization of tachyzoites and bradyzoites has and will continue to help us to identify markers that define bradyzoites. Most laboratories define a bradyzoite by reactivity to the P36 monoclonal antibody (a stage-specific surface antigen), by reactivity to *Dolichos biflorus* agglutinin (a commercially available lectin that specifically bind to the bradyzoite cyst wall), or by the expression of developmentally regulated markers such as heat-shock proteins or glycolytic enzymes.

Most of the knowledge we have gained about the biology of bradyzoite conversion has been obtained from *in vitro* differentiation studies, while the biology of mature tissue cysts and parasite transmission were mostly addressed *in vivo*. Many questions on topics such as tissue tropism, dissemination, long term survival, host–pathogen interactions and co-evolution, in either mature tissue cysts or early bradyzoites, remain unanswered and difficult to address. Here we discuss the cell biology, metabolism, cyst development in the mouse model, long-term survival, and immunobiology of the bradyzoite stage.

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### **Bradyzoite morphology and metabolism: adaptations to an encysted lifestyle**

The establishment of a resistant form often corresponds to morphological and metabolic modifications in order to survive in hostile or variable environments.

#### **Bradyzoite cell cycle requirements**

The development of a bradyzoite starts with changes in the cell cycle and a reduction of the replication rate (Chapter 15 for a detailed description; Bohne *et al.*, 1994; Soète *et al.*, 1994; Jerome *et al.*, 1998; Radke *et al.*, 2003; Dzierszinski *et al.*, 2004). Differentiation necessitates progression of the cell cycle (Bohne *et al.*, 1994; Radke *et al.*, 2001), and a late-S/G2 growth shift limits tachyzoite replication and allows the parasite to enter the bradyzoite differentiation process (Radke *et al.*, 2003). Immature bradyzoites observed *in vitro* divide asynchronously, using both endodyogeny and endopolygeny (Dzierszinski *et al.*, 2004). Mature encysted bradyzoites are non-proliferative forms containing uniform 1N DNA content, and replication occurs only in parasites that re-differentiate back into tachyzoites (Radke *et al.*, 2003).

#### **Bradyzoite and cyst morphology**

Many cytologic and ultrastructural studies have been conducted in order to describe *T. gondii* mature tissue cysts and to determine how bradyzoites develop *in vivo* (described in Chapters 1 and 2). Briefly, cyst characteristics depend on host cell type, parasite strain, and cyst age; young tissue cysts may contain as few as two bradyzoites, while mature tissue cysts measuring between 50 and 100 µm across can contain hundreds of zoites. Older tissue cysts contain both intact and degenerate bradyzoites, in which many organelles are

not present (Ferguson and Hutchinson, 1987). During bradyzoite development, zoites remain polarized and organized cells (described in Chapter 2). One notable difference is the early modification of the parasitophorous vacuole (PV) into an intracellular cyst. Like tachyzoite-containing PVs, tissue cysts are wrapped in host cell filaments (Halonen *et al.*, 1994; 1996; 1998). The cyst wall is a modification of the PV membrane, which becomes an invaginated membrane underlain with an osmiophilic matrix containing membranous vesicles (Ferguson and Hutchinson, 1987; Dubey *et al.*, 1998; Zhang *et al.*, 2001). Although the composition of this 200- to 850-nm-thick structure is not totally known, the cyst wall contains chitin, glycoproteins, and possibly glycolipids (Boothroyd *et al.*, 1997; Zang *et al.*, 2001). Some bradyzoite-specific proteins are secreted early during cyst formation, and generate a cyst matrix with few identified proteins (Zhang and Smith, 1995; Weiss and Kim, 2000; Zhang *et al.*, 2001). The cyst wall and matrix likely protect bradyzoites from environmental conditions in the gut, and may provide an escape from the host immune system, perhaps by interfering with antigen presentation.

Although similar, virulent tachyzoites and mature encysted bradyzoites differ in certain organelles and sub-cellular structures. Bradyzoites show a reorganization of the secretory organelles important for host cell invasion and establishment of the PV. Mature bradyzoites contain rhoptries that are uniformly electron dense, few dense granules and numerous micronemes. The nucleus is localized in a more posterior position. Bradyzoites do not contain lipid bodies, and the presence of white and large amylopectin granules in the cytoplasm reflects an important shift in the carbohydrate metabolism (discussed below). Data regarding the mitochondrion and the cytoskeleton in mature bradyzoites is still fragmentary or simply unavailable. Loss of the apicoplast in some bradyzoites inside large mature tissue cysts isolated from the brain of chronically infected mice can be visualized by immunofluorescence using an antibody recognizing specifically the apicoplast acyl carrier protein and DAPI staining (Dzierszinski *et al.*, 2004). These zoites might correspond to degenerated bradyzoites inside old tissue cysts, as seen by EM (Figure 13 in Dubey *et al.*, 1998). It should be noted that degenerated bradyzoites were not observed in other studies (Ferguson *et al.*, 2005).

Morphological data are available mostly for the late mature encysted bradyzoite stage, and most observations have been made in fixed tissue samples. Several *in vitro* systems have been developed to induce and study *T. gondii* stage conversion (Chapter 16; Lindsay *et al.*, 1991; McHugh *et al.*, 1993; Soète *et al.*, 1994; Tomavo and Boothroyd, 1995; Weiss *et al.*, 1995; Bohne and Roos, 1997; Yahiaoui *et al.*, 1999), but the study of the early bradyzoite stage by a cell biological approach has been largely impaired by the heterogeneous nature of this process (Soète *et al.*, 1993). Bradyzoite development can however be followed *in vitro*, by time-lapse video microscopy on a “vacuole-by-vacuole” basis over several days, using fluorescent reporters fused to parasite proteins or signal sequences targeting the markers into various sub-cellular localizations (Dzierszinski *et al.*, 2004). The study of live immature bradyzoites recapitulates morphological characteristics known from static studies on fixed tissues, while providing a dynamic view and a sense of kinetics. Developing bradyzoites rapidly synthesize the cyst wall, secrete some lectin-binding compounds into the matrix via dense granules, and reorganize secretory organelles in the first few days of the switch. The mitochondrion is maintained in these early stages, while the apicoplast is

occasionally lost (in 10–20% of the vacuoles). The morphological characteristics used to described mature bradyzoites (organization of the cyst wall, accumulation of micronemes, re-localization of the nucleus in a more posterior position, and apparition of amylopectin granules) are established by three days post-induction (Dzierszinski *et al.*, 2004).

### Bradyzoite-specific proteins and metabolism

Changes in gene expression (from either transcriptional or posttranscriptional control) are typical of differentiating organisms. The triggering of bradyzoite differentiation, the switch and regulation of gene expression are described in Chapter 16. We focus here on the description of proteins that have been characterized so far and their potential role in bradyzoite biology. These proteins fall into three main categories: (1) surface antigens, (2) proteins implicated in the biosynthesis of carbohydrates and storage polysaccharide and (3) heat shock proteins.

In the early 1980s, the distinct antigenicity of tachyzoites and bradyzoites was demonstrated (Lunde and Jacobs, 1983). The outer surface of the parasite is decorated with differentially expressed glycosylphosphatidylinositol (GPI)-anchored proteins that are structural homologs (Tomavo *et al.*, 1989; Nagel and Boothroyd, 1989; Tomavo, 1996). The archetypal SAG1 protein is a major and highly immunogenic tachyzoite-specific surface antigen, and all the surface antigens form the SRS (SAG1-related sequence) superfamily (Jung *et al.*, 2004). SRS proteins mediate parasite attachment to the host cell, and elicit host immunity. The development of monoclonal antibodies allowed the first identification of many surface antigens, and served as essential tools in the monitoring of *T. gondii* differentiation (Handman *et al.*, 1980; Tomavo *et al.*, 1991). Upon differentiation, bradyzoites stop expressing the tachyzoite-specific SAG1 (P30) and SAG2 (P22) proteins, but start expressing other antigens, such as SAG4 (P18; Tomavo *et al.*, 1991; Ödberg-Ferragut *et al.*, 1996), BSR4 (P36; Tomavo *et al.*, 1991; Knoll and Boothroyd, 1998), SAG2C/D and SRS9 (Lekutis *et al.*, 2000; Cleary *et al.*, 2002), P34 or the late P21 (Tomavo *et al.*, 1991). The tachyzoite-specific proteins SAG1 and SAG2A elicit a strong immune response upon infection (Godard *et al.*, 1994; Lekutis *et al.*, 2000). The development of ESTs and microarrays (Aijoka, 1998; Manger *et al.*, 1998; Cleary *et al.*, 2002), the sequencing of *T. gondii* genome and its analysis, now reveal that the SRS proteins form actually a huge family of 161 unique sequences, with 47 transcripts detected so far (Jung *et al.*, 2004). Remarkably, tachyzoite and bradyzoite express mostly non-overlapping sets of multiple SRS (Jung *et al.*, 2004). Although antigen variation *per se* has not been shown in *T. gondii*, it is likely that, besides their role in parasite attachment to a very broad range of cell types or in the protection from the environment (Tomavo, 1996) some SRS proteins canalize the immune response towards the tachyzoites, while some parasites differentiate into bradyzoites and establish a chronic infection. This topic is further discussed below in the immunobiological context.

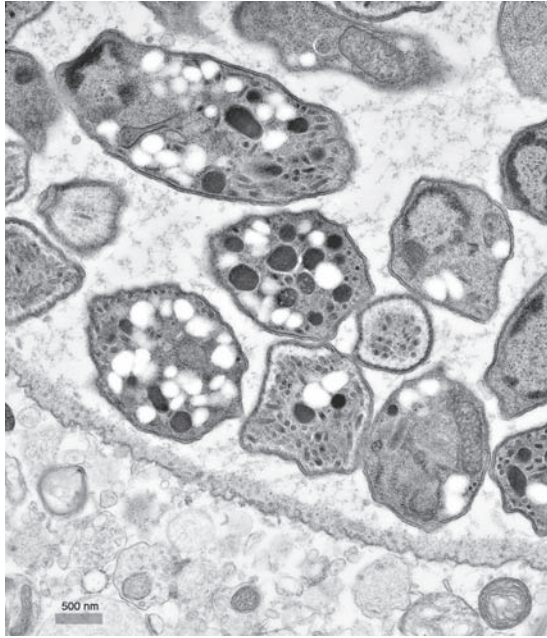
The morphological and biological characteristics of the tissue cyst suggest that the metabolic requirements of the dormant and encysted bradyzoites would differ in some respects from those of the virulent and cytolytic tachyzoites. It should be noted that neither the ability of cysts to import nutrients from the host cell, nor the cyst wall permeability, has been determined so far. Characterization of bradyzoite metabolism in biochemical terms

has been impaired by difficulties in gathering enough biological material, and factors such as host cell contamination. However, Denton *et al.* (1996) were able to compare the activities of phosphofructokinase, pyruvate kinase, lactate dehydrogenase, isocitrate dehydrogenase, and succinic dehydrogenase in tachyzoites and bradyzoites isolated from rodents. Their studies showed that both stages rely on glycolysis and produce lactate. The activity of pyruvate kinase and lactate dehydrogenase were particularly high in bradyzoites, suggesting that lactate production is particularly important in the latent stage. These biochemical studies also suggested that bradyzoites rely predominantly on anaerobic glycolysis rather than TCA cycle (Denton *et al.*, 1996). This is consistent with the activity of chemical compounds that interfere with the mitochondrial respiratory chain functions and induce the differentiation of tachyzoites into bradyzoites (Bohne *et al.*, 1994; Tomavo and Boothroyd, 1995). Several glycolytic enzyme isoforms expressed in *T. gondii* are developmentally regulated: glucose-6-phosphate isomerase, enolase and lactate dehydrogenase (Yang and Parmley, 1997; Manger *et al.*, 1998; Dzierszinski *et al.*, 1999; 2001). A nuclear localization has also been reported for *T. gondii* enolases (Ferguson *et al.*, 2002), and their potential role in development is discussed in Chapter 16 and Ferguson *et al.* (2002). Enzymologic characteristics determined for the recombinant tachyzoite ENO2 and bradyzoite ENO1 demonstrate that ENO2 and ENO1 have similar Michaelis constant ( $K_m$ ), but that ENO2 has a threefold higher specific activity ( $V_{max}$ ) (Dzierszinski *et al.*, 2001), while no biochemical differences were found between LDH1 and LDH2 (Dando *et al.*, 2001). It is therefore tempting to hypothesize that stage-specific expression of glycolytic isoforms may correspond to metabolic rates found in the tachyzoite and bradyzoite stages (Dzierszinski *et al.*, 2001; Tomavo, 2001).

Bradyzoite biology also reflects two major bradyzoite metabolisms: the synthesis of the cyst wall, and the accumulation of storage polysaccharide. First, it was shown that the cyst wall includes *Dolichos biflorus* seed lectin- and succinylated wheat-germ agglutinin (S-WGA)-binding sugars, and that chitinase disrupts the cyst wall, suggesting chitin as a component of the cyst wall (Boothroyd *et al.*, 1997). Furthermore, a glycoprotein CST1 was identified in the cyst wall using mAbs and characterized (Weiss *et al.*, 1992; Zhang *et al.*, 2001), adding to the protein CC2 (Gross *et al.*, 1995). Determining the repertoire of glycosylated molecules present in the cyst wall should provide additional insights in the stability and function(s) of the cyst wall (Coppin *et al.*, 2003). Second, the major carbohydrate substance in *T. gondii* bradyzoites (and also *T. gondii* sporozoites, and *Eimeria* sp. zoites) is amylopectin (Coppin *et al.*, 2003). This storage polysaccharide with plant characteristics is accumulated in the cytoplasm only, shows a crystalline aspect and is a genuine amylopectin composed of linear  $\alpha$ -1,4-glucans with less than 4%  $\alpha$ -1,6 branches (Guerardel *et al.*, 2005). Amylopectin is likely to provide a reserve of energy to the parasite, perhaps essential to the bradyzoite (and the sporozoite) in the extracellular phases of the cycle that occur between hosts (Coppin *et al.*, 2003) (Figure 17.1).

*T. gondii* tachyzoites differentiate into bradyzoites in response to various stresses (pH shift, CO<sub>2</sub> starvation, mitochondrial respiratory chain inhibitors, etc. described in details in Chapter 16). Heat shock proteins (HSPs) are chaperones that are implicated in response to stress, and can be developmentally regulated. Indeed, bradyzoite development is associated with the early expression of the small HSP BAG1 (or HSP30, also





**Figure 17.1** Electron micrograph of a bradyzoite cyst from an infected mouse brain. CBA/J mice were inoculated with a type II Prugniaud strain of *T. gondii*. Four weeks after inoculation, brains were removed, ground with a mortar and pestle, and fixed in 2% paraformaldehyde/2.5% glutaraldehyde followed by postfixation in 1% osmium tetroxide. Cysts were then en bloc stained with 1% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope. Amylopectin are the white granules.

called BAG5; Bohne *et al.*, 1995; Parmley *et al.*, 1995), with the upregulation of HSP70 family members (Lyons and Johnson, 1995; Weiss *et al.*, 1998), with the expression of a differentially spliced form of HSP60 (Toursel *et al.*, 2000), and with the upregulation and the re-localization of HSP90 (Echeverria *et al.*, 2005).

Several other proteins that are differentially expressed in the bradyzoite stage have been reported. The cyst matrix can be detected using mAbs specific against MAG1 (Parmley *et al.*, 1994) or other proteins (Weiss and Kim, 2000). In addition, bradyzoites specifically express a phosphatidylinositol synthase whose role has not been elucidated, but that could be involved in the biosynthesis of GPI anchors found in SRS proteins (Séron *et al.*, 2000), or in the production of signaling molecule precursors. Holpert *et al.* (2001) demonstrated that *T. gondii* possesses P-type  $H^+$ -ATPases (in addition to V-type ATPases), which are predominantly expressed in the bradyzoite stage (Holpert *et al.*, 2001). It was recently shown that the disruption of the gene encoding the P-type  $H^+$ -ATPase PMA1 in a cyst-forming *T. gondii* strain leads to a lower tachyzoite to bradyzoite switching rate (Holpert *et al.*, 2006). Interestingly, PMA1 knock-out parasites develop into fully mature tissue cysts, which successfully establish new acute and then chronic infections in mice infected



either orally or intraperitoneally (Holpert *et al.*, 2006). *T. gondii* possesses a second plasma membrane ( $H^+$ )-ATPase (PMA2), which is also upregulated in bradyzoites (Holpert *et al.*, 2001). The expression of the redundant PMA2 may explain the full development of PMA1 knock-out cysts (Holpert *et al.*, 2006). It will be interesting to see if the two PMA isoforms have complementary functions in the different steps of bradyzoite development.

Studies on bradyzoite biology are benefiting from the genome scale approaches that have now been undertaken: the sequence of *T. gondii* genome that has been determined and is being analyzed (ToxoDB, <http://toxodb.org/>), numerous ESTs (Aijoka, 1998; Manger *et al.*, 1998), SAGE tags, proteomic maps, microarrays, etc (described in Chapter 11), along with the development of comparative genomics (Chapter 12) and molecular and genetic tools (Chapter 14). For example, *in silico* analysis has identified a putative chitinase class I on chromosome Ia. Whole biosynthetic pathways are reconstructed, putative isoforms are determined, and data about stage expression are becoming more complete.

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### Tissue cyst development *in vivo*

To understand how changes in cell biology and metabolism contribute to the biology of bradyzoites, and to valid *in vitro* studies, we need to study bradyzoites *in vivo*.

#### Parasite strains and mouse models

Many studies have examined bradyzoite cyst formation and maintenance in mice. Most of these studies use either ME49 or RRA (Beverley) type II parasite strains since they easily form tissue cysts because type I strains are lethal in mice and fail to establish chronic infection (see Chapter 13 for a discussion of strain types and virulence). Type I strains (e.g. RH lines) can form brain cysts if the mouse is vaccinated with soluble tachyzoite antigen preparation (STAg) and interleukin-12 (Yap *et al.*, 1998) or if the virulence is reduced by targeted disruption of the dense granule protein GRA2 (Mercier *et al.*, 1998). A recently developed bioluminescence-based imaging system examined the growth, dissemination, and reactivation of different *T. gondii* strains in living mice (Saeij *et al.*, 2005). Since the same mouse can be followed throughout its infection, this system has great potential for teaching us much about the biology bradyzoites *in vivo*.

#### Development of tissue cysts

Even though tachyzoites replicate in most host tissues, bradyzoites have a tissue tropism for the central nervous system (CNS) including the retina, and striated muscles. One of the first studies to examine the kinetics of bradyzoite cyst formation in the brains of mice used subcutaneous inoculation of bradyzoite cysts into outbred mice (Ferguson and Hutchison 1987). Tachyzoites were seen in the brain by 11 days post infection (PI). Tachyzoites had disappeared by 21 days PI and only bradyzoite cysts were present. Tissue cysts giving rise to short prepatent periods in cats have been observed in mouse brain three days post-intracerebral infection (Dubey and Frenkel, 1976), but this is not biologically relevant. After oral or intraperitoneal (i.p.) inoculation of bradyzoite cysts, the number of cysts per brain was found to peak between 3 to 4 weeks, and decline afterwards (Blackwell *et al.*, 1993; Ferguson *et al.*, 1994). With inbred mouse strains, large differences were seen in survival rates when mice were orally infected with bradyzoite cysts (McLeod *et al.*, 1984;

1989). Differences were also seen in the susceptibility of inbred strains to acute versus chronic infection after i.p. inoculation of bradyzoite cysts (Suzuki *et al.*, 1993). BALB/c mice died during the acute phase of infection and not in the chronic phase, whereas no mortality was observed with CBA/Ca mice in the acute stage of infection but they began dying 2 months PI.

The initial portal of entry does not influence the susceptibility of many mouse strains, with the exception of C57BL/6 mice. Only 2 of 16 C57BL/6 mice survived after peroral challenge with 100 bradyzoite cysts, whereas 19 of 22 mice survived after i.p. inoculation of the same dose (McLeod *et al.*, 1989). Histopathologic analysis suggested that increased susceptibility resulted from inadequately regulated inflammatory responses that increased tissue destruction.

### Tissue cyst rupture and parasite reactivation

Rupture of tissue cysts and reactivation can lead to acute toxoplasmosis, even in chronically infected mice (Dubey *et al.*, 1998). Although rarely documented, the formation of new tissue cysts in the brain during chronic infection has been reported as the result of leakage and rupture of older cysts (Ferguson *et al.*, 1989). The factors responsible for the apparition of new generations of tissue cysts are not known (Ferguson *et al.*, 1989; Dubey *et al.*, 1998). Reminiscent of the tissue cyst clusters seen in the brain (Dubey *et al.*, 1998), partition of PVs is observed *in vitro* after only 30 hours post-induction of bradyzoite development in fibroblast cells (Dzierszinski *et al.*, 2004). Cysts were able to proliferate by the migration of free zoites and the fission of immature bradyzoite cysts. This cell to cell spreading would increase the long-term survival of bradyzoite cysts. These observations would concord with the increase in tissue cyst number for 4 weeks after infection in the brain (by which time tachyzoites are eliminated by the host immune response).

### Influence of host genetic background

Several elegant studies were performed to determine the host factors that caused susceptibility to chronic infection and cyst development. The genetic background of the host influences cyst counts in mouse brain. MHC class I genes located at H-2L and CD8<sup>+</sup> T cells were found to regulate the number of brain cysts formed after oral inoculation, with L<sup>d</sup> being resistant (low cyst counts) and L<sup>b</sup> being susceptible (high cyst counts) (Brown and McLeod, 1990). Similarly, mice with the b or k allele at the H-2D region had high cyst counts, while mice with the d allele did not (Suzuki *et al.*, 1991). A follow-up study indicated that gene(s) in the D/L region determine whether cyst counts will be high and toxoplasmic encephalitis (TE) will occur, and showed that L<sup>d</sup> gene plays a critical role in resistance against TE (Suzuki *et al.*, 1993). Using mice that have been genetically altered to express the L<sup>d</sup> gene in addition to their own MHC genes, L<sup>d</sup> was definitively shown to decrease the number of brain cysts (Brown *et al.*, 1995). It was later demonstrated that *T. gondii* specific-L<sup>d</sup>-restricted CTLs were generated during the course of infection (Johnson *et al.*, 2002).

The effect of cytokines during chronic infection also depends on the host genetic background. For instance, injection of mAbs to IFN- $\gamma$ , which is a key cytokine in the control of both acute and chronic toxoplasmosis (Chapter 6), augmented inflammatory changes

in the brains of mice with high cyst counts, but not in those with low cyst counts (Suzuki *et al.*, 1991). It was also shown that mice resistant to cysts and TE had little detectable cytokine mRNA in their brains whereas susceptible mice had elevated mRNA for a wide range of cytokines (Brown *et al.*, 1995).

### Long-term survival

One of the most striking features of a bradyzoite cyst is its longevity. Bradyzoite cysts survive throughout the life of their host: hidden from the immune system (discussed below) and non-responsive to anti-microbial agents. Delivery of therapeutics is always challenging since they have to cross biological boundaries to be effective. This is especially true for apicomplexan parasites because drug targets must enter the host cell, cross the PVM, and then enter the parasite. Additional blockades for *T. gondii* bradyzoites include the cyst wall and the blood brain barrier; these have made a chronic infection virtually untreatable (*in vivo* bradyzoites are resistant to all main line therapy). Recent studies have shown that short oligomers of arginine, both unconjugated and conjugated to the drug triclosan, can enter encysted bradyzoites (Samuel *et al.*, 2003). These findings bring hope that clinical treatments for chronic bradyzoite infections may one day be available.

The long-term survival of bradyzoite cysts is dependent on parasite and host cell factors. Along with changing their metabolism to accommodate their dormant lifestyle (this chapter), bradyzoites are likely to be even more efficient than tachyzoites at blocking apoptosis (see Chapter 8), however this has not yet been examined. As previously mentioned in this chapter, bradyzoites are restricted to the CNS and muscle (striated and heart). This tissue-tropism is likely due to the immune privileged nature of the brain (discussed below) and that immune cells do not regularly traffic through muscle tissue. CNS and striated muscle are also inherently resistant to apoptosis as they are post-mitotic. It is interesting that congenital toxoplasmosis is particularly severe early on in pregnancy, the general time frame when programmed cell death is active in the brain of the developing fetus. As the fetal brain becomes more developed during gestation, programmed cell death slows, and congenital toxoplasmosis is not as severe.

Several studies have examined the cells of the CNS in which *T. gondii* will infect, replicate, and encyst. The main cells of the central nervous system are neurons, glia, and cells of the meninges and blood vessels. The function of a neuron is to receive, conduct, and transmit signals. Neurons have a cell body that contains the nucleus, several dendrites that branch from the cell body to receive signals, and usually one axon that conduct signals away from the cell body. Even though the cell body of the CNS neurons is small (10–15  $\mu\text{m}$  across), *T. gondii* will grow and form bradyzoite cysts in neurons both *in vitro* and *in vivo* (Sims *et al.*, 1989; Halonen *et al.*, 1996; Fischer *et al.*, 1997; Creuzet *et al.*, 1998; Lüder *et al.*, 1999). These same studies have also shown that *T. gondii* infects, replicates, and encysts in glia cells, especially astrocytes. Glial cells can be broken into two groups based on their origin: macroglia derive from the neuroectoderm and include astrocytes, oligodendrocytes, and ependyma, while microglia derive from bone marrow. Astrocytes have multipolar, branching cytoplasmic cellular extensions that radiate from the cell body giving them a star-shaped appearance from which they are named. They have diverse functions including detoxifiers, suppliers of nutrients, electrical insulators, barriers controlling the

flow of macromolecules, and even repair and scar formation in the brain. It is believed that astrocytes are the main CNS cells that support the *T. gondii* infection, perhaps because of their larger 100  $\mu\text{m}$  size (Halonen *et al.*, 1996). Microglia are the fixed phagocytic cells of the CNS and are of macrophage or dendritic cell origin. Even though tachyzoites grow well in microglia, these cells may not support bradyzoite cyst formation (Chao *et al.*, 1993; Fagard *et al.*, 1999). Fischer *et al.* (1997) had previously seen that both glial and neuronal cells allowed *T. gondii* encystations, and they speculated that brain-internal spreading of bradyzoites may sustain chronic infection.

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### **Immunobiology: relationships between bradyzoite biology and host immunity**

Tissue cyst long-term survival and persistence in the host reflect the existence of mechanisms developed by bradyzoites to cope with the protective and long-lasting host immune response. On the other hand, experimental neutralization of IFN- $\gamma$  *in vivo* and clinical immunosuppressions induce parasite recrudescence, strongly suggesting that the host immune response plays a role in the maintenance of the dormant form. While host response to infection, immunity against *T. gondii*, pathogenesis and evasion mechanisms are examined in previous chapters (Part 2), we focus here on the effects of bradyzoite biology on host immunity.

#### **Tissue tropism and immune recognition**

Parasites that do not circulate are of course less likely to be detected by immune cells. The brain is considered as an immuno-privileged location, because of its protection by the blood brain barrier, the absence of conventional lymphatic system and low T cell trafficking, graft acceptance, and low levels of MHC class I/II expression (Hickey, 1997). However, it was shown that some brain-derived antigens can initiate inflammatory or protective CNS immune responses; antigen-loaded dendritic cells migrate to secondary lymphoid organs, where they prime CD8<sup>+</sup> T cells that home to the brain (Karman *et al.*, 2004), can activate microglia, and perform immune surveillance (Cabarrocas *et al.*, 2003). Microglia perform both antigen presenting and effector functions, and are able to initiate CNS innate and adaptive CD4<sup>+</sup> T cell responses through Toll-like receptors (Olson and Miller, 2004). Microglia are major effectors in the prevention of tachyzoite replication, but their action on tissue cysts is unknown.

Presentation of bradyzoite antigens (and whether or not the cyst wall interferes with this process) is not elucidated. Using the model antigen  $\beta$ -galactosidase, Kwok *et al.* (2003) showed that  $\beta$ -gal secreted in the cyst matrix failed to prime a specific CD8<sup>+</sup> T cell response in mice infected orally with transgenic tissue cysts. But  $\beta$ -gal secreting bradyzoites did stimulate the expansion of a  $\beta$ -gal-specific CD8<sup>+</sup> T cell population in the brain upon secondary oral infection of mice that were first primed with tachyzoites secreting  $\beta$ -gal (Kwok *et al.*, 2003). The identity of the antigen presenting cells and the mechanisms of bradyzoite antigen presentation to T cells are currently under investigation in several laboratories.

## Bradyzoite SRS proteins and immune diversion

Jung *et al.* (2004) hypothesized that the simultaneous expression of multiple antigenically distinct SRS proteins (discussed earlier in this chapter) would produce variant T cell epitopes, and therefore might impair the development of effector T-cell responses against some SRS antigens. Since *T. gondii* expresses multiple SRS proteins during a single parasite stage, we cannot speak of true antigenic variation. It is however likely that some SRS proteins would focus the immune response towards the tachyzoite stage, while bradyzoites develop in immuno-privileged sites and establish a chronic infection. Some excreted-secreted antigens expressed by both asexual stages and some bradyzoite-specific isoenzymes can elicit some immune response (Prigione *et al.*, 2000; Di Cristina *et al.*, 2004), but proteins expressed by bradyzoites are clearly not immunodominant antigens (Kim and Boothroyd, 2005). Importantly, Kim and Boothroyd (2005) demonstrated that an immune response to bradyzoite-specific SRS proteins (the major SRS9 and other SRS) is not developed during infection in mice and humans, and that the timing (i.e. stage specificity) of SRS protein expression is crucial in the development of a persistent infection and in the containment of immunopathology to the host. The lack of response to bradyzoite-specific SRS might be due to the combination of their expression in the brain, together with other factors to be determined (Kim and Boothroyd, 2005).

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## Other considerations

Even though many protozoan parasites form chronic infections in humans, these stages are poorly understood. Can a molecular understanding of the biology of bradyzoites teach us about the chronic stages of other protozoan parasites? Of the four *Plasmodium* species that cause human malaria, the liver stages of *P. vivax* can form a chronic hypnozoite stage (*P. falciparum* and *P. malariae* complete their liver development within 1 to 2 weeks). Hypnozoites remain in the liver for months to years before re-emerging to produce relapses of malaria. Hypnozoites are not eradicated by any of the standard malaria treatments directed against the blood stages of the parasite, but primaquine therapy can prevent relapse. Another Apicomplexan, *Cryptosporidium parvum*, produces environmentally resistant thick-walled oocysts and autoinfective thin-walled oocysts as part of its lifecycle. The chronic infection of *C. parvum* usually occurs in persons with compromised immune systems (HIV, chemotherapy, malnutrition, or < 2 years of age). Persistent cryptosporidiosis develops when the hepatobiliary system is colonized; this can result in asymptomatic carriage or chronic disease. In a screen to isolate genes that are necessary for the establishment of a chronic *T. gondii* infection, several genes that have orthologs in *Plasmodium* and *Cryptosporidium* have been isolated (Knoll *et al.*, unpublished data). It will be interesting to see if these orthologs complement the *T. gondii* lack of persistence. Similarly, several *Leishmania* species can form persistent chronic infections in humans causing a wide spectrum of disease. Even though *Leishmania* and *T. gondii* are evolutionarily distinct, three different *T. gondii* genes with homology to *L. major* proteophosphoglycan 3 were found to be critical for establishing a *T. gondii* chronic infection (Knoll *et al.*, unpublished data). The function of proteophosphoglycan 3 in *Leishmania* is unknown.

Finally, what are the effects of having a chronic *T. gondii* infection? Studies examining the behavior of mice chronically infected with *T. gondii* have clearly shown an increase in

general movement compared to controls (Hutchison *et al.*, 1980; Hay *et al.*, 1983; 1984). A similar increase in activity was seen in *T. gondii* infected rats, including an enhanced susceptibility to capture in baited traps (Webster *et al.*, 1994; Webster 1994). This increased activity, and decreased avoidance of novel stimuli would increase the chances of the chronically infected animal of being preyed upon. This would obviously be advantageous for *T. gondii* transmission via both sexual and asexual lifecycles. These behavioral changes seen in chronically infected animals may be caused by a slight increase in the levels of the neurotransmitter dopamine (Stibbs, 1985). Do we receive any benefits from having a chronic *T. gondii* infection? *T. gondii* elicits a potent cell-mediated response that can control the growth of other intracellular pathogens including bacteria (Ruskin and Remington, 1968), viruses (Remington and Merigan, 1969), pathogenic fungi (*Cryptococcus neoformans*, Gentry and Remington, 1971), and parasites (*Schistosoma mansoni*, Mahmoud *et al.*, 1976 and *P. yoelii*, Charest *et al.*, 2000). It is unlikely that having a chronic infection has a high metabolic cost to its host since the bradyzoite cysts are essentially dormant, thus *T. gondii* may have evolved as more of a mutualist in warm-blooded animals. This mutualism would provide a safe haven and propagation for the parasite, and protection against intracellular pathogens for the host by continuously priming cell-mediated immunity. The relationship between the *T. gondii* bradyzoite and host, regardless of whether that interaction is parasitic or mutualistic, is a fascinating area of study that will yield many future discoveries.

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## Abstract

Once relegated to a mere supporting role, chromatin has recently taken center stage in performing the regulation of gene expression. Broadly defined as a complex of proteins and nucleic acid, chromatin has stepped into the limelight as a novel control mechanism for eukaryotic gene transcription. It has even been postulated that an epigenetic “code” emerges from the accumulation of certain patterns of post-translational modifications made to chromatin proteins. This chapter includes a primer on chromatin constitution and the enzymatic complexes capable of restructuring it in ways influencing gene expression. I will highlight some of the conserved, as well as the unique features, of chromatin remodeling in *Toxoplasma gondii*, with particular attention paid to how these processes orchestrate changes in the expressed genome that are pertinent to the parasite life cycle.

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## The chromatin factor

Chromatin is inherently repressive to gene expression because it restricts access of the pre-initiation complex to DNA. The primary constituent of chromatin is the nucleosome, a complex of ~146 base pairs of DNA spooled around an octamer comprised of four types of core histone proteins, two copies each of H2A, H2B, H3, and H4 (Luger *et al.*, 1997). The size of the histone octamer (6×11 nm) is sufficient for DNA to make nearly two turns per nucleosome. “Linker” DNA extending between nucleosome core particles is generally limited to only 8–60 base pairs. Histone H1 binds portions of linker DNA, thereby capping the octamer structure and facilitating the formation of higher-order chromatin structures (Wolffe, 1997). The N-terminal tails of the core histones protrude like tentacles from the assembled octamer/DNA complex, free to interact with linker DNA or neighboring nucleosomes. These histone tails are rich in lysine residues, conferring an overall positive charge that can associate with the negatively charged phosphate backbone of DNA. These attributes provide nucleosomes with the capacity to condense chromatin into a highly compact form amenable to the size restrictions of a nuclear compartment. However, these same properties make nucleosomes a significant impediment to cellular factors requiring access to DNA (Lorch *et al.*, 1992). This realization has added a new dimension to the regulation of gene expression, and has important ramifications about the governance of other processes involving DNA, such as recombination, replication, and repair.

## Getting into genes: covalent modification of histones

Histone proteins, particularly H3 and H4, are some of the best conserved proteins among eukaryotes, *Toxoplasma* included (Sullivan, 2003). It stands to reason that eukaryotic cells employ similar mechanisms to circumvent the barrier that histones form to prevent unwarranted access to DNA. It has been known for decades that post-translational modifications, e.g. acetylation, of the histone tails increases production of RNA (Allfrey *et al.*, 1964). In 1995, a histone acetyltransferase (HAT) initially called p55 was identified in the alveolate *Tetrahymena thermophila* (Brownell and Allis, 1995; Brownell *et al.*, 1996). A direct connection between histone acetylation and gene expression activation was established when p55 revealed homology to yeast GCN5 (general control nonderepressible), previously identified as a putative transcriptional coactivator involved in the interactions between certain activators and the transcriptional apparatus (Brownell *et al.*, 1996; Marcus *et al.*, 1994). A multitude of other transcriptional coactivators have since been found to possess intrinsic HAT activity, including p300/CBP (CREB-binding factor), PCAF (p300/CBP-associated factor), MYST-family HATs (named for founding members MOZ, Ybf2/Sas3, Sas2, and Tip60), TAF<sub>II</sub>250, and the p160 family of nuclear receptor coactivator proteins (Sterner and Berger, 2000). As one may surmise, nucleosomal histones devoid of acetyl moieties are associated with gene silencing, and a series of histone deacetylases (HDACs) have subsequently been identified (Thiagalingam *et al.*, 2003). At least 18 HDACs have been identified in higher eukaryotes, categorized into three classes based on similarity to yeast homologs. Class I and II HDACs, similar to yeast RPD3 and HDA1, respectively, are sensitive to the inhibitor trichostatin A (TSA). Class III HDACs (sirtuins, homologs of yeast SIR2) have a structurally distinct catalytic domain that is NAD-dependent and resistant to TSA.

Acetylation is the best characterized modification at present, but several additional transient histone modifications impacting transcription have been documented, including methylation, phosphorylation, ADP-ribosylation, ubiquitination, and sumoylation (Berger, 2002; Nathan *et al.*, 2003). Acetyl or methyl groups can be attached to the  $\epsilon$ -amino group of lysine residues; methyl groups can also be added to arginine residues by PRMTs (protein arginine methyltransferases). It is important to clarify that not all histone modifications activate transcription. For example, histone methylation may be a mark of transcriptional activation or repression (Zhang and Reinberg, 2001). Adding to the complexity of possible modifications bestowed upon histones is the observation that multiple methyl groups can be joined to a single residue. Histone demethylation of lysines is mediated by the nuclear amine oxidase LSD1/BHC110, or lysine-specific demethylase 1 (Lee *et al.*, 2005; Shi *et al.*, 2004). A family of jumonji (JmjC) domain-containing proteins has also been recently found to mediate histone demethylation (Tsukada *et al.*, 2006).

Phosphorylation of H3 is known to play a role in mitotic chromosome condensation (Hsu *et al.*, 2000), but has also been correlated with the activation of immediate-early genes in humans (Chadee *et al.*, 1999), and has been linked to dosage compensation in *Drosophila* (Jin *et al.*, 1999). An increasing number of kinases capable of phosphorylating specific serine and threonine residues of histones are being described, including SNF1, MSK1, and JIL1. As one may expect, phosphatases (e.g. PP1) can act to dephosphorylate histones (Hsu *et al.*, 2000). ADP-ribosylation of histones, particularly H2B, is mediated

by a subclass of poly-ADP-ribose polymerases (PARPs) and results in decondensation (Kraus and Lis, 2003). Conversely, poly-ADP-ribose glycohydrolases (PARGs) break down the polymers and are postulated to restore chromatin to its condensed and transcriptionally repressive state (Kraus and Lis, 2003). C-terminal lysine residues of H2A and H2B are subject to ubiquitination by enzymes such as RAD6; this modification is enriched in transcriptionally active gene-enriched chromatin (Davie and Murphy, 1990). Ubiquitin proteases such as Ubp10 and Ubp8 have roles in silencing and have recently been demonstrated to remove ubiquitin from histone H2B (Gardner *et al.*, 2005). Sumoylation by the UBC9 enzyme primarily occurs on H4 and is linked to transcriptional repression (Shiio and Eisenman, 2003). Presumably, ULP-related proteases function to desumoylate histones as they do other sumoylated substrates.

There are presently two schools of thought explaining how covalent modifications may influence the outcome of gene expression. Traditionally it has been believed that these modifications alter the electrostatic interactions between histones and DNA. Acetylation, for instance, neutralizes the positive charge of the  $\text{NH}_3^+$  on lysine side chains of histones, greatly compromising the ability of the histone to bind DNA (Hong *et al.*, 1993). The accumulation of acetyl groups also abrogates inter-nucleosomal interactions (Fletcher and Hansen, 1996), hampering the condensation of chromatin (Tse *et al.*, 1998). And, consistent with hyperacetylation observed in regions of actively transcribed DNA (Hebbes *et al.*, 1988), the acetylation of histones is correlated with increased binding of transcription factors (Lee *et al.*, 1993). However, *in vivo* crosslinking studies argue that hyperacetylation does not significantly alter the histone-DNA interaction (Stefanovsky *et al.*, 1989). More recent evidence has been marshaled that may help to fully explain the effects of histone modification on gene expression.

Strahl and Allis have voiced an over-arching hypothesis that has been called the “histone code.”

*We propose that distinct histone modifications, on one or more tails, act sequentially or in combination to form a ‘histone code’ that is read by other proteins to bring about distinct downstream events.*

(Strahl and Allis, 2000)

Given the number of transient modifications that can adorn each of the four core histones, the number of potential combinations that could represent an epigenetic “language” is staggering. A key element for a histone code requires the presence of recognition domains that can “decode” the modification patterns. Such modules have in fact been characterized and include the bromodomain, which recognizes acetylated lysines (Dhalluin *et al.*, 1999); the chromodomain and Tudor domain, which may interact with methylated lysines (Bannister *et al.*, 2001; Huang *et al.*, 2006; Huyen *et al.*, 2004); the SANT domain, which preferentially binds non-acetylated histone tails (Yu *et al.*, 2003); and the macro domain, which binds ADP-ribose (Karras *et al.*, 2005). WD40 repeats may also bind methylated lysines, as demonstrated for the transcriptional activator WDR5, whose WD40 repeats recognize di- and tri-methylated H3 at lysine 4 (Wysocka *et al.*, 2005).

Another observation that would be consistent with an operational code is that a number of chromatin remodeling complexes contain multiple types of modifying enzymes. For instance, the *Drosophila* Male Specific Lethal (MSL) complex involved in dosage compensation contains both a MYST HAT as well as the histone kinase JIL-1 (Jin *et al.*, 1999). Studies such as this imply that constellations of modifications occur within a single nucleosome or nucleosomal neighborhood, creating unique binding surfaces that may specify addresses for the myriad of transcription factors in the cell. In some cases, an ordered cascade of gene activation has been elucidated whereby initial histone modifications recruit additional remodelers and, ultimately, general transcription factors (Agalioti *et al.*, 2002).

It must be mentioned that whether or not disparate patterns of modifications constitute a *bona fide* and consistent biological code remains hotly contested. Efforts to identify unambiguous predictive rules used by the putative code are ongoing. The alternative idea argues that the histone modifications do not represent a code, but are simply part of the protein signaling network (Schreiber and Bernstein, 2002). Whatever the case may be, the intense explosion of research in this field has made it clear that nucleosomes are dynamic and covalent modifications of histones have vital roles in gene expression.

### Non-covalent nucleosome remodeling

In addition to the covalent modification of histone proteins, a second broad class of chromatin remodelers exists that restructure the nucleosomal environment using the energy of ATP hydrolysis (Imbalzano, 1998). Non-covalent remodelers include proteins in the SWI2/SNF2 ATPase family that may relocate nucleosomes, alter the topology of nucleosomal DNA, or disrupt higher-order chromatin folding (Peterson, 2002). The founding member of this family is SWI/SNF, or Snf2, a bromodomain-containing ATPase found in screens to identify yeast mutants defective in mating type switching or sucrose fermentation (sucrose nonfermenting) (Neigeborn and Carlson, 1984; Stern *et al.*, 1984). The connection between select SWI/SNF genes and chromatin remodeling was established by Hirschhorn *et al.* (Hirschhorn *et al.*, 1992). There are now three other major recognized classes of ATP-dependent chromatin remodelers that differ in their structural features and complement of associating components: ISWI, Mi-2/CHD, and Ino80 (Mohrmann and Verrijzer, 2005). All contain a hallmark ATPase domain comprised of seven distinct subdomains (subdomains I-IV comprise the DEXDc portion while the remaining comprise the HELICc portion). Distinctive features of ISWI and Mi-2 ATPases include the presence of a SANT domain and chromodomain, respectively. The INO80 (yeast) group is unique in that its ATPase domain has a lengthy insertion separating subdomains IV and V, an attribute also seen in human SRCAP (Snf2-Related CBP-Activator Protein), human p400, and *Drosophila* DOM (*domino*) (Fuchs *et al.*, 2001; Monroy *et al.*, 2001; Ruhf *et al.*, 2001; Shen *et al.*, 2000).

SWI/SNF ATPases can have positive or negative effects on gene expression. Complexes containing SWI/SNF (BRG1 and BRM in human) activate transcription whereas those containing Mi-2 repress it. Consistent with a role in downregulating gene expression, Mi-2 complexes harbor HDACs. ISWI complexes are strongly linked with nucleosome assembly and stabilization (Ito *et al.*, 1997). Several studies point to a critical

role in DNA repair for INO80 (Cairns, 2004), but other studies suggest transcriptional regulation functions as well (Shen *et al.*, 2000). The other SWI/SNFs with elongated ATPase domains have demonstrated roles in transcriptional regulation (Monroy *et al.*, 2001; Ruhf *et al.*, 2001).

Canonical histones can also be replaced with variant versions during deposition or via ATP-dependent remodeling machines (Kobor *et al.*, 2004; Krogan *et al.*, 2004; Mizuguchi *et al.*, 2004), adding yet another level of sophistication to chromatin-mediated regulation of DNA processes. The substitution of these variant histones can have dramatic consequences on the architecture of chromatin. Histone variants of H3 include H3.3, which replaces H3 in actively transcribed genes, and Cse4/CENP-A, which is required for centromere function (Basrai and Hieter, 1995). Incorporation of variant histone H2AZ into nucleosomal arrays appears to compromise condensation, resulting in transcriptional enhancement (Ausio and Abbott, 2002). Inactivated X chromosomes are enriched with macro-H2A (Costanzi and Pehrson, 1998).

Finally, emerging studies from multiple model organisms implicate roles for non-coding RNAs in the control of epigenetic regulation, including dosage compensation, imprinting, and RNA interference (RNAi)-mediated gene silencing (Bernstein and Allis, 2005). Noncoding RNAs have been detected in *Plasmodium* (Upadhyay *et al.*, 2005), and components of the RNAi machinery have been detected in certain apicomplexans (Ullu *et al.*, 2004). However, much work remains to elucidate the function of potential noncoding RNAs in Apicomplexa and whether they contribute to the regulation of gene expression via chromatin remodeling.

In summary, nucleosomes are dynamic; their histones can be extensively modified, relocated, or even replaced. The number of possible ways to influence transcription at the epigenetic level is staggering and undoubtedly a major factor in cellular physiology.

## Beyond transcription

The bulk of this chapter focuses on the influence of chromatin on transcription, but it must be kept in mind that chromatin remodeling activities also play critical roles in DNA repair, replication and cell cycle, and recombination (Ehrenhofer-Murray, 2004; Hassa and Hottiger, 2005; van Attikum and Gasser, 2005). MEC1 kinase in yeast (ATM in humans) is a DNA-damage checkpoint protein that phosphorylates H2A (H2AX in humans) upon recruitment to a double-strand break (Downs *et al.*, 2000), which subsequently attracts additional chromatin remodeling machinery (Downs *et al.*, 2004). A relative of the GCN5-containing SAGA complex (SPT-ADA-GCN5 acetylase), STAGA (SPT3-TAF<sub>II</sub>31-GCN5L acetylase) contains components of the spliceosome as well as a nucleotide excision repair apparatus (Martinez *et al.*, 2001). PARPs are also activated by DNA strand breaks and have been implicated in DNA replication and recombination as well (Petermann *et al.*, 2005). As alluded to above, phosphorylation of the serine-10 residue in H3 is crucial for chromosome condensation and cell-cycle progression (Nowak and Corces, 2004). A variety of SWI/SNF ATPases have also been associated with having roles in DNA repair and recombination, as well as chromatin assembly (Lusser and Kadonaga, 2003; van Attikum and Gasser, 2005).

## Chromatin and disease

Chromatin remodelers are now recognized as essential for a wide variety of DNA processes, which offers increased understanding of several diseases. Profound developmental defects are observed in individuals with Rubinstein-Taybi syndrome and Coffin-Lowry syndrome, both of which are linked to deficiencies in chromatin remodeling machinery. Rubinstein-Taybi syndrome is caused by mutations in the histone acetylase CBP (Petrij *et al.*, 1995). Cells from Coffin-Lowry syndrome patients do not phosphorylate H3 following growth factor stimulation (Sassone-Corsi *et al.*, 1999).

Chromatin remodeling enzymes have been implicated in a wide variety of cancers (Davis and Brackmann, 2003). Leukemia can arise from abnormal fusion proteins produced after chromosomal translocations involving chromatin modifying enzymes (Yang, 2004). Consequently, HDAC inhibitors are being explored as potentially useful in cancer chemotherapy (Minucci and Pelicci, 2006). The human non-covalent remodeler BRG1, a SWI/SNF homolog, is mutated in tumor cell lines (Wong *et al.*, 2000).

Some viruses have been shown to hijack or interfere with chromatin remodeling machinery as well. HATs and HDACs are targets of numerous viruses aiming to modulate host cell acetylation to benefit the infectious process (Caron *et al.*, 2003). SWI/SNF members can also be manipulated by viral agents as evidenced by the ability of the adenovirus DNA-binding protein to bind SRCAP and inhibit CREB-mediated transcription (Xu *et al.*, 2001).

Perturbations in the methylation of cytosine residues of DNA has been linked to several neurodevelopmental disorders, including fragile X, ICF (immunodeficiency, centromere instability, facial anomalies), and Rett syndromes (Robertson and Wolffe, 2000). Recent studies argue that histone methylation and DNA methylation are intimately connected; thus anomalies in the former may have ramifications on the latter resulting in devastating gene expression defects (Ausio *et al.*, 2003; Geiman and Robertson, 2002).

Collectively, the above studies argue that elucidation of chromatin remodeling mechanics may put us in a better position to combat diseases associated with aberrant gene expression. However, a novel pharmacological application involving chromatin remodeling activities may be targeting those indigenous to parasitic protozoa. While these activities are conserved in protozoa, the mediators characterized so far contain striking peculiarities that may open a selective toxicity window (see below). The observation that apicidin, a natural product that has potent, broad-spectrum antiprotozoal activity, targets an HDAC lends support to this idea (Darkin-Rattray *et al.*, 1996). Like many protozoan parasites, *Toxoplasma* has a complex life cycle comprised of multiple life cycle stages. In order to transition between these multiple personalities, a reprogramming of the genome is requisite. It stands to reason that interference with the regulators of parasite gene expression may be profoundly detrimental to the welfare of the pathogen.

Finally, there is value in examining the regulation of gene expression in ancient eukaryotes like *Toxoplasma* besides finding potential ways to destroy it. Studying these processes in *Toxoplasma* may provide insight into the evolution of gene expression modulation. Indeed, one of the seminal contributions to the field emerged from the study of p55 in the ciliated protozoan *Tetrahymena*.



## The chromatin remodeling team in *Toxoplasma*

The completion of several apicomplexan genome sequencing projects has revealed unexpected features with respect to the regulation of gene expression in these parasitic eukaryotes. General (basal) transcription factors associated with the transcriptional pre-initiation complex have been reported in Apicomplexa (Callebaut *et al.*, 2005; Meissner and Soldati, 2005). However, an unexpected observation has been noted by several groups concerning the lack of specific transcription factors in these parasites (Aravind *et al.*, 2003; Templeton *et al.*, 2004) (Sullivan, unpublished observations). Such an observation is paradoxical given the complex life cycles of these organisms, whereby differential gene expression would be anticipated to entail tight control by transcription factors. Despite the paucity of known specific transcription factors, apicomplexans possess a rich repertoire of enzymes associated with epigenetics and chromatin remodeling (Meissner and Soldati, 2005; Sullivan and Hakimi, 2006). Considered together, it can be reasoned that apicomplexans employ an unusually streamlined gene expression regulatory system that relies heavily on epigenetics (Aravind *et al.*, 2003; Bozdech *et al.*, 2003), or transcription factors are present but unrecognizably divergent. Support for the latter idea was recently noted in a bioinformatics study revealing a complement of lineage-specific DNA-binding proteins harboring plant-like AP2 (Apetala2)-integrase domains in Apicomplexa (Balaji *et al.*, 2005). In addition, a novel Myb-related protein of the tryptophan cluster family has been characterized as a transcription factor in *Plasmodium* ssp. (Boschet *et al.*, 2004; Gissot *et al.*, 2005). Of course, these concepts are not mutually exclusive, and how chromatin remodeling operates in the context of putative apicomplexan transcription factors will be an exciting revelation. The near future is likely to reveal much more regarding apicomplexan transcription factors, and the recent progress made in delineating the chromatin remodeling complexes in these parasites may facilitate this goal.

## The histones of *Toxoplasma*

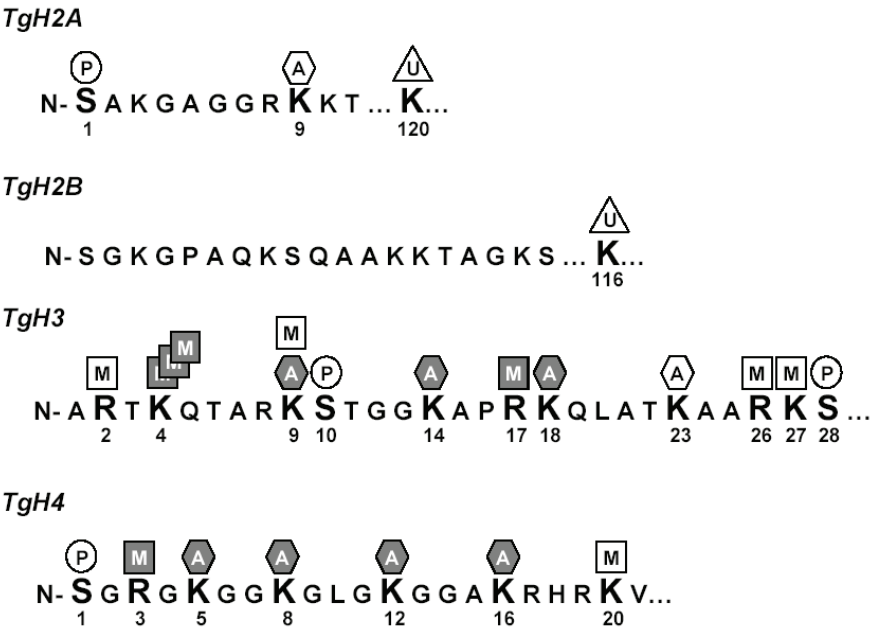
The fundamental subunit of chromatin is remarkably well conserved throughout eukaryotes and *Toxoplasma* is no exception. *Toxoplasma* possesses both H3 and the H3.3 variant present in actively transcribed genes (Sullivan, 2003). In the ToxoDB (ToxoDB.org), H3 corresponds to accession number 55.m00013; H3.3 to 38.00006. Surprisingly, no clear Cse4/CENP-A homolog is readily identifiable in the ToxoDB, although 42.m00059 is H3-like. *Toxoplasma* also possesses a well conserved version of H4 (49.m03134). An abundance of post-translational modifications have been documented for H3 and H4 in other species, and the absolute conservation of the N-terminal ends of TgH3 and TgH4 indicate that these modifications are likely to occur in the parasite (Figure 18.1).

Sequences of H2A and H2B are more variable amongst species. Like most other early eukaryotes, *Toxoplasma* has two variants in addition to the canonical H2A (AY631392, 55.m04926), namely homologs of H2AX (AY573602, 55.m04942) and H2AZ (AF502246, 145.m00002) (Dr. Sergio Angel, personal communication). Not surprisingly, there is no indication of metazoan macroH2A or H2ABBD present. The *Toxoplasma* genome also indicates that three variants of H2B exist, 25.m00008, 541.m02039, and 50.m03422 (Dalmaso *et al.*, 2006). The N-terminal tails of H2A and H2B exhibit variability between species, but some conserved features exist, making it possible that

similar modifications occur in *Toxoplasma*. For example, serine residue 1 of histone H2A (denoted as H2A [S1]) is conserved in TgH2A and TgH2AX and may be susceptible to phosphorylation as shown in other species. Numerous lysines are present in the N-terminal tails of TgH2A and TgH2B that may be acetylated. Finally, lysines shown to be subject to ubiquitination in other species are conserved in the C-terminal tails of TgH2A, TgH2AX, and each TgH2B homolog (Figure 18.1).

Similar to *Plasmodium* (Aravind *et al.*, 2003) and yeast, *Toxoplasma* does not appear to encode the linker histone H1 (Meissner and Soldati, 2005).

In general, there is a great deal of conservation in the *Toxoplasma* histones, including many of the residues known to be associated with at least one type of covalent modification. In later sections, we describe work that validates some of these modifications are indeed exercised by *Toxoplasma*, highlighting the ancient origin of these epigenetic marks and their role in the regulation of gene expression.



**Figure 18.1** Portions of *Toxoplasma* core histone tails and possible modifications. Shown are select amino acid sequences of the four canonical histones from *Toxoplasma*, along with post-translational modifications that have been demonstrated in other species. A shaded modification box above a residue denotes that it has been reported in *Toxoplasma* in an *in vitro* or *in vivo* study; all others are theoretical, based on analogy to other species. A=acetylation; M=methylation; P=phosphorylation; U=ubiquitination. The methyl boxes above H3 [K4] represent this residue may be di- and tri-methylated. There is absolute conservation in the H3 and H4 tails, but considerably less in H2A and H2B homologs. In human H2A, [K5] is also known to be acetylated. TgH2AX (not shown) has a lysine at position 5, but does not have a lysine at position 9. Human H2B can be acetylated at lysines 5, 12, 15, 20, but it is premature to assume the lysines in TgH2B are equivalent.

## The *Toxoplasma* HAT and HDAC families

### GCN5 HATs

The first apicomplexan HAT was discovered independently by two groups, who each cloned a GCN5 homolog from *Toxoplasma* (Hettmann and Soldati, 1999; Sullivan and Smith, 2000). Subsequently, a second, distinct GCN5 was identified in *Toxoplasma* (Bhatti *et al.*, 2006), termed TgGCN5-B (the original is referred to as TgGCN5-A). This was a surprising finding because no other invertebrate has been reported to have more than one GCN5 HAT. The pair of GCN5 HATs also appears to be unique to *Toxoplasma*, as no evidence for more than one GCN5 homolog exists in other apicomplexan genomes.

The HAT and bromodomains of apicomplexan GCN5s are well conserved. The most striking feature is the lengthy N-terminal extension, which varies in length and composition between apicomplexans (Bhatti *et al.*, 2006; Fan *et al.*, 2004b; Sullivan and Smith, 2000). Even the N-terminal extensions between TgGCN5-A and -B (~800 and 625 amino acids, respectively) are highly divergent. Most early eukaryotes, including the alveolate *Tetrahymena*, possess a GCN5 HAT that is comprised of little more than the catalytic region and bromodomain. The N-terminal extensions have no homology to known proteins and are bereft of known protein motifs. We have taken steps to elucidate the role(s) of these peculiar domains by expressing recombinant epitope-tagged forms of each TgGCN5 in *Toxoplasma* for subsequent purification. Using this system, we have found that each N-terminal extension is required for nuclear localization of the respective TgGCN5 (Bhatti *et al.*, 2006). Furthermore, we mapped a 6 amino acid nuclear localization sequence (RKRVKR) on TgGCN5-A that is necessary and sufficient to mediate nuclear translocation via a *Toxoplasma* homolog of importin alpha (TgIMP $\alpha$ ) (Bhatti and Sullivan, 2005). It remains possible that these extensions mediate other protein-protein interactions besides that with TgIMP $\alpha$ , perhaps relevant to the assembly of GCN5 complexes analogous to ADA and/or SAGA. We also employed our purified recombinant TgGCN5-A and -B (with or without their N-terminal extensions) in HAT activity assays. In each case, the N-terminal extension proved to be dispensable for *in vitro* HAT activity on free core histone substrates, but a noteworthy observation was made with respect to the substrate specificity for TgGCN5-A and -B.

TgGCN5-B is capable of acetylating H3 [K9], [K14], and [K18], which is the expected substrate profile for archetypical GCN5 HATs (Bhatti *et al.*, 2006). Surprisingly, TgGCN5-A exhibits a strong proclivity to acetylate H3 [K18] (Saksouk *et al.*, 2005). The HAT domain of each TgGCN5 is extremely well conserved, but four amino acid substitutions were noted downstream in TgGCN5-A that are not found in any other GCN5, including TgGCN5-B (Sullivan and Smith, 2000). Whether any or all of these substitutions explains the disparate substrate capabilities remains to be investigated.

The ADA2-binding domain of GCN5 is found between the HAT and bromodomain. ADA2 is a co-activator protein found in all GCN5-containing complexes, and two ADA2 homologs have been cloned in *Toxoplasma*, termed TgADA2-A and TgADA2-B (Bhatti *et al.*, 2006). This finding is in contrast to other apicomplexan parasites and early eukaryotes, which possess only one ADA2 homolog (Bhatti *et al.*, 2006; Fan *et al.*, 2004a). The unique occurrence of two ADA2 homologs in *Toxoplasma* may be a consequence of

its harboring a pair of GCN5s. We examined whether the two TgGCN5s, which exhibit considerable differences in their ADA2-binding domains, interact differentially with the two TgADA2s. By yeast two-hybrid testing, we observed that TgGCN5-A can only interact with TgADA2-B, whereas TgGCN5-B can interact with both TgADA2s (Bhatti *et al.*, 2006).

Considered together, the data thus far demonstrates that TgGCN5-B is able to fulfill the HAT-mediated obligations of TgGCN5-A, but TgGCN5-A could not replace TgGCN5-B. Given their attributes and the intron/exon structure of the genomic loci (Bhatti *et al.*, 2006), we hypothesize that TgGCN5-A is a gene duplication of TgGCN5-B, possibly adapted to exercise tighter control of a select set of parasite-specific genes dealing with invasion of the host cell. If true, this could be an example of convergent evolution mimicking the dual GCN5 HATs observed in vertebrates.

### MYST HATs

*Toxoplasma* possesses two other HATs that have unequivocal homology to those classified in the MYST family, termed TgMYST-A and -B (Smith *et al.*, 2005). More specifically, each is of the “MYST + CHD” subtype (Utley and Cote, 2003), meaning they harbor a chromodomain (CHD) upstream of the MYST HAT catalytic domain. Each also possesses the conserved cysteine-rich zinc finger CxxCx12HxxxC, which has been shown to interact with the globular region of the nucleosome core and is required for enzyme activity (Akhtar and Becker, 2001). More extensive characterization was carried out on TgMYST-A. The transcript contains two possible ATG start sites that are in-frame with one another, thus potentially encoding for two forms of this protein which would be identical except for the addition of 60 amino acids at the N-terminus. Both the “long” and “short” forms have a preference to acetylate H4, and can do so at any of the four lysine residues in the H4 N-terminal tail (Smith *et al.*, 2005). The histone acetylation profile is consistent with that observed for other MYST family HATs. Western analysis using a peptide antibody (anti-TgMYST-A) demonstrates that the shorter TgMYST-A form is more predominant relative to the longer form, and both are more abundant in tachyzoites relative to bradyzoites (Smith *et al.*, 2005).

Attempts to disrupt the TgMYST-A genomic locus by homologous recombination have not been successful (Smith *et al.*, 2005). While it has not been formally tested if the gene is truly essential, the result is consistent with the fact that the yeast homolog (*Esa1*) is essential. It is also not possible to generate a stable transgenic clone over-expressing TgMYST-A unless the HAT domain is rendered inoperable by a point mutation (Smith *et al.*, 2005). Together, these studies argue that steady-state levels of MYST-A in the parasite must be tightly regulated.

Comparative bioinformatics analysis suggests that *Cryptosporidium* also has two MYST + CHD type HATs, whereas both *Plasmodium* and *Theileria* have only one. Like other early eukaryotes, apicomplexans appear to harbor only MYST + CHD HATs, lacking those reported in more sophisticated organisms, such as HBO1, MOZ, and MORE.

### Other HATs

As one may expect, *Toxoplasma* does not appear to contain homologs of HATs restricted to metazoans, such as p300/CBP and the p160 family of nuclear receptor co-activator HATs. However, Table I lists some additional putative HAT homologs found through bioinformatics analysis using the ToxoDB. We have observed predicted open reading frames encoding potential homologs to Hat1 and Elp3. Hat1 is a universally conserved type-B (cytoplasmic) HAT that targets H4 in a histone-deposition related manner, and Elp3 (ELongation Protein 3) is involved in transcriptional elongation in many eukaryotes. There do not appear to be any clear homologs of yeast Hpa2, Hpa3, or Nut1, whose roles have yet to be characterized.

Some components of TFIID have intrinsic HAT activity, but there is a striking lack of TAFs (TATA-binding protein Associating Factors) in Apicomplexa (Callebaut *et al.*, 2005; Meissner and Soldati, 2005). One exception may be TAF1 (human TAF250/yeast TAF145), for which a putative homolog in *Plasmodium* has been noted *in silico* (PFL1645W) (Callebaut *et al.*, 2005). A *Toxoplasma* gene prediction also possesses a modest match to TAF1 and PFL1645W, predicted to encode a large (2699 amino acid) protein containing a C2HC zinc finger and bromodomain (Table 18.1).

### HDACs

Opposing the HATs are the HDACs, capable of removing the acetyl groups from histones and consequently diminishing transcriptional potency in that region. The first apicomplexan HDAC was identified in *Plasmodium*, as PfHDAC1 (Joshi *et al.*, 1999). PfHDAC1 most resembles an Rpd3 HDAC (class I). A class III HDAC was recently discovered in *Plasmodium* as well, designated PfSir2 (Freitas-Junior *et al.*, 2005). Bioinformatics analysis suggests that *Toxoplasma* has six HDACs: three are class I, two are class II, and one is class III (Table 18.1). By way of comparison, humans have no fewer than 11 class I/II HDACs and at least 7 class III HDACs.

TgHDAC3 (ToxoDB accession number 42.m00014) was the first HDAC to be cloned and characterized in *Toxoplasma* (Saksouk *et al.*, 2005). Recombinant epitope-tagged TgHDAC3 is capable of nuclear localization and exhibits potent histone deacetylase activity that is repressed by known HDAC inhibitors (Saksouk *et al.*, 2005). TgHDAC3 has been co-purified with a number of proteins typically found in co-repressor complexes containing N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors) in higher eukaryotes, such as TgTBL1 (transducin beta-like protein), HSP70-like proteins, and TriC ring complex subunits (Saksouk *et al.*, 2005). While no homologs of N-CoR or SMRT exist in Apicomplexa, several apicomplexan-specific proteins of unknown function were co-purified with TgHDAC3 that deserve further attention.

### Histone methyltransferases

Histone methyltransferases have recently been identified in Apicomplexa: TgCARM1 (Co-activator associated ARGinine Methyltransferase), a homolog of human CARM1/PRMT4, and TgPRMT1. Consistent with their human counterparts, TgCARM1 methylates H3 [R17] and TgPRMT1 methylates H4 [R3] (Saksouk *et al.*, 2005). Attempts to

**Table 18.1** Histone modifying homologs in *Toxoplasma* and *Plasmodium*

Pro/domain	Tg homolog	GenBank/comments	Pf homolog
Histone acetyltransferases			
Gcn5	52.m00008	AAF29981, TgGCN5-A	–
	49.m03346	AAW72884, TgGCN5-B	PF08_0034, AAR88436
MYST	641.m01473	AY578183, TgMYST-A	PF11_0192
	23.m00146	DQ104220, TgMYST-B	–
	Elp3	541.m01178	PFL1345c
TAF1/250/145	64.m00349	ZnF, bromodomain	PFL1645w
Hat1	83.m02144		PFD0795w
Histone deacetyltransferases			
HDAC	42.m00014	TgHDAC3, AAY53803	PFI1260c, AAD22407
	50.m03318	–	
	55.m04722	–	
	74.m00425	PF10_0078	
	20.m03703	Ankyrin repeats	PF14_0690
Sir2	42.m00081		PF13_0152
Arginine methyltransferases			
Hmt1	38.m00018	AY820756, TgPRMT1	PF14_0242
	83.m01256	AY820755, TgCARM1	–
	52.m01547	TgPRMT3	–
	83.m01208	TgPRMT2	–
Skb1/PRMT5	33.m01376	TgSkb1/TgPRMT5	PF13_0323
Lysine methyltransferases			
SET domain	86.m00376	SET1	–
	27.m00875		PFD0190w
	641.m01529		MAL13P1.25
	50.m03139	MYND-type ZnF	–
	583.m05442		–
	42.m03532	HsMLL	PFF1440w
	55.m04720	DmAsh1	MAL13P1.122
Lysine demethyltransferase			
Lsd1/BHC110	63.m00151		MAL8P1.154
	49.m03290		PFL0575w
JmjC	542.m00225		MAL8P1.111



**Table 18.1** Continued

Pro/domain	Tg homolog	GenBank/comments	Pf homolog
	76.m01561		–
	42.m05839		–
	55.m04927		–
Histone kinases			
Snf1	44.m02822		PF14_0516
	80.m02306		–
Histone phosphatase			
PP1	583.m05380	PF14_0142	
ADP-ribosylation			
PARP domain	59.m03538		–
PARG domain	72.m00001		–
	55.m05011		–
Histone ubiquitination/ sumoylation			
Ubiquitin ligase	35.m00006		PF08_0085
	55.m05059		PFE0835w
Ubc9	25.m01826		PFI0740c

Pf (*Plasmodium falciparum*) homologs represent the top hit resulting from a BLASTp analysis of “Pf annotated proteins” at PlasmoDB.org using predicted Tg (*Toxoplasma gondii*) protein sequences. “–” denotes that no homologs were detected or it was a repeat with a lower score; this is not meant to imply that Pf lacks a homolog, but simply that it was not detected by this methodology. Hs=*Homo sapiens*; Dm=*Drosophila melanogaster*. ZnF=zinc finger.

generate a knockout clone of TgCARM1 have been unsuccessful, implying that it may be essential, as noted previously in mice, which die perinatally without CARM1 (Yadav *et al.*, 2003).

Probing of the *Toxoplasma* genomic database with yeast HMT1 reveals two more homologs that may be capable of histone arginine methylation, and a homolog to human Skb1/PRMT5 is also present (M.A. Hakimi, personal communication; Table 18.1). The SET domain (PF00856) is a common element in lysine methyltransferases, some of which target histones (Zhang and Reinberg, 2001). Searching ToxoDB for predicted proteins harboring a SET domain yields 7 hits (Table 18.1). Gene prediction 42.m03532 predicts a very large protein of 7551 amino acids, containing multiple C1 and PHD finger domains and a bromodomain in addition to the SET domain. BLASTp searches indicate this may be a *Toxoplasma* homolog of mammalian Mixed Lineage Leukemia (MLL), linked to transcriptional activation through methylation of H3 [K4]. 55.m04720 is most similar to *Drosophila* Ash1, involved in transcriptional activation via methylation of H3 [K4] and [K9], and H4 [K20] (Beisel *et al.*, 2002). 50.m03139 contains a MYND-type zinc finger, associated with transcriptional silencing and recruitment of HDACs. Finally, *Toxoplasma*

possesses homologs of both types of histone lysine demethylases previously identified in other eukaryotes (Table 18.1).

#### Additional covalent histone modifications in *Toxoplasma*

No published reports exist to date that describe other covalent histone modifications in Apicomplexa. However, the *Toxoplasma* genome sequence suggests that machinery does exist in the parasite to execute these functions (Table 18.1). With regard to potential kinases phosphorylating histones, *Toxoplasma* is predicted to have two Snf1 homologs. Snf1 is associated with the phosphorylation of H3 [S10], a mark of transcriptional activation that works in concert with GCN5 (Lo *et al.*, 2001). Also present are several predicted proteins with significant similarity to phosphatases such as PP1, shown to dephosphorylate histones (Hsu *et al.*, 2000).

By searching the ToxoDB predicted proteins with Pfam domains, we were able to detect one PARP and two PARG candidates (Table 18.1). Whether these enzymes play a role in adding or removing ADP-ribose moieties on histones remains to be verified. Finally, *Toxoplasma* contains several unequivocal homologs of ubiquitin-conjugating enzymes, including Rad6. Ubiquitin carboxyl-terminal hydrolase homologs, similar to Ubp8 and Ubp10 are also present. The strongest match to Ubc9, which mediates histone sumoylation in yeast, is 25.m01826. In summary, *Toxoplasma* contains strong candidates for all of the histone modification machinery reported to date in other eukaryotes.

#### SWI2/SNF2 homologs in *Toxoplasma*

A handful of published reports illustrate that non-covalent chromatin remodelers are also present in the Apicomplexa. The first instance of a SWI2/SNF2 type remodeler was identified in *Plasmodium falciparum* (PfSNF2L), and it resembles the ISWI subclass that contains a SANT domain in addition to the ATPase domain (Ji and Arnot, 1997). Second, a SWI2/SNF2 was cloned from *Toxoplasma* that most closely resembles human SRCAP, hSRCAP (Sullivan *et al.*, 2003). Homologs to this factor (called TgSRCAP) were also noted in *Plasmodium* and *Cryptosporidium* databases (Sullivan *et al.*, 2003). Additional bioinformatics analysis suggests that *Cryptosporidium* and *Plasmodium* have 14 and 11 SWI2/SNF2 ATPases, respectively (Templeton *et al.*, 2004).

#### SRCAP

TgSRCAP best matches the newest strata of the SWI2/SNF2 superfamily, referred to as the Ino80 type (after the yeast homolog). The distinguishing feature of this subclass is the lengthy insert region (or spacer) separating ATPase subdomains IV and V (the DEXDc and HELICc portions). In typical SWI2/SNF2 members, this region is limited to ~160 amino acids, but varies from ~600 to < 1200 amino acids in the members of the Ino80 subclass. The function of the insert is unknown, but has been shown to bind the CBP HAT in hSRCAP. Moreover, hSRCAP enhances CREB-mediated transcription by virtue of its interaction with CBP (Johnston *et al.*, 1999). Outside the ATPase domain, including the intervening spacer region, TgSRCAP bears little homology to hSRCAP or the other Ino80 class members. But surprisingly, TgSRCAP could still specifically enhance CREB-mediated expression in the presence of CBP when co-transfected into HeLa cells (Sullivan

*et al.*, 2003). Genomic sequencing has failed to reveal any CREB or CBP in Apicomplexa, so the role of TgSRCAP in the context of parasite physiology remains unclear.

One clue regarding the role of TgSRCAP in the parasites is that mRNA levels for this protein increase during *in vitro* differentiation, suggesting a possible role in stress and/or differentiation pathways (Sullivan *et al.*, 2003). A yeast two-hybrid screen was performed in attempt to isolate protein(s) that may associate with the spacer region of TgSRCAP and revealed several parasite-specific candidates, some of which have features such as zinc fingers and DNA-binding domains typically found on proteins involved in transcription (Nallani and Sullivan, 2005). The further investigation of TgSRCAP is important; as with many other chromatin remodelers, the TgSRCAP locus is not amenable to disruption and hence may be essential (Sullivan, unpublished).

#### Other SWI2/SNF2s

A Pfam search of ToxoDB for the Snf2\_N domain reveals 16 predicted proteins containing possible SWI2/SNF2 homology. Further analysis shows *Toxoplasma* harbors at least one type of SWI2/SNF2 in each of the four known subclasses (Snf2, Iswi, Mi-2, Ino80). *Toxoplasma* appears to have two strong homologs of the founding member Snf2, but bromodomains are not evident in any of the gene product predictions (Table 18.2). As noted for *Plasmodium*, *Toxoplasma* is also in possession of an Iswi homolog, and in fact may have two, one with DNA-binding AT hooks and another with the conventional SANT domain. We could detect one predicted protein harboring chromodomains in addition to the ATPase domain, making it a probable Mi-2 ortholog.

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### Chromatin remodeling and parasite physiology

#### Antigenic variation

Antigenic variation has not been shown to occur in *Toxoplasma*, but is a biological process critical to *Plasmodium falciparum* and other protozoan pathogens like *Trypanosoma brucei*. Antigenic variation in both of these parasites involves epigenetic regulation, and disruption of the Sir2 HDAC in *Plasmodium* leads to a loss of control in *var* gene expression (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005; Navarro *et al.*, 1999).

#### Gene expression regulation

A significant advance in the study of epigenetics and chromatin remodeling in Apicomplexa is the application of chromatin immunoprecipitation (ChIP). ChIP protocols have recently been developed for *Plasmodium* and *Toxoplasma* (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005; Saksouk *et al.*, 2005). ChIP is a powerful technique that studies DNA–protein interactions, and can be applied to monitor the histone modification profile of select promoters (Das *et al.*, 2004). Another significant advance in the study of epigenetics and chromatin remodeling in Apicomplexa is the use of fluorescence *in situ* hybridization (FISH). FISH has been used to study the regulation of *Plasmodium falciparum* *var* gene expression, helping to demonstrate that subnuclear regions impact transcriptional activity (Figueiredo *et al.*, 2002; Ralph *et al.*, 2005).

**Table 18.2** SWI2/SNF2 homologs in *Toxoplasma* and *Plasmodium*

Pro/domain	Tg homolog	GenBank/comments	Pf homolog
Snf2	641.m01573		PFB0730w
	65.m01174		–
Iswi	59.m00063	AT hook	PF11_0053
	645.m00313	SANT	–
Mi-2	55.m04750	CHD	PF10_0232
Ino80	72.m00005	AAL29689, TgSRCAP	PF08_0048
SNF2 domain	80.m02309		–
	65.m01101	PF13_0308	
	641.m01484	ZnF C3HC4 (RING)	MAL13P1.216
	46.m01723	PHD fingers	PFF1185w, CAG25058
	50.m03086		–
	42.m00128	ZnF C3HC4 (RING); UBA and SAP domains	PFL2440w
	44.m02545		MAL8P1.65
	44.m02726		PF08_0126
	55.m05039		–
	59.m03701		–

\*See legend for Table 18.1. CHD = chromodomain.

We have begun exploring *Toxoplasma* epigenetics using ChIP to chart marks of gene activation and silencing (Saksouk *et al.*, 2005). Since the *Toxoplasma* H3 and H4 N-terminal tails are identical to their mammalian counterparts, many of the commercially available antibodies raised to specific histone modifications can be applied in *Toxoplasma* research. Antibodies to acetylated H3 and H4 have been used in *Toxoplasma* ChIP assays to show that these modifications are a mark of gene expression in the parasite. Similar to the observations made with Sir2 in *Plasmodium*, histone deacetylation correlates with transcriptional inactivation in *Toxoplasma*. Antibodies raised against methylated H3 [R17] demonstrate this modification to also be a mark of gene transcription in *Toxoplasma*. This mark is particularly intriguing since it was found to occur in conjunction with acetylated H3 [K18]. This may be the first example of histone modification “crosstalk” in protozoa. Such an interaction was reported in human cells where p300/CBP acetylates H3 [K18] to facilitate recruitment of CARM1 to methylate H3 [R17] (Daujat *et al.*, 2002). Antibodies raised to di- or tri-methylated H3 [K4] were also examined as indicators of gene activity in *Toxoplasma*; as seen before in yeast, di-methylation of this residue is seen at both inactive and active genes, whereas tri-methylation is observed exclusively at active genes (Saksouk *et al.*, 2005; Santos-Rosa *et al.*, 2002). A summary of other potential epigenetic marks is shown in Figure 18.1.

We have initiated studies to determine the impact of each TgGCN5 on parasite viability. In yeast, its single GCN5 is not essential, but null mutants show retarded growth on nutrient deprived media. Differential gene expression studies performed with the null yeast GCN5 mutant implicate this HAT in the control of amino acid and vitamin synthesis genes as well as other stress response genes (Huisinga and Pugh, 2004). Mammals possess a pair of GCN5 HATs, termed GCN5 and PCAF. Mice lacking GCN5 are embryonic lethal, but those lacking PCAF do not have a detectable phenotype, leading to the argument that GCN5 can compensate for a loss of PCAF (Xu *et al.*, 2000; Yamauchi *et al.*, 2000). We generated a viable TgGCN5-A “knockout” parasite clone ( $\Delta$ TgGCN5-A) by disrupting the genomic locus, and similar studies are under way to do the same for TgGCN5-B. *Toxoplasma* lacking TgGCN5-A do not display any obvious phenotype *in vitro* or in an *in vivo* mouse model, consistent with the possibility that TgGCN5-B may be able to compensate for TgGCN5-A (Bhatti *et al.*, 2006). Proteomics analysis of  $\Delta$ TgGCN5-A and wild-type has revealed that several proteins involved in invasion are depressed in the former; however, no discernable impact on host cell invasion is evident *in vitro*, possibly due to redundancy in the pathways governing parasite invasion (Bhatti *et al.*, 2006). The availability of comprehensive microarrays for *Toxoplasma* in the near future will facilitate differential gene expression studies between  $\Delta$ TgGCN5-A and wild-type parasites under various conditions.

### Parasite differentiation

ChIP can also be used to determine if a particular chromatin remodeling enzyme is present at a given promoter or target gene. Stable transgenic clones expressing epitope-tagged TgGCN5-A and TgHDAC3 were used to show that these chromatin remodelers oppose one another in a stage-dependent manner (Saksouk *et al.*, 2005). As mentioned, histone acetylation/deacetylation are marks of gene activation/inactivation in *Toxoplasma*, as in other eukaryotes. This role has been verified in the context of parasite differentiation; promoter regions of tachyzoite-specific genes (e.g. SAG1 and SAG2A) contain acetylated H3 and H4 during the tachyzoite stage of the life cycle, but bradyzoite-specific genes (e.g. BAG1) are hypoacetylated. Under bradyzoite growth conditions *in vitro*, acetylation of tachyzoite promoters is diminished while acetylation of bradyzoite promoters is increased (Saksouk *et al.*, 2005). Constitutively expressed genes (e.g. TUB and DHFR) exhibit acetylated H3 and H4 during both tachyzoite and bradyzoite stages.

To directly examine if TgGCN5-A takes part in this stage-specific acetylation, ChIP was performed using an antibody to the epitope tag fused to TgGCN5-A. In tachyzoites, TgGCN5-A is directly associated with tachyzoite-specific gene promoters, but absent at bradyzoite-specific promoters. In contrast, ChIP of TgHDAC3 reveals that it is associated with bradyzoite promoters during the tachyzoite stage of the life cycle, and is not bound to tachyzoite promoters (Saksouk *et al.*, 2005).

Specific antibody generated to recognize TgCARM1 was employed in ChIP to show that it specifically mediated methylation of H3 [R17] associated with transcriptional activation in *Toxoplasma* (Saksouk *et al.*, 2005). TgCARM1 appears to mediate this histone modification in both tachyzoites and bradyzoites.

The impact of TgSRCAP on parasite differentiation is incompletely characterized. Consistent with observations of the EST databases, TgSRCAP mRNA is more abundant in developing bradyzoites (Sullivan *et al.*, 2003). Whether this implies a role of TgSRCAP in the process will be interesting to determine.

For more details on the mechanism of tachyzoite to bradyzoite differentiation and regulation of stage-specific gene expression refer to Chapters 16 and 17.

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## Concluding remarks and future directions

Research in the field of chromatin dynamics has exploded in the last decade, but the exploration of chromatin-related phenomena in apicomplexan parasites is still in its infancy. Now that we have had a glimpse into this new terrain, it is obvious there is a lot of novelty that warrants further investigation. The development of powerful techniques such as ChIP for *Plasmodium* and *Toxoplasma* promises to greatly advance this arena of research. Furthermore, comprehensive microarrays for *Toxoplasma* will be available in the near future, allowing ChIP-on-chip studies to be performed that may enable discovery of distinct clusters of genes associated with particular histone modifications in different life cycle stages. The establishment of inducible knockouts in *Toxoplasma* (Meissner *et al.*, 2002) may be employed to facilitate the study of the numerous chromatin remodelers that appear to be essential for parasite welfare.

Given that chromatin remodelers are associated with mediating changes in gene expression pertinent to stage cycle conversion, it is of interest to determine how differentiation is affected if these remodelers are inhibited or knocked out. However, the only chromatin remodeler amenable to disruption thus far has been TgGCN5-A. The  $\Delta$ TgGCN5-A clone does not appear to show any defects, but was made in the highly virulent RH strain, which is not optimal for studying stage conversion. Moreover, it is probable that TgGCN5-B can compensate for the loss of TgGCN5-A. Some recent progress has been made in the development of HAT inhibitors and it will be of interest to determine if they have an observable effect on *Toxoplasma* (Schafer and Jung, 2005). Similarly, apicidin exhibits broad-spectrum activity against apicomplexans at low nanomolar range and targets a parasite HDAC (Darkin-Rattray *et al.*, 1996). Pharmacological interference with histone modifying enzymes could be a novel therapeutic approach to combating apicomplexan diseases.

Another high priority will be the identification of the parasite proteins associating with the chromatin remodeling enzymes. In other eukaryotes, chromatin remodeling enzymes are typically found in massive multi-protein complexes, whose composition may vary in different environmental conditions or developmental stages. Preliminary bioinformatics analysis suggests that many of the well conserved components of these chromatin remodeling complexes do not have homologs in apicomplexan parasites (Sullivan, unpublished observations). Identification of these unique parasite components may help identify the elusive transcription factors, and ultimately connect signal transduction events to the bouncers guarding the doors of gene expression. As these factors are likely to be divergent, they may also represent novel drug targets.



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## Abstract

Acquiring appropriate amounts of suitable lipid species to proper cell compartments is imperative for maintaining the various functions of biological membrane systems. During the intracellular development of *Toxoplasma gondii*, many membrane-containing organelles including the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, apicoplast, dense granules, rhoptries, micronemes, acidocalcisomes, pellicular complex, and the ever-enlarging parasitophorous vacuolar membrane, are formed. Elaborate lipid metabolism and trafficking are required for *T. gondii* replication and persistence in their mammalian hosts. These parasites meet their high demand for the necessary lipid species through synthesis from metabolites produced *de novo* as well as through diversion of prefabricated molecules from host exogenous sources. Unique peculiarities in lipid biosynthetic pathways and in mechanisms of lipid trafficking from host cells to intravacuolar *T. gondii* are likely to provide us with important new therapeutic discoveries in the future.

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## The background

From a mere trickle 10 years ago the flow of publications on lipid metabolism in protozoan parasites has now swollen such that over 100 articles on this topic have appeared in the literature in the last 3 years. There are three main reasons for the current level of interest. First is the recent development of powerful tools to detect and characterize lipids. Novel analytic approaches—in particular, liquid chromatography and mass spectrometry—for lipidomics allow to obtain “lipid profiles” that contain information on the composition and abundance of individual lipids present in a complex lipid mixture (Wenk, 2005). Second is the realization that protozoa have uncommon lipids that can serve as signatures of these pathogens. These findings indicate that unique enzyme systems are operational for the biosynthesis of these specific lipids. Third, and derived from the second observation, is the prospect that parasite lipid metabolic pathways may abound in valid drug targets. Indeed, much recent work has been stimulated by the idea that the sterol and fatty acid biosynthetic pathways are being successfully exploited as antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa (Roberts *et al.*, 2003; Sonda and Hehl, 2006).

Metabolic maps for lipid synthesis in *Toxoplasma gondii* have been recently delineated using bioinformatic tools, e.g. the Kyoto Encyclopedia of Genes and Genomes framework, and have concomitantly revealed parasite auxotrophies for several lipid species. The unique

characteristics of *T. gondii*'s lipid metabolism have attracted a lot of attention with respect to the basic lipidology and have afforded novel targets in lipid biosynthetic pathways for therapeutic intervention. The available data summarized in this chapter represent a stimulating starting point for intensified research on *T. gondii* lipid synthesis, uptake and trafficking. Special attention will be brought to fatty acids as unique models of lipids synthesized by the parasites, and to cholesterol as a prime example of lipids diverted from host mammalian organelles.

## Fatty acids

As a central component of most lipids, the biosynthesis of fatty acids is a critical anabolic pathway in most organisms. In addition to being the major component of membranes, fatty acid lipids are important energy storage molecules, and fatty acyl derivatives have been found to possess a variety of physiological functions. The fundamental process of fatty acid biosynthesis is highly conserved among species. The key feature is the sequential extension of an alkanolic chain, two carbons at a time, by a series of decarboxylative condensation reactions. This process is generally initiated with the carboxylation of acetyl-CoA to yield malonyl-CoA (Smith *et al.*, 2003). The malonate group of malonyl-CoA is transferred to the phosphopantetheine prosthetic group of a small acidic protein called the acyl carrier protein (ACP). Malonyl-ACP is then condensed with acetyl-CoA, reduced, dehydrated, and reduced once again, finally yielding an acyl-ACP. The elongation of the chain occurs by condensing another malonyl-ACP with the acyl-ACP and repeating the reaction cycle.

In nature, there are two basic types of fatty acid synthesis (FAS) architectures. The prototypical FAS I is found in vertebrates and fungi. This pathway is an associated system since it consists of a single gene that produces a multifunctional protein, which contains all of the reaction centers required to produce a fatty acid molecule (Smith *et al.*, 2003). By contrast, plants, bacteria and lower eukaryotes such as yeast and some protozoa, contain two genes that are implicated in fatty acid production, and whose polypeptide products coalesce to form a multifunctional complex (White *et al.*, 2005). This FAS II is a dissociated system wherein each component is encoded by a separate gene that produces a unique protein, which catalyzes a single step in the pathway.

The type I FAS is thought to have evolved by the fusion of a type II complex into a single protein. The multifunctional protein of FAS I is localized in the cytosol. In plants, FAS II takes place in the plastid (chloroplast) since it is derived from a cyanobacterial endosymbiont. The genes for these enzymes are all encoded in the nuclear genome, and the proteins are post-translationally targeted to the plastid, as is common with plastid enzymes in plants and algae (McFadden, 1999). To some extent, FAS can also take place in various subcellular organelles from mammalian cells. The eukaryotic extracytosolic FAS, e.g. intramitochondrial is similar to the prokaryotic type of FAS II in terms of having a set of separate monofunctional enzymes (Miinalainen *et al.*, 2003; Zhang *et al.*, 2003).

FAS I is usually considered to be a more efficient biosynthetic machinery than FAS II because the enzymatic activities are fused into a single polypeptide template and the intermediates do not diffuse from the complex. However, FAS I produces only palmitate, while FAS II is capable of producing a large diversity of fatty acids with different chain lengths. Unsaturated fatty acids, iso- and anteisobranched-chain fatty acids, and hydroxy

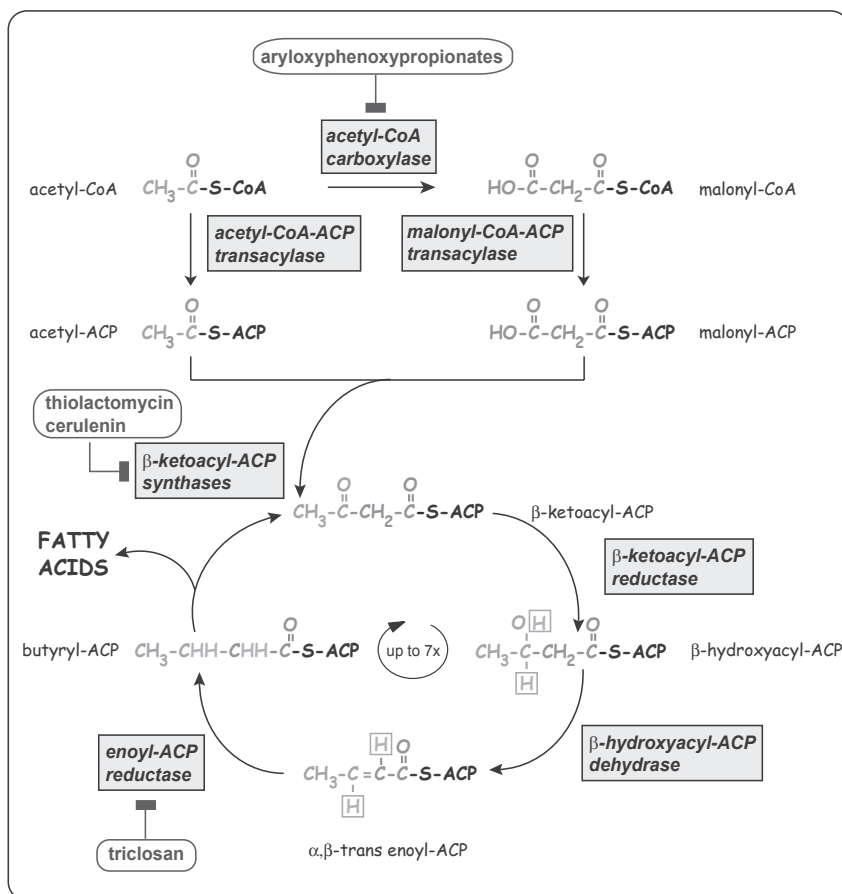
fatty acids are generated by FAS II. In addition, FAS II intermediates are used in the synthesis of key cellular constituents, such as lipoic acid and quorum-sensing molecules. This enormous diversity of products is possible because the ACP intermediates in the type II pathway are diffusible entities that can be diverted into other biosynthetic pathways. Additional information regarding FAS can also be found in Chapter 22.

## Synthesis

A rich content of unsaturated fatty acid chains is reported in *T. gondii* (Gallois *et al.*, 1988), but the global composition in fatty acids remains largely unknown in the parasite. While organisms generally rely on one FAS pathway, either FAS I or FAS II, *Toxoplasma*, surprisingly, harbors all enzyme activities for FAS I (David Roos, personal communication) and for FAS II (reviewed in Seeber *et al.*, 2003). The apicoplast, a secondary chloroplast-like organelle, contains enzymes of the type II complex (Figure 19.1), ascribing a central role of this organelle for *de novo* fatty acid production. Acetyl-CoA is carboxylated to form malonyl-CoA by an acetyl coenzyme A carboxylase (ACC), using bicarbonate as a source of the carboxyl group, biotin as a cofactor, and ATP as a source of energy (Jelenska *et al.*, 2001). Indeed, ACC consists of three major functional domains: the biotin carboxylase domain, the carboxyltransferase domain, and the biotin carboxyl carrier domain containing covalently attached biotin. The first step of the ACC-catalyzed reaction is an ATP-dependent transfer of the carboxyl group from bicarbonate to the biotin residue (first half-reaction). The carboxyl group is then transferred to acetyl-CoA producing malonyl-CoA (second half-reaction). Malonyl-CoA is used for *de novo* fatty acid biosynthesis as well as in fatty acid elongation. Indeed, incubation of radiolabeled malonyl-CoA with *T. gondii* extracts results in the production of palmitate.

ACC is encoded in the nucleus, synthesized in the cytosol before transport to the plastid through a N-terminal transit peptide (Jelenska *et al.*, 2001). The ACC compartmentalization in the apicoplast raises the question of the source of acetyl-CoA used for FAS II. Since acetyl-CoA cannot cross membranes, this molecule has to be produced *de novo* in the apicoplast or has to be transported as another metabolite that could easily be converted into acetyl-CoA. One plausible scenario may involve the transport of phosphoenolpyruvate to the apicoplast via the phosphoenolpyruvate phosphate translocator/triose phosphate/phosphate translocator system, followed by the conversion of phosphoenolpyruvate to pyruvate by a pyruvate kinase. A pyruvate dehydrogenase complex can then generate acetyl-CoA from pyruvate (Ralph *et al.*, 2004; see Chapter 22). Intriguingly, *T. gondii* expresses a second most probably cytosolic ACC, although it possesses the multi-domain type of the ACC prototype found in the cytoplasm of eukaryotes and in plastids of some plants (Jelenska *et al.*, 2001).

Subsequently to the ACC activity, acetyl-CoA and malonyl-CoA are transferred to an ACP by the actions of acetyl-CoA:ACP transacylase and malonyl-CoA:ACP transacylase (FabD), respectively.  $\beta$ -Ketoacyl-ACP is then synthesized from acetyl-ACP and malonyl-ACP by a  $\beta$ -ketoacyl:ACP synthase (FabH).  $\beta$ -Ketoacyl-ACP is reduced by  $\beta$ -ketoacyl:ACP reductase (FabG) to form  $\beta$ -hydroxyacyl-ACP which is dehydrated by  $\beta$ -hydroxyacyl:ACP dehydrase (FabZ) to form  $\alpha,\beta$ -*trans* enoyl-ACP. This is further reduced to butyryl-ACP by the action of enoyl:ACP reductase (FabI). This cycle occurs up to seven



**Figure 19.1** Pathway of type II *de novo* fatty acid biosynthesis in the *T. gondii* apicoplast. The substrate for FAS II, malonyl-CoA, is formed from acetyl-CoA by ACC. Fatty acid elongation consists of rounds of priming ACP with a malonyl moiety followed by condensation, reduction, dehydration, and reduction reactions that add two carbons to the growing acyl chain in each round. So far three major enzymes of *T. gondii* FAS II, acetyl-CoA carboxylase,  $\beta$ -ketoacyl-ACP synthases and enoyl-ACP reductase are promising drug targets. See also Plate 19.1.

times in *T. gondii*. ACP plays a central role in fatty acid biosynthesis by holding the forming acyl chain, whereas FabH and FabZ are involved in the condensation and dehydration steps, respectively, of acetyl addition during acyl chain elongation. Similarly to ACC, ACP and FabH, FabF, and FabI are localized to the apicoplast despite the presence of an unusual “internal” signal peptide in FabH (Waller *et al.*, 1998).

Concerning the FAS I pathway, gene structures reveal the existence of a single large polypeptide that harbors the ACP, FabD, FabH, FabG, FabZ and FabI activities and that is presumably associated with *T. gondii*'s mitochondrion (D. Roos, personal communication) or in a fraction of the cytoplasm enclosed therein (Kohler, 2006). Palmitate is the main

product of parasite FAS I but fatty acids with chains containing more than 16 carbons are also generated. FAS I might provide bulk products to apicoplast FAS II, hence contributing to fatty acid elongation rather than initial lipid synthesis.

## Uptake

*T. gondii* is capable to import some fatty acids from the host cell (Tomavo *et al.*, 1989; Quittnat *et al.*, 2004). Exogenous fatty acids may be used directly for incorporation into complex lipids (see below) or degraded all the way to acetyl-CoA via mitochondrial  $\beta$ -oxidation (see Chapter 22 for more details). Intravacuolar *T. gondii* accumulate the fluorescent fatty acid analog 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid (C4-BODIPY-C9) in large cytosolic punctae, most likely the Golgi/ER, and in a membrane surrounding the parasites, probably the PVM (Charron and Sibley, 2002). Interestingly, when host cells are loaded with fluorescent C4-BODIPY-C9 before infection, the staining is exclusively restricted to a membrane surrounding the parasites. This selective compartmentalization of diverted lipids clearly reflects sorting activities mediated by the parasites to adeptly distribute exogenous lipids into proper organelles.

Intracellular *T. gondii* and host-free parasites are competent to accrue various free fatty acids from their environment with selectivity (Quittnat *et al.*, 2004). Uptake studies with [9,10- $^3$ H]oleic acid, [1- $^{14}$ C]stearic acid, [1- $^{14}$ C]linoleic acid, [1- $^{14}$ C]arachidonic acid and [1- $^{14}$ C]palmitic acid reveal a preferential internalization of palmitate by *T. gondii* over other fatty acids. Inside parasites, exogenous fatty acids are manufactured into triacylglycerols (Quittnat *et al.*, 2004) and cholesteryl esters (Nishikawa *et al.*, 2005) with a two-fold higher incorporation of palmitate compared to other fatty acids. Extracellular parasites incorporate [9,10- $^3$ H]palmitic acid or [9,10- $^3$ H]myristic acid into GPI anchors, such as that of SAG1 (Tomavo *et al.*, 1989). After uptake of [1- $^{14}$ C]butyric acid by intra- or extracellular parasites, the lipid is anabolized into phosphatidylcholine (Charron and Sibley, 2002).

## Applicability to chemotherapy

Research on *Toxoplasma* fatty acid biosynthesis has been accelerating as the type II pathway has become recognized as a target-rich environment for the development of novel chemotherapeutics to combat toxoplasmosis. Indeed, differences in the structure and arrangement of the enzymes within the parasite's FAS II compared to mammalian FAS I offer multiple opportunities for selective therapeutic intervention based on the use of antibiotics or herbicides (Roberts *et al.*, 2003). Inhibition of *T. gondii* growth by selected aryloxyphenoxypropionate herbicides known to interfere with ACC activity has been demonstrated in parasite cultures (Jelenska *et al.*, 2002). *In vitro* and *in vivo* tests with these compounds show that the carboxyltransferase domain of the apicoplast *T. gondii* ACC is the binding target for this class of inhibitors. Expectedly, the cytosolic form of *T. gondii* ACC and human ACC are resistant to aryloxyphenoxypropionates.

Triclosan is a potent inhibitor of type II FabI (Baldock *et al.*, 1996). This drug restricts the growth of *T. gondii* *in vitro* (McLeod *et al.*, 2001). Triclosan blocks the incorporation of radioactive acetate into the fatty acids of Apicomplexa and specifically inhibits their FAS II. Thiolactomycin, a fungal secondary metabolite (Oishi *et al.*, 1982) selectively inhibits

type II FabH of the related apicomplexan parasite *Plasmodium falciparum* that also contains a FAS II complex in the apicoplast and decreases rapidly the growth of the malaria parasite. Cerulenin, a metabolite of *Cephalosporium caerulens*, is an inhibitor of both types I and II FabH (Heath *et al.*, 2001). Cerulenin is found to act synergistically with triclosan in inhibiting FAS II in *P. falciparum*. Thiolactomycin and cerulenin represent potential drugs that may also affect FAS pathways in *T. gondii*, and therefore growth.

## Sterol lipids

Cholesterol is the major sterol molecule ubiquitously present in mammalian cells. This lipid has been selected in the long natural evolution process for its ability to maintain a delicate balance between membrane rigidity (e.g. to allow large cell volumes) and membrane fluidity (e.g. to allow membrane-embedded proteins to function properly; Bretscher and Munro, 1993). Mammalian cells obtain cholesterol both by internalization of plasma low density lipoprotein particles (LDL) or by *de novo* synthesis via the mevalonate pathway in the endoplasmic reticulum (ER) (Goldstein and Brown, 1990). The cholesterol molecule is formed from acetate units. These are joined in a series of reactions to form farnesyl pyrophosphate, a branch point for the biosynthesis of other isoprenoid compounds such as ubiquinone, dolichol, and farnesylated proteins. Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase from the mevalonate pathway is the rate determining enzyme for the entire pathway from acetate to cholesterol. Deposition of excess cellular cholesterol in the form of cholesteryl esters is catalyzed by acyl-CoA:cholesterol acyltransferases (ACAT), ER resident enzymes. Native and exogenous cholesterol has several possible fates: incorporation into membranes, efflux to extracellular acceptors, conversion into cholesteryl esters, or depending on the cell type, metabolism into bile acids or steroid hormones. Rates of cholesterol biosynthesis, LDL internalization, and cholesterol esterification are exquisitely sensitive to cellular levels of free cholesterol. Three possible mechanisms of cholesterol movement include aqueous diffusion, vesicle-mediated transport, and soluble carriers, which may work together or separately to mobilize cholesterol within the cell (Liscum and Underwood, 1995). Evidence has accrued that biological membranes are made of a mosaic of lipids domains. Maintenance of domain structure is critical for cell function. Cholesterol plays a key role in organizing signaling lipids and proteins within these membrane domains (Anderson and Jacobson, 2002).

Parasitic protozoa also contain sterols in their membranes, with cholesterol predominating in many organisms such as *Plasmodium*, *Giardia* and trypanosomes (Roberts *et al.*, 2003). These protozoa are unable to carry out *de novo* cholesterol synthesis, but they are capable of recruiting cholesterol from lipoproteins present in the mammalian host and/or in the culture medium (Furlong, 1989). Extracellular parasites express unique surface receptors that can selectively bind and take up lipoproteins from the medium (summarized in Sehgal *et al.*, 2005). Some kinetoplastid parasites contain ergosterol, a form of sterol alkylated at its side chain as found in yeast, and these parasites are enzymatically equipped to synthesize this sterol *de novo* (Goad *et al.*, 1984). Besides its structural role in membrane architecture, ergosterol plays metabolic roles related to cell cycle regulation and phospholipid metabolism in these parasites (Roberts *et al.*, 2003).



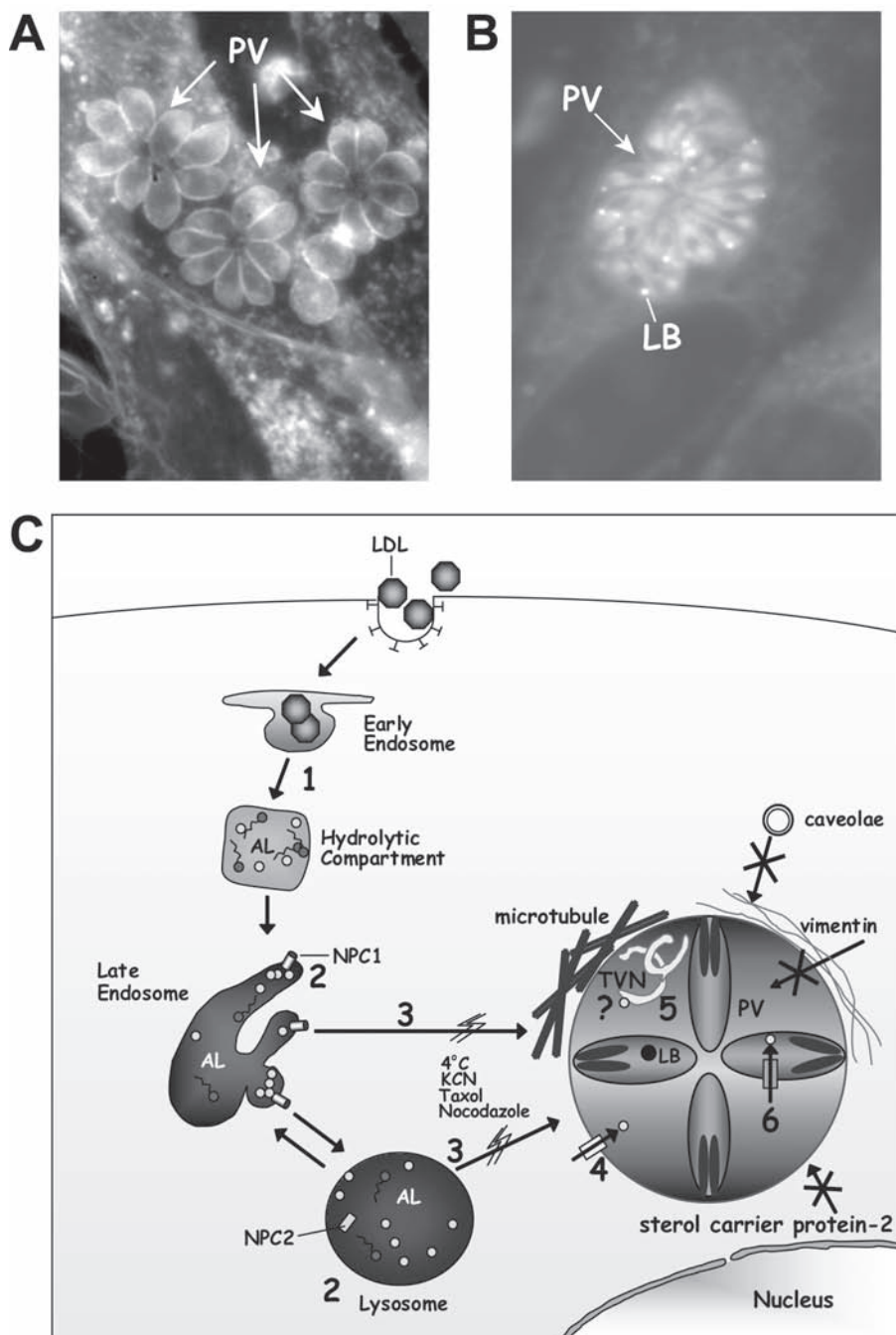
## Uptake

No anabolic pathways has been identified for any steryl lipids in *T. gondii*. Nevertheless, *Toxoplasma* membranes contain  $\beta$ -hydroxy-sterols, as probed by using the polyene antibiotic filipin routinely used to reveal the steady-state distribution of sterols by fluorescence microscopy (Figure 19.2A). A predominantly parasite staining is located to the plasma membrane and the rhoptries, apical secretory organelles whose content is discharged upon parasite invasion (Coppens *et al.*, 2000; Coppens and Joiner, 2003). The dye Nile red, which strongly fluoresces in the presence of steryl esters, detects the presence of cytosolic lipid bodies in the parasite (Sonda *et al.*, 2001; Charron and Sibley, 2002; Quittnat *et al.*, 2004), indicating the ability of cholesterol storage by the parasites (Nishikawa *et al.*, 2005).

Quantitative and qualitative lipid analyses from purified plasma membranes highlight a relatively low cholesterol/phospholipid ratio (Foussard *et al.*, 1991). By contrast, rhoptries contain an unusually high level of free cholesterol. The rhoptry content includes both protein and lipid components, which assemble to form membrane-like structures, leading to the assumption that specific biomolecular interactions between proteins and cholesterol must prevail in these organelles. Rhoptry cholesterol may be relevant for rhoptry function. Although its functional significance is still enigmatic, we can hypothesize that rhoptry cholesterol may be involved in (i) rhoptry protein sorting and transport to mature rhoptries; (ii) signaling platform formation and lipid raft organization in rhoptry membranes; (iii) discharge of proteins from rhoptries and/or (iv) modulation of the function of the rhoptry proteins that can potentially bind to cholesterol (Coppens and Vielemeyer, 2005). *Toxoplasma* rhoptry-derived vesicles are secreted into the host cytoplasm upon invasion (Hakansson *et al.*, 2001). The nascent parasitophorous vacuolar membrane (PVM) surrounding the parasite contains cholesterol (Coppens and Joiner, 2003) but this lipid is mainly derived from host plasma membrane, refuting a substantial role of rhoptry cholesterol as an extracellular effector at the time of invasion.

*T. gondii* diverts LDL-derived cholesterol that has transited through host lysosomes (Coppens *et al.*, 2000; Figure 19.2C) as demonstrated after incubation of infected cells with labeled cholesterol incorporated into LDL. Following endocytosis of the fluorescent cholesterol analog 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3 $\beta$ -ol (NBD-cholesterol) in host cells, the PVM is rapidly labeled (Coppens *et al.*, 2000). The dye is then gradually incorporated into the parasite plasma membranes, intraparasitic structures resembling the Golgi/ER, and lipid droplets (Figure 19.2B). Acquisition of LDL-derived cholesterol requires live parasites since no NBD-cholesterol is accessible to the parasitophorous vacuoles (PV) containing pyrimethamine-killed parasites. Incubation of infected cells with [ $1\alpha,2\alpha(n)^3$ H]cholesteryl oleate incorporated into LDL results in a rapid association of radioactive cholesterol with parasite membranes (Coppens *et al.*, 2000). Quantitative measurements indicate that radioactive cholesterol is taken up by intravacuolar parasites after 10-min incubation with LDL preparations, and is partially converted into cholesteryl esters stored in lipid bodies (~12% of membrane cholesterol; Nishikawa *et al.*, 2005; Sehgal *et al.*, 2005).

Cholesterol trafficking from mammalian lysosomes to the PV requires functional host Niemann–Pick-type C proteins (Sehgal *et al.*, 2005). These are known to mediate cho-



**Figure 19.2** Sterol distribution and uptake by *T. gondii*. (A) Filipin staining of *Toxoplasma*-infected cells to visualize sterol distribution. Three PV are intensively labeled revealing preferential accumulation of cholesterol in the plasma membrane and the apical region of the parasites. (B) Fluorescence labeling of *Toxoplasma*-infected cells with NBD-cholesterol

lesterol egress across the endo-lysosomal membranes (Sleat *et al.*, 2004). This cholesterol traffic is independent of pathways involving the host Golgi and ER. In mammalian cells, LDL-derived cholesterol is transported from lysosomes to the cell surface before redistribution to various cell compartments by undefined mechanisms (Haynes *et al.*, 2000). In contrast to mammalian organelles, the post-endo-lysosomal transfer of cholesterol to the PV vacuole does not involve the host plasma membrane as an intermediate platform (Sehgal *et al.*, 2005). Nevertheless, the PV vacuole is not completely secluded from the host cell cholesterol pathways. Indeed, the PVM vacuole membrane seems to be in dynamic continuity with the host plasma membrane, as exemplified by the transit of cholesterol from the PV vacuole towards the host plasma membrane. Cholesterol movement to the PV vacuole requires temperatures permissive for vesicular transport, metabolic energy and functional microtubules. Protein cholesterol-binding sites on the PVM vacuole membrane and the plasma membrane promote cholesterol delivery to *T. gondii*. These proteins may be analogs of mammalian cholesterol translocators or sensors such as Niemann-Pick type C proteins (Sleat *et al.*, 2004) or ATP-binding cassette proteins (Stefkova *et al.*, 2004) that are present in the *Toxoplasma* genome ([www.ToxoDB.org](http://www.ToxoDB.org)). Within the PV, a tubulo-vesicular network forms at the invaginated posterior end of the parasite shortly after invasion. This network then unfolds throughout the vacuolar space, forming elongated nanotubules that connect with the PVM. This network may participate in the transport of host cholesterol from the PVM to the parasite (Sibley *et al.*, 1995).

In mammalian cells, fine compensatory mechanisms exist to provide the correct amount of cholesterol for organelles (Goldstein and Brown, 1990). When cells are starved for cholesterol, levels of HMG-CoA reductase and LDL receptors are coordinately increased, thus increasing the rate of endogenous sterol synthesis and of lipoprotein uptake. Conversely, in cholesterol-replete cells, the levels of HMG-CoA reductase and LDL receptors decline, thereby lowering sterol production and LDL internalization. In *Toxoplasma*-infected cells, LDL uptake is specifically augmented by 3-fold (Coppens *et al.*, 2000), but surprisingly the HMG-CoA reductase activity is concomitantly upregulated by 4-fold (Blader *et al.*, 2001), which contributes to the rise of cell cholesterol levels from both sources. This obviously demonstrates a dysregulation of host cholesterol metabolism, as evidenced by an

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incorporated into LDL demonstrating cholesterol scavenging by the PV and association with parasite membranes and lipid bodies (LB). (C) Hypothetical model describing the trafficking events of LDL-derived cholesterol to the *Toxoplasma* PV (according to Sehgal *et al.*, 2005). Following LDL receptor-mediated endocytosis, cholesterol (in yellow) is liberated from LDL cholesteryl ester (in red) in early hydrolytic compartments containing the enzyme acid lipase (AL; step 1). Cholesterol then effluxes from the NPC-containing late endosome/lysosome (step 2) before trafficking to the PV (step 3). The post-endo-lysosomal movement of cholesterol to the PV in step 3 is blocked by inhibitors of vesicular transport (double broken lines) but does not require vesicle fusion or host-endo-lysosome fusion with the PV. Neither the host sterol carrier protein-2 nor caveolin is involved; the process is independent of vimentin intermediate filaments. Host cholesterol is delivered to the PV via lipid extractor- or transporter-like proteins on the PVM (step 4), then trafficked within the vacuolar space perhaps in association with the tubulo-vesicular network (TVN) secreted by *T. gondii* (step 5) before internalization into the parasite interior via parasite plasma membrane proteins (step 6), and storage as cholesteryl esters in lipid bodies (LB) (see also Plate 19.2).

uncoupling between LDL internalization and cholesterol biosynthesis. It remains to be elucidated whether these sterol metabolic abnormalities occur as a consequence of the parasite manipulation of host cholesterol regulatory pathways, or the incapability of the host cell to function normally under the stress of the parasite's assault, or both factors acting in concert.

## Storage

*Toxoplasma* is competent to synthesize cholesteryl esters by two ER isoforms of ACAT, named TgACAT1 $\alpha$  and TgACAT1 $\beta$  (Nishikawa *et al.*, 2005). Both enzymes are derived from two full-length cDNA. Similarly to their mammalian counterparts, TgACAT1 $\alpha$  and TgACAT1 $\beta$  sequences have several membrane spanning domains as well as potential sites for N-glycosylation and tyrosine phosphorylation. TgACAT1 $\alpha$  harbors a long hydrophilic serine-rich region at the N-terminus, which is missing in TgACAT1 $\beta$ . When transfected with either TgACAT1 $\alpha$  or TgACAT1 $\beta$ , ACAT-deficient mammalian cells are restored in their capacity to synthesize cholesteryl esters. When a *Saccharomyces cerevisiae* mutant strain lacking neutral lipid production is transformed with TgACAT1 $\alpha$ , ergosteryl esters are produced, although neither ergosterol nor ergosteryl esters have ever been detected in *T. gondii*. The addition of cholesterol to the culture medium induces the synthesis of cholesteryl esters by the transformed mutant yeast strain. This demonstrates that unlike in higher eukaryotes or yeast where one particular sterol ester is predominantly synthesized (Yang *et al.*, 1997), the esterification reaction in *Toxoplasma* is not specific to changes in the sterol side chain since both cholesteryl and ergosteryl esters are produced by the heterogeneously expressed *Toxoplasma* ACATs.

In *Toxoplasma*, both LDL-derived cholesterol and free fatty acids can serve as ACAT activators. The TgACAT1 $\alpha$  mRNA expression, cellular cholesterol esterification and lipid droplet biogenesis appear to be coordinately regulated. Such a mechanism of transcriptional regulation of TgACAT1 suggests a unique sterol regulatory-like element present within the TgACAT1 gene promoters, but absent from human ACAT gene promoters. Lipoprotein depletion causes a progressive consumption of material stored in parasite's lipid bodies. Under conditions of excess LDL, the activity of cholesterol esterification is significantly increased, entailing that the parasites adeptly control the massive supply of cholesterol by producing the storage form of cholesterol.

## Applicability to chemotherapy

The replication rate of intracellular *T. gondii* correlates with the LDL concentration in the medium. Excess cholesterol diverted by the parasite is rapidly neutralized and stored in lipid bodies. Blockade of cholesteryl ester synthesis is deleterious for the parasite, leading to rapid induction of free cholesterol crystallization in parasite membranes and rupture of the plasma membrane (Nishikawa *et al.*, 2005). The higher vulnerability of *T. gondii* toward ACAT inhibitors compared with mammalian cells is probably linked to the absence of cholesterol acceptors (mainly lipoproteins) in the PV vacuole, which are known to desorb excess cholesterol from membranes.

Delivery of sterol analogs or their esters incorporated into LDL could be an alternative strategy to substitute the indispensable cholesterol by structurally related compounds with

growth-reducing activity. The sterol analogs 22,26-azasterol and 24,25-(R,S)-epiminolanosterol, inhibitors of sterol-24-methyl transferase producing 24-alkyl sterols, have potent and selective antiproliferative activity against *T. gondii* (Dantas-Leite *et al.*, 2004). The molecular mechanism of these lipids is unclear since 24-alkyl sterols are not detected in this parasite. It is observed however, that the rapid accumulation of these lipid analogs in diverse membranes alters maintenance, fusogenicity and function of the parasite organelles. It is reported that selected sterol analogs (e.g. cholesteryl chloride, cholestanone, or thio-cholesterol) can affect the growth of various cholesterol-auxotroph organisms (Clayton, 1964). Their antiproliferative properties of these analogs should be promisingly extended to *Toxoplasma*.

## Glycerophospholipids

Glycerophospholipids, also known as phospholipids, are key molecules that contribute to the structural definition of cells and that participate in the regulation of many cellular processes. Phospholipid metabolism is a major activity that cells engage in throughout their growth. (Carman and Zeimet, 1996). In mammalian cells, the most important glycerophospholipids are phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine are synthesized from phosphatidate by the CDP-diacylglycerol pathway, while phosphatidylcholine and phosphatidylethanolamine are also synthesized by the Kennedy (CDP-choline and CDP-ethanolamine) pathway. CDP-diacylglycerol is also used for the synthesis of other phospholipids, including phosphatidylinositol (Carman and Zeimet, 1996; Vance and Vance, 2004).

## Synthesis

Quantification of the phospholipid profile of *Toxoplasma* reveals that phosphatidylcholine is the most prevalent lipid, accounting for about 75% of the total phospholipids (Gupta *et al.*, 2005). The next most abundant lipids are phosphatidylethanolamine (10%), phosphatidylinositol (7.5%), phosphatidylserine (6%) and phosphatidic acids (1%). The parasite is enzymatically equipped to synthesize *de novo* all these aminoglycerophospholipids via the Kennedy pathways. Diphosphatidylglycerol, a mitochondrial cardiolipin representative, and phosphatidylglycerol are also produced by extracellular and intracellular parasites incubated with radioactive acetate (Bisanz *et al.*, 2006). Quantitative data on the rates of phospholipid synthesis reveal that *T. gondii* has an adequate synthetic capacity to produce all of the phosphatidylethanolamine, but only 50% of the phosphatidylserine, and ~5–10% of the phosphatidylcholine as required for a parasite doubling. These findings demonstrate that *T. gondii* must be a choline and/or phosphatidylcholine auxotroph. Indeed it can acquire efficiently their head precursors, e.g. serine, ethanolamine and choline present in its environment for incorporation into complex phospholipids (Gupta *et al.*, 2005; Charron and Sibley, 2002).

## Uptake

Activities of phospholipids uptake by *T. gondii* have been exemplified by using two fluorescent glycerophospholipid analogs, 2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-

bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-phosphatidylcholine) and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3 phosphate (BODIPY-phosphatidic acid) that label parasite compartments after internalization in host cells (Charron and Sibley, 2002). BODIPY-phosphatidylcholine is mobilized to plasma membrane and dispersed small vesicles while BODIPY-phosphatidic acid moves to the parasite compartments similar to those containing fluorescent C4-BODIPY-C9, corresponding presumably to the Golgi/ER and the PVM vacuole membrane. Interestingly, diversion of BODIPY-phosphatidic acid loaded in host cells prior to infection shows a bright fluorescent labeling in the PVM vacuole membrane. By contrast, BODIPY-phosphatidylcholine is completely excluded from parasites that have invaded prelabeled hosts.

Intracellular parasites and host-free parasites are competent to take up [oleic-1- $^{14}\text{C}$ ] L- $\alpha$ -phosphatidic acid, but only parasites inside host cells further metabolize the scavenged lipid into phosphatidylcholine (Charron and Sibley, 2002). *T. gondii* can acquire the phospholipid head group precursors from its environment and use them for the synthesis of major lipids (Gupta *et al.*, 2005). [3- $^3\text{H}$ ]serine internalized by free parasites is metabolized into phosphatidylserine and phosphatidylethanolamine after phosphatidylserine decarboxylation, as well as in minor sphingolipids. Phosphatidylethanolamine is the main polar lipid generated after uptake of [1- $^3\text{H}$ ]ethan-1-ol-2-amine. Like serine, the metabolism of ethanolamine shows a time-dependent increase in lipid synthesis that progressively slows over a 6-hour period. No significant radioactive phosphatidylcholine is detected in *Toxoplasma* membranes after incubation in the presence of either tritiated serine or ethanolamine, suggesting that the parasites, at least in axenical conditions, have a negligible phosphatidylethanolamine methyltransferase activity (Gupta *et al.*, 2005). However, another study conducted on parasites grown in host cells fed with [ $^{14}\text{C}(\text{U})$ ]L-serine or [1,2- $^{14}\text{C}$ ]ethanolamine shows that *T. gondii* can synthesize phosphatidylcholine as a major resultant end product using these labeled precursors (Charron and Sibley, 2002). This discrepancy is probably ascribed to differences in metabolic requirements between parasites released from cells or growing inside cells. When host cells are loaded with [ $^{14}\text{C}(\text{U})$ ]L-serine or [1,2- $^{14}\text{C}$ ]ethanolamine prior to infection, no anabolism of radioactive serine or ethanolamine is observed (Charron and Sibley, 2002). This may be linked to the rapid conversion of serine and ethanolamine into phosphatidylcholine by mammalian cells and the inability of the parasite to scavenge intact phosphatidylcholine from host cells, as corroborated in fluorescence studies (see above).

Incubation of both, extracellular parasites with [1- $^3\text{H}$ ]choline or [ $^{14}\text{C}$ -methyl]choline and intracellular parasites with [ $^{14}\text{C}$ -methyl] choline results in the acquisition of this compound by the parasites and metabolization into phosphatidylcholine (Charron and Sibley, 2002; Gupta *et al.*, 2005). Choline preaccumulated by host cells before infection is also further metabolized into phosphatidylcholine by the parasites, in accordance to the competence of the intravacuolar *T. gondii* to readily take up choline. Various forms of radioactive choline-containing lipids are only observed in intravacuolar parasites. This parallels the observation showing that the metabolism of choline is increased by about 2-fold in host-free parasites incubated in an intracellular-type medium compared to parasites maintained in an extracellular-type medium. This leads to the assumption that choline



metabolism and phosphatidylcholine synthesis are stimulated in response to parasitic invasion and replication within host cells.

In mammalian cells, phospholipids can move between membranes by routes that are independent of the vesicular traffic that carries membrane proteins (Voelker, 2005). Evidence continues to accumulate in support of a system for phospholipid transport that occurs at zones of apposition and contact between donor membranes and acceptor membranes that are unable to synthesize the necessary lipids. This inter-organellar phospholipid transport occurs between the ER and mitochondria or Golgi, and involves special ER regions known as mitochondrial-associated membranes. Of interest, the PVM vacuole membrane quickly becomes physically associated with the ER and mitochondria (Sinai *et al.*, 1997; Sinai and Joiner, 2001). This close apposition of host ER and mitochondria to the PVM vacuole membrane may represent zones of privileged exchanges of lipids between host organelles and the PV vacuole. A direct phospholipid transfer might also occur from host mitochondria to regions of the PVM, which might be analogous to the mitochondrial-associated membranes. Disruption of parasite proteins mediating organellar retention on the PVM results in a failure to recruit host mitochondria to the PV and to acquire host lipids, and ultimately induces parasite growth arrest *in vitro* and in mice (Nakaar *et al.*, 2003).

### Applicability to chemotherapy

The dominance of phosphatidylcholine as a major lipid in *T. gondii* membranes offers great potentialities to disrupt the membrane biogenesis of the parasite. The choline analog *N,N*-dimethylethanolamine is taken up by intracellular parasites as efficiently as choline (Gupta *et al.*, 2005). As a result, abnormal membrane structures accumulate in the new progenies and *T. gondii* growth is progressively arrested, probably due to dramatic phosphatidylcholine depletion and/or toxic phosphatidyltrimethylethanolamine amassing in parasite membranes. In fact, *T. gondii* is highly competent to metabolize dimethylethanolamine and accumulates up to 44% of its phospholipids as phosphatidyltrimethylethanolamine (compared to only 6% of the phospholipid pool for mammalian cells). The phosphatidyltrimethylethanolamine increase in the parasite parallels with a specific decrease in phosphatidylcholine (from 75% to 33% of total phospholipids). In mammalian cells, phosphatidyltrimethylethanolamine is normally produced as a short-lived intermediate in the conversion of phosphatidylethanolamine to phosphatidylcholine (Vance and Vance, 2004). The massive accumulation of phosphatidyltrimethylethanolamine reflects the limited capability of the parasite for phospholipid methylation. Clearly, dimethylethanolamine interferes with choline uptake and metabolism to phosphatidylcholine, resulting in selective alteration in parasite membrane morphology at concentrations non-toxic for the host cell.

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### Glycerolipids

Bacteria, yeast, plants, and animals all have the ability to synthesize glycerolipids, mainly triacylglycerols and diacylglycerols. This is a critical function during periods of nutritional excess and/or nutritional stress (Coleman and Lee, 2004). In higher eukaryotes, triacylglycerols are packaged in circulating lipoproteins for distribution to peripheral tissues where they can be used immediately or stored in cytosolic lipid droplets. Such energy-

dense triacylglycerol stores can free organisms temporally and spatially from the need for an immediate energy supply and provide a reserve depot that can be used when local resources fail or when specific kinds of fatty acids or lipid precursors are required. Triacylglycerol stores can be also partially hydrolyzed to form diacylglycerols, which perform two distinct roles: supporting the biosynthesis/degradation of phospholipids and regulating the protein kinase C activity that controls cell growth. In animals, triacylglycerols are thus energy stores, repositories for fatty acids and precursors for phospholipid biosynthesis, and depots of signaling molecules (van Blitterswijk and Houssa, 1999). In contrast, in bacteria and lower eukaryotes, triacylglycerols are solely synthesized during times of stress or resource depletion, and they are used primarily for phospholipid synthesis. In this view it is not surprising that higher organisms have developed several pathways for triacylglycerol synthesis and regulation, as compared to unicellular organisms. Commonly, the formation of triacylglycerols is catalyzed by the activity of microsomal acyl-CoA:diacylglycerol acyltransferases (DGAT).

### Synthesis

In *T. gondii*, triacylglycerol synthesis occurs via the glycerol-3-phosphate pathway and involves a DGAT, named TgDGAT1 (Quittnat *et al.*, 2004). Fatty acid can be incorporated into *Toxoplasma* diacylglycerol, revealing that this latter lipid is the acyl acceptor. TgDGAT1 contains signature motifs characteristic of the DGAT1 family. TgDGAT1 is an integral membrane protein localized to the ER. When a *S. cerevisiae* mutant strain lacking neutral lipid production is transformed with TgDGAT1, a significant DGAT activity is reconstituted, resulting in the biogenesis of cytosolic lipid inclusions. In contrast to human DGAT1 lacking fatty acid specificity, TgDGAT1 preferentially incorporates palmitate into triacylglycerols. Triacylglycerols are stored in parasite cytosolic lipid bodies. Stored triacylglycerols may be a reservoir of fatty acids utilizable for phospholipid biosynthesis and/or exploitable as respiratory substrates in *Toxoplasma*.

*T. gondii*'s plastid seems to contain the complete pathway to synthesize galactosylglycerolipids from fatty acids and glycerol-phosphate (Marechal *et al.*, 2002) although no homologous gene candidates for chloroplast-like galactolipid syntheses could be identified (Bisanz *et al.*, 2006). These lipids are generally found in plant chloroplast membranes and might be, therefore, synthesized in the parasite apicoplast.

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### Sphingolipids

Glycosylphosphatidylinositols (GPI) are a class of glycolipids that are used by a wide variety of eukaryotic cells to anchor proteins, polysaccharides, and small oligosaccharides to the plasma membrane through covalent linkages (Ferguson and Williams, 1988). Comparison of the chemical structures of GPI membrane anchors of different organisms indicates that the anchors contain a remarkably conserved core glycan structure, suggesting that a common biosynthetic pathway may have been conserved throughout eukaryotic evolution. The transfer of GPI anchors to proteins occurs in the ER with concurrent displacement of a C-terminal hydrophobic peptide, followed by the rapid substitution of the peptide tail by the GPI anchor.

Ceramides are the principal lipid components present in sphingomyelin, complex glycolipids, cerebrosides and gangliosides (Sharma and Shi, 1999). Ceramides are broadly recognized as vital second messengers in the signal transduction process mediated by receptors of many cytokines and growth factors.

### Synthesis

GPI in *T. gondii* serve as membrane anchors for a large number of plasma membrane proteins (Tomavo *et al.*, 1992; Striepen *et al.*, 1997; Zinecker *et al.*, 2001). Their biosynthetic pathway is initiated on the parasite ER with the transfer of *N*-acetylglucosamine to phosphatidylinositol involving a phosphatidylinositol-glycan class A (PIGA)-like protein (Wichroski and Ward, 2003). The GPI core glycan is then assembled via sequential glycosylation of phosphatidylinositol (Tomavo *et al.*, 1992). The *Toxoplasma* PIGA sequence contains a potential transmembrane domain followed by a stretch of mostly hydrophilic residues extending to the C-terminus. A functional copy of PIGA is required for viability, demonstrating that GPI biosynthesis is an essential process in *T. gondii* (Wichroski and Ward, 2003).

Glycosphingolipids, e.g. inositol phosphorylceramide are synthesized *de novo* via the 3-ketosphinganine pathway from serine and palmitoyl-CoA (Azzouz *et al.*, 2002; Sonda *et al.*, 2005) with ceramide as an intermediate. Metabolic studies show that extracellular and intracellular *T. gondii* readily incorporate radioactive acetate into glycosylcerebroside, lactosylcerebroside and globotriosylcerebroside while only intracellular parasites produce globoside (Bisanz *et al.*, 2006).

Extracellular parasites can readily incorporate sugars and amino acids as precursors of sphingolipids. After internalization, [6-<sup>3</sup>H]galactose is metabolized in various glycosphingolipids (e.g. di- and triglycosylated ceramide; Azzouz *et al.*, 2002). Parasite incubation with [3-<sup>3</sup>H]serine leads to the production of labeled ceramide. After uptake, [6-<sup>3</sup>H]glucosamine serves as a GPI glycolipid precursor and is associated with the dominant surface protein, SAG1 (Striepen *et al.*, 1997; Zinecker *et al.*, 2001).

### Uptake

In mammalian cells, NBD-C6-ceramide concentrates at the perinuclear region where the Golgi is located and then is translocated to the plasma membrane, probably after metabolism into sphingolipids and glycosylceramides. Four hours post-incubation with NBD-C6-ceramide, the Golgi of intravacuolar *T. gondii* is stained, suggesting that the parasite can intercept the ceramide pathway of the host cell to acquire exogenous sphingolipids (de Melo and de Souza, 1996).

### Applicability to chemotherapy

GPI-anchored proteins dominate the surface of *T. gondii* and are implicated in both host cell attachment and modulation of the host immune response (Lekutis *et al.*, 2001). Although the GPI core glycan is conserved in all organisms, some differences in additional modifications to GPI structures and biosynthetic pathways have been reported for *T. gondii* (de Macedo *et al.*, 2003). This indicates that the GPI biosynthetic pathway is a potential target for the development of new chemotherapeutics against this parasite. In-

deed, the lethal consequences of *PIGA* disruption in *T. gondii* may result from a deficiency in GPI-anchored proteins, free GPI, or both (Wichroski and Ward, 2003). *In vitro* and *in vivo* studies reveal that sugars and amino acid analogs, synthetic mannoside acceptor substrates and natural compounds specifically interfere with GPI biosynthesis in many different pathogenic organisms (de Macedo *et al.*, 2003). Synthesis of parasite ceramide is dramatically decreased after incubation of intracellular *Toxoplasma* with either threo-phe-nyl-2-palmitoylamino-3-morpholino-1-propanol, a specific inhibitor of glucosylceramide synthesis, or L-cycloserine that blocks the serine palmitoyltransferase activity (Azzouz *et al.*, 2002). The antibiotic aureobasidin A, a potent inhibitor of inositol phosphorylceramide that is absent from mammalian cells, abrogates *T. gondii* replication by the severe reduction of total complex sphingolipids' synthesis (Sonda *et al.*, 2005).

## Isoprenoid derivatives

The posttranslational modification of proteins by isoprenoid residues such as farnesyl and geranylgeranyl, is a major mechanism by which cytosolic proteins interact with cellular membranes (Swiezewska and Danikiewicz, 2005). Isoprenylation is also required for the proper membrane localization and the biological activity of several cellular proteins implicated in the regulation of DNA replication and cell cycling, therefore having important roles in the regulation of cell proliferation. Two isoprenoid pathways coexist in organisms: the mevalonate pathway present in the cytosol of mammalian cells (see above) and the recently described 1-deoxy-d-xylulose-5-phosphate pathway. This latter pathway seems to be restricted so far to bacteria, plastids in plants and Apicomplexa (Jomaa *et al.*, 1999).

## Synthesis

*T. gondii* membranes contains both farnesylated and geranylgeralynated proteins (Ibrahim *et al.*, 2001; Ling *et al.*, 2005). Enzymes of the 1-deoxy-D-xylulose-5-phosphate pathway seem to have an apicoplast localization and may contribute to the production of farnesyl and geranylgeranyl molecules for the parasite (see Chapter 22 for further details on isoprenoid biosynthesis and function).

## Uptake

Alternatively, the parasite can salvage isoprenoids such as [ $1\text{-}^3\text{H}$ ]trans,trans farnesol and [ $1\text{-}^3\text{H}$ ]trans,trans,cis geranylgeraniol, from the medium to produce its prenylated proteins (Ibrahim *et al.*, 2001). This implies the presence of functional protein farnesyltransferase and geranylgeranyl transferase in *T. gondii*. Indeed, a protein farnesyltransferase activity has been detected in the parasite, responsible for the catalysis of isoprene lipid modifications.

## Applicability to chemotherapy

Prenylated proteins are ubiquitously important for the regulation of cell proliferation. Two categories of protein farnesyltransferase inhibitors have been described so far (Qian *et al.*, 1997): isoprene analogues and peptidomimetics based on the consensus CAAX motif that is required for isoprenylation. Peptidomimetics act as alternative substrates *in vitro*, thereby competitively blocking protein farnesylation. Clearly, specific inhibition of *T. gondii* protein farnesyltransferase activity is observed using selected modified heptapep-

tides (Ibrahim *et al.*, 2001; see Chapter 22 for a discussion of the isoprenoid biosynthetic pathway as a drug target).

## The bottom line

Throughout evolution, enzymes and their metabolites have been highly conserved. Parasites are no exception to this but they differ most markedly by the absence of metabolic pathways that are present in the mammalian host. In general, they comfortably rely on host metabolism for their supply of prefabricated components, including lipids or their precursors. In this respect, parasites may be considered “metabolically lazy” but that does not necessarily mean that they have a simple anabolism. In this Chapter, I illustrated that *Toxoplasma gondii* is metabolically highly sophisticated in that, (i) it has retained the genetic capacity to express redundant lipid biosynthetic pathways, and (ii) it has developed efficient mechanisms for host lipid scavenging. This parasite also shows quite unique and amazingly diverse features in lipid metabolism: some lipid pathways share close similarities to yeast pathways whereas some are evolutionary more related to those of bacteria and plants.

From a cell biological viewpoint, no doubt exists that the lipid metabolism of *T. gondii* will likely reveal many more metabolic surprises in the future. In the context of toxoplasmosis chemotherapy, characterization of more lipid-based target molecules and knowledge about mechanisms promoting host lipid delivery to the PV vacuole will hold considerable potential. To this end, rationally designed lipid synthesis inhibitors would represent exciting prospects for the next “generation” of anti-*Toxoplasma* agents.

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## Abstract

Nucleotides and amino acids are of fundamental importance in the replication and development of *Toxoplasma gondii*. Nucleotides provide the key cellular energy source (ATP and GTP), and are particularly essential to the synthesis of DNA (dATP, dGTP, dCTP, dTTP) and the synthesis of RNA (dATP, dGTP, dCTP, dUTP) in rapidly replicating tachyzoites. Nucleotides are the precursors of more complex molecules such as folate. They also provide regulatory roles as intracellular second messengers such as cAMP, provide nucleotide based enzyme cofactors (NAD<sup>+</sup>, FMN, FAD), and control metabolic and gene regulation. In *T. gondii*, nucleotides are synthesized from small molecules and amino acids via pyrimidine biosynthetic pathways, and also are acquired from preformed host nucleobases and nucleosides via salvage pathways. The importance of nucleotide metabolism to *T. gondii* is illustrated by noting that several current clinical strategies for treating toxoplasmosis in humans are based on blocking the accumulation of nucleotides.

The study of nucleotide and amino acid metabolism in *T. gondii* is a challenging area of biology due to the complexity of these parasite pathways as well as the obligatory presence of the mammalian host cell, which also possesses complex and active pathways in nucleotide and amino acid metabolism. Consequently, genetic studies involving the examination of *T. gondii* mutants, or parasite behavior in mutant host cells, have provided particularly valuable information. The investigation of nucleotide and amino acid metabolism in *T. gondii* has also resulted in the establishment of several important genetic selection models that are based on aspects of parasite nucleotide and amino acid metabolism.

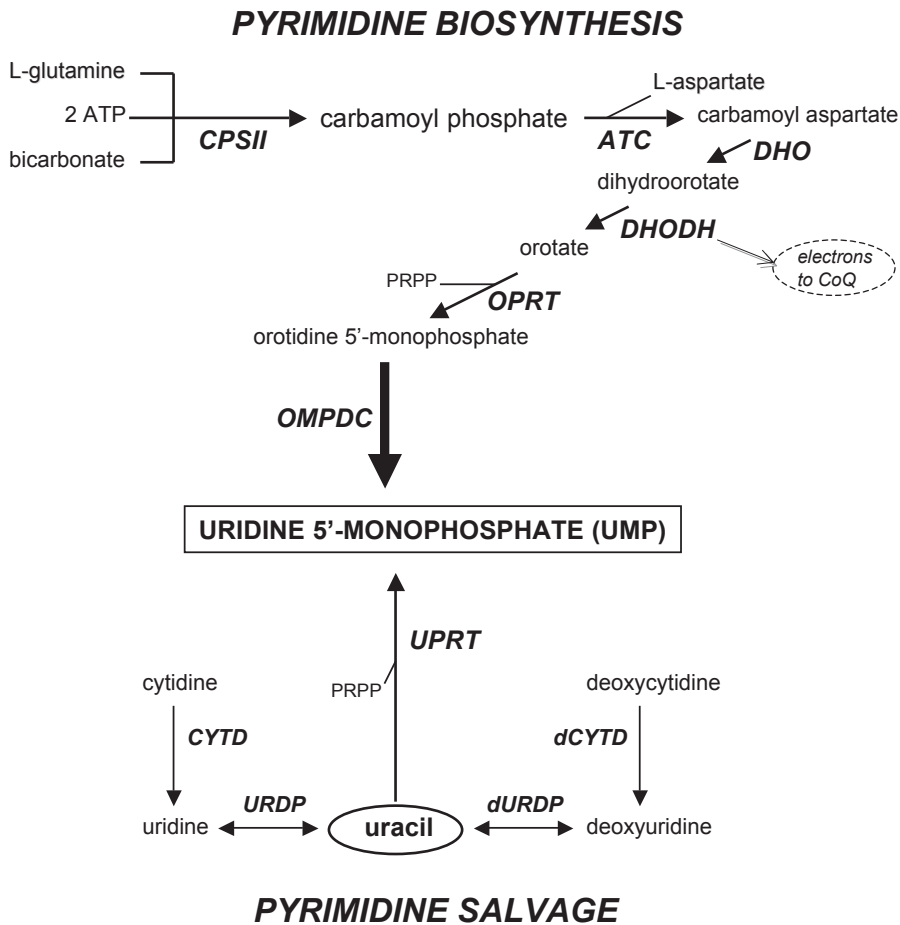
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## Pyrimidines

Pyrimidine nucleotides are essential components of DNA (TTP and CTP) and RNA (UTP and CTP). Pyrimidines also serve as metabolic regulators and they are used to form activated intermediates such as UDP-glucose. *T. gondii* obtains the parental pyrimidine molecule, uridine 5'-monophosphate (UMP), predominantly via pyrimidine biosynthesis. Therefore, *T. gondii* is dependent on an appropriate supply of precursor molecules for the biosynthesis of pyrimidines. These precursor molecules include small molecules such as bicarbonate, purines, and amino acids.

*De novo* pyrimidine synthesis in *T. gondii*

*T. gondii* possesses all six steps of the *de novo* pyrimidine synthetic pathway (Figure 20.1) (Asai *et al.*, 1983; O’Sullivan *et al.*, 1981; Schwartzman and Pfefferkorn, 1981). The pyrimidine biosynthetic pathway begins with 2 molecules of ATP, L-glutamine, and bicarbonate



**Figure 20.1** Pyrimidine biosynthesis and salvage pathways in *T. gondii*. Pyrimidine biosynthesis (top) and pyrimidine salvage (bottom). Solid lines and arrows depict active pathways present in *T. gondii*. Substrates of each enzyme are shown on the side of the solid line and the product(s) of each enzyme activity are shown on the arrowhead side. The enzyme activity responsible for each conversion step is shown in capital italicized text beside the arrowhead line. The primary product of pyrimidine biosynthesis and salvage is uridine 5'-monophosphate (UMP). Abbreviations used are as follows: CPSII; carbamoyl phosphate synthetase II, ATC; aspartate carbamoyltransferase, DHO; dihydroorotase, DHODH; dihydroorotate dehydrogenase, CoQ; mitochondrial coenzyme Q, OPRT; orotate phosphoribosyltransferase, PRPP; 5-phosphoribosyl-1-pyrophosphate, OMPDC; orotidine 5'-monophosphate decarboxylase, UPRT; uracil phosphoribosyltransferase, URDP; uridine phosphorylase, dURDP; deoxyuridine phosphorylase, CYTD; cytidine deaminase, dCYTD; deoxycytidine deaminase. Uridine/deoxyuridine phosphorylase and cytidine/deoxycytidine deaminase activities are likely to be present on a single enzyme, respectively.

that are modified or combined in a sequence of chemical reactions at multiple active sites within the tunnel of carbamoyl phosphate synthetase II (CPSII) to produce carbamoyl phosphate (Holden *et al.*, 1999; Kothe *et al.*, 2005). The carbamoyl phosphate is then fused with L-aspartate in the second step of the pathway by aspartate carbamoyltransferase (ATC) to produce carbamoyl aspartate. Dihydroorotase (DHO) then converts carbamoyl aspartate to dihydroorotate in the third step. Dihydroorotate dehydrogenase (DHODH) synthesizes orotate from dihydroorotate and also creates electrons to coenzyme Q (CoQ) in the mitochondrion. Orotate is joined with 5-phosphoribosyl-1-pyrophosphate (PRPP) in the fifth step by orotate phosphoribosyltransferase (OPRT) to synthesize orotidine-5'-monophosphate (OMP). Finally UMP is produced in the sixth step via the decarboxylation of OMP by orotidine-5'-monophosphate decarboxylase (OMPDC). UMP is phosphorylated to UTP in two sequential steps by UMP kinase and nucleoside diphosphate kinase, and UTP is converted to CTP by CTP synthase (CTPS) in a rate-limiting step. CTPS is the only known route for *de novo* synthesis of cytidine nucleotides. Conversion of ribonucleotides to deoxyribonucleosides occurs at the level of nucleoside diphosphate and is catalyzed by a ubiquitous ribonucleotide reductase (RNR).

#### *Novel architecture, organization and regulation of CPSII in T. gondii*

CPSII catalyzes the rate-limiting step in pyrimidine biosynthesis and controls the flux through the pathway (Evans and Guy, 2004; Jones, 1980). The architecture of the *T. gondii* CPSII is unique. *T. gondii* encodes a novel glutamine-dependent CPSII activity fused with an N-terminal glutamine amidotransferase (GAT) activity (Fox and Bzik, 2003). By contrast, mammalian pyrimidine-specific CPSII is encoded by a gene designated as *CAD* that expresses a multifunctional protein composed of a GAT domain fused via linkers of various lengths in order with CPSII, dihydroorotase (DHO), and aspartate carbamoyltransferase (ATC) (Davidson *et al.*, 1993). In *Saccharomyces cerevisiae* a multifunctional protein contains GAT, CPSII, and ATC, but is missing the DHO activity found on mammalian CAD. Plants, eubacteria, and archaebacteria express a monofunctional CPS with respect to the biosynthetic pathway, and a separate gene encoding GAT (Jones, 1980; Zhou *et al.*, 2000).

*T. gondii* CPSII polypeptide is encoded by 37 exons from a single-copy gene of approximately 32 000 nucleotides present on chromosome X (Fox and Bzik, 2003). Relative to other CPS and GAT domains, the *T. gondii* as well as other CPSII enzymes from the phylum Apicomplexa have unique sites of insertion within GAT domains, within CPS domains, within the linker region fusing the two CPS halves, and within the C-terminal allosteric regulatory domain (Fox and Bzik, 2003).

#### *CPSII in pyrimidine biosynthesis is a validated drug target*

The GAT activity of *T. gondii* CPSII is a possible target of acivicin (Fox and Bzik, 2003). While mammalian CPSII is an allosterically regulated enzyme with activity activated by 5-phosphoribosyl-1-pyrophosphate (PRPP) and suppressed by UTP (Jones, 1980), *T. gondii* CPSII is insensitive to activation by PRPP, but is inhibited by UTP (Asai *et al.*, 1983). *T. gondii* CPSII possesses significant amino acid insertions and divergent amino acid composition in the C-terminal allosteric regulatory domain, suggesting the regulation of parasite CPSII is unique from regulation of host CPSII (Fox and Bzik, 2003).

Genetic inactivation of the *T. gondii* CPSII gene and pyrimidine biosynthesis produced a uracil auxotroph mutant (Figure 20.1) (Fox and Bzik, 2002). The *T. gondii* uracil auxotroph invades host cells and replicates normally *in vitro* in the presence of high concentrations of exogenously supplied uracil. Yet, if uracil is omitted from culture medium, this mutant will invade host cells, but will not replicate. Compared with its virulent parental strain RH (Sabin, 1941), the uracil auxotroph mutant was more than 10 million-fold less virulent, or was completely avirulent in murine infections (Fox and Bzik, 2002). Consequently, the avirulent phenotype produced by disruption of CPSII demonstrates that the *T. gondii* pyrimidine *de novo* synthetic pathway presents a key target for drug development.

*T. gondii* ATC catalyzes the second step in pyrimidine biosynthesis and is a cytosolic monofunctional enzyme (Asai *et al.*, 1983). Recombinant *T. gondii* ATC protein has been produced and characterized. The lack of any detectable regulation on *T. gondii* ATC further supports a key regulatory role for CPSII in the *de novo* pathway of pyrimidine biosynthesis (Mejias-Torres and Zimmermann, 2002). *T. gondii* DHO catalyzes the third step and is a cytosolic monofunctional enzyme that is unresponsive to any nucleotide (Asai *et al.*, 1983). DHODH from *T. gondii* has also been cloned and expressed (Sierra Pagan and Zimmermann, 2003).

#### *Indirect inhibition of pyrimidine biosynthesis*

Atovaquone is an approved drug for treatment of acute toxoplasmosis. Atovaquone is a structural analogue of coenzyme Q (CoQ; ubiquinone) that collapses the membrane potential by inhibition of cytochrome *b* in the *bc*<sub>1</sub> complex (complex III) of the parasite electron transport chain. Blocking electron flow indirectly inhibits mitochondrial membrane associated enzymes such as DHODH that requires electron transfer to CoQ when it oxidizes dihydroorotate to orotate in the fourth step of the pyrimidine biosynthetic pathway (Figure 20.1). DHODH purifies from *T. gondii* in the particulate fraction of tachyzoites and is inhibited by respiratory chain inhibitors (Asai *et al.*, 1983). *T. gondii* mutants resistant to atovaquone can be selected *in vitro* (Pfefferkorn *et al.*, 1993). Characterization of atovaquone resistant mutants identified resistance mutations within the *T. gondii* cytochrome *b* gene (McFadden *et al.*, 2000).

#### *Pyrimidine salvage in T. gondii*

*T. gondii* is unusual among apicomplexa in also possessing potentially significant pyrimidine salvage activities (Figure 20.1). Early labeling studies demonstrated that uracil is highly incorporated into tachyzoites, but did not label host cell nucleic acids (Pfefferkorn and Pfefferkorn, 1977a). *T. gondii* mutants were selected to be resistant to 5-fluorodeoxyuridine (FUDR), and were found to be deficient in their ability to incorporate uridine, deoxyuridine, and uracil (Pfefferkorn and Pfefferkorn, 1977a; Pfefferkorn and Pfefferkorn, 1977b). Correspondingly, these mutants were co-resistant to 5-fluorouracil and 5-fluoruridine. The basis of FUDR resistance is a biochemical defect in a parasite uracil phosphoribosyltransferase (UPRT) activity (Pfefferkorn, 1978).

The parasite cannot directly obtain or salvage phosphorylated nucleotides from the host cell but can transport and incorporate the pyrimidine nucleobase uracil, and the



pyrimidine nucleosides uridine, deoxyuridine, cytidine, and deoxycytidine (Figure 20.1) (Iltzsch, 1993; Pfefferkorn and Pfefferkorn, 1977a; Pfefferkorn and Pfefferkorn, 1977b). *T. gondii* also recovers pyrimidines arising from degradation of parasite nucleic acids through degradation of dUMP, dCMP, and CMP to their corresponding nucleosides by nucleoside 5'-monophosphate phosphorylase (Iltzsch, 1993). All pyrimidine compounds are first catabolized to uracil mediated by parasite activities for uridine phosphorylase, deoxyuridine phosphorylase, cytidine deaminase, and deoxycytidine deaminase. Uracil is the only pyrimidine compound that is directly incorporated by salvage into the parasite pyrimidine pool (Figure 20.1).

*T. gondii* lacks any thymidine kinase (TK) activity making the parasite incapable of salvaging thymidine. *T. gondii* interconverts thymine and thymidine (Iltzsch, 1993), but these compounds are incorporated only into a *T. gondii* strain that has been transformed with a TK gene derived from herpes simplex virus (Fox *et al.*, 2001). Similarly, *T. gondii* cannot salvage cytosine unless the parasite is transformed with a bacterial cytosine deaminase (CD) gene (Fox *et al.*, 1999).

#### *Uracil phosphoribosyltransferase (UPRT) activity in T. gondii*

The UPRT activity expressed by *T. gondii* is largely absent in the mammalian host. In addition to its natural substrate uracil, the *T. gondii* UPRT also recognizes 2,4-dithiouracil and incorporates this uracil analog into parasite nucleic acids. This property of UPRT has been adapted to enable cell specific microarray analysis of mRNA synthesis and decay (Cleary *et al.*, 2005). UPRT also recognizes fluorouracil compounds as well as other uracil analogs that suggest a pathway of drug development based on selective incorporation of toxic analogs by parasite UPRT. This approach has been validated in studies using 5'-fluorouracil, 5'-fluorouridine, FUDR, and emimycin (Pfefferkorn, 1978; Pfefferkorn *et al.*, 1989; Pfefferkorn and Pfefferkorn, 1977a; Pfefferkorn and Pfefferkorn, 1977b). Incorporation of 5'-fluorouracil ultimately blocks the activity of thymidylate synthase and accumulation of thymine nucleotides (discussed below). By contrast, the mechanism of inhibition by emimycin is uncertain. Parasites that are deficient in UPRT activity are completely resistant to the toxic effects of emimycin. While emimycin is incorporated into the nucleotide pools, emimycin 5'-triphosphate is not incorporated into parasite RNA or DNA, and the mechanism of inhibition remains uncharacterized (Pfefferkorn *et al.*, 1989). The crystal structure of *T. gondii* UPRT has already been determined and efforts are under way to identify potential inhibitors, as well as substrates that may be selectively incorporated to induce selective cytotoxicity to *T. gondii* (Schumacher *et al.*, 2002; Schumacher *et al.*, 1998).

Complete disruption of the salvage pathway via disruption of UPRT has no detectable effect on tachyzoite growth or virulence (Donald and Roos, 1995; Pfefferkorn, 1978). Yet uracil is well incorporated into nucleic acids in tachyzoites and represents a functional pathway for obtaining pyrimidines (Fox and Bzik, 2002). The retention of a non-essential gene for the parasite UPRT and other salvage activities suggests that some advantage may be conferred by its expression. Expression of UPRT may confer some minor advantage to intracellular parasites by enabling the recovery and reincorporation of pyrimidines into the UMP pool, or UPRT may play another as yet unidentified role in parasite cell biol-

ogy or metabolism. UPRT behaves as an inactive homodimer composed of two identical subunits in the absence of substrates or its activator GTP. Because GTP binding stabilized the active tetrameric structure of UPRT (Schumacher *et al.*, 2002), it was suggested that UPRT could play some role in balancing pyrimidine and purine nucleotide pools. If so, this balance would be best achieved in the non-mammalian environment because UPRT cannot substantially contribute to parasite pyrimidine nucleotide pools in the mammalian environment (Fox and Bzik, 2002).

### Folate pathways and thymine nucleotide synthesis

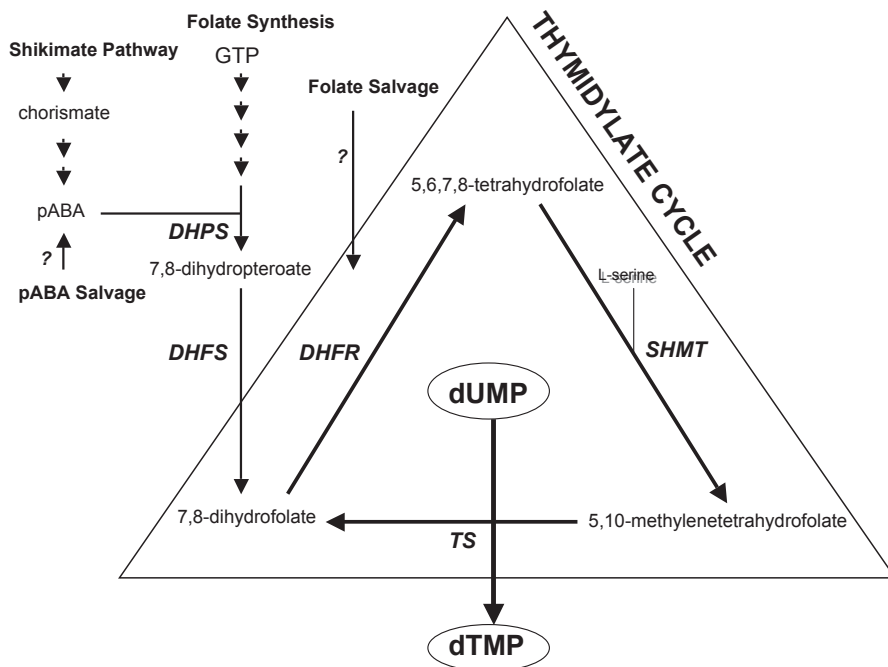
The folate pathway is a key target for antimicrobial agents directed against *T. gondii*. Thymine nucleotides are formed by the methylation of dUMP to produce dTMP in a reaction mediated by thymidylate synthase (TS) in folate metabolism (Figure 20.2). During conversion of dUMP to dTMP a molecule of tetrahydrofolate is oxidized to dihydrofolate. Thus thymidine starvation is induced in *T. gondii* by reducing the pool of tetrahydrofolate via inhibition of DHFR and recycling of dihydrofolate to tetrahydrofolate. During the thymidylate cycle, *T. gondii* relies on TS to produce dTMP from dUMP, on DHFR for recycling of dihydrofolate to tetrahydrofolate, and on serine hydroxymethyltransferase to produce 5,10-methylenetetrahydrofolate from serine (Figure 20.2). While humans depend upon a monofunctional dihydrofolate reductase (DHFR) for DNA replication, the DHFR activity is present on a bifunctional polypeptide with TS activity (DHFR-TS) in parasites from the phylum Apicomplexa and other protozoa (Bzik *et al.*, 1987).

The primary precursors for the *de novo* synthesis of folates are *para*-aminobenzoic acid (pABA) and guanosine 5'-triphosphate (GTP) (Figure 20.2). Humans lack the ability to synthesize folates *de novo* and rely on folate transport from dietary sources. By contrast, *T. gondii* possesses an endogenous folate biosynthetic pathway comprising genes encoding all seven enzymic steps in the *de novo* biosynthesis of 7,8-dihydrofolate (Figure 20.2). While the folate salvage capacity of *T. gondii* is not yet clearly defined, the parasite appears to have putative transporters capable of transporting host pABA and folate (Chaudhary, 2005). In addition to potential salvage from the host, a second pathway to pABA exists as a product of the shikimate pathway via chorismate. *T. gondii* encodes several enzymes of this pathway (Roberts *et al.*, 1998).

### Antifolate chemotherapy and antifolate resistance

Antifolates such as pyrimethamine (PYR) that targets DHFR, and sulfa drugs that target dihydropteroate synthase (DHPS) have long been in clinical use for the treatment of *T. gondii* infections (Figure 20.2). In addition to DHFR-TS, DHPS is also a bifunctional enzyme with hydroxymethylpterin pyrophosphokinase activity in *T. gondii* (Pashley *et al.*, 1997). Current treatment for *T. gondii* infection employs a strategy using both PYR and sulfadiazine. While *T. gondii* has not developed resistance to antifolates, the long-term use of this therapy to treat toxoplasmosis in AIDS patients has proven to be difficult due to significant adverse clinical reactions (Haverkos, 1987).

By modeling *Plasmodium falciparum* resistance mutations at codons equivalent to amino acids 59 and 108 (Bzik *et al.*, 1987), high-level PYR resistance was obtained in *T. gondii* DHFR-TS (Donald and Roos, 1993; Roos, 1993). Plasmids conferring high-



**Figure 20.2** The thymidylate cycle, thymine nucleotide synthesis and pathways to folate. The thymidylate cycle is enclosed by a large triangle. Solid lines and arrows depict active pathways present in *T. gondii*. Substrates of several enzyme activities are shown at the start of the solid line and the product(s) are shown on the arrowhead side. The enzyme activity responsible for selected conversion steps is shown in capital italicized text beside the arrowhead line. All *de novo* pathways shown appear to be present in *T. gondii*, but folate salvage and pABA salvage are indicated with a question mark because these areas have not been well investigated. Polyglutamated forms of folate are not shown. dTMP synthesis is shown at the bottom of the thymidylate cycle. Abbreviations used are as follows: DHPS; dihydropteroate synthase, DHFS; dihydrofolate synthetase, SHMT; serine hydromethyltransferase, DHFR; dihydrofolate reductase, TS; thymidylate synthetase.

level PYR resistance in *T. gondii* were used as a model to investigate the evolution and mechanisms associated with PYR and cycloguanil resistance in *Plasmodium falciparum* (Reynolds and Roos, 1998). This same approach was also used to investigate evolutionary fitness of DHFR-TS mutations associated with PYR resistance (Fohl and Roos, 2003).

Modeling studies of *T. gondii* DHFR-TS have suggested that the long linker domain connecting DHFR to TS donates a helix that crosses to the second DHFR domain of the homodimer complex and contacts the outer shell of the DHFR active site (Belperron *et al.*, 2004; O'Neil *et al.*, 2003). Genetic studies of *T. gondii* DHFR-TS revealed mutations within the linker domain that inactivated pyrimethamine resistance as well as enzyme activity *in vitro* and *in vivo* (Belperron *et al.*, 2004). These observations suggest that the novel long linker domain of DHFR-TS in parasites from the phylum Apicomplexa may represent a drug target.

Genetic selection models based on enzymes of pyrimidine metabolism

The isolation of both a genomic DNA version as well as a cDNA version of the PYR resistant DHFR-TS enabled an assessment of general homology requirements for recombination in *T. gondii*. The cDNA version of *T. gondii* DHFR-TS lacks numerous introns contained in the genomic DNA version and plasmids carrying the DHFR-TS cDNA incorporate randomly into the genome of the parasite (Donald and Roos, 1993). By contrast, plasmids carrying a large genomic DNA version of *T. gondii* DHFR-TS can integrate into the homologous DHFR-TS gene locus (Donald and Roos, 1994; Donald and Roos, 1995). These studies established methods and tools for random insertional mutagenesis, as well as gene replacement approaches in *T. gondii*.

Subsequently, high-level PYR resistant DHFR-TS was converted into a trifunctional enzyme by the incorporation of either a *Herpes simplex* virus TK gene, or a bacterial cytosine deaminase (CD) gene. The TK and CD genes were inserted as in-frame coding regions into the linker domain of *T. gondii* DHFR-TS. The DHFR-CD-TS plasmid confers high-level resistance to PYR (positive selection), and stable PYR resistant parasite clones are killed (negative selection) by treatment of parasites with low doses of the normally non-toxic prodrug 5-fluorocytosine (Fox *et al.*, 1999). This study established DHFR-CD-TK as a trifunctional enzyme capable of positive and negative selection in *T. gondii*. Similarly, construction of a plasmid encoding the trifunctional enzyme DHFR-TK-TS on a single polypeptide enabled positive selection by high-level resistance to PYR, and negative selection in sub-micromolar doses of ganciclovir (Fox *et al.*, 2001). The DHFR-TK-TS plasmid was used in positive and negative selection experiments to obtain the avirulent uracil auxotroph mutant by targeted knock out of the CPSII gene (Fox and Bzik, 2002). In the phylum Apicomplexa, the *T. gondii* CPSII is the most amenable for genetic dissection of CPSII activities and regulation. The availability of an attenuated CPSII knock out mutant parasite, as well as the *T. gondii* CPSII gene sequences enables a new genetic scheme for positive selection based on complementation (Fox and Bzik, 2002; Fox and Bzik, 2003).

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## Purines

Purines are crucial to all cells as required components of nucleic acids, a cellular energy source, and cofactors or substrates for specific aspects of cellular metabolism. All aspects of the *T. gondii* life style including motility dependent invasion of host cells are powered by nucleotides (Kimata and Tanabe, 1982). Remarkably, *T. gondii* is incapable of *de novo* synthesis of the purine ring (Krug *et al.*, 1989; Perotto and Keister, 1971; Schwartzman and Pfefferkorn, 1982). Therefore the parasite has adopted elaborate pathways to steal purines from the host cell, as well as having incorporated a variety of enzymes for salvaging host purine nucleobases and nucleosides into the parasite nucleotide pools. The transport and salvage pathways necessary for purine acquisition have long been viewed as a significant weakness of the parasite that may be targeted for chemotherapy. *T. gondii* is an obligate intracellular parasite that inhabits and replicates within a parasitophorous vacuole within the cytoplasm of the host cell. Consequently, while exogenous purines most likely would need to pass through the host cell plasma membrane, purines in the host cell cytoplasm would only need to pass through the parasitophorous vacuole membrane and the parasite plasma membrane before gaining access to the parasite enzymes in the parasite cytosol that can salvage and incorporate host purines into the parasite nucleotide pools (Ngo *et al.*, 2000).

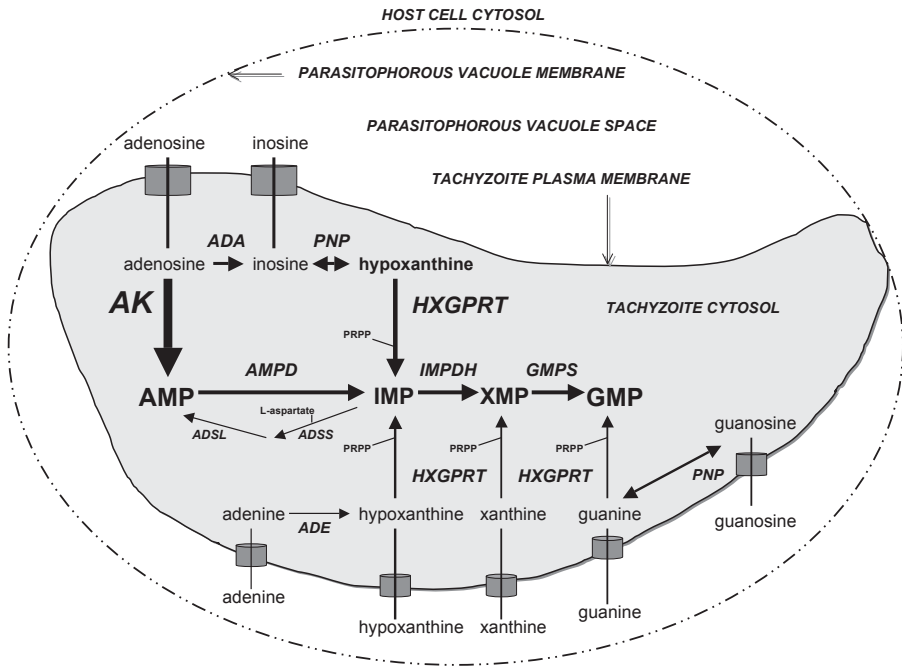
## Capture and transport

Nucleoside and nucleobase transporters fall into two general groups, sodium-dependent concentrative nucleoside transporters (CNT) and non-concentrative equilibrative nucleoside transporters (ENT). Mammals and bacteria possess CNT and ENT transporters while the protozoa (so far) only have ENT-type transporters (de Koning *et al.*, 2005; Landfear *et al.*, 2004). Analysis of the *T. gondii* genome reveals that the parasite possesses genes for putative purine permeases that are predicted to be members of the ENT transporter family (Chaudhary, 2005). To date, genes corresponding to other families of purine transporters have not been identified among the protozoa.

Current models propose that *T. gondii* purine transporters on the parasite plasma membrane are a required mechanism of purine acquisition from the host. For *T. gondii*, this model suggests that host cell derived purines could passively or actively accumulate in the parasitophorous vacuole space (Figure 20.3). However, few studies have yet experimentally addressed whether there is any specific requirement for host cell purine transporters for the successful capture of purines by replicating tachyzoites.

### *An early model of purine acquisition in T. gondii*

The first working model of purine acquisition by *T. gondii* was based on host cell ATP, a purine present at several mM in host cytosol (Plagemann *et al.*, 1988). This model was supported in early dual-label experiments showing that extracellular *T. gondii* readily incorporated the nucleoside component of ATP or AMP into nucleic acids, but did not incorporate the phosphate moiety (Schwartzman and Pfefferkorn, 1982). The identification of the parasitophorous vacuole surrounding intracellular tachyzoites as a passive permeation barrier suggested that the high concentration of cytosolic or mitochondrial derived host ATP potentially could permeate into the parasitophorous vacuole space and equilibrate at mM concentrations (Schwab *et al.*, 1994). The discovery of a remarkably abundant nucleoside triphosphate hydrolase (NTPase) activity secreted into the parasitophorous vacuole space suggested host ATP may be hydrolyzed to AMP within the vacuolar space (Asai *et al.*, 1995; Bermudes *et al.*, 1994; Sibley *et al.*, 1994b). Consequently, it was postulated that *T. gondii* might obtain its purine requirement from the flux created through permeation of ATP into the vacuolar space, conversion of ATP to AMP by vacuolar NTPase activity, and conversion of AMP to adenosine by a putative parasite plasma membrane 5'-ectonucleotidase. Since the pool of cytosolic host cell adenosine is very small at ~1  $\mu$ M (Plagemann *et al.*, 1988), utilization of host ATP pools was an attractive model to concentrate adenosine in the parasitophorous vacuole space. The first adenosine transporter characterized for *T. gondii*, TgAT1, was described as a non-concentrative low-affinity ( $K_m$  ~120  $\mu$ M) adenosine transport system that likely would need a higher concentration of adenosine than the 1  $\mu$ M present in host cell cytosol for physiological significance (Schwab *et al.*, 1995). Subsequent work has indicated that intracellular tachyzoites as well as the parasitophorous vacuole space, or membrane, have no detectable 5'-ectonucleotidase activity (Ngo *et al.*, 2000). Therefore *T. gondii* has no access to intracellular pools of host nucleotides, suggesting that host cell derived purine nucleosides and nucleobases are the most important purine sources for intracellular *T. gondii* (Figure 20.3).



**Figure 20.3** Model of purine salvage and incorporation pathways in *T. gondii*. The host cell cytosol is shown outside of the parasitophorous vacuole membrane. The tachyzoite form of *T. gondii* is shown inside the parasitophorous vacuole space. Purine compounds accessible to the salvage machinery of the tachyzoite cytosol are shown inside the parasitophorous vacuole space. Purine transporters in the tachyzoite plasma membrane (shown as cylinders) transport purine compounds present in the parasitophorous vacuole space into the cytosol of the tachyzoite. *T. gondii* has significant metabolic machinery for the interconversion and salvage of host purine nucleobases and nucleosides into the adenylate (AMP) and guanylate (GMP) nucleotide pools. Solid lines and arrows depict active pathways present in *T. gondii*. Substrates of each enzyme activity are shown on the side of the solid line and the product(s) of each enzyme activity are shown on the arrowhead side. The enzyme activity responsible for each interconversion step is shown in capital italicized text beside the arrowhead line. Adenosine kinase (AK) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) represent the major pathways for salvage and incorporation into the nucleotide pool. Additional abbreviations used are as follows: PRPP; 5-phosphoribosyl-1-pyrophosphate, ADA; adenosine deaminase, ADE; adenine deaminase, PNP; purine nucleoside phosphorylase, ADSS; adenylosuccinate synthetase, ADSL; adenylosuccinate lyase, AMPD; AMP deaminase, IMPDH; inosine 5'-monophosphate dehydrogenase, GMPS; GMP synthetase.

*Purine transporters in T. gondii*

TgAT1 is a low affinity transporter for adenosine, inosine, and guanosine nucleosides (Schwab et al., 1995). Low-affinity transport of adenosine by TgAT1 is blocked by dipyrri-damole and inhibited by excess inosine, formycin B, or hypoxanthine, but not pyrimidines. Nitrobenzylthioinosine (NBMPR), an inhibitor of adenosine transport in mammalian cells did not block adenosine transport by TgAT1. Selection and characterization of ad-



enine arabinoside resistant (ara-A) mutants identified mutations at two genetic loci, one corresponding to adenosine kinase (the activator of ara-A cytotoxicity) and the second in a gene, designated TgAT, characterized as a putative 11 membrane-spanning region protein of the ENT family (Chiang *et al.*, 1999). Expression of TgAT in *Xenopus laevis* oocytes reconstituted low-affinity adenosine transport function similar to the previously characterized TgAT1 suggesting that TgAT represents the cloned gene of TgAT1 (Chiang *et al.*, 1999; Schwab *et al.*, 1995). Remarkably, disruption of the TgAT gene is not lethal (Chiang *et al.*, 1999).

In addition to TgAT, high affinity nucleoside and nucleobase transporters were identified in extracellular tachyzoites of *T. gondii* (De Koning *et al.*, 2003). Kinetic evidence revealed the presence of a high-affinity adenosine ( $K_m \sim 0.49 \mu\text{M}$ ) and inosine ( $K_m \sim 0.77 \mu\text{M}$ ) transporter that was also a high-affinity and broad-spectrum transporter capable of transporting a large number of different purine and pyrimidine nucleosides. This second adenosine transport system was designated as TgAT2 (De Koning *et al.*, 2003). The high-affinity and broad specificity of TgAT2 suggested it could be an efficient route for uptake of various therapeutic nucleosides (De Koning *et al.*, 2003). This work also revealed a third transport system, designated TgNT1 that selectively transports purine nucleobases. TgNT1 is a high-affinity transport system for hypoxanthine ( $K_m \sim 0.91 \mu\text{M}$ ) as well as guanine and xanthine. The presence of TgAT2 and TgNT1 on the tachyzoite plasma membrane of extracellular tachyzoites indicates a significant capacity for *T. gondii* tachyzoites to salvage even low concentrations of host purine nucleobases and nucleosides that may permeate into the parasitophorous vacuole space in the intracellular environment (Figure 20.3).

The *T. gondii* genome reveals TgAT and three additional ENT orthologs designated as TgNT1, TgNT2, TgNT3 (Chaudhary, 2005). Other than TgAT (Chiang *et al.*, 1999), the specific functional role of each of these ENT orthologs in purine transport is currently unknown.

TgAT, TgNT1 and TgNT3 are expressed in tachyzoite and bradyzoite stages (Chaudhary, 2005). In infected host cells, TgAT and TgNT3 primarily localize to the parasite plasma membrane, and TgNT1 localized as punctate labeling to the cytosol but did not co-localize with any other marker of a known parasite organelle (Chaudhary, 2005). The cytosolic and non-organellar localization of TgNT1 is highly unusual for a transporter that is typically associated with transport across biological membranes. The characterization of a family of *T. gondii* nucleoside and nucleobase transporter genes now enables a more detailed functional characterization of their potential roles in purine acquisition by the intracellular tachyzoite.

#### *Purine transport in the T. gondii infected host cell*

Remarkably, *T. gondii* parasitized host cells selectively transport NBMPR (Al Safarjalani *et al.*, 2003), and NBMPR is selectively cytotoxic to intracellular *T. gondii* (el Kouni *et al.*, 1999). The activation of NBMPR cytotoxicity is associated with the ability of *T. gondii* derived enzyme extracts, but not host cell derived enzyme extracts, to phosphorylate NBMPR to its nucleoside 5'-monophosphate. Adenosine kinase (AK) deficient *T. gondii* failed to phosphorylate NBMPR to its nucleoside 5'-monophosphate showing parasite

AK to be the major pathway to selective incorporation and cytotoxicity (Al Safarjalani *et al.*, 2003; Rais *et al.*, 2005). Remarkably, *T. gondii* parasitized host cells also selectively transport non-physiological  $\beta$ -L-enantiomers of purine nucleosides,  $\beta$ -L-adenosine,  $\beta$ -L-deoxyadenosine, and  $\beta$ -L-guanosine. Notably, uninfected host cells do not transport NBMPR or the  $\beta$ -L-nucleosides. NBMPR also inhibits the transport function of the host cell nucleoside transporter ENT1 (Gupte *et al.*, 2005). Dipyridamole, another inhibitor of nucleoside transport, inhibited transport of NBMPR and  $\beta$ -L-nucleosides into parasitized host cells. Transport of NBMPR and  $\beta$ -L-nucleosides in the parasitized host cell required a functional TgAT transporter (Al Safarjalani *et al.*, 2003; Chiang *et al.*, 1999). These observations address the requirement for transport of purines into the intracellular tachyzoite, but do not explain why the parasitized host cell selectively transports these compounds, whereas the non-parasitized host cell does not. Therefore infection with *T. gondii* confers parasite-specific purine transport mechanisms to the host cell.

#### *Model of novel purine transport mechanism(s) in T. gondii infected host cells*

The novel transport capacity of the *T. gondii* parasitized host cell may open new avenues for understanding transport functions and biology, as well as opportunities for drug development. The novel transport mechanisms specific to the *T. gondii* parasitized host cell may arise from the recently reported equilibratory high-affinity adenosine transporter (De Koning *et al.*, 2003), a concentrative ion-dependent channel, a tubulovesicular membrane system interconnecting the parasitophorous vacuole to the host cell periphery, or a duct for transport of macromolecules that bypasses the host cell plasma membrane (Gero *et al.*, 2003). The elegant electrophysiological description of the mechanism of parasitophorous vacuole formation in *T. gondii* suggested that after *T. gondii* invasion and vacuole formation a fission pore remnant is left on the host cell surface (Suss-Toby *et al.*, 1996). Shortly after invasion of the host cell, a protein and membrane rich intravacuolar network (tubulovesicular network) derived from electron-dense granules is formed in the parasitophorous vacuole space (Mercier *et al.*, 1998; Mercier *et al.*, 2002; Sibley *et al.*, 1995). Three-dimensional imaging of *T. gondii* within recently formed vacuoles revealed fibrous and tubular material that connects the parasite plasma membrane on intracellular tachyzoites within the parasitophorous vacuole to the remnant of the fission pore at the host cell plasma membrane (Schatten and Ris, 2004). Collectively, these observations suggest that the transport of nutrients such as purines to the parasite in the parasitophorous vacuole may be facilitated by additional mechanisms beyond simple diffusion of host cell cytosol nutrients through proposed pores in the parasitophorous vacuole membrane. Further studies are necessary to define specific roles to each of the identified *T. gondii* purine transporter ENT gene orthologs (Chaudhary, 2005), the requirement for host cell transporter function(s), the role of the tubulovesicular network established in the parasitophorous vacuole space, and the mechanisms established within the parasitized host cell that promotes the permeation of host purines into the parasitophorous vacuole space and the replicating tachyzoite.

#### *Purine metabolic pathways in T. gondii*

In early studies, [ $^3\text{H}$ ] hypoxanthine labeling of *T. gondii* infected Lesch-Nyhan mutant human host cells deficient in host hypoxanthine-guanine phosphoribosyltransferase activ-

ity revealed that only parasites were labeled and no detectable incorporation occurred in host cell nucleic acids (Pfefferkorn and Pfefferkorn, 1977c). Therefore within the *T. gondii* parasitized host cell, the flux of purines is unidirectional from the host cell to the tachyzoite (Figure 20.3).

#### *Current model of purine interconversion and salvage pathways in T. gondii*

Our current understanding of purine salvage pathways in *T. gondii* is illustrated in Figure 20.3. Purine acquisition in this parasite has generally been a highly investigated area of biology due to the early recognition that interruption of this pathway would starve the parasite of essential nucleotides. In parasites from the phylum Apicomplexa, *T. gondii* appears to possess the most extensive pathways for acquisition of purines (Chaudhary *et al.*, 2004).

A comprehensive analysis of *T. gondii* biochemical activities involved in salvage, interconversion and incorporation of host purines was assessed in viable non-replicating extracellular tachyzoites (Krug *et al.*, 1989). This study employed high concentrations of radiolabeled purines to maximize their transport and incorporation. Hence, it should be recognized that such high levels of host purines are unlikely to be available to the intracellular replicating tachyzoites. This study reported that the purine bases hypoxanthine, xanthine, guanine, and adenine were incorporated indicating the presence of a hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) enzyme as well as an adenine phosphoribosyltransferase (APRT) (Krug *et al.*, 1989). Subsequent studies have demonstrated *T. gondii* has no detectable APRT activity or gene (Chaudhary *et al.*, 2004). Adenine was incorporated only one-half as efficiently as hypoxanthine.

The purine nucleosides adenosine, inosine, guanosine, and xanthosine were incorporated into nucleotide pools and nucleic acids. Adenosine was incorporated more than 12-fold as well as any other purine nucleoside or nucleobase, and suggested a major parasite adenosine kinase (AK) activity to be the primary route to AMP. Correspondingly in parasite extracts AK activity was greater than 15-fold more active than the next most active enzyme *in vitro*. Hypoxanthine was incorporated at 8.3%, inosine at 8.2%, xanthine at 5.6%, guanine at 4.6%, adenine at 3.9%, guanosine at 2.5%, and xanthosine at 0.3% of the rate at which adenosine was incorporated (Krug *et al.*, 1989). While xanthosine incorporation was detected, this host purine is considered to be insignificant to *T. gondii*.

Guanine, guanosine, xanthine, and xanthosine labeled only guanylate nucleotides. Therefore *T. gondii* has no pathway from guanylate to adenylate nucleotides (Krug *et al.*, 1989; Pfefferkorn *et al.*, 2001). Adenosine, inosine, hypoxanthine, and adenine labeled adenylate and guanylate nucleotides pools at approximately equal ratios.

Activities were detected for guanine deaminase (GUAD), adenine deaminase (ADE), adenosine deaminase (ADA), and AMP deaminase (AMPD) (Krug *et al.*, 1989). While the GUAD activity may be present, the GUAD reported in early studies is not shown on the current pathway depicted in Figure 20.3 because this activity was low, there is abundant host GUAD that could contaminate tachyzoite preparations, and no gene ortholog is yet detected for a *T. gondii* GUAD (Chaudhary *et al.*, 2004). While a putative gene ortholog for ADE has been identified, this pathway appears to be a minor pathway for incorporation of purine nucleotides (Krug *et al.*, 1989; Chaudhary *et al.*, 2004). Although

no gene ortholog for the ADA activity has been yet reported (Chaudhary *et al.*, 2004), the *T. gondii* ADA activity was validated in studies using mutant host cell deficient in host ADA (Krug *et al.*, 1989). Purine nucleoside phosphorylase (PNP) activities were detected only for guanosine and inosine. Once hypoxanthine is available, it is converted to IMP by HXGPRT. AMP is made in two steps from IMP by adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ADSL), and GMP is made in two steps by inosine 5'-monophosphate dehydrogenase (IMPDH) and GMP synthetase (GMPS). Interconversion of adenylate and guanylate nucleotides occurs only in the direction of adenylate to guanylate nucleotides via AMP deaminase (AMPD) (Figure 20.3) (Krug *et al.*, 1989; Pfefferkorn *et al.*, 2001).

Apart from AK no other nucleoside kinase or phosphotransferase activities were detected. There is no GMP reductase. Genome analysis indicates gene orthologs for nine purine metabolic enzymes (AK, HXGPRT, ADE, PNP, AMPD, ADSS, ADSL, IMPDH, GMPS) (Chaudhary *et al.*, 2004). Therefore *T. gondii* possesses a minimum of 10 enzymes involved in interconversion or incorporation of host purines. *T. gondii* can transport and incorporate at least seven host purine nucleobases and nucleosides, adenine, adenosine, inosine, hypoxanthine, xanthine, guanine, and guanosine. Structurally, *T. gondii* possesses two major biochemical routes to incorporate host purines into the parasite nucleotide pools via AK and HXGPRT (Figure 20.3). After salvage of host purines to AMP and GMP the parasite possesses the kinases to construct the necessary nucleoside diphosphate (ADP and GDP) and triphosphate forms (ATP and GTP). Conversion of the purine ribonucleotides to deoxyribonucleotides occurs at the level of nucleoside diphosphate by RNR.

### Adenosine kinase and hypoxanthine-xanthine-guanine-phosphoribosyltransferase

Direct salvage of host adenosine to AMP via AK appears to be the major pathway of purine nucleotide incorporation (Figure 20.3) (Chaudhary *et al.*, 2004; Krug *et al.*, 1989; Ngo *et al.*, 2000). Yet, parasites disrupted in their AK gene and activity replicate normally most likely by salvage through HXGPRT (Pfefferkorn and Pfefferkorn, 1976; Pfefferkorn and Pfefferkorn, 1978; Sullivan *et al.*, 1999). *T. gondii* AK has been expressed in *E. coli* for biochemical, kinetic, and structural studies that have revealed significant differences between the parasite AK and the mammalian AK, which may be exploited for drug design (Darling *et al.*, 1999; Schumacher *et al.*, 2000a; Schumacher *et al.*, 2000b).

HXGPRT represents the second major route of incorporation of host purines. Yet, parasites disrupted in their HXGPRT gene and activity replicate normally most likely by salvage through AK (Donald *et al.*, 1996; Pfefferkorn and Borotz, 1994). Therefore, *T. gondii* possesses a functionally redundant purine salvage pathway (AK and HXGPRT) with the capacity to meet the purine requirement by using an assortment of potential host cell purine nucleobases and nucleosides (Figure 20.3). The crystal structure and enzyme mechanisms of *T. gondii* HXGPRT have been characterized and HXGPRT is under investigation as a potential drug target (Heroux *et al.*, 1999a; Heroux *et al.*, 1999b; Schumacher *et al.*, 1996).

### *Selection of AK and HXGPRT deficient parasites*

HXGPRT and AK activities cannot be simultaneously disrupted in *T. gondii*, suggesting that these are the only functional routes to purine nucleotides in the parasite (Chaudhary *et al.*, 2004). A parasite possessing no functional endogenous *T. gondii* genes for AK and HXGPRT is viable when complemented by a functional APRT gene from *Leishmania donovani* (Chaudhary *et al.*, 2004). Therefore the AK and HXGPRT pathways are the only functional routes for purine incorporation in *T. gondii*.

This genetic study also demonstrated that single gene knock outs of parasite AK or HXGPRT have a small but detectable defect in fitness as determined by growth rate of tachyzoites. AK deficient parasites exhibit a fitness defect in growth rate of 7.6% per generation, while HXGPRT deficient parasites exhibit a fitness defect in growth rate of 3.7% per generation (Chaudhary *et al.*, 2004). This significant finding suggests that purine acquisition is likely to be rate limiting to the parasite growth rate *in vitro*. In the case of the AK knock out, the same host supply of adenosine would be available as in the wild type parent, thus the flux of adenosine, when diverted by lack of AK activity, to inosine to hypoxanthine to IMP (Figure 20.3), then to guanylate and adenylate nucleotides is not sufficient to fully support the normal parasite growth rate. Similarly, in the HXGPRT knock out, transport and incorporation of host adenosine through the AK pathway is insufficient to fully support parasite replication. Therefore, multiple host purine nucleobases and/or nucleosides as well as both the AK and HXGPRT pathways of incorporation of host purines into the nucleotide pools of *T. gondii* appears to be required for supporting the maximum replication rate of tachyzoites during their intracellular growth. Based on the very high activity of AK in *T. gondii* (Krug *et al.*, 1989), a bottleneck for purine acquisition may exist due to the limited availability and/or transport of host purine nucleosides and nucleobases in the intracellular environment.

### *Purine nucleoside phosphorylase*

The gene for *T. gondii* PNP has been cloned and active PNP expressed in *E. coli* (Chaudhary, 2005). *T. gondii* PNP recognizes guanosine and inosine as good substrates, and xanthosine and adenosine as poor substrates. In contrast to human PNP, the parasite enzyme is unusual in recognizing the deoxynucleosides as poor substrates. The parasite PNP shows no activity to 5'-methylthiopurine compounds, but the enzyme, like human PNP, is inhibited by immucillin-H.

Immucillin-H has no effect on the growth of wild type parasites. By contrast, immucillin-H blocks replication of parasites deficient in AK activity (Chaudhary, 2005). Exogenously supplied hypoxanthine antagonizes the growth inhibition of AK deficient parasites by immucillin-H. Based on the described pathways of purine acquisition (Figure 20.3), it appears that if the host supplied significant quantities of adenine and hypoxanthine, immucillin-H may not inhibit parasite replication even in an AK deficient parasite (Figure 20.3). Therefore host purine nucleosides, rather than host nucleobases, appear to be the more important host purine source for replication of intracellular tachyzoites.



### *Isoforms of HXGPRT*

A novel feature of the *T. gondii* purine salvage pathway is the expression of two forms of HXGPRT from a single gene locus by alternatively spliced mRNA (Donald *et al.*, 1996; White *et al.*, 2000). The two isoforms differ by a 49 amino acid segment comprising an extra exon in isoform-II. Both isoforms behave in a kinetically similar manner, although isoform-II is slightly less efficient in recognizing guanine as a substrate (White *et al.*, 2000). Isoform-I is cytosolic, while the longer version isoform-II is localized to within the inner membrane complex (IMC) of the tachyzoite (Chaudhary *et al.*, 2005). The 49 amino acid insert at the N-terminus of isoform-II is required for the localization to the IMC. The mechanism of IMC localization was identified to be palmitoylation that occurred at three adjacent cysteine residues within a 49 amino acid insert. Mutation of these three cysteines blocked palmitoylation and localization to the IMC (Chaudhary *et al.*, 2005). The biological basis of functional redundancy of HXGPRT in *T. gondii* is not obvious because both isoforms are functionally competent HXGPRT activities (Donald *et al.*, 1996; Donald and Roos, 1998).

### Genetic selection based on HXGPRT

Early studies using the inhibitor 6-thioxanthine established the parasite HXGPRT as both a potential drug target and a gene that would be amenable for both positive and negative genetic selection in *T. gondii* (Pfefferkorn and Borotz, 1994). The mechanism of growth inhibition by 6-thioxanthine is based on activation of 6-thioxanthine to 6-thioxanthine 5'-monophosphate by parasite HXGPRT (Pfefferkorn *et al.*, 2001). Unlike mercaptopurine in mammals (Elion, 1989), 6-thioxanthine and its nucleotide product 6-thioxanthine 5'-monophosphate is not a substrate for *T. gondii* GMPS and is not incorporated into nucleic acids (Figure 20.3). The mechanism of inhibition is parasitostatic and appears to primarily involve inhibition of parasite IMPDH (Pfefferkorn *et al.*, 2001).

*T. gondii* mutants deficient in HXGPRT are completely resistant to the toxic effects of 6-thioxanthine (Pfefferkorn and Borotz, 1994). Once 6-thioxanthine resistance is selected by knock out of HXGPRT, then parasites with a functional HXGPRT can be positively selected by growth in mycophenolic acid (MPA) and xanthine or guanine (Pfefferkorn and Borotz, 1994). This selection scheme is based on the ability of MPA to specifically inhibit IMPDH, blocking the conversion of IMP to XMP (Figure 20.3). The identification of the HXGPRT gene enabled a test of this biochemical prediction and resulted in the establishment of a robust genetic selection scheme for positive and negative selection using the selection principles described above (Donald *et al.*, 1996). The HXGPRT selection scheme established the first genetic system for hit and run mutagenesis in *T. gondii* where a stable pseudodiploid could be established during positive selection, then negative selection is used to force out the HXGPRT gene to create a subtle or major mutation within the gene locus of interest (Donald and Roos, 1998).

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## Amino acids

Amino acids are essential for protein synthesis and serve in numerous aspects of cellular metabolism. Relatively little experimental work has directed at this area of parasite biology. Nonetheless, several areas of interesting parasite biology, including nucleotide metabolism,



have been connected with amino acid metabolism. In *T. gondii*, amino acids are required for pyrimidine biosynthesis (Figure 20.1), thymine nucleotide synthesis (Figure 20.2), and conversion of IMP to AMP (Figure 20.3). The genome of *T. gondii* has enabled new insights into novel aspects of amino acid metabolism.

### Amino acid acquisition in *T. gondii*

Surprisingly, the architecture of CPSII activity in *T. gondii* revealed the presence of a single glutamine-dependent CPSII gene and activity (Fox and Bzik, 2002; Fox and Bzik, 2003). The existence of a single CPSII in *T. gondii* is highly unusual for a eukaryotic organism. In many prokaryotes, a single CPS polypeptide is typically found and this CPS activity is responsible for producing carbamoyl phosphate, the precursor molecule for both pyrimidines and arginine. Consequently, disruption of *E. coli* CPS produces a dual pyrimidine and arginine auxotrophy (Beckwith *et al.*, 1962). In most eukaryotes two distinct CPS genes and activities are found, a glutamine-dependent CPSII selectively linked with pyrimidine biosynthesis and a mitochondria associated CPSI dedicated to arginine biosynthesis (Davis, 1986). The carbamoyl phosphate produced by CPSI in many eukaryotes is sequestered in the mitochondria for immediate conversion to citrulline via ornithine carbamoyltransferase. Arginine is produced from citrulline in two steps by the sequential actions of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) (Davis, 1986).

The availability of a CPSII knock out mutant in *T. gondii* enabled a functional determination of whether the sole CPSII in apicomplexa is responsible for pyrimidine and arginine biosynthesis (Fox *et al.*, 2004). *T. gondii* CPSII and the carbamoyl phosphate product are dedicated to pyrimidine biosynthesis (Figure 20.1). *T. gondii* has a natural arginine auxotrophy. The arginine auxotrophy of *T. gondii* is rescued by supplementing growth media with either arginine or citrulline. Using mutant host cells it was demonstrated that rescue with citrulline was dependent on the presence of host cell AS and AL activities. These experiments demonstrated the absence of any arginine biosynthetic enzyme activities in *T. gondii* and this conclusion has been verified by the absence of corresponding gene orthologs in the *T. gondii* genome.

The natural arginine auxotrophy and arginine depletion in *T. gondii* infection have been linked with the differentiation of tachyzoites to bradyzoite containing cysts *in vitro* (Fox *et al.*, 2004). These observations suggest that local depletion of arginine by inducible nitric oxide synthase during the host immune response to *T. gondii* infection may promote signaling or formation of slow growing bradyzoites or cyst development and maintenance (Fox *et al.*, 2004; Wu and Morris, 1998).

Curiously, a second amino acid auxotrophy of *T. gondii* has also been linked to host immune response and control of infection. Early work demonstrated a natural tryptophan auxotrophy of *T. gondii*, which was associated with reduced growth rate of tachyzoites during tryptophan depletion elicited by induction of interferon gamma during the host immune response to *T. gondii* infection (Pfefferkorn, 1984; Pfefferkorn *et al.*, 1986). Notably, tryptophan prototrophy has also been used as a strategy to establish another model for positive selection in *T. gondii* (Sibley *et al.*, 1994a).

Collectively, these studies suggest a fundamental link in host immune responses leading to local depletion of amino acids at sites of *T. gondii* infection as a general mechanism to either slow parasite growth or trigger differentiation of rapidly replicating tachyzoites into slow growing encysted bradyzoite forms.

#### Amino acid metabolism in *T. gondii*

Humans require nine amino acids in their diet and can synthesize the remaining 11 amino acids required for protein synthesis. Mammals, primarily in liver tissue, can synthesize arginine *de novo* from CPSI, OCT, AS and AL activities and arginine is now considered to be a non-essential amino acid (Wu and Morris, 1998). In addition to arginine, humans can synthesize alanine, serine, and glycine (derived from glycolysis), glutamic acid, glutamate, proline, aspartic acid and asparagine (derived from the TCA cycle), and tyrosine and cysteine (derived from essential amino acids phenylalanine and methionine, respectively, from diet).

Parasites from the phylum Apicomplexa as well as other protozoan parasites have diminished amino acid biosynthetic capabilities compared with the human host (Chaudhary and Roos, 2005). In the phylum Apicomplexa, *T. gondii* has the least diminished amino acid biosynthetic capability and most resembles the human host. Compared with the human host *T. gondii* is auxotrophic only for arginine and cysteine. Uniquely, lysine is not an essential amino acid in *T. gondii* because the parasite can synthesize lysine from aspartic acid derived in the TCA cycle by the diaminopimelate pathway (Chaudhary and Roos, 2005). The acquired ability of *T. gondii* to synthesize lysine suggests an increased need for this amino acid, or its limited availability in host tissues. *T. gondii* may also be capable of salvaging lysine from the host cell, but if the parasite lacked this capability then the diaminopimelate pathway represents a potential target. Collectively, these observations suggest that significant adaptations of the different parasites are likely to be related to their potentially available nutritional amino acid resources.

#### The origin of polyamines in *T. gondii*

The origin of polyamines in *T. gondii* is not clearly resolved at this time. The parasite may transport ornithine, putrescine or other polyamines. *T. gondii* appears to lack ornithine decarboxylase (ODC) activity (Seabra *et al.*, 2004), and growth of tachyzoites is unaffected by difluoromethylornithine (DFMO) (Chaudhary, 2005). However, the *T. gondii* genome does reveal the presence of a gene ortholog with homology to the arginine/ornithine decarboxylase (ADC/ODC) gene family. If functional this gene ortholog would represent a potential ADC based on lack of parasite sensitivity to DFMO (Chaudhary, 2005). Currently, no gene ortholog can be identified for any member of the related arginase/agmatinase gene family, or any other recognized gene member of the polyamine biosynthetic pathway (Chaudhary, 2005). Collectively, these data suggests that *T. gondii* is incapable of polyamine biosynthesis and most likely relies upon direct transport and salvage of preformed polyamines supplied by the host cell.

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# Part V

## **Organelles: Sorting, Content, and Function**

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# Protein Targeting to the Apicoplast

21

Marilyn Parsons, Amy DeRocher, and Jean E. Feagin

## Abstract

The apicoplast is a relict chloroplast that is present in *Toxoplasma gondii* and many other apicomplexans. Cytologic and phylogenetic analyses indicate that this organelle evolved via a secondary endosymbiotic event which partnered the progenitor apicomplexan with an algal cell containing the chloroplast ancestor of the apicoplast. Distinct from primary plastids, which are enveloped by two membranes, the apicoplast is bounded by multiple membranes. This chapter discusses how proteins encoded in the parasite nuclear genome are targeted to the lumen of the apicoplast. The first step is specified by an N-terminal signal sequence that directs the protein into the endoplasmic reticulum. Cleavage of the signal sequence reveals a transit peptide that directs the protein to the apicoplast. Current data suggest that trafficking bypasses the Golgi, hence distinguishing the apicoplast from other destinations in the *T. gondii* secretory system.

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## Introduction

In *Toxoplasma gondii*, as in many other apicomplexans, the secretory system sorts proteins to novel organelles, including the dense granules, micronemes, rhoptries, and the apicoplast. The apicoplast is of key interest because not only is it unique to Apicomplexa, but its functions are essential to parasite survival. Most of these functions are accomplished by nucleus-encoded apicoplast targeted (NEAT) proteins. Unlike other organelles, which are bounded by one or two membranes, the apicoplast is bounded by multiple membranes. Hence the molecular mechanisms of protein targeting to the organelle lumen are likely to include novel features. The highly polarized nature of the *T. gondii* tachyzoite, coupled with its simple asexual division cycle leading to two daughters (endodyogeny), has facilitated microscopic analysis of the apicoplast. Coupled with well-developed transfection tools, these advantages have allowed key features of protein targeting to the apicoplast (discussed in this chapter), as well as apicoplast division (discussed elsewhere), to be elucidated in *T. gondii*. Relevant studies in *Plasmodium falciparum* will also be discussed here, particularly with respect to any features which differ in the two organisms.

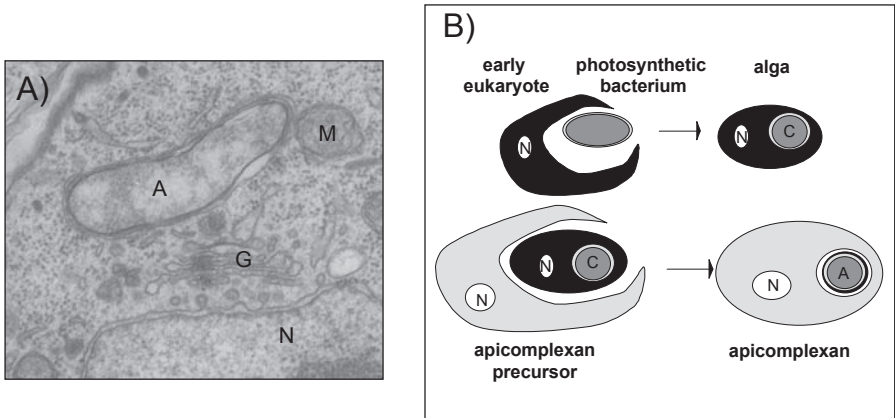
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## The identification and origin of the apicoplast

Although it was originally seen in electron microscopic analyses of apicomplexan parasites [discussed in Siddall (1992)], the function and origin of the apicoplast remained enigmatic

for many years. Figure 21.1A shows a transmission electron microscopy image of a *T. gondii* parasite with the multimembraned organelle clearly visible in its typical position between the nucleus and apical organelles such as the rhoptries. Molecular biological studies aimed at identifying the mitochondrial genome of malaria parasites discovered an unusual DNA that has features reminiscent of chloroplast genomes, with inverted repeats of ribosomal DNA and subunits of a prokaryote-like RNA polymerase (Wilson *et al.*, 1996). EF-Tu, a translational elongation factor which is found in chloroplast as well as bacterial genomes, is also encoded on this genome, as are several other unidentified open reading frames. All of these together mark the DNA as organellar, and more related to chloroplast than mitochondrial genomes. Key experiments using *in situ* hybridization coupled with transmission electron microscopy localized the genome to the multimembraned organelle of *T. gondii* (McFadden *et al.*, 1996; Kohler *et al.*, 1997). The organelle was dubbed the apicoplast based on its presence in phylum Apicomplexa and its genetic relationship to plastids, including chloroplasts. Each *T. gondii* cell contains one apicoplast.

The multiple membranes provide a clue as to the evolutionary origin of the apicoplast, diagrammed in Figure 21.1B. Strong evidence supports the contention that primary plastids arose as the result of one ancient endosymbiotic event in which an ancestral eukaryote engulfed a photosynthetic bacterium, most likely a cyanobacterium. This bacterium remained within the endosome, co-existing with its host in a mutually beneficial relationship that eventually led to the loss of most of the bacterial genes or their transfer to the host nucleus. The resulting chloroplast envelope has two membranes. The inner membrane is clearly derived from the cyanobacterium (Nassoury and Morse, 2005). The outer membrane has galactolipids, which are characteristic of the cyanobacterial outer membrane



**Figure 21.1** Structure and origin of the apicoplast. (A) Transmission electron micrograph of *T. gondii* tachyzoites. Organelles marked include: apicoplast (A), Golgi (G), mitochondrion (M), and nucleus (N). Note the multiple membranes of the apicoplast. Image courtesy of J.F. Dubremetz, reprinted with permission from John Wiley and Sons (Dubremetz, 1995). (B) Model for origin of secondary plastids. Apicoplast (A), chloroplast (C), and nucleus (N) are indicated (other organelles are not shown). Note the transition from 2 to 4 membranes surrounding the plastid, resulting from the secondary endosymbiosis.



(Nassoury and Morse, 2005) and the protein TOC 75, which is clearly of cyanobacterial origin (Reumann *et al.*, 1999). This membrane also contains phosphatidylcholine, which is characteristic of endomembrane systems, but it is non-fusogenic with endosomal vesicles (Nassoury and Morse, 2005). Taken together, the most parsimonious explanation is that the outer membrane of the chloroplast originated from the outer membrane of the cyanobacterium, and the membrane of the endosomal vesicle was lost. Unlike the chloroplasts of green plants, the *T. gondii* apicoplast appears to be bounded by more than two membranes [see images in (Kohler *et al.*, 1997; Ferguson *et al.*, 2005)]. A multimembraned plastid is also seen in several other groups of eukaryotes, including dinoflagellates, euglenoids, cryptomonads, and chlorarachniophytes (Cavalier-Smith, 2003; Palmer, 2003). Of these, dinoflagellates are thought to be the most closely related to apicomplexans (Zhang *et al.*, 2000). Intriguingly, cryptomonads and chlorarachniophytes contain a nucleomorph, a nuclear remnant tucked between the second and third membranes. The nucleomorph contains genes related to those found in algal nuclear genomes (Gilson and McFadden, 2002). Putting these points together, cryptomonad and chlorarachniophyte plastids are clearly the result of a secondary endosymbiosis in which a eukaryote engulfed an alga already bearing a chloroplast. By extension, other multi-membraned plastids are thought to be the results of secondary endosymbiosis. Such plastids are termed secondary or complex plastids. These conclusions are generally supported by phylogenetic analysis of sequence relationships [reviewed in Waller and McFadden (2005)]. It is still debated whether the parent of the apicoplast was a red or green alga, but the preponderance of evidence supports a red algal ancestry of the apicoplast [reviewed in (Waller and McFadden, 2005)].

According to the secondary endosymbiosis model, the inner two membranes of the apicoplast correspond to the inner and outer chloroplast membranes, the third (periplastid) membrane corresponds to the ancestral algal plasma membrane, and the outermost membrane corresponds to the phagocytic vacuole membrane. In some algae with secondary chloroplasts, one membrane has been lost, leaving a total of three bounding membranes. Transmission electron microscopy indicates that the *T. gondii* apicoplast is bounded by four membranes. One recent report suggests that the apicoplast is a primary plastid with only two convoluted membranes (Kohler, 2005), but this proposal is difficult to reconcile with the mechanism of targeting discussed below.

As noted above, the original cyanobacterial endosymbiont lost most of its genes as it became a chloroplast, but many genes were transferred to the nucleus (the complement that remains in the chloroplast genome varies among species). The proteins encoded by the transferred genes are translated in the cytosol yet many of them function in the chloroplast. Ultimate destinations include the stroma (chloroplast lumen), thylakoids (sites of photosynthesis not present in apicoplasts), and the inner and outer envelope membranes. Stromal and thylakoid proteins must penetrate two membranes to take up residence in the chloroplast. Molecular studies, discussed further below and reviewed in Soll and Schleiff (2004), have determined that the most common means of routing luminal proteins to the chloroplast occurs via a transit peptide located at the N-terminus of the protein. This transit peptide, which has conserved characteristics but not a specific sequence, interacts with receptors on the outer membrane of the chloroplast that mediate the first step in translocation into the chloroplast. Following import into the chloroplast lumen, the transit

peptide is cleaved by processing peptidase. Trafficking to the apicoplast and other secondary plastids is more complex since they have additional bounding membranes that must be crossed. Passage across the third membrane—presumably the endosymbiont's original cell membrane—is little understood. However, since the fourth membrane is derived from a phagocytic vacuole, proteins directed to the secondary plastids might need to navigate the endomembrane system.

## The bipartite targeting sequence

The apicoplast genome in both *P. falciparum* and *T. gondii* is only 35 kb and encodes primarily genes for functions related to the genetic systems of the organelle, that is, transcription and translation [(Wilson *et al.*, 1996); the *T. gondii* apicoplast genome sequence is available at <http://www.toxodb.org>]. Hence it is clear that most biosynthetic or metabolic functions of the apicoplast must be mediated by nucleus-encoded apicoplast-targeted (NEAT) proteins. Several investigators embarked on searches of the nascent genome databases, looking for genes related to plastid functions. Among those found initially were genes encoding the ribosomal proteins L28 and S9, acyl carrier protein (ACP), and  $\beta$ -hydroxyacyl-ACP dehydratase (the last two are involved in the type II pathway for fatty acid biosynthesis) (Waller *et al.*, 1998). Subsequently additional enzymes in the fatty acid synthesis pathway (Jelenska *et al.*, 2001; Foth *et al.*, 2005), as well as several proteins involved in heme biosynthesis (van Dooren *et al.*, 2002) and iron-sulfur cluster biosynthesis (Seeber, 2002) have been shown to be targeted to the apicoplast of either *T. gondii* or *P. falciparum*. The DOXP pathway for isoprenoid biosynthesis is also localized to the apicoplast in *P. falciparum* (Jomaa *et al.*, 1999) and most likely in *T. gondii* [see discussion in Wiesner and Seeber (2005)]. The metabolic functions of the apicoplast are briefly discussed below and are more fully addressed in Chapter 22 as well as by Ralph *et al.* (2004a). The sequences of these proteins showed the presence of N-terminal extensions as compared to their bacterial counterparts. These extensions seemed a likely location for topogenic information. A closer examination of the N-terminus indicated that NEAT proteins bear a predicted signal sequence for protein targeting to the endoplasmic reticulum (ER). This region is followed by a segment that shows little sequence relationship between the proteins, but is reminiscent of the transit peptide that targets protein to the chloroplast. This bipartite configuration is similar to that seen in proteins targeted to secondary plastids of other organisms [reviewed in (Nassoury and Morse, 2005)].

As yet, no *in vitro* systems have been developed for protein import into the apicoplast. The approach taken to study protein targeting to this organelle has relied heavily on the use of transfection and fluorescence microscopy. In such experiments, gene fusions are constructed with full-length or partial NEAT protein coding regions fused to reporters such as green fluorescent protein (GFP) or to epitope tags. These are cloned into *T. gondii* expression plasmids and transfected into the parasites by electroporation. The fusion proteins are then visualized by fluorescence microscopy using intrinsic fluorescence or antibodies to the reporter. In most cases, it is possible to perform these experiments using transient transfections due to the relatively high transfection rate in *T. gondii*. Furthermore, proteins produced from transient transfections typically show the same subcellular localization as those of proteins produced from low copy integrated plasmids, and are less prone to

overexpression artifacts. When the N-terminal extension of ACP was placed at the amino terminus of GFP, the protein was routed to the apicoplast, as shown by co-localization with the apicoplast genome (Waller *et al.*, 1998). An example of such an experiment is shown in Figure 21.2A, in which the GFP is detected by its intrinsic fluorescence, and the apicoplast genome is revealed by staining with the DNA dye DAPI. This work has been replicated for several other NEAT proteins expected to reside in the apicoplast lumen, leading to the general conclusion that the topogenic information for targeting to the apicoplast resides in the N-terminal extension [reviewed in (Waller and McFadden, 2005)].

Little is known about apicoplast membrane proteins. No characterizations have been published at this time but, by analogy with chloroplasts, it is expected that transporters are required to import substrates and export products. Similarly, the apicoplast import apparatus is expected to consist of multiple polypeptides, many of which are likely to be transmembrane proteins. Again, by analogy with chloroplasts, multiple mechanisms for targeting of membrane proteins can be expected. Some chloroplast membrane proteins have an N-terminal transit peptide, similar to other nuclearly encoded chloroplast proteins, but others have an N-terminal sequence resembling an ER signal sequence and some lack a cleaved N-terminal targeting sequence. It is unclear whether targeting specificity of proteins that lack a transit peptide is conferred by cytosolic chaperonins, membrane proteins such as Toc75, membrane lipids unique to the chloroplast, or some combination of these (Hofmann and Theg, 2005). While it has not yet been characterized, Waller and McFadden (2005) noted that the *P. falciparum* genome encodes a likely homolog of Tic22, a component of the import complex which resides between the two chloroplast membranes. The predicted protein bears a bipartite extension. Similarly, a catalog of predicted apicoplast sequences in *P. falciparum* includes a sugar phosphate transporter which also bears a bipartite extension (Foth *et al.*, 2003). A related, but distinct, transporter in *T. gondii* (and its *P. falciparum* ortholog) lacks such a targeting sequence (Karnataki *et al.*, 2007; Mullin *et al.*, 2006). These molecules are localized to the apicoplast.

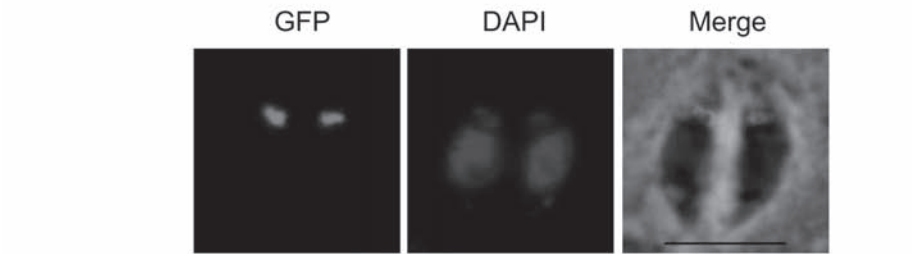
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### The first step in targeting: entry into the ER

As noted above, the N-terminal portion of the targeting sequence looks like a signal sequence, having a positively charged N-terminal region and being rich in hydrophobic amino acids. Several experiments have demonstrated that the N-terminal region is indeed a signal peptide. If this region of *T. gondii* ribosomal protein S9 (DeRocher *et al.*, 2000) or of *P. falciparum* ACP (Waller *et al.*, 2000) is cloned at the amino terminus of GFP, then the fusion protein is secreted when expressed in the parasite. Hence this region can target proteins to the secretory system and behaves as a bona fide signal sequence. Conversely, if the signal sequence of apicoplast ferredoxin NADP<sup>+</sup> reductase (FNR) is replaced by one from the dense granule protein GRA8, the resulting bipartite sequence can target yellow fluorescent protein (YFP) to the apicoplast (Harb *et al.*, 2004). Furthermore, this region is essential for targeting of these proteins. If only the transit sequence of *P. falciparum* ACP is fused to GFP, the resulting fusion protein is localized to the cytosol (Waller *et al.*, 2000). A similar construct, fusing the transit sequence of *T. gondii* S9 to GFP, produced a fusion protein that unexpectedly trafficked to the mitochondrion rather than to the apicoplast or the cytosol. Analysis of the TgS9 transit peptide showed a predicted amphipathic helix,

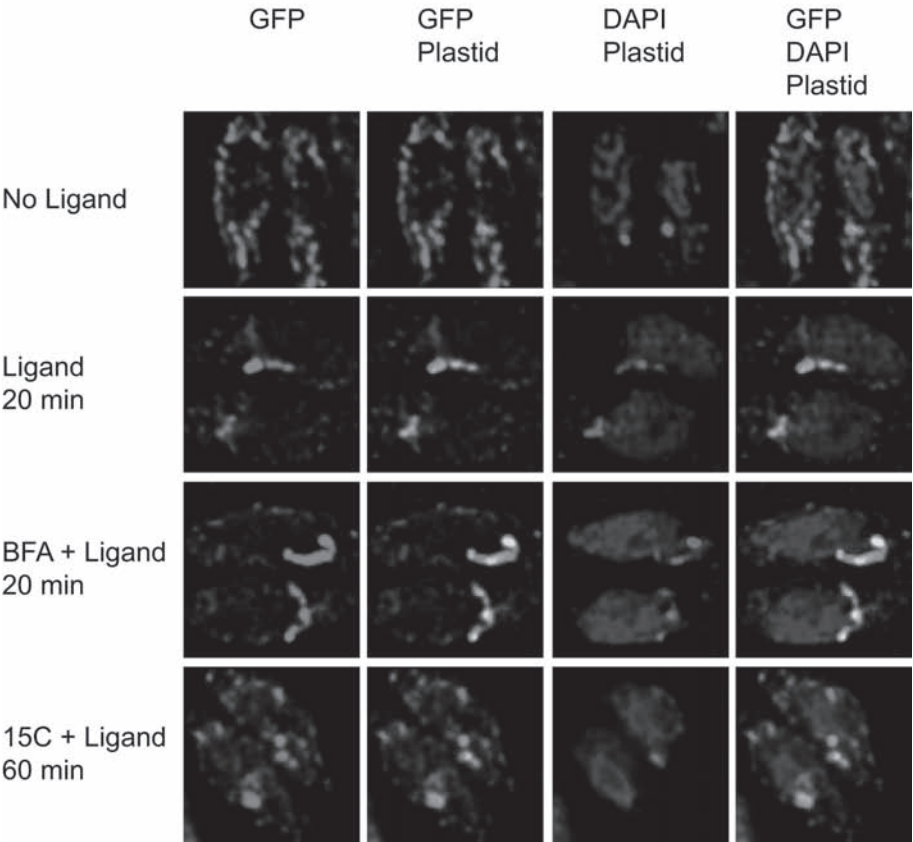
A

S+T(ACP)-GFP



B

S+T(S9)-CAD-GFP



rendering region similar to mitochondrial targeting sequences. Since mitochondrial targeting occurs directly from the cytosol, failure to sequester the *T. gondii* S9 fusion protein to the ER leaves it available for trafficking to the mitochondrion. (DeRocher *et al.*, 2000; Yung *et al.*, 2001) Thus, a signal sequence to gain access to the secretory system is a prerequisite for targeting of NEAT stromal proteins to the plastid.

Another line of evidence that targeting occurs via the ER comes from experiments in which an ER retrieval sequence was added to NEAT GFP reporters. An example of such a study is work with the diatom *Phaeodactylum tricornutum*, which also contains a secondary plastid. A full-length plastid targeting sequence fused to GFP directed the fusion protein to the chloroplast even when an ER retrieval sequence was added. However, the same retrieval sequence, when fused with a truncated but functional plastid targeting sequence, caused the fusion protein to be retained in the ER (Apt *et al.*, 2002). Likewise, when an ER retrieval sequence (HDEL) is appended to the C-terminus of *T. gondii* ACP-GFP proteins, the fusion protein is still targeted to the apicoplast but transit peptide cleavage is slowed somewhat (DeRocher *et al.*, 2005), suggesting a delay in localization to the apicoplast. Results with *T. gondii* superoxide dismutase (SODB2) were more complex. Although the native protein is dually targeted to the apicoplast and mitochondrion (Dominique Soldati, personal communication), the predicted bipartite targeting sequence, when fused to GFP, routes the fusion protein to the mitochondrion only. When an arginine at position 12 was mutated to alanine, the fusion targeted to the apicoplast. Adding an HDEL sequence to this fusion resulted in retention of the fusion protein in the ER (Brydges and Carruthers, 2003). The experiments described above indicate that the ER retrieval system and the putative transit peptide receptor can compete for substrates, so the relevant molecules must at least transiently reside in the same compartment. This latter result implies that NEAT proteins pass through the ER en route to the apicoplast. The canonical receptor for retrieval of soluble proteins from the Golgi to the ER is ERD2. Interestingly, recent studies have revealed that in addition to ERD2, apicomplexans possess two additional ERD2-like sequences which could function in other compartments (Pfluger *et al.*, 2005).

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**Figure 21.2** Analysis of NEAT protein trafficking to the apicoplast. (A) Topogenic information lies in the bipartite extension. The N-terminal extension containing the signal and transit peptide (S+T) of ACP was fused to GFP and expressed in *T. gondii*. Shown are the GFP fluorescence, DAPI-stained DNA, and a merged image. Note the small apicoplast genome that colocalizes with the GFP reporter above the nucleus. Bar, 5  $\mu$ M. Similar results were originally published by Waller *et al.* (1998). See also Plate 21.2. (B) Regulated aggregation used to study targeting of proteins to the apicoplast. The signal and transit sequence of ribosomal protein S9 were fused to GFP bearing four CAD repeats [S+T(S9)-CAD-GFP]. The CAD domain causes protein aggregation when synthetic ligand AP21998 is withdrawn. Ligand was removed for 48 hours (top panels) and then added back for 20 or 60 minutes (lower panels), with or without inhibition of Golgi trafficking by brefeldin A (BFA) or low temperature. Shown are the localization of GFP (revealed by anti-GFP, green), the plastid (revealed by Quantum red streptavidin, which binds the apicoplast marker acetyl CoA carboxylase), and DNA (revealed by DAPI staining). For the 15°C incubation, cells were precooled to this temperature before adding ligand. Data and images courtesy of Dr. Amy DeRocher. See also Plate 21.2.

## Transit peptides

Full-length NEAT proteins are not observed, indicating that the signal sequence is cleaved rapidly upon import into the ER. This is typical for N-terminal signal sequences. Once the signal sequence is cleaved, the transit peptide is exposed at the newly created N-terminus. The transit region is also required for targeting of NEAT proteins, since as noted above, its deletion results in secretion. Thus there are at least two separate targeting events required for NEAT proteins: entry into the ER and targeting from the ER to the apicoplast. The molecular machinery that recognizes the transit peptide and organizes subsequent trafficking to the apicoplast must require initial steps that occur within the secretory system.

The transit peptide of chloroplast proteins is responsible for the specific targeting of luminal proteins to the organelle and these transit peptides are rapidly cleaved upon import. The peptides are variable in length, from 25 to over 100 aa, and show little conservation of sequence (Bruce, 2001). However, there is a "typical" structure for most transit peptides of plants. At the N-terminus are about 10 uncharged amino acids, followed by a central region enriched for hydroxylated amino acids (serine and threonine) and poor in acidic residues. The final block is a region rich in basic amino acids (Claros *et al.*, 1997). An amphipathic structure is not required, although it may be adopted in some cases. For some proteins, import into chloroplasts *in vitro* is facilitated by phosphorylation and subsequent interaction with molecular chaperones 14-3-3 and HSP70 (May and Soll, 2000).

How similar are the transit peptides of apicoplast proteins to those of chloroplasts? Analysis of the sequence of proteins known to be targeted to the *T. gondii* apicoplast has revealed that the signal sequence is followed by a region that is rich in basic residues and in hydroxylated amino acids. This region is also poor in acidic residues, like the transit peptides of chloroplasts. Indeed, the transit peptide of *T. gondii* ribosomal protein S9 allows *in vitro* import of proteins into pea chloroplasts (DeRocher *et al.*, 2000). The *P. falciparum* transit peptides vary from those of *T. gondii* and plants. They tend to be enriched for lysine and asparagine whereas plant transit peptides more commonly employ arginine (Ralph *et al.*, 2004a). This difference appears to result from the high AT content of the *Plasmodium* genome as compared to the more balanced composition in most plants (Ralph *et al.*, 2004a). Hydroxylated residues do not appear to be essential for targeting in *P. falciparum*, since their replacement by alanine in the *P. falciparum* FabH ( $\beta$ -ketoacyl-ACP synthase III) transit peptide did not alter targeting of a GFP fusion protein (Waller *et al.*, 2000). As discussed by Ralph *et al.* (2004b), this difference could arise from the fact that cytosolic phosphorylation of serine and threonine, which can be important for enhancing the distinction between chloroplast and mitochondrial targeting, is not needed in Apicomplexa. Apicomplexan NEAT proteins bypass the mitochondrial import apparatus by virtue of their signal sequence. On the other hand, an HSP70 binding site in the ACP transit peptide is important for targeting to the *P. falciparum* apicoplast (Foth *et al.*, 2003).

Several studies have dissected the key components of apicoplast transit peptides. The first set of these showed that large segments of the *T. gondii* S9 transit peptide could be deleted while still maintaining targeting (DeRocher *et al.*, 2000; Yung *et al.*, 2001). Depending on the nature of the deletion, different localization patterns are observed. As noted above, deletion of the transit peptide leads to secretion. Partial deletion of the S9 or FNR transit peptides can lead to apparent apicoplast localization, secretion, or localization



to other components of the secretory system such as rhoptries (Yung *et al.*, 2003; Harb *et al.*, 2004). Because the four membranes of the apicoplast are tightly juxtaposed and the organelle is quite small (200–300 nm in diameter), it is challenging to determine whether trafficking to the region of the apicoplast indicates complete targeting to the lumen or to an intermediate compartment. Nevertheless, with deconvolution microscopy, and especially with mutants with enlarged apicoplasts, it is possible to discriminate targeting to the apicoplast lumen from targeting to the membranes (He *et al.*, 2001).

The most complete analysis in *T. gondii* to date dissected the transit peptide of FNR (Harb *et al.*, 2004). This protein has a 150 aa N-terminal extension that contains a typical signal sequence followed by a transit peptide. Analysis of a panel of deletion constructs showed that the last 45 aa of the transit peptide are completely dispensable for targeting YFP to the apicoplast. Further deletions resulted in a new phenotype in which the YFP appeared to be localized to the periphery of the apicoplast coupled with processing at a different site. Deconvolution microscopy revealed that the YFP is closely juxtaposed, but not overlapping with ACP, a marker of the apicoplast lumen. It is not clear whether this localization represents molecules accumulating in a novel structure very close to the apicoplast; molecules that have stalled in the import process, perhaps residing between membranes; or molecules residing in a specific subcompartment of the apicoplast. By testing constructs lacking different regions of the transit peptide, these authors were able to identify two separate regions that could target a reporter molecule to this compartment, dubbed the apicoplast periphery. Other deletions resulted in retention in the ER, targeting to secretory organelles such as rhoptries, or secretion. From this work it appears that multiple functions are specified by different regions of the transit peptide (Harb *et al.*, 2004). These functions include mediating release from the ER, targeting to the apicoplast periphery, efficient import, and sites for cleavage by the processing enzymes.

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## ER to apicoplast targeting and import

Targeting to the apicoplast appears to be very efficient. When intact signal and transit sequences are attached to a reporter, fluorescence microscopy reveals the reporter only in the apicoplast. However, it has been challenging to study how proteins are targeted from the ER to the apicoplast.

Several models of how proteins traffic from the ER to the apicoplast have been proposed. The first model is that NEAT proteins are sorted in the Golgi or trans-Golgi network, as is common for proteins targeted to other destinations in the secretory system. This trafficking pathway has been demonstrated in *Euglena*, which has a secondary chloroplast derived from a green algal ancestor (Sulli and Schwartzbach, 1995). In these organisms, the bipartite targeting sequence has an ER stop-transfer domain in the presequence. Hence the mature domain is exposed to the cytoplasm while the precursor is transported through the endomembrane system (Sulli *et al.*, 1999). Apicoplast transit peptides do not contain stop-transfer sequences.

Several groups have reported that brefeldin A, which is an inhibitor of Golgi trafficking, has no discernable effect on apicoplast protein localization of GFP tagged proteins in *T. gondii* [(DeRocher *et al.*, 2005), unpublished studies cited in (Joiner and Roos, 2002)]. All fluorescence, whether intrinsic or revealed by anti-GFP antibodies, was in the

apicoplast even when brefeldin A was present for several hours. However, the presence of pre-existing apicoplast-targeted GFP complicated these experiments. To overcome this problem, a conditional aggregation system was adapted for use in *T. gondii* (DeRocher *et al.*, 2005). This system [available from Ariad Pharmaceuticals ([www.ariad.com](http://www.ariad.com))] employs a mutant version of the FK506 binding protein which is reiterated four times in a cassette (Rollins *et al.*, 2000). These domains aggregate unless a synthetic ligand is present. The original authors used this system to regulate secretion of the hormone insulin (Rivera *et al.*, 2000). Insulin tagged with the conditional aggregation domain (CAD) accumulated in the ER. When ligand was added, the tagged insulin was released and secreted from the cell. When CAD was linked to a NEAT GFP reporter, the fusion protein localized to the apicoplast when ligand was present (Figure 21.2B). However, when ligand was absent, the protein accumulated in the ER. Importantly, the accumulated protein was released and properly targeted once the ligand was added. Neither brefeldin A nor low temperature incubation, which also inhibits Golgi trafficking, impeded the localization of the released protein to the region of the apicoplast. However, brefeldin A did block the cleavage of the transit peptide. It is not clear at which step brefeldin A is acting, although the possibility that it directly inhibits the processing enzyme was eliminated (DeRocher *et al.*, 2005). These data argue that protein traffic to the apicoplast bypasses the Golgi.

In the second model, the apicoplast lies within the ER and proteins reach the organelle directly, not through vesicle-mediated steps (Joiner and Roos, 2002). In several organisms including *Ochromonas*, whose secondary plastids are derived from a red algal ancestor, the outer membrane of the plastid is clearly contiguous with the ER, and ribosomes can be seen on part of the plastid outer membrane (Gibbs, 1979; Nassoury and Morse, 2005). However, neither ER contact with the outer membrane nor ribosomes studding the outer membrane have been observed in *Toxoplasma*, and direct evidence supporting this model has not, to our knowledge, been published.

The third model suggests vesicular trafficking directly from the ER. Vesicle traffic bypassing the Golgi has been described. One example is the cysteine protease of germinating seeds, which is targeted to storage bodies directly from the ER (Toyooka *et al.*, 2000). Another example is the differential sorting and retrieval of endocytosed proteins (Maxfield and McGraw, 2004). Recent studies show vesicles bearing an apicoplast membrane protein (Karnataki *et al.*, 2007), supporting a model involving vesicular trafficking.

Once a protein transits the outermost membrane of the apicoplast, it still has three membranes to traverse before reaching the lumen of the organelle. It is broadly assumed that the translocation machinery of the inner two membranes of the apicoplast is derived from the chloroplast protein import machinery. Import into the lumen of primary chloroplasts is mediated by two hetero-oligomeric complexes: the translocons of the outer (Toc) and inner (Tic) chloroplast membranes. It has also been postulated that part of the translocon might be duplicated and inserted into the periplastid membrane in secondary plastids. For a more thorough review of the functions and addition proteins in the Toc and Tic complexes, see Soll and Schleiff (2004). Three components of the Toc complex have been well characterized in plants: Toc34, Toc75, and Toc 159 (Becker *et al.*, 2004; Wallas *et al.*, 2003). Toc34 and Toc159 are both GTPases, and GTP hydrolysis by Toc159 provides the motive force for translocation across the outer membrane through a  $\beta$ -bar-

rel channel formed by Toc75. Proteins in the translocon of the inner membrane, Tic110, Tic22 and Tic20, physically associate with the Toc complex (Kouranov *et al.*, 1998). Tic22 is in the intermembrane space, while Tic110 and Tic20 are integral membrane proteins and may construct the channel of the inner membrane translocon. Homologs of several chloroplast translocon genes have been identified in the genome of the photosynthetic bacterium *Synechocystis*, including *Toc75*, *Tic55*, *Tic22*, and *Tic20* (Reumann *et al.*, 1999; Reumann and Keegstra, 1999) although other components (e.g. Toc 159 and Tic 110) have not been found. Surprisingly, the proteins that were most strongly conserved between *Synechocystis* and *Pisum sativum*, *Toc75* and *Tic55*, do not have readily identifiable counterparts encoded in the apicomplexan genomes. A recent review indicates that potential homologs of *Tic22* and *Toc34* have been detected in the *P. falciparum* database (Waller and McFadden, 2005). These components apparently are more divergent in *T. gondii*, since the Tic and Toc components are not readily identified by database mining.

The motive force for translocation across the inner membrane of chloroplasts is hydrolysis of stromal ATP. Thus the plastid-localized ATP-hydrolyzing proteins ClpC and/or HSP60 might act as molecular ratchets to pull proteins through the translocon into the chloroplast. ClpC is encoded by the apicoplast genome (Wilson *et al.*, 1996). Tic110 can associate with ClpC (Nielsen *et al.*, 1997) and imported proteins associate with HSP60. Other, less well-characterized proteins that are part of the translocons are reviewed elsewhere (Soll and Schleiff, 2004). In addition to specific interactions with the Toc complex, chloroplast transit peptides often can bind unique lipids present in the outer membrane of the chloroplast, although the relevance of this to import is not clear (Bruce, 1998). More recently, studies have shown that transit peptide interactions with soluble proteins or chaperones in the cytosol may be important in guiding the protein to the Toc complex (May and Soll, 2000). In the case of the apicoplast, any such interactions would probably occur within the secretory system, most likely in the ER.

In addition to protein targeting to the lumen of the apicoplast, there are presumably specific mechanisms to ensure that the appropriate proteins are targeted to each membrane and inner membrane space of the apicoplast, a total of eight distinct compartments. How this nuanced targeting is achieved has not been explored.

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### Transit peptide processing

Once a chloroplast protein is translocated into the stroma, the transit peptide is rapidly cleaved to yield the mature protein. Indeed, at steady state it is difficult to detect the precursors of chloroplast proteins [for example, see (Shanklin *et al.*, 1995)]. In contrast, *T. gondii* precursors containing transit peptides are easily seen at steady state (Waller *et al.*, 1998; Vollmer *et al.*, 2000), and pulse-chase analysis indicates that the  $t_{1/2}$  for cleavage can be more than one hour (DeRocher *et al.*, 2005). It is not clear whether this lag in cleavage results from a lengthy journey to the apicoplast lumen or slow cleavage once fully internalized. In studies of the TgFNR transit peptide, deletion of as few as 10 amino acids changed the site of processing, although import was maintained (Harb *et al.*, 2004). Some work indicates the possibility of additional, earlier processing steps. For example, evidence for cleavage was noted in some FNR-GFP fusions that targeted to the distinct compartment called the apicoplast periphery (see above). Indeed, an earlier study had indicated an

initial cleavage of the S9 transit peptide near its N-terminus in addition to the cleavage that produces the mature protein (Yung *et al.*, 2001). As noted above, BFA indirectly inhibits transit peptide processing (DeRocher *et al.*, 2005). A homolog of the higher plant transit peptide processing protease has been identified in the *P. falciparum* genome, and the N-terminal sequence of the protein encoded by its cDNA is predicted to direct this protein to the apicoplast (van Dooren *et al.*, 2002). These authors also sequenced the N-terminus of an ACP-GFP fusion targeted to the apicoplast. Interestingly the sequence context of the experimentally determined apicoplast processing site VNF↓L (where ↓ indicates the cleavage site) is markedly different than the consensus chloroplast processing site from higher plants [(I/V)X(A/C)↓A], highlighting the evolutionary distance between these organisms.

### Implications for the apicoplast proteome

The identification and analysis of apicoplast targeting sequences has allowed the generation of neural-network (Zuegge *et al.*, 2001) and rule-based algorithms (Foth *et al.*, 2003) for identifying candidate apicoplast proteins in *P. falciparum*. In the case of *P. falciparum*, approximately 550 candidates were identified—or somewhat less than 10% of the predicted ORFs. For *Theileria parva*, the number was 345 of the 4035 predicted protein coding genes (Gardner *et al.*, 2005). How likely is it that ~10% of the ORFs indeed encode proteins targeted to the apicoplast? A significant fraction of these candidates are orthologs of chloroplast proteins and hence very likely to be apicoplast targeted. However, approximately 70% of the predicted apicoplast-localized sequences from *P. falciparum* are hypothetical proteins with no identified function. Estimates of the proteome of chloroplasts, 1700 to 3400 proteins, suggest that 6–12% of the nuclear gene models encode chloroplast-targeted proteins (Van Wijk, 2004). It is reasonable that apicoplasts, which lack the chloroplast photosynthetic machinery and all of the allied functions, would have far fewer proteins.

It is clear that the sorting algorithms are not completely sensitive or specific. The chloroplast algorithms TargetP and Predotar are estimated to have sensitivities of 82–90% and specificities of 69–82% (Van Wijk, 2004). The most difficult discrimination is likely to be that between mitochondrial presequences and chloroplast transit peptides. That distinction may be somewhat better in apicomplexans since many apicoplast-targeted proteins have a signal sequence. However, apicoplast protein prediction in *T. gondii* is complicated by the need to correctly predict intron splicing so that the targeting algorithms are applied to the correct protein sequence. As gene models improve, this difficulty should diminish. In any case, the identification of these candidate apicoplast proteins paves the way for focused experimental analyses. The importance of such studies is demonstrated by the case of SODB2, as discussed above. Another example where experimental verification is essential concerns the *P. falciparum* proteins that have both predicted apicoplast targeting sequences and a short internal motif for targeting to the host cell. Like apicoplast targeting, the first step for host cell targeting is entry into the ER. Proteins destined for the host cell cytosol or membrane are secreted into the parasitophorous vacuole. Once there, the internal motif directs proteins across the vacuolar membrane (Marti *et al.*, 2004; Hiller *et al.*, 2004). *P. falciparum* has a family of protein kinases that contain such host targeting motifs and several of them also have predicted apicoplast targeting sequences (Ward

*et al.*, 2005). Given their usual roles, it is likely that the protein kinases are destined for the host cell rather than the apicoplast, and this is indeed the case for the two that have been tested (Nunes *et al.*, 2007). Finally at least one apicoplast membrane protein appears to lack a signal sequence and transit peptide (see above) and so would not be identified by these algorithms. In conclusion, informatics has yielded an invaluable set of candidate NEAT proteins, but experimental assessment will be necessary to validate and adjust the repertoire.

Knowledge of the evolutionary origins of the apicoplast and the characteristics of apicoplast targeting sequences has allowed the identification of candidate apicoplast proteins, whose localization has been verified. These proteins function in pathways which are quite distinct from those of humans, suggesting that they would make excellent drug targets (Wiesner and Seeber, 2005). This proposal is bolstered by the fact that inhibitors of key enzymes in these pathways inhibit growth of *T. gondii* and *P. falciparum* *in vitro* (see Chapter 22). Examples include thiolactomycin [(Waller *et al.*, 1998), unpublished data cited by (McFadden and Roos, 1999)] and triclosan (McLeod *et al.*, 2001; Perozzo *et al.*, 2002) which both inhibit the type II pathway for fatty acid synthesis. This pathway is not present in the human host. Similarly, the DOXP pathway for isoprenoid synthesis is not present in mammals. Fosmidomycin, an antibiotic which inhibits DOXP reductase, is toxic to the malaria parasite (Jomaa *et al.*, 1999), although it shows little activity against *T. gondii*. Since *T. gondii* possesses a DOXP reductase gene, resistance may be mediated by specific changes in the target.

Pathways acquired from the secondary endosymbiont, but which are not localized to the apicoplast, may also make excellent targets since they too are often highly diverged from human counterparts. One example of this in Apicomplexa is the shikimate pathway for aromatic amino acid synthesis (Roberts *et al.*, 1998). This is a biosynthetic pathway normally used by plants but not by animals. Chorismate synthase, the initial enzyme in the pathway, is the target of the herbicide glyphosate and *T. gondii* in culture is sensitive to this compound (Roberts *et al.*, 1998). Work with shikimate pathway inhibitors in *P. falciparum* has confirmed the potential of these enzymes as drug targets (McRobert *et al.*, 2005; McConkey, 1999). Chorismate synthase is cytosolic rather than apicoplast-localized (Fitzpatrick *et al.*, 2001) but the genes for the pathway are presumed to have been acquired by lateral transfer from the endosymbiont chloroplast or nucleus. Indeed, one study suggests that approximately 18% of nuclear genes in *Arabidopsis thaliana* are derived from the endosymbiont that gave rise to the chloroplast (Martin *et al.*, 2002). Thus an algal origin is predicted for many apicomplexan genes.

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## Concluding remarks

Protein targeting to the apicoplast shares several features with protein targeting to plastids of other organisms. Like protein targeting to primary plastids, luminal targeting is dependent on a transit peptide sequence. Like protein targeting to other secondary chloroplasts, the transit peptide is preceded by a signal sequence, and the targeted proteins appears to have similar interactions with ER retrieval mechanisms. Several critical questions concerning targeting to the apicoplast remain unresolved: What is the route from the ER to the apicoplast? What is the mechanism to transport proteins across the periplastid membrane?



What is the machinery for translocating proteins across the different membranes? How are proteins directed to the multiple distinct compartments of the apicoplast? How are the products synthesized by the biosynthetic pathways of the apicoplast exported? Clearly there is a lot yet to learn about this exciting but enigmatic organelle!

## Acknowledgments

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# The Metabolic Functions of the Mitochondrion and the Apicoplast

22

Frank Seeber and Dominique Soldati

## Abstract

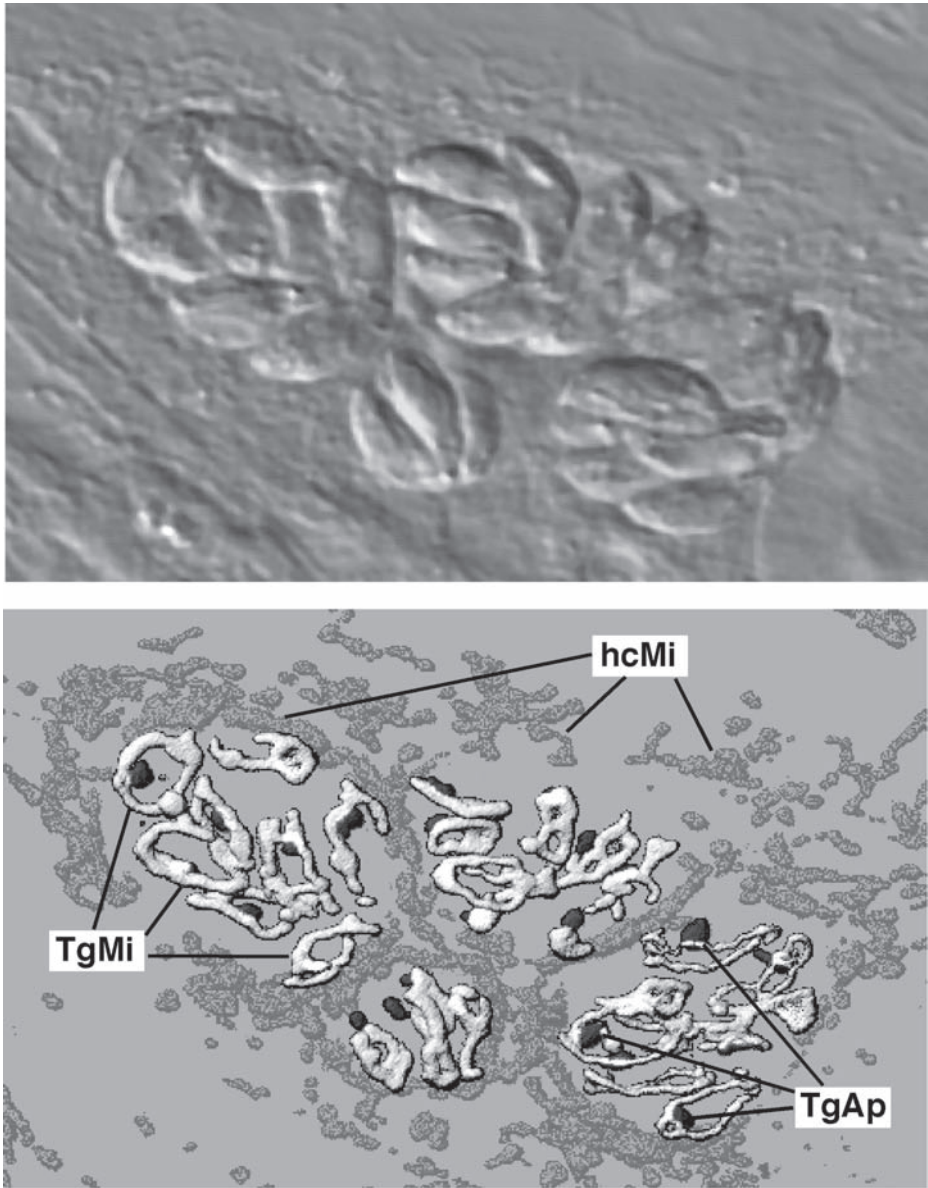
*Toxoplasma gondii*, like most Apicomplexa, possesses a single endosymbiotic mitochondrion and a plastid-derived organelle called the apicoplast both of which host conserved as well as unanticipated metabolic pathways. In this chapter we give a short overview of the current knowledge of those pathways that have either been experimentally characterized to operate in these compartments, or where solid bioinformatical evidence gives clear indications for their localization in the respective organelles. Besides well-known tasks like the tricarboxylic acid cycle or oxidative phosphorylation the mitochondrion also harbors unexpected pathways like the methylcitrate cycle for the detoxification of propionate. The plastid-derived pathways of fatty acid, isoprenoid biosynthesis and partial heme biosynthesis are the known major metabolic tasks in the apicoplast, but auxiliary pathways for the generation of reducing equivalents or co-factors in this organelle have shown surprising deviations from previous knowledge.

These maintained metabolic functions of these two organelles are coming to light as rich sources of potential targets for anti-parasitic drug discovery.

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## Introduction

Now that the complete genome sequences of several Apicomplexa, including *Toxoplasma gondii*, are known, comparative genomics allows us to fill some biochemical gaps in already known organellar pathways of the mitochondrion and the apicoplast. On the other hand, metabolic functions appear to be localized in the two organelles that have not previously been suspected to exist in *T. gondii*, while some other pathways are absent. It is worth mentioning that the Apicomplexa represent the first group of organisms that possess a mitochondrion but are devoid of peroxisomes, organelles known to play an essential role in lipid metabolism and free radical detoxification in eukaryotes (Schluter *et al.*, 2006). The mitochondrion and the apicoplast are physically associated, as are the host cell mitochondria with the parasitophorous vacuolar membrane surrounding intracellular tachyzoites (see Figure 22.1), which is likely a prerequisite for the exchange of some metabolites between these various compartments (see below). Consequently one of the considerable obstacles for the biochemical and proteomic characterization of the apicoplast and mitochondrion in *T. gondii* is the lack of appropriate methods to separate these two organelles into distinct fractions that can then be analyzed further (He *et al.*, 2001). Nevertheless,



**Figure 22.1** Visualization of host cell and parasite mitochondria and apicoplast. Human fibroblasts infected with tachyzoites (top) were fluorescently tagged using either GFP or antibodies. The confocal images of the different color channels were volume-rendered and the different images further processed in Photoshop (bottom). hcMi (dark gray), host mitochondria; TgMi (light gray), *T. gondii* mitochondrion; TgAp (black), *T. gondii* apicoplast. Note the accumulation of host mitochondria surrounding the PVM.





through the use of transgenic approaches the fate and behavior of presumed organellar proteins can be quite easily studied in *T. gondii*. The combination of inhibitor studies and knockout strategies for nuclear-encoded genes of organellar proteins should contribute to the unfolding picture of the complex interplay between mitochondrion, apicoplast and the rest of the parasite and host cell.

Although this chapter deals with biochemistry, we do not show panels of reaction schemes or chemical formulae. Instead we refer to several comprehensive internet resources at the end of the chapter where this information can be found and retrieved. A schematic overview of the described pathways and their confirmed or inferred interactions is given in Figure 22.2.

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## The mitochondrion

The mitochondrion performs a large number of reactions in eukaryotic cells, including the transduction of energy from nutrient-derived substrates into ATP, the Krebs cycle, heme biosynthesis, the  $\beta$ -oxidation of fatty acids, metabolism of certain amino acids and the biosynthesis of iron-sulfur clusters.

The mitochondrion of *T. gondii* is a single tubular structure with discernible cristae (Seeber *et al.*, 1998; Melo *et al.*, 2000; Köhler, 2006). In general, apicomplexans are known to possess the smallest mitochondrial genomes of all organisms, with a size of about 6–7 kb, with some forming concatamers (Gray *et al.*, 2004). In the case of *Plasmodium* and *Theileria* spp the genome possesses only three protein coding genes (cytochrome oxidase subunit I and II and apocytochrome b; Feagin, 2000). The mitochondrial genome of *T. gondii* has not yet been described in the literature despite the completion of a draft genome sequence. The difficulty in assembling this organellar genome is likely due to the presence of numerous dispersed sequences in the nuclear genome, which contain fragments of the genes for *coxI* and *cob* (Ossorio *et al.*, 1991). However, indirect evidence indicating the existence of a functional mitochondrial genome is substantial and includes the sensitivity of *T. gondii* to mitochondrial translation inhibitors (Beckers *et al.*, 1995) and the existence of a tRNA import system (Esseiva *et al.*, 2004). More recently, however, the mitochondrial genome has been experimentally assembled and confirmed to code for the same three genes as observed for the other apicomplexans (D. Roos, personal communication).

## Main metabolic pathways

### Oxidative phosphorylation and respiration

Vercesi *et al.* provided the first direct biochemical evidence that the respiratory chain and oxidative phosphorylation are functional in *T. gondii* tachyzoites, although the terminal respiratory pathway appears to be different from that in the mammalian host (Vercesi *et al.*, 1998). Like *Plasmodium* (Uyemura *et al.*, 2004), *T. gondii* does not have a multi-enzyme rotenone-sensitive complex I. Instead, the parasite possesses two mitochondrion-localized isoforms of a so-called alternative NADH oxidoreductase with 43% amino acid identity to each other (Saleh *et al.*, 2007). These mitochondrial alternative NADH dehydrogenases (EC 1.6.5.3) can donate reducing equivalents from NADH to ubiquinone A, and a recent pharmacological study validated the mitochondrial *Plasmodium* homolog of type II NADH

dehydrogenase (PfNDH2) as a potential chemotherapeutic target against malaria (Biagini *et al.*, 2006). Additionally, the complex III of the electron transfer chain of apicomplexans has been the focus of significant efforts for the development of new anti-parasitic drugs. The action of atovaquone was shown to be due to the complete inhibition of the respiratory chain and disruption of the mitochondrial membrane potential. Cytochrome b was formally identified as the target of atovaquone in *Plasmodium* (Srivastava *et al.*, 1999), and subsequently a mutant cyt b protein was shown to be a source of resistance to the drug in *T. gondii* (McFadden *et al.*, 2000). Treatment of tachyzoites with atovaquone and other inhibitors of mitochondrial respiration have been shown to promote stage conversion (Bohne *et al.*, 1994), which might be linked to the fact that bradyzoites appear to lack a functional respiratory chain (Denton *et al.*, 1996). Interestingly, atovaquone-resistant mutants were reported to be hypersensitive to clindamycin, a drug that inhibits apicoplast translation. This suggests that both organelles might influence the process of differentiation (Tomavo and Boothroyd, 1995). For a more detailed description of the *Plasmodium* mitochondrial metabolism, see the reviews of Vaidya and Mather (2005) and Krungkrai (2004).

#### *Tricarboxylic acid cycle (Krebs cycle) and the generation of acetyl-CoA*

The tricarboxylic acid (TCA) cycle, in conjunction with oxidative phosphorylation, provides most of the energy used by aerobic cells, but in addition it is also an important source of precursors of many other biomolecules such as amino acids, nucleotide bases and heme. Little is known about the TCA cycle in *T. gondii*. Although all the enzymes of this pathway are present in the genome, their precise localization and biochemical characteristics remain to be defined. Earlier work provided evidence for the enzymatic activity of succinate dehydrogenase (SDH, EC 1.3.5.1) and NADP<sup>+</sup>-dependent isocitrate dehydrogenase (ICDH, EC 1.1.1.42) in both tachyzoites and bradyzoites (Denton *et al.*, 1996). TgICDH1 is targeted to the apicoplast while TgICDH2 localizes to the mitochondrion of *T. gondii* (Pino *et al.*, in preparation), and both enzymes provide NADPH instead of NADH (similar to the single plasmodial ICDH; Chan and Sim, 2003; Wrenger and Müller, 2003). This characteristic indicates that the TCA cycle (and thus respiration) should not contribute significantly to energy production unless there is a transhydrogenase that would convert NADPH to NADH. Instead, the TCA cycle might be more important for the provision of precursor molecules like succinyl-CoA for heme biosynthesis.

The pyruvate dehydrogenase complex (PDH) links glycolysis to the TCA cycle by converting pyruvate to acetyl-CoA, which is then fed into the cycle. Strikingly, and like in *Plasmodium* (Foth *et al.*, 2005), the PDH complex is absent in the mitochondrion of *T. gondii*. The parasite possesses a single copy for each of the genes encoding the four subunits E1 $\alpha$ , E1 $\beta$ , E2 and E3. The components of the E1 subunit (pyruvate dehydrogenase, EC 1.2.4.1), which usually occurs as a heterotetramer ( $\alpha_2\beta_2$ ) of distinct E1 $\alpha$  and E1 $\beta$  subunits, and E2 (dihydrolipoamide S-acetyltransferase, EC 2.3.1.12) are exclusively targeted to the apicoplast (Crawford *et al.*, 2006; W. Bohne and D. Soldati, unpublished results). The mitochondrial E3 subunit is known as dihydrolipoamide dehydrogenase or LipDH (EC 1.8.1.4) and is commonly shared between the PDH complexes, the branched-chain ketoacid dehydrogenase complex (BCKDH), the  $\alpha$ -ketoglutarate dehydrogenase complex (KGDH) and the glycine cleavage complex (GCV) (Crawford and Roos, unpublished).

The absence of PDH in the mitochondrion raises the pertinent question as to how mitochondrial acetyl-CoA is generated. Possible sources could be  $\beta$ -oxidation of fatty acids (FAs) and/or the degradation of branched-chain amino acids in the mitochondrion.

FAs are first converted into acyl-CoA by long-chain-fatty-acid-CoA ligase (EC 6.2.1.3) before degradation can start. The breakdown of FAs requires four enzymes: (i) acyl-CoA dehydrogenase (EC 1.3.99.3), (ii) 2,3-enoyl-CoA hydratase (EC 4.2.1.17), (iii) 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and (iv) 3-ketoacyl-CoA thiolase (EC 2.3.1.16). Sequences coding for enzymes related to long-chain-fatty-acid-CoA ligase and the reactions (i), (ii) and (iv) are present in the genome of *T. gondii*, and several putative short chain dehydrogenases can be found that might include a 3-hydroxyacyl-CoA dehydrogenase (iii). Note, however, that these enzymes could also be involved exclusively in branched-chain amino acid degradation. The presumed mitochondrial localization of these enzymes cannot be judged by predictive algorithms in all cases, but an alternative peroxisomal location can be excluded since *T. gondii* lacks peroxisomes (Ding *et al.*, 2004; Schluter *et al.*, 2006). The substrates for  $\beta$ -oxidation could be FAs produced by fatty acid synthesis in the apicoplast and which might have easy access through the close association of both organelles (see "Methylcitrate cycle"). Alternatively, free FA can be taken up from the host cell (Charron and Sibley, 2002; see also Chapter 19) but the chemical identity of the FAs remains to be established for both scenarios. Still, direct biochemical evidence for  $\beta$ -oxidation in *T. gondii* is lacking. Anecdotally, its activity in *P. falciparum* has been reported to be low or even absent (Holz, 1977; Palacpac *et al.*, 2004), which would be in agreement with acyl-CoA dehydrogenase not being present in the genome of *P. falciparum*.

Branched-chain amino acid degradation in general also yields acetyl-CoA as an end product. *T. gondii*'s genome contains a homolog of branched-chain amino acid transferase (BCAT, EC 2.6.1.42), an enzyme that deaminates valine, leucine and isoleucine. The resulting branched chain  $\alpha$ -keto acids are then decarboxylated by the BCKDH complex consisting of subunit E1 (EC 1.2.4.4), the E2 subunit dihydrolipoyl transacylase (EC 2.3.1.168) and E3 subunit dihydrolipoamide dehydrogenase (EC 1.8.1.4) which has been localized to the *P. falciparum* mitochondrion (McMillan *et al.*, 2005). The resulting branched-chain acyl-CoAs (3-methylbutanoyl-CoA from leucine, 2-methylpropanoyl-CoA from valine, and 2-methylbutanoyl-CoA from isoleucine) are formed irreversibly. Some of the genes coding for enzymes leading to the subsequent degradation of branched chain amino acids are partly shared also with  $\beta$ -oxidation and are unambiguously present in *T. gondii*'s genome (see above). They include enoyl-CoA hydratase (EC 4.2.1.17), methylmalonate-semialdehyde dehydrogenase (MMSA, EC 1.2.1.27) and 3-hydroxyisobutyrate dehydrogenase (3HIDH, EC 1.1.1.31) involved in the degradation of valine to propionyl-CoA. Hydroxymethylglutaryl-CoA lyase (HMGCL, EC 4.1.3.4) is involved in the degradation of leucine to produce acetyl-CoA and acetoacetate. Finally, acetyl-CoA C-acyltransferase (THIK, EC 2.3.1.16) is involved in the degradation of isoleucine and leads to the conversion of 2-methyl-acetoacetyl-CoA into propionyl-CoA and acetyl-CoA. All these enzymes are predicted to localize in the mitochondrion but this requires experimental confirmation.

### Heme biosynthesis

Heme (haem) or other metal-tetrapyrroles are required as prosthetic groups for a number of essential proteins in nature, such as hemoglobin, catalase, peroxidase, nitric oxide synthase, guanylate cyclase and the large group of cytochromes. *T. gondii* certainly requires heme for its various cytochromes in the mitochondrion, its cytosolic heme-type catalase (Ding *et al.*, 2000) and possibly for its guanylate cyclase.

Biosynthesis of the tetrapyrrole backbone of heme differs substantially between most bacteria, algae and plants on one side and animals, fungi or  $\alpha$ -proteobacteria on the other side (Dailey, 1990). The latter group uses succinyl-CoA and glycine as precursors to generate  $\delta$ -aminolevulinate (ALA), a reaction that is catalyzed by  $\delta$ -aminolevulinate synthase (ALAS, EC 2.3.1.37, so-called Shemin pathway). In contrast, the alternative route (C5-pathway) starts with glutamate which becomes attached to a modified aminoacylated tRNA (Glu). This activated glutamate is then converted via two enzymatic steps first to glutamate-1-semialdehyde and then to ALA. From here on both pathways utilize the same principle steps to synthesize the heme group from 8 ALA molecules via enzymatic reactions catalyzed by seven proteins abbreviated HemB to HemH (see "Internet resources" for details).

The complexity of this pathway is further augmented by the distribution of individual steps to different cellular compartments in different organisms (except bacteria). In plants and algae ALA synthesis as well as probably all other steps occur in the plastid. Heme (or its precursor protoporphyrin IX) required in the mitochondria is then imported. In animals, however, the precursor succinyl-CoA derives from the TCA cycle, and consequently heme synthesis starts in the mitochondrion. ALA is then transferred to the cytoplasm where the synthetic steps up to coproporphyrinogen III occur. This latter compound then enters the organelle again where the final assembly of the heme molecule occurs at the inner mitochondrial membrane (Dailey, 2002).

The situation in Apicomplexa is even more complex. As one might expect because of the acquisition of a plastid, heme synthesis is neither purely animal nor purely plastid-like but seems to be a unique mixture of both. Through a combination of biochemical and cell biological assays, combined with genomic and phylogenetic analyses of both *P. falciparum* and *T. gondii*, a consensus picture has emerged (Wu *et al.*, 2003; reviewed in Ralph *et al.*, 2004; Wilson, 2005). Like in animals, ALA synthesis starts in the mitochondrion, with glycine and succinyl-CoA as precursors. The enzymes for the next four steps are presumably located in the apicoplast, so ALA has to be transported to the neighboring organelle. HemD (EC 4.2.1.75) has been difficult to identify in the genome of *P. falciparum*, so its location there is unclear. In *T. gondii* both HemD and HemE (EC 4.1.1.37) seem to possess a plastid targeting sequence and HemF (EC 1.3.3.3) was reported to be cytosolic, while the last two enzymes, HemG (EC 1.3.3.4) and HemH (EC 4.9.11), are most likely mitochondrial in *T. gondii* (Wu *et al.*, 2003). A phylogenetic analysis of several heme biosynthetic enzymes demonstrates the mosaic character of this pathway in terms of origin from either  $\alpha$ -proteobacteria (mitochondrial origin), cyanobacteria (plastid origin) or host cell nucleus (Obornik and Green, 2005).

### *Fatty acid biosynthesis (type I and type II) and generation of lipids*

Biosynthesis of fatty acids (FAs) is a vital anabolic process that serves to provide short to long chain alkylcarboxylic acids for a variety of purposes. They are mainly required as building blocks of lipids, as an energy store, as precursors for other molecules, including second messengers and cofactors, etc. More details on this and FA synthesis can be found in Chapter 19 (lipid synthesis and uptake).

The overall reaction of FA synthesis is as follows:



The principal synthesis can either be executed by a single polypeptide chain (type I fatty acid synthase, FAS I; EC 2.3.1.85) or by several individual enzymes acting in concert (type II fatty acid synthase, FAS II).

Although FA synthesis in animal cells was long thought to be exclusively performed by the cytosolic FAS I (Smith *et al.*, 2003), recent investigations have proven that in addition, components of a putative type II-like FAS machinery are also localized to the mitochondria of humans and probably most eukaryotes (Cronan *et al.*, 2005a; Zhang *et al.*, 2005). One very likely reason for mitochondrial FA synthesis is to provide octanoyl-ACP, the precursor for lipoic acid (LA) synthesis via lipoic acid synthase (LIAS), also a mitochondrial enzyme. However, this is not the case for *T. gondii* and *P. falciparum*, since their FAS II is exclusively apicoplast-localized (see “Lipoic acid biosynthesis” for further discussion). Consequently, the capability of the Apicomplexans to synthesize FAs is quite unique and complex. The available complete genomes (*Plasmodium* spp, *T. gondii*, *Theileria* spp, *C. parvum*, *Eimeria tenella*) have revealed a surprising divergence with regard to the ability to synthesize FAs. Whereas *Theileria* has obviously lost its FAS machinery entirely (Gardner *et al.*, 2005; Pain *et al.*, 2005), *P. falciparum* possesses only a functional FAS II apparatus located exclusively in the apicoplast (Gardner *et al.*, 2002; Gornicki, 2003). *C. parvum*, on the other hand, has lost its plastid-derived organelle and therefore contains no FAS II but instead has gained (or retained) a giant cytosolic FAS I (Zhu, 2004). Lastly, *T. gondii* (and most likely also *E. tenella*) completes the possible permutations by having both, FAS II in the apicoplast (Waller *et al.*, 1998; Jelenska *et al.*, 2001) and an extremely large FAS I (> 10 000 kDa) that seems to be mitochondrion-associated (M.J. Crawford, G. Zhu, and D. Roos, personal communication). Its exact ultrastructural localization (very close attachment to or inside the mitochondrion) has not been resolved but a strictly intra-mitochondrial FAS I would pose the question how such a huge protein would get into the organelle. A putative cytosolic ATP-citrate lyase (ACL, EC 2.3.3.8) identifiable in the genome of *T. gondii* (and *E. tenella*), which can convert citrate into acetyl-CoA would argue for a cytosolic localization of FAS I. Mitochondrion-derived citrate can be shuffled out of the organelle through a dicarboxylate/tricarboxylate exchanger, which can be identified in *T. gondii*'s genome. A possible solution to this dilemma might come from an ultra-structural study describing some cytosolic invaginations enclosed by mitochondrial membranes (Köhler, 2006).

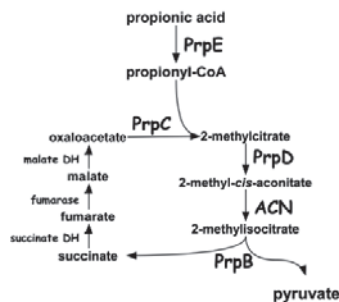


One obvious reason for most Apicomplexa to have their own FAS II is the high demand for lipids and especially phospholipids to build the membranes for their secretory organelles, the pellicular complex, and the growing parasitophorous vacuolar membrane (Charron and Sibley, 2002; Krishnegowda and Gowda, 2003; Coppens and Vielemeyer, 2005; Bisanz *et al.*, 2006). *Theileria*, on the other hand, which does not have to maintain a PVM and has direct access to the host cell cytosol could afford to rely entirely on host-derived FAs. According to a recent study in *T. gondii*, membrane lipids seem to be synthesized *de novo* in three locations: the apicoplast, the endoplasmic reticulum and the mitochondrion (Bisanz *et al.*, 2006), but how FAs can be transported out of the apicoplast is yet unclear.

In *T. gondii*, FAS I probably serves for the generation of very long chain fatty acids (FA elongase). This would be in agreement with the fact that inhibition of FAS II by triclosan kills the parasite (McLeod *et al.*, 2001), indicating that FAS I cannot complement the loss of the metabolites generated by FAS II. In addition, work by B. Striepen's lab indicates that a conditional knockout mutant for the apicoplast acyl carrier protein (ACP) in tachyzoites of *T. gondii* grows at a significantly reduced rate and eventually dies (Mazumdar *et al.*, 2006).

### Methylcitrate cycle

*T. gondii* harbors the complete set of genes specific for a biochemical pathway otherwise found only in bacteria and fungi: the methylcitrate cycle (MCC, see Figure 22.3) (Uchiyama *et al.*, 1982; Pronk *et al.*, 1994). Analyses of various apicomplexan genomes further uncover striking metabolic disparities between parasites (Chaudhary and Roos, 2005). Only the members of the Coccidia (*T. gondii*, *Neospora caninum*, *Eimeria tenella* and *Sarcocystis neurona*) possess the MC pathway, while the Haemosporida (*Plasmodium* species), the Piroplasmida (*Theileria* species) and the Cryptosporidia are lacking it. The MCC in conjunction with enzymes of the TCA cycle ultimately converts propionate into pyruvate, thereby allowing bacteria and fungi to use propionate as carbon source (Brock *et al.*, 2000; Brock *et al.*, 2002). Bacteria and fungi can also use  $\beta$ -oxidation and catabolism



**Figure 22.3** Overview of the enzymatic reactions catalyzed by the methylcitrate cycle in conjunction with Krebs cycle enzymes. Abbreviations: ACN, aconitase; DH, dehydrogenase; PrpB, methylisocitrate lyase; PrpC, methylcitrate synthase; PrpD, methylcitrate dehydratase; PrpE, propionyl-CoA synthetase.

of branched-chain amino acids as carbon sources. As detailed above degradation of some amino acids and fatty acids yields both acetyl-CoA and propionyl-CoA. Propionyl-CoA is a toxic metabolic intermediate, which can be detoxified by oxidation to pyruvate via the methylcitrate cycle (Brock and Buckel, 2004; Brock, 2005).

*T. gondii* tachyzoites express five genes associated with the MCC: propionyl-coenzyme A synthetase (PrpE, EC 6.2.1.17); 2-methylcitrate synthase (PrpC, EC 2.3.3.5); aconitase (Acn, EC 4.2.1.3); 2-methylcitrate dehydratase (PrpD, EC 4.2.1.79) and methylisocitrate lyase (PrpB, EC 4.1.3.30). Preliminary subcellular localization data indicate that PrpE, PrpC, Acn, and PrpB enzymes are targeted to the mitochondrion (D. Soldati, unpublished). In contrast to *Plasmodium*, *T. gondii* hosts several amino acid degradation pathways that lead to the production of acetyl-CoA and/or propionyl-CoA. In addition to the pathway of branched-chain amino acid degradation (see above), *T. gondii* possesses enzymes implicated in the degradation of methionine, including cystathionine  $\beta$ -synthase (CBS, EC 4.2.1.22) which produces cystathionine from homocysteine, and cystathionine  $\gamma$ -lyase (CGL, EC 4.4.1.1) producing 2-oxobutyrate, which can be converted into propionyl-CoA. The probable use of valine, isoleucine and methionine as carbon sources also yields propionyl-CoA. The presence of the MCC in *T. gondii* allows the conversion of propionyl-CoA into pyruvate, which then can directly replenish the intermediates of the TCA cycle in the mitochondrion by the action of pyruvate carboxylase (PC, EC 6.4.1.1), converting it into oxaloacetate. In this context the MCC might fulfill the crucial task of detoxifying propionyl-CoA by converting it into pyruvate (Matsuishi *et al.*, 1991). The oxaloacetate produced by PC acts as carrier to shuffle the acetyl-CoA produced into the mitochondrion to the cytosol. *T. gondii* also possesses a putative cytosolic alanine transaminase (EC 2.6.1.2), enabling the parasite to generate even more pyruvate from alanine, if needed.

Amino acid degradation products can be assimilated as carbon source for anaplerotic reactions like FAS as opposed to an exclusive dependence on the glycolysis-derived metabolites PEP and pyruvate (as it is the case for the intraerythrocytic stages of *Plasmodium*). Consistent with this hypothesis is the fact that the hexose import machinery of *T. gondii* has a much higher affinity for glucose than the corresponding plasmodial transporters (Joet *et al.*, 2002), suggesting that the glucose concentration within the host cell surrounding *T. gondii* is generally lower than in the modified erythrocytes in which *Plasmodium* resides. Indeed for *Plasmodium*, the supply of hexoses is likely sufficient (at least in part) due to an increased, parasite-induced permeability of the host cell (Staines *et al.*, 2004) and to the fact that glucose enters the intracellular parasite via an equilibrative and not an active process (Kirk *et al.*, 1996).

### Iron–sulfur cluster biosynthesis

Iron-sulfur clusters ([Fe-S]) are modular units in which iron and sulfur atoms are coordinated in different ways, usually attached to a protein backbone via cysteine sulfur linkages (Johnson *et al.*, 2005). [Fe-S]-containing proteins, because of their enormous range in redox potentials, are mostly involved in electron transfer reactions and are vital for most organisms. However, [Fe-S] also constitute substrate binding and catalysis sites in proteins or possess regulatory and sensing functions, as in aconitase.

The core pathway of their generation is relatively conserved between prokaryotes and eukaryotes, where it is located in the mitochondria (Barras *et al.*, 2005; Lill and Mühlenhoff, 2005). However, plastid-harboring eukaryotes (including Apicomplexa) have a second site of [Fe-S] synthesis in the apicoplast (Ellis *et al.*, 2001; Seeber, 2002). In the apicoplast of *T. gondii* and *P. falciparum* at least four such proteins are presently known: ferredoxin (see “Ferredoxin redox system”), lipolic acid synthase (see “Lipoic acid biosynthesis”), LytB and GcpE (see “Isoprenoid biosynthesis”) (Ralph *et al.*, 2004; Seeber *et al.*, 2005). The fundamental aspects of [Fe-S]-assembly in plastids seem to be similar but the molecular players are different compared to the mitochondrion (Balk and Lobreaux, 2005; Fontecave *et al.*, 2005) (see below).

The individual steps of [Fe-S] synthesis are only given as a basic overview of the process (see Barras *et al.*, 2005; Johnson *et al.*, 2005; Lill and Mühlenhoff, 2005 for details). It requires the initial uptake of chelated iron into the mitochondrion (or plastid), a process that is still ill-defined in all organisms, and the provision of elemental sulfur. The latter derives from L-cysteine through the action of a desulfurase, yielding sulfide and L-alanine. Both elements are then assembled into [2Fe-2S] or other types of clusters on distinct “scaffold proteins,” coordinated to cysteine residues of that protein. Since both iron and sulfide ions are highly toxic in their free form this process has to be highly coordinated. Accessory proteins like chaperones and a redox system consisting of the mitochondrial-type ferredoxin and its reductase are also involved in this and subsequent steps. Finally, the clusters are released from the scaffold proteins and transferred to either mitochondrial apo-proteins or transported into the cytosol via a special transport system. Here several other proteins are involved in the assembly, maturation and/or transport of [Fe-S]s onto apo-proteins localized in the cytosol or nucleus.

[Fe-S] cluster biosynthesis in apicomplexan mitochondria seems not to deviate from this general route as apicomplexan genomes contain most genes related to the bacterial and yeast enzymatic (ISC-like) machinery required for this process (Seeber, 2002; Mather *et al.*, 2007). Notably, a homolog for frataxin, a presumed iron chelator, is present in *C. parvum* but not in *T. gondii* or in *Plasmodium*. The scaffold proteins IscA and IscU from *T. gondii* have been epitope-tagged and demonstrated to target into the mitochondrion (D. Soldati, unpublished). However, no biochemical analysis has been published for any of these proteins in any Apicomplexa. Predicted mitochondrial [Fe-S] proteins of *T. gondii* are the components of the respiratory chain, aconitase, mitochondrial-type ferredoxin and possibly ferrochelatase.

It is now an accepted view that the so-called SUF proteins presumably constitute the sole [Fe-S] synthesis machinery in plant plastids (Balk and Lobreaux, 2005), and by extension also in the apicoplast. The SufS/SufE complex seems to act as a combined desulfurase and sulfur “transporter” to the scaffold protein SufA where the [Fe-S] is assembled (there is another likely scaffold protein in plastids called cNFU). This step also requires reducing electrons, and although this aspect has not been addressed by any study, one possible electron donor could be the plastid-localized ferredoxin (Seeber *et al.*, 2005). The role of the three other SUF proteins, SufB, C and D, which form a complex, is still unclear. Proposals for their function are chaperone activity, involvement in energy generation for iron acquisition and stimulation of the desulfurase reaction (Fontecave *et al.*, 2005).

The picture for the apicoplast pathway is even sketchier, although the essential gene products are predicted to be present in the apicoplast of *T. gondii* and *P. falciparum* (Ellis *et al.*, 2001; Seeber, 2002). Notably, SufB is one of the two proteins encoded by the apicoplast genome in *Plasmodium*, *T. gondii* and *E. tenella*, but it is not sufficient to explain the maintenance of the apicoplast genome in general since *Theileria* obviously lost SufB entirely but still contains [Fe-S] proteins in the plastid (Gardner *et al.*, 2005).

### Ubiquinone biosynthesis

As the name suggests, ubiquinone (coenzyme Q) is a ubiquitous co-factor in nature and is essential for processes such as the transfer of electrons from complex I or II to complex III in the respiratory chain. It consists of a benzoquinone backbone to which an isoprenoid side chain of varying length, depending on the organism, is attached (Turunen *et al.*, 2004). A homolog of 4-hydroxybenzoate nonaprenyltransferase (EC 2.5.1.39) with a predicted mitochondrial targeting sequence is present in *T. gondii*. It attaches the isoprenoid chain to 4-hydroxybenzoate (4-HB) and thus provides a link between the generation of isoprenoid precursors in the apicoplast and mitochondrial ubiquinone synthesis. Most of the enzymes required for the subsequent conversion of 4-HB to 2,3-dimethoxy-5-methyl-*p*-benzoquinone can be identified in the genome. The full shikimate pathway, which could give rise to the 4-HB precursor chorismate, is also present in *T. gondii*, but contrary to earlier notions it is cytosolic and not plastid-resident as in plants (Keeling *et al.*, 1999; Campbell *et al.*, 2004). How chorismate could be converted to 4-HB is unclear since no chorismate lyase has yet been identified in the genome.

### Auxiliary pathways

#### *Protein biosynthesis in the mitochondrion and apicoplast*

*T. gondii* is sensitive to a number of inhibitors of eukaryotic protein synthesis like cycloheximide or emetine. In addition, several antimicrobials (clindamycin, azithromycin, chloramphenicol, thiazolyl peptide antibiotics) known to target the prokaryotic-like protein synthesis machinery are active against Apicomplexa, including *T. gondii*, and have been used as curative drugs against these parasites for some time (Wiesner and Seeber, 2005). The targets of some of these inhibitors have been determined experimentally as the translational apparatus of the apicoplast. For example, clindamycin-resistant *T. gondii* mutants contain a point mutation in the apicoplast large-subunit rRNA at a position known to be the binding site of this drug in *E. coli* (Camps *et al.*, 2002).

Direct evidence for the translation of genes encoded in the plastid genome has been provided in *P. falciparum* with the translation of the elongation factor Tu (Chaubey *et al.*, 2005). The anti-parasitic effects of tetracycline and its derivatives also provide experimental evidence that the mitochondrial protein synthesis is active and essential in *T. gondii* and *P. falciparum* (Prapunwattana *et al.*, 1988; Kiatfuengfoo *et al.*, 1989; Beckers *et al.*, 1995; Budimulja *et al.*, 1997), although direct proof is still missing. A recent study implied that both the mitochondrion and the apicoplast of *P. falciparum* are targets of minocycline, a potent tetracycline derivative (Lin *et al.*, 2002). Taken together, the presence of a functional translational machinery in the apicoplast and most likely also in the mitochondrion is evi-

dent. What is less clear, however, is how a complete set of the 20 required aminoacyl-tRNA synthetases (aaRS) in the two organelles as well as in the cytosol is achieved. Theoretically, each compartment needs a full range of tRNAs and aaRSs, but currently only 34 potential genes have been found in *T. gondii* instead of the 60 that would be required. The same ambiguity exists in other Apicomplexans, with *P. falciparum*'s genome containing apparently only 35 putative aaRS genes. A limited set of aaRS genes has also been observed in other organisms, and in *Arabidopsis thaliana* at least 15 aaRSs are shared between mitochondria and plastids, and 5 between cytosol and mitochondria due to the dual targeting of these proteins (Duchene *et al.*, 2005).

N-formyl methionine is a universal initiator in bacterial protein synthesis and most mature proteins do not retain their initial N-terminal amino acid. *T. gondii* possesses single genes coding for methionyl-tRNA fMet-formyltransferase (MTF, EC 2.1.2.9) and peptide deformylase (PDF, EC 3.5.1.27), although the two enzymes are expected to be present in both the apicoplast and the mitochondrion. Again it is plausible to assume that the product of each single gene is delivered to both organelles by a mechanism of bimodal targeting, alternative translation initiation or alternative RNA splicing.

### *Lysine biosynthesis*

Surprisingly, *T. gondii*'s genome contains several genes of the lysine biosynthesis pathway that convert aspartate into lysine and should thus not be auxotrophic for this amino acid, in contrast to its mammalian host (Chaudhary and Roos, 2005). Lysine biosynthesis appears to be unique to *T. gondii* (it is absent in the coccidian *E. tenella*) and encompasses aspartate kinase (EC 2.7.2.4), aspartate-semialdehyde dehydrogenase (EC 1.2.1.11), dihydrodipicolinate synthase (EC 4.2.1.52) and dihydrodipicolinate reductase (EC 1.3.1.26). Localization of this pathway in *T. gondii* is unclear, and current data do not allow its unambiguous designation to the four known pathways leading from tetrahydrodipicolinate to meso-diaminopimelate (Hudson *et al.*, 2006). If it follows the so-called succinyl-DAP pathway then a mitochondrial localization would be likely since succinyl-CoA would be required. On the other hand, lysine biosynthesis is localized to the plastids in plants, so this question needs further investigation.

### *Precursors for nucleotide biosynthesis*

*T. gondii* is capable to synthesize pyrimidines, and plasmodial dihydroorotate dehydrogenase (EC 1.3.99.11), which catalyzes the fourth step in the pyrimidine biosynthetic pathway, has been localized to the mitochondrion by *in vivo* studies (Krungrak, 1995). The predicted *T. gondii* gene possesses a mitochondrial targeting signal. This enzyme therefore connects this organelle with an important cytosolic pathway for the parasite. Pyrimidine biosynthesis is detailed in Chapter 20.

Another set of proteins links the mitochondrion to the folate pathway and thymine nucleotide synthesis. A glycine cleavage complex (GCV) together with serine hydroxymethyltransferase (SHMT) is presumed to be present in *P. falciparum* (Salcedo *et al.*, 2005). Its function is probably the degradation of glycine to provide precursors for folate biosynthesis. GCV catalyses the reversible oxidative cleavage of glycine into CO<sub>2</sub> and NH<sub>3</sub>, with transfer of the remaining methylene (CH<sub>2</sub>-) moiety to tetrahydrofolate (THF) form-

ing N<sup>5</sup>,N<sup>10</sup>-methenyl THF and production of NADH. Four enzymes are assembled to form the GCV: P-protein or glycine decarboxylase (EC 1.4.4.2), H-protein, which contains a lipoamide prosthetic group, T-protein or tetrahydrofolate aminomethyltransferase (EC 2.1.2.10) and L-protein or dihydrolipoamide dehydrogenase (EC 1.8.1.4). Tagged H-protein of the GCV from *T. gondii* is targeted to the mitochondrion and lipoylated (M. Crawford and D. Roos, personal communication); and therefore the complex is likely also active in *T. gondii*. However, contrary to *P. falciparum* only a single SHMT gene (with no apparent organellar targeting sequence) is detectable.

### *Antioxidant system*

Reactive oxygen species (ROS) including hydrogen peroxide, the superoxide radical and the hydroxyl radical, are produced as by-products of oxygen metabolism in all cells. *T. gondii* is well equipped with superoxide dismutases (SOD), catalase, glutathione peroxidases (GPx) and peroxiredoxins (Prx) that form an antioxidant network. *T. gondii* catalase is a cytosolic enzyme acting downstream of the cytosolic SOD1. Like most aerobic eukaryotic cells, *T. gondii* also possesses mitochondrion-targeted SODs (TgSOD2 and TgSOD3) and peroxidases (TgPRX3) to safely dispose of oxygen radicals that are generated in cellular respiration and metabolism (Ding *et al.*, 2004). No anti-oxidant enzymes have been reported to localize to the apicoplast so far. However, this site of biosynthesis of fatty acids, isoprenoids, iron-sulfur clusters and heme is on high demand for antioxidant protection and a need for redox cycling of co-factors. Recently a superoxide dismutase (TgSOD2) and the product of an alternative spliced transcript coding for a thioredoxin-dependent peroxidase gene (TgTPX1/2) has been localized both in the mitochondrion and the apicoplast of tachyzoites (Pino *et al.*, in preparation). Dissection of the TgSOD2 protein sequence reveals that its bipartite N-terminal targeting sequence is similar to conventional apicoplast-targeting leader sequences and that it is necessary but not sufficient to target the protein to the apicoplast. This mechanism of bimodal targeting is possibly extended to other proteins including the aminoacyl-tRNA synthetases.

### *Missing pathways*

There is no genetic evidence for a urea cycle operating in *T. gondii*, which in other eukaryotes is divided between the mitochondrion and the cytosol. Ammonia is probably metabolized via mitochondrial glutamate dehydrogenase (EC 1.4.1.2) and/or glutamine synthetase (EC 6.3.1.2) but nothing is known about ammonia efflux. Another pathway that is apparently not present in *T. gondii*'s mitochondrion is the glyoxylate cycle, which is a variation of the TCA cycle that generates succinate and glyoxylate from isocitrate by the action of isocitrate lyase.

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## **Apicoplast**

Initially, the organelle later called apicoplast was described morphologically in several species in the 1960s and 1970s, and given various names that essentially referred to the multiple membranes surrounding this structure (Siddall, 1992). In the early 1990s it became evident that a circular 35 kb extrachromosomal DNA of *P. falciparum* coded for an RNA polymerase that was more similar to those of chloroplasts than to that of bacteria



(Gardner *et al.*, 1991). This gave the first hint to an evolutionary connection between algae and parasites (Wilson *et al.*, 1994). Then in 1996/97 two groups provided molecular evidence that the circular 35 kb genome and the enigmatic organelle described 30 years earlier co-localized (McFadden *et al.*, 1996; Köhler *et al.*, 1997). Several reviews have covered the discovery and biology of the apicoplast in depth (McFadden *et al.*, 1997; Roos *et al.*, 1999; Foth and McFadden, 2003; Waller and McFadden, 2005; Wilson, 2005).

Obviously, the apicoplast arrived in an ancestor of extant Apicomplexa through secondary endosymbiosis, most likely of a red alga (Harper *et al.*, 2005). However, there is still some debate as to how green algal phylogenetic traces in the genome can be reconciled with this view (Funes *et al.*, 2004). One possible explanation could be horizontal gene transfer to either the donor or acceptor of the plastid at one point in time (Huang *et al.*, 2004). It seems that almost all Apicomplexa have retained the plastid, with the notable exception of *Cryptosporidium* spp. (Abrahamsen *et al.*, 2004), and that its presence and function is crucial for their survival, as shown first for *T. gondii* (Fichera and Roos, 1997). This functional conservation is reflected by the fact that most drugs affecting plastid-localized targets are effective against many apicomplexan species (like *T. gondii* and *P. falciparum*; Wiesner and Seeber, 2005) due to considerable sequence homology in the respective proteins. It is therefore not uncommon to use *T. gondii* as a model to study or deduce *P. falciparum* apicoplast metabolism and *vice versa* (see below).

Compared to plastids of other organisms the apicoplast appears to harbor the most reduced plastid genome known, containing mainly genes involved in transcription and translation (Wilson and Williamson, 1997, but see “Iron–sulfur cluster biosynthesis” for the plastid-encoded *SufB*). The majority of proteins constituting the proteome of the apicoplast, however, are nuclear-encoded and transported to the organelle via the secretory pathway using a bipartite targeting domain at the N-terminus (see Chapter 21 for details). Bioinformatic predictions from the complete genome data of *P. falciparum* allowed the compilation of a list of 545 putatively apicoplast-localized proteins, of which > 70% are of unknown function (Ralph *et al.*, 2004). Nevertheless, the early realization that plant-derived metabolic pathways of the apicoplast should present ideal candidates for new drug targets has accelerated the knowledge of this organelle’s function enormously.

## Main metabolic pathways

### *Isoprenoid biosynthesis*

Isoprenoids are a large class of molecules important for a variety of cellular processes, including cell signaling and post-translational protein modifications (see Chapter 19). Two very different biosynthetic pathways are known in nature that lead to the two initial building blocks of isoprenoids: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The so-called mevalonate pathway is found in animals, fungi and archaeobacteria and starts from mevalonate, a precursor derived from the degradation of leucine, whereas the second pathway uses pyruvate and glyceraldehyde-3-phosphate as starting material to form DOXP (1-deoxy-D-xylulose 5-phosphate). This synthesis route is therefore called DOXP pathway (sometimes also MEP pathway), and it is present in plants, algae and most eubacteria (Eisenreich *et al.*, 2004). In 1999 it was reported to be

also present and functional in the apicoplast of *P. falciparum* (Jomaa *et al.*, 1999). All seven genes coding for the enzymes involved in the formation of IPP and DMAPP can be found in the genome of *T. gondii*, although no thorough biochemical investigations have been published for any of them. However, there is preliminary evidence for the activity of the second enzyme in the pathway (DOXP reductoisomerase, Dxr) in *T. gondii* extracts (J. Wiesner, personal communication). Fosmidomycin, a drug that in *P. falciparum* inhibits Dxr both *in vitro* and *in vivo* (Jomaa *et al.*, 1999; Cassera *et al.*, 2004), has no effect on *T. gondii* growth, although the mentioned Dxr activity in *T. gondii* extracts can be inhibited by this drug. The likely explanation is that the very hydrophilic compound has insufficient access to the intracellular parasite. As will be discussed in “Ferredoxin redox system” the last enzymatic steps of the DOXP-pathway are critically dependent on this redox system.

Isoprenoids seem to be made exclusively in the apicoplast by the DOXP route since no genes for a cytosolic mevalonate pathway are present in the genomes of Apicomplexa. Consequently, IPP/DMAPP must be transported to other sites in the parasite where isoprenoids are required, like the cytosol (e.g. for prenylation of proteins, Chakrabarti *et al.*, 1998; Ibrahim *et al.*, 2001; or as precursors for dolichols) and the mitochondrion (e.g. for side chain modification of quinones, de Macedo *et al.*, 2002). Other important metabolic routes dependent on the DOXP pathway in *P. falciparum* are vitamin B6 synthesis in the cytosol via DOXP-derived pyridoxal phosphate (Cassera *et al.*, 2004; Wrenger *et al.*, 2005) and possibly thiamine pyrophosphate (TPP) synthesis from the same precursor in the apicoplast (Ralph *et al.*, 2004). TPP is an essential co-factor for the apicoplast-resident pyruvate dehydrogenase complex subunit E1 and for DOXP synthase itself. Another potential isoprenoid consumer in the plastid is tRNA isopentenylation. These modified tRNAs are likely required to correctly translate the genes of the organelle’s own genome, which in several Apicomplexa contain numerous stop codons (Ralph *et al.*, 2004).

### *Fatty acid biosynthesis (type II)*

For its discussion see “Fatty acid biosynthesis (type I and type II) and generation of lipids.”

### *Heme biosynthesis*

As mentioned, the heme biosynthetic pathway also operates partly in the apicoplast (for details see *Heme biosynthesis* above). To what extent intermediate metabolites might be required for other processes in the apicoplast is currently unknown.

## Auxiliary pathways

### *Carbon sources*

The non-photosynthetic apicoplast needs to import hexoses and triose phosphates from the cytosol as carbon source. The *Plasmodium* genome indicates the presence of a phosphoenolpyruvate/phosphate translocator (PPT) predicted to be targeted to the apicoplast that would allow the import of phosphoenolpyruvate (PEP). Once in the apicoplast, PEP is converted into acetyl-CoA by the action of a pyruvate kinase (PYK, EC 2.7.1.40) and the PDH complex (Ralph *et al.*, 2004). Unexpectedly, *T. gondii* does not possess a gene

coding for PPT. An additional cytosolic source of carbon is dihydroxyacetone phosphate (DHAP), which in plants is imported by the triose phosphate transporter (TPT). In the apicoplast, DHAP can be converted to glyceraldehyde-3-phosphate (GA3P) by triose phosphate isomerase (TPI, EC 5.3.1.1) and enter the DOXP pathway for isoprenoid synthesis (Ralph *et al.*, 2004). Both *Plasmodium* and *Toxoplasma* possess a gene coding for a TPT likely targeted to the apicoplast. Since some TPTs are capable of transporting PEP (Neuhaus and Wagner, 2000), it is plausible that *T. gondii*'s TPT serves simultaneously as a transporter for DHAP and PEP.

### Energy and reducing power sources

The mechanism(s) that supplies the apicoplast with energy has not been elucidated to date. There is no evidence of an import mechanism for ATP or hexoses but there are several individual glycolytic enzymes that are encoded by pairs of genes, one of which is predicted to target to the apicoplast. TgGAPDH1 (EC 1.2.1.12) localizes to the cytosol while TgGAPDH2 is a large 995 amino acids long polypeptide that exhibits a N-terminal bipartite targeting signal and localizes to the apicoplast (D. Soldati, unpublished). Interestingly, this large protein shows a domain of weak homology to phosphoribulokinase (PRK, EC 2.7.1.19), an enzyme of the Calvin cycle in the chloroplast of plants. PRK and CP12 form a complex with GAPDH and the activity of these enzymes is regulated by the NADPH-mediated dissociation of the PRK/CP12/GAPDH complex (Wedel and Soll, 1998). Import of DHAP and conversion to GA3P by triose phosphate isomerase (TPI), followed by its conversion to 1,3-diphosphoglycerate (1,3-DPGA) by GAPDH generates NAD(P)H. TPT could export 1,3-DPGA in exchange for another molecule of DHAP, thereby creating an electron shuttle (Ralph *et al.*, 2004). Alternatively, *T. gondii* possesses a second gene (carrying a putative bipartite targeting signal) coding for phosphoglycerate kinase (EC 2.7.2.3), which can convert 1,3-DPGA into 3-phosphoglycerate. This reaction generates ATP and thus might be a second way of producing energy in the apicoplast, besides the cleavage of PEP into pyruvate and ATP by pyruvate kinase.

A second potential source of reducing power in the apicoplast is suggested by the unexpected presence of two enzymes of the TCA cycle in the apicoplast that mediate the conversion of citrate to  $\alpha$ -ketoglutarate, leading to the production of NADPH. Aconitase (EC 4.2.1.3), encoded by a single enzyme, is bimodally targeted to the mitochondrion and the apicoplast, and the second gene codes for an isoform of isocitrate dehydrogenase (ICDH1, EC 1.1.1.42). TgICDH2, in contrast, is localized to the mitochondrion (Pino *et al.*, unpublished data). Whether citrate is present in the apicoplast, and how it gets there is currently unknown.

### Iron–sulfur cluster biosynthesis

The apicoplast as a second site for [Fe-S] biosynthesis has been discussed above.

### Ferredoxin redox system

The apicoplast contains a protein redox system consisting of the small acidic [2Fe-2S] protein ferredoxin (Fd) and its reductase, ferredoxin NADP<sup>+</sup> reductase (FNR, EC 1.18.1.2). In plants and cyanobacteria, Fd receives electrons from photosystem I and transfers them via FNR to the electron acceptor NADP<sup>+</sup>, providing NADPH for the Calvin cycle. The

identification of this protein pair in *T. gondii* was therefore somewhat surprising (Vollmer *et al.*, 2001), given the absence of a photosynthetic apparatus in the apicoplast. However, apicoplast FNR provides reduced Fd under consumption of NADPH (Pandini *et al.*, 2002). The key question is what the electron acceptors in the apicoplast are (Seeber *et al.*, 2005). Currently, the only experimentally confirmed Fd-requiring enzyme is (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate reductase, also called LytB. It is involved in the last step of the isoprenoid biosynthesis pathway (see above) and was shown *in vitro* to receive electrons from reduced *P. falciparum* Fd (Röhrich *et al.*, 2005). The enzyme acting before LytB, GcpE, is also a likely recipient for Fd's electrons (Okada and Hase, 2005; Seemann *et al.*, 2006). It is conceivable that other proteins will be discovered that receive electrons from Fd, like liponic acid synthase or compounds of [Fe-S] biosynthesis (Seeber *et al.*, 2005). The Fd/FNR system might also contribute to the redox balance in the organelle (Krapp *et al.*, 2002). It is therefore assumed to be an essential pathway in the apicoplast.

### *Lipoic acid biosynthesis*

Lipoic acid (LA), a dithiol-containing medium-chain fatty acid, is an essential co-factor for most organisms. LA is covalently attached to specific lysine residues in the E2-subunits of 2-oxo acid dehydrogenase complexes, which include pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched-chain 2-oxo acid dehydrogenase (BCDH) (Perham, 2000; Cronan *et al.*, 2005b; see also "Tricarboxylic acid cycle"). In addition, the H-protein of the glycine decarboxylase system is also lipoylated (Douce *et al.*, 2001; see "Precursors for nucleotide biosynthesis"). Protein-bound LA serves as a swinging arm, transferring an activated acyl group to coenzyme A. Non-lipoylated proteins are enzymatically inactive, and consequently this pathway is essential in organisms relying e.g. on a functional citric acid cycle. In eukaryotes, lipoylation takes place only in mitochondria, while plants and algae also modify a plastid isoform of PDH-E2 with LA (Mooney *et al.*, 2002).

LA can be synthesized *de novo* from octanoyl-acyl carrier protein (generated by FAS II, see 2.1.4), via the action of two enzymes, lipoyl (octanoyl)-acyl carrier protein:protein transferase (LipB) followed by lipoic acid synthase (LipA) (Cronan *et al.*, 2005b). The latter is an [Fe-S] protein and incorporates two sulfur atoms into the protein-attached octanoyl group most likely using a mechanism similar to that of biotin synthase (Layer *et al.*, 2004). Alternatively, free LA can be coupled to lipoyl domains by a single ATP (or GTP)-dependent protein, lipoate protein ligase (LplA) (Cronan *et al.*, 2005b).

Plant and algal plastids contain LipA and LipB but no LplA, and this is also true for the apicoplast of *T. gondii* and *P. falciparum* (Thomsen-Zieger *et al.*, 2003; Wrenger and Müller, 2004). In contrast, eukaryotic mitochondria (including those of plants) typically contain all three enzymes. Remarkably, both *T. gondii* and *P. falciparum* contain only LplA in the mitochondrion, but no LipA/LipB (Thomsen-Zieger *et al.*, 2003; Wrenger and Müller, 2004). It could be shown recently that lipoylation of the apicoplast-localized PDH-E2 subunit in *T. gondii* is critically dependent on a functional FAS II pathway as a donor for octanoyl-ACP, demonstrating LipA/LipB activity in this organelle (Crawford *et al.*, 2006). Since pharmacological disruption of apicoplast LA synthesis does not affect lipoylation in the parasite mitochondrion, however, the conclusion is that mitochondrial LA

is taken up from the host, rather than being acquired from the apicoplast. Consequently, LA is attached to the parasite's mitochondrial acceptor proteins by virtue of *T. gondii*'s LplA. Phylogenetic analyses also reveal that *T. gondii* has lost the mitochondrial lipoate synthase and transferase genes it once harbored. Thus, the parasite has to scavenge lipoic acid from the host due to the unique compartmentalization of the LA pathway (Crawford *et al.*, 2006). However, the actual source of host LA under normal circumstances is currently unknown (LA is usually only present as protein-bound form in most cells). This finding raises the question as to whether the close interaction of host mitochondria with the parasitophorous vacuole is connected to lipoic acid acquisition from the host (see also Chapter 31).

Whether the dithiol LA in its free or protein-bound form also acts as an antioxidant in *T. gondii* is currently unknown, but such a role has been advocated in *P. falciparum* (Müller, 2004).

### Missing pathways

The majority of proteins are imported into mitochondria and chloroplasts by the activity of the Tim-Tom and Tic-Toc complexes. While components of the Tim-Tom complexes are present in *T. gondii*'s genome, the machinery for import of proteins into the apicoplast remains to be identified.

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## Conclusions

### Shaping of the organellar metabolism during parasite evolution

Endosymbiosis of the apicoplast precursor not only resulted in the acquisition of new metabolic pathways previously not present in the recipient cell (e.g. photosynthesis) but also in the duplication of several pathways already operating. Through a process of "reductive evolution" (best characterized in intracellular bacteria, Wernegreen, 2005) those genes that are superfluous should be lost because for example the products of metabolic routes can be acquired from the host.

Comparative genomics can give invaluable insights into the shaping of such pathways and processes over time and under different environmental conditions. A limited comparison between the parasitic green alga *Helicosporidium* sp. and the free living non-photosynthetic green alga *Prototheca wickerhamii* with *P. falciparum* has been published (Borza *et al.*, 2005). Not surprisingly, *P. falciparum* has the most reduced metabolic activities, sharing only fatty acid, isoprenoid and heme biosynthesis with the two algal plastids, whereas in *Helicosporidium*, in contrast to Apicomplexa, some amino acid synthesis pathways are still present in the plastid (de Koning and Keeling, 2004). Comparing selected organellar pathways of *T. gondii* with the genome of the free-living marine diatom *Thalassiosira pseudonana* (Armbrust *et al.*, 2004) (the only other completely sequenced chromalveolate with a secondary plastid of red-algal origin, phylogenetically related to Apicomplexa) is also insightful. The diatom has two pathways generating isoprenoids (plastid-localized DOXP and cytosolic MEP pathways, respectively), the latter probably required for the generation of cholesterol, which in the case of *T. gondii* can be scavenged from the host (see Chapter 19). Contrary to *T. gondii*, it has as a urea cycle in the mitochondrion but

shows no genomic evidence for FAS I or MCC (F. Seeber and D. Soldati, unpublished observation). Nevertheless, MCC proteins PrpB and PrpD are also present in the ciliate *Tetrahymena thermophila*, and these two genes are phylogenetically very closely related to the *T. gondii* enzymes. This suggests that the methylcitrate cycle has been lost in many protists rather than being acquired in a small subset of them by lateral gene transfer (B. Foth and D. Soldati, unpublished).

Another interesting example highlighting the complexity of these gains, losses and mergers is the lipoic acid biosynthesis pathway. It was present in the mitochondrion of the free living ancestor cell of all chromalveolata before the endosymbiosis of a red alga (since *T. pseudonana* still has two sets of LipA/B genes for targeting into both organelles) but was subsequently lost in Apicomplexa (Crawford *et al.*, 2006). However, the driving force for this gene loss was presumably not a new second site for LA synthesis in the apicoplast since LA seems to be unable to leave the organelle and is thus not available for the mitochondrion. Rather, it was the possibility to scavenge this essential co-factor from the host cell (which the free-living *T. pseudonana* cannot), so in this case an intracellular habitat was the prerequisite for the reduction in genome content and not the endosymbiosis of the plastid.

### Exploiting pathways in the two apicomplexan organelles as drug targets

The main approach for the development of low side effect drugs against infectious organisms is to exploit the differences in metabolism between intruder and host. Apicomplexan parasites, although eukaryotic in nature, offer also a variety of attractive targets in this respect (see special issue of Current Drug Targets 2007, 8, 1–88). In general, it should be easier to design specific inhibitory compounds directed against parasite-specific molecules rather than targeting proteins present in both organisms, although this is not necessarily a prerequisite. Host and parasite mitochondria differ substantially, as pointed out above, either in some important structural aspects or in kinetic aspects of enzymes common to both.

Nevertheless, unique pathways restricted to the mitochondrion, like the MCC of *T. gondii*, or to the apicoplast offer the most attractive overall target for future developments, especially for the treatment of malaria, due to the absence of comparable proteins in the host (McFadden and Roos, 1999; Ralph *et al.*, 2001; Seeber, 2003; Wiesner and Seeber, 2005). One example is the isoprenoid biosynthesis pathway inhibitor fosmidomycin, which is in pre-clinical testing as a combination therapy with clindamycin against malaria (Wiesner and Seeber, 2005). The enoyl-reductase inhibitor triclosan, targeting the FAS II pathway, is another compound that has been advocated as a future drug against malaria, although reservations against the use of FAS II inhibitors in humans have been expressed due to the fact that their mitochondria also possess a mitochondrial FAS II-like pathway (Zhang *et al.*, 2005). Other pathways important for the maintenance of the apicoplast like DNA replication, transcription and translation, have already been exploited in the past by using antibiotics like clindamycin, doxycycline or ciprofloxacin, targeting the respective prokaryotic machinery of the apicoplast (Wiesner and Seeber, 2005). Several other unique protein targets like the ferredoxin redox system, the SUF system of [Fe-S]



synthesis or the transport machinery for the nuclear-encoded apicoplast-resident proteins are just emerging as rewarding drug targets, not to mention the nearly 70% of proteins with unknown functions in the plastid of *P. falciparum* (Ralph *et al.*, 2004). The challenge is not so much to define new drug targets but to develop compounds that are safe, effective and affordable.

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## Internet resources

Resource	Internet address	Comments
<b>General metabolism</b>		
KEGG (Kyoto Encyclopedia of Genes and Genomes)	<a href="http://www.genome.ad.jp/kegg/">http://www.genome.ad.jp/kegg/</a>	Many search options and links; best known for its pathways that are directly linked to different organisms
BRENDA (Comprehensive Enzyme Information System)	<a href="http://www.brenda.uni-koeln.de/">http://www.brenda.uni-koeln.de/</a>	Very comprehensive cross-kingdom information about enzymes, their substrates/inhibitors and biochemistry; with direct links to PubMed
ENZYME (Enzyme nomenclature database at ExPASy)	<a href="http://www.expasy.org/enzyme/">http://www.expasy.org/enzyme/</a>	Search by EC number or enzyme name; links to other databases
Biochemical Pathways (Roche/ExPASy)	<a href="http://www.expasy.org/cgi-bin/search-biochem-index">http://www.expasy.org/cgi-bin/search-biochem-index</a>	The original "Boehringer Biochemical Pathways;" still a valuable resource with reaction schemes; with direct links to ENZYME
IUBMB-Nicholson Minimaps	<a href="http://www.tcd.ie/Biochemistry/IUBMB-Nicholson/mintro.html">http://www.tcd.ie/Biochemistry/IUBMB-Nicholson/mintro.html</a>	Another site with pathway maps
<b>Parasite-specific metabolism</b>		
ToxoDB	<a href="http://toxodb.org/">http://toxodb.org/</a> <a href="http://v3-0.toxodb.org/include/fpPathwayReconstr.html">http://v3-0.toxodb.org/include/fpPathwayReconstr.html</a>	Comprehensive genome database for <i>T. gondii</i> with many links Metabolic Pathway Reconstruction for <i>T. gondii</i> (database was under construction at the time of writing. Direct links to KEGG)
Metabolic Pathways of the Malaria Parasite (Maintained by Hagai Ginsburg)	<a href="http://sites.huji.ac.il/malaria/">http://sites.huji.ac.il/malaria/</a>	This site is a very useful starting point for searches on a specific parasite pathway (has many links to other databases); naturally some pathways are different or missing compared to <i>T. gondii</i>
PlasmoCyc (part of the BioCyc Pathway/Genome Database Collection)	<a href="http://biocyc.org/PFA/server.html">http://biocyc.org/PFA/server.html</a>	Useful start in combination with other databases; many links to other organisms
PlasmoDB	<a href="http://plasmodb.org/">http://plasmodb.org/</a>	Comprehensive genome database for <i>Plasmodium</i>

All mentioned *T. gondii* enzymes/proteins (predicted or confirmed) involved in the various pathways have been compiled in a table, together with their links to ToxoDB. It can be accessed at <http://staff-www.uni-marburg.de/~seeber/toxobook/metabol/>



M.-H. Huynh and V.B. Carruthers

## Abstract

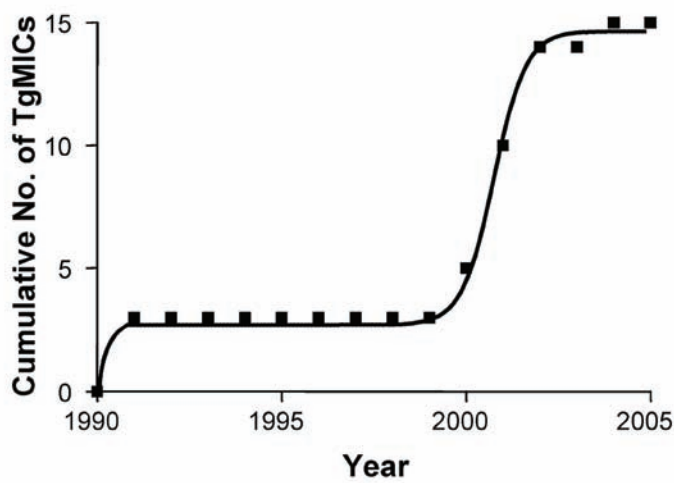
Contents of the microneme organelles play essential roles in the survival and propagation of *T. gondii* parasites. Microneme proteins (MICs) are involved in gliding motility, host cell invasion, and virulence in the mouse model of disease. Almost all MICs are comprised of multiple adhesive motifs, and the diversity of MICs results from combining different numbers and types of modules. The proper synthesis, folding, trafficking, targeting, secretion, and proteolytic processing of these proteins are all critically important in the lytic cycle of this parasite. This chapter will focus on the conserved motifs of the MICs, which may have implications for the broad host cell range of *Toxoplasma*. We will also discuss trafficking of MICs through the secretory pathway and their secretion onto the parasite surface, as well as the cooperative functions of these proteins and complexes in cell invasion and virulence.

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## Microneme gene discovery

A defining characteristic of apicomplexans including *Toxoplasma* is the presence of highly specialized organelles located at the elaborate apical pole of these parasites. One set of organelles is the micronemes, the contents of which have been shown to be necessary for host cell attachment and invasion (Soldati *et al.*, 2001; Carruthers, 2002). Micronemes were identified as early as 1967 in *Toxoplasma*, while the first ultrastructural description of micronemes by electron microscopy occurred soon thereafter (Jacobs, 1967; Scholtyseck and Mehlhorn, 1970). Since micronemes are a conserved feature of the phylum, and microneme proteins show extensive structural similarity, investigations of other Apicomplexa such as *Plasmodium*, *Cryptosporidium*, *Eimeria*, and *Sarcocystis*, also provide insight for *Toxoplasma*, and vice-versa. Indeed the first clear evidence that microneme proteins were directly involved in host cell attachment came from *Plasmodium* studies in which antibodies that inhibited parasite adhesion to erythrocytes also recognized microneme proteins from *Plasmodium* merozoites (Miller *et al.*, 1988). The contents of the *Toxoplasma* micronemes were initially recognized by specific apical immunofluorescence of antibodies produced against parasite lysates or from enriched fractions (Achbarou *et al.*, 1991). Novel micronemal proteins (MICs) have also been identified by microsequencing of proteins in microneme enriched sub-cellular fractions or secretory products (Brydges *et al.*, 2000; Hoff *et al.*, 2001; Harper *et al.*, 2004b). However, the main contributor to the dramatic recent

acceleration of MICs discovery (Figure 23.1) has been the development and expansion of the expressed sequence tag (EST) database and the *Toxoplasma gondii* genome project, which have permitted the identification of novel MICs through sequence homology to existing MICs of *Toxoplasma* or its kin (Wan *et al.*, 1997; Brecht *et al.*, 2001; Meissner *et al.*, 2002). The *Toxoplasma* DNA databases are also crucial for identifying MICs from proteomic studies. Micronemes can be stimulated to release their contents into the culture medium, resulting in an excreted-secreted antigen (ESA) fraction. Proteomic analysis of the induced ESA fraction by two-dimensional electrophoresis/matrix assisted laser desorption time-of-flight (2DE/MALDI-TOF) mass spectroscopy and multi-dimensional protein identification technology (MudPIT) indicated that 24% of the proteins have a putative role in attachment and invasion and are likely MICs, while 54% are of unknown function (Zhou *et al.*, 2005). These proteins with unassigned roles could represent additional uncharacterized MICs. Despite the power that a near-complete genome provides in accelerating gene identification and characterization, a notable caveat is that examining a sequence for homology or similarity to known genes of a family may result in false negatives. That is, the absence of conserved microneme sequences or motifs does not rule out a protein as being a microneme protein; this must be considered during analysis of sequence information from proteomic and genomic databases. One example is P40, a protein that was initially identified in ESA by N-terminal microsequencing (V.B.C., unpublished) and then later re-identified in ESA MudPIT and shown to partially localize to the micronemes when expressed as a YFP fusion protein (Zhou *et al.*, 2005). Although our understanding of the role of MICs has been advanced tremendously in the last decade, the exact contribution of only a handful has been clearly demonstrated, and functional analysis of the other known MICs and the newly identified molecules remains to be completed.



**Figure 23.1** Discovery of *Toxoplasma* MICs. The cumulative number (No.) of *Toxoplasma* MICs is plotted as a function of the year in which they were first described in the primary literature. The accelerated pace of discover coincides with the availability of EST (1997) and genomic (2002) datasets. A second growth phase of verified MICs is anticipated in the near future from ongoing homology-based searches and proteomic studies.

## Host cell attachment and invasion: adhesive motifs and domains

Currently there are 15 well-defined MICs (Table 23.1) that have been shown to occupy *Toxoplasma* micronemes based on localization of either the endogenous protein or tagged recombinant protein expressed in transgenic parasites. Designations of MICs have been based on their chronological discovery (TgMIC1, TgMIC2, etc.), while others such as

**Table 23.1** Properties of *Toxoplasma* MIC proteins











MIC	Domains/Motifs	Orthologues	Characteristics/Functions
TgMIC1		NcMIC1, EtMIC3	<ul style="list-style-type: none"> <li>• Binds host cells, via lactose</li> <li>• No proteolytic processing</li> <li>• Associates with TgMIC4 and TgMIC6</li> <li>• C-term. galectin-like domain required for TgMIC6 folding and transport</li> <li>• mic1ko: invasion and virulence defect</li> <li>• mic1-3ko: severely impaired virulence</li> <li>• Fourmaux et al. (1996) Mol. Biochem. Parasitol. <b>83</b></li> </ul>
TgMIC2		NcMIC2, EtMIC1 <i>Plasmodium</i> TRAP	<ul style="list-style-type: none"> <li>• Binds host cells via heparin, ICAM-1</li> <li>• N-term. processed by MPP2</li> <li>• C-term. processed by MPP1 (rhomboid-like)</li> <li>• Associates with TgM2AP in heterohexamers</li> <li>• C-term. tail contains microneme targeting signals, binds to aldolase for invasion and motility</li> <li>• mic2kd: invasion and gliding defect, avirulent</li> <li>• Wan et al. (1997) Mol. Biochem. Parasitol. <b>84</b></li> </ul>
TgMIC3		NcMIC3	<ul style="list-style-type: none"> <li>• Synthesized as a dimeric proprotein</li> <li>• Propeptide cleavage required for adhesion</li> <li>• Associates with TgMIC8</li> <li>• mic3ko: no invasion or virulence defect</li> <li>• Garcia-Reguet et al. (2000) Cell. Microbiol. <b>2</b></li> </ul>
TgMIC4		NcMIC4, EtMIC5	<ul style="list-style-type: none"> <li>• Weak cell-binding activity</li> <li>• N-, C-term. processing</li> <li>• Associates with TgMIC1 and TgMIC6</li> <li>• mic4ko: no invasion or virulence defect</li> <li>• Brecht et al. (2001) J. Biol. Chem. <b>276</b></li> </ul>
TgMIC5			<ul style="list-style-type: none"> <li>• N-term. propeptide</li> <li>• mic5ko: increased surface proteolysis; no invasion or virulence defect</li> <li>• Brydges et al. (2000) Mol. Biochem. Parasitol. <b>111</b></li> </ul>
TgMIC6			<ul style="list-style-type: none"> <li>• N-term. propeptide</li> <li>• C-term. processing by MPP1 (rhomboid-like)</li> <li>• C-term. microneme targeting motifs</li> <li>• mic6ko: mistargeting of TgMIC1 and TgMIC4 to dense granules</li> <li>• Reiss et al. (2001) J. Cell Biol. <b>152</b></li> </ul>
TgMIC7			<ul style="list-style-type: none"> <li>• Bradyzoite-specific expression</li> <li>• Sorting signals imply a role as an escorter</li> <li>• Meissner et al. (2001) J. Cell Sci. <b>115</b></li> </ul>
TgMIC8			<ul style="list-style-type: none"> <li>• One lectin-like domain and 10 EGF-like domains</li> <li>• Contains conserved intramembranous cleavage sequences for rhomboids</li> <li>• Escorts TgMIC3</li> <li>• Meissner et al. (2001) J. Cell Sci. <b>115</b></li> </ul>
TgMIC9			<ul style="list-style-type: none"> <li>• Bradyzoite-specific expression</li> <li>• Sorting signals imply a role as an escorter</li> <li>• Meissner, M et al. (2001) J. Cell Sci. <b>115</b></li> </ul>
TgMIC10		NcMIC10, SnMIC10	<ul style="list-style-type: none"> <li>• <math>\alpha</math>-helical protein, highly immunogenic</li> <li>• No obvious motifs, does not bind cells</li> <li>• Hoff et al. (2001) Exp. Parasitol. <b>97</b></li> </ul>

Table 23.1 Continued

MIC	Domains	Orthologues	Characteristics/Functions
TgMIC11		NcMIC11, EtMIC11, SnMIC11	<ul style="list-style-type: none"><li>• Internal propeptide cleaved to produce two chains tethered by a disulfide bond</li><li>• <math>\alpha</math>-helical protein with some structural similarities to TgMIC10</li><li>• Harper et al. (2004) Int. J. Parasitol. <b>34</b></li></ul>
TgMIC12		EmMIC4, EtMIC4	<ul style="list-style-type: none"><li>• Contains 31 EGF-like and 12 TSR repeats</li><li>• C-term. microneme targeting signal</li><li>• Contains conserved intramembranous cleavage sequences for rhomboids</li><li>• Opitz et al. (2002) EMBO J. <b>21</b></li></ul>
TgAMA1		PfAMA1, EtAMA1	<ul style="list-style-type: none"><li>• N-term. propeptide</li><li>• C-term. intramembrane cleavage</li><li>• Interacts with TgRONs to form moving junction during invasion</li><li>• ama1kd: severe invasion defect</li><li>• Donahue et al. (2000) Mol. Biochem. Parasitol. <b>111</b></li></ul>
TgM2AP		EtMIC2, NcM2AP	<ul style="list-style-type: none"><li>• N-term. propeptide</li><li>• C-term. processed by MPP2</li><li>• Associates with TgMIC2</li><li>• Propeptide necessary for the complex to exit Golgi-associated compartment; removal required for efficient mobilization from the micronemes</li><li>• m2apko: invasion defect, slight reduction in virulence</li><li>• Rabenau et al. (2001) Mol. Microbiol. <b>41</b></li></ul>
TgSUB1		NcSUB1, PfSUB1/2	<ul style="list-style-type: none"><li>• Subtilisin-like serine protease</li><li>• N-term. propeptide</li><li>• C-term. processed by MPP2</li><li>• GPI-anchored</li><li>• Miller et al. (2001) J. Biol. Chem. <b>276</b></li></ul>

Cleaved during maturation

Cleaved on parasite surface

Propeptide

Transmembrane

Globular

A/I-domain

TSR-like

EGF-like

Chitin-binding-like

Apple/PAN-like

Protease

TgAMA1 and TgSUB1 were named based on similarity to orthologs in other Apicomplexa (Donahue *et al.*, 2000; Miller *et al.*, 2001). Most of the characterized MICs contain extracellular modules and motifs that are also found in adhesive proteins of higher eukaryotes, such as thrombospondin type I repeats (TSRs), integrin-like A-domains, and epidermal growth factor-like (EGF-like) repeats. These domains most likely function in binding to host cell surface molecules. This important first step for establishing an attachment zone with the host cell initiates the invasion process and the lytic cycle. Tight binding to the host is necessary as the parasite actively penetrates into the cell and invaginates the host plasma membrane to form a parasitophorous vacuole (PV), in which asexual replication occurs. During invasion, many MICs are seen on the parasite surface in the vicinity of the moving junction (MJ), a constriction that forms between the parasite and host membranes, and which excludes most MICs and host membrane proteins from entering the PV. For example, TgMIC2 has been shown to bind to heparan sulfate proteoglycans and ICAM-1 (Harper *et al.*, 2004a; Barragan *et al.*, 2005), while TgMIC1 is a lactose-binding



lectin (Lourenco *et al.*, 2001); both are associated with the externally exposed region of the MJ, are capped towards the posterior of the parasite through invasion, and subsequently cleaved from the surface. The breadth and complexity of the different MICs and motifs likely contribute to the promiscuity of this parasite in its ability to bind to different host cell receptors and invade all nucleated cells studied. Each of the adhesive domains will be examined in greater detail as follows.

## TSRs

Thrombospondin-1 (TSP-1) is a glycoprotein adhesion molecule, but has pleiotropic functions involving a broad range of cellular processes including cell proliferation, migration, apoptosis, and neuronal development (Adams, 1997; Bornstein *et al.*, 2004). TSP-1 is a homotrimer, with each monomer featuring N- and C-terminal globular domains, a procollagen-like domain, and three repeated sequence motifs, designated type 1, 2, and 3 (Lawler and Hynes, 1986). The TSRs have been identified in multiple protein families, and the presence of TSRs in *C. elegans* revealed that this is an ancient module that arose before the evolutionary separation of nematodes and chordates (Hutter *et al.*, 2000). TSRs have been demonstrated to bind to extracellular molecules such as collagen V (Takagi *et al.*, 1993) and fibronectin (Sipes *et al.*, 1993) as well as cellular molecules, including heparan sulfate and other glycosaminoglycans (Incardona *et al.*, 1996; Yu *et al.*, 2000), and receptors such as CD36 and  $\beta$ 1 integrins (Lawler and Hynes, 1989; Dawson *et al.*, 1997). Since both cell- and matrix-binding have been established for TSRs (reviewed in Adams and Tucker, 2000), it has been hypothesized that the presence of these repeats plays a significant role in the activities of these diverse proteins. One or multiple TSR modules are present in many apicomplexan MICs including *Plasmodium* thrombospondin-related adhesive protein (TRAP), circumsporozoite protein (CS), and circumsporozoite- and TRAP related protein (CTRP); *Eimeria* EtMIC1 and EtMIC4; *Cryptosporidium* TRAP-C1 and sporozoite cysteine-rich protein (SCRIP); *Neospora* NcMIC2, and *Toxoplasma* TgMIC1, TgMIC2, and TgMIC12. The solved crystal structure of TSR revealed an anti-parallel, three- $\beta$ -stranded fold forming an extended conformation (Tan *et al.*, 2002). A positively charged groove was proposed to be the site of interaction with ligands and receptors. Based on this structure, proteins containing several TSR repeats—many of which are also transmembrane proteins—would extend out from the parasite surface after secretion from the micronemes and be ideally positioned to engage host surface receptors for attachment and invasion. The similarity between micronemal TSR domains and host cell molecules may be a form of mimicry, allowing the parasite to bind to natural counterpart receptors on the target cell surface. Studies have provided evidence that members of the apicomplexan TSP family can bind to host ligands and are also involved in substrate-dependent gliding motility (Robson *et al.*, 1995; Spaccapelo *et al.*, 1997; Sultan *et al.*, 1997). Genetic disruption of TgMIC1 reduces invasion efficiency (Cerede *et al.*, 2005) and both TSR-like domains have been shown to be necessary for TgMIC1 cell adhesion properties (Saouros *et al.*, 2005), providing supporting evidence of the host-cell binding function of the TSRs. In addition to cell binding, TSRs have other functions. In TgMIC2, they are implicated in the tight association with its partner protein MIC2 associated protein (TgM2AP; J.M. Harper and V.B.C., unpublished), while in TgMIC1 they also recruit and interact with TgMIC4 in the TgMIC1-4-6 complex (Saouros *et al.*, 2005).

## A/I-domains

The integrins are a family of cell-surface molecules that mediate adhesion in a large number of cell-matrix and cell–cell interactions (Danen and Sonnenberg, 2003). Each integrin is a heterodimer composed of one  $\alpha$ -chain and one  $\beta$ -chain. I-domains, also referred to as A-domain for von Willebrand Factor type A, can be found in both chains, although I-domain-containing  $\alpha$  chains are only found in vertebrates and the primitive chordate *Halocynthia roretzi* (Miyazawa *et al.*, 2001; Whittaker and Hynes, 2002). Proteins with A/I-domains exist in Eukaryota, Eubacteria, and Archaea, signifying that this is an ancient motif (Tuckwell, 1999). These domains are often involved in protein-protein interactions and in the formation of multiprotein complexes. These A/I-domains consist of a  $\beta$ -sheet sandwiched by multiple  $\alpha$ -helices and usually contain a non-contiguous sequence of amino acids that make up a metal ion-dependent adhesion site (MIDAS) (Whittaker and Hynes, 2002). Although the MIDAS site is crucial for binding in certain cases, such as in type VI dimer formation (Ball *et al.*, 2003), it does not play a significant role in others, such as the binding of the snake venom molecule EMS16 with its collagen receptor, integrin  $\alpha_2\beta_1$  (Horii *et al.*, 2004). Amongst the Apicomplexa, proteins in the TRAP family also possess at least one A/I-domain (e.g. *Plasmodium* TRAP, *E. tenella* EtMIC1, and *T. gondii* TgMIC2). The A/I-domains from at least two of these proteins have been shown to have adhesive properties, PfTRAP and TgMIC2, both of which bound to heparin in a MIDAS-independent manner (McCormick *et al.*, 1999; Harper *et al.*, 2004a).

## Apple/PAN domains

Apple domains, also known as PAN domains, have been identified in the soluble plasma glycoproteins coagulation factor XI (FXI), plasma pre-kallikrein, and a number of nematode proteins. These domains are composed of six conserved cysteine residues which form disulfide bridges and a resultant structure resembling the outline of an apple. FXI circulates in a complex with H-kininogen, and the FXI Apple 3 (A3) domain has been shown to bind to platelets in the presence of H-kininogen and zinc or prothrombin and calcium (Ho *et al.*, 2000; Baglia *et al.*, 2004). The A3 domain also binds to heparin and factor IX (the substrate of activated FXI) (Ho *et al.*, 1998; Sun *et al.*, 1999). FXI exists as a homodimer, and the Apple 4 domain is required for dimer formation. H-kininogen binding sites are found in both pre-kallikrein and FXI, and they have been localized to a conserved Apple domain found in both proteins. This high binding specificity is likely conserved in other Apple domain-containing molecules. Two *Toxoplasma* MICs with Apple/PAN domains have been identified thus far, TgMIC4 and TgAMA1. TgMIC4 contains 6 tandem Apple domains, and as mentioned previously, is in a heterocomplex with TgMIC1 and TgMIC6. While the first two Apple/PAN domains of TgMIC4 interact with TgMIC1, TgMIC4 also has weak host cell binding capabilities, which is greatly increased in the presence of TgMIC1 (Saouros *et al.*, 2005). Based on the conservation between orthologs, domains in TgAMA1 were inferred to form Apple/PAN domains from the crystal structure of *P. vivax* AMA1, in which domains I and II were recognized as belonging to the PAN superfamily (Pizarro *et al.*, 2005). The function and binding affinities of TgAMA1 domains are less well defined, although recent data has shown that they participate in MJ formation in cooperation with rhoptry neck proteins (Alexander *et al.*, 2005), as discussed in greater detail below.

## CBL domains

Plants synthesize an array of defense proteins (chitinases), that reversibly bind to chitin, a  $\beta$ -1,4-linked biopolymer of *N*-acetylglucosamine (GlcNAc) that constitutes an important structural component of fungal cell walls (Raikhel *et al.*, 1993). Chitin-binding like (CBL) domains are 30–43 residue motifs first identified and best characterized in wheat germ agglutinin and contain eight disulfide-linked cysteines along with several conserved aromatic residues (Wright *et al.*, 1991). The cysteines ensure proper protein folding while the aromatic residues interact with GlcNAc. These domains are also known as hevein domains, the component in natural rubber latex that is recognized by latex-allergic patients (Alenius *et al.*, 1996). Chitin-binding proteins with lectin (or agglutinin) properties are capable of cross-linking GlcNAc-containing polymers due to the presence of these domains. In *T. gondii*, TgMIC3 and TgMIC8 both contain a CBL-lectin domain at their N-termini, followed by several EGF-like domains (Garcia-Réguet *et al.*, 2000; Meissner *et al.*, 2002). Binding of the lectin-like domain of TgMIC3 to host cell surfaces was dependent upon its dimerization through interaction of the last 65 amino acids (Cerede *et al.*, 2002). By disrupting the aromatic residues in the CBL domain that are hypothesized to be critical for adhesive function, this domain was shown to be important for parasite virulence (Cerede *et al.*, 2005). TgMIC8 exists as a monomer, but fusion of the TgMIC3 dimerization domain to the extracellular domains of TgMIC8 resulted in dimerization and binding of the chimera to cell surfaces, indicating that the CBL-domain of TgMIC8 may have binding activity when in a dimeric form (Cerede *et al.*, 2002).

## EGF-like domains

EGF stimulates the replication of ectodermal and mesodermal cells, especially epithelial and fibroblast cells (Carpenter and Cohen, 1979; Yamada *et al.*, 1997; Wong and Guillaud, 2004). EGF binds to and activates the EGF receptor (EGFR) family which initiates intracellular mitotic signaling cascades. Recent research on EGF and EGFR has focused on their roles in cancer (Shelton *et al.*, 2005) and neurogenesis (Vescovi *et al.*, 1993; Baldauf and Reymann, 2005). EGF-like motifs are involved in protein-protein interactions, such as the binding of the developmentally important receptor Notch with Delta on an opposing cell and the interaction of factor VII EGF modules with its tissue factor receptor (Banner *et al.*, 1996; Artavanis-Tsakonas *et al.*, 1999). Apicomplexan molecules containing EGF-like domains were first identified in glycosylphosphatidylinositol (GPI)-anchored proteins in *Plasmodium* ookinetes and merozoites (Kaslow *et al.*, 1988; Blackman *et al.*, 1991). In *T. gondii*, TgMIC3, 6, 7, 8, 9, and 12 contain multiple EGF-like motifs in their extracellular domains (reviewed in Soldati *et al.*, 2001). TgMIC3, 6, 8 and 12 are expressed in the tachyzoite stage, while TgMIC7 and 9 are mainly expressed in bradyzoites. TgMIC3 contains EGF-like and lectin-like domains and binds to all nucleated cells tested as well as the tachyzoite surface (Garcia-Réguet *et al.*, 2000). Receptor-binding is attributed to the lectin-like domain, whereas the EGF-like domains may promote the proper conformation of the protein in order to expose the binding motif. Additionally, the EGF-like domains may be involved in complex formation since TgMIC8, a transmembrane adhesin containing ten EGF-like domains, associates with TgMIC3 for delivery to the micronemes. Similarly, TgMIC6 is a transmembrane protein with three EGF-like motifs; the third EGF domain

interacts directly with one of its associated proteins, TgMIC1 (Saouros *et al.*, 2005). As will be discussed later in the chapter, secretion from the micronemes of this arsenal of proteins with diverse adhesive motifs likely contributes to the broad host cell range of this parasite and to its virulence in the mouse infection model.

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### Soluble and transmembrane MICs

Many of the characterized MICs exist in multimeric complexes, consisting of one transmembrane (TM) MIC and one or two other soluble MICs. Three complexes have so far been identified: TgMIC1-4-6, TgMIC2-M2AP, and TgMIC3-8. TgMIC2, TgMIC6, and TgMIC8 each contain a stretch of hydrophobic residues that anchor the complexes to the parasite membrane after secretion. These TM MICs are also referred to as escorts since they accompany and guide the other soluble members to the micronemes based on the targeting signals in their C-terminal tails (Reiss *et al.*, 2001; Meissner *et al.*, 2002). In TgMIC2, this signal is provided by two tyrosine-based sorting motifs capable of directing a heterologous protein to the micronemes (Di Cristina *et al.*, 2000). Genetic disruption of any of the TM MICs results in retention of the other members of the complex along the secretory system or in mistargeting to the default secretory pathway, which in *T. gondii* is secretion via the dense granules. When the level of TgMIC2 expression is experimentally reduced, TgM2AP colocalizes with the dense granules and is secreted into the PV (Huynh and Carruthers, 2006). Similarly, TgMIC6 knockout parasites show a complete misrouting of TgMIC1 and TgMIC4 to the dense granules (Reiss *et al.*, 2001). Nonetheless, escorts still depend on their cargo for proper trafficking since soluble proteins in the complexes are required for protein folding, as is the function of the galectin-like domain of TgMIC1 (Saouros *et al.*, 2005), or necessary for exiting an endosomal compartment associated with microneme biogenesis, as shown for the TgM2AP propeptide (Harper *et al.*, 2006).

Both soluble and transmembrane MICs contain adhesive domains that can bind to host surface molecules and receptors. Together, these proteins act as a bridge connecting host receptors with the parasite intrapellicular actin-myosin system. One such molecule is TgMIC2, whose C-terminal tail participates in gliding motility by binding to aldolase, which in turn interacts with actin filaments and what is termed the glideosome, consisting of myosin A (TgMyoA), its associated light chain (TgMLC1), gliding-associated protein 45 (TgGAP45), and the myosin receptor TgGAP50 (Jewett and Sibley, 2003; Gaskins *et al.*, 2004). The glideosome is anchored in the outer face of the inner membrane complex of mature parasites, while TgMIC2 is anchored within the plasma membrane. During invasion, the extracellular domain of TgMIC2 is bound to host cell receptors while the cytosolic tail is linked to the motility machinery of the actinomyosin system. As TgMIC2 caps toward the posterior end of the parasite, this connection pulls the host plasma membrane over the parasite, forming the PV. The link between TgMIC2 and the glideosome is severed by the proteolysis of TgMIC2 from the parasite surface near the end of invasion, which also encloses the parasite within the PV.

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### Importance of proteolysis

Characterization of known MICs has indicated that proteolytic processing, either during trafficking through the secretory pathway, post-exocytosis, or both, is critical for the

proper functioning of most MICs. All MICs are synthesized with a signal peptide that is co-translationally removed in the endoplasmic reticulum. Additionally, more than half of the MICs possess a propeptide that is proteolytically cleaved during transport to the micronemes and removal of this transient element affects protein complex stability, secretion, and adhesion. For example, our recent experiments have shown for TgM2AP that although propeptide cleavage is not necessary for the TgMIC2-M2AP complex to exit the processing compartment, its removal helps stabilize the complex and facilitates its subsequent release from the micronemes (Harper *et al.*, 2006). In the case of TgMIC3, the propeptide directly precedes the adhesive lectin-like domain. Expression of proTgMIC3 or mature TgMIC3 in animal cells revealed that the propeptide auto-regulates the adhesive activity of TgMIC3 (Cerede *et al.*, 2002). This is presumably a means of avoiding inappropriate interaction with endogenous carbohydrate receptors in the early secretory pathway prior to cleavage and packaging in the micronemes. Given the diversity of proMIC proteins, it seems likely that additional roles for propeptide cleavage will also emerge. Indeed, a number of other MICs contain propeptides, including TgMIC5, TgMIC6, TgAMA1, TgSUB1, and TgMIC11, which contains an internal propeptide that, after proteolysis, results in two peptides that are held together by disulfide bonds.

Analogous to the removal of the TgMIC3 propeptide, the N-terminal extension of TgMIC2 has been reported to regulate binding of its A/I domain with the intercellular adhesion molecule 1 (ICAM-1) (Barragan *et al.*, 2005), a cell surface glycoprotein in the immunoglobulin superfamily. The full-length TgMIC2 molecule did not bind to ICAM-1, while removal of the N-terminal extension of approximately 45 residues activated binding. Interestingly, the presence or absence of the N-terminal extension did not affect *in vitro* binding of the TgMIC2 A/I-domain to heparin (Harper *et al.*, 2004a), suggesting that this activation mechanism may be specific to ICAM-1. A hypothetical surface protease termed microneme protein protease 2 (MPP2) is responsible for processing the N-terminal extension of TgMIC2 (Carruthers *et al.*, 2000b), as well as cleaving C-terminal peptides from TgM2AP, TgMIC4, and TgSUB1 (Brecht *et al.*, 2001; Zhou *et al.*, 2004). Apart from reports that MPP2 activity is blocked by a subset of serine and cysteine protease inhibitors and activated by cytochalasin B (Carruthers *et al.*, 2000b; Zhou *et al.*, 2004) little is known about this protease. It remains to be determined whether MPP2 plays a general role in activating MIC protein function, or whether it regulates other processes.

During the final seconds of invasion, TgMIC2 is processed within the transmembrane domain by a microneme protein protease 1 (MPP1) (Carruthers *et al.*, 2000b), which liberates the molecule from the parasite surface in addition to disrupting receptor binding (Carruthers *et al.*, 1999; Barragan *et al.*, 2005). C-terminal MPP1 intramembrane cleavage also occurs in TgMIC6, TgMIC8, TgMIC12 (Reiss *et al.*, 2001), and TgAMA1 (Donahue *et al.*, 2000; Howell *et al.*, 2005). MPP1 was proposed to be a rhomboid protease due to the ability of *Drosophila* Rhomboid-1 to process TgMIC2, TgMIC6, and TgMIC12 (Urban and Freeman, 2003). A family of rhomboid proteases have now been identified in *T. gondii*, although there is some contention regarding which rhomboid family member most likely represents MPP1 (Brossier *et al.*, 2005; Dowse *et al.*, 2005). Regardless, post-exocytotic C-terminal processing of TgMIC2 is required for invasion as shown by Brossier and colleagues who demonstrated that a TgMIC2 C-terminal cleavage-resistant mutant



strain failed to efficiently invade cells (Brossier *et al.*, 2003). Interestingly, these parasites showed increased adhesion to host cells, presumably because of the elevated levels of surface TgMIC2, which accumulated at the posterior end of the parasite due to capping. This suggests that MPP1-dependent shedding is not only important during the final stages of invasion but also for avoiding the posterior accumulation of MIC complexes and inappropriate adhesion at this end. These examples underscore the importance of proteolytic processing, both within the parasite and on its surface.

## MIC redundancy

Given the large repertoire of MICs and the diversity of adhesive domains that each possesses, it might be expected that there is a level of redundancy in their function, such as receptor recognition and binding. On the other hand, *T. gondii* infects a large number of host cell types, possibly due to the variety of binding motifs and the ability to engage different types of receptors. The different invasion pathways utilized by the *Plasmodium* merozoite may serve as a useful paradigm, since many molecules involved in gliding motility and invasion in *Plasmodium*, such as the adhesins and actinomyosin machinery, have orthologs in other members of the Apicomplexa, including *Toxoplasma* (Soldati *et al.*, 2004). This functional conservation was illustrated by the ability of inhibitors of *T. gondii* invasion, identified in a high-throughput screen of small molecules, to also effectively block *Plasmodium knowlesi* invasion (Carey *et al.*, 2004).

*P. falciparum*, the parasite that causes the most virulent form of malaria in humans, utilizes multiple ligands and receptors in invading host cell erythrocytes, and is capable of switching invasion pathways (Dolan *et al.*, 1990; Barnwell and Galinski, 1998). Merozoite invasion pathways have primarily been characterized based on results from enzyme treatments: sialic acid-dependent/trypsin-sensitive (glycophorin A), sialic acid-dependent/trypsin-insensitive (glycophorin B), and sialic acid-independent/trypsin-sensitive (receptor X) (Dolan *et al.*, 1994; Fu *et al.*, 2005). Some parasite strains mainly use ligands that bind to sialic acid-containing erythrocyte receptors, while others bind to receptors independent of sialic acid. The first *P. falciparum* ligand identified that bound erythrocytes with high affinity was erythrocyte-binding antigen 175 (EBA-175) (Camus and Hadley, 1985). EBA-175 binding to glycophorin A is the dominant sialic acid-dependent invasion pathway in some strains (Sim *et al.*, 1994), while in parasites with deleted or truncated versions of EBA-175, ligands that are capable of binding to receptors independent of sialic acid are utilized to mediate invasion (Duraisingh *et al.*, 2003). Additionally, a member of the family of genes homologous to EBA-175, EBA-140 (BAEBL), binds to glycophorin C and functions in yet another, less significant invasion pathway through this receptor (Maier *et al.*, 2003). Multiple ligand–receptor interactions not only increase the probability of successful invasion by presenting alternative or compensatory pathways, it could be required to attain a sufficiently high affinity of receptor binding necessary for invasion. Interestingly, parasites are capable of switching between sialic acid-dependent and -independent invasion (Reed *et al.*, 2000). Stubbs and colleagues showed that parasites can switch from sialic acid-dependent to -independent invasion reversibly, adapting to host receptor availability and selective pressures for invasion (Stubbs *et al.*, 2005). Switching receptor usage is yet



another strategy to evade host receptor polymorphisms and immune mechanisms, and provides an advantage to the survival and propagation of the parasite.

Specific ligand–receptor binding and invasion pathways are poorly defined in *T. gondii* compared to *Plasmodium*. Studies thus far have identified several receptors and host cell components that are recognized by the parasite: heparan sulfate proteoglycans (Carruthers *et al.*, 2000a; Harper *et al.*, 2004a), sialic acid (Monteiro *et al.*, 1998), laminin (Furtado *et al.*, 1992), ICAM-1 (Barragan *et al.*, 2005), and lactose (Lourenco *et al.*, 2001). The host binding capabilities of many MICs have been demonstrated, but precise host receptors have not been characterized in most cases. As described above, MICs feature a broad range of adhesive domains that can potentially recognize an array of host cell components to facilitate attachment and invasion. Similar to *Plasmodium*, the adhesion repertoire may offer *Toxoplasma* an advantage in adapting to host receptor polymorphisms. Contrary to the specific invasion of erythrocytes by *Plasmodium* merozoites however, *Toxoplasma* can invade any nucleated cell, indicating a much greater plasticity in host cell recognition, possibly attributable to the broad range of adhesive MICs. This variety of molecules also suggests that one or more MICs may compensate for the loss of another. In support of this, genetic disruption of one MIC gene, which, because of interdependence, effectively disables an entire complex of two to three MICs in some cases, either has no effect on host cell invasion efficiency or does not completely inhibit invasion; certain MICs cause a more significant reduction than others (Reiss *et al.*, 2001; Huynh *et al.*, 2003; Cerede *et al.*, 2005; Mital *et al.*, 2005). *In vitro* invasion efficiency assays measure the ability of the parasite to enter host cells within a limited time period. However, it may not always reflect the virulence of the parasite. For instance, genetic disruption of *TgM2AP* resulted in an 80% reduction in attachment and invasion efficiency, while in mouse virulence assays, this led to a slight, but statistically significant one day delay in time-to-death (Huynh *et al.*, 2003; Harper *et al.*, 2006). In a conditional knockout of MIC2 that expresses ~5% of endogenous MIC2 levels, attachment and invasion efficiency is also decreased by ~75%, but these parasites are non-lethal in the mouse infection model, even at infection dosages that are greater than 100X the lethal dose of the parental strain (Huynh and Carruthers, 2006).

MICs can also function synergistically. By generating a double knockout of *TgMIC1* and *TgMIC3*, essentially disrupting both *TgMIC1*-4-6 and *TgMIC3*-8 complexes, Cerede and colleagues found that the *mic1ko* led to an approx. 50% reduction in *in vitro* host cell invasion, while the *mic3ko* had no effect (Cerede *et al.*, 2005). Both individual knockouts caused a minimal delay in time-to-death in some mice, but the double knockout led to more than 80% survival of infected mice. This indicates that these two molecules/complexes act synergistically in effecting virulence, but not in the invasion process, since the invasion efficiency of the double knockout was similar to the *mic1ko* alone. These combined results indicate some redundancy in the function of the MICs in host cell invasion, but the ability or inability to rapidly and effectively enter host cells can have severe consequences in virulence. This delay may leave the parasite extracellular for longer time periods and exposed to host immune facilitators capable of clearing the parasite. Based on the few examples available, the degree of parasite impairment in invasion and virulence is

dependent on the particular MIC molecule, and this may reflect a host receptor and tissue tropism encountered during *in vivo* dissemination.

In addition to supporting multiple invasion pathways for infection of different cell types, a second function of the diverse MIC protein repertoire is to create a high affinity binding interface during cell invasion. Parasites visualized by video microscopy glide along surfaces at 5–10  $\mu\text{m/s}$  but slow down approximately 5-fold during invasion, suggesting that they encounter considerable resistance while actively penetrating into a target cell. This resistance is probably due in part to the host cell's cortical cytoskeleton, which likely contributes to the prominent constriction that deforms the parasite during entry. The multiplicity of adhesins may be needed to reach a sufficiently high receptor binding affinity to offset this resistance, allowing the parasite to achieve an adequate grip and avoid slippage as it pulls itself into the host cell. The loss of synergistic MICs or a key microneme protein may result in insufficient receptor binding and a reduction in invasion efficiency.

Additional evidence supporting a role for MICs in forming a strong binding interface recently came from conditional expression of TgAMA1. By reducing the expression levels of TgAMA1 to < 1% of endogenous levels, the MJ (defined as an interaction of < 6 nm between the host cell and parasite) did not form, and invasion was substantially impaired (Mital *et al.*, 2005). Interestingly, parasites were capable of adhering to the host cells but they failed to form a highly intimate association. Additional mechanistic insight came from co-immunoprecipitation experiments showing that TgAMA1 associates with several  $\rho$ hoptry neck (RON) proteins on the parasite surface (Alexander *et al.*, 2005). Visualization of the complex during invasion suggests that TgAMA1 and TgRON2-4-5 collaborate to form a ring-like structure near or within the MJ that the parasite slides through during entry. If the cytoplasmic tail of TgAMA1 associates with the glideosome then this complex may contribute to parasite penetration. The intimate association of the complex with the MJ also implies a role in excluding host surface markers as a means of precluding PV fusion with host organelles. The identification of the TgAMA1-RON ring provides important new insight into the elaborate mechanism of *Toxoplasma* invasion and opens novel avenues of future investigation to determine the temporal and spatial relationships amongst MICs during active parasite entry.

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## Summary and conclusions

The emergence of *Toxoplasma* DNA databases are greatly facilitating the pace of MICs discovery using homology-based queries or searches with mass spectroscopy data. The current 15 member microneme repertoire includes proteins with a wide array of domains involved in protein-protein or protein-carbohydrate interactions, many with structural similarity to vertebrate adhesive proteins. Receptors for *Toxoplasma* invasion are still poorly characterized and incomplete but the available data suggests that tachyzoites use multiple receptors and invasion pathways, in a manner akin to *Plasmodium* merozoites. Despite their abundant variety, *Toxoplasma* MICs are only partially redundant. Some (e.g. TgMIC1-4-6 and TgMIC3-8 complexes) act synergistically implying that they contribute to overlapping invasion pathways, while others (TgAMA1-RON2-4-5 and TgMIC2-M2AP complexes) are effectively indispensable for entry. Assembly of MICs into multimeric complexes ensures correct trafficking to the micronemes and delivery to the parasite surface during inva-

sion. Proteolysis appears to regulate stability and secretion of MIC complexes, along with modulating their adhesive activity on the parasite surface. Transmembrane MICs have the opportunity to simultaneously connect with host receptors and the parasite glideosome to power entry into a target cell. Cooperation between TgAMA1 and TgRON proteins at the MJ contributes to the intimate binding interface at the MJ and may also work to exclude host markers from entering the PV, thereby promoting intracellular survival of the parasite. With a rapidly lengthening list of invasion related proteins, future challenges will include developing more effective and efficient systematic approaches to understanding their individual and cooperative contributions to parasite entry.

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# Proteomic Analysis of the Rhoptry Organelles of *Toxoplasma gondii*

24

Jonathan M. Wastling and Peter J. Bradley

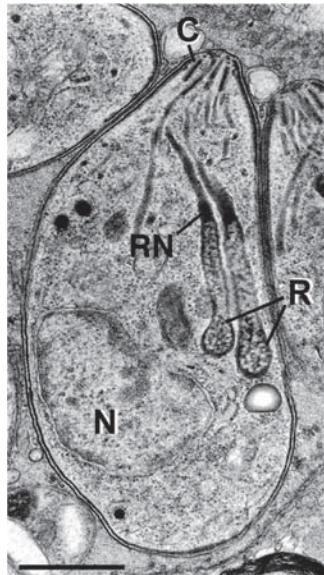
## Abstract

Rhoptry organelles are part of the defining features of the phylum Apicomplexa. The contents and function of the rhoptries have been the focus of considerable attention in an effort to understand this most important of all apicomplexan characteristics: the ability to invade a host cell. *Toxoplasma gondii* is able to parasitize a wide variety of cells, during which these specialized secretory organelles play a central role. The recent development of proteomic methods for *T. gondii* and the availability of genome sequence to underpin a proteomic database represent an unprecedented opportunity to characterize protein composition and function in this parasite. For the rhoptry organelles specifically, this has enabled the identification of a large number of novel proteins and the subsequent discovery of a completely new class of rhoptry proteins which localize to the neck of the organelle. This rapid expansion in our understanding of the composition of rhoptries has led to new insights into their structure and function and set the foundation for a more comprehensive understanding of how they contribute to the process of invasion and intracellular survival in this parasite.

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## Introduction

Of all of the extraordinary sub-cellular features common to apicomplexan parasites, perhaps the most unusual organelles are the rhoptries. The rhoptries are named after the Greek word meaning “club” and are indeed club-shaped organelles that are approximately 2  $\mu\text{m}$  in length, roughly a third of the length of the parasite (Figure 24.1). The number of rhoptries varies depending on the apicomplexan and life cycle stage, with *Toxoplasma* containing 8–12 rhoptries whereas *Plasmodium* contains just two of the organelles. The organelles are bounded by a single membrane and are composed of a bulbous body structure which tapers into a thin duct-like rhoptry neck that extends apically to the conoid and through which the contents of the rhoptries are secreted during host cell invasion. The rhoptry bodies and rhoptry necks are distinct sub-compartments within the organelles, with rhoptry proteins typically localized to one or the other sub-organelle location. In electron micrographs, the *Toxoplasma* rhoptry bodies have a distinctive mottled appearance while the neck portion is electron dense (Figure 24.1). The mottled appearance of the body portion of the organelle is dependent on the presence of the rhoptry protein ROP1, as ROP1 knockout parasites have a uniformly electron dense appearance. The means by which the sub-compartments



**Figure 24.1** Transmission electron micrograph of an intracellular *Toxoplasma gondii* tachyzoite. The rhoptries are the long club-shaped organelles that extend from the conoid (C) along the length of the parasite to near the parasite nucleus (N). The rhoptries are composed of a bulbous rhoptry body (R) that has a mottled appearance which tapers into thin electron-dense rhoptry necks (RN) which serve as ducts through which the contents of the organelle are secreted. Scale bar is 1  $\mu$ m. Image courtesy DJP Ferguson, Oxford University.

are divided is not known as no apparent membrane exists between the rhoptry bodies and necks. Even the pH of the rhoptry bodies and necks differs, with the bulbous bodies having a low pH of  $\sim 5.5$  whereas the necks are a more neutral pH (Shaw *et al.*, 1998). Secretion from the rhoptries occurs at the onset of invasion and parasitophorous vacuole formation, but the precise trigger which causes release of the contents of the organelle is not known. The contents and function of the rhoptry organelles have long been a matter of debate. With the evolution of molecular methods and more recently, post-genomic techniques, significant advances have been made in understanding their composition and molecular function. In this chapter we describe how proteomic analysis of the rhoptry constituents from *T. gondii* has led to the discovery of novel rhoptry proteins and discuss how these proteins might contribute to the success of this parasite.

### Rhoptry composition

Rhoptries contain both proteins and lipids. Until recent proteomic analysis of these organelles (Bradley *et al.*, 2005) there were 8 known rhoptry proteins of *Toxoplasma*. In the absence of homology to known proteins, the rhoptry proteins identified were designated ROP proteins (ROP1, ROP2, ROP4, ROP8, and ROP9) (Sadak *et al.*, 1988; Ossorio *et al.*, 1992; Beckers *et al.*, 1994; Beckers *et al.*, 1997; Reichmann *et al.*, 2002). In addition, two rhoptry proteases (a subtilisin-like protein TgSUB2 (Que *et al.*, 2002) and a cathepsin

B-like protein (Toxopain-1) (Miller *et al.*, 2003), had been identified as well as a sodium-hydrogen exchanger named TgNHE2 (Karsasov *et al.*, 2005). All eight proteins localize to the bulbous body portion of the organelle rather than the duct-like rhoptry necks. Based on cDNA and genomic sequencing, the predicted amino acid sequence for all eight known rhoptry components includes a typical signal peptide characteristic of eukaryotic secreted proteins. ROP1 and ROP9 are soluble rhoptry proteins whose function is as yet unknown (Ossorio *et al.*, 1992; Soldati *et al.*, 1995; Reichmann *et al.*, 2002). ROP2, ROP4, and ROP8 are members of a family of membrane-associated proteins at least one of which (ROP2) has been implicated in recruitment of host cell mitochondria to the exterior face of the parasitophorous vacuole membrane (PVM) (Sadak *et al.*, 1988; Beckers *et al.*, 1994; Beckers *et al.*, 1997; Sinai *et al.*, 2001). Host-mitochondrial association is accomplished by the ROP2 protein having its processed N terminus exposed to the host cell cytosol where it is recognized as a mitochondrial import signal (Sinai *et al.*, 2001). Host mitochondria recognize this sequence and attempt to import the ROP2 protein which is firmly anchored in the PVM, thus tethering the mitochondria to the PVM. The precise function of the host mitochondria at the PVM has not been directly demonstrated, but the mitochondria are likely to serve as a source of lipids that must be salvaged by the parasite for intracellular growth and survival (for more detail see Chapter 19). ROP2 appears to be essential to parasite intracellular survival since antisense RNA knockdowns of ROP2 disrupt the rhoptries and parasite growth, demonstrating that ROP2 function(s) cannot be performed by other members of the rhoptry family (Nakaar *et al.*, 2003). The TgSUB2 and Toxopain-1 proteases have been implicated in processing of rhoptry proteins (see section VI and Chapter 30 for more details), whereas TgNHE2 is suggested to function in organellar osmotolerance (Que *et al.*, 2002; Miller *et al.*, 2003; Karasov *et al.*, 2005).

In addition to protein constituents, the rhoptries contain lipids which are packaged into what appear to be lamellar sheets within the lumen of the organelle (Bannister *et al.*, 1986; Foussard *et al.*, 1991). The lipids appear to be localized to the bulbous body portion of the organelle. The rhoptry lipids are unusually high in their cholesterol content with a cholesterol:phospholipids ratio of 1.3–1.5:1 (Foussard *et al.*, 1991; Kaneko *et al.*, 2001). This ratio exceeds what is expected for a lipid bilayer and suggests that the cholesterol is packaged into some sort of crystalline array within the rhoptries. Lipids are released along with other contents of the rhoptries during invasion, although their contribution to the developing parasitophorous vacuole (PV) appears to be minimal as the bulk of the lipids forming the nascent vacuole are acquired from the host plasma membrane (Suss-Toby *et al.*, 1996). Furthermore, depletion of rhoptry cholesterol does not affect invasion, while depletion of cholesterol from the host plasma membrane impairs invasion (Coppens *et al.*, 2003). Thus the precise role of rhoptry lipids both in the parasite and during invasion is unclear.

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### Analysis of rhoptry proteins in the post-genomic era

It was always anticipated that the handful of proteins described above did not represent the full complement of rhoptry protein constituents. Indeed, the discovery of other potential rhoptry gene fragments suggested that the repertoire of rhoptry proteins might be substantial (Hajj *et al.*, 2006). The completion of the genome sequence heralded a new era in gene

discovery for *Toxoplasma*. It was initially supposed that bioinformatic analysis of the newly available apicomplexan genome sequences, including that for *T. gondii*, might reveal many additional members of the “rhoptry family.” Unfortunately, this approach proved far less successful than was originally anticipated because of what became apparent as a notorious lack of homologous proteins and/or conservation among apicomplexan rhoptry proteins. Moreover, discovery in the genome of fragmented areas of homology with known rhoptry constituents was by no means proof that these sequences coded for gene products that ended-up in the organelle. Furthermore, the paucity of rhoptry sequences available meant that it was impossible to determine a rhoptry targeting signal that could be used to identify additional proteins associated with the compartment. Meanwhile, whilst bioinformatic approaches were proving less successful than was hoped in the search for additional members of the rhoptry family, protein sequencing methods were being dramatically revolutionized by the development of proteomics.

Proteomics is now a well established technique in the repertoire of tools with which to study parasites and their hosts. Yet little over a decade ago the possibility of rapidly sequencing nanogram quantities of hundreds of individual parasite proteins would have appeared totally unfeasible. Even if technically possible, the cost would have far exceeded most ordinary research budgets. Today, like its forerunners of DNA sequencing and microarray analysis, large-scale and affordable analysis of parasite proteomes has reached the status of an established, albeit still evolving tool of functional genomics. Based on key developments in both bioinformatics and mass spectrometry, proteomics essentially entails the matching of two data sets, one experimental and one hypothetical, to enable the rapid identification of proteins. The experimental data set consists of mass spectrometry data, which in its simplest form contains the precise masses of peptides obtained from proteins digested with an enzyme such as trypsin. Measured using a matrix assisted laser-desorption/ionization mass spectrometer (MALDI), the individual masses of each peptide give rise to a unique peptide mass fingerprint. Alternatively, peptides can be further fragmented in an electrospray ionization mass spectrometer to produce tandem mass spectrometry data, or MS/MS data. Again, a unique fingerprint is obtained, this time for each individual peptide. In both cases, the experimental MS data can then be matched using an appropriate algorithm to a corresponding hypothetical peptide mass database. Hypothetical mass databases are dependent on the existence of a substantial quantity of genome sequence, or EST data and are derived from translations of all possible open reading frames into protein sequences. Calculated masses of the peptides from *in silico* enzymatic digests of the resulting protein sequences are compiled to form the hypothetical peptide mass database. Precise matches between the hypothetical and experimental peptide databases can then be used to identify the open reading frame corresponding to each protein.

Many early parasite proteome projects centered on attempts to resolve whole proteomes using a single separation technique such as multi-dimensional liquid chromatography followed by tandem mass spectrometry (MS) (commonly referred to as Multidimensional Protein Identification Technology or “MudPIT”) (Florens *et al.*, 2002; Lasonder *et al.*, 2002), or two-dimensional electrophoretic separation of proteins followed by MS analysis of individually resolved spots (Cohen *et al.*, 2002). These first attempts to resolve entire proteomes were only partially successful since in each case they relied on a single approach



to protein separation. However, it is now generally recognized that no single separation technique is sufficient to resolve the complete proteome of even relatively simple organisms, including protozoan parasites. Two principles have emerged to guide experimental design in parasite proteomics. First, the nature of the proteins to be analyzed will dictate the most appropriate protein separation method(s) to be used. For instance, the soluble portion of a proteome is readily amenable to resolution by two-dimensional electrophoresis (2-DE), whereas hydrophobic proteins are poorly resolved by this method and are better handled by non-gel based methods. Second, the proteome of any cell consists of a heterogeneous mixture of soluble and hydrophobic proteins, some of which may be present in large stable quantities, whilst others are in transitory and almost undetectable amounts. An obvious way to overcome the challenge of complexity is to simplify the mixture of proteins before analysis. This principle has led to the development of "sub-proteomics." Sub-proteomics relies on the preparation or enrichment of a smaller fraction of the total proteome, a strategy employed for example by Zhou *et al.* (2005) to analyze the protein repertoire of the excretory-secretory products of *T. gondii* (Zhou *et al.*, 2005). This approach is highly useful when the study is restricted to the proteins of a particular organelle in which a method for purifying the organelle has been established (Yates *et al.*, 2005). Thus, with sub-proteomic and organellar proteomic experiments, reproducible sample preparation, immunolocalization to verify the origin of newly discovered proteins and careful bioinformatic interpretation of proteomic data are important adjuncts to successful studies.

### Characterizing the proteome of *Toxoplasma* rhoptries

The development of proteomic methods for *T. gondii* (Cohen *et al.*, 2002) and the availability of genome sequence to underpin a proteomic database presented a unique opportunity to characterize protein composition and function in this parasite. In a recent study, we undertook a comprehensive analysis of the proteome of the rhoptries of *T. gondii* tachyzoites in which we identified a large number of novel rhoptry proteins and characterized a completely new class of rhoptry protein which localizes to the neck of the organelle (Bradley *et al.*, 2005).

Successful execution of the rhoptry proteome project required the development of a protocol for obtaining highly purified preparations of rhoptry organelles in quantities sufficient for MS analysis. This was achieved by further development of a Percoll gradient method of Dubremetz *et al.* (Leriche *et al.*, 1991; Hehl *et al.*, 2000) used to isolate a fraction enriched for rhoptries. Analysis of the fraction obtained by the original protocol using 2-DE revealed that it contained substantial proportions of contaminants, mainly dense granules, mitochondria and plastids. To improve on the initial enrichment, this fraction was further subjected to sucrose gradient floatation as described by Bradley *et al.* (Bradley *et al.*, 2005). Subsequent analysis of 1-D SDS PAGE gels of this fraction by immunoblotting using antibodies to known proteins from the rhoptries, dense granules and mitochondria, showed that this new sucrose floatation step was able to enrich for the rhoptries whilst effectively removing the vast majority of dense granules and contaminating mitochondria (Bradley *et al.*, 2005). Thus, we had the ideal components for an organellar proteomic study including (1) a highly purified preparation of an important organelle in the parasite,

(2) sufficient genomic and EST sequence for mass spectrometric analysis (3) a situation whereby only a few proteins had previously been identified from this compartment.

### The hydrophobic nature of the rhoptry contents

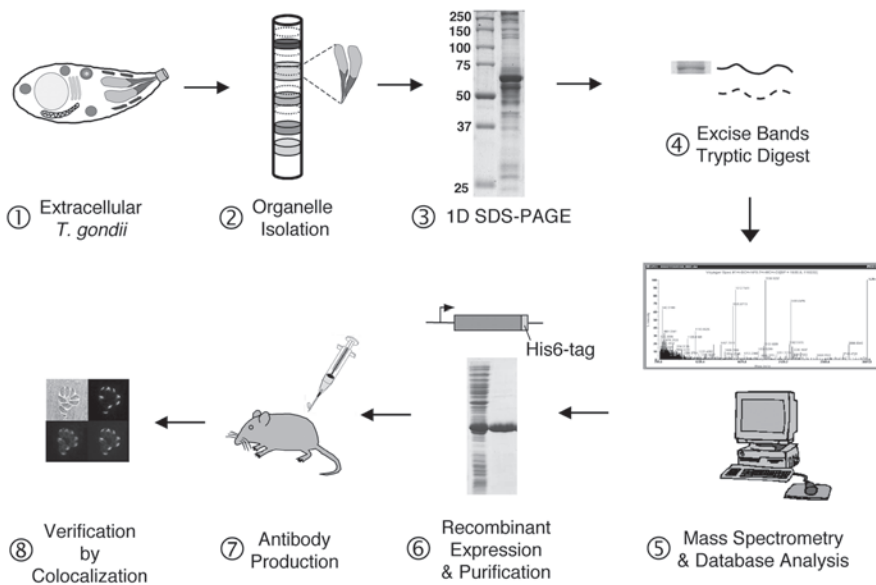
Analysis of enriched rhoptry fractions by 2-DE demonstrated the hydrophobic nature of rhoptry contents (Bradley *et al.*, 2005). When a highly enriched rhoptry fraction was subjected to 2-DE, the representation of proteins on the gel was poor, presumably because the hydrophobic nature of many rhoptry proteins made them less amenable to solubilization in the isoelectric focusing step of 2-DE. Thus, although soluble proteins such as ROP9 can easily be detected in a 2-DE separation, membrane associated rhoptry proteins such as the ROP2–8 family were not represented well on the gel. The hydrophobic nature of additional rhoptry proteins was further confirmed by sodium carbonate extraction (Fujiki *et al.*, 1982; Bradley *et al.*, 2005). This procedure releases proteins that are soluble or associated with membranes through ionic interactions, leaving behind proteins that are anchored in the membranes by transmembrane domains or other strong interactions such as glycosylphosphatidylinositol anchors. Sodium carbonate extraction of partially enriched rhoptry fractions showed the predicted partitioning of known membrane-associated proteins (ROP2/3/4), but importantly also indicated that the majority of novel rhoptry proteins are likely to be found within the membrane fraction and thus not amenable to analysis by 2-DE (Bradley *et al.*, 2005).

### Protein separation and identification of rhoptry proteins by mass spectrometry

A variety of options are available for the separation and proteomic analysis of hydrophobic proteins involving combinations of SDS-PAGE and liquid chromatography, or various forms of non-gel based proteomics using liquid chromatography alone. For the analysis of the rhoptry proteome we chose to use conventional one-dimensional SDS-PAGE followed by the excision of 51 contiguous gel slices, each of which was subjected to in-gel trypsin digestion and then tandem MS (MS/MS) to obtain peptide fragmentation data suitable for proteomic database searching. The resulting MS/MS data were used to search the most current version of ToxoDB ([www.toxodb.org](http://www.toxodb.org), version 3.0), a database of *Toxoplasma* genomic, cDNA sequences and protein prediction models compiled from sequences representing a total of ~10 times the size of the *Toxoplasma* genome and thus likely to contain virtually the entire genome. MS data was searched against a single file containing ESTs from ~69 000 cDNA clones, 4954 Glimmer HMM protein predictions, 8336 TigrScan protein predictions and 7588 TwinScan protein predictions for *Toxoplasma*. For each identified protein, the exact position of each peptide was checked manually against alignments of the genome with ESTs and protein prediction models using a generic gene model organism data base construction set (GBrowse [www.gmod.org/](http://www.gmod.org/)) available for *T. gondii* at [www.toxodb.org](http://www.toxodb.org).

As expected, several of the known rhoptry proteins were readily identified in this proteomic analysis, including members of the ROP2/4/8 family and ROP9. In addition, 38 previously unidentified candidate novel rhoptry proteins were detected in the fraction. A combination of approaches was used to determine the true localization of the novel

proteins identified including epitope tagging and the production of antibodies against peptides and recombinant proteins. Of 13 randomly chosen proteins that were tested using these methods, 12 were indeed found to be localized to the rhoptries. These results indicate that a large percentage of the remaining proteins will also be rhoptry-localized and validate the purification and analysis scheme (a schematic of the complete approach is shown in Figure 24.2). One exciting subgroup of rhoptry proteins found by antibody validation were those that localized to the duct-like neck portion of the organelle and established the first genes identified that encode rhoptry neck proteins in *Toxoplasma*. A summary of all confirmed rhoptry proteins from proteomic analyses and previous studies is shown in Table 24.1. For each of the confirmed proteins, the table shows the calculated molecular weight (MW), isoelectric point (pI), the presence of a predicted signal peptide (SP), pro-domain processing, and function when known. As expected, most of the rhoptry proteins contain a signal peptide for entrance into the secretory pathway. Many of the rhoptry proteins also have an unusually high pI, a feature noted when several of the first rhoptry proteins were identified (Leriche *et al.*, 1991).



**Figure 24.2** Schematic for proteomic analyses of the *Toxoplasma* rhoptries. (1) Extracellular parasites that have freshly lysed out of host cells are used as starting material to minimize host cell contamination. (2) The parasites are lysed in isotonic sucrose to maintain intact organelles and the organelles fractionated by percoll and sucrose gradients (only a single gradient is shown here for simplicity). (3), (4) The purified organelles are then separated by 1D SDS-PAGE and individual bands are excised from the gel and digested with trypsin. (5) The tryptic fragments are collected and subjected to LCMS/MS and database analysis to identify the proteins in each gel slice. (6) Novel proteins identified by mass spectrometry are then expressed as His6-tag fusions and purified by nickel-agarose chromatography. (7), (8) The purified proteins are injected into mice for polyclonal antibody production and co-localization verified by immunofluorescence assays.

**Table 24.1** Confirmed rhoptry proteins of *Toxoplasma gondii*

Name	MW (kDa)	pI	SP	Pro- peptide	Function	References
ROP1	43	5.8	1–23	Y	Penetration enhancing factor? (NE)	(Ossorio <i>et al.</i> , 1992; Soldati <i>et al.</i> , 1995; Soldati <i>et al.</i> , 1998; Bradley <i>et al.</i> , 1999; Bradley <i>et al.</i> , 2001; Striepen <i>et al.</i> , 2001; Bradley <i>et al.</i> , 2002)
ROP2*	64	7.8	1–26	Y	PVM-Host mitochondria assoc. (LE)	(Beckers <i>et al.</i> , 1994; Sinai <i>et al.</i> , 2001; Nakaar <i>et al.</i> , 2003)
ROP4*	64	8.5	1–33	Y	Unknown (NE)	(Carey <i>et al.</i> , 2004)
ROP5*	61	9.8	1–24	Y	Unknown	(Bradley <i>et al.</i> , 2005)
ROP7*	63	6.7	1–33	Y	Unknown	(Hajji <i>et al.</i> 2006)
ROP8*	65	9.2	1–35	Y	Unknown	(Beckers <i>et al.</i> , 1997)
ROP9 <sup>1</sup>	35	7.1	1–19	N?	Unknown	(Reichmann <i>et al.</i> , 2002)
ROP10	61	4.2	1–27	?	Unknown	(Bradley <i>et al.</i> , 2005)
ROP11*	58	7.7	1–26	?	Unknown	(Bradley <i>et al.</i> , 2005)
ROP12	25	4.5	1–20	?	Unknown	(Bradley <i>et al.</i> , 2005)
ROP13	45	9.4	1–26	?	Unknown	(Bradley <i>et al.</i> , 2005)

ROP14	122	9.0	No	?	Unknown	(Bradley <i>et al.</i> , 2005)
ROP15	34	8.6	1-21	?	Unknown	(Bradley <i>et al.</i> , 2005)
ROP16	76	9.0	1-23	?	Putative kinase	(Bradley <i>et al.</i> , 2005)
RON1	127	4.9	1-49	?	Unknown	(Bradley <i>et al.</i> , 2005)
RON2	156	9.7	1-24	?	Moving junction complex	(Alexander <i>et al.</i> , 2005; Bradley <i>et al.</i> , 2005; Lebrun <i>et al.</i> , 2005)
RON3	223	9.3	1-26	?	Unknown	(Bradley <i>et al.</i> , 2005)
RON4	107	6.3	1-27	?	Moving junction complex (LE)	(Alexander <i>et al.</i> , 2005; Bradley <i>et al.</i> , 2005; Lebrun <i>et al.</i> , 2005)
TgSUB2	141	5.7	1-28	Y	Rhoptry protein maturase (LE)	(Miller <i>et al.</i> , 2003)
Toxopain	62	5.3	1-34	Y	Rhoptry protein maturase	(Que <i>et al.</i> , 2002)
Toxofilin	27	9.6	1-23	N	Actin/PP2C binding?	(Poupel <i>et al.</i> , 2000; Delorme <i>et al.</i> , 2003; Bradley <i>et al.</i> , 2005)
TgNHE2 <sup>1</sup>	87	6.2	1-17	N?	Ion homeostasis? (NE)	(Karasov <i>et al.</i> , 2005)
TgBRP1	18	9.8	1-36	?	Bradyzoite/merozoite specific (NE)	(Schwarz <i>et al.</i> , 2005)
Rab11	25	10.6	No	N	Membrane trafficking GTPase	(Bradley <i>et al.</i> , 2005)

Table 24.1 shows the calculated molecular weight (MW), isoelectric point (pI), presence of a predicted signal peptide, processing of a pro-domain, and function of confirmed rhoptry proteins when known. The MW and pI are calculated from the primary translation product including signal peptide when present. NE, not essential, \*ROP2 family proteins, <sup>1</sup>MW and pI for ROP9 and TgNHE2 are calculated from the third potential start codon which indicates the best predicted signal peptide for these proteins.

Unexpectedly, proteomic analysis also identified the known *Toxoplasma* proteins Rab11 and toxofilin as present in the rhoptry fraction (Bradley *et al.*, 2005). These findings were confirmed by immunolocalization using antibodies raised against purified recombinant proteins and in the case of toxofilin, parasites engineered to express a C-terminal HA-tagged version of the protein.

Finding toxofilin in the rhoptries was surprising because this protein had previously been localized largely to the parasite apical cytoplasm and shown to interact with parasite actin and a protein phosphatase 2C (Poupel *et al.*, 2000; Delorme *et al.*, 2003). The demonstration that toxofilin was in fact localized to the rhoptries resolved the presence of a predicted signal peptide at the N-terminus of the protein and accounted for its apical location (Poupel *et al.*, 2000). If this is the case however, it is not clear how this protein may interact with the two *Toxoplasma* proteins to which it has previously been shown to bind, actin and a protein phosphatase 2C. Toxofilin lacks a predicted transmembrane domain so it is probably contained in the lumen of the rhoptries where it is unlikely to be able to interact with the *Toxoplasma* actin and protein phosphatase 2C which both lack signal peptides and are thus predicted to be cytoplasmic. One intriguing possibility is that toxofilin could be secreted into the cytoplasm of the host following rhoptry release during invasion where it interacts with host actin and/or a protein phosphatase 2C. While host localization could not be detected during host cell invasion or later during infection, the antibodies used may not have been able to detect small quantities of this protein which could rapidly diffuse in the vast host cytoplasm.

Rab11 is a member of a family of small GTPases involved in the regulation of vesicular trafficking in eukaryotic cells (Stenmark *et al.*, 2001). In mammalian cells, Rab11 is involved in trafficking of recycling endosomes, regulating exocytosis and cholesterol homeostasis (Mohrmann *et al.*, 1999; Stenmark *et al.*, 2001; Holttä-Vuori *et al.*, 2002). These features are common to the cholesterol-rich rhoptries of *Toxoplasma*, which are believed to be formed via the convergence of the secretory and endocytic pathways and thought to be related to secretory exosomes or lysosomes (Holttä-Vuori *et al.*, 2002; Coppens *et al.*, 2003; Ngo *et al.*, 2004). The identification of Rab11 in the rhoptry proteome and verification by antibody localization indicates that *Toxoplasma* Rab11 is a regulator of trafficking of protein and/or lipids to the rhoptries. As expected, the Rab11 protein does not contain a predicted signal peptide but instead contains a C-terminal CCXX motif that indicates that this protein is membrane-associated by geranyl-geranyl modifications of the two cysteines (Gromov *et al.*, 1998). Rab11 would then be predicted to be localized to the cytoplasmic face of the rhoptry membrane, similar to the orientation seen for Rab proteins in other systems. The rhoptry localization of Rab11 may also provide insight into the origin of these unique secretory organelles.

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## Bioinformatic analysis of the rhoptry proteome

As might be expected for such specialized organelles, many of the proteins identified in the *T. gondii* rhoptry proteome appear to be unique to either *Toxoplasma* or to the Apicomplexa (Bradley *et al.*, 2005). For example, nine of the newly identified proteins in the rhoptry proteome appear to have homologs in *Plasmodium* but are not present in non-apicomplexan organisms. *T. gondii* rhoptry proteins thus appear to fall broadly into one



of three categories (a) rhoptry proteins specific to the Sarcocystidae (b) rhoptry proteins conserved throughout the Apicomplexa (c) proteins common to eukaryotic cells and not specific to the Apicomplexa such as kinases, phosphatases and proteases. Twelve of the proteins identified contain putative protein kinase domains. At least nine of these have some homology to the ROP2 family of rhoptry proteins that have been reported previously to have a kinase domain (Carey *et al.*, 2004). Proteins with homology to protein phosphatase 2C and serine and insulinase-type proteases were also identified.

### Rhoptry proteins specific to the Sarcocystidae

This group of rhoptry proteins appear to have no homolog currently identified in any other organism outside of genera that are extremely closely related to *Toxoplasma*. This group includes *Neospora caninum* and *Sarcocystis* spp, but does not include *Eimeria* and more distantly related apicomplexans such as *Plasmodium*. These proteins may have drifted to an evolutionary point where their homologs are no longer recognizable in other families, or they could be truly restricted to the Sarcocystidae. The evolutionary pressure for the emergence of these proteins may have derived from some specialized property of these organisms, such as their ability to form tissue cysts, their broad host range in mammals, or ability to invade a particular cell type. Thus, the majority of the verified rhoptry proteins in *Toxoplasma* (ROP1, ROP2 family proteins, ROP9, toxofilin, ROP12, ROP13, ROP15, and ROP16) do not have apparent homologs in *Plasmodium* spp. and are thus likely to be specific to *Toxoplasma* and its close family members. Similarly, among the 38 novel *Toxoplasma* rhoptry constituents, no clear homologs could be detected for many known rhoptry proteins of *Plasmodium* including RhopH2–3, RAP1–3, PF148, and RAMA1 (Anthony *et al.*, 2000; Baldi *et al.*, 2002; Lobo *et al.*, 2003; Topolska *et al.*, 2004). Homologs for these genes are also not apparent in the near-complete *Toxoplasma* genome, and so they are likely to be truly restricted to *Plasmodium* and its close relatives. The fact that the *Toxoplasma* tachyzoite and *Plasmodium* merozoite rhoptry proteins are so unique with respect to each other suggests that these organelles are exquisitely adapted to their respective target cells.

### Rhoptry proteins conserved throughout the Apicomplexa

The rhoptry proteome contains a second class of proteins that appear to be conserved throughout the Apicomplexa, including *Plasmodium* spp., but not found in organisms outside this phylum. As previously noted, there are nine proteins, five of which have been verified as being derived from the rhoptries by antibody co-localization studies (ROP14, RON1, RON2, RON3, and RON4). The suggestion is therefore that these proteins are likely to be involved in rhoptry functions common to all Apicomplexa, such as host cell invasion and/or creation of the PVM. One would also expect that proteins in this class would include structural and/or regulatory components of the rhoptries that are common to all apicomplexans. These structural or regulatory proteins should be relatively easy to distinguish as they would probably remain resident in the rhoptries and not be released during invasion. It is intriguing that all four verified *Toxoplasma* rhoptry neck (RON) proteins have homologs in *Plasmodium*, indicating that rhoptry neck proteins are apparently more highly conserved than other rhoptry proteins which perhaps perform more specialized tasks. Indeed, a subset of the rhoptry neck proteins has recently been shown

to play a key role in invasion that is likely common to all apicomplexans (see Section VII). Moreover, the neck of the rhoptry organelle has been suggested to serve as a duct for the release of microneme proteins, and thus RON proteins may facilitate fusion of the two compartments for secretion (Bannister *et al.*, 2003). Determining precise sub-cellular and sub-organellar localization of the remaining *Plasmodium* homologs identified in the *Toxoplasma* rhoptry proteome will be an important step in determining common functions of this organelle across the Apicomplexa.

Kinases, phosphatases and proteases common to eukaryotic cells

The third class of proteins observed in the rhoptry proteome consists of proteins with functions that are common to most eukaryotic cells such as kinases, phosphatases, and proteases. Both ROP4 and toxofilin have been shown to be phosphoproteins and represent potential substrates for rhoptry-localized kinases and phosphatases (Delorme *et al.*, 2003; Carey *et al.*, 2004). In addition to rhoptry proteins, potential targets of these kinases and phosphatases include parasite proteins released from the other secretory organelles (micronemes and dense granules), parasite surface proteins, and host proteins. Such host proteins would most likely interact at the cytoplasmic face of the PVM, where rhoptry proteins have been shown to be localized following their release during invasion. It is also possible that the rhoptry kinases and phosphatases access the host cytoplasm directly during invasion in the form of so-called e-vacuoles (Hakansson *et al.*, 2001). Access to the host cytoplasm separate from the parasitophorous vacuole could then enable these effectors to reach a wide array of host proteins. Molestina and Sinai have shown recently that an unidentified parasite kinase is responsible for the phosphorylation of host IKB $\alpha$  localized at the PVM in infected cells (Molestina *et al.*, 2005). The putative kinases identified in the rhoptry proteome represent good candidates for the parasite kinase activity described; of the 38 novel proteins identified, 12 have homology to protein kinases. The bulk of these proteins are also members of the ROP2/3/4 family of proteins of which several members have homology to protein kinases but lack the key residues necessary for kinase activity and thus can be excluded as potential kinases of host IKB $\alpha$ . The presence of a predicted protein phosphatase 2C in the rhoptry proteome further indicates that phosphorylation will likely play an important role in rhoptry function, either within the rhoptries themselves or upon release into the host cell or nascent PV during invasion.

The presence of a serine protease and an insulinase-like metalloprotease in the rhoptry fraction indicates that, in addition to the previously identified rhoptry proteases Toxopain-1 and TgSUB2 (Que *et al.*, 2002; Miller *et al.*, 2003), other proteases may be important constituents of the rhoptries. These additional proteases may function in the processing of rhoptry proteins or in processing of host or parasite proteins upon secretion of the rhoptries during invasion. Of these, the insulinase-like metalloprotease is at present more tentatively associated with the rhoptries as it is also found in the excreted/secreted fraction of Zhou *et al.* which is largely devoid of rhoptry proteins (Zhou *et al.*, 2005), so definitive localization of this protein is awaited.

## Proteolytic processing of rhoptry proteins

An intriguing characteristic common to many rhoptry proteins is that they are synthesized as larger precursors ("pre-pre-proteins") that are proteolytically processed into the mature forms of the proteins en route to the rhoptries (Sadak *et al.*, 1988; Soldati *et al.*, 1998; Carey *et al.*, 2004; Hajj *et al.*, 2005). The first processing event is the removal of what appears to be a typical eukaryotic signal peptide ("or pre-sequence") upon entry into the endoplasmic reticulum. The second processing event is the cleavage of a pro-domain that results in the mature form of the protein. The latest point in the secretory pathway that the pro forms of rhoptry proteins are detected is in the pre-rhoptries, which are nascent rhoptries found in forming daughter parasites during the process of endodyogeny (Soldati *et al.*, 1998; Carey *et al.*, 2004). The pH of the pre-rhoptries is lower than the mature rhoptries, which may be necessary for processing of the pro-domains (Shaw *et al.*, 1998). Thus, pro-domain cleavage is believed to occur prior to delivery to the mature rhoptries although processing and rapid degradation in the mature rhoptries themselves cannot be excluded.

The pro-mature processing site for the rhoptry protein ROP1 has been experimentally determined and occurs after the glutamic acid residue at position 83 in the sequence 80-SFVE<sup>^</sup>APVR (P4-P4') (Bradley *et al.*, 1999). Two rhoptry localized proteases have been identified that are candidates for rhoptry protein maturases, the cathepsin B-like protease Toxopain-1 and the subtilisin-like TgSUB2 (Que *et al.*, 2002; Miller *et al.*, 2003). Inhibitors of both subtilisins and cathepsin B affect the ultrastructure of the rhoptries and also affect parasite replication, indicating that these proteolytic activities are critical to rhoptry function and intracellular survival (Shaw *et al.*, 2002). TgSUB2 appears most likely to be the ROP1 processing enzyme; it associates with ROP1 and is autocatalytically processed at sites similar to that identified for ROP1 (Miller *et al.*, 2003). Mutagenesis of these sites blocks processing and indicates the consensus site for processing is SΦXE at the P4'-P1' positions (where Φ is a large hydrophobic residue and X is any residue). Preliminary results also suggest that ROP2 family proteins associate with TgSUB2 and may be substrates for TgSUB2 *in vitro* (Binder *et al.*, 2004). If TgSUB2 is indeed a rhoptry protein maturase, its preference for glutamic acid at the P1 position is atypical as this class of enzymes generally cleave following a basic residue or if acidic, an aspartic acid residue (Bergeron *et al.*, 2000; Withers-Martinez *et al.*, 2004). This difference for substrate specificity and the fact that TgSUB2 appears to be essential indicates that this protease may be an ideal therapeutic target (Miller *et al.*, 2003). The precise rhoptry target(s) of toxopain are not yet clear, but gross inhibition with cysteine protease inhibitors appears to affect ROP2 processing in addition to rhoptry structure and invasion (Que *et al.*, 2002).

Pro-domains of proteins serve a variety of functions including sub-cellular targeting, maintaining inactivity of zymogens, inhibition of activity of mature proteins and enhancement of secretion (Shinde *et al.*, 1993; Suciu *et al.*, 1996; Huete-Perez *et al.*, 1999). While the precise role(s) of the pro-domains of rhoptry proteins is not currently known, evidence exists for both the targeting and maturation/activation of rhoptry proteins. For the soluble rhoptry protein ROP1, the pro-domain is sufficient for targeting reporter proteins to the organelle (Bradley *et al.*, 2001; Striepen *et al.*, 2001). However, the ROP1 pro-domain is not necessary for rhoptry localization, indicating that duplicate targeting sequences are

present in the mature form of the protein (Soldati *et al.*, 1998). Cleavage of the ROP1 pro-domain is not required for rhoptry targeting or secretion from the parasite during host cell invasion (Bradley *et al.*, 2002). The precise function of ROP1 is not known, thus an additional potential role for the pro-domain in regulating activity has not been assessed.

Evidence for the role of the pro-domain in regulating rhoptry protein function is seen in the ROP2 protein. Processing of the pro-domain of the PVM-associated ROP2 reveals an N-terminal mitochondrial-type targeting sequence that is exposed to the host cytoplasm and capable of binding host mitochondria that are intimately associated with the parasitophorous vacuole membrane (Sinai *et al.*, 2001). The role of additional pro-domains in regulating rhoptry protein activity will become clear as functions of these proteins become better understood. Likewise, the identification of pro-domains will be useful for determining common sequences that play roles in targeting to this specialized organelle. While the novel proteins identified by proteomics provide an excellent panel of proteins to examine processing events, it should be noted that detection of such processing events may be problematic because several of the proteins migrate aberrantly on SDS-PAGE due to repeat sequences and/or charge distribution within these polypeptides (e.g. the mass of mature ROP1 is ~33 kDa but migrates at ~60kDa) (Ossorio 1992; Bradley *et al.*, 1999; Bradley *et al.*, 2005).

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### Rhoptry neck proteins and the moving junction

One of the most exciting findings of proteins in the rhoptry proteome is the subgroup of proteins that localize exclusively to the duct-like rhoptry necks (Bradley *et al.*, 2005). To distinguish these from rhoptry body proteins that have been called ROPs, rhoptry neck proteins have been named RONS (Rhoptry Neck). As previously noted, four RON (RON1–4) proteins were confirmed using specific antibodies raised against these proteins which localized to the necks of the organelle. To exclude the possibility that these proteins were staining a compartment other than the rhoptry necks, localization was confirmed for one of these, RON4, by immunoelectron microscopy.

Upon rhoptry release during invasion, the rhoptry neck protein RON4 is localized to the moving junction, a structure that forms the interface between the surface of the parasite and the host cell plasma membrane (Alexander *et al.*, 2005; Lebrun *et al.*, 2005) (Figure 24.3). As its name implies, the junction moves along the surface of the invading parasite, at all times connecting the host and parasite plasma membranes (Aikawa *et al.*, 1978; Michel *et al.*, 1980). The moving junction is believed to serve as the molecular sieve which allows host plasma membrane lipids to enter the forming parasitophorous vacuole membrane but excludes host transmembrane proteins (Mordue *et al.*, 1999). This sieving function is likely to be essential for the failure of the parasitophorous vacuole to fuse with any component of the host endocytic system and avoid lysosomal destruction (Jones *et al.*, 1972; Sibley *et al.*, 1985; Mordue *et al.*, 1999). While the moving junction has been seen in transmission electron micrographs of both *Toxoplasma* and *Plasmodium* as long as thirty years ago, only RON4 in *Toxoplasma* and MCP1 in *Plasmodium* have been localized specifically to the moving junction (Aikawa *et al.*, 1978; Michel *et al.*, 1980; Klotz *et al.*, 1989; Bradley *et al.*, 2005; Lebrun *et al.*, 2005).

RON4 is part of a complex of rhoptry neck proteins that includes RON2 and a protein identified in the rhoptry proteome as TgTwinscan\_4705, which we have recently shown to localize to the rhoptry necks and have named RON5 (Alexander *et al.*, 2005; Bradley *et al.*, 2005; Lebrun *et al.*, 2005) Cheng and Bradley, unpublished). All three proteins are believed to be present at the moving junction although localization has only directly been shown for RON4 due to the reagents available for detecting these proteins (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). Intriguingly, the RON complex associates with the microneme protein AMA1 which is secreted onto the surface of the parasite and also localizes to the moving junction during invasion (Alexander *et al.*, 2005). The interaction between AMA1 and the RON complex is surprisingly strong, with the ability to form a complex in detergent-containing lysates following release from the individual secretory compartments (Alexander *et al.*, 2005). The release of the micronemes may occur via fusion with the rhoptry necks which would allow the AMA1 protein access to the RON complex prior to interacting on the surface of the parasite (Bannister *et al.*, 2003). This collaboration of the *Toxoplasma* micronemes and rhoptries then provides a model for the junction moving along the parasite surface, with AMA1 anchoring the junction at the parasite plasma membrane via its transmembrane domain (Alexander *et al.*, 2005) (Figure 24.3).

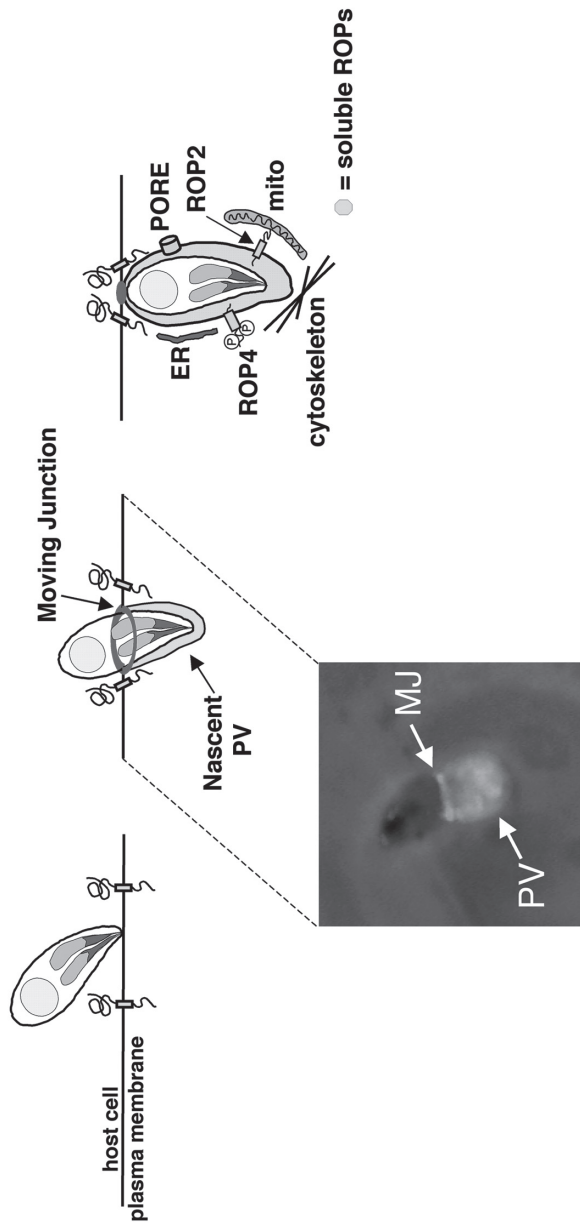
How the moving junction complex is anchored in the host cell plasma membrane is currently unclear. RON4 is a ~107 kDa protein that contains an interesting 44 amino acid repeat but lacks transmembrane domains and is thus unlikely to be membrane anchored (Alexander *et al.*, 2005; Bradley *et al.*, 2005). At the moving junction, RON4 can be detected with antibodies in the absence of permeabilization demonstrating that it is exposed to the exterior of the host–pathogen interface (Alexander *et al.*, 2005). Its partners in the complex, RON2 and RON5, both contain hydrophobic portions that may function as transmembrane domains which could anchor the junction in the host plasma membrane (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). Alternatively, additional parasite and/or host proteins may be present in the moving junction that serve to attach the junction to the host plasma membrane.

Some of the important questions still to be answered regarding rhoptry protein function at the moving junction include (1) are additional parasite or host proteins required at the moving junction? (2) where do these proteins reside relative to the host and parasite plasma membranes? (3) how are host proteins sieved-out of the nascent parasitophorous vacuole? (4) what is the trigger for release of the RON proteins from the rhoptries for formation of the moving junction complex? It will be particularly interesting to determine if host proteins are required, or if the parasite supplies all of the constituents necessary for host cell invasion. If indeed no host proteins are required, this would help explain the remarkable ability of *Toxoplasma* to invade virtually any mammalian cell.

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## Rhoptry function in additional life cycle stages

In our discussion of the rhoptries of *Toxoplasma* so far we have referred to the rhoptry proteome as if it is a relatively stable entity, rather like the genome of the parasite. In fact this is anything but the case. All proteomes are highly dynamic and reflect the biological context of the cell at the time of analysis. Due to the important roles of the rhoptries in invasion and vacuole formation, almost all of the information regarding rhoptry composition and



**Figure 24.3** Rhotry protein secretion during host cell invasion. Rhotry body (ROP) proteins are shown in green and rhotry neck (RON) proteins are shown in red. Once the parasite has firmly attached to the host cell via microneme proteins released at the apical end of the parasite (not shown), rhotry protein release occurs simultaneously with initiation of the formation of the nascent parasitophorous vacuole (PV). RON proteins are secreted into the moving junction which is seen as a ring-shaped structure that moves posteriorly along the invading parasite, always in contact with the host and parasite plasma membranes. The moving junction serves as a molecular “sieve,” allowing host lipids to enter the forming PV while excluding host transmembrane proteins. ROP proteins are secreted into the nascent PV and to the parasitophorous vacuole membrane (PVM) where they make contact with the host cytoplasm. ROP2 recruits host mitochondria to the PVM and ROP4 is likely to be phosphorylated by host cell kinases. Other host-pathogen interactions that may be mediated by the rhotries or other secretory organelles include ER and cytoskeletal interactions and insertion of a pore for exchange with the host cytoplasm (for more detailed analysis of the PVM see Martin and Sinai chapter in this book). Invasion is completed and the non-fusogenic parasite-containing-PV pinches-off from the host plasma membrane and intracellular replication begins. Inset shows an immunofluorescence image of a partially invaded parasite stained with anti-ROP4 (red) highlighting rhotry neck proteins at the moving junction and anti-ROP1 (green) showing rhotry body proteins released into the nascent PV. See also Plate 24.3.



function has been determined from the invasive tachyzoite form of the parasite. Recently, a single rhoptry protein has been identified that is not found in tachyzoites, but instead in *Toxoplasma* bradyzoites and merozoites (Schwarz *et al.*, 2005). This protein has been named Bradyzoite Rhoptry Protein 1 (BRP1). BRP1 is a predicted 18kDa protein that contains a signal peptide and has a high isoelectric point (10.6) similar to many rhoptry proteins of the tachyzoite stage. Its sequence does not provide clues to its function other than a coiled-coil domain, its stage specificity, and that it is unique to *Toxoplasma* (and close relatives such as *Neospora caninum*). It is not apparently found in distantly related Apicomplexa such as *Plasmodium*.

The finding of BRP1 in *Toxoplasma* merozoites and bradyzoites indicates a possible function in invasion of the gut epithelium. However, BRP1 knockout parasites show no gross phenotype using *in vitro* assays to examine bradyzoite development or *in vivo*, in which BRP1 knockout cysts encountered the gut epithelium of mice following oral administration of tissue cysts. It is thus likely that its role may be confined to the merozoite stage, or that another *Toxoplasma* protein can carry out the function of BRP1 in its absence. Most importantly, the finding of a bradyzoite/merozoite specific rhoptry protein indicates that a completely new complement of rhoptry proteins may exist in additional life cycle stages of the parasite. A proteomic approach to identify the protein constituents at these stages seems like an ideal first step for understanding the role of the rhoptries during additional life cycle stages.

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# Dense Granules of the Infectious Stages of *Toxoplasma gondii*: Their Central Role in the Host–Parasite Relationship

# 25

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and David J.P. Ferguson

## Abstract

The infectious forms (tachyzoite, bradyzoite, merozoite, sporozoite) of *Toxoplasma gondii* contain a variable number of dense granules. These granules are part of the apical complex and certain of the dense granule proteins are believed to be involved in the modification and function of the parasitophorous vacuole. In this chapter, the dense granules and their proteins will be described and discussed in the different stages relatively to their sub-cellular location and possible functions throughout the parasite cycle. In addition, the importance of dense granule proteins in the host immune response as well as their potential uses in the design of both new diagnostic reagents and a possible vaccine against toxoplasmosis will be reviewed.

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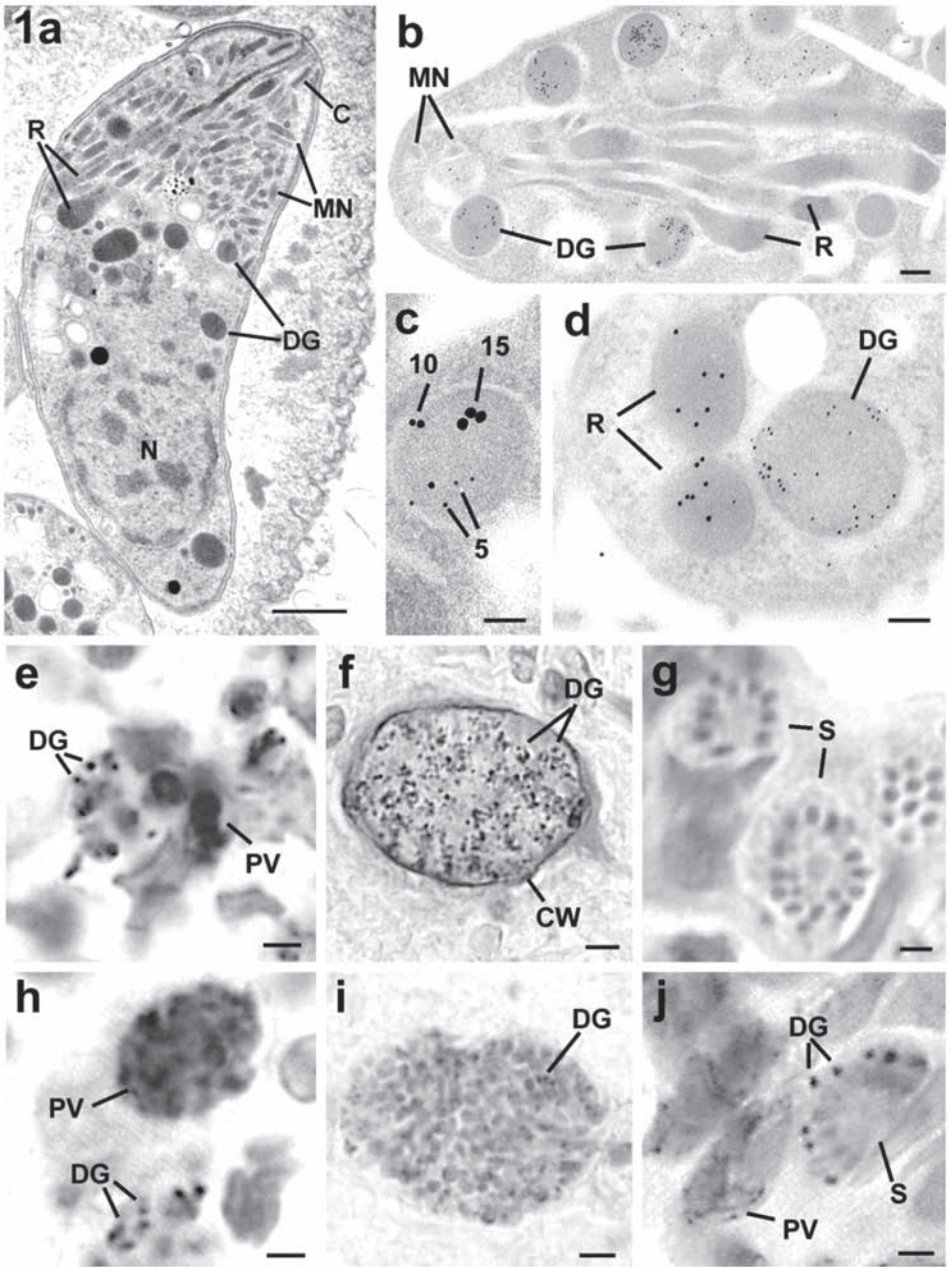
## Introduction

The term “dense granule” (DG) as used in this chapter, refers to membrane bound granules with electron dense contents present in the infectious forms (tachyzoites, bradyzoites, merozoites and sporozoites) of *Toxoplasma gondii* (Figure 25.1a-d). Such granules are common cytoplasmic structures that have been identified by electron microscopy (EM) in numerous cell types. The electron density of the contents usually reflects high protein concentration similar to those of the secretory granules of both exocrine and endocrine cells. However, the identity and function of the dense granule proteins (DGPs) cannot be inferred from the DG appearance.

In the case of *T. gondii*, there are examples of electron dense organelles in other stages of the life cycle that are unrelated to the DGs of the infectious stages. Indeed, two types of dense microspheres have been identified within the cytoplasm of the female macrogamete and are secreted to form distinct parts of the oocyst wall; a loose outer veil and the outer layer of the oocyst wall (Ferguson *et al.*, 1975, 2000). However, the proteins involved in this process are unknown and will not be discussed further in this chapter.

The specific DGs of the infectious stages are considered part of the apical complex that characterize the Phylum Apicomplexa, although they can be located throughout the cytoplasm (Figure 25.1a). The apical complex consists of the micronemes, rhoptries and DGs plus, in the Family Coccidia, the truncated cone termed the conoid (Figure 25.1a). These various organelles play distinct but important roles in the different steps of invasion and parasitophorous vacuole (PV) formation (Dubremetz *et al.*, 1993, Carruthers and Sibley, 1997). In this chapter, only this tightly defined group of DGs will be discussed.





**Figure 25.1** (a) Electron micrograph of a longitudinally sectioned bradyzoite showing the apical organelles consisting of the conoid (C), micronemes (MN), rhoptries (R) and dense granules (DG). N—nucleus. Bar is 0.5µm. (b) Immuno-electron micrograph of a tachyzoite labeled with anti-GRA1 showing numerous gold particles over the dense granule (DG) while the micronemes (MN) and rhoptries (R) are negative. Bar: 100 nm. (c) A tachyzoite section triple labeled with anti-GRA2 (5 nm gold), anti-GRA4 (10 nm gold) and anti-GRA6 (15 nm gold) and showing a dense granule labeled with all three markers. Bar: 100 nm. (d) Cross-section through



With the development of molecular techniques and the application of immuno-cyto-chemical techniques, it has been possible to identify specific proteins located in the DGs: these DG proteins have been shown to be involved in the formation of the PV (Figures 25.1 and 25.2). In this chapter, we will focus on DG formation, secretion and on the role of DGPs throughout the parasite life cycle. The importance of DGPs in the host immune response as well as their potential utilization in the design of both new diagnostic reagents and effective vaccine against toxoplasmosis will also be reviewed.

## Biogenesis of DGs and exocytosis

In all infectious stages, the DGs are microspheres of approximately 200 nm in diameter and enclosed by a unit membrane. The number of DGs varies between the different infectious stages. The largest numbers (approx. 15) have been observed in the tachyzoites and sporozoites with intermediate numbers (approx. 8–10) in the bradyzoites (Figure 25.1a) and few (3–6) in the merozoites. This may correlate with the number of DGPs expressed and the type of PV formed (discussed below).

Concerning the biogenesis of the DGs, it has been shown using expression of reporter proteins, that any soluble protein preceded by a signal peptide, is delivered to DGs by bulk flow and further secreted into the PV (Karsten *et al.*, 1998). DGs thus constitute the secretory default pathway for soluble proteins. Protein condensation in mammalian secretory vesicles usually occurs via subtle changes in the forming granule (mild acidification or increased concentration of calcium) while trafficking through different compartments of the secretory pathway. Since DGs do not constitute an acidic compartment, protein condensation is unlikely to result from acidification. Most of the DGPs identified so far are predicted to be type I transmembrane proteins. However, within the DGs, DGPs occur as both soluble and aggregated forms (reviewed in Mercier *et al.*, 2005). The delivery of some DGPs seems to depend on a peculiarity of their transmembrane domain (TMD) (Karsten *et al.*, 2004). Whether the sorting of proteins to DGs involves additional sorting pathways is currently under investigation.

The existence of sub-populations of DGs storing specific DGPs, was examined by double or triple labelings with specific antibodies: all DGs exhibited multiple labelings. This indicates the storage of multiple DGPs within the same DGs (Figure 25.1c,d) (Sibley *et al.*, 1995, Ferguson *et al.*, 1999, Labruyère *et al.*, 1999).

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the anterior of a merozoite double labeled with anti-ROP2 (10 nm gold) and anti-NTPase (5 nm gold) showing the specific labeling of the rhoptries (R) and dense granules (DG). Bar: 100 nm. (e–j) Section of tachyzoites in lung of an acutely infected mouse (e, h), tissue cysts in the brain of a chronically infected mouse (f, i) and the coccidian stages in the small intestine of an infected cat (g, j) immuno-stained with anti-GRA1 (e, f, g) and anti-NTPase (h, i, j). Note the strong staining of the dense granules (DG) and parasitophorous vacuole (PV) for both GRA1 and NTPase of the tachyzoites (e, h). In the tissue cyst, there is strong staining of the dense granules (DG) and cyst wall (CW) for GRA1 (f) but reduced labeling of the dense granules and no labeling of the cyst wall for NTPase (i). In the coccidian stages, the merozoites in mature schizonts (S) and parasitophorous vacuoles (PV) were negative for GRA1 (g) while the dense granules (DG) and parasitophorous vacuole were labeled for NTPase (j). Bar: 1 µm (e, g, h, j) and 5 µm (f, i). (Images supplied by David Ferguson, Oxford University; copyright retained.)

The secretion of the DGs has been difficult to capture: fusion of the DG membrane with the parasite plasma membrane (PPM) takes place sub-apically, at supposed gaps between the plates forming the inner membrane complex (Dubremetz *et al.*, 1993). In an artificial secretory system using serum as inducer, secreted DGPs remain in the soluble fraction after 100 000 *g* ultracentrifugation, indicating that they would be secreted as soluble proteins only (Lecordier *et al.*, 1999; Adjogble *et al.*, 2004).

DG secretion appears to respond to signals associated with both constitutive and regulated pathways of secretion. In favor of constitutive secretion, DG fusion with the target PPM is assisted by small GTPases of the Rab family and by soluble accessory factors (*N*-ethylmaleimide Soluble Factor (NSF), Soluble NSF Associated Protein REceptor/Soluble NSF Associated Protein machinery) (Chaturvedi *et al.*, 1999) and augmented by the ADP-ribosylation Factor 1 (Liendo *et al.*, 2001). Furthermore,  $\text{Ca}^{2+}$  intracellular increase, which usually triggers fusion of mammalian dense core granules with the plasmalemma, has no effect on DG exocytosis (Chaturvedi *et al.*, 1999; Liendo *et al.*, 2001) while resulting in secretion of the micronemes (Carruthers and Sibley, 1999). In contrast, the burst of DG secretion into the PV occurring shortly after its formation (Dubremetz *et al.*, 1993, Carruthers and Sibley, 1997), the fact that brefeldin A has no effect on the release of pre-stored DGPs (Coppens *et al.*, 1999) and that DG secretion was shown to be quantitatively and specifically induced by heat-inactivated serum (Darcy *et al.*, 1988; Coppens *et al.*, 1999), would be in favor of a regulated secretion. Two mechanisms driving DG secretion are thus hypothesized. Since two distinct populations of DGs were never observed, the type of secretion might be related to the compaction stage of DGPs: peripheral or unaggregated proteins might be constitutively released throughout the intravacuolar development of the parasite, while a regulated mechanism, still uncharacterized, would take place shortly after invasion, when DGPs would be required for setting up a functional vacuole.

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## Characterization of the DGPs

### Molecular characterization of DGPs

Among the DGPs characterized, 9 proteins are of relatively small molecular weight (20–40 kDa), with a N-terminal signal peptide and either a TMD or amphipathic alpha-helix(es) bordered by two hydrophilic domains (Henriquez *et al.*, 2005; reviewed in: Mercier *et al.*, 2005) (Table 25.1). Since these proteins do not present significant homology with proteins of known function, they were named GRA proteins (Sibley *et al.*, 1991). Several proteins with enzymatic activity or with homology with proteins already described were also localized in the DGs, including 2 serine protease inhibitor isoforms, 3 Nucleoside Tri Phosphatase (NTPase) isoforms, a 14.3.3 protein and one protein with acid phosphatase activity (Metsis *et al.*, 1995 and Mercier *et al.*, 2005). It is likely that the recent advances in genomic and proteomic techniques will help to identify new DGPs (Zhou *et al.*, 2005).

### Regulation of DGP gene expression

The *DGP* genes are single copy genes, which do not assemble in clusters but are distributed throughout the whole nuclear genome of *T. gondii* (Mercier *et al.*, 2005). As judged by their

Table 25.1 Distribution of DGPs throughout the life cycle of *Toxoplasma gondii*

	GRA1	GRA2	GRA3	GRA4	GRA5	GRA6	GRA7	GRA8	GRA9	NTPasess	TgPI
<b>TK</b> DG PV	23 K + (sol.) sol. matrix loose assoc.MNN	28 K + Amph. $\alpha$ -hel. MNN	30 K + TMD MNN, PVM, PVE	40/41 K + TMD MNN	21 K + TMD PVM, PVE	32 K + TMD MNN	29 K + TMD PVM, PVE	40 K + TMD PVM, PVE	41 K + Amph. $\alpha$ -hel. MNN	67 K + Sol. matrix Loose assoc. MNN	38/42 K + Sol. matrix
<b>BZ</b> DG cyst	+ CWM	+ CWM (faint) CWma	+ CWM	+ -	+ CWM	+ CWM CWma	+ CWM	+ -	+ +	reduced -	+ ND
<b>SZ</b> DG PV1 PV2	+ (25-30 K) - +	+  (small qty) - +	- + +	+ - +	+ -/+ +	+ - +	+ ND ND	+ ND ND	ND ND ND	- + +	+ ND ND
<b>MZ</b> DG PV	- -	- -	- -	- -	- -	- -	+ +	- -	ND ND	+ +	ND ND

Amph.  $\alpha$  hel.: amphipathic  $\alpha$ -helix(ces); CWma: cyst wall matrix; qty: quantity; CWM: cyst wall membrane; K: kDa; MNN: membranous nanotubular network; ND: not determined;

PVE: parasitophorous vacuole extensions; PVM: parasitophorous vacuole membrane; sol.: soluble; TMD: transmembrane domain

representation in the dbESTs, mRNAs coding for GRA1, GRA2, GRA6 and GRA7 are particularly abundant (Ajioka, 1998).

Few studies have examined the control of *DGP* gene expression. A common heptanucleotidic motif (WGAGACG) constitutes the minimal functional element driving transcription of *GRA1*, *GRA2*, *GRA5* and *GRA6* at the tachyzoite stage. This motif functions in an orientation-independent and additive manner (Mercier *et al.*, 1996). In silico analysis highlighted the presence of one to three of these motifs in the 300 bp located upstream of the transcription start site of all the other *GRA* genes, with the exception of *GRA9*, where the first motif is located at 40–50 bp upstream of the transcription start site (Mercier *et al.*, 2005). However, this motif is not absolutely essential for expression of DGPs since expression of NTPases is independent of WGAGACG (Nakaar *et al.*, 1999). In addition, this motif is not restricted to *DGP* genes (Soldati *et al.*, 1995, Matrajt *et al.*, 2004).

Data concerning the regulation and/or level of expression of DGPs in the different stages are limited although microarrays analysis comparing the levels of expression in the tachyzoite and tissue cyst (bradyzoite) showed that expression of the *GRA1*, *GRA5* and *GRA8* mRNAs is suppressed during encystation whereas the transcripts encoding *GRA2*, *GRA3*, *GRA4*, *GRA6* and *GRA7* are stable (Cleary *et al.*, 2002). Yet both the *GRA1* and *GRA5* proteins are detected both in the bradyzoite and in the cyst wall (Torpier *et al.*, 1993). Transcriptome and proteome analyses of the different parasite stages should help to clarify the situation.

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## Expression of DGPs and their targeting within the PV of the different developmental stages

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### Tachyzoite

The involvement of DGPs in the adaptation of the PV has only been examined in detail during tachyzoite development. During host cell (HC) invasion, the tachyzoite becomes enclosed by an invagination of the HC plasmalemma, which forms a relatively loose PV bounded by a unit membrane. Shortly after invasion, a burst of secretion of the DGs into the PV is observed (Dubremetz *et al.*, 1993; Carruthers *et al.*, 1997). This secretion coincides with specific structural changes of the PV that are characteristic of tachyzoite development (Figure 25.2a,b). These changes involved the development of a membranous nanotubular network (MNN) constituted of two membrane bilayers forming convoluted tubules (200–500 nm in length and 30–35 nm in diameter) within the vacuolar space (Figure 25.2b) (Sibley *et al.*, 1986; Magno *et al.*, 2005). These membranous tubules appear to form from multilamellar structures in a posterior invaginated pocket of the parasite (Sibley *et al.*, 1995). The MNN further extends into the vacuolar space to connect the parasites to the PVM (Sibley *et al.*, 1995; Magno *et al.*, 2005). An intimate association of strands of rough endoplasmic reticulum (rER) and mitochondria of the HC with the PVM (Figure 25.2b) was shown to be mediated by certain proteins secreted from parasite rhoptries into the PV (Sinai and Joiner, 2001; Sinai *et al.*, 1997). It has recently been proposed that PVM invaginations associated with host microtubules result in the formation of Host Organelle

Sequestering Tubulo-structures (HOSTs), which may serve as conduits for the delivery of host endo-lysosomes into the PV (Coppens *et al.*, 2006).

Following secretion into the PV, most of the DGPs are detected associated with the vacuolar membranous structures (Figure 25.1e,h). By immuno EM, GRA2, GRA4, GRA6 and GRA9 and some GRA1, GRA3, GRA7, NTPases were detected at the MNN (Figure 25.2c) (reviewed in Mercier *et al.*, 2005). In these membranes, GRA2, GRA4 and GRA6 were shown to establish privileged interactions (Labruière *et al.*, 1999). In contrast, GRA3, GRA5, GRA7 and GRA8 are preferentially detected at both the PVM and its membranous extensions within the HC cytoplasm (reviewed in Mercier *et al.*, 2005). It has recently been suggested that GRA7 is associated with the protein coat decorating the HOST structures (Coppens *et al.*, 2006).

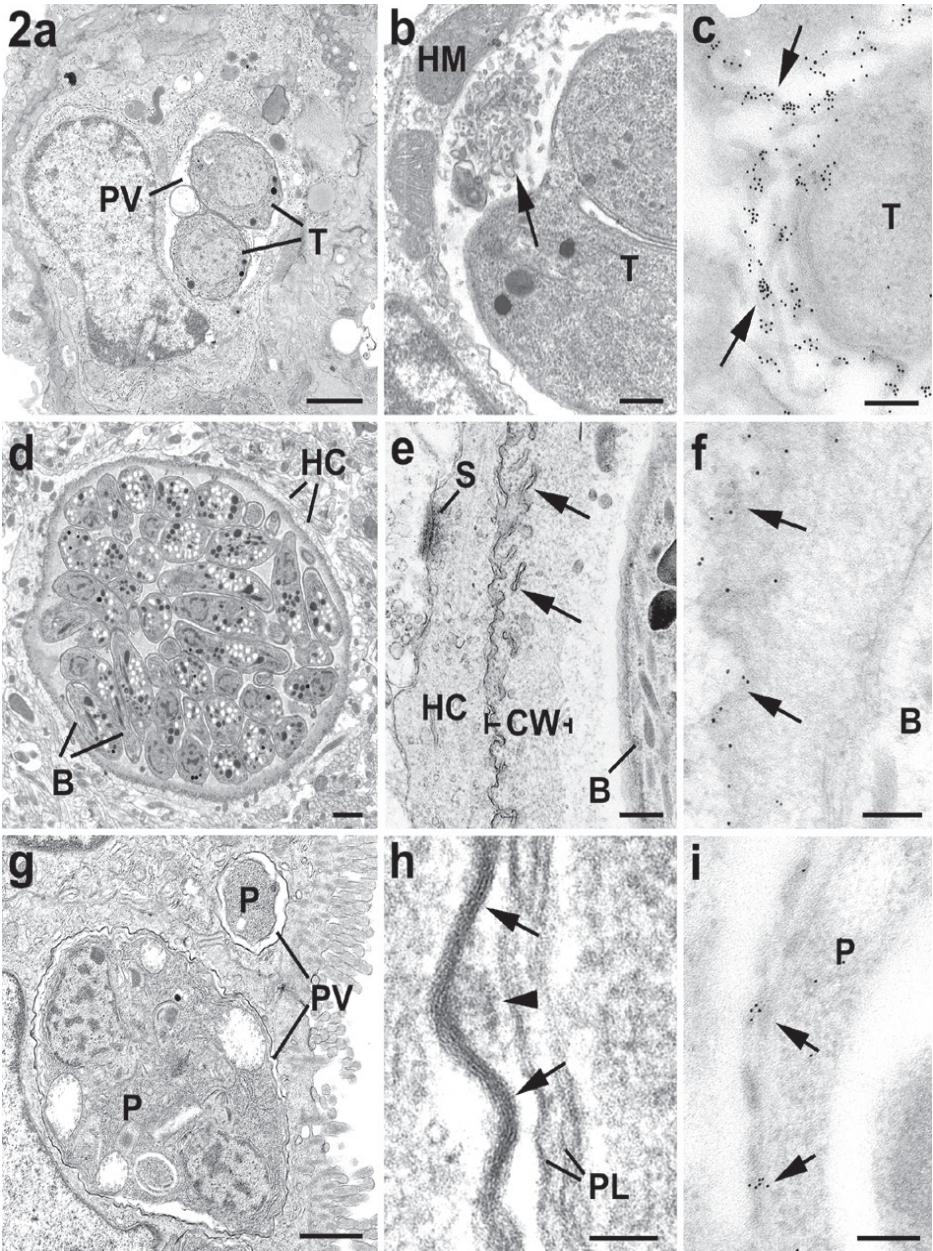
DGPs exhibit various types of membrane association. While both GRA1 and NTPases exhibit a very loose association to the MNN (Sibley *et al.*, 1994, 1995), GRA4 is only displaced by urea treatment (Labruière *et al.*, 1999), suggesting an association based mainly on hydrogen bonds. In contrast, GRA2, GRA3 and GRA5–9 are totally displaced from their respective membranes by non ionic detergents only, indicating membrane spanning domains stabilized by hydrophobic interactions (reviewed in Mercier *et al.*, 2005). In the case of both GRA2 (Mercier *et al.*, 1998a) and GRA5 (Lecordier *et al.*, 1999), the respective putative membrane domains (the GRA2 amphipathic  $\alpha$ -helices and the GRA5 TMD) were shown to be responsible for membrane association. Furthermore, GRA5 was determined to be a type I transmembrane protein in the PVM (Lecordier *et al.*, 1999). GRA1, NTPases, TgPIs are thus the only DGPs which remain primarily soluble within the tachyzoite PV lumen throughout parasite development (Sibley *et al.*, 1994; 1995; Pszenny *et al.*, 2002).

These data raise the following unsolved questions: (1) how are typical membrane proteins like the GRA proteins stored and secreted as apparent soluble components, (2) what are the mechanisms that allow selective targeting and association with specific vacuolar membrane structures (MNN or PVM), (3) what are the mechanisms regulating association of the GRA proteins within the PV membranes.

### Bradyzoite/tissue cyst

Bradyzoites are formed in intracellular tissue cysts found predominantly within muscle cells and within cells of the central nervous system (mainly neurons) (Figure 25.2d). The changes that characterize cyst formation occur immediately after the tachyzoite enters the HC (Ferguson and Hutchison, 1987). The PV is limited by a unit membrane with numerous shallow invaginations, some showing vesicle formation on their inner aspect. Formation of an underlying layer of moderately electron dense fine granular material contributes to the wall of the tissue cyst (Figure 25.2e) (Ferguson and Hutchison, 1987). Unlike the tachyzoite PV, the bradyzoite PV is devoid of the MNN and associated HC rER or mitochondria (Ferguson, 2004). Within the bradyzoite, the DGs contain all the DGPs identified in the tachyzoite although there is evidence for a reduced expression of NTPase (Figure 25.1f,i) (Nakaar *et al.*, 1998, Ferguson *et al.*, 1999a). Furthermore, GRA4, GRA8 and NTPases (Figure 25.1i) were not detected in the cyst wall, which might indicate an arrest of secretion of these particular DGPs, their degradation or their modification during





**Figure 25.2** Electron micrographs of tachyzoites in the lung of an acutely infected mouse (a–c), bradyzoites in tissue cyst in the brain of a chronically infected mouse (d–f) and coccidian stages in the small intestine of a cat (g–i). (a) Macrophage with two tachyzoites (T) present within a loose parasitophorous vacuole (PV). Bar: 1  $\mu$ m. (b) Detail of a parasitophorous vacuole containing developing tachyzoites (T). Note the extensive tubular array (arrow) within the vacuole and a number of host cell mitochondria (HM) adhering to the outer surface. Bar: 100 nm. (c) Part of a tachyzoite (T) contained in a parasitophorous vacuole labeled with anti-



encystment. In contrast, the other GRA proteins were shown to be present in the cyst wall, in a similar location to that observed within the tachyzoite PV (Figure 25.1f, 25.2f) (Torpier *et al.*, 1993; Ferguson, 2004). Whether DGPs are involved in a structural role during cyst wall formation or in communication with the HC remains to be investigated. Evidence for DGPs moving beyond the cyst wall is limited although it has been reported that GRA7 might traffic into the HC (Fischer *et al.*, 1998; Ferguson *et al.*, 1999b).

### Merozoite

During the coccidian development of *T. gondii* in the enterocytes of the cat, the ultra-structural appearance of the PV is very different from that observed for the tachyzoite PV (Ferguson, 2004). The parasites are located in a tightly fitting PV limited by a thickened membrane with a laminated appearance consisting of three closely applied unit membranes (Figure 25.2g,h). The PV lacks the MNN but does have cone-like densities protruding into the PVM (Figure 25.2h) (Ferguson, 2004). There is no association of the HC rER or mitochondria. In the limited studies available, the majority of DGPs are not expressed (Figure 25.1g) with only two, GRA7 and NTPase, having been identified within the DGs (Table 25.1 and Ferguson, 2004). These proteins are released into the PV shortly after invasion and locate in the PV (Figure 25.1j, 2i) but the level of staining drops as the parasites mature (Ferguson *et al.*, 1999a, b). The absence of MNN and mitochondrial/rER association may explain the lack of expression of the other GRA proteins.

### Sporozoite

The DGs of the sporozoite appear to contain GRA1–8 with the exception of GRA3 and NTPase (Tilley *et al.*, 1997; Ferguson 2004). *In vitro*, sporozoites entering a HC, form an unusual large vacuole (PV1) devoid of DGPs (Tilley *et al.*, 1997) but leave this PV to enter a new HC, forming a typical tachyzoite-like PV (PV2). Formation of this PV2 is correlated to the expression of the typical tachyzoite DGPs (Tilley *et al.*, 1997; Speer *et al.*, 1998). In fact, GRA3, GRA5 and NTPases are first to be detected, followed by GRA1, GRA2, GRA4 and GRA6 (Tilley *et al.*, 1997). However PV1 could be an *in vitro* artifact since *in vivo*, sporozoites invading the gut form typical tachyzoite-like PVs (Dubey *et al.*, 1998).

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GRA6: numerous gold particles associate with the tubular structure (arrows). Bar: 100nm. (d) Low power magnification through a tissue cyst containing numerous bradyzoites (B) and located within a neural cell (HC) in the brain parenchyma. Bar: 1µm. (e) Section through the periphery of a tissue cyst located within a neurone showing the numerous invaginations of the limiting membrane (arrows) into an underlying layer of granular material forming the cyst wall (CW). B—bradyzoite; S—synapse. Bar: 100 nm. (f) Similar section to that in e labeled with anti-GRA5 showing numerous gold particles associated with the cyst wall (arrows). Bar: 100 nm. (g) Low power magnification showing two coccidian stages (P) within parasitophorous vacuoles (PV) in the apical cytoplasm of enterocytes. Bar: 1µm. (h) Enlargement showing the laminated structure of the electron dense membrane limiting the parasitophorous vacuole (arrows). Note a conical structure protruding into the membrane (arrowhead). PL—parasite pellicle. Bar: 100nm. (i) Similar area stained with anti-NTPase showing gold particles associated with the parasitophorous vacuole (arrows). Bar: 100nm. (Images supplied by David Ferguson, Oxford University; copyright retained.)

To date, the *in vivo* evidence is that bradyzoites and sporozoites default to tachyzoite-like development when entering a new HC. This would be consistent with the tachyzoites, bradyzoites and sporozoites expressing the almost full repertoire of DGPs (Table 25.1) since these would be required to adapt the PV for optimal tachyzoite development and facilitated parasite proliferation in many cell types. In contrast, merozoites undergo limited proliferation and rapidly differentiate into the sexual stages in the enterocytes of the cat small intestine; the limited repertoire of DGPs within the merozoite may be related to this requirement.

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## Host-parasite interactions of DGPs: role in the pathophysiology of toxoplasmosis

### Possible functions of the DGPs within the PV

To date, the function of most DGPs remains unknown. Their burst of secretion and their selective targeting within the PV suggest that they would contribute significantly to the making of this new compartment and/or would have important functions in the PV metabolism (Dubremetz *et al.*, 1993).

BLAST searches performed on GRA proteins did not reveal significant homologies between the DGPs or between the DGPs and other proteins found in the databases. However, certain specific motifs suggest potential biochemical properties. Two EF-Hands predicted in GRA1 suggested calcium-binding properties, which were subsequently confirmed (Cesbron-Delauw *et al.*, 1989). A C-terminal ER retrieval motif in GRA3 (Henriquez *et al.*, 2005), a P-loop involved in ATP/GTP fixation in GRA4 and an RGD adhesion motif in GRA7 (Mercier *et al.*, 2005) would require further investigation.

In order to examine the functions of the DGPs, RH genetic knock-out (KO) parasites were created by a double event of homologous recombination at the considered locus. Analysis of GRA2, GRA6 or GRA2-GRA6 KOs showed that these proteins contribute to the formation of the MNN: (1) GRA2 induces the formation of the MNN membranous tubes from membranous vesicles found in the PV shortly after invasion and (2) GRA6 stabilizes the membranous tubules (Mercier *et al.*, 2002). The function of PVM-associated DGPs is not clear since a GRA5 KO did not lead to any obvious change in the phenotype (Mercier *et al.*, 2001). However, GRA7 was recently shown to induce tubulation of liposomes *in vitro* through the formation of homo-oligomers, and to be necessary for the formation of PV HOST membrane tubes. This suggests that GRA7 and possibly other secreted proteins may be involved in the sequestration of host endocytic vesicles in the vacuolar space (Coppens *et al.*, 2006).

Studies investigating the presentation of heterologous proteins engineered to be secreted into the PV indicate that proteins could escape from the PV into the HC cytosol (Kwok *et al.*, 2003; Pepper *et al.*, 2004; Gubbels *et al.*, 2005). Whether DGPs exert their function only within the PV or whether some of them must be actively exported into the HC cytosol to be functional, will require further investigation.

Despite their homology with characterized proteins, the function of the other DGPs is also not clearly established. NTPases are essential apyrases (Asai *et al.*, 1983; 1995; Nakaar *et al.*, 1999). However, their activity would be tightly repressed throughout parasite

multiplication. Indeed, *in vitro* activation of NTPases requires dithiols (Asai *et al.*, 1983; Bermudes *et al.*, 1994). Yet, the PV is a reducing compartment. With the PVM becoming more permeable as parasites develop, *in vivo* activation of NTPases would occur just before parasite egress (Stommel *et al.*, 1997; Silverman *et al.*, 1998), so that the ATP pumped from the HC could be degraded and provide the energy necessary for parasite motility.

*In vitro*, TgPIs are capable of neutralizing a broad spectrum of digestive proteases. *In vivo*, they might protect the parasite from gastrointestinal tract enzymes during the natural course of infection (Morris *et al.*, 2002).

### DGPs and parasite virulence

The importance of DGPs in pathogenesis was explored using gene KO approaches. Despite numerous attempts, it was impossible to KO or knock-down the *GRA1*, *GRA4* or the *NTPases* genes, suggesting that some DGPs are indispensable for intracellular survival of tachyzoites (Nakaar *et al.*, 1999; Braun and Cesbron-Delauw, unpublished; Travier and Mercier, unpublished). Targeted deletion of *GRA2*, *GRA5* or *GRA6* did not reduce parasite multiplication under normal conditions of growth *in vitro* (Mercier, 1998b, 2001, 2002) whereas knocking-out *GRA7* reduced parasite growth under serum deprivation (Coppens *et al.*, 2006). Whereas deletion of *GRA5* did not reduce parasite virulence in mice (Mercier *et al.*, 2001), those infected with the *GRA2*, *GRA6* or *GRA2-GRA6* KO survive acute infection and develop a chronic infection characterized by a specific antibody (Ab) response and formation of brain cyst-like structures (Mercier *et al.*, 1998b, Mercier *et al.*, unpublished).

It has been reported that attenuation of a virulent strain by prolonged passages in culture, resulted in the reduced expression of *GRA7* (Neudeck *et al.*, 2002) and possibly other secreted proteins (Nischik *et al.*, 2001). However, such abnormal selection pressure will probably result in many molecular changes and it is not clear if the reduced virulence is a direct consequence of the down regulation of particular GRA proteins.

### Role of the DGPs in the immune response

#### *Are DGPs immunoregulators during the early phase of infection?*

Up to now, most of the studies on the role of DGPs in the immune response have used antigen (Ag) mixtures such as the Soluble Tachyzoite Antigen (STAg) fraction (Hakim *et al.*, 1991) or serum induced Excreted-Secreted Ags (ESAs), the latter being enriched in DGPs (Darcy *et al.*, 1988). Sher and co-workers showed that two STAg proteins control production and secretion of Interleukin-12 (IL-12) by mouse dendritic cells during the first days of infection via the recruitment of both the Toll Like Receptor 11 and the Chemokine Receptor CCR5 (Aliberti *et al.*, 2003; Yarovsky *et al.*, 2005). Diana *et al.* (2005) completed these results, showing that *Toxoplasma* ESAs, via their binding to the CCR5, control the recruitment of immature human dendritic cells. Although the known *T. gondii* CCR5 ligand cyclophilin 18 has not been specifically shown to be a DGP, it is likely that this and similar STAg and ESA proteins localize to DGs.

### *Immunogenicity of DGPs during acute and chronic infection*

At the beginning of the infection, circulating antigens (CAGs) are likely to contain high concentrations of DGPs as the result of active secretion by tachyzoites or bradyzoites. Numerous studies have demonstrated that serum-induced ESAs, which contain mostly DGPs, induce a protective antibody (Ab) response in mice, rats and humans (Darcy *et al.*, 1988; Decoster *et al.*, 1988) as well as a T cell protective response in rats (Duquesne *et al.*, 1990; Zenner *et al.*, 1999).

In terms of mechanisms by which DGPs could be involved during the different phases of the immune response, data are fragmentary. Fatoohi *et al.* (2004) showed that STAgS induce CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells in infected pregnant women. The STAgS 26–40 kDa fraction as well as recombinant GRA1, GRA6 and GRA7, efficiently induce these CD4<sup>+</sup> CD25<sup>+</sup> T cells, which would thus be involved in the protection of the fetus in the case of acquired immunity during pregnancy (Fatoohi *et al.*, 2002). Prigione *et al.* (2000) reported that ESAs and more particularly, GRA2 elicit CD4<sup>+</sup> T cells presenting a TH0 pattern (production of both interferon (IFN)- $\gamma$  and interleukin-4) during the chronic stage of toxoplasmosis.

Together, these data suggest that GRA proteins could play an important role in the long term stimulation of the immune response.

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## **Potential of DGPs in diagnosis and/or vaccine development**

### DGPs as diagnostic reagents

Because of their high immunogenicity, DGPs were considered as potential diagnostic reagents. Many studies have attempted to include them in new generation diagnostic tests which combine both secreted and/or surface antigens produced as recombinant proteins in bacteria (Redlich and Muller, 1998; Jacobs *et al.*, 1999; Lecordier *et al.*, 2000; Aubert *et al.*, 2000; Ferrandiz *et al.*, 2004; Pietkiewicz *et al.*, 2004).

Avidity of specific immunoglobulins G, which determines the strength with which Abs bind to their Ags, shifts from low avidity to high avidity at approximately 5 months post-infection. Low avidity IgGs should thus detect recent seroconversions (Pfrepper *et al.*, 2005). Beghetto *et al.* (2003) showed that the combination of both GRA3 and GRA7 in such an avidity test, detects 85% of positive sera. Pfrepper *et al.* (2005) who tested 11 Ags including GRA4, GRA6, GRA7 and GRA8 showed that low avidity IgGs recognize GRA7 and GRA8 before recognizing MAG1 and eventually SAG1. In contrast, IgGs of high avidity, indicative of a chronic infection, are directed against SAG1 and GRA7 and never against ROP1 or GRA8.

Based on these results, it is clear that the DGPs (along with other proteins) could find their place in a cocktail of commercially available recombinant proteins for the development of more selective diagnostic kits.

### Identification of strain differences

At present, there is a limited number of genetic markers used to classify the various parasite isolates into the three canonical groups (groups I, II and III) of different virulence in mice (Howe and Sibley, 1995). Therefore, any additional polymorphism would assist in better

distinction between isolates and understanding their biological variations. To date, two alleles at the *GRA4* locus (Meisel *et al.*, 1996) and 9 alleles in the *GRA6* coding sequence, with a specific deletion in the *GRA6* C-terminus in avirulent isolates (Fazaeli *et al.*, 2000) have been identified. A peptide corresponding to the C-terminus of *GRA6*, which can react with sera of mice infected with either type I or type III isolates but not with types II, can now be used in combination with peptides derived from *GRA3*, *GRA7* and *SAG2* to discriminate type II from non type II isolates in mice (Kong *et al.*, 2003). This study opens new avenues for the typing of *Toxoplasma* isolates based on *DGP* polymorphism.

### Inclusion of DGPs in potential vaccines

Immunization with *GRA2* (Sharma *et al.*, 1984; Brinkmann *et al.*, 1993; Cesbron-Delauw *et al.*, 1996) or with BCG expressing *GRA1* (Supply *et al.*, 1999) protected mice significantly against a lethal infection by *T. gondii* and reduced congenital transmission in the rat model (Zenner *et al.*, 1999).

DNA vaccines leading to expression of *GRA1*, *GRA7* and *ROP2* induced a strong Ab response and decreased the brain cyst burden after challenge with the virulent strain IPB-G (Vercammen *et al.*, 2000). The immunization by plasmid DNA encoding *GRA1* favors the development of a CD8<sup>+</sup> cytotoxic T cell response as well as a CD4<sup>+</sup> T cell response producing IFN- $\gamma$  (Scorza *et al.*, 2003).

Finally, *GRA4* has been of interest since it is implicated in mucosal immunity (Mevelec *et al.*, 1992). Immunization of mice with the recombinant protein was shown to (1) induce either a (TH2 like) mucosal and systemic response or a TH1 response depending on the adjuvant and (2) to provide partial resistance against oral infection (Mevelec *et al.*, 1998; Martin *et al.*, 2004). Interestingly, naked *GRA4* DNA induced partial protection against a non-lethal challenge (Desolme *et al.*, 2000). In combination with DNA encoding GMCSF (which facilitates recruitment and activation of Ag presenting cells) and a mutated version of *SAG1*, *GRA4* DNA provided very promising results (Mevelec *et al.*, 2005).

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### Future directions

To better understand the different steps required for maturation of the PV compartment, several directions should be explored, such as the dynamics of PV modifications relatively to intracellular parasite development and/or the possible trafficking and functioning of DGPs beyond the PVM (Haldar *et al.*, 2005; Marti *et al.*, 2005). More fundamental work is also required to further explore the promising results obtained recently in the field of immunoregulation of the host response by DGPs during the early phase of infection.

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# Calcium Homeostasis and Acidocalcisomes in *Toxoplasma gondii*

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## Abstract

Calcium ion ( $\text{Ca}^{2+}$ ) is used as a major signaling molecule in *Toxoplasma gondii*.  $\text{Ca}^{2+}$  is critical for conoid extrusion, microneme secretion, gliding motility, and invasion of host cells, and its cytosolic concentration is regulated by the concerted operation of a number of transporters present in the plasma membrane, endoplasmic reticulum, mitochondria, and acidocalcisomes. Recent findings have shed light on the function of these transporters and the roles that they play in cellular metabolism and have shown that acidocalcisomes, electron-dense acidic organelles rich in calcium and polyphosphate, are linked to several functions, including polyphosphate metabolism, and calcium homeostasis in *T. gondii*.

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## Introduction

Calcium is a ubiquitous intracellular messenger that controls a variety of cellular functions. The cytosolic  $\text{Ca}^{2+}$  concentration [ $\text{Ca}^{2+}$ ]<sub>i</sub> is maintained at very low levels (of the order of  $10^{-7}$  M) compared to the concentration in the extracellular medium (about  $10^{-3}$  M). The cytosolic calcium level is responsible for the regulation of  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -controlled enzymes. The total calcium inside the cell is much higher than  $10^{-7}$  M, but the bulk of this calcium is either bound to proteins, polyphosphate, membranes or other cellular constituents, or is sequestered inside intracellular organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus, and nuclei (Irvine, 1986).

In eukaryotic cells  $\text{Ca}^{2+}$  homeostasis is achieved by the concerted operation of several  $\text{Ca}^{2+}$  transporting systems located in the plasma membrane, endoplasmic reticulum, nucleus, and mitochondria. The plasma membrane possesses calcium channels for  $\text{Ca}^{2+}$  influx, and a  $\text{Ca}^{2+}/\text{Na}^{+}$  exchanger and a  $\text{Ca}^{2+}$ -ATPase (PMCA) for the active extrusion of the cation (Carafoli, 1987). The endoplasmic reticulum and the nuclear membrane also possess a  $\text{Ca}^{2+}$ -ATPase (SERCA) for the influx of the cation and a  $\text{Ca}^{2+}$  channel for the efflux. In contrast to the plasma membrane, nucleus, and the endoplasmic reticulum, mitochondria do not possess  $\text{Ca}^{2+}$ -ATPases. The cation moves into the organelle through a uniport mechanism driven electrophoretically by the inside-negative membrane potential, while the efflux pathway appears to promote the electroneutral exchange of matrix  $\text{Ca}^{2+}$  by external  $\text{Na}^{+}$  or  $\text{H}^{+}$  (Nicholls *et al.*, 1984).

Several of these homeostatic mechanisms have been demonstrated to occur in *T. gondii*. In addition, it has been clearly demonstrated that calcium is critical for parasite invasion



of host cells and is also probably involved in its egress from the host. These two events are critical steps in the lytic cycle of *T. gondii* (Black and Boothroyd, 2000).

### Cytosolic $\text{Ca}^{2+}$ concentration ( $[\text{Ca}^{2+}]_i$ ) and the role of the plasma membrane in $\text{Ca}^{2+}$ homeostasis

The  $[\text{Ca}^{2+}]_i$  in tachyzoites is about  $70 \pm 6$  nM when measured in the absence of extracellular  $\text{Ca}^{2+}$  (with the  $\text{Ca}^{2+}$  chelator EGTA added to the medium) and  $100 \pm 9$  nM in the presence of 1 mM extracellular  $\text{Ca}^{2+}$ , as detected in fura 2-loaded cells (Moreno and Zhong, 1996). These concentrations are in the range observed in many studies with eukaryotic cells (Gryniewicz *et al.*, 1985).

A sequence with homology to a voltage dependent calcium channel is present in the *T. gondii* genome (20.m03897) (Chen, 2006) (<http://orthomcl.cbil.upenn.edu/cgi-bin/OrthoMclWeb.cgi>). The demonstration for this gene product to be functional as a calcium channel awaits further work. There is no direct evidence for receptor-operated ( $\text{Ca}^{2+}$  influx after receptor stimulation) or store-operated  $\text{Ca}^{2+}$  channels ( $\text{Ca}^{2+}$  influx occurring as a consequence of depletion of intracellular stores) (Tsien and Malinow, 1990; Tsien and Tsien, 1990) in *T. gondii*.

The active export of calcium from eukaryotic cells is accomplished by the action of a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and a  $\text{Ca}^{2+}$ -ATPase (PMCA). In this regard, a gene encoding for a protein of 501 amino acids has recently been annotated as a sodium/calcium exchanger (25.m01788) (Chen, 2006). Further biochemical studies would be important to demonstrate if this gene product functions as a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in *T. gondii*. It is possible that this gene encodes for a  $\text{Ca}^{2+}/\text{H}^+$  exchanger, as it occurs in yeast since there are no reports of the presence of  $\text{Na}^+/\text{Ca}^{2+}$  exchangers in early eukaryotes (Pozos *et al.*, 1996).

A PMCA-type  $\text{Ca}^{2+}$ -ATPase (*TgA1*) has been characterized and located in the plasma membrane and acidocalcisomes of *T. gondii* (Luo *et al.*, 2001). Biochemical evidence for calmodulin (CaM) stimulation of this pump has been reported (Bouchot *et al.*, 2001) although *TgA1* appears to lack a typical CaM-binding domain. This might suggest the presence of a different domain able to bind CaM. A gene coding for a second putative PMCA has been found in the *T. gondii* genome (44.m02812) (Chen, 2006). The deduced amino acid sequence (1200 aa) shows 45% identity with *TgA1* (Luo *et al.*, 2001). It is possible that the expression of these genes could be stage specific.

### $\text{Ca}^{2+}$ -binding proteins

Cytosolic  $\text{Ca}^{2+}$  can either interact with soluble  $\text{Ca}^{2+}$ -binding proteins or become sequestered into intracellular organelles where it binds to storage proteins. Calmodulin (CaM) is a cytosolic  $\text{Ca}^{2+}$  binding protein that acts as a  $\text{Ca}^{2+}$  receptor while the calsequestrin, and calreticulin families are endoplasmic reticulum proteins that store  $\text{Ca}^{2+}$ . *T. gondii* CaM is a small (16 kDa) acidic calcium-binding protein with four calcium-binding sites (EF hands) and a high level of identity (92.5%) with human CaM (Seeber *et al.*, 1999). Immunofluorescence analysis (Pezzella-D'Alessandro *et al.*, 2001) and immunogold electron microscopy (Song *et al.*, 2004) using antibodies reactive against CaM from other species, have identified the presence of CaM in the apical end of tachyzoites together with actin and myosin. The CaM inhibitors calmidazolium and trifluoperazine significantly



reduced parasite invasion *in vitro* and caused changes in the tachyzoites shape (Pezzella *et al.*, 1997). However, calmidazolium, can also increase  $[Ca^{2+}]_i$  and stimulate microneme secretion independently of its effect on CaM (Wetzel *et al.*, 2004). Another calcium-binding protein, P24, was identified in tachyzoites, and shown to be a major antigen and component of the dense granules (Cesbron-Delauw *et al.*, 1989).

No studies have been reported on  $Ca^{2+}$  storing proteins, like calreticulin or calsequestrin, in *T. gondii*. However, a calnexin gene (whose product is in the endoplasmic reticulum of eukaryotic cells) has been found in the *Toxoplasma* genome (583.m05347) (Chen, 2006). In addition, several sequences with similarity to CaMs, as well as to myosin light chains (see Chapter 28 for more details) have been found in the *T. gondii* genome.

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## Calcium storage

### Endoplasmic reticulum

The presence of a SERCA-type  $Ca^{2+}$ -ATPase in *T. gondii* was first demonstrated by experiments using fura 2-loaded tachyzoites in which thapsigargin, a very specific inhibitor of this pump when used at low concentrations (Thastrup *et al.*, 1990), was shown to increase  $[Ca^{2+}]_i$  in tachyzoites (Moreno and Zhong, 1996). More recently, Nagamune *et al.* (Nagamune, 2005a; Nagamune, 2005b) provided molecular evidence for the presence of this pump in *T. gondii*. The gene encoding this enzyme complemented yeast deficient in  $Ca^{2+}$  pumps providing genetic evidence of its function.

$Ca^{2+}$  release from the endoplasmic reticulum of eukaryotic cells is mediated by ryanodine (RyR) and inositol 1,4,5-trisphosphate ( $InsP_3$ ) channels. RyR are activated by a rise in  $[Ca^{2+}]_i$  ( $Ca^{2+}$ -induced  $Ca^{2+}$  release, CICR). In addition there are RyR-like channels activated by cyclic ADP-ribose (cADPR), sphingosine and a distinct  $Ca^{2+}$ -release pathway activated by nicotinic acid adenine dinucleotide phosphate (NAADP). The enzyme that generates the second messengers  $InsP_3$  and diacylglycerol, *T. gondii* phosphoinositide-specific phospholipase C, was recently cloned, sequenced and expressed in *E. coli* and its enzymatic characteristics were investigated (Fang, 2005). The enzyme was found to be located in the plasma membrane. Pharmacological studies provided evidence for the presence of an  $InsP_3$ /ryanodine-sensitive stores in *T. gondii* (Lovett *et al.*, 2002). Ethanol increased  $InsP_3$  and  $[Ca^{2+}]_i$  and this pathway was sensitive to inhibitors of  $InsP_3$ R channels. *T. gondii* also responded to agonists of cADPR-gated channels such as ryanodine and caffeine (Lovett *et al.*, 2002). cADPR cyclase and cADP hydrolase activities, which control cADPR levels, were found in *T. gondii* (Chini *et al.*, 2005). Microsomes loaded with  $^{45}Ca^{2+}$  released  $Ca^{2+}$  when treated with cADPR, and the RyR antagonists 8-bromo-cADPR and ruthenium red blocked this response. *T. gondii* microsomes also responded to  $InsP_3$ , but the inhibition profiles of these calcium-release channels were mutually exclusive (Chini *et al.*, 2005). There is no molecular evidence for the presence of  $InsP_3$ R or RyR channels in *T. gondii*, but this could be due to lack of homology with the channels of animal cells, as it is the case in plants (Nagata *et al.*, 2004).

## Nucleus

It has been reported that the movement of  $\text{Ca}^{2+}$  in the nucleus may be restricted and require a SERCA-type pump. The nuclear membrane of *T. gondii* is continuous with the endoplasmic reticulum (Hager *et al.*, 1999) and a similar composition in channels and pumps would be expected.

## Mitochondria

$\text{Ca}^{2+}$  changes in mitochondria parallel cytosolic  $\text{Ca}^{2+}$  changes. Biochemical evidence for mitochondrial  $\text{Ca}^{2+}$  uptake is available in malaria parasites (Uyemura *et al.*, 2000) and preliminary evidence suggests the presence of a uniport mechanism in *T. gondii* (Vercesi and Moreno, unpublished observations). However, nothing is known about how mitochondrial  $\text{Ca}^{2+}$  is released.

## Acidocalcisomes

These acidic calcium-storage organelles were found in a diverse range of organisms from bacteria to man (Docampo *et al.*, 2005). The main characteristics of these novel organelles are their acidic nature, high density (both in weight and by electron microscopy), and high content of pyrophosphate, polyphosphate (poly P), calcium, magnesium, and other elements (Docampo *et al.*, 2005). Acidocalcisomes are similar to the volutin or metachromatic granules first described a hundred years ago (Kunze, 1907) in Coccidia, and detected in *T. gondii* for their ability to stain red when treated with toluidine blue (metachromasia) (Mira Gutierrez, 1966). They were also named “black granules” (Bonhomme *et al.*, 1993).

Acidocalcisomes are the largest store for  $\text{Ca}^{2+}$  in *T. gondii* (Bouchot *et al.*, 1999; Luo *et al.*, 2001; Moreno and Zhong, 1996). Their acidity is easily demonstrated through the incubation of tachyzoites with the weak base Acridine Orange (AO) and subsequent observation by fluorescence microscopy. A report by Shaw *et al.* (Shaw *et al.*, 1998) has pointed out that when using DAMP (3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine) to detect acidic compartments, the only acidic compartments of tachyzoites at the electron microscope level, are mature and forming rhoptries and that there is no labeling of other organelles. However, no orange staining of the rhoptries is observed by fluorescence microscopy (Moreno *et al.*, in preparation), indicating that they are not as acidic as the acidocalcisomes. In addition, even if the rhoptries are acidic they are probably not as acidic as the acidocalcisomes.

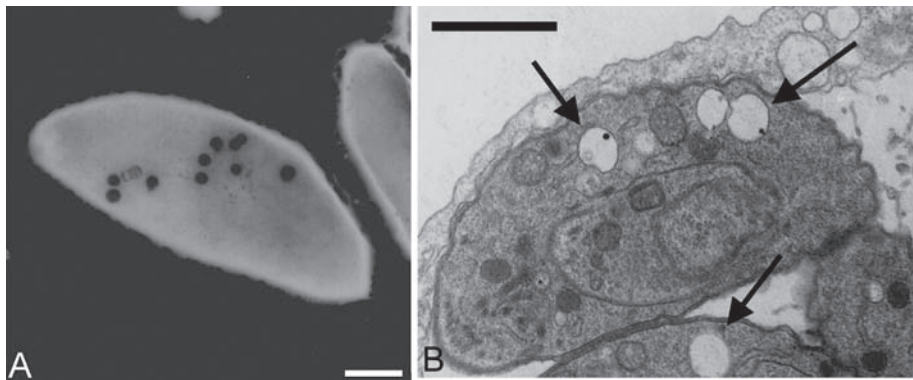
In thin sections, the acidocalcisomes of *T. gondii* appear as empty vesicles occasionally bearing an electron dense material that sticks to the inner face of the membrane (Figure 26.1B). Acidocalcisomes can be seen in electron spectroscopic images of whole cells directly dried on Formvar-coated grids (Figure 26.1A). The advantage of this type of preparation is the observation of the whole parasite (and whole organelles) without the addition of fixatives and other chemicals used in the routine procedures for transmission electron microscopy. This reduces significantly the extraction of material from the acidocalcisomes and, therefore, allows the observation of the organelle in its “native” state (Luo *et al.*, 2001). In these preparations, acidocalcisomes can be seen as spherical electron dense organelles randomly spread throughout the cell body (Figure 26.1A). Approximately 10 acidocalcisomes, with diameters varying between ~150 and ~400 nm, are observed per cell.

X-ray microanalysis (Luo *et al.*, 2001) of these organelles revealed considerable amounts of oxygen, sodium, magnesium, phosphorus, potassium, calcium and zinc concentrated in these compartments, similarly to what has been reported previously in the acidocalcisomes of trypanosomatids (LeFurgey *et al.*, 2001; Miranda *et al.*, 2000; Miranda *et al.*, 2004; Rodrigues *et al.*, 1999; Scott *et al.*, 1997).

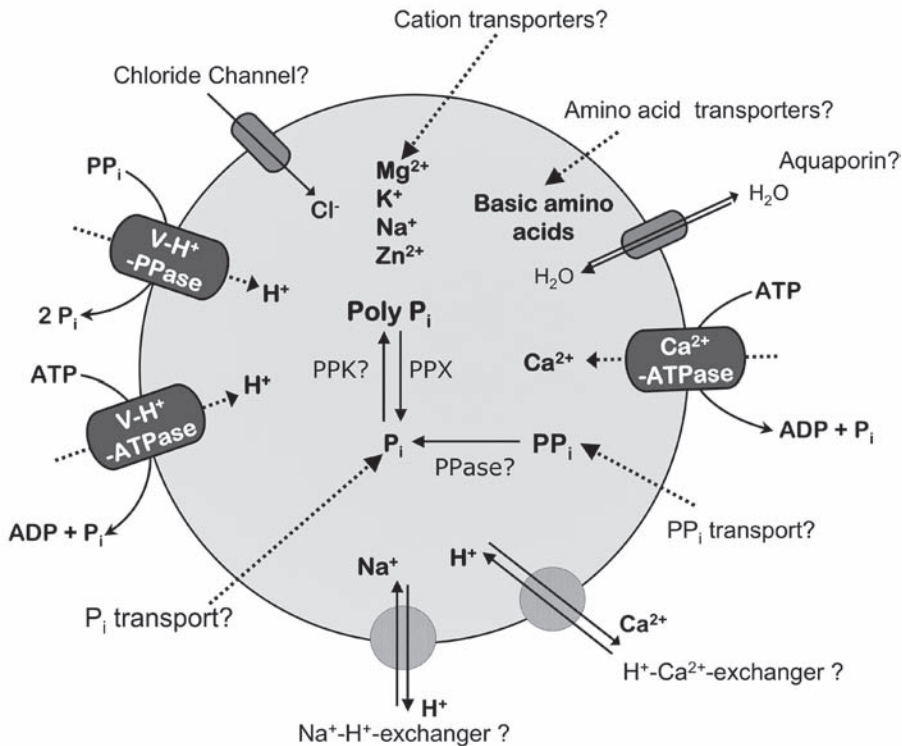
*T. gondii* acidocalcisomes possess a PMCA (*TgA1*), involved in  $\text{Ca}^{2+}$  influx, which is similar to vacuolar  $\text{Ca}^{2+}$ -ATPases of other unicellular eukaryotes (Bouchot *et al.*, 2001; Luo *et al.*, 2001), and two proton pumps, a vacuolar  $\text{H}^{+}$ -ATPase ( $\text{V-H}^{+}$ -ATPase) and a vacuolar  $\text{H}^{+}$ -pyrophosphatase ( $\text{V-H}^{+}$ -PPase) (Figure 26.2), involved in their acidification (Moreno *et al.*, 1998; Rodrigues *et al.*, 2000; Drozdowicz *et al.*, 2003; Luo *et al.*, 2001). The mechanism for  $\text{Ca}^{2+}$  release from acidocalcisomes is unknown. However, a gene with similarity to a previously described two-pore channel 1 (TPC1), the *Arabidopsis thaliana*  $\text{Ca}^{2+}$ -dependent  $\text{Ca}^{2+}$ -release channel (Furuichi *et al.*, 2001), has been found in the genome of *T. gondii* (583.m05406) (Chen, 2006) and it is possible that the gene product could be located in acidocalcisomes. The predicted protein sequence has 12 transmembrane spanning regions distributed in two domains typical of this kind of channels, which are present in the plant vacuoles.

The  $\text{Ca}^{2+}$  content of acidocalcisomes is very high (probably in the molar range), but most of it is bound to poly P and can be released only upon alkalization (Moreno and Zhong, 1996) or after poly P hydrolysis (Rodrigues *et al.*, 2002).

Mutants deficient in *TgA1* showed a decreased virulence *in vitro* and *in vivo* due to their impaired ability to invade host cells (Luo *et al.*, 2005). Biochemical analysis revealed that the tachyzoite poly P content was drastically reduced, and that the basal  $\text{Ca}^{2+}$  levels were increased and unstable. Microneme secretion under the conditions of stimulation by ionophores was altered. Complementation of *null* mutants with *TgA1* restored most functions (Luo *et al.*, 2005).



**Figure 26.1** Transmission electron microscopy of tachyzoites of *T. gondii*. (A) Electron spectroscopic image (ESI) of *T. gondii*. Scale bar, 1  $\mu\text{m}$ . The black spheres correspond to acidocalcisomes. (B) Thin section of a *T. gondii* tachyzoite showing several acidocalcisomes in different regions of the cell, with different degrees of preservation of the electron dense material (arrows). Scale bar, 1  $\mu\text{m}$ .



**Figure 26.2** Schematic representation of a typical acidocalcisome.  $Ca^{2+}$  uptake occurs in exchange for  $H^+$  by a reaction catalyzed by a vacuolar  $Ca^{2+}$ -ATPase. A  $H^+$  gradient is established by a vacuolar  $H^+$ -ATPase and a vacuolar  $H^+$ -pyrophosphatase ( $V\text{-H}^+ \text{-PPase}$ ).  $Ca^{2+}$  release could occur in exchange for  $H^+$  and is favored by sodium-proton exchange. An aquaporin allows water transport. Other transporters (for example, for  $Mg$ ,  $Zn$ , inorganic phosphate ( $P_i$ ) and pyrophosphate ( $PP_i$ ), basic amino acids) are probably present. The acidocalcisome is rich in pyrophosphate, short- and long-chain polyphosphate (poly  $P$ ), magnesium, calcium, sodium and zinc. An exopolyphosphatase ( $PPX$ ), a pyrophosphatase ( $PPase$ ) and a polyphosphate kinase ( $PPK$ ) may also be present. A question mark was added to indicate the lack of biochemical evidence for their presence.

The  $V\text{-H}^+ \text{-ATPase}$  activity was first identified in *T. gondii* by its sensitivity to bafilomycin  $A_1$ , a specific inhibitor of this proton pump when used at low concentrations (Bowman *et al.*, 1988). Using intact tachyzoites loaded with the fluorescent calcium indicator fura 2, bafilomycin  $A_1$  caused the release of calcium from an intracellular compartment of *T. gondii* (Moreno and Zhong, 1996). The  $V\text{-H}^+ \text{-ATPase}$  was also shown, by immunofluorescence microscopy, to localize in acidocalcisomes and in the plasma membrane where it has a role in regulating intracellular pH homeostasis (Moreno *et al.*, 1998).

A  $V\text{-H}^+ \text{-PPase}$  activity was also found in *T. gondii* (Rodrigues *et al.*, 2000). This enzyme was also shown to localize to the acidocalcisomes of *T. gondii* (Drozdowicz *et al.*, 2003; Luo *et al.*, 2001; Rodrigues *et al.*, 2000) (Figure 26.2). The gene encoding the *T. gondii* enzyme (*TgVP1*) was cloned and sequenced and a truncated version of the enzyme

(without the *N*-terminal) could be functionally expressed in yeast (Drozdzowicz *et al.*, 2003). Interestingly, the V-H<sup>+</sup>-PPase-specific staining of *T. gondii* assumes a transverse radial distribution soon after the parasite has made contact with the host cell. A collar-like structure is generated that migrates along the length of the parasite in synchrony with, and immediately anterior to, the apicobasally propagating penetration furrow (Drozdzowicz *et al.*, 2003). Upon completion of infection, the V-H<sup>+</sup>-PPase-associated fluorescence disperses before reappearing again at the anterior apex of the intracellular tachyzoite (Drozdzowicz *et al.*, 2003). In recent work a chimera of the *T. gondii* V-H<sup>+</sup>-PPase with or without the *N*-terminal extension of *T. cruzi* V-H<sup>+</sup>-PPase at its *N*-terminus showed improved expression levels enough to complement yeasts deficient in the soluble pyrophosphatase (Drake *et al.*, 2004). The acidocalcisomal enzyme belongs to the K<sup>+</sup>-stimulated group of V-H<sup>+</sup>-PPases (type I) (Drozdzowicz *et al.*, 2003; Rodrigues *et al.*, 2000), and has been successfully used as a marker for acidocalcisome purification, because this protein is abundantly concentrated in these organelles (Rodrigues *et al.*, 2002).

A number of genes identified in the genome of *T. gondii* could potentially encode additional acidocalcisome transporters. Some of these genes are: a Ca<sup>2+</sup>/H<sup>+</sup> exchanger similar to those present in the vacuole of yeast and plants that has been annotated, probably erroneously as indicated above, as a Ca<sup>2+</sup>/Na<sup>+</sup> antiporter (20.m03897); a putative phosphate transporter (49.m03192); two putative chloride channels (57.m01751 and 80.m02270); a neutral and basic amino acid transporter (583.m05611); a Zn<sup>2+</sup> transporter (52.m01632), and Na<sup>+</sup>/H<sup>+</sup> exchangers (129.m00252 and 541.m01159) (Chen, 2006). Aquaporins (AQP1: AAP33053; AQP2: AAQ84549), which are present in acidocalcisomes of *T. cruzi* (Montalvetti, 2004) and polyphosphate synthases (or vacuolar transporter chaperones, VTCs) (VTC1: AAR14678; and VTC2: AAQ94606), which are present in acidocalcisomes of *T. brucei* (Fang *et al.*, unpublished results) have also been identified in the genome of *T. gondii*. Some or all of these transporters could also be located at the parasite plasma membrane.

All acidocalcisomes described so far have been found to contain high levels of phosphorus as inorganic pyrophosphate (PP<sub>i</sub>) or polyphosphate (poly P). *T. gondii* acidocalcisomes are rich in short chain poly P such as poly P<sub>3</sub> (Moreno *et al.*, 2001; Rodrigues *et al.*, 2000).

PP<sub>i</sub> is formed as a byproduct of many biosynthetic reactions (synthesis of nucleic acids, coenzymes, proteins, activation of fatty acids, and isoprenoid synthesis) and its hydrolysis by inorganic pyrophosphatases makes these reactions thermodynamically favorable. However, none of these pathways have been found in *T. gondii* acidocalcisomes. It is possible that acidocalcisomal PP<sub>i</sub> is a by-product of the hydrolysis of poly P or an intermediate for its synthesis. Three reactions are known to use PP<sub>i</sub> in *T. gondii*, the phosphofructokinase (Peng *et al.*, 1995), the V-H<sup>+</sup>-PPase responsible for acidification of acidocalcisomes (Rodrigues *et al.*, 2000) (Drozdzowicz *et al.*, 2003), and an inorganic pyrophosphatase (Luo and Moreno, unpublished results). As found in several mammalian tissues (Ho *et al.*, 2000), it is plausible that the transport of PP<sub>i</sub> through the acidocalcisomal membrane mediated by a specific transporter occurs in *T. gondii*.

The storage of phosphate as poly P reduces its osmotic effects. Short and long chain poly P levels rapidly decrease upon exposure of tachyzoites to agents that mobilize Ca<sup>2+</sup>



such as calcium ionophores (ionomycin), alkalinizing agents ( $\text{NH}_4\text{Cl}$ ) or inhibitors of the  $\text{V-H}^+$ -ATPase (bafilomycin  $\text{A}_1$ ) (Rodrigues *et al.*, 2002). This would suggest a role for poly P in the adaptation of the parasites to environmental stress.

In addition to the proton and calcium pumps, another enzymatic activity has been detected in acidocalcisomes of *T. gondii*, a polyphosphatase (Rodrigues *et al.*, 2002).

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## **$\text{Ca}^{2+}$ functions in *T. gondii***

### Motility

Gliding motility is essential for active invasion of host cells by *T. gondii* (Dobrowolski and Sibley, 1996). Time-lapse microscopy experiments of parasites loaded with Fluo 4 have revealed that during parasite motility, cytosolic calcium levels undergo periodic increases (as occurs in muscle cells during contractions) and that  $\text{Ca}^{2+}$  released from intracellular stores and not originating from the extracellular pool is involved in these changes (Lovett and Sibley, 2003).

### Conoid extrusion

Conoid extrusion (which occurs during host cell invasion) is stimulated by agents that increase  $[\text{Ca}^{2+}]_i$  like ionomycin, calcium ionophore A23187, and the ATPase inhibitor thapsigargin (Mondragon and Frixione, 1996; Monteiro *et al.*, 2001). Ionophore-induced conoid extrusion can be prevented by preincubation of tachyzoites with the intracellular  $\text{Ca}^{2+}$  chelator BAPTA-AM but not by agents that reduce extracellular  $\text{Ca}^{2+}$ , like EGTA (Mondragon and Frixione, 1996).

### Microneme secretion, attachment, and invasion of the host cell

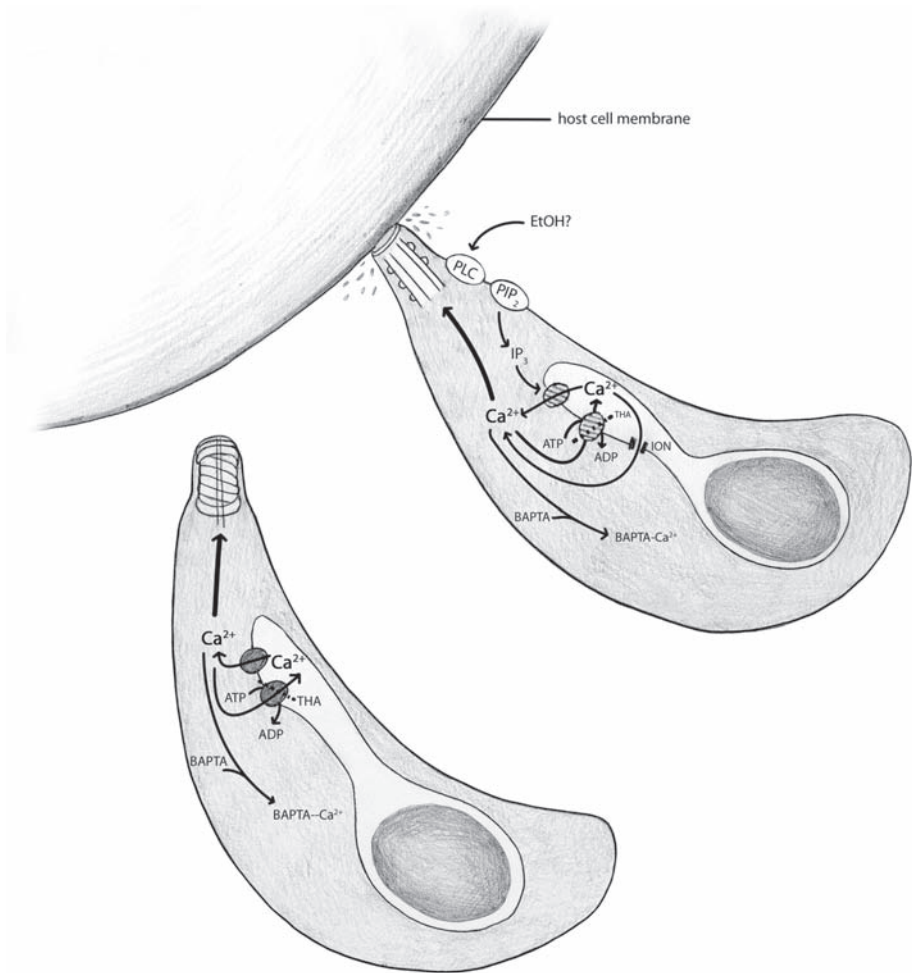
Micronemes, rhoptries, and dense granule contents are released by invading parasites and participate in attachment, vacuole formation, and intracellular survival, respectively (Carruthers and Sibley, 1997). Micronemes are the first secretory organelles to be discharged. Microneme proteins participate in attachment to host cell surfaces (Brecht *et al.*, 2001; Carruthers *et al.*, 2000; Fourmaux *et al.*, 1996; Garcia-Reguet *et al.*, 2000) and the transmembrane microneme proteins such as the adhesin MIC2 may link parasite and host cell membrane during invasion (Lovett *et al.*, 2002). Increases in intracellular  $\text{Ca}^{2+}$  mediate microneme secretion even in the absence of host cells, as demonstrated by treatment of cells with calcium ionophores like ionomycin or A23187, thapsigargin (Carruthers and Sibley, 1999), ethanol (Carruthers *et al.*, 1999b; Matthiesen *et al.*, 2003), or the anti-calmodulin agent calmidazolium (Wetzel *et al.*, 2004) (Figure 26.3).

The cytosolic  $\text{Ca}^{2+}$  concentration of *T. gondii* tachyzoites increases during their interaction with host cells, as demonstrated by digital fluorescence microscopy of parasites loaded with Fura-2 (Vieira and Moreno, 2000). When  $\text{Ca}^{2+}$  transients are prevented by loading the cells with BAPTA-AM at concentrations able to chelate intracellular  $\text{Ca}^{2+}$ , but not with the chemical analog half-BAPTA-AM that does not chelate  $\text{Ca}^{2+}$ , a decrease in host invasion by tachyzoites is observed (Vieira and Moreno, 2000). Inhibition of microneme release by chelation of intracellular calcium with BAPTA-AM, also inhibits host cell invasion (Carruthers *et al.*, 1999a) (Figure 26.3). In addition, time-lapse microscopy



of parasites loaded with the  $\text{Ca}^{2+}$  indicator Fluo-4 demonstrated the role of calcium in gliding motility and invasion (Lovett and Sibley, 2003). In summary, these results indicate that a  $\text{Ca}^{2+}$  increase that occurs upon attachment of tachyzoites to the host cell surface is possibly associated to conoid extrusion, microneme secretion, and invasion.

The  $\text{Ca}^{2+}$  increase needed for invasion is coming from an intracellular store. Previous reports using EGTA to chelate extracellular  $\text{Ca}^{2+}$  indicated that this was also important for invasion (Pezzella *et al.*, 1997). More recent work has established that this effect is due



**Figure 26.3** Schematic representation of the signaling pathways involved in calcium mobilization during invasion.  $\text{Ca}^{2+}$  is pumped into the endoplasmic reticulum (ER) by a thapsigargin (THA)-sensitive SERCA-type  $\text{Ca}^{2+}$ -ATPase. An increase in cytosolic  $\text{Ca}^{2+}$  stimulates conoid extrusion and microneme secretion. BAPTA chelates  $\text{Ca}^{2+}$  and prevents conoid extrusion and microneme secretion. Ethanol stimulates a mechanism not completely characterized that leads to an increase in intracellular  $\text{Ca}^{2+}$  and stimulation of microneme secretion. It has been proposed that its effect is through the stimulation of a phospholipase C leading to an increase in  $\text{IP}_3$ , which will act on the ER to release  $\text{Ca}^{2+}$  into the cytosol (Illustration by Cheryl Esther Reese).

to acidification of the culture medium by EGTA and not to chelation of  $\text{Ca}^{2+}$  (Lovett and Sibley, 2003).

Early studies had shown that ryanodine and caffeine enhanced  $\text{Ca}^{2+}$  release and microneme secretion, and that ethanol, which is known to cause  $\text{Ca}^{2+}$  release, stimulated an increase in inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) (Lovett *et al.*, 2002) (Figure 26.3). In addition, xestospongine C, an  $\text{InsP}_3$  receptor antagonist, inhibited microneme secretion and blocked parasite attachment and invasion of host cells (Lovett *et al.*, 2002). These studies suggested that  $\text{InsP}_3$  was acting as a second messenger and that *T. gondii* possessed an intracellular  $\text{Ca}^{2+}$  release channel with properties of the  $\text{InsP}_3$ /ryanodine receptors (RyR) superfamily. More recent work has established that another second messenger involved in intracellular  $\text{Ca}^{2+}$  release, cyclic ADP ribose (cADPR), is also present in *T. gondii*. When this pathway is inhibited by incubation of the parasites with the non-hydrolysable analogue 8-Br-cADPR or with the RyR inhibitor dantrolene there is a decrease in microneme secretion and gliding motility (Chini *et al.*, 2005). These results indicate that both  $\text{InsP}_3$  and RyR channels are important for efficient motility and cellular invasion, suggesting that they may work co-operatively, as in other systems (Chini *et al.*, 2005).

### Egress from the host cell

Changes in intracellular  $\text{Ca}^{2+}$  have also been proposed to occur during *T. gondii* egress from the host cells (Black *et al.*, 2000; Endo *et al.*, 1982; Arrizabalaga and Boothroyd, 2004). Addition of the  $\text{Ca}^{2+}$  ionophore A23187 to infected macrophages stimulates the movement and egress of tachyzoites resulting in host cell lysis (Endo *et al.*, 1982). This parasite egress is temperature dependent (Black *et al.*, 2000). Microinjection of intracellular  $\text{Ca}^{2+}$  stimulates the exit of the parasites (Schwab *et al.*, 1994). A *T. gondii* mutant defective in a  $\text{Na}^+/\text{H}^+$  exchanger located on the parasite's plasma membrane has been shown to be altered in its response to the  $\text{Ca}^{2+}$  ionophore A23187. These mutant cells have increased levels of intracellular  $\text{Ca}^{2+}$ , which explains their decreased sensitivity to A23187 (Arrizabalaga *et al.*, 2004).

The *T. gondii* PI-PLC has been proposed to have a role in parasite egress from the dying host on the basis of studies with the PI-PLC inhibitor U73122 (Moudy *et al.*, 2001). It was shown that permeabilized *Toxoplasma*-infected cells preincubated with U73122 but not with the inactive analog U73343, prevented parasite egress in the presence of extracellular buffer, and it was proposed that parasite egress depended on the intracellular  $\text{Ca}^{2+}$  increase stimulated by the decrease in the external  $\text{K}^+$  concentration (Moudy *et al.*, 2001). Since the inhibitor U73122 apparently affects PI-PLC activity in mammalian cells through its effects on heterotrimeric G proteins (Thompson *et al.*, 1991), and these have not been described in *T. gondii*, direct measurements of the products of TgPI-PLC activity ( $\text{InsP}_3$ , diacylglycerol) will be necessary to confirm this proposal.

Dithiotreitol (DTT, 5 mM) was also shown to activate egress of previously nonmotile intravacuolar parasites. This was accompanied by an increase in intra-parasitophorous vacuole (PV) fluorescence ratio of Indo 1-loaded infected human fibroblasts. Chelation of extracellular  $\text{Ca}^{2+}$  by EGTA and BAPTA-AM prevented parasite activation and  $\text{Ca}^{2+}$  increase, although ionomycin was still able to increase  $\text{Ca}^{2+}$  in the PV, and motility and egress of the parasite (Stommel *et al.*, 2001). DTT is known to activate the nucleoside

triphosphate hydrolase (NTPase) of the parasite, and a link with this effect was suggested although no direct evidence of this link was provided (Stommel *et al.*, 2001).

## Conclusions

The regulation of the cytosolic  $\text{Ca}^{2+}$  concentration in *T. gondii* has similarities and differences with the process that occurs in other eukaryotic cells. Acidocalcisomes are distinct calcium-storage organelles present in *T. gondii*, in which calcium is mostly bound to poly P. No information is available on second messengers involved in  $\text{Ca}^{2+}$  release from these organelles and further studies are necessary to understand the function of acidocalcisomes in  $\text{Ca}^{2+}$  homeostasis in *T. gondii*.  $\text{PP}_i$ , poly P, and cations are accumulated in large amounts in acidocalcisomes but their transport mechanism and their functions in *T. gondii* are largely unknown.  $\text{Ca}^{2+}$ -ATPases are present but apparently different from their mammalian counterparts. The PMCA-type  $\text{Ca}^{2+}$ -ATPase, which also localizes in acidocalcisomes, does not possess a typical calmodulin-binding domain. There are several well established roles for  $\text{Ca}^{2+}$  in *T. gondii*, such as its role in conoid extrusion, microneme secretion, invasion of host cells, and egress from host cells. With the information provided by microbial genome sequencing and further work in the field we will be able to discover other functions and exploit such information to design effective therapeutic agents to specific pathways within *T. gondii*.

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# Part VI

## **Cytoskeleton, Signaling, Motility, and Invasion**

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# Tubulin, Microtubules, and Microtubule-associated Structures in *Toxoplasma gondii*

27

Naomi S. Morrisette

## Abstract

*Toxoplasma gondii* uses tubulin to build both conventional structures (spindles and flagella) and unconventional structures (conoid fibers are a nine-protofilament tubulin ribbon). *Toxoplasma* microtubules determine parasite shape and apical polarity and are essential to the sexual cycle which relies upon flagellated male gametes. Unlike most eukaryotes, *Toxoplasma* tachyzoites contain two independent microtubule organizing structures: the apical polar ring organizes subpellicular microtubules and centrioles are associated with spindle microtubules. This chapter describes the distinct properties of *Toxoplasma* tubulin and the unique structures and functions that rely upon *Toxoplasma* microtubules.

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## Introduction

Tubulins are a group of highly conserved proteins that are essential to the survival of all eukaryotes. In organisms ranging from protozoan parasites like *Toxoplasma gondii* to their metazoan hosts,  $\alpha$ - and  $\beta$ -tubulin associate to form a stable heterodimer subunit and these subunits polymerize to build microtubules (Nogales, 2000). Microtubules are critical components of many basic cellular processes including mitosis, membrane trafficking and motility. Both  $\alpha$ - and  $\beta$ -tubulin are ubiquitous proteins in eukaryotes, as is  $\gamma$ -tubulin, a conserved and essential component of microtubule organizing centers (MTOCs). Several additional tubulins ( $\delta$ ,  $\epsilon$ ,  $\iota$ ,  $\eta$ ) are restricted to organisms with cilia and flagella (Dutcher, 2001; Dutcher, 2003). As described below, *Toxoplasma* tachyzoites have centrioles (albeit atypical in structure) and *Toxoplasma* male gametes have flagella templated by apparently conventional basal bodies; consistent with these observations, the *Toxoplasma* genome contains genes for  $\gamma$ -,  $\delta$ - and  $\epsilon$ -tubulins as well as for  $\alpha$ - and  $\beta$ -tubulin. Although tubulin and microtubules are universal components of eukaryotes, their organization, regulation and use in apicomplexan protozoa represent intriguing departures from our understanding of these cellular elements in model organisms.

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## Organization of microtubules and associated structures in *Toxoplasma*

### Spindle and subpellicular microtubules

The invasive forms of *Toxoplasma* (sporozoites, tachyzoites and bradyzoites) have two discrete populations of microtubules: subpellicular microtubules and spindle microtu-

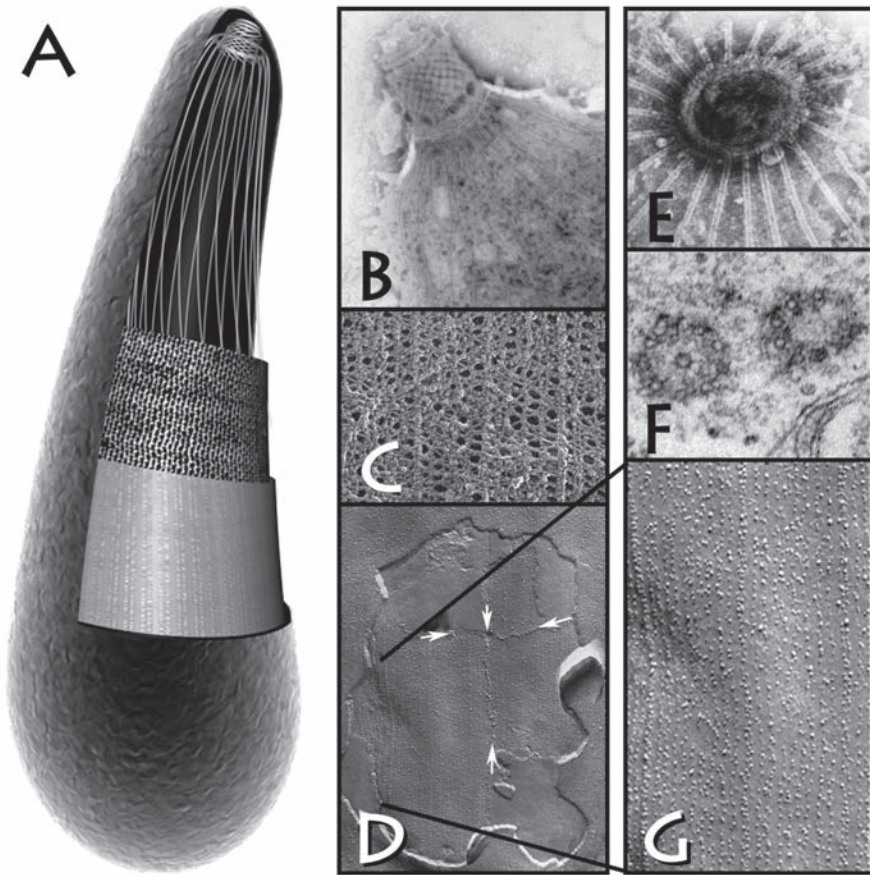
bules (Morrisette and Sibley, 2002b; Nichols and Chiappino, 1987; Shaw *et al.*, 2000). Subpellicular microtubules are non-dynamic; they maintain both apical polarity and the characteristic crescent shape of the parasite by interacting with the pellicle, a composite structure formed by the association of the plasma membrane with the underlying inner membrane complex. Spindle microtubules form an intra-nuclear spindle to coordinate chromosome segregation. Both populations are critically important to parasite survival and replication.

*Toxoplasma* replicates by endodyogeny, a process that couples mitosis with budding, the internal formation of daughter cells (described below). Spindle microtubules originate in the cytoplasm proximal to centrioles. These microtubules pass through pores located in an invaginated region of the nuclear envelope to mediate chromosome segregation. *Toxoplasma* centrioles have an unconventional form consisting of a central tubule surrounded by nine singlet microtubules which is quite distinct from the canonical “9 + 0” microtubule triplets observed in most centrioles (Figure 27.1F).

Subpellicular microtubules are intimately associated with the cytosolic face of the parasite pellicle, extending in a spiral from the apical tip to below the nucleus (approximately two-thirds of the total body length). These microtubules impose both an elongated serpentine shape and characteristic apical polarity on *Toxoplasma* and other apicomplexan parasites (Figure 27.1A,B). *Toxoplasma* tachyzoites have 22 subpellicular microtubules that are approximately 5  $\mu\text{m}$  long and are evenly distributed around the circumference of the cell body (Nichols and Chiappino, 1987). Subpellicular microtubules are organized by lateral association with the apical polar ring (APR), a circular MTOC that is unique to apicomplexan parasites (Figure 27.1E). Attachment of subpellicular microtubules to the APR is supported by blunt projections of the APR, which form a cogwheel pattern in transverse views (Nichols and Chiappino, 1987; Russell and Burns, 1984; Russell and Sinden, 1982). Consistent with other MTOCs, the plus end of subpellicular microtubules is distal to the APR (Russell and Burns, 1984). Subpellicular microtubules are not dynamic and are unusually stable after isolation (Hu *et al.*, 2002a; Morrisette *et al.*, 1997).

Apicomplexan parasites are bounded by a distinctive three-layered pellicle which consists of the plasma membrane and a closely apposed inner membrane complex (Figure 27.1D) made of flattened vesicles joined in a patchwork fashion by sutures (Dubremetz and Torpier, 1978; Porchet and Torpier, 1977). The inner membrane complex (IMC) originates at the APR, leaving the extreme apical region of the parasite enclosed by only plasma membrane. It extends to the posterior of *Toxoplasma*, where the individual vesicle plates join in a turbine-shaped structure. The IMC has a critically important role in segregation of organelles into daughter cells (described below) and is closely associated with microfilaments, microtubules and an intermediate filament-like network.

Freeze fracture studies of *Toxoplasma* and other apicomplexans has shown that IMC membranes are characterized by uniform organization of intramembranous particles (IMPs) (Dubremetz and Torpier, 1978; Morrisette *et al.*, 1997; Porchet and Torpier, 1977). IMPs are thought to represent the transmembrane domains of integral membrane proteins. IMPs in the IMC are organized into a highly regular two dimensional lattice (Morrisette *et al.*, 1997). Within the lattice, there are double and single rows of IMPs (Figure 27.1G). The IMPs that appear in double rows correspond in number and arrange-



**Figure 27.1** Organization of the *Toxoplasma* membrane cytoskeleton and microtubule organizing centers. (A) Cut-away schematic of the *Toxoplasma* membrane cytoskeleton which consists of the conoid and subpellicular microtubules, the IMC network filaments, the IMC containing an intramembranous particle (IMP) lattice and the overlying plasma membrane. (B) A detergent extracted, negative stained conoid and subpellicular microtubules. (C) A glycerol and detergent extracted, freeze-dried replica reveals the regular array of IMC filaments. (D) Freeze-fracture exposes the plates of the IMC joined by sutures (arrow) and containing IMP rows. (E) A detergent extracted, negative stained apical polar ring (APR) nucleates 22 subpellicular microtubules. (F) *Toxoplasma* centrioles consist of nine single microtubules surrounding a central tubule. (G) An enlarged portion of the freeze-fracture image in D illustrates the double (arrows) and single IMP rows. Panel A was drawn by Paul Warfel and was originally published in (Sibley, 2004); panels B, D and F are from (Morrisette *et al.*, 1997), panel C is from (Morrisette and Sibley, 2002a) and panel F is from (Morrisette and Sibley, 2002b). Panel E is an unpublished micrograph by the author.

ment to the underlying subpellicular microtubules. These double rows are interspersed with a number of single particle rows. Both the double and single IMP rows show continuity across the plates of the IMC, which represent topologically distinct vesicles.

The organization of the particle rows suggests an intimate association with both the subpellicular microtubules and a second, non-microfilament cytoskeletal network. Fourier analysis of isolated subpellicular microtubules reveals that they are coated with a microtubule associated protein (MAP) that binds with a 32 nm periodicity (Morrissette *et al.*, 1997). Akin to the MAP repeat along subpellicular microtubules, the double IMP rows overlying the microtubules also exhibit a 32 nm periodicity that is revealed by Fourier analysis of freeze-fracture images. This suggests that the MAP coordinates the close interaction of the subpellicular microtubules with the IMC. The 32 nm longitudinal repeat extends to the single rows of IMPs, creating a two-dimensional lattice, with the second dimension at an angle of approximately 75° to the longitudinal rows. The organization of the IMP rows persists to the posterior of the parasite, past the region associated with subpellicular microtubules. Moreover, the integrity of the particle lattice is not destroyed by disruption of actin or microtubules suggesting that other IMC-associated cytoskeletal filaments organize this structure.

The IMC is intimately associated with a non-actin, non-tubulin filamentous network that extends from the APR to the parasite posterior (Figure 27.1C). This network can be observed after glycerol or deoxycholate-extraction of *Toxoplasma* or other apicomplexans (D'Haese *et al.*, 1977; Mann and Beckers, 2001). The network filaments have a diameter of 8–10 nm and the extracted network maintains the same general shape as the parasite. Two novel proteins (TgIMC-1 and TgIMC-2) which are predicted to form coiled coils localize to the IMC network (Mann and Beckers, 2001; Mann *et al.*, 2002). TgIMC-1 is composed of ~30% valine and glutamic acid and shares a 12 amino acid VPV repeat with the articulins, a group of proteins that form a membrane skeleton in *Euglena* and other protists. IMC-1 orthologs have been recently identified in other apicomplexans and gene knock-outs indicate that the IMC network is critically important to shape maintenance, motility and invasion (Khater *et al.*, 2004).

### The conoid

Apicomplexan parasites are named for their distinctive polarized apex which contains a number of unique organelles that coordinate invasion of host cells. The apical complex contains secretory organelles (micronemes and rhoptries) which release soluble components that facilitate host cell invasion (Black and Boothroyd, 2000; Morrissette and Sibley, 2002a). The apical complex of some apicomplexans, including *Toxoplasma*, also includes the conoid, a pointed or cone-shaped structure at the extreme apex of these parasites (Hu *et al.*, 2002b; Nichols and Chiappino, 1987). The conoid has a diameter of 380 nm, and consists of fibers wound into a spiral. There are two ~400 nm long, closely associated microtubules in the center of conoid (Figure 27.1B). These microtubules are tightly bound together and are eccentric to the longitudinal axis of the conoid due to their attachment to two preconoidal rings. Recent work indicates that the conoid filaments are a novel polymer form of tubulin (see below). Since only a subset of apicomplexan parasites have a conoid structure, it has been suggested that the conoid may play a mechanical role in invasion by parasites that must penetrate the robust barrier of the intestinal epithelium of vertebrates (Morrissette and Sibley, 2002a).



### Flagellar microtubules

Although apicomplexan parasites (including *Toxoplasma*) replicate asexually for the bulk of their life cycles, these parasites undergo differentiation to gametes that fuse to form a transient diploid zygote. The male gamete (microgamete) is flagellated and swims to the female gamete (macrogamete) in order to carry out fertilization. The anterior ends of apicomplexan microgametes are pointed and contain three basal bodies in close proximity to the apical pole (Scholtyseck *et al.*, 1972). The basal bodies nucleate three flagella that extend past the nucleus and away from the apical end. Two of the flagella are long and free from the gamete body. The third flagellum is shorter and is attached to the surface of the gamete at its anterior end. Additional microtubules originate in the region of the basal body and extend to the posterior end of the microgamete. In contrast to the atypical centrioles observed in other stages, the basal body of male gametes has a typical triplet microtubule structure with nine-fold symmetry and the flagellar axoneme contains a conventional “9+2” arrangement of doublet microtubules surrounding the central pair microtubules (Morrissette and Sibley, 2002a; Scholtyseck *et al.*, 1972).

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### Tubulin genes, tubulin drug binding, and tubulin structures

#### Tubulin genes and MAPs in *Toxoplasma*

Although *Toxoplasma* was originally thought to have single-copy  $\alpha$ - and  $\beta$ -tubulin genes, recent studies have exploited the *Toxoplasma* genome project to identify two additional  $\alpha$ - and two additional  $\beta$ -tubulin genes (Hu *et al.*, 2004b; Nagel and Boothroyd, 1988). The two new  $\beta$ -tubulin genes are 97% identical to the  $\beta$ 1-gene, but have a highly divergent 23 amino acid carboxy-terminal tail. The new  $\alpha$ -tubulin genes are significantly different from  $\alpha$ 1 (68% and 40% identical). In particular, the  $\alpha$ 3 isoform has an extra 40 amino acids at the carboxy-terminus. These different tubulin isoforms are expressed at quite different levels during different stages of the *Toxoplasma* life-cycle (Hu *et al.*, 2004b). One possible role for the recently discovered tubulin genes (particularly the  $\alpha$ -tubulin genes) is in the construction of specialized structures such as the conoid fibers or the flagellar apparatus of male gametes. The related apicomplexan parasite, *Plasmodium*, has two unlinked  $\alpha$ -tubulin genes (Akella *et al.*, 1988; Delves *et al.*, 1990; Holloway *et al.*, 1990; Holloway *et al.*, 1989). The  $\alpha$ -tubulin-I gene is expressed throughout the parasite differentiation cycle. The  $\alpha$ -tubulin-II gene is also required throughout the lifecycle, but is especially highly expressed in male gametes (Kooij *et al.*, 2005; Rawlings *et al.*, 1992). A monoclonal antibody that specifically identifies  $\alpha$ -tubulin-II labels the flagellar axoneme of male gametes and mutation of  $\alpha$ -tubulin-II causes defective male gametogenesis (Guinet *et al.*, 1996; Rawlings *et al.*, 1992).

Microtubule associated proteins (MAPs) are critically important to the highly organized structure of *Toxoplasma* and other apicomplexan parasites. Diffraction of isolated subpellicular microtubules from *Toxoplasma* suggests that they are heavily coated with a MAP that bind at 32 nm intervals (Morrissette *et al.*, 1997). Fourier analysis of conoid fibers also suggests association with additional proteins (Hu *et al.*, 2002b). Monoclonal antibodies that identify a putative *Toxoplasma* MAP associated with subpellicular microtubules cross-react with *Plasmodium*, suggesting that these proteins are conserved within

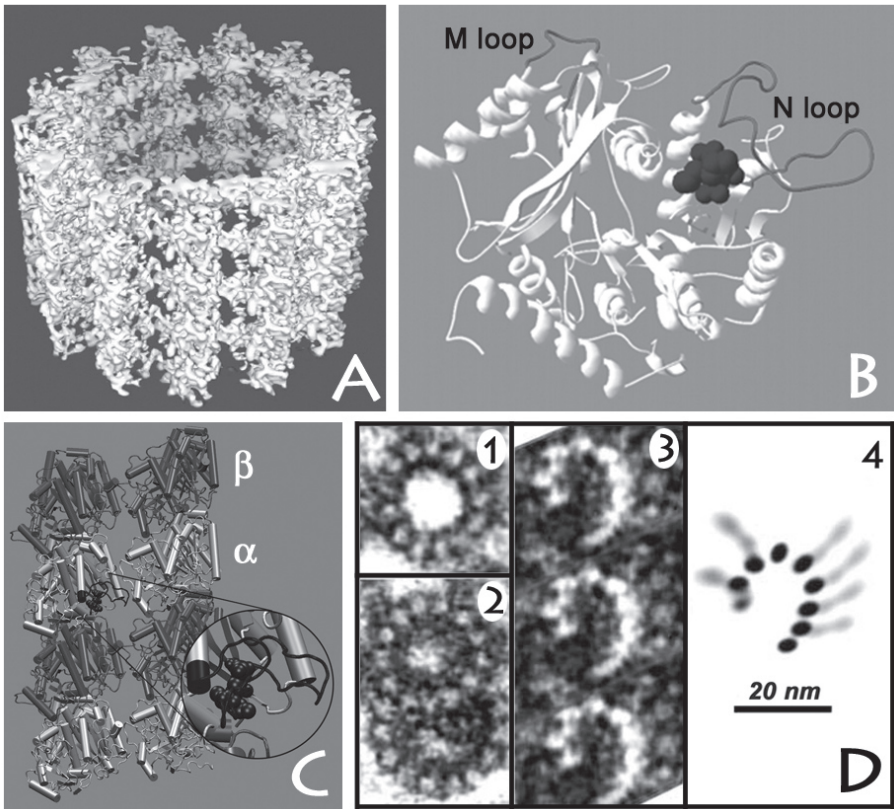
the Apicomplexa (Morrisette *et al.*, 1994; Morrisette and Roos, 1998). The *Toxoplasma* genome project has sequences annotated as encoding kinesin and dynein microtubule motors as well as the plus-end microtubule binding protein EB1 (for more details see Chapter 28 on molecular motors). Proteomic analysis of the isolated *Toxoplasma* cytoskeleton using MudPIT (Multidimensional Protein Identification Technique) analysis has identified many new proteins associated with the *Toxoplasma* cytoskeleton. These include TgDLC, a dynein light chain that localizes to the ends of microtubules and conoid fibers, two calmodulin-like proteins that target exclusively to the conoid and TgCentrinII which localizes to the centrioles and the pre-conoidal rings (Hu *et al.*, 2004a; Hu *et al.*, 2005; Hu *et al.*, 2006; Nishi *et al.*, 2005).

### Tubulin sensitivity to dinitroanilines

Microtubules typically contain 13 protofilaments, a substructure formed by the longitudinal head to tail association of  $\alpha$ - $\beta$ -tubulin heterodimers (Nogales, 2000). The lateral association of protofilaments forms a microtubule, which consists of a 24 nm wide cylinder with a hollow lumen (Figure 27.2A). Both  $\alpha$ - and  $\beta$ -tubulin contain conserved M and N loops which mediate interactions between adjacent protofilaments and both subunits bind GTP.  $\alpha$ -tubulin bound GTP is non-hydrolyzable and non-exchangeable, but  $\beta$ -tubulin hydrolyzes GTP in a polymerization-dependent fashion. Hydrolysis causes a conformational change in the location of the  $\beta$ -tubulin M loop that decreases the affinity of dimers for each other, increasing the likelihood of tubule disassembly. The importance of protofilament–protofilament interactions for microtubules is illustrated by the action of the microtubule-stabilizing drug taxol (Snyder *et al.*, 2001).  $\alpha$ -tubulin contains an eight amino acid insert missing from the analogous region of  $\beta$ -tubulin which stabilizes the  $\alpha$ -tubulin M-loop to promote lateral association between protofilaments. Taxol binds to  $\beta$ -tubulin and mimics the  $\alpha$ -tubulin insert, stabilizing the  $\beta$ -tubulin M loop independent of bound nucleotide state (Snyder *et al.*, 2001). As described below, dinitroanilines act in a converse fashion by disrupting the association of the  $\alpha$ -tubulin N loops to inhibit protofilament association and therefore prevent microtubule polymerization (Morrisette *et al.*, 2004).

Microtubule polymerization can be affected by a wide variety of compounds that shift the equilibrium between tubulin dimers and polymers to destabilize or stabilize microtubules (Jordan *et al.*, 1998). Tubulins from different organisms often display distinct susceptibilities to these drugs due to subtle differences in tubulin amino acid sequence. Dinitroanilines (oryzalin, ethafluralin and trifluralin) have been used in herbicide formulations for many years. These compounds disrupt plant and protozoan microtubules, including those in *Trypanosoma* spp., *Leishmania* spp., *Entamoeba* spp., *Plasmodium falciparum*, *Cryptosporidium parvum* and *Toxoplasma gondii* (Bogitsh *et al.*, 1999; Chan and Fong, 1990; Chan *et al.*, 1991; Makioka *et al.*, 2000; Morrisette and Roos, 1998; Stokkermans *et al.*, 1996; Traub-Cseko *et al.*, 2001). Dinitroanilines are ineffective against vertebrate or fungal microtubules and do not bind to these tubulins (Edlind *et al.*, 1996; Hugdahl and Morejohn, 1993; Morejohn *et al.*, 1987; Murthy *et al.*, 1994).

Dinitroaniline-resistance in *Toxoplasma* is universally associated with  $\alpha$ -tubulin mutations and a wide variety of single point mutations confer resistance. Over 30 different  $\alpha$ -tubulin mutations have been identified in dinitroaniline-resistant *Toxoplasma* lines



**Figure 27.2** Tubulin structures in *Toxoplasma*. (A) Microtubules typically contain 13 protofilaments, a substructure formed by the head to tail association of  $\alpha$ - $\beta$ -tubulin heterodimers and the lateral association of protofilaments forms a 24 nm wide cylinder with a hollow lumen. (B) Dinitroaniline compounds such as oryzalin bind to the  $\alpha$ -subunit of the tubulin dimer beneath the N loop. (C) Oryzalin binding beneath the  $\alpha$ -tubulin N loop disrupts its interaction with the M loop of the adjacent protofilament. (D) Cross-sections of a conventional 13 protofilament singlet microtubule (1) and a flagellar doublet microtubule (2) are compared with the nine protofilament tubulin ribbon of the conoid filaments (3). A schematic of the tubulin ribbon and associated protein “arms” is shown in (4). Panel A is from (Li *et al.*, 2002), panel C is from (Morrisette *et al.*, 2004) and panel D is from (Hu *et al.*, 2002b). Panel B is an unpublished image by the author.

(Morrisette *et al.*, 2004; Morrisette, unpublished data). Transformation with the individual point mutation genes is sufficient to confer resistance to oryzalin in *Toxoplasma*. In pseudodiploids, wild-type and resistant  $\alpha$ -tubulins are co-expressed, leading to low levels of oryzalin resistance (0.5  $\mu$ M). Allelic replacement of wild-type tubulin with a gene bearing a point mutation confers resistance to higher (1–5  $\mu$ M) concentrations of oryzalin. The individual substitutions are distributed throughout the linear sequence of  $\alpha$ -tubulin but when mapped onto a model of *Toxoplasma* tubulin based on the crystal structure of bovine tubulin they cluster in three regions: the M loop, the N loop and a core region. These mutations are predicted to fall into two categories based on their underlying mechanism

of action. The mutations that localize to the M and N loops are hypothesized to increase dimer-dimer affinity within the microtubule lattice. These mutant tubulins may continue to bind oryzalin, but the increased affinity of the tubulin subunits will compensate for drug destabilization. Other  $\alpha$ -tubulin mutations cluster in a core region. Six of these mutations are in amino acids that define the dinitroaniline binding site identified by computational methods and the other point mutations are located directly behind this predicted binding site. Mutant  $\alpha$ -tubulins with altered core residues are predicted to have decreased affinity for dinitroanilines due to a directly or indirectly altered drug binding site.

Computational modeling and analysis of resistance mutations in *Toxoplasma* indicate that dinitroanilines bind to and act on  $\alpha$ -tubulin. Dinitroanilines docked to multiple conformations of *Toxoplasma* tubulin derived from a molecular dynamics simulation predict a consistent binding site with an affinity of approximately 23 nM. This value is in agreement with experimentally determined values obtained with purified plant tubulin (Hugdahl and Morejohn, 1993; Murthy *et al.*, 1994). The dinitroaniline binding site is defined by Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243 and Phe244 (Figure 27.2B). Several resistance mutations (Val4Leu, Phe24His, His28Gln, Thr239Ile and Arg243Cys or Arg243Ser) map to residues within this site (Morrissette *et al.*, 2004; Morrissette, unpublished observations). Parallel analysis of vertebrate tubulin does not identify an analogous binding site on vertebrate  $\alpha$ -tubulin, consistent with the selective action of these compounds.

Both the binding site and the mutations indicate a mechanism for microtubule disruption by dinitroanilines. The dinitroaniline binding site is situated beneath the N loop between protofilaments in the microtubule (Figure 27.2C). Dinitroaniline binding beneath the N loop is likely to inhibit protofilament-protofilament interactions, causing microtubule disruption. Recent molecular dynamics simulations of parasite  $\alpha$ -tubulin alone or bound to a dinitroaniline indicates that in the absence of drug the N loop is highly mobile, but when the drug binds, this motility is highly curtailed and the N loop is drawn in closer to the core of the protein (Mitra and Sept, 2006). These data represent the first case of a drug that acts on  $\alpha$ - rather than  $\beta$ -tubulin function. Since micromolar concentrations of dinitroanilines selectively inhibit growth of many protozoan parasites, it is clear that small molecules can discriminate between vertebrate and protozoan tubulins, offering the promise of microtubules as a therapeutic target for parasitic infections.

*Toxoplasma* tubulin forms microtubules and nine-protofilament ribbons. *Toxoplasma* spindle and subpellicular microtubules are constructed of typical 13 protofilament microtubules. The two inter-conoidal microtubules also contain 13 protofilaments, but the conoid itself is constructed of a novel tubulin polymer (Hu *et al.*, 2002b). As described above, the conoid is a unique filamentous structure found at the extreme apex of some apicomplexan parasites, including *Toxoplasma* (Figure 27.1B). The conoid is composed of fourteen 430 nm long fibers that follow a left-handed helical path to complete a half turn from the apex to the base of the conoid. Although the conoid contains tubulin, conoid filaments are not arranged in a closed tubule but exist as a nine protofilament ribbon that is folded into a non-symmetric “comma” shape (Figure 27.2D). The concave side of these filaments faces the conoid interior, with the comma tail pointing toward the

conoid base. In images where the cross-sections of conoid fibers are averaged, the comma shape of the nine protofilaments is visible, as are additional arms, which likely represent MAPs (Figure 27.2D).

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## Role of microtubules in replication, polarity, shape, motility, and fertilization

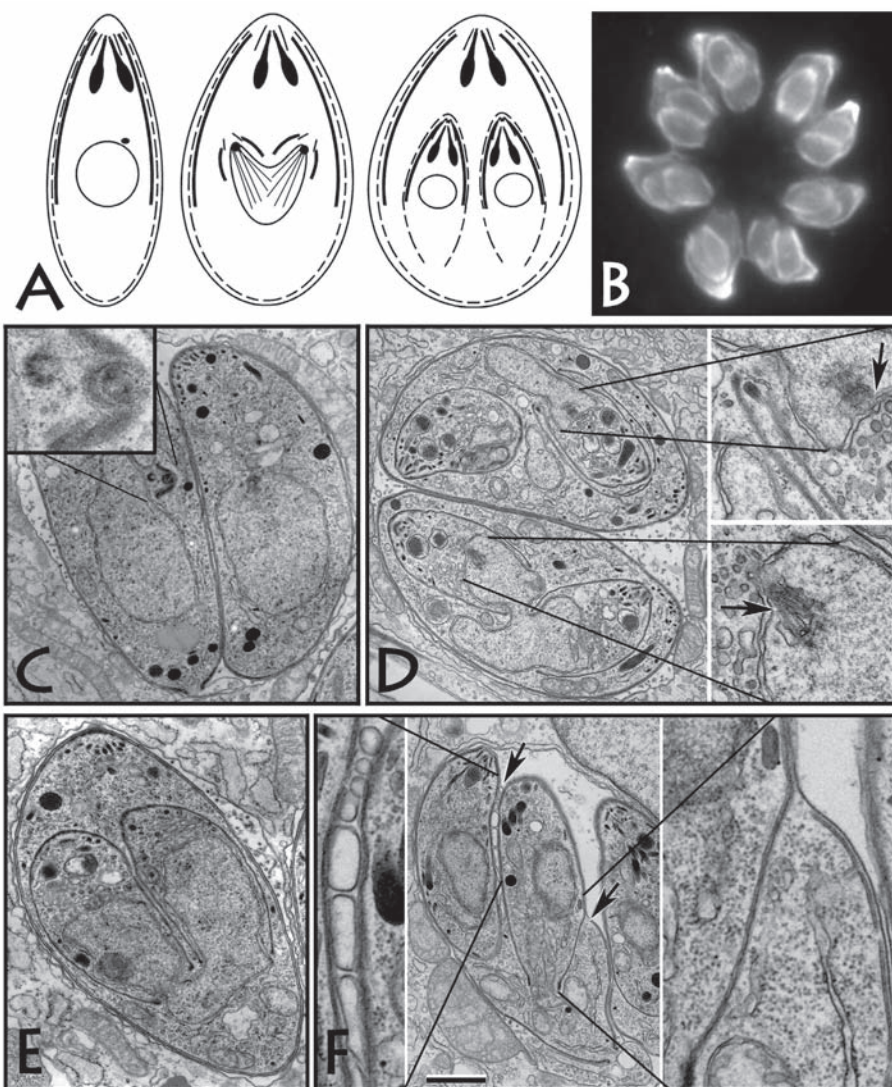
### *Toxoplasma* replication

Replication in *Toxoplasma* occurs by endodyogeny which creates two daughter parasites (Sheffield and Melton, 1968). Nuclear division is a cryptomitosis (the nuclear membrane remains intact) and karyokinesis (chromosome movement) occurs without chromosomal condensation (Morrisette and Sibley, 2002a). In endodyogeny, two daughter parasites are formed within an intact, fully polarized mother parasite (as schematically drawn in Figure 27.3A). This preserves the capacity of replicating *Toxoplasma* to invade throughout the cell cycle. The internal daughter cells are delimited by IMC and associated subpellicular microtubules and each contains (in addition to the nucleus, mitochondrion, Golgi and plastid) a complete set of apical organelles (conoid, rhoptries, micronemes) that are essential to invasion (Figure 27.3B). When the daughter cells are fully mature, the maternal apical complex is disassembled and the daughter parasites bud from the mother, adopting her plasma membrane (Figure 27.3F).

During endodyogeny, spindle microtubules form an intra-nuclear spindle to coordinate chromosome segregation (Morrisette and Sibley, 2002b). In contrast to the linear organization of most spindles, *Toxoplasma* spindle microtubules are inserted into the nucleus at an acute angle that persists during chromosome segregation (Figure 27.3D). *Toxoplasma* tachyzoites expressing YFP-tagged  $\alpha$ -tubulin have been used to measure the tubulin content of parasite spindles (Swedlow *et al.*, 2002). These measurements suggest that the spindle is approximately 1.2  $\mu\text{m}$  long and contains 10 or 11 microtubules. Since *Toxoplasma* has 14 chromosomes, this is approximately consistent with a mitotic spindle consisting of 1 microtubule per chromosome. *Toxoplasma* spindles originate in the nucleus and pass through an electron-dense invagination of the nuclear membrane to coordinate mitosis. The spindle poles are associated with cytoplasmic centrioles which have an unconventional shape, consisting of nine singlet microtubules surrounding a central tubule (Figure 27.1F). Although most centrioles have a “9 + 0” structure of triplet microtubules, centrioles containing singlet microtubules are found in *Caenorhabditis* testes and *Drosophila* embryos. However, *Toxoplasma* centrioles are still distinct since these other singlet centrioles lack a central tubule (Morrisette and Sibley, 2002a; Morrisette and Sibley, 2002b).

*Toxoplasma* centrioles contain centrin, a conserved centriole marker which has been exploited to follow centriole behavior during endodyogeny. Early in replication the centriole pair migrates from the apical side to the posterior side of the nucleus, separates and returns to the apical region (Hartmann *et al.*, 2006). *Toxoplasma* expresses a 33 kDa homolog of SF-assemblin (TgSFA), a fiber-forming protein which organizes basal body associated rootlet microtubules in green algae such as *Chlamydomonas*. In *Toxoplasma*, SFA localizes to centrioles shortly after duplication but moves to the tip of the developing daughter buds, suggesting that TgSFA has a role in the organization of nascent subpellicular microtubules





**Figure 27.3** *Toxoplasma* replication by endodyogeny. (A) A schematic of the steps in endodyogeny. Interphase *Toxoplasma* has a juxtanuclear centriole, the centriole duplicates and is found at the poles of the intranuclear spindle. Each pole is surmounted by a daughter apical complex consisting of a conoid, IMC and subpellicular microtubules. The IMC extends to enclose maturing daughter parasites. (B) Immunofluorescence with a *Toxoplasma* tubulin specific antibody reveals daughter cell buds within each mother parasite in a rosette. (C) Electron microscopy captures early endodyogeny showing a juxtanuclear daughter bud in close association with a centriole (enlarged in inset). (D) *Toxoplasma* spindle microtubules (arrows) are inserted into an invagination of the nuclear membrane. (E) Extension of the daughter buds encloses Golgi bodies and the dividing nucleus. (F) Thin section of daughter parasites emerging from the maternal cell at the completion of endodyogeny. In the outward-facing areas of the daughter cells, escape involves the coordinated dissociation of the maternal IMC from the plasma membrane and association of the daughter IMC onto the plasma membrane



(Lechtreck, 2003). *Toxoplasma* centrioles are associated with segregation of the apicoplast, a non-photosynthetic plastid, which carries out essential parasite functions (Striepen *et al.*, 2000). Apicoplast division is characterized by an elongated, dumbbell-shaped intermediate, with the apicoplast ends linked to the separating centrioles. Force generated by the mitotic spindle and the IMC of forming daughter cells mediates organelle scission.

Daughter bud growth proceeds from the apical end, encapsulating the endoplasmic reticulum and duplicated Golgi as the IMC and associated subpellicular microtubules lengthen (Pelletier *et al.*, 2002). The MORN motif is characteristic of proteins that mediate membrane junctions and TgMORN1 participates in endodyogeny as a component of the apical complex, the spindle pole and a cap at the tip of the growing daughter parasites (Hu *et al.*, 2004a; Hu *et al.*, 2005). The IMC network is assembled at an early stage in the development of daughter parasites. Late in daughter cell development, proteolysis removes the carboxyl terminus of the major network component, TgIMC1, converting the network from a detergent-labile to a detergent-resistant state (Mann *et al.*, 2002). This transformation increases network stability, substituting an assembly competent, loose structure with a rigid configuration that provides tensile strength in the mature parasite.

*Toxoplasma* spindle microtubules and subpellicular microtubules have distinct susceptibilities to disruption, most likely as a consequence of the microtubule length required to build functional spindles or subpellicular microtubules (Morrisette and Sibley, 2002b). Although extracellular parasites are refractory to the effects of microtubule-disrupting drugs, during intracellular growth, parasite microtubules are dynamic and are sensitive to disruption by colchicine or dinitroanilines. *Toxoplasma* tachyzoites treated with 0.5  $\mu\text{M}$  oryzalin (a dinitroaniline) can form a spindle and undergo nuclear division but have truncated subpellicular microtubules. As a consequence of shortened subpellicular microtubules, parasites are round rather than elongated in shape and lose the ability to reinvade host cells. In 2.5  $\mu\text{M}$  oryzalin, *Toxoplasma* loses all microtubules and cannot carry out any microtubule-dependent functions including mitosis, although they continue to replicate DNA and synthesize proteins (Shaw *et al.*, 2000; Shaw *et al.*, 2001; Stokkermans *et al.*, 1996).

When *Toxoplasma* tachyzoites are removed from 0.5  $\mu\text{M}$  oryzalin, the parasites return to replicate as crescent-shaped, invasive normal parasites, indicating that spindle function is intact during drug treatment. When oryzalin is washed out from *Toxoplasma* tachyzoites treated with 2.5  $\mu\text{M}$  drug, subpellicular microtubules are restored and the parasites attempt to bud daughter parasites (Morrisette and Sibley, 2002b). Since the polyploid nuclear mass cannot be correctly segregated, daughter parasites contain large aggregates of DNA or lack nuclei altogether. *Toxoplasma* microtubule populations are individually nucleated by two distinct microtubule-organizing centers (MTOCs). The APR drives *Toxoplasma* budding, whereas nuclear division is controlled by the centrioles. This arrangement permits *Toxoplasma* to replicate while maintaining a fully differentiated phenotype.

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(arrow and enlarged inset). Between the two daughter cells, scission involves membrane fusion events to create new plasma membrane (arrow and enlarged inset). Scale bar = 1  $\mu\text{m}$ . Panel B, D and F are from (Morrisette and Sibley, 2002b) and panels A, C and E are unpublished images by the author.

Although this unusual organization grants greater cell cycle flexibility to *Toxoplasma*, it abolishes the checks for coregulation of nuclear division and cytokinesis found in other eukaryotes.

### *Toxoplasma* polarity, cell shape, motility, and invasion

*Toxoplasma* treatment with dinitroanilines disrupts parasite microtubules and allows us to consider microtubule functions in parasite survival. As in all eukaryotes, the loss of spindle microtubules is associated with disrupted mitosis (Morrissette and Sibley, 2002b; Shaw *et al.*, 2000; Shaw *et al.*, 2001; Stokkermans *et al.*, 1996). However, in low levels of dinitroaniline, *Toxoplasma* spindle microtubules are intact, but subpellicular microtubules are truncated and cannot impose a normal elongated shape (Morrissette and Sibley, 2002b). These resulting round parasites cannot invade host cells, indicating that an elongated shape or rigid pellicle is required for invasion. The complete disruption of subpellicular microtubules is associated with the loss of apical polarity (Morrissette and Roos, 1998; Stokkermans *et al.*, 1996). These results indicate that the subpellicular microtubules are essential for shape and polarity.

Motility and invasion by apicomplexan parasites is dependent upon the glideosome, an actomyosin based motile system that is linked to the capping and shedding of secreted adhesins (Keeley and Soldati, 2004; Soldati and Meissner, 2004). In contrast to many examples of myosin motility, recent evidence indicates that the *Toxoplasma* myosin motor remains fixed and the actin filaments move. In the current model of *Toxoplasma* motility, MyoA in complex with its associated light chain and two novel proteins localizes to the IMC facing the plasma membrane (Gaskins *et al.*, 2004). One of the novel proteins, TgGAP50, is an integral membrane glycoprotein which immobilizes MyoA with its myosin head domain projecting toward the plasma membrane. Since MyoA is immobilized by the IMC, the actin and adhesin complex moves, resulting in a forward translocation of the parasite. One correlate of the glideosome model is that immobilized myosin requires IMC rigidity to move actin filaments. *Toxoplasma* parasites with truncated subpellicular microtubules are polarized but are incapable of invading new host cells (Morrissette and Sibley, 2002b). One possible interpretation of this is that the subpellicular microtubules provide a rigid framework to immobilize myosin in the IMC and truncated microtubules do not provide sufficient rigidity. The IMC-1 network filaments also contribute to cell shape and rigidity for gliding motility. When the *Plasmodium* IMC1 ortholog is knocked out, the resulting parasites are mis-shapen with a displaced nucleus and greatly impaired motility and invasion (Khater *et al.*, 2004).

### Male gametes and fertilization

All apicomplexan parasites have a transient sexual stage as a feature of their life cycles (Morrissette and Sibley, 2002a). In *Toxoplasma*, differentiation to gametocytes (gamete precursors), microgametes (male) and macrogametes (female) only occurs in the intestinal epithelium of cats. In contrast, gamete differentiation of the related apicomplexan parasite *Plasmodium* is completed in the intestinal epithelium of the *Anopheles* mosquito vector and can proceed *in vitro*. Much greater advances have been made in understanding gametogenesis in *Plasmodium*, and since it is likely that many of these findings will also be true in *Toxoplasma*, the results are presented here.

*Plasmodium* gametocytes are triggered to transform into gametes by environmental cues and microgametes must locate and fertilize the macrogametes within 30 minutes of exflagellation. Gamete fusion results in the formation of a diploid zygote that undergoes meiosis and develops into a motile ookinete. Recent work has characterized the specific proteomes of *Plasmodium* gametes by using stage and sex-specific promoters to express GFP markers to specifically label *Plasmodium* microgametes and macrogametes for isolation by cell sorting (Khan *et al.*, 2005). Male gametes are the only parasite stage to build flagella for motility. Flagellar axoneme-associated proteins are overwhelmingly represented in the microgamete proteome. These conserved flagellar proteins include dynein heavy and light chains, kinesins, centrin, tctex1, radial-spoke proteins, outer dynein arm-docking-complex protein and PF16 (Khan *et al.*, 2005).

## Conclusions

The conservation of tubulins and microtubules in eukaryotic cells should imply that these proteins carry out similar functions in all settings. Although many properties of tubulin and microtubules are conserved (flagella and spindles), other functions (shape, polarity, motility and invasion) are specialized for the unique niche occupied by *Toxoplasma* and other apicomplexan parasites. Microtubules are a well-established therapeutic target for treatment of diverse medical conditions including cancer and parasitic nematode infection. The unusual organization, regulation and use of microtubules in *Toxoplasma* and other apicomplexan parasites distinguish parasite tubulin and microtubules from metazoan tubulin and microtubules. In the future, we may be able to exploit these distinctive properties of tubulin and roles for microtubules to develop novel anti-parasitic therapies.

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## Abstract

Molecular motors are biological engines that convert chemical energy into movement. Three types of motor molecules—myosins, kinesins, and dyneins—are ubiquitously found in eukaryotes and serve a multitude of fundamental biological functions such as locomotion, nuclear and cell division, and the intracellular transport of molecules, vesicles, and organelles. The almost complete genome sequence of *Toxoplasma gondii* reveals a repertoire of 11 myosin heavy chains, 15 kinesin heavy chains, and 10 dynein heavy chains, making this parasite the apicomplexan with the largest known inventory of all three motor types known to date. Of these, only a few *T. gondii* myosins have been experimentally investigated: TgMyoA for example has been shown to be indispensable for the gliding motility of tachyzoites, a form of cell locomotion that does not involve cellular shape change or cell protrusions and that is essential for the spreading through host tissues and for host cell invasion and egress, whereas two other myosins (TgMyoB/C) may be involved in parasite cell division. Here, we present an overview over all molecular motor heavy chains that can be gleaned from the *T. gondii* genome and summarize what is known about the few molecular motors on which research has been published. For the majority of *T. gondii* motor molecules that have not yet been studied experimentally we focus on their classification into existing classes and families and discuss possible functions based on this classification and on apparent protein domain structures. We conclude that *T. gondii* probably employs its diverse arsenal of molecular motors both for general functions like nuclear and cell division, flagellar movement, and organellar transport, as well as for apicomplexan-specific adaptations to their lifestyle as intracellular parasites such as gliding motility.

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## Molecular motors and their cytoskeletal tracks

Molecular motors are proteins that are able to convert chemical energy into movement, a feature they share with common man-made engines. Myosins, kinesins, and dyneins are the three major groups of eukaryotic molecular motors involved in biological motility and transport processes, and all three types of motors move along cytoskeletal tracks. The function of these biological motors is to move cargo molecules, vesicles, cell organelles, or whole cells, and they are thus involved in diverse biological processes such as mitosis and cell division, signal transduction, organellar positioning, and locomotion. In all cases, chemical energy released by the hydrolysis of ATP is converted into mechanical work, and

an initially small spatial displacement is mechanically amplified into a usable movement with a step size of up to 36 nm (in the case of myosin V) (Mehta *et al.*, 1999).

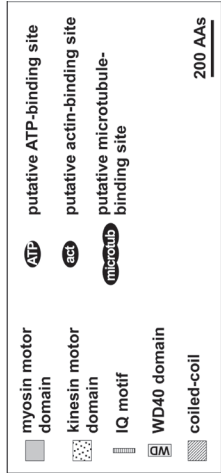
All molecular motors feature distinct protein domains. The actual motor domain with its inherent ATP-hydrolysis activity is situated in what is usually also referred to as the “head,” whereas other protein domains include “neck,” “tail” and/or “stalk” domains (see Figure 28.1A). The motors may be functionally active as monomers (e.g. class I myosins), dimers (e.g. class II myosins), or trimers (e.g. axonemal outer arm dyneins), and dimerization for example is often achieved by tail or stalk domains that are capable of forming coiled-coils (Schliwa and Woehlke, 2003).

Myosins move along actin filaments, whereas both kinesins and dyneins use microtubules as molecular tracks. Actin filaments (F-actin) consist of globular actin (G-actin) monomers and exhibit polarity with a fast growing plus-end and an oftentimes depolymerizing minus-end. In comparison to other organisms, actin of *T. gondii* polymerizes at relatively low G-actin concentration and generates very short filaments. For more information on *T. gondii* actin and actin dynamics please refer to Chapter 29. Microtubules are also polar assemblies with a growing plus-end and a shrinking minus-end, but—unlike actin—they represent hollow tubes that are made up of more than a dozen protofilaments which in turn consist of  $\alpha$ - and  $\beta$ -tubulin heterodimers. For more details on microtubules please see Chapter 27.

Some motors tend to dissociate rapidly—after every step—from their respective track, while others are capable of “walking” a considerable distance along a track without detaching from it. Such processive movement is often found in dimeric motors where the two molecules attach to and detach from their track asynchronously, ensuring that the dimer

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**Figure 28.1** Opposite. (A) Schematic representation of a myosin, a kinesin, and a dynein heavy chain of *T. gondii*. The “motor domain” in the three types of molecular motors is located in the “head” of myosins and kinesins and in the “head ring” of dyneins. Cargo binding is achieved by domains referred to as “tail” (myosins and dyneins) and “stalk” (kinesins). In dyneins, the microtubule-binding “stalk” protrudes from the heptameric head ring that is formed by the six AAA domains in conjunction with probably the C-terminus of the molecule. The first AAA domain contains a highly conserved ATP-binding P-loop motif that is probably responsible for the ATPase activity leading to movement. The nucleotide-binding sites of domains AAA2, AAA3, and AAA4 are less well conserved, while domains AAA5 and AAA6 lack a readily recognizable P-loop motif. The short neck of the *T. gondii* kinesin has been mapped according to (Endow, 1999), whereas the AAA and stalk regions of the dynein have been determined using the *Chlamydomonas reinhardtii* flagellar outer arm dynein  $\gamma$  (GenBank accession no. Q39575) as reference. (B) Schematic representation of the *T. gondii* myosin heavy chain repertoire. TgMyoB and TgMyoC represent differentially spliced products of the same gene and differ only in the C-terminus. Roman numbers indicate the classification into one of 24 myosin classes (see (Foth *et al.*, 2006)). See the text for details. (C) Alignment of putative IQ motifs of select apicomplexan class XIV myosins with the general IQ motif consensus sequence. All putative IQ motifs derive from the homologous position in the myosin neck. The conserved amino acid positions of the consensus are highlighted by arrow heads, and the two negatively highlighted arginines of the TgMyoA sequence denote the residues that are essential for correct interaction with the myosin light chain (Hettmann *et al.*, 2000). Abbreviations: AAA, ATPases associated with a variety of cellular activities; ATS1, alpha-tubulin suppressor 1; Bb, *Babesia bovis*; Cp, *Cryptosporidium parvum*; Et, *Eimeria tenella*; Gp, *Gregarina polymorpha*; Pf, *Plasmodium falciparum*; RCC1, regulator of chromosome condensation 1; Ta, *Theileria annulata*; Tg, *Toxoplasma gondii*; TH4, myosin tail homology 4.



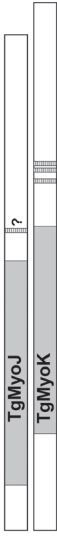
**Kinesin** (TgTwinScan\_1498, Kinesin-3)



**Dynein** (TgTwinScan\_2019, OADγ)



**B VI**



**XIV**



**XXII**



**XXIII**



**XXIV**



unclassified



TgMyoA	irAQahhRRhnlvdn
EtMyoA	irTQahhRRhivDh
PfMyoA	lrVQahhRRKmwAq
BbMyoA	vrLQahhRRhivEs
TaMyoA	vrLQahhRRvlnEg
CpMyoA	vrIQagLRRvldIe
GpMyoA	vrAQaltRRrlaGk
TgMyoD	trICanvRRklvQa
EtMyoD	srICahhRRkltQh
TgMyoB/C	trLEsnhRRhlePd
TgMyoE	vrIQsqiRKarvIm
TgMyoH	trVQslmRFYqIrK
EtMyoH	vrLQslmRFYqIrK
consensus	IQxxxxRxxxxK

does not completely dissociate from its track over considerable periods of time. Another difference is the directionality of the motor molecules: most myosins (except class VI) move towards the plus-end of actin, and most kinesins (except the kinesin-14 family) towards the plus-end of microtubules. In contrast, dyneins are generally minus-end-directed motors (Schliwa and Woehlke, 2003). But beyond this basic outline, molecular motors also have a tendency to mix and match *in vivo*, both functionally and structurally. Some biological functions e.g. are carried out cooperatively by different types of motors: both myosins and kinesins appear to be involved, either at the same time or sequentially, in the intracellular transport of certain organelles (Schliwa and Woehlke, 2003). On the other hand, some myosins are able to directly bind to microtubules (e.g. class X myosins via MyTH4 and FERM domains (Weber *et al.*, 2004)), while some kinesins directly interact with actin (Kuriyama *et al.*, 2002).

This chapter presents an overview over what is currently known or can be inferred from the almost completely sequenced genome of *T. gondii* regarding myosins, kinesins, and dyneins, and from a comparison with the fellow apicomplexan genera *Plasmodium*, *Theileria*, and *Cryptosporidium* whose full genome sequences are already available (Abrahamsen *et al.*, 2004; Gardner *et al.*, 2005; Gardner *et al.*, 2002). In particular, we will focus on the actual motor molecules, i.e. the heavy chains of these motors, and discuss accessory molecules such as light chains only briefly. Other proteins like helicases, translocases, and the F1-ATPase that are sometimes also referred to as molecular motors will not be considered here. For more general information on myosins, kinesins, and dyneins, the reader is referred to one of many excellent reviews (Asai and Wilkes, 2004; Berg *et al.*, 2001; Hirokawa, 1998; Hirokawa and Takemura, 2004; Koonce and Samsø, 2004; Miki *et al.*, 2005; Oiwa and Sakakibara, 2005; Pfister *et al.*, 2006; Sakato and King, 2004; Schliwa and Woehlke, 2003; Sellers, 2000; Vale, 2003; Vale and Milligan, 2000; Vallee *et al.*, 2004; Welte, 2004; Wozniak *et al.*, 2004; Yildiz and Selvin, 2005).

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## Actin-based motors: myosins

### The myosin repertoire

Myosin heavy chains commonly feature three domains: a well conserved head domain—the actual “motor”—that interacts with actin, exhibits ATPase activity, and generates movement, a short neck domain that commonly binds calmodulin or other myosin light chains via its IQ motif(s), and a highly variable tail domain that usually binds to the “cargo” and determines the functional specificity of the motor (Figure 28.1A). The *T. gondii* genome contains 10 genes encoding such myosin heavy chains. Since one of these genes gives rise to two gene products (TgMyoB/C) by differential splicing (Delbac *et al.*, 2001; Heintzelman and Schwartzman, 1997), *T. gondii* has a repertoire of at least 11 different myosin heavy chains at its disposal (Figure 28.1B) (Foth *et al.*, 2006). Three of these myosins (TgMyoA, TgMyoC, TgMyoD) have so far been shown to bind actin in an ATP-dependent manner, and TgMyoA and TgMyoD have also been demonstrated to possess ATPase activity, indicating that these molecules represent functional myosin motors (Delbac *et al.*, 2001; Heintzelman and Schwartzman, 1999; Herm-Götz *et al.*, 2006; Hettmann *et al.*, 2000).

One additional sequence (TgTwinScan\_0256) exhibits clear similarity to myosin heavy chains but is generally very divergent (only ~20%/40% sequence identity/similarity compared to the TgMyoA head domain) and in particular lacks sequence conservation in key motifs that are otherwise highly conserved also among apicomplexan myosins (e.g. the ATP- and actin-binding motifs, GESGAGKT and HFIRCIKPN, respectively). The existence of likely orthologs of this myosin-like protein e.g. in *P. falciparum* and *C. parvum* (GenBank accession numbers NP\_701557 and EAK89094) suggests that these molecules serve a conserved function throughout the Apicomplexa. Intriguingly, their significantly divergent nature indicates that they may act as static cytoskeletal elements rather than as molecular motors.

Since the *T. gondii* genome sequencing project is close to finished we do not expect the repertoire of myosin heavy chain genes to significantly increase in the future, but alternative gene splicing—which is frequently encountered in myosins (Arner *et al.*, 2003; Lin *et al.*, 2005; Standiford *et al.*, 1997)—may *de facto* cause a significant expansion of the range of myosin motors available to *T. gondii*. The 11 myosin heavy chains of *T. gondii* compare with six apparently *bona fide* myosins present in each of the genomes of *P. falciparum* and *Cryptosporidium parvum* (Abrahamsen *et al.*, 2004; Gardner *et al.*, 2002) and with three such molecules in *Theileria annulata* (Pain *et al.*, 2005) and represents the largest number of myosins found in any apicomplexan genome so far.

Previously, myosin heavy chains discovered in apicomplexans had been named independently of one another by various researchers (Chaparro-Olaya *et al.*, 2005; Heintzelman, 2004; Heintzelman and Schwartzman, 1997; Heintzelman and Schwartzman, 2001; Lew *et al.*, 2002; Matuschewski *et al.*, 2001), eventually leading to a rather confusing situation where in some cases the same letters designated non-orthologous myosins in different genera (e.g. TgMyoC and PfMyoC) (Chaparro-Olaya *et al.*, 2005). To remedy this situation, we have recently proposed a systematic naming convention for apicomplexan myosin heavy chains with the aim to designate orthologous myosins across all apicomplexans by the same capital letter (e.g. TgMyoA, PfMyoA, etc.) (Foth *et al.*, 2006). This scheme is based on orthology to the *T. gondii* myosins—for the simple reason that *T. gondii* features the largest myosin repertoire of any apicomplexan examined so far—and has led to the renaming of a few myosins of *P. falciparum*, *Theileria parva* (Table 28.1), and *Babesia bovis* (Foth *et al.*, 2006).

## Myosin phylogeny and classification

Myosin heavy chains are commonly classified based on phylogenetic analysis of their conserved head domain which usually yields more than 650 amino acid positions that can be used for such analyses. Currently, there are 24 myosin classes recognized (Foth *et al.*, 2006), with class II myosins, the myosin type responsible for muscle contraction, being referred to as “conventional” myosins. Three myosins of *T. gondii* (TgMyoA and the differential splice products TgMyoB and TgMyoC) were the first apicomplexan myosins to be described in detail and were classified in their own class XIV (Heintzelman and Schwartzman, 1997). Orthologs of TgMyoA have since been found in all available apicomplexan genomes, whereas TgMyoB/C is only known from *T. gondii* and its fellow coccidians *Neospora caninum* (accession number CF371489) and *Eimeria tenella* (Foth

**Table 28.1** Myosin heavy chains of *Toxoplasma gondii*

Myosin class <sup>a</sup>	Myosin sub-class <sup>a</sup>	Name <sup>a</sup>	Accession Number or ToxoDB gene model	Ortholog in <sup>b</sup> <i>P. falciparum</i> <i>T. annulata</i>	
VI	–	TgMyoJ	TgTwinScan_6580	AAN36373 (PFL1435c)	–
	–	TgMyoK	TgTwinScan_3990	AAN35999 (PF11_0416)	–
XIV	XIVa	TgMyoA	AAC47724	CAD52556 (PF13_0233)	CAI73593
		TgMyoD	AAD21243	–	–
	XIVb	TgMyoB TgMyoC	AAL30896 AAL30895	–	–
		TgMyoE	AAF25495	–	–
	XIVc	TgMyoH	TgTwinScan_6293	–	CAI72989
XXII	–	TgMyoF	DQ131541	CAD52416 (MAL13P1.148)	CAI73326
XXIII	–	TgMyoG	DQ131540	–	–
XXIV	–	TgMyoI	TgTwinScan_5760	–	–
Functional myosin motor?			TgTwinScan_0256	NP_701557? (PFL0975w)	CAI74579 and CAI74578? (Tp: EAN32688)

<sup>a</sup>Myosin classification and nomenclature according to Foth *et al.* (2006).  
<sup>b</sup>Listed are accession numbers for orthologs found in *Plasmodium falciparum*, *Theileria annulata* (Tp refers to *Theileria parva*), and *Cryptosporidium parvum*. PlasmoDB identifiers (<http://plasmodb.org>) are given in parentheses for *P. falciparum*.  
<sup>c</sup>Refers to the former name of some *P. falciparum* and *T. parva* myosins that have recently been renamed such that orthologous myosins are designated by the same letter across different apicomplexan genera (Foth *et al.* 2006) (with the only exceptions of PfMyoB and PfMyoE that are



<i>C. parvum</i>	Former name <sup>c</sup>	IQ motifs	Myosin tail domains <sup>d</sup>	Comments
EAK89304	MyoD	0	–	Metazoan class VI myosins: monomer and/or dimer; move towards minus-end of actin filaments; various possible functions including endocytosis, maintaining Golgi complex morphology, vesicle formation and transport, mitosis, and cell migration (by plasma membrane protrusions)
EAK87907	MyoF	2–3	(coiled-coil)	
EAK90372	–	1	No tail	TgMyoA is essential for gliding motility, host cell invasion and egress
–	–	0	No tail	Possible function for gliding motility in bradyzoites?
–	–	0–1?	–	Probably involved in cell division; more specifically, TgMyoC may function in the formation of the inner membrane complex during cell division
–	–	1	No tail	Function unknown
EAK90414	MyoB	8	ATS1/RCC1	Yeast ATS1 and mammalian RCC1 have been implicated in cell division-related functions
EAK90169	MyoC	3–6	WD40	WD40 repeats are thought to serve as site for protein–protein interactions and in the formation of multi-protein complex assemblies
–	–	1	MyTH4	In conjunction with a FERM domain, the MyTH4 domain may directly bind to microtubules
CAD98475	–	2	(N-terminal SH3-like) coiled-coil	Function unknown
EAK89094?	–	4	ATS1/RCC1	Head domain is very divergent suggesting that it may not be functioning as a myosin motor

**not** orthologs of TgMyoB and TgMyoE, respectively).

<sup>a</sup>As predicted for the respective *T. gondii* myosin by SMART (<http://smart.embl-heidelberg.de/>) and the Conserved Domain Database and Search Service, v2.06 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>); abbreviations and accession numbers (SMART, COG, and Pfam databases): ATS1=alpha-tubulin suppressor 1, COG5184; MyTH4=myosin tail homology 4, SM00139; N-terminal SH3-like, PF02736; RCC1=regulator of chromosome condensation 1, PF00415; WD40, SM00320.

*et al.*, 2006). Two more *T. gondii* myosins were identified by Hettmann and colleagues (TgMyoD, TgMyoE) and were quickly recognized as closely related class XIV myosins (Hettmann *et al.*, 2000; Hodge and Cope, 2000). Class XIV was then subdivided into two groups, with TgMyoA, its orthologs from other apicomplexans, and the closely related TgMyoD comprising subclass XIVa, and the *T. gondii* myosins TgMyoB/C and TgMyoE being placed in subclass XIVb (Lew *et al.*, 2002).

Eventually, the significant advancement of the *T. gondii* genome sequencing initiative revealed that this parasite harbors six more myosins (TgMyoF-K). These new myosins and their homologs from other apicomplexans proved to be surprisingly resilient to being classified into the existing myosin classification scheme, and several methodological adjustments were necessary to arrive at reproducible phylogenetic trees that could place these myosins into groups with significant statistical support (Foth *et al.*, 2006). We found that the phylogenetic analysis of myosin head domains was improved considerably by including a large number of sequences, by carefully identifying and excluding from the analysis “rogue” sequences that could specifically disrupt certain clusters in the phylogeny because of their divergent nature (Sanderson and Shaffer, 2002), and by employing more sophisticated phylogeny inference software (e.g. MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) or PHYLIP’s ProtDist (Felsenstein, 2004) in combination with the Jones-Taylor-Thornton model of amino acid substitution (Jones *et al.*, 1992)) that takes the empirical likelihood of change from a given amino acid to any other amino acid into account (Foth *et al.*, 2006). As had already been suspected for some myosins of *Plasmodium* (Chaparro-Olaya *et al.*, 2005; Lew *et al.*, 2002), five of the six most recently discovered *T. gondii* myosins turned out not to belong to the “classical” apicomplexan myosin class XIV. Instead, two myosins (TgMyoJ, TgMyoK) were placed in the highly intriguing myosin class VI, while the three myosins TgMyoF, TgMyoG, and TgMyoI are among the founding members of the three newly established myosin classes XXII, XXIII, and XXIV (Table 28.1) (Foth *et al.*, 2006). Only TgMyoH was again classified as a class XIV myosin, albeit as a distinctly different one that—together with orthologs from *Eimeria*, *Cryptosporidium*, *Theileria*, and *Babesia*—falls into its own new subclass XIVc (Table 28.1) (Foth *et al.*, 2006). Interestingly, two of these newly classified *T. gondii* myosins (TgMyoF and TgMyoH) exhibit protein domains in their tail domains (WD40 and ATS1/RCC1) that had not been encountered as part of myosin heavy chains before (see section *Myosin structure and function*).

In contrast to the earlier notion that apicomplexan myosins are so distinct that they belong to one (or more) class(es) exclusively made up of apicomplexan motors, our phylogenetic analyses place some apicomplexan sequences in myosin classes for which representatives are also known from other taxonomic groups. In addition to the respective apicomplexan myosins, classes VI, XIV, and XXII also feature myosins from metazoan animals, from the ciliate *Tetrahymena thermophila*, and from the diatom alga *Thalassiosira pseudonana*, respectively (Foth *et al.*, 2006). The high similarity of apicomplexan myosins with those of ciliates and diatoms does not come as a surprise since it has recently emerged that the Apicomplexa are probably close relatives of both ciliate protozoa and a group of organisms called the Chromista, a heterogeneous assemblage of mostly unicellular algae that also includes the diatoms. Together with dinoflagellate algae, the apicomplexans are

thus thought to be part of the ancient evolutionary lineage Chromalveolata (Adl *et al.*, 2005; Bhattacharya *et al.*, 2004; Cavalier-Smith, 1999; Cavalier-Smith, 2000; Harper *et al.*, 2005; Yoon *et al.*, 2002).

## Myosin structure and function

### *Class VI: TgMyoJ and TgMyoK*

Class VI is one of the relatively few myosin classes that have been studied in great detail. Studies on the corresponding motors of humans and animals have shown that these are the only myosins known so far to move towards the minus-end of actin filaments, not the plus-end as is usually the case (Buss *et al.*, 2004). *In vitro*, these myosins have been found to be able to function both as a non-processive monomeric motor and as a processive dimer, and their mode of action *in vivo* may be dependent on cargo load (Park *et al.*, 2006). In addition to a frequently encountered N-terminal SH3-like domain of unknown function and coiled-coil forming stretches in the tail domain, the characteristic feature of metazoan class VI myosins is a ~50 amino acid insertion between the head domain and the single IQ motif that is responsible for the minus-end-directed motility (Menetrey *et al.*, 2005). Do the apicomplexan class VI myosins possess a homologous sequence insertion between head domain and IQ motif?

As has been shown for TgMyoA, apicomplexan myosins appear to bind myosin light chains courtesy of “degenerate” IQ motifs that do not fully conform to the usual motif consensus (IQxxxRGxxxR/K; Figure 28.1C) (Herm-Gotz *et al.*, 2002; Hettmann *et al.*, 2000), making it difficult to reliably predict potential IQ motifs in apicomplexan myosins. Furthermore, some of the currently identified apicomplexan class VI myosins (including those of *T. gondii*) have not yet been annotated experimentally by cDNA sequencing. Despite these uncertainties we note that the head domains of TgMyoJ, TgMyoK, and of several orthologous apicomplexan myosins are predicted to be separated from the first IQ motif by an insertion of between ~50 and several hundred amino acids length (our unpublished data). Yet these predicted insertions do not share any significant sequence conservation with those of the metazoan class VI myosins, casting doubt on whether these insertions are actually homologous. This certainly also leaves the question unanswered whether the apicomplexan class VI myosins move towards the plus- or minus-end of actin filaments. Metazoan class VI myosins have been implicated in a number of cellular functions such as endocytosis, maintenance of Golgi complex morphology, vesicle formation and transport, mitosis, and cell migration via plasma membrane protrusions (filipodia, lamellipodia) (Buss *et al.*, 2004), but the function of their apicomplexan counterparts still awaits investigation.

### *Class XIVa: TgMyoA, gliding motility, and host cell invasion*

Apicomplexan parasites are capable of gliding motility on solid substrates, i.e. cell locomotion that does not involve cellular shape change or cell protrusions (see e.g. Hakansson *et al.*, 1999). This ability also allows the parasites to actively enter and egress from host cells, and to spread through host tissues. It had been suggested early on that an actin-myosin system might be involved in this process (Endo *et al.*, 1988; King, 1981; King, 1988;

Russell, 1983; Russell and Sinden, 1981; Schwartzman and Pfefferkorn, 1983; Yasuda *et al.*, 1988), and many studies employing drugs that interfere with actin dynamics (e.g. cytochalasins, jasplakinolide, and latrunculin B) or inhibit myosin ATPase function (e.g. BDM = 2,3-butanedione monoxime) confirmed for several apicomplexan genera that this was indeed the case (Chen and Fan-Chiang, 2001; Dobrowolski *et al.*, 1997; Dobrowolski and Sibley, 1996; Hakansson *et al.*, 1999; Matuschewski *et al.*, 2001; Poupel and Tardieux, 1999; Schwartzman and Pfefferkorn, 1983; Shaw and Tilney, 1999; Stewart and Vanderberg, 1991; Wetzel *et al.*, 2005). Of this multitude of experiments, work carried out with *T. gondii* was particularly revealing as to the molecular players that power apicomplexan gliding motility and host cell invasion.

First, elegant experiments employing either mutant, cytochalasin D-resistant *T. gondii* parasites or cytochalasin D-resistant host cells unambiguously proved that it is the parasite's actin (and not the actin of the host cell) that is essential for host cell invasion (Dobrowolski and Sibley, 1996). In particular, it is the sole "conventional" actin of *T. gondii* (ACT1) (Gordon and Sibley, 2005) that is responsible for the susceptibility of host cell invasion to the inhibitory effect of cytochalasin D (Dobrowolski and Sibley, 1996). Second, using a tetracycline-inducible gene expression system in *T. gondii* that allows to generate conditional knockouts even of essential genes, Meissner *et al.*, clearly demonstrated that the myosin motor TgMyoA is essential for gliding motility, host cell invasion, and egress of tachyzoites, and—not surprisingly—also for virulence in mice (RH strain). In contrast, it did not affect intracellular replication or secretion of micronemal proteins (Meissner *et al.*, 2002).

TgMyoA is a very small myosin heavy chain (93 kDa) that belongs to class XIVa (Foth *et al.*, 2006; Lew *et al.*, 2002). It is a fast, single-headed myosin with kinetic and mechanical characteristics that are similar to fast skeletal muscle myosins (class II) and has been shown to move in steps of 5.3 nm and at a velocity of 5.2  $\mu\text{m/s}$  towards the plus-end of actin filaments (Herm-Gotz *et al.*, 2002). Its movement along actin is non-processive with a low duty ratio, i.e. it spends a short time bound strongly to actin filaments during its ATPase cycle (Herm-Gotz *et al.*, 2002). It lacks any significant tail domain but, courtesy of a degenerate IQ motif in its neck (Figure 28.1C) (Herm-Gotz *et al.*, 2002), binds to a myosin light chain that has some similarity to calmodulin (TgMLC1; see section *Myosin accessory molecules*). This TgMyoA neck domain and in particular the two arginine residues within the putative IQ motif (Figure 28.1C) are essential for the distinct subcellular localization of this motor at the cell periphery of tachyzoites (Hettmann *et al.*, 2000).

Both TgMyoA and TgMLC1 are part of the so-called "glideosome," the complex molecular machinery that integrates the motility-specific actin-myosin system with the remainder of the cytoskeleton and controls its activity (Keeley and Soldati, 2004; Opitz and Soldati, 2002). As such, it encompasses the myosin motor, the actin track on which the motor moves, as well as various bridging molecules that link the myosin motor and the actin track to the rest of the parasite's cytoskeleton and the extracellular substrate, respectively. Considering their functional role during invasion, even proteases like e.g. MPP1 and MPP2 (Carruthers *et al.*, 2000) may be considered part of a broadly defined glideosome (Opitz *et al.*, 2002). Two novel glideosome-associated proteins, TgGAP45 and TgGAP50, have recently been identified (see also section *Myosin accessory molecules*), with

the integral membrane protein TgGAP50 serving as the “receptor” that anchors a pre-assembled complex consisting of TgMyoA, TgMLC1, and TgGAP45 in the inner membrane complex (IMC) (Gaskins *et al.*, 2004). We suggest to refer to this heterotetrameric complex (TgMyoA, TgMLC1, TgGAP45, TgGAP50) as the MyoA “motor complex” to distinguish it from the initially more broadly defined glideosome (Opitz *et al.*, 2002).

Importantly, for gliding motility to occur, the current model proposes that the MyoA motor complex is somehow firmly anchored to the subpellicular microtubule cytoskeleton of the parasite (the details of this interaction are currently unknown), while actin is firmly attached to a solid substrate outside the cell via adhesive proteins (e.g. microneme protein TgMIC2) and connecting linker proteins (e.g. aldolase) (Soldati *et al.*, 2004). In this constellation, movement of TgMyoA relative to actin leads to locomotion of the whole parasite. This same process is also responsible for host cell invasion, except that here the interaction of TgMyoA with the adhesin/linker/actin complex concentrates on a ring-like structure around the circumference of the parasite that forms a tight connection with the host cell that is being invaded. This moving junction is formed between the apical tip of the parasite and the host cell plasma membrane at the start of the invasion process, and movement of TgMyoA relative to actin translocates the moving junction from the apical to the posterior end of the parasite, thereby mechanically forcing the parasite into the host cell. For further details regarding the glideosome and the invasion process in general, the reader is referred to the other chapters of Part 6 in this book or to one of many recent reviews (Carruthers and Blackman, 2005; Fowler *et al.*, 2004; Kappe *et al.*, 2004; Keeley and Soldati, 2004; Sibley, 2004; Soldati and Meissner, 2004). Two short movies are available online that illustrate the current model of apicomplexan host cell invasion (Soldati *et al.*, 2004) (doi:10.1016/j.pt.2004.09.009).

In summary, TgMyoA currently represents the best-understood apicomplexan myosin motor, and it has been firmly established that it is, in conjunction with actin, essential for gliding motility, host cell invasion, and egress of *T. gondii* tachyzoites. MyoA appears to be abundantly expressed throughout all life stages in both *T. gondii* and *P. falciparum*, but it is not yet clear whether other molecular motors also contribute to cell motility—perhaps specifically in other stages of the life cycle—and whether or how they may possibly contribute to the ability of *T. gondii* to generate three different types of motion, i.e. circular gliding, upright twirling, and helical rotation (Hakansson *et al.*, 1999).

#### Class XIVa: TgMyoD

Myosin D of *T. gondii* is structurally very closely related to TgMyoA with the two motors sharing 55% and 70% amino acid sequence identity and similarity, respectively. MyoD probably derives from a MyoA gene duplication event in the Coccidia since it has so far been found only in *T. gondii* and *Eimeria tenella* (on contig00020965 of Sanger's assembly\_2005\_10\_13). As is the case with TgMyoA, its localization around the tachyzoite cell periphery (which is somewhat less sharply defined than in TgMyoA) is effected by its C-terminal neck domain, and its kinetic characteristics are similar to those of fast skeletal muscle myosin (Herm-Gotz *et al.*, 2006; Hettmann *et al.*, 2000). But both semi-quantitative RT-PCR and Western blot analysis show that TgMyoD expression in bradyzoites is approximately 10-fold higher than in tachyzoites (Delbac *et al.*, 2001; Herm-Gotz *et*

*al.*, 2006). Interestingly, the two arginine residues of TgMyoA's putative, near C-terminal IQ motif (see above) are conserved in the corresponding sequence of TgMyoD (Figure 28.1C), although this potential TgMyoD IQ motif does not even contain a glutamine in the second position (it contains a cysteine instead). It is unclear what the consequences of such amino acid changes on the potential binding of myosin light chains might be. But given that *T. gondii* contains five putative myosin light chains (see section *Myosin accessory molecules*) one may speculate whether TgMyoD interacts with a different light chain than TgMyoA—perhaps one that is bradyzoite-specific—and whether this could account for the not completely convincing subcellular localization observed in experiments that were performed on tachyzoites (Hettmann *et al.*, 2000).

In stark contrast to TgMyoA, a genetic knockout of TgMyoD in the RH strain has yielded no apparent phenotype regarding tachyzoite motility, host cell invasion, or virulence in mice (Herm-Gotz *et al.*, 2006). Intravacuolar growth of the parasites was also not affected, ruling out a role for TgMyoD in tachyzoite cell division (Herm-Gotz *et al.*, 2006). The simplest explanation of these results would be that TgMyoD functions mostly or exclusively during a non-tachyzoite life cycle stage. Unfortunately, because the RH strain does not differentiate into bradyzoites *in vitro* and because experimentation with the cyst-forming strain Prugniaud is much more challenging than with RH, a potential functional role of TgMyoD in bradyzoites e.g. in gliding motility and host cell invasion has not yet been experimentally investigated (Herm-Gotz *et al.*, 2006).

#### *Class XIVb: TgMyoB/C and TgMyoE*

In *T. gondii*, one myosin gene has been shown to give rise—by differential splicing of one intron—to two different myosin heavy chains (TgMyoB and TgMyoC) that differ only in the C-terminus of their tail domains (Delbac *et al.*, 2001) (Figure 28.1A). Despite the fact that both myosins are identical over their entire head and neck domains and part of their tail domains, they feature different subcellular localization, different solubility, and slightly different expression patterns in tachy- and bradyzoites: according to epitope-tagging experiments, TgMyoB is localized in the cytoplasm and associated with punctate, cytoplasmic structures, is mostly soluble in PBS, and appears to be expressed only weakly in the bradyzoite stage and hardly at all in tachyzoites. In contrast, TgMyoC shows a very distinct subcellular localization at the posterior and anterior polar rings, is very resistant to solubilization also with detergent (2% Triton X-100), and is more strongly expressed than TgMyoB in both tachy- and bradyzoites (Delbac *et al.*, 2001). Interestingly, overexpression of these myosins did not affect host cell invasion or nuclear division but led to remarkable cell division phenomena: cytokinesis was slowed down, dividing parasites formed abnormal residual bodies, the normally regular arrangement of parasites within a parasitophorous vacuole (rosette) was significantly disturbed, and the epitope-tagged myosins were distributed very unevenly between daughter cells. Furthermore, in dividing tachyzoites TgMyoC appears to be present at the growing edge of the inner membrane complex. It was therefore concluded from these experiments that TgMyoB/C probably plays a role in cell division (Delbac *et al.*, 2001). Support for such a function has recently emerged from the study of an unrelated, novel protein (MORN1) that colocalizes with TgMyoC—and actin (Patron *et al.*, 2005)—at the polar rings and that has been implicated in daughter cell formation



(Gubbels *et al.*, 2006). Like TgMyoC (Delbac *et al.*, 2001), MORN1 can be seen to move as a ring with the leading edge of the extending IMC along the length of the developing daughter cells during endodyogeny. Striepen and coworkers speculate that one function of TgMyoC may be to constrict the posterior polar ring at the end of cytokinesis (Gubbels *et al.*, 2006).

TgMyoE is the least-well studied class XIVa/b myosin of *T. gondii*, and the only experimental data available so far indicate that TgMyoE is expressed in bradyzoites but not at all in tachyzoites (Delbac *et al.*, 2001). According to phylogenetic analyses TgMyoE has been classified in subclass XIVb together with TgMyoB/C (Foth *et al.*, 2006; Lew *et al.*, 2002), despite the fact that it is structurally more similar to the class XIVa myosins TgMyoA and TgMyoD in that it completely lacks a tail domain and has a putative, degenerate IQ motif at its C-terminus (Figure 28.1B). Its function remains unknown at this point.

We note that MyoB (see e.g. Chaparro-Olaya *et al.*, 2003) and MyoE of *Plasmodium* do not represent direct orthologs of TgMyoB and TgMyoE, respectively, and that their orthology in relation to *T. gondii* myosins is unclear. The nomenclature of these two *Plasmodium* motors is thus the only exception to the general rule whereby the same letter in a myosin name denotes orthologous molecules across apicomplexans (Foth *et al.*, 2006).

#### Class XIVc: TgMyoH

TgMyoH belongs to the only recently described subclass XIVc, and unambiguous orthologs to this *T. gondii* myosin are present in *Eimeria tenella*, *Cryptosporidium parvum*, *Theileria annulata*, and *Babesia bovis* (Foth *et al.*, 2006). In contrast to all other class XIV myosins of *T. gondii*, TgMyoH features a long neck domain with 8 predicted IQ motifs and a tail domain with some homology to ATS1 and related RCC1 proteins (Pfam and COG accession numbers PF00415 and COG5184) (Figure 28.1B) which are thought to act as guanine-nucleotide exchange factors. Mammalian RCC1 is chromatin-bound and promotes the activation of the small nuclear GTPase Ran which has several cell division-related functions e.g. in nucleocytoplasmic transport, mitotic spindle assembly, and nuclear envelope formation, while the yeast protein ATS1 is thought to be involved in coordinating the microtubule state during the cell cycle (Hutchins *et al.*, 2004; Kirkpatrick and Solomon, 1994; Shields *et al.*, 2003). In the absence of any experimental data, one can only speculate whether TgMyoH might also play a role during mitosis, cytokinesis, or other cell-cycle events. The low number of TgMyoH-specific ESTs in the database suggests that this myosin is not very abundantly expressed.

In contrast to the vast majority of non-class XIV myosins, all class XIV myosins including TgMyoH contain a serine or threonine residue at a conserved site in the myosin head domain (position 585 in chicken skeletal muscle myosin II, GenBank accession number AAB47555) (Foth *et al.*, 2006). Since myosin heavy chain phosphorylation is known to regulate myosin characteristics in other systems—e.g. filament (dis)assembly and localization of class II myosin (Ben-Ya'acov and Ravid, 2003; Yumura *et al.*, 2005)—it is tempting to speculate that this class XIV-specific polymorphism may also serve a regulatory control mechanism.

Interestingly, myosin-like proteins of *T. gondii* (TgTwinScan\_0256) and other apicomplexans contain a similar ATS1/RCC1-like domain as TgMyoH (Figure 28.1B),

suggesting that these proteins may share a common evolutionary origin. This notion is supported by the fact that the divergent myosin-like proteins also feature the class XIV-specific polymorphism just mentioned above. Finally, some myosins of the ciliate *Tetrahymena thermophila* (classified in subclass XIVd) also possess an ATS1/RCC1-like domain in their myosin tail (Foth *et al.*, 2006), thus making these apicomplexan and ciliate myosins (and myosin-like proteins) the only myosins in which such protein domains have been found to date.

#### *Class XXII: TgMyoF*

The *P. falciparum* ortholog of TgMyoF was originally suggested to belong to myosin class V because the compact arrangement of 6 IQ motifs in its neck domain is a characteristic feature of this class (Vale, 2003). Myosins of classes V and XI (class XI is the plant equivalent of class V) form dimers and move processively along actin in big individual steps of about 36 nm. They are thus able to transport their cargo—mRNAs, proteins, vesicles, vacuoles, or organelles—unidirectionally across relatively long distances through the cell cytoplasm without detaching from their actin track (Tominaga *et al.*, 2003; Vale, 2003). Both class V and class XI myosins feature a “dilute” domain in their tail which was named after a pigmentation mutation in mice (Mercer *et al.*, 1991).

Our recent phylogenetic analyses show that TgMyoF and its orthologs from other apicomplexans and the diatom alga *T. pseudonana* are possibly somewhat related to classes V and XI but are sufficiently different from these and in fact from all other previously described myosin classes to be grouped into their own new class XXII (Foth *et al.*, 2006). In addition, none of the class XXII myosins possesses a dilute domain, whereas the apicomplexan members of this class feature 4–6 WD40 repeats in their tail domain. WD40 repeats can adopt a highly regular beta-propeller fold and are found in many proteins of very different functions where they are thought to serve as site for protein-protein interactions and in the formation of multi-protein complex assemblies (Smith *et al.*, 1999; van Nocker and Ludwig, 2003). Interestingly, the apicomplexan class XXII myosins are the first known examples of myosin heavy chains containing WD40 repeats, yet these repeats have previously been found to be part of myosin heavy chain kinases where they may target these enzymes to their substrate via direct interaction with class II myosin (Steimle *et al.*, 2001). The *P. falciparum* ortholog of TgMyoF appears to be highly expressed throughout the intraerythrocytic life stages (Le Roch *et al.*, 2004; Le Roch *et al.*, 2003), but localization and function of class XXII myosins are currently unknown.

#### *Classes XXIII and XXIV: TgMyoG and TgMyoI*

MyTH4 domains (short for Myosin Tail Homology 4) are found in a wide range of myosin classes. In conjunction with FERM domains (also referred to as ERM or B41), they have been shown in a metazoan class X myosin to be able to directly bind to microtubules (Weber *et al.*, 2004). Given the highly structured subpellicular microtubule cytoskeleton of apicomplexan parasites, a myosin motor capable of directly interacting with both microtubules and actin would be very fascinating indeed. But whether the class XXIII myosin TgMyoG, which possesses a MyTH4 but no FERM domain, is able to interact with

microtubules is unknown. Interestingly, a TgMyoG ortholog has so far only been found in the coccidian *E. tenella* (Foth *et al.*, 2006).

The class XXIV myosin TgMyoI remains the most enigmatic myosin of *T. gondii*: apart from two IQ motifs, no other recognizable protein domains are present in its tail domain. The only apparent ortholog of this myosin is found in *Cryptosporidium*. This CpMyoI is predicted to contain an N-terminal SH3-like domain of unknown function and coiled-coil forming regions in its tail domain, indicating that this myosin may be dimeric. Again, localization and biological function of these myosins are at present unclear.

In our phylogenetic analyses, myosins of both class XXIII and class XXIV were sometimes found associated with myosins from the diatom alga *T. pseudonana* (Foth *et al.*, 2006), but the determination of the precise relationship between these motor molecules requires further investigation.

### Myosin accessory molecules

*T. gondii* myosin A, the motor that is essential for tachyzoite motility and host cell invasion (Meissner *et al.*, 2002), has been shown to associate with a myosin light chain called TgMLC1 (Herm-Gotz *et al.*, 2002). TgMLC1 is somewhat similar to calmodulin, a calcium-sensitive protein that serves as a light chain for many myosins itself and that is very highly conserved across distant evolutionary lineages (human and *T. gondii* calmodulin exhibit 99% amino acid similarity). Like calmodulin, TgMLC1 contains several putative calcium-binding motifs (EF-hands), and both calmodulin and a TgMLC1-ortholog are also present in several other apicomplexan genomes, e.g. in *Eimeria tenella*, in *Plasmodium*, and in *Cryptosporidium*. In *Plasmodium*, the TgMLC1-ortholog is referred to as MyosinA Tail domain Interacting Protein (MTIP) and has been shown experimentally to be associated with MyoA (Bergman *et al.*, 2003; Green *et al.*, 2006). In addition to calmodulin and TgMLC1, the *T. gondii* genome reveals four further putative myosin light chains that are not present in either *Plasmodium* or *Cryptosporidium* (whereas several MLC1 paralogs are also apparent in the genome of the fellow coccidian *E. tenella*). Interestingly, all five MLC paralogs of *T. gondii* feature EF-hand motifs that conform much less to the general consensus sequence than the four EF-hands of calmodulin, while the IQ motifs present in the neck domains of apicomplexan myosin heavy chains also deviate from the general consensus sequence (Figure 28.1C) (Herm-Gotz *et al.*, 2002; Hettmann *et al.*, 2000). This may suggest that apicomplexan myosin heavy and light chain interactions and their regulation are unusual compared to other organisms (Bahler and Rhoads, 2002).

In addition to TgMyoA and the myosin light chain TgMLC1, the myosin A motor complex also comprises the two novel glideosome-associated proteins TgGAP45 and TgGAP50, an amino-terminally N-myristoylated and an integral membrane protein, respectively. This motor complex is thus a heterotetrameric protein assembly that is anchored in the inner membrane complex by TgGAP50 (Gaskins *et al.*, 2004) (see section *Class XIVa: TgMyoA, gliding motility, and host cell invasion*). Orthologs of both GAP proteins are found in other apicomplexan genomes, and GAP45 and GAP50 have recently been shown to also be part of the MyoA motor complex in *P. falciparum* (Baum *et al.*, 2006). It thus appears that the myosin A-based motility system is highly conserved throughout the Apicomplexa.

## Microtubule-based motors

Microtubules form a well-defined cytoskeleton in *T. gondii*, and microtubule-based movement is known or thought to be essential for many biological functions and features of this parasite such as nuclear and cell division, cell shape and polarity, host cell invasion, and motility of both non-sexual stages (tachyzoites, bradyzoites) and gametes. For a detailed discussion of microtubular structures and functions, please see Chapter 27.

According to our searches the *T. gondii* genome encodes about 15 kinesins and 10 dyneins, but despite their obvious importance for the parasite virtually no experimental data are available on any of these motors. In contrast, some detailed work has been carried out on microtubule motors of *P. falciparum* (Fowler *et al.*, 2001; Moores *et al.*, 2003; Moores *et al.*, 2002; Shipley *et al.*, 2004). Below we present a tentative classification of *T. gondii* kinesins and dyneins (see Tables 28.2 and 28.3) based on our phylogenetic analyses (data not shown) and very briefly review the hallmark features and functions of homologous motor molecules from other organisms. While in general orthologous proteins are likely to perform the same or similar functions in different organisms, it is important to keep in mind that many biological processes in apicomplexan parasites are either highly divergent or even unique to these organisms. We also note that the *T. gondii* kinesin and dynein heavy chain sequences discussed below are currently based solely on gene predictions and that their annotation has not been confirmed experimentally.

### Kinesins

Kinesins used to be classified into only three categories (KinN, KinI, and KinC) depending on where the ATP- and microtubule-binding sites containing motor domain was located within the molecule: at the N-terminus (KinN), internally (KinI), or at the C-terminus (KinC). This simple classification was then replaced by a more complex scheme based primarily on phylogenetic analysis—see also Dagenbach and Endow (2004)—where the former KinN kinesins have been subdivided into 12 families while the KinI and KinC sequences are now placed in families Kinesin-13 and -14, respectively (Lawrence *et al.*, 2004; Miki *et al.*, 2005). Recently, this classification has again been updated by a study that included a wide range of protist kinesins, including those of the apicomplexans *Plasmodium falciparum*, *Theileria annulata*, and *Cryptosporidium parvum* (Wickstead and Gull, 2006).

Our kinesin inventory of the *T. gondii* genome has yielded 15 kinesin heavy chains in comparison to eight such motors found in *P. falciparum* and *Cryptosporidium*, and four kinesins in *T. annulata* (Wickstead and Gull, 2006). According to our analyses, 10 of the 15 *T. gondii* kinesin heavy chains can be placed confidently into 7 of the 14 previously described kinesin families, whereas the classification of another five *T. gondii* kinesins remains speculative or unknown (Table 28.2).

### Kinesin-2

Motors of this family have been implicated in organelle transport, mitosis, and anterograde intraflagellar transport (Haraguchi *et al.*, 2006; Miki *et al.*, 2005). *T. gondii* features one kinesin heavy chain belonging to this family, but a homolog of the kinesin associated protein 3 (KAP3) that may form a heterotrimer with these kinesins is not apparent in the *T. gondii* genome.

### Kinesin-3

Two sequences of *T. gondii* are placed in this kinesin family by phylogenetic analysis, and both motors contain the family typical forkhead-associated (FHA) domain followed by a coiled-coil forming region in their tail (see Figure 28.1A). The FHA domain may serve as a site for protein-protein interactions with phosphorylated proteins (Durocher and Jackson, 2002) and/or facilitate dimerization of this motor (Lee *et al.*, 2004). Possible functions include vesicle and organelle transport (Miki *et al.*, 2005).

### Kinesin-4/10

The classification of one *T. gondii* kinesin in this family is speculative. We list this family as a “combined” family because the kinesin-10 family appears in some analyses, including our own, as paraphyletic and not monophyletic (as one would expect from a “natural” group) since it frequently encompasses the kinesin-4 clade (Lawrence *et al.*, 2002; Wickstead and Gull, 2006). There are both motile and non-motile kinesins within this family, and some members serve organelle transport while others bind to chromosomes and function in mitosis (Matthies *et al.*, 2001; Miki *et al.*, 2005; Sekine *et al.*, 1994; Yajima *et al.*, 2003).

### Kinesin-5

Inhibitors like monasterol that are specific against the vertebrate kinesin Eg5 arrest cells in mitosis (Cochran *et al.*, 2005). Eg5 is involved in spindle formation and probably pushes antiparallel microtubules between spindle poles apart (Kapitein *et al.*, 2005). Kinesins belonging to this family are found in *T. gondii*, *P. falciparum* and *C. parvum*.

### Kinesin-7

As for kinesin-4/10, the classification in this kinesin family of one *T. gondii* sequence and its *P. falciparum* ortholog is speculative, but the presence of some predicted coiled-coil forming regions is at least consistent with this classification. The kinesin-7 family member CENP-E is associated with centromeres, is required for correct positioning of chromosomes at the metaphase plate, and regulates mitotic checkpoint signaling (Mao *et al.*, 2005; Wood *et al.*, 1997).

### Kinesin-8

Two *T. gondii* kinesins belong to this family for which reported functions include the control of microtubule stability and spindle morphology during meiosis, mitosis, and cytokinesis (Gandhi *et al.*, 2004; Gatt *et al.*, 2005). Orthologs to at least one of the *T. gondii* kinesin-8 members are found across several apicomplexan lineages (Table 28.2). Intriguingly, the *Plasmodium* ortholog (PFA0535c) of one of these two *T. gondii* kinesin-8 motors (TgTwinScan\_2915) is specifically expressed in male but not in female gametocytes and may in fact represent the only kinesin whose expression is strongly upregulated in this life stage (Khan *et al.*, 2005).

### Kinesin-9

A kinesin of this family from the flagellated green alga *Chlamydomonas reinhardtii* has been shown to be essential for normal flagellar motility (Yokoyama *et al.*, 2004). Given

**Table 28.2** Kinesin heavy chains of *Toxoplasma gondii*

Kinesin family <sup>a</sup>	ToxoDB gene model or Sequence identifier <sup>a</sup>	Ortholog in <sup>b</sup>		
		<i>P. falciparum</i>	<i>T. annulata</i>	<i>Cryptosporidium</i>
Kinesin-2	TgTwinScan_0841	–	–	–
Kinesin-3	TgTwinScan_1498	–	–	–
	TgTwinScan_2516	–	–	–
Kinesin-4/10 ?	TGG_994300 (nucleotides 125904-128013)	–	–	–
Kinesin-5	TgTwinScan_0560	CAB11138 (PFC0770c) [PfKLP3]	–	Cp: CAD98462 [Cp_boursin]
Kinesin-7 ??	TgTwinScan_7533	AAN36198 (PFL0545w) [PfKLP7]	–	–
Kinesin-8	TgTwinScan_4288	CAB39023 (PFC0860w) [PfKLP4]	CAI72998 [TaKLP2]	Ch: XP_665996 (Cp: AQ855704)
	TgTwinScan_2915	CAD49067 (PFA0535c) [PfKLP5]	–	–
Kinesin-9	TgTwinScan_5692 and TgGlmHMM_3127	–	–	–
	TgGlmHMM_2656	–	–	–
Kinesin-13	TgTwinScan_3589	PfKinI: AAN36517 (PFL2165w) [PfKLP8]	CAI74297 [TaKLP1]	Cp: EAK89501 [CpKip3]
Kinesin-14	TgTwinScan_3487	–	–	Ch: EAL37275 ?? (Cp: AQ854850) ??
?	TgTwinScan_5295	CAD50998 (PF07_0104) [PfKLP2]	–	–
?	TgTwinScan_2213	–	–	–
?	TgGlmHMM_0863	–	–	–

<sup>a</sup>Presented is our preliminary classification of *T. gondii* kinesin sequences into existing kinesin families according to Lawrence *et al.* (2004), Miki *et al.* (2005), and Wickstead and Gull (2006) by phylogenetic analysis of the conserved head domains which were added to an existing collection of kinesin sequences (Dagenbach and Endow, 2004; <http://www.proweb.org/kinesin/KinesinAlign.html>). Phylogenetic bootstrap analysis was carried out with ClustalX v1.83 (Chenna *et al.*, 2003). Uncertain classification is indicated by question marks.

<sup>b</sup>Listed are accession numbers for orthologs found in *Plasmodium falciparum*, *Theileria annulata*, and *Cryptosporidium* (Cp=*C. parvum*; Ch=*C. hominis*). PlasmoDB identifiers (<http://plasmodb.org>)



Protein domains <sup>c</sup>	Likely structure and function of kinesins belonging to this kinesin family
– FHA, coiled-coil FHA, coiled-coil, FERM, C2 –	Dimeric or heterotrimeric including a non-motor subunit (e.g. KAP3); organelle and intraflagellar transport and role in mitosis Monomeric or homodimeric; forkhead-associated domain (FHA) may facilitate interactions with phosphorylated proteins; vesicle and organelle transport Kinesin tail with coiled-coil and sometimes with WD40 repeats (“kinesin-4”) or with helix-hairpin-helix DNA-binding motif (HhH) near C-terminus (“kinesin-10”); predicted to bind directly to chromosomes and to function in cell division (e.g. KIF22, KID, NOD) or in organelle movement
coiled-coil	Homotetrameric; specific neck (also found in apicomplexan Kin-5 sequences) and BimC box domain; push apart antiparallel microtubules between spindle poles during mitosis
coiled-coil	Often long coiled-coils; important for timing of anaphase onset during mitosis (e.g. CENP-E controls mitotic checkpoint signaling)
coiled-coil	May control microtubule stability and spindle morphology during mitosis, cytokinesis and meiosis (e.g. Kip3)
–	– coiled-coil Probably involved in motility of flagella and cilia
–	–
–	Some kinesins of this family have an N-terminal extension (“internal kinesin/motor”); the <i>P. falciparum</i> ortholog (KinI) has been studied in detail and shown to have microtubule depolymerizing activity; may function in mitosis
coiled-coil (C-terminal kinesin motor)	Monomeric or dimeric; structurally reversed: the catalytic motor domain is C-terminal, leading to reversed motility towards the MT minus end; some members contain CH or MyTH4 and FERM domains; shown to depolymerize microtubules (e.g. Kar3 in yeast); may function in mitosis or meiosis
–	?
coiled-coil	?
–	?

are given in parentheses for *P. falciparum*. Square brackets denote protein names according to Wickstead and Gull (2006) for easy comparison.

<sup>c</sup>As predicted for the respective *T. gondii* kinesin by SMART (<http://smart.embl-heidelberg.de/>) and the Conserved Domain Database and Search Service, v2.06 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>); abbreviations and accession numbers (SMART and CDD databases): C2 = Protein kinase C conserved region 2, cd00030; FERM (B41), SM00295; FHA = Forkhead associated domain, cd00060.

**Table 28.3** Dynein heavy chains of *Toxoplasma gondii*

Dynein type <sup>a</sup>	Subtype <sup>a</sup>	ToxoDB Gene Model <sup>a</sup>	Ortholog in <sup>b</sup> <i>P. falciparum</i>	<i>T. annulata</i>	<i>C. parvum</i>	Protein domains <sup>c</sup>
Cytoplasmic	1	TgTigrScan_6048	CAD51040 (MAL7P1.162)	–	EAK88498	–
Axonemal: outer arm dynein	OAD $\alpha$	TgGlmHMM_4104	NP_701100 (PF11_0240) and/or chr12. genefinder_23r?	–	–	IPT, IG_FLMN (in N-terminal dynein tail)
	OAD $\beta$	TgGlmHMM_0727	AAN35421 (PF10_0224)	–	–	–
	OAD $\gamma$	TgTwinScan_2019	CAD51738 (PFI0260c)	–	–	–
Axonemal: inner arm dynein	IAD 1 $\alpha$	TgTwinScan_4570	–	–	–	–
	IAD 1 $\beta$	TgTwinScan_2758	–	–	–	–
	IAD (various)	TgTigrScan_7440	–	–	–	–
		TgTwinScan_7524	–	–	–	–
		TgGlmHMM_3163	–	–	–	–
		TgGlmHMM_4944	–	–	–	–

<sup>a</sup>Presented is our preliminary classification of *T. gondii* dynein sequences into existing dynein types following the nomenclature of *Chlamydomonas reinhardtii* dyneins (see e.g. Pazour *et al.*, 2006) by phylogenetic analysis of the conserved head ring domains. Phylogenetic bootstrap analysis was carried out with ClustalX v1.83 (Chenna *et al.*, 2003).

<sup>b</sup>Listed are accession numbers for orthologs found in *Plasmodium falciparum*, *Theileria annulata*, and *Cryptosporidium parvum*. PlasmoDB identifiers (<http://plasmodb.org>) are given in parentheses for *P. falciparum*.

<sup>c</sup>As predicted for the respective *T. gondii* dynein—in addition to AAA domains and coiled-coils—by SMART (<http://smart.embl-heidelberg.de/>) and the Conserved Domain Database and Search Service, v2.06 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>); abbreviations and accession numbers (SMART database): IG\_FLMN = filament-type immunoglobulin domains, SM00557; IPT = ig-like, plexins, transcription factors, SM00429.

that apicomplexan parasites have flagellated microgametes it is not surprising to find two kinesins of this family in *T. gondii* while it is unclear why orthologs appear to be absent from other members of the Apicomplexa.

### Kinesin-13

Kinesins of this family like MCAK (mitotic centromere-associated kinesin) do not move along microtubules but instead destabilize and depolymerize them from their ends (Moore and Wordeman, 2004; Moore *et al.*, 2005). The *P. falciparum* homolog (PfKinI) has been studied in great detail, and its biochemical characteristics and three-dimensional structure have contributed significantly to understanding the functional mechanism of these kinesins (Moore *et al.*, 2003; Moore *et al.*, 2002; Shipley *et al.*, 2004) whose main functional role is in mitosis.

### Kinesin-14

The characteristic feature for these kinesins is a C-terminal motor domain that causes reversed motility towards the minus-end of microtubules (Endres *et al.*, 2006). Interestingly, this family is divided into two groups (14A and 14B) (Lawrence *et al.*, 2004; Miki *et al.*, 2005), and while our phylogenetic analysis suggests the *Cryptosporidium* homolog to belong to the 14A family, the *T. gondii* homolog is firmly placed into group 14B. Despite its apparently close relationship to plant KCBP kinesins (kinesin-like calmodulin-binding protein) the *T. gondii* homolog does not appear to possess MyTH4 or FERM domains typical for the plant motors (Reddy and Reddy, 1999). Kinesin-14 motors have been reported to function in mitosis and cytokinesis, to transport vesicles and organelles, and to catalyze microtubule depolymerization (Hirokawa and Takemura, 2004; Sproul *et al.*, 2005; Zhu *et al.*, 2005).

### Dyneins

Dynein motors move towards the minus end of microtubules. They are more complex than kinesins and myosins because they usually comprise several heavy, intermediate, light intermediate, and light chains. The dynein heavy chains are bigger (molecular mass > 500 kDa) than either kinesin or myosin heavy chains (Figure 28.1A) and fall into two major groups, the cytoplasmic and the axonemal dyneins. The cytoplasmic dynein heavy chains are homodimers that may move processively. They have a number of essential roles in mitosis and serve the retrograde transport of organelles and vesicles, in particular in nerve axons and within flagella. In contrast, the axonemal dynein heavy chains occur as monomers, heterodimers or heterotrimers and form the inner and outer dynein arms in cilia and flagella. They serve as the essential motors that drive ciliar and flagellar movement and are thus responsible for a diverse range of biological functions from single cell locomotion to the transport of fluids and mucus by ciliated epithelia. Interestingly, dyneins can “shift gears,” i.e. they may change step size and mechanical force as they move along microtubules depending on cargo load and ATP availability (Mallik *et al.*, 2004).

We have currently identified 10 dynein heavy chains in the *T. gondii* genome, which is more than is apparent in the genomes of *P. falciparum* (6–9 dyneins), *T. annulata* (two dynein-like proteins), and *C. parvum* (one dynein). This relative dearth of dyneins (and in fact

also of kinesins) in the completely sequenced genomes of *Theileria* and *Cryptosporidium* is consistent with the lack of flagellated cells in these two genera (Briggs *et al.*, 2004). The 10 genes of *T. gondii* appear to encode full-length dynein heavy chains including 6 AAA domains and a microtubule-binding stalk between the fourth and fifth AAA domain (Figure 28.1A). This makes *T. gondii*—as far as we know—the apicomplexan with the largest gene repertoire for each of the three types of motor molecules, i.e. myosins, kinesins, and dyneins.

In contrast to some previously published phylogenetic analyses of dyneins (Asai and Wilkes, 2004; Pazour *et al.*, 2006) we have assembled a dataset of full-length sequences and used the entire motor domain of each dynein (from the first AAA domain to the end of the sequence) to generate an alignment with over 2500 usable amino acid positions. Subsequent phylogenetic analysis with ClustalX resulted in a well-resolved tree (data not shown) on which we have based our classification of the 10 *T. gondii* dyneins (Table 28.3; see below). Unfortunately, the nomenclature of certain axonemal outer arm dyneins (OADs) is confusing since it arose from biochemical work long before any gene sequences were known. Orthologs of two OADs (OAD $\alpha$  and OAD $\gamma$ ) are thus labeled inversely across different species: OAD $\gamma$  of the green alga *Chlamydomonas reinhardtii* is orthologous to the protein called OAD $\alpha$  of the ciliate *Tetrahymena* and the sea urchin, whereas OAD $\alpha$  of *C. reinhardtii* is the ortholog of *Tetrahymena*'s OAD $\gamma$  (an ortholog of this dynein does not exist in metazoans). In contrast, the OAD $\beta$  is orthologous across both metazoans and protists (Toba *et al.*, 2004). In having to choose which naming convention to follow we decided to adopt the OAD nomenclature of *C. reinhardtii* because the OADs of this model organism are the ones that are the best annotated and characterized, and because the human OAD orthologs have recently been described following the *C. reinhardtii* model (Pazour *et al.*, 2006).

### Cytoplasmic dyneins

Of the two cytoplasmic dynein subtypes that are known (cytoplasmic dynein 1 and 2), the apicomplexans *T. gondii*, *P. falciparum*, *T. annulata*, and *C. parvum* all possess orthologs of the more widespread cytoplasmic dynein 1. In general, these motors have been implicated in various aspects of mitosis, in positioning and functioning of the nucleus, the Golgi apparatus, and the endoplasmic reticulum, and in the retrograde transport of organelles in neuronal axons. Interestingly, the cytoplasmic dynein 1 of *Plasmodium* is specifically expressed in female but not male gametocytes (Khan *et al.*, 2005). In contrast, cytoplasmic dynein 2, which is usually associated with retrograde intraflagellar transport (IFT) (Pfister *et al.*, 2006), is apparently absent in *T. gondii* and other apicomplexans (see also the brief discussion in section *Concluding remarks*).

### Axonemal outer arm dyneins

The flagellar axoneme contains highly ordered arrays of outer dynein arms (ODAs) that provide the bulk of the bending force needed to generate the beating movement of these organelles. Each axonemal ODA of *C. reinhardtii* represents a complex arrangement of three different types of outer arm dynein heavy chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and various supplementary proteins (Sakato and King, 2004). All three ODA dynein types are present in

both *T. gondii* and *P. falciparum* and have been classified based on the nomenclature of the *C. reinhardtii* OADs (Pazour *et al.*, 2006; Sakato and King, 2004). Although motility in most life stages of apicomplexans is based on an actin-myosin system, flagella are essential for the motility of their microgametes. Reassuringly, all three different ODA dynein types have recently been found in *Plasmodium* to be highly and specifically expressed in male gametocytes, which in general accumulate many of the proteins that are needed by the very rapidly differentiating microgametes (Khan *et al.*, 2005).

#### *Axonemal inner arm dyneins*

Inner dynein arms (IDAs) are thought to be responsible for the modulation of flagellar movement, e.g. by generating specific waveform patterns. They are more heterogeneous than ODAs, and a typical flagellum contains three different IDA types called I1, I2, and I3. The I1 arm represents a heterodimer of inner arm dynein heavy chains 1 $\alpha$  and 1 $\beta$ , whereas the I2 and I3 elements consist of a variety of single-headed dyneins (Asai and Wilkes, 2004; Sakato and King, 2004). This general diversity of inner arm dyneins is well reflected in *T. gondii* which contains one each of dynein type 1 $\alpha$  and 1 $\beta$ , and four additional dyneins that are likely to contribute to IDAs. Surprisingly, our analyses have not uncovered any evidence for the existence of IDA dyneins in *P. falciparum*, despite the presence of ODA dyneins.

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### Concluding remarks

The ancient eukaryotic cenancestor—the last common ancestor to all living eukaryotes—already possessed the three types of molecular motors myosins, kinesins, and dyneins, and while a few individual evolutionary lineages have altogether lost their myosins or dyneins, not a single eukaryote is known that exists without a kinesin motor (Richards and Cavalier-Smith, 2005; Wickstead and Gull, 2006). Apicomplexan parasites (for which a number of genomes have now been completely sequenced) are thus no exception, and their biology relies heavily on all three types of motor molecules. Incidentally, *T. gondii* is so far the apicomplexan with the largest known repertoire of each of the three motor types.

Regarding the myosins, only four of the at least 11 different motors have been investigated experimentally, and they have been shown to function in gliding motility, host cell invasion, and probably also in cell division. It seems likely that more isoforms and splice variants of the currently known or predicted molecular motors will be discovered in the future, thereby increasing an already great diversity of motor molecules that are bound to be involved in a multitude of functions, both housekeeping and parasite-specific.

*T. gondii* features clearly defined microtubular structures including a basket-shaped subpellicular cytoskeleton, a conoid, nuclear spindle, and flagella, and contains a total of 25 genes encoding kinesin and dynein heavy chain motors that could interact with these cytoskeletal elements. Amazingly, to date there are virtually no experimental data available for any of these motors at all, yet a couple of functional aspects may be inferred from comparisons with other organisms. The apparent absence from *T. gondii* and other apicomplexans of cytoplasmic dynein 2—the motor normally responsible for retrograde intraflagellar transport (IFT)—is remarkable and may be accounted for by the short life span of the only flagellated apicomplexan life stage, the microgamete (Ferguson, 2002;

Janse and Waters, 2004). This very brief use of flagella throughout the apicomplexan life cycle may have rendered retrograde IFT for flagellar maintenance and its associated genes redundant during evolution. In contrast, a kinesin-2 for anterograde IFT is still evident in *T. gondii*, but not in *Plasmodium*. This intriguing discrepancy may be explained by the different types of axoneme biogenesis in these two parasites. Axonemes of *Plasmodium* are assembled in the cytosol before a compartmentalized flagellum is extruded from the cell body (Sinden *et al.*, 1976), and it thus appears plausible that the cytosolic assembly and the short life span of the mature flagella may not necessitate any dedicated IFT. *T. gondii* on the other hand follows a more conventional blueprint for axoneme formation whereby developing flagella grow into the parasitophorous vacuole, a considerable distance away from the cell body (Ferguson *et al.*, 1974), a process for which at least anterograde IFT may still be indispensable. In contrast, the complete lack of flagellated cells in the *Cryptosporidium* and *Theileria* life cycles is mirrored by the apparent absence in their genomes of any genes encoding IFT motors or axonemal dyneins (Briggs *et al.*, 2004).

Some particularly intriguing aspects of the *T. gondii* motor arsenal now include myosins that feature protein domains (WD40 repeats, ATS1/RCC1-like domain) that had not been encountered as part of such motors before, a myosin-like protein that is well conserved throughout the Apicomplexa and that may have evolved from the also highly conserved MyoH motor to serve as a static cytoskeletal element rather than a motor molecule, and the absence of a cytoplasmic dynein-2. We have therefore no doubt that the functional characterization of *T. gondii*'s diverse store of molecular motors will teach us many more fascinating cell biological lessons in the future.

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# Actin Dynamics and Motility in Apicomplexans

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## Abstract

Actin-based motility controls substrate-dependent gliding, tissue migration, and cell invasion by apicomplexan parasites. Although motility depends on the action of a small myosin motor, the polymerization of new actin filaments controls the timing and speed of motility. Additionally, motility is directional, implying that actin filaments, which are normally polarized, must also be aligned with the longitudinal axis of the parasite. Actin dynamics in apicomplexans are highly unusual and the majority of actin is kept in an unpolymerized state in resting cells. Actin polymerization occurs permissively yet results in short filaments that are inherently unstable. The relatively small component of actin-binding proteins in apicomplexans attests to the streamlined nature of actin-based motility in these cells. Structural differences in apicomplexan actin may render it ideally suited for rapid cycles of assembly and disassembly, which likely represents an important adaptation for gliding motility. The concerted action of this actin-based motility system drives forward motion by retrograde translocation of cell surface adhesins. This unique form of motility is responsible for pathogenesis and presents numerous potential targets for therapeutic intervention.

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## Motility and invasion by apicomplexans

Apicomplexans are most closely related to ciliates and dinoflagellates (Baldauf *et al.*, 2000); however, unlike these free living protozoa, apicomplexans do not utilize cilia or flagella for motility (with the exception of male gametes, which are flagellated). The phylum Apicomplexa contains several notable human pathogens including *Plasmodium* spp., causative agents of malaria, *Cryptosporidium* spp., responsible for severe diarrheal disease, and *Toxoplasma gondii*, an opportunistic pathogen. Despite their diversity, apicomplexans use a common means of actin-based motility for migrating through tissues and for powering cell invasion (Sibley, 2004). *Toxoplasma* provides a model system to explore the molecular basis of gliding motility due to its ease of experimental use, excellent tools for cellular and molecular biological study, and well-developed genetic systems (Roos *et al.*, 1994). Genetic and biochemical studies reveal that motility and cell penetration by *Toxoplasma* are driven by polymerization of new actin filaments in the parasite (Dobrowolski and Sibley, 1996). *Toxoplasma* invasion of host cells is completely independent of the host actin cytoskeleton (Morisaki *et al.*, 1995), thus distinguishing it from host-mediated uptake. Active entry is

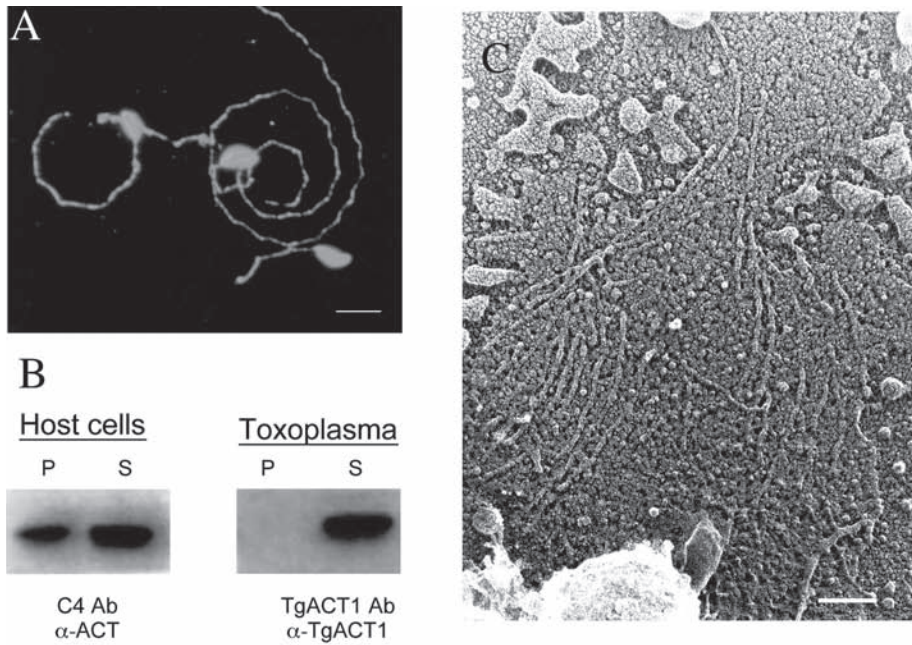
shared by apicomplexans and it is mechanistically distinct from the process of bacterial or viral uptake, or the crawling behavior of amoeba or vertebrate cells (Sibley, 2004).

### Comparison of gliding motility in apicomplexans

Apicomplexans move by gliding, a contact-dependent process that deposits a characteristic trail of surface membrane on the substrate (Figure 29.1A). Sporozoites of *Cryptosporidium* (Arrowood *et al.*, 1991), *Eimeria* (Russell, 1983) and *Plasmodium* (Stewart and Vanderberg, 1988) all exhibit gliding motility *in vitro*. Gliding motility is also exhibited by gregarines, an early branch of the phylum that contains a diverse array of parasites of invertebrates (King, 1988). Even prior to the molecular identification of the various components, studies done in gregarines were instrumental in proposing a model where an actin-myosin motor propels forward motion by translocation of cell surface adhesive proteins (King, 1981). These studies were also among the first to recognize that “gliding” is a misnomer since the small size of protozoan parasites dictates that forward movement is dependent on continuous traction rather than inertia (Preston and King, 1996). Gliding motility by apicomplexans occurs at rates of 1–10 microns/s and occurs independent of cilia or flagella. There are functional similarities between apicomplexan gliding and motility exhibited by pennate diatoms (Poulsen *et al.*, 1999) in that both proceed by translocation of cell surface adhesins and rely on an actin-dependent motor. While occurring at a similar rate, gliding by apicomplexans is mechanistically distinct from bacterial gliding. In *Myxococcus*, gliding motility is similar to twitching motility, which relies on type IV pili extension and retraction (Youderian, 1998). In contrast, other bacteria (cyanobacteria, mycoplasmas, and cytophaga-flavobacteria) rely on an ATP-binding cassette transporter and a set of cell surface lipoproteins (McBride, 2004).

Gliding in *Toxoplasma* is characterized by two basic behaviors called “circular” or “helical” based on the pattern of the track formed by the parasite (Frixione *et al.*, 1996; Håkansson *et al.*, 2001). During circular gliding, the parasite moves counterclockwise in a circular pattern, while it moves in a clockwise spiral during helical gliding. Notably, only helical gliding, in which the organism rotates along the long axis of the body while moving forward, results in cell invasion (Håkansson *et al.*, 1999). In contrast circular gliding results in the parasite remaining in essentially the same spot on the substrate. Similar behaviors are exhibited by *Plasmodium* sporozoites (Vanderberg *et al.*, 1990), *Eimeria* sporozoites (Russell and Sinden, 1981), and *Cryptosporidium* sporozoites (Wetzel *et al.*, 2005). *Cryptosporidium* sporozoites also use actin-dependent motility to the parasite to invade host cells (Wetzel *et al.*, 2005), despite the fact that later in development it recruits host cell actin to form a pedestal beneath the vacuole (Clark and Sears, 1996).

Gliding motility also plays a role *in vivo* where its manifestations are somewhat more variable, reflecting the more complex environment of tissue. Passage of *Toxoplasma* across cellular barriers such as polarized epithelial monolayers requires active motility and occurs without significant damage to the host cell monolayer (Barragan and Sibley, 2002). This process is important for dissemination *in vivo* and more virulent strains show an enhanced ability to cross monolayers *in vitro* and to disseminate to deep tissues *in vivo* (Barragan and Sibley, 2002). Sporozoites of *Plasmodium* cause extensive cellular damage and migrate through multiple hepatocytes before adopting an intracellular niche (Mota *et al.*, 2001).



**Figure 29.1** Gliding motility involves changes in actin polymerization. (A) Gliding motility by *Toxoplasma* tachyzoites deposits trails on the substrate. Stained with antibody to SAG1 and visualized with fluorescently conjugated goat anti-mouse secondary antibody. Bar = 5 microns. Used with permission (Sibley, 2003). (B) Separation of globular (s = supernatant) and filamentous actin (p = pellet) by sedimentation at 100 000g for 1 hour as described previously (Dobrowolski *et al.*, 1997). Host cells contain substantial F-actin while the majority of actin in parasites is not sedimented at 100 000g and is thus likely monomeric or in small complexes. Western blotting performed with anti-actin mAb C4 (left) or rabbit anti-TgACT1, right. (C) Demonstration of actin filaments beneath the plasma membrane of *Toxoplasma*. Parasites were allowed to glide as in A then gently sonicated off the substrate. The resulting membrane patches were rapidly frozen and used to generate platinum replicas that were examined by EM. Bar = 100 nanometers. Used with permission from (Sahoo *et al.*, 2005).

Intravital microscopy has revealed that following deposition of sporozoites in the skin during mosquito feeding, they are highly motile within the interstitial space but eventually enter blood vessels and are transported via the circulation (Frischknecht *et al.*, 2004). A subset of sporozoites also traverse the skin but are transported in lymphatics to regional lymph nodes, where their encounter with dendritic cells may be influential in mounting an immune response (Amino *et al.*, 2006). Similar imaging techniques have revealed that during entry into the liver, sporozoites glide along the surface of sinusoidal epithelial cells, migrate slowly through Kupfer cells, penetrate through multiple hepatocytes, and finally invade and develop within a host hepatocyte (Frevert *et al.*, 2005). Motility also plays a role in the insect during malaria infection of mosquitoes where active motility is needed for ookinete migration (Vlachou *et al.*, 2004) and for penetration of the salivary glands by sporozoites (Kappe *et al.*, 2003).

## A molecular mechanism for gliding motility

Apicomplexan motility relies on a unique actin-dependent, myosin motor beneath the plasma membrane that translocates cell surface adhesins in a conveyor-belt like fashion from the anterior to posterior end of the cell (Sibley, 2004). The small myosin TgMyoA provides the force for propulsion and this motor is anchored in the inner membrane as part of a complex (Gaskins *et al.*, 2004; Meissner *et al.*, 2002). The concerted action of this actin-based myosin motor is responsible for the rearward translocation of cell surface adhesins such as MIC2 and TRAP (Brossier and Sibley, 2005). A number of microneme proteins contain conserved transmembrane and cytoplasmic domains (MIC6, MIC8, and MIC12 in *Toxoplasma*) and hence may be translocated by a similar process. Motility by *Toxoplasma* also involves repeated oscillations of intracellular calcium (Lovett and Sibley, 2003), which regulates microneme secretion (Carruthers *et al.*, 1999). *Toxoplasma* responds to agonists of IP<sub>3</sub> receptors and to ryanodine receptors, suggesting the presence of both types of calcium release channels (Chini *et al.*, 2005; Lovett *et al.*, 2002). Following release at the anterior surface of the parasite, translocation of MIC2 and TRAP occurs by a specific molecular interaction between the cytoplasmic domain of the adhesin and the actin-binding protein aldolase that connects to actin filaments beneath the membrane (Buscaglia *et al.*, 2003; Jewett and Sibley, 2003). The process of rearward translocation culminates in processing of MIC2, and possibly other adhesins with similar TM domains, by proteolytic cleavage within the membrane, likely accomplished by one of several rhomboids present on the parasite cell surface (Brossier *et al.*, 2005; Dowse *et al.*, 2005).

## The actin cytoskeleton in apicomplexan parasites

Actin is encoded by a single copy gene in *T. gondii* and only a single isoform is known (Dobrowolski *et al.*, 1997). Malaria is known to express two different actins, PfACTII was reported to be expressed in gametocytes and PfACTI in all other stages (Wesseling *et al.*, 1988; Wesseling *et al.*, 1989). However, examination of the expression profile data from microarrays (<http://www.plasmodb.org/>) indicates that PfACTII is expressed early in erythrocytic development while PfACTI is expressed late and both genes are expressed in gametocytes. Apicomplexan actins are remarkably conserved, being ~80% identical to vertebrate actins. However, as discussed below, parasite actins have conserved molecular differences that impart very unique structural and kinetic features.

Although filamentous actin is essential for motility and cell invasion by apicomplexans (Dobrowolski and Sibley, 1996), the majority of actin does not sediment when centrifuged at 100 000 g (Figure 29.1B), a technique that has typically been used to define filamentous vs. globular actin (Dobrowolski *et al.*, 1997). Recent reports indicate that actin in malaria merozoites also fails to sediment at 100 000 g, but that a large fraction of the actin pellets at 500 000 g (Schmitz *et al.*, 2005). Examination of this 500 000 g pellet revealed short actin-like filaments of approximately 100 nm in size, suggesting that short filaments in the parasite may only sediment at higher speeds (Schmitz *et al.*, 2005). A variety of actin-binding proteins also pellet with *P. falciparum* actin in parasite lysates centrifuged at 500 000 g (Schmitz *et al.*, 2005), suggesting that much of the actin is complexed with other components. Further studies on the molecular mass and composition of such complexes may reveal important attributes of parasite actin dynamics.

Several observations suggest that the transition from non-motile to motile parasites requires that actin filaments be rapidly assembled in a localized region, and then recycled to prevent unwanted motility. Filamentous actin is difficult to detect in parasites by conventional EM, but short actin filaments are found beneath the plasma membrane in gliding parasites (Figure 29.1C). Treatment with the actin-stabilizing drug jasplakinolide (JAS) results in an overabundance of actin filaments (Shaw and Tilney, 1999). While this treatment was originally described to block motility and invasion (Poupel and Tardieux, 1999), further analysis indicated that parasites were actually hyperkinetic but that directional control was disrupted (Wetzel *et al.*, 2003). JAS treated parasites accumulate tangled mats of actin filaments and undergo sudden reversal of direction and rapid spinning in one place (Wetzel *et al.*, 2003). These observations indicate that while filamentous actin is essential for motility its assembly must be carefully regulated to allow productive forward motion.

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### Actin regulation and unique kinetic properties of parasite actin

Studies on model organisms have provided X-ray crystal structures (Kabsch *et al.*, 1990), mapped crucial residues by extensive mutagenesis studies (Belmont *et al.*, 1999; Whitacre *et al.*, 2001), and defined the kinetics of filament assembly and turnover (Pollard *et al.*, 2000; Pollard and Borisy, 2003). Actin undergoes self-polymerization that is influenced by divalent cations ( $Mg^{2+}$ ), favored at higher salt concentrations, and dependent on protein concentration. The actin filament is polarized, and polymerization occurs more rapidly at the plus end, also called the barbed end. Filament turnover is a dynamic process that is influenced by a variety of actin-binding proteins that allow the cell to maintain pools of unpolymerized actin (G-actin) and to assemble filamentous actin (F-actin) for structural support or to drive motility (Pollard *et al.*, 2000). Despite extensive studies, findings from these model systems do not adequately explain the unusual dynamics of actin in parasites.

The rapid turnover of actin in apicomplexans could reflect differences in regulation or be due to inherent properties of the actin itself that affect polymerization. To distinguish between these possibilities, TgACT1 was expressed in baculovirus and purified to homogeneity to study its kinetic properties *in vitro* (Sahoo *et al.*, 2005). An inherent property of actin is the ability to spontaneously assemble into filaments above a critical concentration ( $C_c$ ), which varies from 0.1 to 0.6 micromolar at the barbed (+ end) and pointed (– end) end of the molecule, respectively (average  $C_c$  is ~0.12 millimolar) (Pollard *et al.*, 2000). TgACT1 purified from the baculovirus system displayed a typical dependence of salt,  $Mg^{2+}$  and ATP to polymerize and underwent a slow annealing step followed by rapid elongation, characteristic of actin. TgACT1 was able to polymerize very efficiently even at low concentrations and it displayed a  $C_c$  3–4 fold lower than mammalian actin. This demonstrates that in the absence of other proteins, TgACT1 is highly permissive for polymerization.

Actins from different organisms can often co-polymerize *in vitro* and previous studies have shown that *Acanthamoeba* and *Tetrahymena* actins can assemble with vertebrate actin (Gordon *et al.*, 1976; Hirono *et al.*, 1990). However, TgACT1 does not readily copolymerize with vertebrate actin and only forms small clusters under conditions that normally



result in formation of long filaments by conventional actins (Sahoo *et al.*, 2005). The small foci formed by TgACT1 weakly stain with phalloidin, consistent with a previous report indicating that malaria actin binds phalloidin less strongly than yeast actin (Schuler *et al.*, 2005). TgACT1 filaments visualized by EM are short (~100 nanometers), unbranched, approximately 9 nanometers in width, contain repeated cross-striations, and display a slight helical twist characteristic of conventional actin (Sahoo *et al.*, 2005). The average length of mammalian actin formed under similar conditions is more than 10 times this length, suggesting that the short filaments formed by TgACT1 could be due to structural and/or functional differences.

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### Lessons from divergent actins

The unusual dynamics of TgACT1 indicates that there are likely underlying structural differences between mammalian and parasite actins. Phylogenetic analysis of actins identified many key differences specific to apicomplexans and their close relatives ciliates and dinoflagellates (Baldauf *et al.*, 2000). Projecting the amino acid sequence of TgACT1 on to the crystal structure of ADP muscle actin revealed that the majority of conserved substitutions occurred on the surface of the exposed domains of the actin monomer that may affect the monomer-monomer interactions (Sahoo *et al.*, 2005). Modeling TgACT1 using the Holmes filament model as a template (Kabsch *et al.*, 1990) also showed many of the residues conserved among apicomplexans fall on the outside surface of the filament and thus are likely to affect interactions with actin-binding proteins (Sahoo *et al.*, 2005). As well, several substitutions are predicted to affect both the filament structure and stability. In muscle actin, a strong ionic interaction between residues R39 in subdomain 2 and E276 in subdomain 3 (muscle numbering) is thought to stabilize lateral interactions within the filament. Although K40 is a conserved change in *T. gondii*, E276 is replaced with R277, leading to a highly unfavorable electrostatic interaction. In addition, according to the Holmes model, this region (262–272 in muscle actin) is predicted to bind in the pocket formed by adjacent monomers and stabilize the two strands of the filament. The sequence at the tip of this hydrophobic loop is FIGM (266–269) in muscle actin while in TgACT1 the corresponding residues are FLGK (267–270). The K at position 270 is expected to affect the hydrophobic interactions made by the rest of the loop, further destabilizing the interactions between the two strands of the actin filament.

The properties of TgACT1 exhibited *in vitro* suggest that it spontaneously polymerizes rapidly, yet it is prone to disassembly or fragmentation. These features make it ideally suited for rapid assembly and disassembly. Since TgMyoA is a non-processive motor (Herm-Gotz *et al.*, 2002), it requires a high local concentration of filaments to productively translocate its cargo. Alternatively, if TgMyo is anchored in the inner membrane as a dense array of motor proteins and it does not need to be processive in order to translocate the short actin filaments that have been detected in *Toxoplasma* and *Plasmodium*. Binding between the transmembrane adhesin and aldolase may bundle and hence stabilize actin filaments during motility. Once this cargo has reached its destination, release of the adhesin-aldolase complex may assure rapid turnover due to the inherent instability of TgACT1 filaments. This simple process facilitates directional migration, prevents unwanted movement, and regenerates subunits for future rounds of assembly. Structural alterations suggest that



TgACT1 is unstable due to weaker interactions between filament subunits. Interestingly, the TgACT1 filament instability was restored in the presence of JAS and phalloidin, known to stabilize the cross-subunit interaction (Kuang and Rubenstein, 1997). The inherent instability of TgACT1 may be an important adaptation to increase turnover. The extreme sensitivity of apicomplexan parasites to agents like JAS suggests that targeting actin dynamics in apicomplexans may be an effective strategy to prevent motility and hence infection.

The actin regulatory system in apicomplexans is remarkably streamlined compared to plant, yeast, or animal cells. Relatively few actin-binding proteins are identified in apicomplexans and these include profilin, actin-depolymerizing factor (ADF), coronin, and capping protein (CapZ) (Table 29.1). ADF is highly expressed in apicomplexans and the function of this protein in regulating filament turnover is considered further below. Coronin has been described in *P. falciparum* (Tardieux *et al.*, 1998) and previous studies on actin-binding proteins in *P. knowlesi* identified what are likely homologs of CapZ  $\alpha/\beta$  (Tardieux *et al.*, 1998). Apicomplexans contain a single profilin gene (Table 29.1), which is likely involved in sequestering G-actin and in nucleotide exchange. Profilin may also work in concert with formin homology (FH2) domain containing proteins to influence actin dynamics by promoting actin polymerization from the barbed-end, as described in other systems (Evangelista *et al.*, 2003; Higgs and Peterson, 2005). Exhaustive BLAST search analyses of apicomplexan genomes reveals they do not contain homologs of thymosin  $\beta$ 4, or the severing proteins gelsolin and severin, the cross-linking proteins  $\alpha$ -actinin or tropomodulin, or a variety of more specialized actin-binding proteins (Table 29.1) (Bear *et al.*, 2001; Cooper and Schafer, 2000; Pollard and Borisy, 2003). Previous studies have described an actin binding protein called toxofilin in *T. gondii* and suggested it is involved in regulating actin dynamics in the parasite (Poupel *et al.*, 2000). However the localization of this protein in the rhoptry fraction as shown by proteomic analysis (Bradley *et al.*, 2005), suggests an intriguing alternative, that it is injected into the cytosol of the host cell where it may act on the host cytoskeleton.

### ADFs in *Plasmodium* and *Toxoplasma*

The actin depolymerizing factor (ADF)/cofilin family is a group of small, highly conserved actin-binding proteins that are found ubiquitously in eukaryotes, and are essential for regulating the turnover of actin filaments *in vivo* (Moon and Drubin, 1995). While single-celled organisms typically possess one member of this family, metazoans can have multiple isoforms, with *Arabidopsis* containing up to 12 ADF genes (Bamburg, 1999). Most apicomplexans contain a single ADF homolog, except for *Plasmodium* that contains two ADF genes (Table 29.1).

Initial characterization of proteins in this family revealed their ability to decrease the low-shear viscosity of F-actin and the average length of actin filaments as visualized by electron microscopy (Cooper *et al.*, 1986; Mabuchi, 1983; Maciver *et al.*, 1991). Severing of filaments also increases the number of filament ends for assembly, consistent with kinetic data showing an increase in polymerization in the presence of ADF/cofilins (Cooper *et al.*, 1986; Nishida *et al.*, 1984). A role for severing was supported by structural data showing that ADF/cofilins bind to two adjacent subunits along the long pitch of the two-strand

**Table 29.1** Summary of actin-binding proteins conserved in Apicomplexans

Protein	Activity	<i>Toxoplasma gondii</i> <sup>a</sup>	<i>Plasmodium falciparum</i>	<i>Cryptosporidium hominis</i>
Arp2/3 complex	Nucleates pointed end	Absent <sup>b</sup>	Absent	Absent
ALP1 <sup>b</sup>	Unknown	AAW23163	AAN35700	EAK88581
ADF	F-actin turnover G-actin-binding	AAC47717	ADF1 AAY86358 ADF2 AAY86357	EAK88221
Capping protein	Caps barbed end	$\alpha$ AAU93918 $\beta$ AAU93916	$\alpha$ CAD51646 $\beta$ CAD51540	$\alpha$ - <sup>c</sup> $\beta$ EAK88546
Coronin	Cross-links F-actin	AAU93915	CAA05244	EAL37692 <sup>d</sup> EAL35113 <sup>d</sup>
Formins	Nucleates barbed end	20.m05986 <sup>d,e</sup> 20.m03963 31.m00924	NP_703650 <sup>f</sup> NP_701549	EAL37124 <sup>d</sup> EAL37373
Profilin	Sequesters G-actin Nucleotide turnover	AY937257 AAX47289 <sup>g</sup>	CAD51999	EAL35531
Toxofilin	Binds G-actin, increases turnover	CAB72264	No ortholog	No ortholog
$\beta$ 4 thymosin	Sequesters G-actin	Absent	Absent	Absent
Severin	Severs filaments	Absent	Absent	Absent
Gelsolin	Ca <sup>2+</sup> activated severing	Absent	Absent	Absent
$\alpha$ -Actinin	Cross-links F-actin	Absent	Absent	Absent
Filamin	Cross-links F-actin	Absent	Absent	Absent
Tropomodulin	Cross-links F-actin	Absent	Absent	Absent

<sup>a</sup>Entries from NCBI unless otherwise noted.

<sup>b</sup> Additional actin-like proteins in apicomplexans summarized in (Gordon and Sibley, 2005).

<sup>c</sup>Alpha subunit is not highly conserved.

<sup>d</sup> Closest orthologs to the *Plasmodium* proteins are listed.

<sup>e</sup> <http://ToxoDB.org> Draft 3 annotation.

<sup>f</sup>(Higgs and Peterson, 2005), <sup>g</sup>identical GenBank entries.

actin helix causes a change in the twist of the filament by  $3\text{--}5^\circ$  (McGough *et al.*, 1997). The altered pitch is thought to increase the stress on the filament leading to fragmentation (Carlier *et al.*, 1997; Pope *et al.*, 2000).

In addition to their ability to bind and sever filaments, ADF/cofilins may also increase the off rate of subunits from the pointed end of the filament (Carlier *et al.*, 1997; Maciver *et al.*, 1998; McGough *et al.*, 1997). Addition of ADF/cofilins to F-actin has been shown to increase the pointed end off-rate by 25–30 fold, leading to rapid treadmilling of filaments (Carlier *et al.*, 1997; Maciver *et al.*, 1998). While there is some controversy over how ADF/cofilins regulate filament turnover, mutational analysis indicates that both severing and depolymerizing activities can be uncoupled (Pope *et al.*, 2000). The extent to which severing vs. enhanced turnover occurs varies depending on the organism and whether heterologous or homologous actin is used to study the interaction (Blanchoin and Pollard, 1999).

ADF/cofilins have an established role in regulating actin filament turnover in other systems and it seems likely that they fulfill a similar function in the *Apicomplexa*. Previous studies on ADF from *Toxoplasma* support a role in actin turnover (Allen *et al.*, 1997). TgADF is present as a single copy gene in *Toxoplasma*, and purified TgADF expressed as recombinant protein in *E. coli* is able to increase turnover of rabbit actin filaments *in vitro* in a concentration dependent manner (Allen *et al.*, 1997). TgADF also forms complexes with actin monomers similar to other ADF/Cofilin members.

*Plasmodium* contains two ADF genes, ADF1 and ADF2, but only ADF1 is expressed at a significant level and it is essential in the pathogenic erythrocytic stage (Schuler *et al.*, 2005). Surprisingly, recombinant PfADF1 lacked binding to F-actin and had no effect on turnover of bovine actin filaments *in vitro*. Whether this is due to the use of heterologous actin, or is simply due to an unusual property of PfADF1 is uncertain. Recombinant PfADF1 was found to interact preferentially with ADP-bound actin monomers. However, in contrast to other ADF/cofilins, PfADF1 rendered *Plasmodium* actin monomers more susceptible to thermal denaturation, suggesting that it destabilized G-actin (Schuler *et al.*, 2005). PfADF1 was also found to stimulate nucleotide exchange on both *Plasmodium* actin and mammalian actin. This again contrasts with the normal property of ADF to stabilize ADP-G actin and resist nucleotide exchange (Blanchoin and Pollard, 1998; Nishida, 1985). Since apicomplexans possess homologs of profilin and cyclase associated protein (CAP), which normally enhance nucleotide exchange on actin (Blanchoin and Pollard, 1998; Nishida, 1985) it is unusual that ADF would also exhibit this function. A detailed analysis of *Toxoplasma* ADF will be important to determine if these unusual properties of PfADF are unique to *Plasmodium* or if they represent another mechanism by which apicomplexans have adapted their actin cytoskeleton to undergo rapid cycles of filament assembly and disassembly.

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## Actin-related proteins in Apicomplexans

While apicomplexans typically contain only a single isoform of actin, they contain a diverse array of actin-like proteins (ALPs). Analysis of several recently completed apicomplexan genomes reveals that apicomplexans contain 10 different phylogenetically related groups of ALPs (Gordon and Sibley, 2005). Of these, only 3 groups are conserved with

traditional actin-related proteins (Arps) identified in all other eukaryotes (Schroer *et al.*, 1994), including Arp1, Arp4, and Arp6. Arp1 is an essential component of the dynactin complex involved in vesicle transport (Schroer, 2004), while Arp4 and Arp6 are involved in chromatin remodeling (Blessing *et al.*, 2004). The most surprising finding is that none of the apicomplexans encode either an Arp2 or Arp3 protein homolog (Gordon and Sibley, 2005; Muller *et al.*, 2005). The remaining groups of actin like proteins (ALPs) are either conserved throughout phylum (i.e. ALP1 and 2) or specific to one or more genus such as the *Toxoplasma*-specific ALP8 and ALP9 (Gordon and Sibley, 2005).

Arps are characterized by four subdomains surrounding a central nucleotide-binding pocket that forms an actin fold (Kabsch and Holmes, 1995). The larger Arp family is subdivided into 11 separate groups based on a hierarchical divergence from actin, as well as sequence similarity within each group (Poch and Winsor, 1997; Schroer *et al.*, 1994). Arps typically associate in larger protein complexes with defined functions. For example, Arp2 and Arp3 are the major components of the Arp2/3 complex, which regulates actin polymerization (Higgs and Pollard, 2001; Mullins *et al.*, 1998; Mullins *et al.*, 1997). Arp1 and Arp11 are components of dynactin, a protein complex necessary for trafficking vesicles along microtubules (Eckley *et al.*, 1999; Eckley and Schroer, 2003; Vaughan, 2005). Arps 4–9 are nuclear Arps that serve as various components of the chromatin/heterochromatin remodeling machinery (Blessing *et al.*, 2004; Kato *et al.*, 2001; Olave *et al.*, 2002).

The Arp2/3 complex is made up of 7 subunits that regulate actin polymerization (Mullins *et al.*, 1998). Actin filament formation begins with a slow phase during formation of a trimer that constitutes a critical nucleus for subsequent elongation by successive addition of monomers (Cooper *et al.*, 1983). When the Arp2/3 complex is activated, the Arp2 and Arp3 proteins undergo a conformational change that mimics the actin nucleation event, making the addition of monomers and the elongation of the actin filament more thermodynamically favorable (Volkman *et al.*, 2001). Gliding motility in apicomplexans is dictated by the parasite's ability to quickly transform from a non-motile state to one that is organized and primed for motility (Wetzel *et al.*, 2003). It is therefore unusual that these parasites lack a similar control over rapid actin polymerization that is common and essential to other eukaryotes.

Comparative genome analysis reveals that apicomplexans not only lack Arp2 and Arp3 proteins, but that the majority of the subunits are also absent (Gordon and Sibley, 2005). Neither *Toxoplasma* nor *Theileria* encode orthologs to any of the 7 subunits of this complex, while *Plasmodium* and *Cryptosporidium* both encode orthologs to the ARPC1/p41 subunit. ARPC1 is the largest subunit and it serves as an adaptor between the Arp2/3 complex and its activator proteins (Pan *et al.*, 2004), but it is not involved in the overall cohesiveness of the complex (Gournier *et al.*, 2001). *Cryptosporidium* also encodes a conserved ARPC4/p20 subunit. The ARPC4 subunit forms a stable heterodimeric complex with ARPC2/p34 and these two proteins form the structural core of the complex and are essential for its overall integrity (Gournier *et al.*, 2001). It is unusual that *Cryptosporidium* retains a close homolog to one subunit yet completely lacks the other. Other alveolates, such as the closely related but deeper branching ciliate lineages (Baldauf *et al.*, 2000) encode a canonical Arp2/3 complex. Thus it is likely that the ARPC1/p41 and ARPC4/p20 subunits in *Plasmodium* and *Cryptosporidium* represent remnants of an

Arp2/3 complex that may have been present in these parasites at one time. Since then, they have either lost the complex completely or the subunits have diverged to the extent that they are no longer recognizable.

The loss of a functional Arp2/3 in apicomplexans may be compensated for by the low critical concentration of parasite actin. Alternatively other conserved ALPs may fulfill a similar function. Since the mechanism of gliding motility is highly similar among apicomplexans, a conserved actin-like protein likely fulfills such a role. Future studies will reveal if the apicomplexan ALPs play roles in regulation of actin polymerization during gliding motility or if they participate in other biological processes.

In addition to motility, actin is required for several other cellular processes such as vesicular trafficking (Schafer, 2002) and transcriptional regulation (Olave *et al.*, 2002). Apicomplexans encode conserved Arp1, Arp4, and Arp6 homologs, which are widely conserved among eukaryotes and function in vesicle transport and chromatin remodeling, respectively. While their functions have not been addressed in apicomplexans, the high degree of conservation allows us to make some predictions about possible roles based on findings in model systems.

The Arp1 protein is a major constituent of the dynactin complex (Schafer *et al.*, 1994), which associates with microtubules to tether vesicular cargo to cytoplasmic dynein, a microtubule motor protein (Gill *et al.*, 1991). Dynactin is made up of 11 different protein subunits that are divided into two domains: the Arp1 rod and a flexible arm region (reviewed in (Schroer, 2004)). The proteins in the Arp1 rod are most widely conserved among eukaryotes (Schroer, 2004). Of these, *Toxoplasma* and *Plasmodium* encode 5 conserved subunits (Arp1, p62, p25, p27, and dynamitin). *Cryptosporidium* encodes Arp1, p62, p25, and p27; however, a protein similar to dynamitin was not identified. Curiously, *Theileria* does not appear to encode any proteins in the dynactin complex including Arp1. The presence of a conserved dynactin complex in most apicomplexans suggest this complex may be involved in transport of vesicles along microtubules toward the anterior end of the cell (Morrisette and Sibley, 2002). See Chapter 28 in this volume for more details on microtubule-dependent motors.

Arp4 and Arp6 have recently been shown to be the most widely conserved proteins among eukaryotes (Muller *et al.*, 2005). In accordance with this, all the apicomplexans encode relatively conserved Arp4 and Arp6 orthologs. Arp4 and Arp6 are nuclear Arps involved in transcriptional regulation (Blessing *et al.*, 2004; Boyer and Peterson, 2000; Mizuguchi *et al.*, 2003; Olave *et al.*, 2002). Arp4 associates with several different chromatin-modifying and -remodeling protein complexes including the SWI2-SNF2 family of chromatin-remodeling complexes and the NuA4 histone acetyltransferase (Blessing *et al.*, 2004; Doyon and Cote, 2004; Shen *et al.*, 2000). Arp6 is found in association with Arp4 to form the SWR1 complex, a subgroup of the SWI2/SNF2 family (Blessing *et al.*, 2004; Michel *et al.*, 1979; Mizuguchi *et al.*, 2003; Ueno *et al.*, 2004). Chromatin-remodeling plays an important role in gene expression in apicomplexans (Saksouk *et al.*, 2005) and it is likely that nuclear Arps contribute to this process. See Chapter 18 in this volume for more details on chromatin remodeling.

## Remaining mysteries

Despite progress in understanding the molecular basis of motility, significant gaps exist in our knowledge about how actin assembly is regulated in parasites. At present it is unclear if parasite actins require a nucleation factor and if pointed-end or barbed-end nucleation predominates during motility vs. other cellular activities. It is also unclear how directionality is provided to assure that motility will propel the parasite forward. TgMyoA is a plus end directed motor, and gliding motility always occurs in a directional manner (forward), implying that short actin filaments must be oriented along the long axis of the parasite. This process may be facilitated by the arrangement of myosin motor complexes anchored in the IMC. The inherent instability of parasite actin filaments suggests they may also require a stabilizing force *in vivo*, and yet this action must clearly be regulated. The profound effect of JAS, which stabilizes actin filaments, indicates the importance of these dynamic processes in controlling apicomplexan motility. The molecular machinery for actin-based motility in apicomplexans is highly dissimilar to that found in conventional model systems. Hence, future studies on the role of apicomplexan actin-binding proteins and ALPs in parasites will be necessary to decipher the mechanisms of gliding motility.

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## Abstract

The protozoan parasite *Toxoplasma gondii* is an obligate intracellular organism that survives in a variety of hosts. It undergoes a complex life cycle and has a highly specialized series of unique secretory organelles that enable its successful invasion of host cells. The study of proteases of *T. gondii* is relatively recent, but ongoing studies from several laboratories suggest that proteases perform critical functions during the development of the parasite. It appears that regulated proteolysis of the secreted products is essential for efficient invasion by *T. gondii*. Proteases are implicated in regulation of biogenesis of secretory organelles as well as the assembly and disengagement of adhesive complexes secreted by micronemes. Proteases are important for assembly of the cytoskeleton, targeting to the apicoplast or mitochondria and other essential physiological processes. Several unique aspects of proteolysis are emerging that may eventually facilitate development of novel chemotherapeutic agents.

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## Introduction

Because the Apicomplexa are obligate intracellular parasites, their survival depends on their ability to invade host cells, avoid degradation by host cell machinery and propagate intracellularly. The Apicomplexa are named for the unique set of secretory organelles that are intimately associated with these functions. Several lines of evidence suggest that proteases are important in the creation of a hospitable environment for these parasites.

The genome of *T. gondii* has offered many insights into the biology of *T. gondii*. An initial analysis of the *P. falciparum* genome revealed over 90 genes with homology to well-characterized protease families (Wu *et al.*, 2003; Wang and Wu, 2004), and *T. gondii* is likely to encode at least as many proteases ([www.toxodb.org](http://www.toxodb.org)). Based upon the successes in development of protease inhibitors for treatment of HIV and cardiovascular diseases, proteases have been investigated as potential chemotherapeutic targets in many pathogens. A variety of classes of proteases are now being characterized in *T. gondii* in light of studies that indicate that regulated proteolysis is an integral part of secretory organelle biogenesis. Regulated proteolysis is a critical aspect of successful invasion of host cells by all Apicomplexa. In addition many important housekeeping functions are likely to be regulated by proteolysis.

It was initially assumed that the repertoire of *T. gondii* proteases, particularly those involved in invasion, would mirror that of other Apicomplexa including *Plasmodium* species.



The validity of this assumption is being tested currently, but based upon initial comparative analyses of genomes, there are significant differences in the repertoire of proteases, particularly those potentially involved in invasion.

An incomplete list of the classes of proteases likely to be expressed in *T. gondii* is detailed in Table 30.1. In most cases these have been inferred to be proteases based upon homology to proven proteases rather than being experimentally validated. Chemical biology strategies using libraries of small molecule inhibitors are also being used to dissect the contributions of cysteine and serine proteases to various biological events including host cell invasion, replication and host cell egress (Ward *et al.*, 2003; Carey *et al.*, 2004). Thus it is anticipated that there will be a more comprehensive understanding of the contribution of proteolysis to the biology of *T. gondii* in the near future.

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### Secretory pathway of the Apicomplexa

The secretory pathway in the Apicomplexa is highly polarized and specialized for the invasion process, which is initiated at the apical end of the parasite. *T. gondii* replicates by endodyogeny, a unique form of replication in which daughter cells are assembled within a mother cell. Proteins are synthesized in the endoplasmic reticulum (ER) before being trafficked to the Golgi complex. From the trans Golgi network, the specialized secretory granules are formed. Proteolytic processing of some microneme and rhoptry contents proteins occurs within the secretory pathway in “pre” compartments, before final targeting.

Proteins are also targeted to the apicoplast (a unique chloroplast-like organelle) and to the mitochondrion. Although the details of apicoplast targeting are being worked out, proteins targeted to the apicoplast initially enter the secretory pathway. Targeting of many proteins to the apicoplast or mitochondrion is mediated by a transit peptide. An essential part of trafficking to these endosymbiotic organelles is recognition and subsequent cleavage of transit peptides. While the proteases responsible for these activities have not been identified, orthologs to similar proteases in other systems can be identified in Apicomplexan genomes (van Dooren *et al.*, 2002) as well as in *T. gondii*.

### Regulated secretion in Apicomplexa

Successful host cell invasion involves the sequential release of micronemes, rhoptries and dense granules (Carruthers and Sibley, 1997). Micronemes have properties reminiscent of the dense core granules of the ciliates and neuroendocrine cells. Rhoptries are similar to lysosomal secretory organelles and thus are hypothesized to be derived from multivesicular bodies derived from both secretory and endocytic pathways (Ngo *et al.*, 2004; Yang *et al.*, 2004). Dense granules are the constitutive secretory pathway for soluble proteins in *T. gondii*. Remarkably micronemes, rhoptries and dense granules have little overlap in their contents.

Biogenesis of regulated secretory organelles is not fully understood in any system. Aggregation of organellar contents in a calcium and pH dependent manner is thought to be the major mode of organellar assembly. Proteolysis appears to regulate biogenesis and maturation of both dense core granules (Zhou *et al.*, 1999; Kuliawat *et al.*, 2000; Turkewitz, 2004) and lysosome-related secretory organelles (Berson *et al.*, 2003). Biogenesis of micronemes and rhoptries appears to be regulated by proteolysis as well. Dense granule content



proteins are not proteolytically cleaved, but both endogenous protease inhibitors (Morris *et al.*, 2002; Morris and Carruthers, 2003) and cathepsin C orthologs (Que *et al.*, 2007) localize to dense granules and are secreted into the parasitophorous vacuole.

### Proteolytic processing in the Apicomplexan secretory pathway

Although the exact molecular actors are still being defined, it is clear that proteases play an essential role in host cell invasion by the Apicomplexa. (reviewed in Carruthers, 1999; Carruthers, 2004; Kim, 2004; Soldati, 2004; Carruthers and Blackman, 2005; Dowse and Soldati, 2005). Early observations that serine protease inhibitors DCI and AEBSF (Conseil *et al.*, 1999) and cysteine protease inhibitors (Que *et al.*, 2002) prevent *T. gondii* tachyzoites from penetrating host cells have now been pursued by several groups. *In vivo* data with the serine protease inhibitor AEBSF also support this contention (Buitrago-Ray *et al.*, 2002). There are also reports that 1–10 phenanthroline affects invasion, suggesting that a metalloprotease may be involved in invasion (Song and Nam, 2003). Two different metalloproteases were identified in rhoptry and ESA proteomic efforts (Bradley *et al.*, 2005; Zhou *et al.*, 2004; Zhou *et al.*, 2005) with other members in the genome.

A drawback of these inhibitor data is the lack of specificity for many of the inhibitors used. Although serine and cysteine proteases have been implicated by these studies, it is likely that more than one protease has been affected. For example, the cysteine protease inhibitor PRT2253F inhibits invasion and replication (Que *et al.*, 2002). Although it inhibits the rhoptry protease toxopain I (Que *et al.*, 2002), PRT2253F's effects upon invasion may be due to inhibition of toxopain I or another cysteine protease, perhaps the Cathepsin L ortholog TgCPL.

Conversely, lack of inhibition by canonical inhibitors does not rule out a role of proteases of a particular class. Plasmepsin V, an aspartyl protease of *P. falciparum* localizes to the secretory pathway and appears to be essential (Klemba *et al.*, 2005). Surprisingly, it does not appear to bind pepstatin, the classic aspartyl protease inhibitor that does inhibit the other characterized plasmepsins (Klemba *et al.*, 2005). Therefore, it and a potential *T. gondii* ortholog, TgAP5, may have an important, but as yet unappreciated role in maturation of microneme or rhoptry content proteins.

Despite these caveats, proteases clearly play essential roles in regulation of the secretory pathway, biogenesis of organelles and the invasion process (see Table 30.2). Much of the data discussed in this chapter is unpublished and reflects the rapidly evolving nature of the field.

### Proteolysis during microneme formation and secretion

Micronemes are the smallest of the secretory organelles. Involved in the early stages of invasion, they contain many adhesins that help in the tight attachment of the parasite to the host cell. Microneme secretion is a calcium-dependent process and is enhanced by manipulations that increase intracellular calcium (Carruthers and Sibley, 1999; Carruthers *et al.*, 1999). (For more detail about micronemes see Chapter 23.)

Microneme proteins assemble as macromolecular adhesion complexes. Microneme proteins that are type I integral membrane proteins contain microneme-targeting signals in their C-terminal domains (Di Cristina *et al.*, 2000; Reiss *et al.*, 2001). The membrane

**Table 30.1** Classes of proteases in *Toxoplasma gondii*

Class	Name	Processing	Localization	Substrate
Aspartyl (formerly toxomepsins)	AP1	N and C term	Apical dispersed; IMC during endodyogeny	
	AP2			
	AP3	N and C term	Punctate apical	
	AP4			
	AP5			
	AP6			
Cysteine	Toxopain I (CPB or TCP1)	Cathepsin B-like	rhoptry	Rhoptry proteins?
	CPL	Cathepsin L-like	“pre-microneme”?	Microneme propeptides? K/R;L/F;X
	CPC1–2	Cathepsin C-like	Dense granule	Dipeptides from N termini
	CPC3	Cathepsin C-like		
Serine-subtilase	TgSUB1	Autocatalytic, cleaved upon secretion	Microneme, released into ESA	Activates MPP2/3 vs is MPP2/3
	TgSUB2	Autocatalytic	Rhoptry	ROP1; ROP2 family?
	TgSUB3–12			
Serine rhomboid	TgROM1		microneme	
	TgROM2		Golgi?	
	TgROM3			

Function	Apicomplexan orthologs	Other features	Inhibitors	Reference
Essential??	Plasmepsins 1-!V	Type II		Soldati, unpublished,
	Eimepsin	GPI anchor		Soldati, unpublished,
Essential??	Plasmepsin X	Tachyzoite: Type II		Soldati, unpublished,
	Eimepsin	GPI		Soldati, unpublished,
	Plasmepsin V	Signal peptide; C term TM domain		Soldati, unpublished,
Stage specific	Plasmepsin VII	Sporozoite EST, probably not in tachyzoites		Soldati, unpublished,
Rhoptry biogenesis; probably essential	Not in Plasmodium			(Que <i>et al.</i> , 2002)
Essential??; Activation of microneme proteins/adhesins	Falcipain 1, 2, 2',3	Type II	LHVS	Soldati, unpublished Carruthers, unpublished,
CPC1 and CPC2 redundant?; Degradation peptides in parasitophorous vacuole	DPAP1	Exopeptidase;	Gly-Phe-Methylketone	(Que <i>et al.</i> , 2007)
	DPAP1	Sporozoites, not tachyzoites		(Que <i>et al.</i> , 2007)
MPP2/3??	PfSUB1	GPI anchor	PMSF?	(Miller <i>et al.</i> , 2001)
Essential??		TM domain	Subtilisin Inhibitor III?	(Miller <i>et al.</i> , 2003)
		Not all expressed in tachyzoites		Kim, unpublished
	PfROM1	Active in mammalian cell assay		(Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005)
		Sporozoite> tachys		(Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005)
	PfROM3	sporozoite		(Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005)

Table 30.1 is continued overleaf

Table 30.1 Continued

Class	Name	Processing	Localization	Substrate
Metallo	TgROM4		Plasma membrane	
	TgROM5		Plasma membrane and posterior	TM domains of MIC2, 6, 8, 12; MIC2
	TgROM6		Mitochondria	
		insulinase	Rhoptry neck Microneme? (in ESA)	
		Stromal Processing Peptidase	Apicoplast	Transit peptide
		Mitochondrial Processing Peptidase	Mitochondrion	Transit peptide

proteins form a complex with soluble microneme proteins (Reiss *et al.*, 2001). Proper assembly of these complexes is required for exit from the ER/Golgi and targeting to micronemes (Huynh *et al.*, 2003; Reiss *et al.*, 2001).

The MIC2/M2AP complex is one of the three major adhesive complexes mediating invasion (Brossier *et al.*, 2003; Huynh *et al.*, 2003), and MIC2 appears to be essential. Parasites deficient in MIC1 (MIC1/4,6 complex) and MIC3 (MIC3/8 complex) are attenuated, suggesting that the microneme adhesion complexes have redundant functions (Reiss *et al.*, 2001; Meissner *et al.*, 2002; Cerede *et al.*, 2005). It is also possible that adhesive complexes are more important in different developmental stages or with particular host cell types. The *in vivo* phenotype of MIC protein disruption has sometimes been more pronounced than *in vitro* studies illustrating the limitations of testing phenotypes in cultured cells using a limited number of assays (Cerede *et al.*, 2005).

Proteolysis within the secretory pathway appears to regulate maturation of microneme contents and formation of micronemes, much as it appears to regulate formation of secretory granules in other eukaryotes (Zhou *et al.*, 1999; Kuliawat *et al.*, 2000; Turkewitz, 2004). Although not needed for trafficking, cleavage of the prodomain does appear to facilitate productive interaction of partners within the adhesive complex and may also confer additional microneme targeting properties (Cerede *et al.*, 2002).

Most microneme complexes have one member that is proteolytically cleaved during transit through the secretory pathway. The prodomain is required for trafficking of M2AP, MIC3 and MIC5 (Striepen *et al.*, 2001; Cerede *et al.*, 2002), but surprisingly, cleavage of the prodomain may not be required for microneme targeting (Harper *et al.*, 2006; V. Carruthers, unpublished). For MIC5 and M2AP the site of cleavage appears to be prior to arrival in the micronemes in a post-Golgi “pre-microneme” compartment.

Function	Apicomplexan orthologs	Other features	Inhibitors	Reference
MPP1??	PfROM4	Not active in mammalian assay	DCI?	(Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005)
MPP1??	PfROM4	Active in mammalian assay	DCI?	(Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005)
				Soldati, unpublished (Bradley <i>et al.</i> , 2005) (Zhou <i>et al.</i> , 2005)
	PfSPP	Function not proven but inferred based upon sequence		(van Dooren <i>et al.</i> , 2002)
	PfMPP $\alpha$ , PfMPP $\beta$	Function not proven but inferred based upon sequence		(van Dooren <i>et al.</i> , 2002)

Proper cleavage is probably required for proper maturation and function of microneme proteins. Proteolytic cleavage of MIC3 is required for its adhesive properties (Cerede *et al.*, 2002) and deletion of its prodomain results in MIC3 that is retained within the secretory pathway. M2AP that is not cleaved traffics to the micronemes but does not associate with MIC2 as effectively and does not restore virulence of M2AP disruptants (Huynh *et al.*, 2003; Huynh *et al.*, 2006; Harper *et al.*, 2006; V. Carruthers, unpublished). Pulse chase analyses and inhibitor studies suggest there are at least 2 processing activities that cleave microneme prodomains. The kinetics of cleavage of M2AP and MIC5 are significantly different (Carruthers, 2004).

As invasion occurs, *T. gondii* microneme proteins are secreted onto the parasite's surface and move towards the posterior end before being shed off the surface by a number of unidentified proteases (Carruthers *et al.*, 1999; Carruthers *et al.*, 2000; Zhou *et al.*, 2004; reviewed in Kim, 2004). The interaction of the treadmilling proteins with the actin-myosin complex provides the force for host cell invasion (Kappe *et al.*, 1999; Jewett and Sibley, 2003). Mutation of sites cleaved during invasion does not affect trafficking to micronemes (Opitz *et al.*, 2002) but probably does affect the interaction with other members of the adhesive complex or host cell components (Harper *et al.*, 2004; Barragan *et al.*, 2005).

There are at least 2–3 proteolytic activities that occur on the surface (Carruthers *et al.*, 2000; Zhou *et al.*, 2004). These activities have been named microneme protein proteases 1–3 with classification based upon differential sensitivity to protease inhibitors. Initial cleavage events mediated by MPP2/3 may be part of the maturation of adhesions (Barragan *et al.*, 2005) or part of regulated degradation of secretory complexes (Carruthers and Blackman, 2005). As invasion occurs, more extensive proteolysis occurs which is likely to be part of a regulated proteolytic cascade that first activates and then inactivates adhe-

**Table 30.2** Possible biological functions of toxoplasma proteases

	Function	Location of cleavage	Candidate protease	Candidate substrate
Invasion	Maturation of adhesins	“Premicroneme”	CPL	MIC3, M2AP, MIC6, AMA1
	Prodomain cleavage	“Premicroneme”		MIC5
	Microneme Processing Protease 2	Parasite surface	TgSUB1	MIC2, M2AP, MIC4
	Microneme Processing Protease 3	Parasite surface	TgSUB1	MIC2
	Shedding of adhesins	Parasite surface, posterior	TgROM4 TgROM5	TM domains of MIC2, 6, 8, 12; AMA1
	General invasion	?	ROMs, SUBs	
Organellar biogenesis	Rhoptry formation	“Pre-rhoptry”?	Toxopain I aka CPB or CP1	Rhoptry proteins
	Rhoptry formation	“Pre-rhoptry”?	TgSUB2	Rhoptry proteins ROP1; ROP2 family
	Vacuole	Parasitophorous vacuole	CPC1–2	Dipeptides from N termini
	Mitochondria		TgROM6	
	Mitochondria Apicoplast			Transit peptides
Cytoskeletal formation	Inner membrane complex	Inner membrane complex	Calpain?	IMC

sive complexes, much in the way complement activation or the coagulation pathways are regulated. The adhesive complexes are shed from the parasite surface as invasion proceeds. Microneme adhesion complexes are discarded and generally do not enter the host cell with the invading parasite (Zhou *et al.*, 2004).

Microneme proteases

TgSUB1 is a microneme-associated subtilase (Blackman *et al.*, 1998; Miller *et al.*, 2001) homologous to PfSUB1. TgSUB1 is secreted in a calcium-dependent manner from the parasite, like other *T. gondii* microneme proteins. TgSUB1 is unique in that it contains a GPI anchor (Binder and Kim, unpublished), yet is targeted to micronemes rather than the parasite’s surface. Other known GPI anchored proteins are expressed on the surface and substitution of a GPI anchor for the transmembrane domain and cytoplasmic tail redirects microneme proteins to the cell surface (Reiss *et al.*, 2001). TgSUB1 may be part



Inhibitors?	References
LVHS, E64d, Cathepsin L Inhibitor II	(Cerede <i>et al.</i> , 2002); Carruthers unpublished
DFP	Carruthers, Kim unpublished
ALLN; ALLM; chymostatin, PMSF	(Carruthers <i>et al.</i> , 2000; Zhou <i>et al.</i> , 2004) Carruthers, Kim unpublished
None known, activity missing in SUB1 knock-out	(Carruthers <i>et al.</i> , 2000; Zhou <i>et al.</i> , 2004) Carruthers, Kim unpublished
DCI, TLCK	(Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005; Howell <i>et al.</i> , 2005)
DCI, AEBSF	(Consiel <i>et al.</i> , 1999)
Partial block of ROP2/3/4 processing (PRT2253F); impaired rhoptry biogenesis PRT2253F and Cathepsin Inhibitor III	(Que <i>et al.</i> , 2002; Shaw <i>et al.</i> , 2002)
Subtilisin inhibitor III?	(Miller <i>et al.</i> , 2003; Shaw <i>et al.</i> , 2002)
Gly-Phe-Methylketone	Reed, unpublished
	Soldati, unpublished
	(van Dooren <i>et al.</i> , 2002)
	(Mann and Beckers, 2001)

of complex that is targeted to the micronemes, but coimmunoprecipitation experiments do not reveal obvious interacting protein partners.

There is probably considerable redundancy in microneme proteases. A knockout of TgSUB1 is viable and has no obvious defect in invasion (Binder and Kim, unpublished). Despite this, the knockout lacks both MPP2 and MMP3 activity, reflecting the likely redundancy of invasion pathways (Lagal, Binder, Carruthers and Kim, unpublished). Catalytically inactive TgSUB1 Ser 490Ala is partially processed, probably at different sites from active enzyme (Binder and Kim, 2004). Thus it appears other proteases interact with TgSUB1 during its transit through the secretory pathway. Among these candidate proteases are uncharacterized members of the subtilase family that are expressed in tachyzoite forms (Table 30.1).

Similarly, extensive mutation of M2AP and MIC5 cleavage sites is necessary to abrogate cleavage (Carruthers, unpublished). This suggests either that the processing

protease has relatively lax specificity or that there are other proteases that can process microneme proteins if the usual enzyme cannot. Similar observations have been reported in *Tetrahymena* (Bradshaw *et al.*, 2003; Turkewitz, 2004).

MPP1, the protease responsible for shedding microneme adhesive complexes (Carruthers *et al.*, 2000) has the unusual property of cleaving microneme proteins within their transmembrane domains as verified by mass spectrometry analysis of the cleavage site of MIC6 and then later MIC2 (Opitz *et al.*, 2001; Zhou *et al.*, 2004). TgAMA1 is also shed by intramembrane proteolysis, but surprisingly, the malaria ortholog PfAMA-1 is released by juxtamembrane cleavage by PfSUB2, a subtilase that is also responsible for shedding of MSP1 (Howell *et al.*, 2005; Harris *et al.*, 2005)

Intramembrane proteolysis has been recently described as being important for a variety of signaling events including quorum sensing, notch signaling and cholesterol signaling (Brown *et al.*, 2000). Integral membrane proteases such as presenilin (aspartyl) and S1P (metallo) are responsible but most do not cleave until another protease has processed the substrate. The rhomboid family of intramembranous serine proteases is now thought to be responsible for the cleavage of microneme proteins TgMIC2, TgMIC6, and TgAMA1. Rhomboids cleave near the within the transmembrane domain in a region of small, helix breaking amino acids such as alanine and glycine (Urban and Freeman, 2003), properties that are consistent with the cleavage sites.

### Proteolysis during rhoptry formation

Rhoptries are long club-shaped organelles that secrete proteins through their elongated necks at the apical tip of the parasite (for more details see Chapter 24). In *Toxoplasma*, secretion of the micronemes precedes rhoptry exocytosis (Carruthers and Sibley, 1997), but biological triggers of rhoptry secretion have not been identified. Data are now emerging that rhoptry bulb proteins (ROPs) and rhoptry neck proteins (RONs) have different functions. Rhoptry neck proteins appear to be secreted first in association with microneme protein AMA1, and are localized to the moving junction where there is tight apposition of host and parasite membranes (Alexander *et al.*, 2005; Lebrun *et al.*, 2005).

Rhoptries have internal membranes and type I transmembrane proteins ROP2/3/4 and TgSUB2 localize to the lumen of rhoptries rather than their periphery (Miller *et al.*, 2003; Ngo *et al.*, 2004). Proteolysis of organellar contents is commonly seen, but the proteases responsible have not been definitively identified.

Most characterized *T. gondii* rhoptry proteins are proteolytically cleaved during transit in the secretory pathway with the exception of ROP5 and ROP9. In *T. gondii* only the cleavage site ROP1 has been definitively mapped (Bradley and Boothroyd, 1999). Surprisingly, trafficking of ROP1 does not seem to be dependent on proteolytic processing since unprocessed ROP1 is correctly trafficked to rhoptries and secreted into the parasitophorous vacuole during invasion (Bradley and Boothroyd, 2001; Bradley *et al.*, 2002). The prodomain of ROP1 and ROP4 can target a heterologous protein (VSG; GFP) to the rhoptry (Bradley and Boothroyd, 2001). It is unknown whether proteolytic processing of other rhoptry proteins affects their trafficking.

### Proteases of rhoptries

Subtilases and cathepsins are hydrolases classically associated with lysosomal compartments and orthologs are present in *T. gondii* rhoptries (Que *et al.*, 2002; Miller *et al.*, 2003). TgSUB2 localizes to the rhoptry secretory organelle. Furthermore, TgSUB2 shares a common cleavage site motif found among many rhoptry proteins, including the ROP1 and ROP2 family members (Miller *et al.*, 2003). This motif, SØXE, is present at 3 sites in TgSUB2 and site directed mutagenesis of these sites alters TgSUB2 autoprocessing (Miller *et al.*, 2003; Thathy *et al.*, unpublished). TgSUB2 coimmunoprecipitates with ROP1, ROP2 and ROP4 (Miller *et al.*, 2003; Thathy and Kim, unpublished data).

Genetic studies suggest that TgSUB2 is essential and may play an important role in biogenesis of the rhoptry secretory organelle (Miller *et al.*, 2003). Knockout of TgSUB2 was unsuccessful, and TgSUB2 antisense parasites displayed impaired rhoptry formation, accumulation of vesicular structures and impaired replication (Thathy *et al.*, unpublished).

Further studies by the group of Dr. Dubremetz have uncovered a large family of ROP2 related proteins. The majority of these proteins is proteolytically processed and has the proposed TgSUB2 cleavage motif. ROP5, which is not processed, has a deletion in the region of the motif (El Hajj *et al.*, 2007; JF Dubremetz, unpublished).

Antisense depletion of the rhoptry cathepsin B homolog, CPB (previously named toxopain-1), also leads to impaired rhoptry formation (Que *et al.*, 2002). An inhibitor of toxopain I, partially inhibits ROP2/3/4 protein processing and also results in abnormal rhoptry formation. Cathepsin inhibitor III and subtilisin inhibitor III were reported to impair rhoptry biogenesis (Shaw *et al.*, 2002). Thus it appears that toxopain TgCPB (cysteine) and TgSUB2 (serine) are candidate cysteine and serine proteases with a role in rhoptry protein maturation.

### Proteases involved in organellar assembly

Both the apicoplast and mitochondria have proteases that are required for proper trafficking and organellar biogenesis (van Dooren *et al.*, 2002). Some of the classes of proteases involved include metalloprotease and rhomboid serine proteases. One of the six rhomboids, TgROM6 localizes to the mitochondrion (Dowse and Soldati, unpublished). Orthologs of stromal processing proteinase (apicoplast transit peptide cleavage) and mitochondrial processing proteinases (mitochondrial transit peptide cleavage), metalloproteases of the pitrylsin family (van Dooren *et al.*, 2002) are present in the genomes of apicomplexans including *T. gondii*.

### Cytoskeletal assembly

The inner membrane complex (IMC) is lined by a meshwork of intermediate filament-like proteins, termed the subpellicular network, consisting of IMC 1, 2, 3 (Mann and Beckers, 2001; Morrisette *et al.*, 2002). During the last stages of assembly of the IMC during endodyogeny, a C-terminal proteolytic cleavage occurs within IMC 1 that results in an increase in rigidity of the nascent daughter cytoskeleton (Mann *et al.*, 2002). The subpellicular network appears to be formed *de novo* in each daughter cell. As the daughter forms develop within the mother, the subpellicular network is stable, but at the end of replica-

tion its IMC proteins are degraded rapidly. Thus there is a very tight temporal control of proteolysis that accompanies cytoskeletal formation and endodyogeny

The proteases involved and their mode of regulation remain areas of investigation. One candidate, an aspartyl protease called TgAP1, localizes to the IMC of daughter cells (M. Shaw and D. Soldati, unpublished). *P. falciparum* plasmepsin II (PfPMII) and plasmepsin IV (PfPMIV) are inhibited by “calpain inhibitor” ALLN, with an  $IC_{50}$  of 1  $\mu$ M for PfPMIV (Kim *et al.*, 2006). As TgAP1 is a homolog of PfPMIV, we can speculate that TgAP1 may also be inhibited by ALLN or ALLM, another related “calpain inhibitor.”

Another candidate IMC processing protease is the calpain ortholog (Twinscan 0400). Calpains are proteases that are regulated by calcium, associated with the cytoskeleton and inhibited by ALLM and ALLN (Perrin and Huttenlocher, 2002).

### Other proteases

Proteases have universal functions during development, cell cycle progression, and gene regulation. *T. gondii* has a full complement of proteases associated with the proteasome and other degradative functions. Most of these have not yet been investigated.

### Endogenous protease inhibitors.

In addition to a large repertoire of secreted proteases, *T. gondii* also encodes a number of proteinaceous protease inhibitors (serpins). These are of the Kazal family and localize primarily to the dense granules (Pzenny *et al.*, 2000; Pzenny *et al.*, 2002; Morris *et al.*, 2002; Morris and Carruthers, 2003). Recombinant serpins are able to inhibit trypsin and chymotrypsin activities (Morris *et al.*, 2002). The function of these inhibitors is unknown and could be for protection against host degradative enzymes or possibly as a regulator of endogenous protease activity.

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## Classes of proteases in *T. gondii*

Proteases are classified according to families and clans based upon the amino acid residues used for cleavage of the peptide bond. Many different families use serine or cysteine. In most cases these protease clans appear to have little homology and are examples of convergent evolution.

### Serine proteases (rhomboids and subtilases)

Serine proteases have been implicated to play an important part of invasion in *Toxoplasma* and *Plasmodium*. Rhomboids (Urban and Freeman, 2003) and subtilases are likely to be important (Kim, 2004; Binder and Kim, 2004), and are distributed throughout the Api-complexa. Studies suggest that both classes of proteases play key roles in invasion.

Rhomboids are polytopic serine proteases that cleave substrates within membranes at helix-breaking residues. *T. gondii* has at least 6 rhomboid proteases. The exact functions and substrates are still not established, but current data suggest that a rhomboid protease is responsible for the MPP1 activity leading to intramembranous cleavage and shedding of microneme adhesins MIC2, AMA1, MIC6 and other microneme proteins with C-terminal transmembrane domains. ROM4 and ROM5 are the best candidates for this function. ROM5 has been shown to be active against MIC2 and the transmembrane

domains of MIC6 and MIC12 in a mammalian cell-based cleavage assay (Brossier *et al.*, 20005; Dowse *et al.*, 2005). It localizes to the plasma membrane and the posterior of the parasite, fitting the expected localization pattern of MPP1. ROM4 does not have activity in a heterologous system (Brossier *et al.*, 2005; Dowse *et al.*, 2005) but does localize to the plasma membrane, and it shows the greatest homology with rhomboids of *Plasmodium* that are hypothesized to have similar functions in malaria (Brossier *et al.*, 2005; Dowse *et al.*, 2005; Dowse and Soldati, 2005). ROM1 (and the *Plasmodium* ortholog) localizes to micronemes and is also a possible candidate for MPP1, but current models postulate that MPP1 is a resident parasite surface protease that is constitutively active (Opitz *et al.*, 2001; Brossier *et al.*, 2005). ROM2 and ROM3 (as is the *Plasmodium* ortholog ROM3) are predominantly seen in sexual stages (Brossier *et al.*, 2005; Dowse *et al.*, 2005).

Subtilases are synthesized as pre-proproteins. Their N-terminal signal sequence directs them to the ER. In most cases, the propeptide is cotranslationally cleaved in a rapid autocatalytic process. The propeptide remains non-covalently attached to the subtilase and acts as an inhibitor as the protease traffics through the secretory pathway. Three subtilases have been identified in *P. falciparum*, PfSUB-1, PfSUB-2 and PfSUB-3. (reviewed in Withers-Martinez *et al.*, 2004). PfSUB-2 localizes to micronemes, and appears to be an essential malaria sheddase responsible for release of adhesive proteins MSP1 and AMA1 from the merozoite during invasion (Howell *et al.*, 2003; Harris *et al.*, 2005). PfSUB1 also appears to be essential and localizes to dense granules (Blackman *et al.*, 1998). The localization of PfSUB3 is unknown, but this subtilase is not conserved in all species of malaria ([www.plasmodb.org](http://www.plasmodb.org)) and does not appear to be essential in blood stage parasites (Harris *et al.*, 2005).

In *T. gondii*, two subtilases have been cloned, TgSUB1 (Miller *et al.*, 2001) and TgSUB2 (Miller *et al.*, 2003). A total of 12 potential subtilases are present in the *T. gondii* genome, although RT-PCR experiments and examination of ESTs suggest that not all are expressed in tachyzoites, and that several are developmentally stage-specific (Squires, Lagal and Kim, unpublished).

### Aspartic protease

*Toxoplasma gondii* possesses six putative aspartyl proteases, named TgAP1 to TgAP6. The function of these protease is currently unknown. Several attempts to disrupt TgAP1 have failed suggesting that one at least one family member may be essential (D. Soldati, unpublished), and genetic studies with other family members are under way.

There are ten plasmepsins (*Plasmodium* aspartic proteases) in the *P. falciparum* genome. Plasmepsins I-IV, V, IX, X are expressed in intraerythrocytic stages (Banerjee *et al.*, 2002) and of these four, PMI, II, IV and HAP (histo-aspartic protease or PMIII), are the major aspartic acid proteases of the food vacuole (Banerjee *et al.*, 2002). Plasmepsins I-IV are type II transmembrane proteins that mature after transit to the plasma membrane.

TgAP1–6 all have orthologs in *Plasmodium* spp. Three of these, TgAP1, TgAP3, and TgAP5 are expressed in tachyzoites. TgAP1 has a similar type II membrane topology as PfPMI-IV, and both TgAP1 and TgAP3 undergo multiple processing steps. As mentioned earlier, TgAP5 is predicted to have a C-terminal TM domain like its *Plasmodium* homolog

PfPMV, TgAP2 and TgAP4, orthologs of *Eimeria tenella* eimepsin and PfPMVI, are predicted to be sexual stage proteases and are predicted to encode a GPI-anchoring signal.

### Cysteine proteases

Cysteine proteinases are named for the active site cysteine that is essential for catalysis. The CA family prototype is papain, which is classically inhibited by E64 (Barrett and Rawlings, 2001). The major cysteine proteases located in the food vacuole of *P. falciparum* are cathepsin L-like papain family members falcipain 2/2' and falcipain 3 (Shenai *et al.*, 2000; Sijwali *et al.*, 2001). Falcipains are type II integral membrane proteins that probably traffic to the food vacuole in vesicles similarly to plasmepsins, but their maturation is thought to be autocatalytic and stimulated by the acidic environment of the food vacuole (Dahl and Rosenthal, 2005; Singh *et al.*, 2006). Although not yet established, falcipains may activate both themselves and the plasmepsins (Rosenthal, 2004).

The food vacuole hemoglobins are synthesized earlier in the life cycle than proteases involved in invasion. Falcipain I is synthesized late in the life cycle, supporting the hypothesis that this protease has a prominent role in invasion. Early studies with E64-derived protease inhibitors suggested falcipain I was involved in merozoite invasion (Greenbaum *et al.*, 2002). Unexpectedly, gene disruption studies did not reveal a phenotype in blood stages, but parasites did exhibit defects in oocyst formation (Eksi *et al.*, 2004). These data could be reconciled if there are other proteases that are able to compensate for falcipain function upon gene disruption.

*T. gondii* possesses a single CatL ortholog TgCPL that is predicted to be a type II transmembrane protein. Initial studies suggest that this protein is essential and plays a role in maturation of microneme protein M2AP within the secretory pathway (D. Soldati, unpublished; V. Carruthers unpublished). The prodomain cleavage site of several microneme proteins is similar so CPL may be a major microneme prodomain processing protease with specificity for MIC3, MIC6, and AMA1. Recombinant TgCPL is activated under acidic conditions and shows typical cathepsin L specificity with a leucine in the P2 position (V. Carruthers, unpublished). It localizes to a post-Golgi compartment that is hypothesized to be a "pre-microneme" where prodomain processing occurs prior to transit to or maturation of micronemes.

*Toxoplasma gondii* has 3 cathepsin C orthologs currently being studied by the Reed group (Que *et al.*, 2007). Cathepsin C is classically a secreted protease that cleaves N-terminal dipeptides. CPC3 appears to be sporozoite-specific, while CPC1 and CPC2 are expressed in tachyzoites. These localize to dense granules and are secreted into the parasitophorous vacuole. Disruption of one Cat C is accompanied by upregulation of the second Cat C, suggesting that they have partially redundant functions. The biological substrates of CatC are not yet identified, but one possible substrate is ROP1, which is secreted into the PVM during invasion but disappears approximately 24 hours into intravacuolar development. Studies with Cat C inhibitors suggest that at least one of the CPCs is essential (Que *et al.*, 2007).

A malaria Cathepsin C ortholog, dipeptidyl aminopeptidase I (DPAP 1) is involved in hemoglobin degradation (Klemba *et al.*, 2004). *Plasmodium falciparum* also has two other DPAP/Cathepsin C orthologs that have somewhat different expression patterns (by mi-



croarray and proteomic studies) from DPAP1 (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003). DPAP 1 is a food vacuole protease, but proDPAP I accumulates in the PV, suggesting that its trafficking involves a complex pathway (Klemba *et al.*, 2004). Thus the trafficking pathway of the cathepsin C orthologs may have similarities in the Apicomplexa.

Toxopain is a cathepsin B ortholog that localizes in the rhoptries and appears to be essential (see rhoptry proteases above). *Plasmodium* species do not encode a Cathepsin B ortholog.

*P. falciparum* does encode a large family (9) of papain-like proteases termed SERAs that do not have orthologs in *T. gondii*. Some members of this family have a substitution of a serine for the catalytic cysteine but at least one has been demonstrated to have serine protease activity (Hodder *et al.*, 2003). These proteins localize to the parasitophorous vacuole (Miller *et al.*, 2002) and are implicated in merozoite egress from parasitophorous vacuole or sporozoite egress from oocysts (Aly and Matuschewski, 2005). At least some members of this family appear to be essential (Miller *et al.*, 2002).

## Metalloproteases

Metalloproteases are named because of their dependence upon divalent cations, usually zinc, for activity. Using proteomics approaches, metalloproteinases have been identified from excreted-secreted antigen (ESA) fractions enriched in microneme proteins (Zhou *et al.*, 2005) as well as rhoptry fractions (Bradley *et al.*, 2005). The function of these secreted proteases is not known. Metalloproteases are involved in mitochondrial protein processing or stromal processing peptidases within the apicoplast (van Dooren *et al.*, 2002) as well as a number of other essential biological functions. Orthologs of many classes of metalloproteases are present in the *T. gondii* genome, but their importance and function is unknown.

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## Conclusion

During assembly of its organelles, *T. gondii* must synthesize and target specific contents, including proteases, to appropriate and unique compartments. Several classes of proteases are anticipated to play essential roles in biogenesis of microneme and rhoptry secretory organelles and other critical processes including invasion. The sequence of the *T. gondii* genome is being complemented with EST and SAGE studies (Li *et al.*, 2003; Radke *et al.*, 2005) as well as proteomic efforts. While most proteases of *T. gondii* are of unknown function, interest and investigations have rapidly increased and will likely provide fascinating insights into the biology of this parasite.

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# The *Toxoplasma gondii* Parasitophorous Vacuole Membrane: Transactions at the Parasite–Host Interface

31

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## Abstract

The obligate intracellular protozoan *Toxoplasma gondii* establishes its replication permissive niche within the infected host cell. This niche, the parasitophorous vacuole (PV), is delimited from the host cell cytoplasm by the PV membrane (PVM). In this chapter we highlight the roles of the PVM in the remodeling of host cell architecture, nutrient acquisition, the manipulation of signaling and touch upon the potential roles in the parasite developmental cycle. We further present the PVM as a unique and dynamic “organelle” found only within the infected cell where it is established outside the parent organism. Despite its importance little is known about the biology of the PVM. There has, however, been a recent renewal of interest in the PVM, the study of which has become more tractable with the application of both classical approaches as well as genomic and proteomic analyses. With this chapter we discuss the diverse activities associated with the PVM and present pressing questions that remain to be elucidated regarding this enigmatic organelle.

Study of the PVM has been quite limited. Despite its location at the interface between the intracellular parasite and host cytoplasm, the PVM has been somewhat refractory to extensive molecular, cell biological and biochemical analyses. Several issues regarding the PVM have conspired to complicate its in depth analysis. Key among these is the fact that the PVM is formed only in infected cells thus limiting the availability of material. In addition, the recruitment and high affinity binding of host mitochondria and endoplasmic reticulum (ER) to the PVM precludes biochemical purification (Sinai *et al.*, 1997). In reality the “purification” of PVM-enriched membranes represents a refined ER and mitochondrial preparation within which the PVM fraction is of relative low abundance (Sinai *et al.*, 1997). In this chapter we will address our present knowledge regarding the biogenesis of the PVM, activities ascribed to it and begin to probe the molecular basis for some of these activities.

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## Biogenesis of the PVM

Unlike a typical phagosome membrane formed by conventional phagocytosis or macropinocytosis (Desjardins *et al.*, 1994; Jutras and Desjardins, 2005), the *T. gondii* PVM is formed during the active invasion of the host cell by the parasite (Dobrowolski and Sibley, 1996). Several features of this invasion process directly impact on the protein composition of the vacuolar membrane. The early freeze-etch microscopic studies of Porchet-Hennere

demonstrated that the early vacuolar membrane was devoid of intramembrane particles suggesting the selective exclusion of membrane proteins in the course of invasion (Porchet-Hennere and G., 1983; Porchet-Hennere *et al.*, 1985). A significant and important refinement to the model came from the studies from the Sibley lab who demonstrated that the exclusion of membrane proteins at the time of invasion was in fact selective for proteins containing single transmembrane domains while permitting inclusion of lipid-anchored proteins (Charron and Sibley, 2004; Mordue *et al.*, 1999) and certain polytopic membrane proteins (Charron and Sibley, 2004). The exclusion of host proteins associated with the formation of the nascent vacuole is mediated by a unique structure formed at the site of parasite invasion. First characterized morphologically in the context of *Plasmodium* and *Toxoplasma* entry, the "moving junction" appeared as an area of increased electron density at the interface between the parasite surface and the host plasma membrane (Aikawa *et al.*, 1978; Michel *et al.*, 1980). Additional studies revealed the moving junction to be critical in parasite invasion resulting in the significant deformation of the transiting organism (Aikawa *et al.*, 1977; de Souza, 2005; Michel *et al.*, 1980; Nichols and O'Connor, 1981), exclusion of host markers (Charron and Sibley, 2004; Mordue *et al.*, 1999; Suss-Toby *et al.*, 1996) and the capacity to strip opsinizing antibodies from the parasite surface (Dubremetz *et al.*, 1985). The first insights into the molecular composition of the moving junction have emerged in recent publications from the Boothroyd (Alexander *et al.*, 2005) and Dubremetz (Lebrun *et al.*, 2005) laboratories. These studies demonstrate that the parasite delivers a complex of proteins (Alexander *et al.*, 2005; Lebrun *et al.*, 2005) recently identified as residents in the rhoptry necks (Bradley *et al.*, 2005) to the site of invasion. Upon secretion into the moving junction, these rhoptry neck, or RON, proteins with the microneme protein AMA1 serve as the anchor points on the parasite and host plasma membrane sides of the interaction as the organism squeezes itself through the constriction (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). The nascent PVM forms the leading edge of the invading parasite eventually engulfing the organism as it is completely internalized and detached from host plasma membrane.

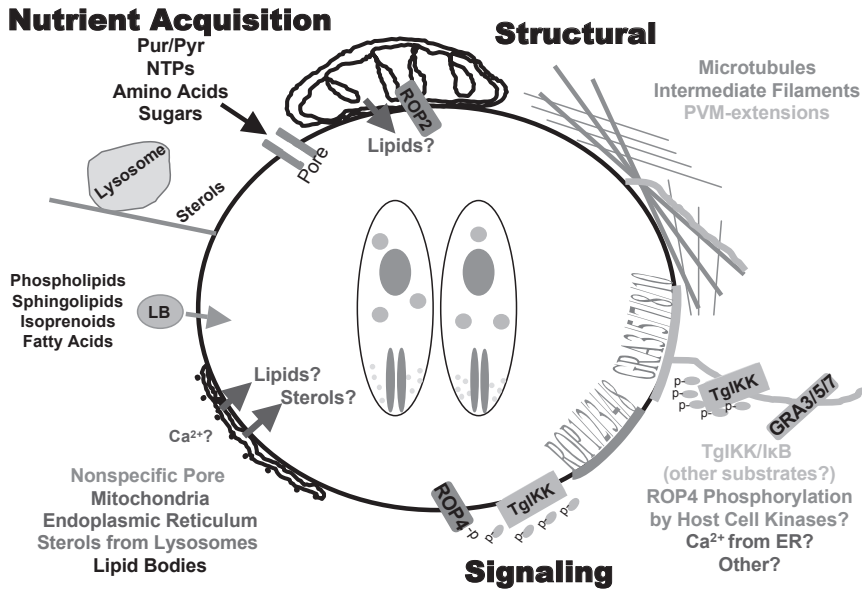
Stripped of most host proteins, the lipid bilayer forming the nascent vacuole appears to be derived predominantly from the host plasma membrane (Suss-Toby *et al.*, 1996). This was elegantly demonstrated by the Ward laboratory using real-time electrophysiological analyses of invading parasites (Suss-Toby *et al.*, 1996). Their studies convincingly show that the parasite contributes little if any lipid to the new vacuole (Suss-Toby *et al.*, 1996). In some sense this is surprising given that the discharge of the rhoptry contents includes not just proteins but also lipids rich in phosphatidylcholine and cholesterol (Coppens and Joiner, 2003; Coppens and Vielemeyer, 2005; Foussard *et al.*, 1991).

The newly formed PVM is rapidly modified by the temporally and spatially regulated release of proteins from the parasite rhoptries and dense granules (Carruthers and Sibley, 1997; Carruthers, 1999; de Souza, 2005; Dubremetz *et al.*, 1993); the first proteins to appear in the PVM are derived from the rhoptries (Carruthers and Sibley, 1997; Dubremetz *et al.*, 1993). Among the proteins secreted are ROP1 (Ossorio *et al.*, 1992), ROP2 (and ROP2 family members) (Beckers *et al.*, 1994) as well as a number of recently identified rhoptry proteins (Bradley *et al.*, 2005). The presence of lipid in the rhoptry matrix suggests

that several PVM-localizing proteins may be already prepackaged in vesicles that may fuse with the forming vacuolar membrane (Hakansson *et al.*, 2001).

Within minutes of complete internalization, a large scale secretion event at the posterior end of the parasite results in the release of a number of dense granule proteins (Sibley *et al.*, 1986). Several of these GRA proteins including GRA3,5,7, 8,9 and 10 localize to the PVM (Adjogble *et al.*, 2004; Ahn *et al.*, 2005; Bermudes *et al.*, 1994; Carey *et al.*, 2000; Henriquez *et al.*, 2005; Jacobs *et al.*, 1998; Lecordier *et al.*, 1999) (reviewed in Mercier *et al.*, 2005) (Figure 31.1). A subset of these proteins have been shown to integrate into the PVM with exposure to the host cell cytoplasm despite being soluble proteins in the secretory pathway (Ahn *et al.*, 2005; Carey *et al.*, 2000; Henriquez *et al.*, 2005; Jacobs *et al.*, 1998; Lecordier *et al.*, 1999). This property of post-secretion membrane integration is poorly understood although the length and composition of the transmembrane domains appear to be important factors (Karsten *et al.*, 2004). Unlike rhoptries, where the prevailing wisdom holds that discharge is limited to parasite invasion, dense granules form the basis of the constitutive and default secretory pathway (Karsten *et al.*, 1998; Mercier *et al.*, 2005). What is not known is whether the relative concentrations of GRA proteins loaded into the secretory granules vary at different phases of the parasite's intracellular residence. One might expect such changes to occur at the time of the developmental switch from a tachyzoite to bradyzoite (Tomavo *et al.*, 1991; Weiss and Kim, 2000) when components required for the formation of the cell wall (Zhang *et al.*, 2001) would be needed. Although our knowledge of the PVM of tachyzoites is in its infancy, even less is known about the PVM in bradyzoites (Ferguson, 2004; Weiss and Kim, 2000) or sporozoites (Dubey *et al.*, 1998; Ferguson *et al.*, 1999; Kasper and Ware, 1985; Speer *et al.*, 1995). The bradyzoite PVM likely forms the template for the assembly of the highly glycosylated cyst wall (Weiss and Kim, 2000; Zhang *et al.*, 2001). Antigenically early cysts contain GRA5 which is also found in tachyzoites (Lecordier *et al.*, 1993) suggesting that several GRA proteins secreted into the PVM by tachyzoite stage parasites may establish a structural and functional template for subsequent cyst formation (Figure 31.1). Morphologically, the cyst wall appears to incorporate not only the PVM but also elements of the associated organelles and the host cytoskeleton (Matsubayashi and Akao, 1963; Weiss and Kim, 2000). These interactions are established during the tachyzoite stage as discussed below. The sporozoite PVM is a transient structure within which redifferentiation to a tachyzoite occurs (Speer *et al.*, 1995). Interestingly, the sporozoite PVM has only a limited subset of GRA proteins and, unlike the tachyzoite PVM (see below), is not permeable to the host cell cytoplasm (see below) (Speer *et al.*, 1995).

The seminal studies of Jones and Hirsch (Jones and Hirsch, 1972; Jones *et al.*, 1972) were the earliest to demonstrate that the *T. gondii* tachyzoite vacuole failed to fuse with lysosomes (Figures 31.1 and 31.2A). This seminal observation, confirmed and refined in multiple subsequent studies, indicates that the *T. gondii* PVM is non-fusogenic with regard to components of the endocytic and exocytic pathways (De Carvalho and deSouza, 1989; DeMelo and Souza, 1996; Joiner *et al.*, 1990; Mordue and Sibley, 1997) of membrane traffic and fails to acidify (Mordue and Sibley, 1997; Sibley *et al.*, 1985). The capacity to establish and maintain the non-fusogenic state appears to be intrinsic to the PVM as demonstrated by the elegant experiments of Hakansson and colleagues (Hakansson *et al.*,



**Figure 31.1** Activities associated with the *T. gondii* PVM. The *T. gondii* PVM possesses a number of activities which can be grouped into those involved in Nutrient Acquisition, Structural and Signaling activities. Soluble metabolites including amino acids, sugars and precursors of nucleic acids are acquired across the PVM-pore activity. Lipids are likely scavenged from multiple sources including lipid bodies (LB) and PVM-associated organelles. The bulk of the parasites' requirements for cholesterol are met by the host lysosomal pathway. Structural Interactions: The PVM and as yet unknown PVM functions establish interactions with the host microtubule and intermediate filament based cytoskeletons. Modifications, apparent as projections extending from the PVM and decorated with selected GRA proteins as well as the TgIKK activity, suggest a potential degree of organization. In addition intimate interactions with host mitochondria and endoplasmic reticulum serve as an additional structural modification of the PVM. Signaling Activities: As the interface between the parasite and host, the PVM serves as a signaling platform. At least one parasite derived kinase activity, the *Toxoplasma* derived I kappa B kinase activity (TgIKK) localizes to the PVM and phosphorylates host I kappa B alpha. In addition ROP4 and family members are phosphorylated in infected cells. The contribution of other ROP2 family members, ROP13, ROP16, and ROP18, to signaling modification is discussed in the text. Finally the intimate association of host ER may suggest parasite directed modulation of  $Ca^{2+}$  signaling although no evidence in direct support of this exists. Proteins resident in the PVM derive from rhoptries (ROP) and dense granules (GRA). Of these only ROP2 has been linked to a function, that of organelle association. The diversity of activities at the PVM suggests multiple effectors remain to be identified. See also Plate 31.1.

2001). In this study the authors used the microfilament depolymerizing agent cytochalasin D to block the entry of partially invaded parasites. Remarkably, while the parasites were frozen at the host plasma membrane in the process of invasion, PVM vesicles defined by the presence of rhoptry proteins and termed e-vacuoles were found to have retained their non-fusogenic property indicating that the PVM alone is responsible for this property (Hakansson *et al.*, 2001).

## Reorganization of host cell architecture by the *T. gondii* PV

It is perhaps expected that a growing space filling lesion such as a pathogen containing vacuole would perturb the host cell's architecture. Thus, several studies have examined the effect of *T. gondii* development on the host cytoskeleton. Studies have focused on microtubules (Andrade *et al.*, 2001; Cintra and De Souza, 1985; Melo *et al.*, 2001; Schwartzman *et al.*, 1985; Sehgal *et al.*, 2005) and intermediate filaments (Cintra and De Souza, 1985; Halonen and Weidner, 1994) both of which appear to form a scaffold or overcoating of the vacuole (Figures 31.1 and 31.2B,C). The distortion of the host cytoskeleton in infected cells is actually dynamic as evidenced from recent studies from the Coppens laboratory (Sehgal *et al.*, 2005). Microtubules play an important role in the distribution of organelles and certain lipid bodies (Hayden, 1988; Stebbings, 1990). In addition, studies implicate intermediate filaments in the organization of lipid bodies within the cell (Evans, 1992; Franke *et al.*, 1987). These interactions are likely to be important in several parasite-directed mechanisms for nutrient acquisition as discussed below.

Could there be specific parasite proteins on the PVM that serve to engage the host cytoskeleton? One interesting observation is that e-vacuoles migrate along microtubules (Hakansson *et al.*, 2001). That e-vacuoles contain rhoptry proteins suggests a mechanism for this directed movement in the cell by specific interactions between as yet unknown parasite proteins and either microtubules or more likely microtubule associated proteins and/or motors (Hakansson *et al.*, 2001). An even more dramatic reorganization results in the recruitment of the microtubule organizing center (MTOC) to the PV surface (Coppens *et al.*, 2006). This appears to be a directed event as extensions that label with several GRA antibodies (Bermudes *et al.*, 1994; Coppens *et al.*, 2006; Lecordier *et al.*, 1999) tend to radiate toward the MTOC, often circumventing the host nucleus (Coppens *et al.*, 2006) (see below).

## Association of the PVM with host organelles

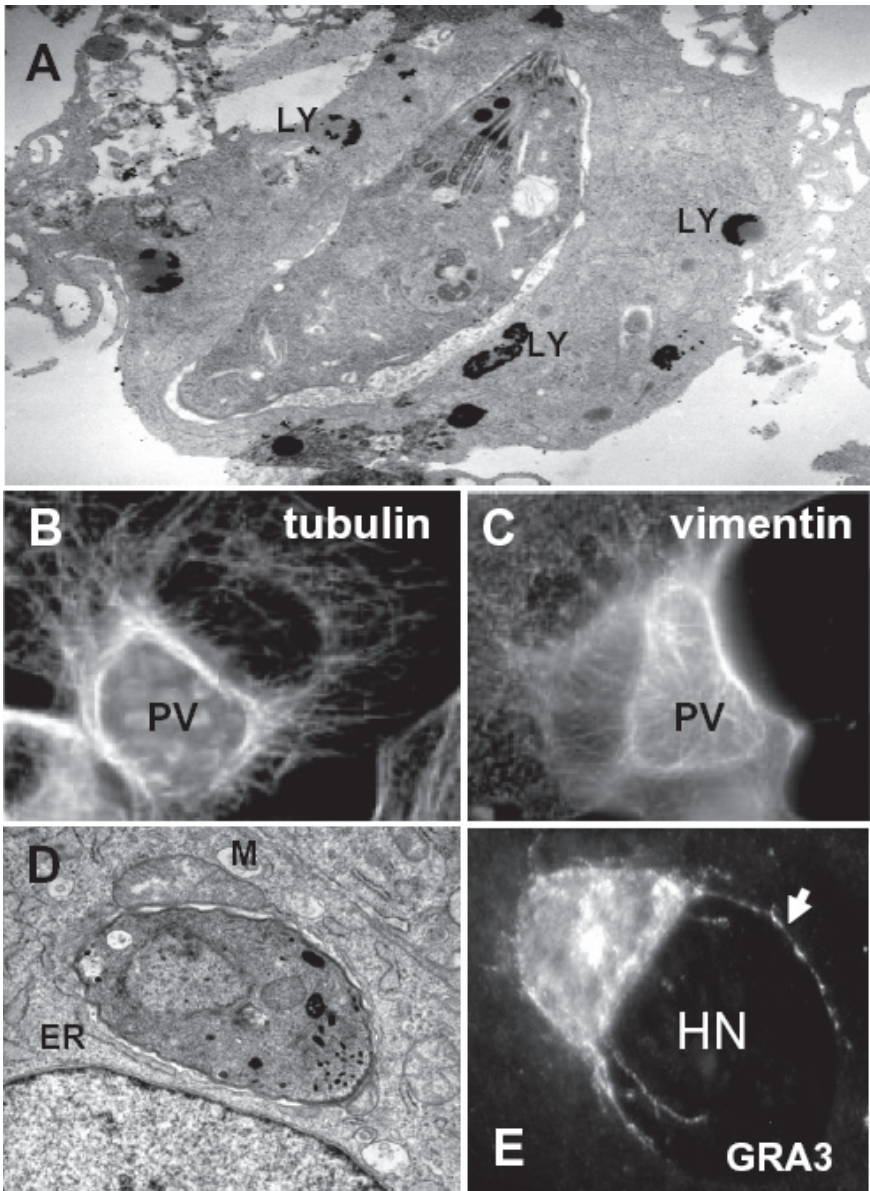
Morphologically, a feature defining the *T. gondii* PVM is the intimate association with host mitochondria and the endoplasmic reticulum (de Melo *et al.*, 1992; de Souza, 2005; Magno *et al.*, 2005b; Melo and de Souza, 1997; Schatten and Ris, 2004; Sinai *et al.*, 1997) (Figures 31.1 and 31.2D). A key element of this interaction is the tight apposition of the host organelle membranes with the PVM but the absence of membrane fusion; measurements indicate separations that average 12 and 18 nm respectively for PVM-mitochondrial and PVM-ER associations (Sinai *et al.*, 1997). It is notable that the extent of PVM-organelle association is limited to the surface area of the vacuole but does not change with the increase in vacuolar volume (Sinai *et al.*, 1997). The extent of the PVM-organelle association and the relative distribution has been quantified morphometrically and appear to vary, particularly for the extent of PVM-mitochondrial association, depending on the cell type and approach used for quantification (Magno *et al.*, 2005b; Sinai *et al.*, 1997). This variation very likely reflects the relative concentration of these organelles in the cell, particularly in the juxtanuclear area where the vacuoles tend to reside (Sinai *et al.*, 1997). All indications point to the PVM-organelle association being a highly stable and irreversible interaction (Sinai *et al.*, 1997). Experiments indicating PVM proteins co-fractionate with organelle markers in sucrose floatation gradients despite harsh treatments

like carbonate and urea extractions suggest that the PVM and associated organelles are inseparable (Sinai *et al.*, 1997).

The kinetics of PVM-organelle association are very rapid and detectable within a minute of parasite entry (Sinai and Joiner, 2001). This property correlates with those for the discharge of rhoptries at the time of parasite invasion (Carruthers and Sibley, 1997; Carruthers, 1999). Both the nascent PVM and e-vacuoles contain ROP2 (Beckers *et al.*, 1994; Hakansson *et al.*, 2001). Furthermore, the topology of ROP2 in the PVM was elucidated in the elegant experiments of Beckers and colleagues as having the N-terminus exposed to the host cytoplasm (Beckers *et al.*, 1994). What drew attention to ROP2 in host organelle recruitment was the examination of the N-terminus of mature ROP2 following processing in the secretory pathway en route to the rhoptry. Mature ROP2, which begins at or close to aa98 (Sadak *et al.*, 1988), reveals a curious stretch of amino acids from aa98–127. This, now N-terminal stretch, includes a positively charged amphipathic helix, a high incidence of Ser and Thr and a poor representation of prolines and bulky amino acids (Beckers *et al.*, 1994; Sinai and Joiner, 2001), a motif largely conserved in other ROP2 family members. Together, these features are remarkably reminiscent of a mitochondrial import signal (Horwich *et al.*, 1985; von Heijne, 1990) suggesting the subversion of mitochondrial import as a potential mechanism for the recruitment of mitochondria to the PVM. The capacity of the host cytoplasm exposed domain of ROP2 (ROP2hc) to be partially translocated across the mitochondrial outer membrane in a manner dependent on the putative N-terminal signal sequence (aa98–127) coupled with the targeting of ROP2hc to mitochondria following expression in mammalian cells strongly argue for ROP2 as the mediator of PVM-mitochondrial association (Sinai and Joiner, 2001). Interestingly, fusion of the 30aa N-terminal signal to GFP directs the chimeric protein to the mitochondria following the transient transfection of host cells (Sinai and Joiner, 2001). Deletion of aa98–127 results in redirection of ROP2hc to the endoplasmic reticulum (Sinai and Joiner, 2001) although its significance is unclear. In addition, ROP2hc interacts with organellar membrane with high affinity and is not extractable by carbonate (pH 11.5) treatment (Sinai and Joiner, 2001). Thus the high affinity and irreversible binding of mitochondria to the PVM can be explained by the establishment of a “molecular rivet” linking the two organelles (Sinai and Joiner, 2001). The role of ROP2 in PVM-mitochondrial association is further strengthened by the marked reduction in the extent of mitochondrial association in parasites expressing a ROP2 antisense construct (Nakaar *et al.*, 2003). In addition ROP2 antisense expressing parasites exhibit significant growth retardation suggesting an important role for PVM-mitochondrial association (Nakaar *et al.*, 2003).

The mechanism for PVM-ER association remains elusive. While deletion of aa98–127 from ROP2hc results in localization and high affinity binding to the ER (Sinai and Joiner, 2001), the question of whether this represents an actual interaction in infected cells remains an open question. A recent paper suggests that the presence of a putative ER-retrieval motif on GRA3 may indicate a role in PVM-ER association (Henriquez *et al.*, 2005). This is unlikely as the C-terminus of GRA3, and therefore the ER-retrieval motif, is likely to be in the lumen of the vacuole and not exposed to the host cytoplasm (Henriquez *et al.*, 2005).





**Figure 31.2** PVM-activities associated with the establishment of infection. (A) Resistance of the *T. gondii* PVM to phagolysosomal fusion. Infection of macrophages previously loaded with BSA-gold in the lysosomes (Ly) results in the establishment of the PV that resists phagolysosomal fusion noted by the lack of delivery of BSA-gold to the parasite containing compartment. (B and C) Reorganization of host cytoskeletal elements including microtubules (B) and vimentin intermediate filaments (C) around the parasite containing vacuole (PV). (D) Intimate association of host mitochondria (M) and endoplasmic reticulum (ER) with the PVM by electron microscopy. (E) Distribution of the dense granule marker GRA3 at the PVM exhibiting PVM extensions (arrow) several of which extend around the host nucleus (HN). Images of the reorganization of cytoskeletal elements are kindly provided by Dr. Isabelle Coppens.

The recruitment of host organelles to the vacuolar membrane is a property restricted to a few intracellular pathogens and suggests a highly specialized function (Sinai and Joiner, 1997). These may include structural roles, roles in signaling and in nutrient acquisition. The contributions either demonstrated or predicted for these unique interactions in these processes are discussed below in the context of the biology of the *T. gondii* PVM.

### Role of the PVM in nutrient acquisition

At its very basic level a parasite may be fundamentally defined as an organism that feeds at the expense of another. In this context the obligate intracellular *Toxoplasma* must acquire its nutrients from the host cytoplasm, organelles and the extracellular milieu (such as seen with the scavenging of LDL-cholesterol) (Coppens *et al.*, 2000) across the PVM (Sinai and Joiner, 1997). The well recognized auxotrophies for purines, certain amino acids and particular lipids (reviewed in (Sinai and Joiner, 1997; Sonda and Hehl, 2005)) have been validated by the examination of the parasite genome and the determination of metabolic pathway maps (<http://www.toxodb.org/include/fpPathwayReconstr.html>). For more details see Chapters 19 and 20. As the physical barrier delimiting the replication permissive niche, the PVM must contain specific activities to facilitate access to these critical nutrients.

As purine auxotrophs, *Toxoplasma* have evolved an elaborate purine salvage pathway (Krug *et al.*, 1989). While the enzymology of purine utilization within the parasite had been elucidated, the question of how purines crossed the PVM was once an open question (Schwartzman and Pfefferkorn, 1982). The association of mitochondria was once suggested as a source of this nutrient by invoking a direct transfer across the points of contact (Schwartzman and Pfefferkorn, 1982). The actual solution to this enigma emerged over a decade ago with the discovery that the PVM is a permeable membrane with a non-selective pore that allows free equilibration of soluble molecules of under 1300 daltons between the vacuolar space and the host cytoplasm (Schwab *et al.*, 1994). The presence of the pore provided a mechanism not only for the access of purines but also other building blocks like amino acids, sugars and other low molecular weight metabolites (Schwab *et al.*, 1994) (Figure 31.1). Remarkably, despite the evidence for the pore existing for over a decade, nothing is known about its molecular composition—a testament to the difficulties associated with the study of the PVM.

While providing a mechanism for the scavenging of low molecular weight soluble molecules, the PVM pore fails to address how sterols and lipids might be acquired. Auxotrophies for cholesterol (Coppens *et al.*, 2000; Sehgal *et al.*, 2005) and certain lipids (Charron and Sibley, 2004; Gupta *et al.*, 2005; Nishikawa *et al.*, 2005; Sonda and Hehl, 2005; Thomsen-Zieger *et al.*, 2003) have been established. How are cholesterol and these lipids scavenged by the parasite? A few years ago the prevailing wisdom held that the *Toxoplasma* PVM was entirely divorced from the pathways of membrane traffic within the infected cell (Sinai and Joiner, 1997). The studies of Coppens *et al.*, however, demonstrated that intracellular *Toxoplasma* efficiently redirect the trafficking of cholesterol in LDL from lysosomes in a parasite directed manner (Coppens *et al.*, 2006; Coppens *et al.*, 2000). More recent work has demonstrated a critical function for the remodeling of the architecture and

organization of microtubules in this unusual scavenging mechanism (Coppens *et al.*, 2006; Sehgal *et al.*, 2005).

The acquisition of cholesterol from the lysosomes does not appear to involve overt lysosomal fusion at the PVM as this is a lethal event (Joiner *et al.*, 1990; Jones and Hirsch, 1972). Therefore, an alternative mechanism for the scavenging of LDL-cholesterol from host lysosomes must be present. Mammalian cells not only acquire cholesterol from the serum in LDL particles but have the capacity for *de novo* cholesterol synthesis in the ER (Soccio and Breslow, 2004). Although the PVM does associate with the host ER, the primary route of cholesterol scavenging is the endocytic pathway of the host cell (Coppens *et al.*, 2000), a pathway until recently considered not to intersect the *Toxoplasma* vacuole (Sinai and Joiner, 1997). Recent work from the Coppens group demonstrates a highly specialized microtubule driven mechanism to sequester lysosomes in the cell (Coppens *et al.*, 2006) in effect reversing a long held paradigm.

Cholesterol is not the only lipid for which the parasite is auxotrophic (Charron and Sibley, 2004; Gupta *et al.*, 2005; Nishikawa *et al.*, 2005; Sonda and Hehl, 2005; Thomsen-Zieger *et al.*, 2003). Several recent studies clearly demonstrate that the parasite scavenges diverse lipids from lipid bodies (Charron and Sibley, 2002; Nishikawa *et al.*, 2005; Quittnat *et al.*, 2004; Sonda and Hehl, 2005) suggesting an as yet unidentified mechanism of transport across the PVM. Such activities may include lipid transporters (Borst *et al.*, 2000; Pohl *et al.*, 2005), receptors for lipid binding proteins and flippases (Devaux, 2002). Evidence for parasite proteinaceous mediators of cholesterol transport to the parasite has recently emerged from an elegant protease-susceptibility study on isolated PVs in the Coppens laboratory (Sehgal *et al.*, 2005). This work describes a methodology to efficiently isolate *Toxoplasma* in vacuoles using the differential accessibility of antibodies against the outer leaflet of the PVM and either luminal antigens or the parasite surface to control for the integrity of the vacuoles (Sehgal *et al.*, 2005). The standardization of such experimental approaches is significant as it opens the door to study transport processes across the PVM and identify the molecular basis underlying them.

The recruitment and high affinity association of host mitochondria and ER with the PVM suggest a likely source of scavenged lipids (Sinai and Joiner, 1997; Sinai *et al.*, 1997). Both host organelles have well established functional and enzymatic relationships in lipid metabolism (Vance and Vance, 2004). The best studied of these is the highly specialized ER elements that make up the mitochondrion associated membrane (MAM) fraction (Vance, 1990; Vance and Shiao, 1996). Morphologically, morphometric analyses reveal striking similarities in the comparison of MAM-mitochondrion interactions and those between the PVM and associated organelles (Sinai *et al.*, 1997; Vance, 1990; Vance and Shiao, 1996). Perhaps the most compelling evidence for PVM-mitochondrial association being involved in the scavenging of a specific lipid comes from the Seeber laboratory and the analysis of the *Toxoplasma* genome (Thomsen-Zieger *et al.*, 2003). Analysis of the genome reveals that *T. gondii* is unable to synthesize the mitochondrial lipid lipoic acid (Crawford *et al.*, 2006; Thomsen-Zieger *et al.*, 2003). This lipid is synthesized exclusively in the mitochondria of eukaryotes where essentially all of it is found (Gueguen *et al.*, 2000; Morikawa *et al.*, 2001). Despite being unable to synthesize mitochondrial lipoic acid directly, this lipid is present in the parasite mitochondrion suggesting it was scavenged

from PVM-associated mitochondria (Crawford *et al.*, 2006; Thomsen-Zieger *et al.*, 2003). Notably, the *T. gondii* apicoplast can synthesize lipoic acid but fails to deliver it to the positive mitochondrion (Crawford *et al.*, 2006).

### **The *T. gondii* PVM as a signaling platform**

The *Toxoplasma* infected cell is fundamentally remodeled at the level of transcription (Blader *et al.*, 2001) and, by extension, the proteome (Wastling *et al.*, 2005). Many of these changes are due to the parasite-mediated subversion of critical cellular transcription regulators. Work in our laboratory (Molestina *et al.*, 2003; Molestina and Sinai, 2005b) and that of others (Blader *et al.*, 2001; Brenier-Pinchart *et al.*, 2000; Denney *et al.*, 1999; Kim *et al.*, 2001) has found that the activation of the host transcription factor NF $\kappa$ B accompanies infection in several cell types. Among the consequences of this change is a major contribution to the anti-apoptotic state established in infected cells (Payne *et al.*, 2003). NF $\kappa$ B is activated by diverse stimuli following the phosphorylation of its inhibitor I $\kappa$ B at two critical residues (Ser 32/36 for I $\kappa$ B $\alpha$ ) resulting in its subsequent ubiquitination and degradation (reviewed in (Ghosh and Karin, 2002)). This phosphorylation is mediated by the I $\kappa$ B kinase (IKK) complex which has been the only activity shown to phosphorylate both Ser 32 and 36 on I $\kappa$ B $\alpha$  (Karin and Delhase, 2000; Karin *et al.*, 2004). In studies to define the molecular mechanism of NF $\kappa$ B activation, we examined the phosphorylation status of I $\kappa$ B $\alpha$  in *T. gondii* infected cells. Remarkably, Phospho-I $\kappa$ B $\alpha$  (Ser 32/36) was found decorating the *T. gondii* PVM (Molestina *et al.*, 2003; Molestina and Sinai, 2005a) and occasionally in projections emanating from the PVM (Figure 31.1). The fact that this labeling pattern was detected in cells entirely devoid of all the host IKK activity (IKK $\alpha$ -/- $\beta$ -/-) suggested the presence of a unique parasite derived kinase activity, designated TgIKK, at the *T. gondii* PVM (Molestina and Sinai, 2005a). Biochemical evidence for such an activity capable of phosphorylating recombinant I $\kappa$ B $\alpha$ -chimeras *in vitro* can be detected both in parasite extracts as well as the PVM-enriched fractions of *Toxoplasma* infected cells (Molestina and Sinai, 2005a). TgIKK activity appears at the PVM coincident with the first round of parasite replication (6–9 hours post infection) and plays a role in the sustaining activation of the NF $\kappa$ B pathway (Molestina and Sinai, 2005b). The kinetics with which I $\kappa$ B $\alpha$  phosphorylation is observed at the PVM suggests the TgIKK activity or a factor required to activate it is likely to be secreted from the dense granules as opposed to the rhoptries (Molestina and Sinai, 2005b).

In addition to active kinases, recent data suggest that ROP2 and ROP2-family members such as ROP4 possess the signature of having been an ancestral kinase (Carey *et al.*, 2004; El Hajj *et al.*, 2006) with at least three members, ROP16, ROP17, and ROP18, possessing all the motifs required for kinase activity (El Hajj *et al.*, 2006). Although not itself a kinase, ROP4, and possibly other members, appear to be substrates for kinases as they are efficiently phosphorylated in infected cells (Carey *et al.*, 2004) (Figure 31.1). Recent work indicates two members of the ROP2 family, ROP16 and ROP18, possess all the motifs necessary for kinase activity (El Hajj *et al.*, 2006), although the molecular identity of their target proteins is not known. Regardless, both activities are essential for parasite virulence (Taylor *et al.*, 2006; Saeij *et al.*, 2006; El Hajj *et al.*, 2007), and one of these proteins, ROP18, localizes to the PVM (Taylor *et al.*, 2006; El Hajj *et al.*, 2007). The



kinase ROP16 is translocated to the host cell nucleus (Saeij *et al.*, 2007). Either way, the apparently PVM-localized phosphorylation of key parasite proteins may have signaling implications for both the parasite as well as the host. More germane to this chapter, the TgIKK activity and evidence suggesting other potential kinases indicate that the *T. gondii* PVM serves as a “signaling platform” from which many additional pathways in the host cell may be directly manipulated.

## The organization and composition of the PVM

To better understand the organization of the PVM, it is important to consider the structural basis of the PV. Electron microscopic studies clearly indicate that the vacuolar space contains a proliferation of membrane tubules referred to as the PV network (reviewed in (Mercier *et al.*, 2005)). The network is structurally (Magno *et al.*, 2005a; Sibley *et al.*, 1995) and antigenically (Labruyere *et al.*, 1999; Lecordier *et al.*, 1995; Mercier *et al.*, 2005; Mercier *et al.*, 1998; Mercier *et al.*, 1993; Sibley *et al.*, 1995) distinct from the PVM but does exhibit some degree of connectivity to it. The fact that dense granule proteins exhibit differential localization patterns between the network and PVM while rhoptry proteins appear to localize to the PVM alone suggests a degree of organization in secreted protein trafficking (Labruyere *et al.*, 1999; Lecordier *et al.*, 1995; Mercier *et al.*, 2005; Mercier *et al.*, 1998; Mercier *et al.*, 1993; Sibley *et al.*, 1995). Recent studies have examined the organization of the *T. gondii* PV using scanning and transmission electron microscopy of thick preparations. The application of new sample preparation techniques coupled with high resolution electron microscopy reveal a remarkably detailed picture of vacuolar organization (Magno *et al.*, 2005a; Schatten and Ris, 2004). In these stunning images the parasites appear to be decorated with the network structure which forms a mesh-like pattern with connections to the PVM (Magno *et al.*, 2005a; Schatten and Ris, 2002; Schatten and Ris, 2004). This organization has been suggested to provide a degree of mechanical support from within the lumen of the vacuole (Magno *et al.*, 2005a). The growth of the vacuole and its resident parasites is under a high degree of regulation that must be responsive to signals from within the lumen of the vacuole and the host cytoplasm. The changes implicit in regulating the growth of the vacuole are dynamic as morphometric analyses demonstrate that a typical vacuole increases its surface area four fold and its volume eight fold during a 24 hour period post infection (Magno *et al.*, 2005a). The growth of the vacuole appears to be linked to parasite replication as “spacious” vacuoles are generally not observed. One reported instance of an apparent dysregulation of vacuole growth appears in a clone of parasites selected for resistance to artemisinin (Berens *et al.*, 1998).

The PVM is not a uniform membrane. This heterogeneity is evident on a gross level by the differences in the distribution and extent of PVM-organelle association (Magno *et al.*, 2005b; Sinai *et al.*, 1997). In addition, there may be functional subdomains within the vacuolar membrane. A particularly dramatic “modification” of the PVM is membranous projections emanating from many but not all vacuoles. These PVM-extensions are observed by the localization of a number of dense granule proteins including GRA3 (Bermudes *et al.*, 1994) (Figure 31.2E), GRA5 (Lecordier *et al.*, 1999), GRA7 (Coppens *et al.*, 2006) and GRA8 (Carey *et al.*, 2000). The staining pattern evident in these projections appears to be discontinuous—akin to beads on a string (for GRA3, Figure 31.2E). While

an artifact of fixation cannot be ruled out, these proteins may form discrete complexes of unknown function. At least one activity, TgIKK, is localized to PVM-extensions (data not shown) suggesting a potential signaling role. Host microtubules appear to be guiding PVM-extensions that may be important in the recruitment of the MTOC to the PVM (Coppens *et al.*, 2006).

## Identification of novel activities at the PVM

Over the last few years there has been a burst of interest in the study of PVM structural, nutrient scavenging and signaling interactions with the host cell. Given the position of *Toxoplasma* as a model system for Apicomplexa, the dissection of PVM functions could have significant impact on the understanding of experimentally less tractable systems. In one respect, however, the understanding of membrane systems extracorporeal to the parasite is much better developed in *Plasmodia* (Halдар *et al.*, 2005). The complete absence of an endomembrane system in the mammalian erythrocyte allows for the easy detection of parasite modifications and delivery of parasite proteins across the Plasmodial PVM (Halдар *et al.*, 2005). Recent studies have even identified a critical motif targeting proteins across this PVM (Hiller *et al.*, 2004; Marti *et al.*, 2005; Marti *et al.*, 2004).

Do such systems exist in *Toxoplasma* infected cells? Several lines of evidence suggest that *Toxoplasma* may in fact secrete proteins directly into the host cell and the host plasma membrane. This has been demonstrated for the dense granule protein GRA7 (Fischer *et al.*, 1998) and in a recent paper using a heterologous reporter based on the Cre-recombinase system (Gubbels *et al.*, 2005). In the latter system, the Cre-recombinase was able to cross the PVM and reach the host cell nucleus to trigger loxP mediated recombination that resulted in the expression of GFP (Gubbels *et al.*, 2005). The result clearly shows that parasite proteins may get into the host cytoplasm where they could modulate host cell activities. More recently, at least two enzymatically active proteins secreted from rhoptries have been localized to the host cell nucleus. These include a protein phosphatase 2C (Gilbert *et al.*, 2007) and ROP16 (Saeij *et al.*, 2007), the polymorphisms of which have been shown to be associated with acute virulence in *T. gondii* (Taylor *et al.*, 2006; Saeij *et al.*, 2007). While the mechanism for protein export to the host cytoplasm remains to be elucidated, such an activity would likely originate at the PVM.

The recent completion of the *Toxoplasma* genome (<http://www.toxodb.org/>), as well as proteomic studies cataloging the secretory organelles (Bradley *et al.*, 2005; Zhou *et al.*, 2005), have and will continue to advance our understanding of PVM. We have recently undertaken a proteomic analysis of the PVM using a set of unique reagents. By purifying PVM-containing host organelle fractions in rabbit fibroblasts as immunogens, we have successfully generated a pair of high titer multivalent polyclonal antisera against the *T. gondii* PVM (Martin and Sinai, manuscript in preparation). These reagents are being used to identify PVM proteins by both MALDI-TOF and multi-dimensional protein identification technology (MuDPIT) (Aebersold and Mann, 2003). Activities suggested to localize at the PVM by these studies include those involved in structural maintenance, including predicted high molecular weight glycosylated proteins (proteophosphoglycans and sugar modifying enzymes), those with signaling roles (kinases, phosphatases), protein chaperones, additional enzymatic activities including both proteases and protease inhibitors. The



majority of proteins, however, are hypothetical proteins with unknown function or even functional homologies, contributing further to the enigma that is the PVM (Martin, Liu, Lynn, and Sinai, unpublished).

The recent application of new techniques and resources to the study of the PVM will undoubtedly chip away at the difficulties in the study of this unique organelle. Bioinformatic and proteomic studies have and will continue to identify and functionally classify the activities at the PVM. Among the activities of particular interest and importance are: in the role of nutrient acquisitions, (1) identification of the PVM-pore and (2) identification of sterol and lipid transporters in the PVM; in the roles of structural interactions: (3) identification of PVM components involved in interactions with the host cytoskeleton and (4) Identification of the mediator(s) of PVM-ER association; in the role of a signaling platform: (5) identification of the gene(s) encoding the TgIKK activity and other signaling components and (6) identification of a putative protein-export system from the PV to the host cell cytoplasm; in parasite differentiation: (7) definition of the roles of PVM localized tachyzoite proteins involved in differentiation and the formation of the cyst wall; in the organization of the PVM: (8) physical organization and “compartmentalization” of the PVM and its correlation with function and (9) dynamics of the PVM and the PVM-proteome during intracellular growth and stage differentiation.

The list above is certainly ambitious. However, for the first time the technology and resources to tackle them are either available or in development. As the true interface between the parasite and the host, an understanding of the transactions across this unique organelle is central to defining the biology of the parasite. In addition, diverse enzymatic activities likely located at the PVM will provide new targets for therapeutic intervention that could limit nutrient access to the parasite or affect their capacity to manipulate the functions of infected immune effector cells. Finally, as activities at the PVM are extended to the bradyzoite vacuole, the potential for targeting that pharmacologically intractable but clinically relevant form of the parasite may be revealed.

In conclusion, it is our belief that we are on the verge of flurry of new discoveries regarding the fascinating biology of the *T. gondii* PVM and hope this chapter has made a case for the PVM as a unique organelle within the infected cell.

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# Index

## A

- Abortion 93
- Acetyl-CoA carboxylase 348, 397
- Acyl carrier protein (ACP) 251, 346–348, 426
  - bipartite targeting sequence 394–399, 402
  - knockout 417
  - visualizing apicoplast 305
- Acidocalcisomes 19–20, 493–503
  - definition 496
  - staining 496
  - ultrastructure 19, 20
- Actin 483, 526, 528–539, 555–566
  - actin binding proteins 562
  - actin-like proteins 563
  - actin-related proteins 564
  - toxofilin 464
- ACT1 560
- Actin-depolymerizing factor 561
- Adenosine kinase (AK) 378, 379
- Adenosine transporters 375
- Adults 72, 98, 227
  - IgG assays 42
  - prospective treatment studies 46
- Aldolase
  - gliding motility 444, 535, 558, 560
- AMA1 440, 469, 578, 580
  - essential gene function 251
  - moving junction 21, 448, 469, 592
  - protease processing 445, 578–589, 582–584
  - protein complex 448, 449, 469
- Amino acid metabolism 366, 380, 382
- Amylopectin 306, 307, 308
- Animal infections 3, 194, 228
  - bradyzoite 11
  - experimentally induced 75–83
  - food/domestic animals 104, 161
    - game animals 104
  - marine animals 12–13
    - oocyst 12–13
    - tachyzoite 4
    - type strains 160
- Animal models/studies 59, 69, 159
  - atypical strains 233
  - colonization 232
  - congenital toxoplasmosis 95
  - ocular toxoplasmosis 74
  - South American isolates 196
  - tracking parasites 247
- Anti-apoptotic 149, 311
  - direct inhibition 151
    - decreased caspase activation 151
    - IAPs 151
  - I $\kappa$ B 152, 178
  - indirect mechanisms 149
    - GM-CSF 149
    - HSP65 150
    - NF $\kappa$ B 152, 178
- Antifolate chemotherapy 370
- Antioxidant system 422
- Apical complex 518
  - biogenesis, development 23
  - endodyogeny 23, 519
  - organelle replication 272, 517
  - secretory organelles 475, 512
  - taxonomy 210
- Apical rings
  - tachyzoite 4
  - ultrastructure 19
- Apical polar ring 510
- Apicidin 326
- Apicoplast 4, 17, 18, 209, 348, 391
  - bipartite targeting sequence 394
  - bounding membranes 393
  - division 24, 26, 274, 286, 305, 519
  - drug sensitivities 248, 428, 349–350
  - evolutionary aspects 214–216, 423
  - fatty acid synthesis 347–349
  - genome 394

- glycerolipid synthesis 358
- isoprenoid derivative synthesis 360
- historical aspects 422
- metabolism 409
- ultrastructure 17, 20, 27
- protein synthesis
- protein trafficking 391–404, 572
- proteome 402
- transit peptide processing 401
- Apoptosis 80–81, 143–155, 311
  - death receptor pathway 146
  - effector mechanism 153–154
  - FasL in eyes 84
  - granzyme/perforin pathway 146
  - impact on infection 143
  - leukocyte triggering 147
  - lymphocyte 147
  - mitochondrial pathway 145
  - NFκB 152
  - parasite survival 153
  - pathogenesis 154–155
  - protozoan programmed cell death 146–147
    - growth factor deprivation 147
    - nitric oxide/NO 146–147
- PVM 175
  - regulation in mammalian cells 145
- splenocyte 149
- subversion of host 177–178, 311
- Aquaporins 499
- Arginine 381
- Articulins 512
- Asexual cycle 228
  - prior to gametogenesis 26
- Astrocytes 311
- Atovaquone 37, 52, 73, 368, 413
  - immunocompromised treatment
    - ocular disease 47, 70
  - laboratory mutation cytochrome b gene 47
  - tissue cyst 47
- Azithromycin 73
  - ocular disease 46
  - tissue cyst 47

## B

- Bafilomycin A1 498
- Biallelic population structure (Adam and Eve) 195, 231
- Bipartite targeting sequence 425
- Blood-brain barrier 118, 311, 312
- Bradyzoites *see also* tissue cyst 303, 304–314, 471
  - behavior 313
  - bradzoite rhoptry protein (BRP1) 184–185, 287, 294
  - cell cycle 304
  - cyst wall 305

- definition 7–8
- dense granule proteins 481
- development 305, 309
- differentiation 337
- Dolichos biflorus* agglutinin 304
- energy metabolism 307
- glycolytic enzyme 307
- kinase 136, 174
- metabolism 306
- morphology 304
- parasitophorous vacuole 305
- phosphatase 136, 174
- role in life cycle 10
- stage-specific surface antigens 306
- tissue cyst 6
- Bradyzoite specific genes 287
  - BAG1 279, 287, 307
    - promoter 224, 289, 292
    - cell cycle 289
    - cyst formation 293
  - BAG5 291
  - BSR4 287, 306
  - MAG1 287, 308
- Brefeldin A 478

## C

- Calcium-binding proteins 494
- Calcium ionophore 502
- Calcium ionosphere 500
- Calcium ions 494
  - functions 500–503
  - Na<sup>+</sup>/Ca<sup>2+</sup> exchanger 494
  - PMCA type CA<sup>2+</sup>-ATPase 493
  - SERCA type CA<sup>2+</sup>-ATPase 493, 501
  - storage 495
- Calmidazolium 500
- Calmodium 494
- Calmodulin 539
- Calnexin 495
- Carbamoyl phosphate synthetase II (CSP II)
  - pyrimidine biosynthesis 366–368
  - genetic selection 372
  - amino acid acquisition 381
- Carbohydrate metabolism 202–203, 305
- Cats (felids; *see also* definitive host) 3–5, 8–10, 74, 165
  - coccidian development 24
  - cross 197, 230, 236, 241, 255
  - epidemiological outbreaks 228
  - feces exposure 94, 104, 228
  - inbreeding 230
  - life cycle (sexual cycle) 3–4, 8–12, 159, 184, 185, 193–195, 229, 309
  - ocular toxoplasmosis 74, 78
  - oocysts 8, 228, 290
  - risk factor 39, 40

- Cat feces 9, 40
  - dogs 10
  - preventable infection 104
- Cattle (cows, beef) 3, 39, 40, 74, 104, 161
- CBL domains 443
- CCR5
  - IL-12 production 113–114
  - lipoxin A<sub>4</sub> 131
  - migration of CD8+ T cells 113
- CD4+ T cell 77, 79, 115
  - cell death 147, 153
  - class II antigen presentation 117, 129, 132
  - congenital toxoplasmosis 96
  - encephalitis 118
  - immunocompromised patients 45
  - mouse model 116, 130–131
  - ocular parasite burden 83–84
  - pathological T cell response 118
- CD8+ T cell 77, 79, 112, 115, 127–128
  - CCR5 113
  - cell death 147, 153–154
  - class I antigen presentation 117, 129
  - congenital toxoplasmosis 96
  - encephalitis 118–119
  - immune privilege 84
  - mouse model 116, 129–131, 310, 312
  - ocular parasite burdens 83–84
  - Th-1 response 95
- Cell cycle 265–279
  - conditional mutants 253, 269
  - development 278–279
    - bradyzoite 285–286, 289–290
  - endodyogeny 274
  - genetic complementation 270
  - microarray analysis 179
  - nuclear DNA 266
  - organelle replication 272
  - parasite replication 268–269
  - promoter regulation 244
  - regulation 274–278
    - bi-modal arrest 275
    - checkpoints 275, 276
    - cyclins 275
  - S-phase 272
  - tachyzoite 271
- Cell mediated immunity 111, 314
  - adaptive immunity 116–118
- Centrin 517
- Centrioles
  - mitosis 23, 517
  - sexual development 26
  - organelle replication 274
- Centrocones 18
  - mitosis 23
    - biogenesis, development 23–24
- Centrosomes
  - mitosis 23, 278
    - biogenesis, development 23–24, 274, 278
- Cerebral toxoplasmosis *see also* encephalitis
  - correlation with ocular disease 74
- Cerebral calcifications 79
- Cerulenin 350
- Chemical mutagenesis 253, 269, 294
- Chemokine receptor 465, 113, 485
- Chemokines 112, 119, 131, 132
- Chemotherapy 45–47, 311
  - immunosuppressed 47
  - new drugs 47
  - ocular disease 69, 75
  - targets
    - apoptosis 144
    - fatty acid biosynthesis 345, 348, 349
    - cholesterol ester synthesis 354
    - choline metabolism 357
    - GPI biosynthesis 359
    - farnesyltransferase 360
      - anti-folate 370–371
    - purine transport, salvage 372, 378–380
    - pyrimidine biosynthesis 367
      - mitochondrion 428–429
    - protein kinases 174
    - apicoplast 214, 403, 428–429
    - transcription factors 338
- Chickens 8, 232
- Children 38, 66, 72
  - chemotherapy 46, 50
  - congenital effects, diagnosis 94, 99
  - EIA test 42
  - long-term sequelae 70
  - South America 38
- Cholesterol 176–177, 346, 350–361, 594
  - acquisition 176–177
  - chemotherapy 354–355
  - lack of synthesis machinery 176
  - microtubules 175
  - rhodopy content 457, 464, 592
  - PVM 598–599
  - signaling 580
  - storage 354
  - uptake 351–354
- Chorioretinitis *see also* Ocular disease 62, 72–73, 233
  - other brain infections 99
  - stage differentiation 286
- Chromatin remodeling 321–339
  - acetylation 322
  - methylation 322
  - nucleosome remodeling 324
- Chromosomes 192, 198–201
  - cell cycle 271, 276
  - chromosome Ia
  - chromosome IX 200

- SNF gene 199
  - chromosome XI 200
    - UPRT gene 199
  - chromosome XII 200
    - ARA-A gene 199
      - conservation 201
      - evolution 200–201
      - haploid content 196, 197
      - nuclear DNA content 266
      - oral infectivity 201, 232
      - organelle replication 272
      - transfection 242
      - virulence 234–236
      - virulence locus chromosome VIIa 159, 164–166
  - Ciprofloxacin 428
  - Clarithromycin
    - immunocompromised treatment
      - ocular disease 70
  - Clindamycin 61, 64, 428
    - immunocompromised treatment
      - ocular disease 70
      - ocular disease 46
      - pregnancy treatment 70
  - Coccidia
    - classification 3
    - definitive host cat 5, 8
  - Congenital toxoplasmosis 93–105
  - Conoid 18, 25, 30, 512, 516
    - biogenesis, development 24
    - daughter formation 26
    - tachyzoite 4
    - taxonomy 210–211
    - ultrastructure 17–20, 210
  - Conoid extrusion 500
  - Corticosteroids 69, 70, 72, 73, 75
  - Cryptomitosis 517
  - Cyclic ADP ribose 502
  - Cytokines 96, 97, 115, 118–120, 130–132, 135–136, 148–150, 154, 161, 174, 176, 178–179, 310, 311, 359
  - Cytochalasin D 534
  - Cytoskeleton 511
- D**
- Definitive host 5, 8, 24, 194, 265, 285
    - Isospora 4
    - model 78, 290
    - ocular disease outbreaks 65
    - oocyst cycle 11
    - oocyst environmental survival 11–12, 228
    - role in cross 197
    - tachyzoite cycle 11
    - tissue cyst assay 290, 309
  - Dendritic cells (DCs) 115, 117, 127, 129, 162, 178, 312, 557
    - gene expression changes after infection 180, 182
    - IL-12 production 161, 485
    - immature preference for infection 132
    - paralysis 131
    - recruitment 112
    - T-lymphocyte apoptosis 148
  - Dense granules 18, 22, 30, 128, 172–173, 475–487, 592
    - biogenesis 24, 477
    - bradyzoite 29, 184, 185, 305
    - exocytosis, secretion 21, 173, 175, 184, 477
    - PV 21, 173, 184
    - tachyzoite 4
    - ultrastructure 17, 20
  - Dense granule proteins 478–480, 572, 593
    - diagnosis 486
    - exocystosis 477
    - function 484
    - polymorphism 487
    - post-secretory trafficking 479
    - PV 127, 173
    - soluble tachyzoite antigen 485
    - stage-specific expression 479
    - trafficking to the parasitophorous vacuole 480
    - transcription regulation 480
    - vaccine 486
    - virulence 485
  - Diagnosis 37, 48–50, 71
    - biological 99
    - congenital 93, 98–99, 105
    - EIA test 42
    - fetus 45
    - IgG avidity index 43, 52
    - immunocompromised 45
    - immunological 101–102
    - newborns (neonates) 43, 44–45, 100, 102
    - pregnant women 41
    - prenatal 100
  - Dihydrofolate-thymidylate synthase (DHFR-TS) 244, 250, 254, 370
    - complementation 253
    - insertional mutagenesis 252
    - promoter mapping 269, 294
    - selectable marker 247–249
  - Dinitroalanes 514, 519
  - Dithiotreitol 502
  - Dolphins (porpoises, whales) 12, 13, 228
  - Doxycycline 428
  - DOXP pathway 423
  - Dyneins 545–548
    - flagella 545
    - flagellar axonema 546
    - inner dynein arms 547
    - intraflagellar transport 547



outer arm dyneins 546  
 Dynein heavy chains 544

## E

EGF-like domains 443  
 Egress 502  
*Eimeria* 3  
 Encephalitis 118–119, 227, 233  
   apoptosis 154–155  
   B-cells 116  
   chronic disease 83  
   congenital disease 99  
   IL-10 83, 131  
   lipoxin A<sub>4</sub> 131  
   MHC Class I allele 310  
   ocular disease 74  
   perforin mediated lysis 154  
   SAG1 bradyzoite expression 130  
   stage differentiation 286  
   type X in otters 195  
 Endodyogeny 510, 517, 518  
   definition 5  
 Endoplasmic reticulum 172, 397  
   gene products 202  
   host cell ER 175  
   retrieval sequence 397  
   tachyzoite 4  
   ultrastructure 17, 20, 23  
 Endosymbiont 17, 20, 209, 392  
   evolutionary implications 214, 215  
 Enolases 307  
 Epidermal growth factor-like 440  
 Epigenetics 327  
 Evacuoles 21, 173–175  
 Excystation 4, 9, 28  
 Expressed sequence tags (ESTs)  
   comparative genomics 209, 212–214  
   gene transfer, evolution 214–217  
   gene identification, expression  
     CDKs 275  
     GRAs 480  
     MICs  
     MyoH 537  
     proteomics 458, 460  
     SRCAP  
   SNP discovery, uses 197, 231  
   stage specific 287, 288, 306, 309, 575, 583

## F

Fatty acid metabolism 346, 416  
   acetyl-CoA 346, 424  
   acetyl coenzyme A carboxylase 347  
   apicoplast 347  
   FabG 347  
   FabH 347  
   FabI 347

FabZ 347  
 FAS I 346  
 FAS II 346  
 Ferredoxin 419, 425  
 Fertilization 4, 11, 28, 230, 513, 517, 520  
 Fetus 8, 71, 111, 127  
   diagnosis 46, 100–103  
   ocular disease 74, 77, 78  
   placenta 96–98  
   treatment, transmission 70, 94–95, 103, 104  
 Filipin 352  
 Fluorescent protein 246, 247, 267, 269, 293, 305, 394–395  
 5-Fluorocytosine 249  
 5-Fluorouridine 197  
 Folate 258  
 Folate pathways 370, 421  
 Folate synthesis 371  
 Folinic acid 49, 51  
   congenital treatment 103–104  
 Forward genetics 159, 163, 196, 242, 252, 255, 270  
 Fosmidomycin 424, 428

## G

Gametocyte, gametes 26, 194, 278, 520  
   microgametogony 26  
   macrogametogony 27  
   near diploid 272  
 Gamonts 10  
 GAP45 444, 534, 539  
 GAP50 444, 520, 534, 539  
 Ganciclovir 372  
 GCN5 329, 337  
 Genetic  
   complementation 253–255, 270  
   cross 163–166, 197–198, 218, 235, 255  
   markers 164, 196–197, 199  
 Genetic linkage map 183, 196–197, 199  
   *P. falciparum* 196  
 Genetic mapping, 166, 167, 183, 194  
 Genome 120, 159, 193–204, 211, 213, 241–256, 265  
   apicoplast 216  
   basic comparative features 199–200  
   cell cycle 271–272, 275  
   comparative, phylogeny 218–221  
   complementation 253, 270  
   complex, multigene traits, 164, 167  
   drug resistance 197, 199  
   EST 212  
   gene targeting 249–250  
   gene transfer 214–217  
   insertional mutagenesis 252  
   map unit 197

- metabolic pathways 202–204
- mitochondrial 216
- organellar 214
- polymorphism 200
- promoters, trapping 242–243, 252, 289
- relative size 196
- RFLP markers 164
- sequencing 197
- SNP markers 232
- SSU-rRNA 211
- transcription 288–289, 335
- Gliding motility 21, 500, 525, 531, 533–536, 547, 555, 557
  - comparative 556, 565
  - IMC 520
  - microneme proteins 437, 441, 444, 446
  - molecular mechanism 558
    - actin like protein 563–565
- Glycerolipids 357–358
- Glycolysis 202, 382, 413, 418
  - enzymes stage specific expression 287, 307
- Glycolytic enzymes 182, 304, 425
  - HIF1 regulation 181
- Glycosylphosphatidylinositol (GPI) 358, 443, 575
  - chemotherapy 359–360
  - protein preparation 460
  - proteases 578–584
  - PVM 128
  - surface proteins 287, 306, 309, 349
  - synthesis 359
- Goats 3, 8, 161
- Golgi complex 18
  - biogenesis, development 24, 27
  - gene products 202
  - organelle replication 272, 274
  - tachyzoite 4
  - ultrastructure 17, 19–20
- GRA1 480, 484, 487
- GRA1-8 185
- GRA2 309, 480, 484
- GRA3 173, 483, 596, 601
- GRA4 487
- GRA5 480, 484, 593, 601
- GRA6 480, 484
- GRA7 127, 173, 174, 480, 483, 484, 485, 487, 601
- GRA8 480, 601
- GRA9 480
- Guinea pigs 8, 75

## H

- Heat shock proteins 145, 146, 91
  - developmentally regulated 304, 306, 307
  - element 292
  - enolase 292

- HSP65 150
- HSP70
  - bradyzoite 244, 291–292
  - cell survival 150
- HSP90
  - cell survival 150
- Heme biosynthesis 394, 409, 415–417, 424
  - evolution 427
  - mitochondrion 412
  - TCA cycle 413
- Heparan sulfate proteoglycans 447
- Histone deacetylases (HDACs) 322, 324, 329, 331–333, 338
  - Parasite differentiation 337
  - Plasmodium* antigenic variation 335
- Histone acetylase 326
- Histone acetyltransferases (HATs) 322, 332, 326, 556, 338
  - chromatin remodeling 329–331
  - gene expression 337
- Histone methyltransferases 331–334
- Histone proteins 321, 327
  - covalent modifications 322, 328
  - modifying enzymes 332–335
- HIV-AIDS 7, 45, 62, 72, 127, 227
  - encephalitis 74, 227
  - evolution 236
  - materno-fetal infection 115
  - ocular disease 67, 69, 74
  - T cell mediated immunity 116
  - trimethoprim-sulfamethoxazole prophylaxis 47, 70
  - type I strains 160
  - type II strains 65, 160
  - seroprevalence in Asia 38
- HLA
  - placenta 97
- Horses 8
- Hypnozoites 313
- Hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) 374, 377, 378
  - isoforms 380
  - positive/negative selection 380

## I

- IgA 48–49
  - antibody 42
  - congenital immunological diagnosis 101, 103
  - congenital infection 44
  - EIA test 41
- IgG 37, 48–49, 103
  - acute and chronic infection 116
  - adult assay 42
  - avidity index 43–44, 52

- childbearing age 94
- congenital immunological diagnosis 101–103
- DGPs 486
- EIA test 41
- gold standard congenital 42
- neonate 44
- newborn assay 43
- pregnant women 41
- seroprevalence 38
- IgM 37, 68, 62
  - acute and chronic infection 116
  - congenital immunological diagnosis 101–103
  - EIA test 41–42
  - neonate 44
  - newborn assay 43
  - pregnant women 41
- IFN- $\gamma$  79–84, 95–97, 111–119, 128–131, 134, 176–179, 290, 310, 406
  - Astrocytes 119
  - B-cells 116
  - CD4+ cells 116–118, 130, 487
  - CD8+ cells 95–96, 116–117, 119, 128, 154, 487
  - chronic infection 310
  - CNS anti-parasite 119
  - congenital toxoplasmosis/Materno-fetal transmission 96–97
  - GTPase induction 115, 128
  - IL-10 130, 180
  - IL-12 83, 111–114, 116–118, 148, 179
  - IL-6 84
  - immune privilege 84
  - infection 83, 113
  - JAK/STAT 176
  - Lipoxin A<sub>4</sub> 131
  - MHC class I 178–179
  - MHC class II 178–179
  - nitric oxide/NO/iNOS 83, 86, 115, 119, 290
  - NK cell production 96, 112–114, 127, 178
  - parasite killing/reactive oxygen 114–115
  - pathological response 118, 130
  - SAG1 activation 129–130
  - SRS9 130
  - stage specific responses 130
  - STAT1 134
  - STAT1 activation 179
  - T-cell immunity 116–117
  - T-cells 112, 114, 127, 154, 178, 487
  - Th0 406
  - Th1 response 95, 127
  - TNF- $\alpha$  83–84, 116, 119
  - tryptophan 176
- I $\kappa$ B 133
  - anti-apoptotic 178, 600
  - immune evasion 133–134, 178
  - Theileria parva* 132
  - vacuolar membrane 152
- I $\kappa$ B kinase 132, 136, 152, 600
  - comparison to STAT3 activation 135
- Immune evasion 127–137, 171, 178
  - induction of anti-inflammatory mediators 130
  - IL-10 130–131, 132
  - Lipoxin A<sub>4</sub> 131
  - TGF $\beta$  131
  - interference with proinflammatory signaling 132
- Immune response 96, 111–120, 154, 265, 266
  - acute disease pathology 161–163
  - adaptive cell mediated, Th1 116–119, 161–162, 180
  - amino acid depletion 381–382
  - apoptosis 143–155, 178
  - dense granule proteins 475, 477, 485–486
  - GPI-anchored proteins 359
  - HIF1 181–182
  - innate resistance 112–116
  - metabolic changes, arginine, tryptophan 381
  - MHC 178–179
  - ocular disease 83–84
  - stage specific 184, 310, 312–313
  - time course 180
- Inflammatory response
  - animal ocular disease 74
  - apoptosis 143, 144, 149, 150, 152, 153
  - dose response 310
  - immunoevasion 130, 137
  - ocular disease 84, 85
  - tissue cyst, bradyzoite 7, 303
- Innate immunity, response, system 96, 113, 114, 127
- Interleukin-1 (IL-1) 119
  - astrocytes 119
- Interleukin 4 (IL-4) 118
  - pathological response 118
- Interleukin 5 (IL-5) 118
  - pathological response 118
- Interleukin 6 (IL-6) 83–84, 119, 134, 180
  - activation by MAPK pathway 134
  - astrocytes 119
  - deficiency, increased retinitis, vitritis 83
  - IFN- $\gamma$  83–84
  - immune privileged site 84
  - nitric oxide/NO/iNOS 84
  - TNF- $\alpha$  84
  - upregulation in HFFs, DCs 180
- Interleukin 10 (IL-10) 82–84, 118–119, 130–136, 180
  - anti-inflammatory 118, 130–131

- astrocytes 119
  - deficiency and increased eye pathology 83
  - downregulation of TNF- $\alpha$ , IL-12 135
  - independent activation of STAT3 135–136
  - limiting immune necrosis in eye 84
  - receptor 180
  - SAG1 induced hyperproduction 130
  - Interleukin 12 (IL-12) 83–84, 111–119, 127, 130–135, 148, 161–162, 179, 405
    - Astrocytes 119
    - B-cells 116
    - dendritic cell production 112, 113, 161
    - dendritic cell paralysis 112, 131
    - dendritic cell CCR5 131
    - dendritic cell lipoxin A<sub>4</sub> 131
    - dense granule proteins (DGPs) 405
    - Fas/FasL regulation of 148
    - G-coupled protein 113
    - IL-10 downregulation 130
    - immune evasion 134
    - induction by CCR5 113
    - induction by TLR signaling 113, 132
    - infection 83
    - IFN- $\gamma$  83 111–114, 116–118, 148, 179
    - interference with proinflammatory signaling 132–133
    - MyD88 deficiency 114
    - NF $\kappa$ B 133–134
    - NK cell activation 112, 114
    - response to antigens 113
    - RH 134–135
    - P38MAPK 114, 135
    - pathological T-cell response 118
    - PTG 134
    - production by neutrophils 112, 161
    - proximal signaling events 113
    - suppression by STAT3 135
    - synergizing with co-factors 114, 116
    - T-cell immunity 116, 161
    - toll-like receptor 13
    - toxoplasmosis/Th1 host response 111–113, 127, 161
  - Interleukin 15 (IL-15) 114, 118
  - Interleukin 18 (IL-18) 114
  - Intracellular mechanisms 133–135
    - MAPK 134–135
    - Rel/NF $\kappa$ B 133–134
    - STAT3 135
    - parasite niche 128–130
    - signalling cascade interference 132–133
  - Inner membrane complex (IMC) 18, 273, 510–520, 531, 581–582
    - biogenesis, development 24, 26
    - cell cycle 276, 536
    - dense granule fusion 478
    - glideosome anchor 444, 535, 539
    - HXGPRT localization 380
    - protein targeting 255
    - ultrastructure 17, 19, 581–582
    - visualization 269
  - Intermediate host 5
  - Invasion 21, 136, 437–449, 455–471, 475–484, 495–503, 520, 533–547, 555–559, 571–585, 591–596
    - actin dynamics 555–559
    - actin motility 128
    - apical complex 512–521
    - calcium homeostasis 493–503
    - dense granule proteins 475–484
    - differentiation 290
    - exocytosis, PV, TVN 21, 23, 136, 172–176, 184, 287, 351
    - host cell choline, phosphatidylcholine stimulation 356–357
    - immunosuppression 132
    - microneme secretion 437–449
    - molecular motors 533–547
    - proteases 571–585
    - pVM 591–596
    - rhoptry proteins 455–471
    - STAT3 activation
    - strain, host cell dependent 5
    - TgGCN5 control 330, 337
  - Intramembranous particles 510, 511
  - Ionomycin 500
  - Iron-sulfur cluster biosynthesis 418
  - Isoprenoid synthesis 403, 425, 499
  - Isospora* 3
  - Isoprenoid biosynthesis 423
- J**
- Jasplakinolide 559
- K**
- Kinesins 540–545
    - domains 543
    - functions 541
  - Kinetochores 23
- L**
- Lactase dehydrogenase 307
  - Lactose 447
  - Laminin 447
  - Lateral gene transfer 426
  - Life cycle 3–4, 159, 193, 228, 279, 303, 455
    - asexual end-point stages 271, 275
    - cats, sexual reproduction, perpetuation 10, 12, 234
    - chromatin remodeling 321, 326, 327, 338
    - clonal population structure 195–196, 230
    - dense granule proteins 477, 479
    - differentiation 337, 513

- heteroxenous 159, 193
- immune evasion 128, 136
- in tissue culture 256
- intermediate hosts 12
- myosin expression 535–536
- oocyst 216
- proteases 571, 584
- rhoptry function 469
- taxonomy 210
- tubulin expression 513, 520
- Lipids 345–361
  - FAS II 349
  - rhoptries 351
  - storage 354
  - therapeutic targets 354, 357, 360
  - uptake 349, 351, 355, 360
- Lipoic acid biosynthesis 416, 426, 599
- Lysine biosynthesis* 421
- M**
- M2AP
  - diagnostic assay 43
  - invasion efficiency 447
  - protease processing 444–445, 576–579, 584
  - protein complex 441, 444, 448
- Macrogametocyte
  - properties 11
- Macrogamont
  - properties 10
- Mass spectrometry 345
  - microneme proteins 580
  - proteomics 358, 460, 461
- ME49 strain 80–84, 130, 217, 219, 220, 309
  - clone B7 197
  - FUDR 197
- Merozoite 9, 10, 483
- Methylcitrate cycle 417
- MHC class I 310
- MIC1, 439
  - invasion efficiency 441, 447
  - protein complex 441–444, 447–448
- MIC2 439–441, 558, 580
  - conditional knockout, invasion 447
  - C-terminal tail, gliding 444, 535, 558
  - diagnostic assay 43, 102
  - escorter function for M2AP 444
  - essential gene, function 251, 576
  - N-terminal extension, ICAM-1 445
  - protease processing 445, 577–580
  - protein complex 444, 448
  - TRAP family member 442
- MIC3 439
  - knockout, invasion 447, 584
  - protease processing 445
  - protein complex 443–444, 448
  - tachyzoite expression 443
- MIC4 439
  - protease processing 445, 578
  - protein complex 444, 448
- MIC5 439
  - protease processing 445, 578, 579
- MIC6 439
  - escorter function for MIC1, MIC4 444
  - protease processing 445, 558, 578, 580, 583, 584
  - protein complex 444, 448
  - tachyzoite expression 443
- MIC7 439
  - bradyzoite expression 443
- MIC8 439
  - escorter function 444
  - protein complex 443–444, 448
  - tachyzoite expression 443
- MIC9 439
  - Bradyzoite expression 443
- MIC12 440
  - diagnostic assay 43
  - protease processing 445, 583
  - tachyzoite expression 443
- Microgamete 513
- Microgametocyte 9
  - properties 10–11
- Microgamont
  - properties 10
- Microneme 30, 128, 173, 437
  - biogenesis, development 24
  - bradyzoite 29, 305–306
  - comparison of invasive stages 184
  - exocytosis, secretion 21, 173, 174
  - promoters 244
  - secretion 500
  - tachyzoite 4
  - taxonomy 210
  - ultrastructure 17, 20, 210
- Microneme proteases 578
- Microneme proteins (MICs) 438–449, 572
  - functional redundancy 446
  - proteolysis 444
  - receptors 447
  - transmembrane/cytosolic domains 444
- Micro pore 25, 172
  - biogenesis, development 24, 26
  - tachyzoite 4
  - ultrastructure 19
- Microtubules 69, 172, 509–521
  - associated proteins 513
  - biogenesis, development 24, 26, 269
  - cholesterol acquisition 175, 177
  - endodyogeny 23
  - evacuole trafficking 173, 175
  - flagellar 513

- microtubules organizing centers (MTOCs) 274, 519, 595
- mitosis 23, 274, 278
- redistribution in host cell 175, 177
- spindle 509, 517
- subpellicular microtubules 509, 520
- tachyzoite 4
- ultrastructure 17, 19
- Mitochondria 496
- Mitochondrion 18, 172, 215, 409, 572, 581
  - apicomplexan evolution 215–216
  - biogenesis, development 26–27
  - branched-chain amino acid degradation 414
  - cytochrome B phylogeny 212
  - gene products 202
  - host cell mitochondria 23, 24, 29, 175–176, 286
  - apoptosis 144, 145–146, 149, 151, 176
  - inhibitors, bradyzoite development 290
  - metabolism 409
  - protein synthesis 420
  - oxidative phosphorylation 412
  - tachyzoite 4
  - tricarboxylic acid cycle 413
  - ultrastructure 17, 20
- Mitosis
  - cell cycle 270–279
    - organelle replication 272
  - microtubules 509–510, 517, 519, 520
  - molecular motors 525–546
- Mitotic spindle 23, 276
  - apicoplast division 274
  - microtubules 517, 519
  - molecular motors 537
- MLC1 539
- MORN 519
- MORN1 536
- Motility 520, 555
  - flagellar 545
- Motors 525–548
- Mouse model
  - congenital disease 95, 97
  - T-cell immunity 116
  - virulence 159, 160, 163, 251
    - type strains 161
- Moving junction 440
- MPP1 445
- MPP2 445
- MTOC 510
- MyD88
  - TLR signaling 113–114
  - IL-12 113–114
    - downstream signaling 114, 135
- MyoA 520, 529, 533, 535
- MyoC 536
- MyoD 535
- MyoF 538
- MyoG 538
- MyoH 537
- MyoI 538
- Myosin light chain 534, 539
- Myosins 526, 528–539
  - domains 530–531
  - functions 533
  - phylogeny and classification 529–533
- N**
- Neospora* 4
- Neutrophils 31, 115, 127
  - Apoptosis 149
  - IL-12 storage 112
- NFκB 128
  - anti-apoptotic 174, 178, 600
  - immune evasion 133–134
  - Theileria parva* 132, 135
  - TLR4 independent 133
- Nitric oxide
  - apoptosis 146, 150
  - down regulation by IL-10 130
  - immune response 83
  - inducible nitric oxide synthase (iNOS) 115, 128, 132, 179
  - macrophage, Microglia 119, 179
  - TLR block 132
  - tryptophan starvation 176
- NK cell 115, 145, 154
  - apoptosis 146, 147
  - cell-mediated resistance 113
  - IL-12 112
  - RAG-2 knockout 96
- NTPase 478, 484, 483
- Nucleoside transporters 373
- Nucleus 18, 22, 25, 30, 172, 215
  - biogenesis, development 24, 26–29, 291, 305–306
    - enolase 292
    - HSP90 291
  - bradyzoite 7, 29, 286, 291
  - calcium homeostasis 493, 496
  - cell cycle, division 271, 274, 278, 517–518, 546, 564
  - host cell nucleus 137
    - NFκB 133–134, 178
    - ROP16 601
    - STAT 134, 174
    - vimentin, PVM 175, 595, 597, 602
  - in cat intestinal epithelia 10
  - mitosis 23
  - RNA knock-down 251
  - tachyzoite 4
  - ultrastructure 17, 19, 20, 456, 496, 510
    - endosymbiosis 392, 403, 415



- visualization of replication 269
- Nucleus-encoded-apicoplast targeted (NEAT) proteins 391–394, 397
- Nutrient acquisition 173, 204
  - amino acids, nucleotides 366–382
  - tryptophan 176, 180
  - lipids 345–361
    - cholesterol 176
  - PVM 591, 594, 595, 598–600, 603

## O

- Ocular disease (ocular toxoplasmosis) 59–85, 62–63, 75
  - atypical strains 196
  - bone marrow transplantation 70
  - C57BL/6 murine immune response 83, 84
  - histopathology 74
  - immune privilege 84
  - in animals 74
  - murine acquired 78
  - murine congenital model 78
  - postnatally acquired 59
  - TGF- $\beta$  84
  - treatment 46
  - type strains 40, 65
- Oocyst *see also* sporozoite 3, 8, 9, 112, 165, 193–194, 218, 228, 271, 287
  - cell cycle 279
  - coccidian key stage 3–4, 211
  - cycle in cats 10–12, 194
  - cycle in intermediate host 12, 28
  - definition 8
  - differences in infectivity 8
  - energy storage 203, 216
  - genetic cross 197
  - oocyst wall, post-fertilization 11, 27–28
  - prepatent period 10, 11, 194
  - risk factors 104
  - shedding by congenitally infected kittens 12
  - sporulation 28, 39
  - survival in environment 11
  - water, food contamination 8, 12–13, 39, 94
  - vaccination to prevent shedding 10
  - virulence, ocular disease 52, 65
- Oral infection 130, 312, 487
  - animal ocular disease 75
  - apoptosis 147
  - stage conversion 29
- Oral infectivity 159, 234, 304
  - between intermediate hosts 196, 218
- Otters 12, 13, 195, 228
- antigen presentation 117, 129, 247
- bradyzoite development 286, 290
  - rhoptry protein BRP1
  - MAG1 287
- composition 602
- dense granule proteins 173–174, 185
- host GTPase 115
- intracellular development 21, 23, 24
- membrane protein composition 128, 137
- nutrient acquisition 598
- recruitment of host organelles 595
- sporozoites 12
- tachyzoite development 28, 128
- tubulovesicular network 601
- Parasitophorous vacuole membrane (PVM) 21, 601–603
- Pathological T cell responses 119
- PCR 250
  - diagnosis
  - fetus 45, 99–100
  - immunocompromised 45, 47
  - genotyping 229
  - insertional mutagenesis 252
  - microarray verification 179
  - SSU-rRNA 211
  - stage-specific gene expression 287, 535, 583
- Pellicle 25
  - apicoplast division 274
  - biogenesis, development 24, 26
  - invasion 520
  - microgamont 10
  - tachyzoite 4
  - ultrastructure 510, 530
- Perforin (granzyme) 119, 145, 146, 154
- Phalloidin 560
- Pigs (swine, pork) 6, 8, 39, 74, 104, 161
- Plasmalemma 18
  - tachyzoite 5
  - bradyzoite cycle 10
  - ultrastructure 19
  - biogenesis, development 24, 26
  - sporulation 28
- Plasmodium 174, 227
  - apicoplast 214
  - CDKs 275
  - cyclins 275
  - gametocyte near diploid 272
  - GTFs 289
  - merozoites 278
  - population biology 230
  - SSU-rRNA 211–212
- Plasmodium berghei* 213
- Plasmodium chabaudi* 213
- Plasmodium falciparum* 193, 201, 213, 219
  - comparative genome size 199
  - developmental gene regulation 288

## P

- PAN domains 442
- Parasitophorous vacuole (PV) 5, 6, 18, 25, 127, 136, 152, 172, 353, 480, 482

- growth and development 268
  - inbreeding 230
  - linkage map 196
  - pyrimethamine resistance 248
  - sporozoite stage 244
  - synchronization 268
  - toxoplasma model
  - Plasmodium gallinaceum* 213
  - Plasmodium knowlesi* 21, 213
  - Plasmodium reichenowi* 213
  - Plasmodium spec.* 146, 147
  - Plasmodium vivax* 213
  - Plasmodium yoelli* 213
  - Polar rings 30, 536
    - tachyzoite 4
      - ultrastructure 19
    - taxonomy 210
    - MTCO 274
  - Polyamine metabolism 382
  - Polyphosphate 499
  - Population genetics 197, 227–237
    - clonal population structure 229–230, 233
    - exotic, atypical strains 232
    - sexual recombination 231–232
  - Prevention
    - atovaquone 52
    - cardiac transplants 45
    - congenital infection
      - pyrimethamine-sulfadiazine-folinic acid, spiramycin 103
      - primary prevention 105
    - late-onset eye disease 47
  - Proteases 571–585
    - aspartic proteases 583
    - cysteine proteases 584
    - inhibitors 582
    - metalloproteases 585
    - microneme protein processing 573
    - rhomboids 582
    - subtilases 582
  - Proteomics (proteome) 438, 455–471, 514
    - 15-lipoxygenase activity 131
    - cytoskeletal proteins 514
    - dense granule proteins 478
    - metalloproteases 585
    - microneme proteins 438
    - organelle specific 409
    - PVM proteins 591, 602–603
    - rhostry neck proteins 468–469
    - rhostry proteins 456–458, 459–468, 561
    - stage specific 309
  - Purine metabolism 372
    - purine transporters 374, 376
    - salvage 374, 377, 598
  - Purine nucleoside phosphorylase (PNP) 379
  - Pyrimethamine 37, 45, 49, 51, 61, 64, 71, 72, 73, 102
    - congenital, pregnancy treatment 103–105
      - teratogenic 103
    - immunocompromised treatment 45, 47
    - neonatal treatment 44
    - ocular disease 46
      - experimental 75
    - prenatal treatment 100
    - pregnancy treatment 45
      - ocular disease 70
    - resistance DHFR-TS 248, 250, 254, 370
  - Pyrimidines metabolism 365
    - 5-fluorodeoxy-uridine (FUDR) 368
  - carbamoyl phosphate synthetase II 367
    - de novo pyrimidine synthesis 366
    - salvage 366, 368
    - uracil phosphoribosyltransferase (UPRT) 368
    - carbamoyl phosphate synthetase II 367
  - Pyruvate dehydrogenase complex (PDH) 413
- ## Q
- Quantitative Trait Loci (QTL) 164
    - mapping drug resistance 199
    - mapping virulence 164, 168, 236
      - chromosome VIIa 166
      - type I × type III cross 166, 236
      - type II × type II cross 236
- ## R
- Rab11 464
  - Rabbits 8, 75
  - Reactivation, recrudescence 7, 59, 111, 227, 310
    - congenitally acquired infection 59
    - differentiation into tachyzoites 278
    - encephalitis 118
    - eradication of bradyzoites 70
    - immune competent 118
    - immunocompromised 116, 118, 160
    - immunosuppression 77
    - ocular disease 85, 77
    - prevention by Th1 response 128
    - spontaneous CNS disease 161
  - Recent origin 159, 195
  - Recombinant antigen
    - adult assay 42–43
    - congenital immunological diagnosis 101
    - diagnosis in pregnant women 41
    - EIA test 41
    - intracellular tachyzoites 137
    - newborn assay 43
  - Recombinant strains, parasites, genotypes 40, 195, 229, 231, 232
    - exotic, atypical strains 232
    - fecundity 231
    - ocular disease 65

- promoter trapping 294
  - type I x Type III cross 164, 197
  - type II x Type III cross 166, 197
  - virulence 234–235, 237
  - Recombination 159
    - chromosome Ia 201
    - co-infection 194
    - comparative frequency 196–197
    - complementation 270
    - establishment of sexual cycle 194
    - gateway system 253
    - homologous allelic replacement 249, 250, 255
    - limitations for mapping 199
    - non-homologous plasmid insertion 242
    - oral infectivity 218
    - plasmid stability 253
    - positional cloning 199
    - strain diversity 229–233
    - targeting essential genes 251
    - virulence 166, 234–236
  - Restriction fragment length polymorphism (RFLP)
    - genotyping recombinants 164, 165, 197
    - population variation 195, 229, 232
  - Retinoblastoma *see also* Ocular disease 46, 51, 67, 78, 93
    - animal studies 69, 74–78
    - clindamycin 46
    - congenital disease 99
    - histopathology 74
    - immunocompromised 66, 67, 69
    - pyrimethamine-sulfadiazine treatment 46
    - trimethoprim-sulfamethoxazole treatment 46
    - type I strains 40
    - type II strains 40
    - type II strains 40
  - RH strain 75, 79, 81, 83, 84, 132, 133–135, 160, 179, 244, 248, 253, 270–273, 277, 289, 309, 338
    - DGP knockouts 484
    - Myo knockouts 534, 536
    - transgenic TK+ 268–269, 271, 277
    - UPRT deficient 294
  - Rhomboid 580
  - Rhoptries 18, 22, 30, 128, 172–173, 285, 448, 456–471, 572, 592
    - biogenesis, development 24
    - bradyzoite 7, 29, 305
    - comparison of invasive stages 20, 184, 286
    - exocytosis, secretion 21, 173, 174
    - Proteins 255
    - proteome preparation 460–464
    - PV 21, 137, 173
    - rhoptry neck (RON) 20, 448
    - taxonomy 210
    - tachyzoite 4
    - ultrastructure 17, 20, 210
  - Rhoptry neck 21, 173
  - Rhoptry neck proteins 448, 465
    - moving junction 468
  - Rhoptry proteins 456, 457
    - bioinformatics 464
    - host mitochondria 468
    - kinases, phosphatases and proteases 466
    - parasitophorous vacuole 457
    - processing 467
  - RNA polymerases 288, 232, 422
  - RON4 468
  - ROP1 173, 467, 580
  - ROP2 23, 126, 174, 251, 457, 596
  - ROP2/3/4 173, 174, 580
  - ROP7 173
  - ROP16 600
  - ROP18 600
  - RNA mediated knock-down 251–252
- ## S
- SAG *see* surface antigen
  - SAG1 related sequence (SRS) 184
    - bradyzoite specific
      - SRS9 130, 287
    - tachyzoite specific
      - SRS1 287
      - SRS2 287
      - SRS3 287
  - Sarcocystis 4
  - Sarcocystidae 465
  - SAG1-related Sequence (SRS) 184, 303, 306
    - GPI anchors 308
    - SRS1–3 287
    - SRS9 130
    - Bradyzoite antigen 287, 306, 313
  - Schizogony 10
  - Seals (sea lions, walruses) 12, 13, 228
  - Sexual cycle 10, 193, 227, 228
    - fecundity 231
    - genetic diversity 229
    - initiation by infective stages 194
    - initiation by single clone 194
  - SF-assemblin 517
  - Sheep (lamb) 3, 8, 39, 74, 98, 104
  - Shikimate pathway 371
  - Sialic acid 447
  - Single nucleotide polymorphisms (SNPs)
    - biallelic population structure 195
    - evolution of type strains 232
    - identification by ESTs 197
  - Sphingolipids 358
    - glycosylphosphatidylinositols (GPI) 358
  - Spindle formation 541

Spindle pole  
 mitosis 23  
 organelle replication 272, 274

Spiramycin 45, 48–49, 51, 71  
 congenital, pregnancy treatment 45, 70,  
 103–104

Sporozoite *see also* oocyst 9, 483  
 role in life cycle 11–12

Sporulation 4

SRS proteins 306, 313

Stage conversion 29, 31, 287  
 contribution of host cell 290  
*in vitro* 305, 413  
 ultrastructure 29

SufB 420

SUB1 578

SUB2 581

Sulfadiazine 37, 45, 71, 72–73, 102, 103  
 congenital treatment 103–104  
 immunocompromised treatment  
 ocular disease 70  
 neonatal treatment 44  
 ocular disease  
 experimental 75  
 pregnancy treatment 45, 70  
 prenatal treatment 100

Sulfamethoxazole 37, 61, 64  
 immunocompromised treatment 45, 47  
 ocular disease 70  
 ocular disease 46, 59

Surface antigen (SAG)  
 SAG1 62, 235, 243, 287, 291, 306  
 expression cloning 255  
 GPI anchor 349  
 IgG assay 42, 43, 102, 486  
 O-glycosylation 203  
 stage differentiation 129–130, 279, 306,  
 337  
 tachyzoite promoter 244, 289  
 T-cell, B-cell immunity 120  
 vaccination 96, 97

SAG2 62, 287  
 stage differentiation 129

SAG2 C/D Bradyzoite 306

SAG2A tachyzoite 306, 337  
 serotyping 233

SAG3 62  
 virulence 163

SAG4 62, 287  
 bradyzoite surface marker 292, 306

SAG5 62, 287

SWI/SNF 324, 336

## T

*T. gondii* 3

Tachyzoite

definition 4–5  
 role in life cycle 11

Taxol 514

T cells 77, 81, 82, 111, 116–119, 148  
 apoptosis in infection 144, 147, 153, 155  
 $\gamma\delta$  118  
 HIV patients 45  
 IL-2 requirement 117, 148  
 IL-5, IL-7 growth, homeostasis 118  
 recognition of infected cells 84, 129  
 recruitment in CNS 119, 312  
 regulation by astrocytes 119  
 resistance in SCID mice 114  
 transgenic mice 247

T cell mediated immunity 116–118

Temperature sensitive (ts)  
 essential functions 252  
 growth mutants (cell cycle) 253, 255,  
 269–270, 275

Tetracycline (Tet)  
 regulatable promoters 244–245, 251, 253

TgA1 497

TgNHE2 457

TgSUB2 456, 467

Thapsigargin 500

Thiolactomycin 349

Thrombospondin-1 (TSP-1) 441

Tissue cyst 309, 312

Tissue tropism 312

TNF- $\alpha$  79, 81–82  
 anti-microbial 115  
 apoptosis 149  
 astrocytes 119  
 CD-28 114  
 CNS anti-parasite 119  
 encephalitis 119  
 Fas/FasL 145  
 IFN- $\gamma$  83–84, 114–116, 119, 130, 132  
 IL-1 114, 119, 135  
 IL-10 130  
 IL-12 114, 116, 132–133, 135  
 IL-15 114  
 IL-18 114  
 IL-6 84, 119  
 infection 83  
 macrophage 132, 135  
 MAPK/p38MAPK 133, 135  
 NF $\kappa$ B 133  
 nitric oxide/NO/iNOS 83–84, 119, 132  
 SRS9 130  
 STAT1 116  
 STAT3 135  
 synergize co-factors 114  
 TLR 132, 133

TNF-receptor (TNF-R) 146–149  
 apoptosis 146–149

- Toll like receptors (TLRs) 136
    - dendritic cell activation 132
    - IL-10 135
    - IL-12 production 113–114
    - MAPK phosphorylation 135
    - neutrophil dysfunction 133
    - NFκB signaling 133
    - TLR4 132
    - TLR 11 114
  - Toxofilin 464
  - Transcription 325
  - Transcription
    - host
      - anti-apoptotic genes 152
      - HIF1α 181–182
      - MAPK activation 135
      - NFκB 133–134, 152
      - STAT1 134, 179
      - STAT3 127, 174, 183
      - STAT6 174, 183
      - TNFα in neutrophils 133
    - toxoplasma 243, 254
      - absence of known transcripton factors 243
      - bradyzoite specific genes 285, 295
      - cis-acting elements 292
      - control of gene regulation 243–244, 288
      - monocistronic 242
      - tetracycline inducible promoter 245
      - TGLZTR 292
  - Transcriptome 242, 253, 480
  - Transmission 127, 159, 166, 174, 212, 228, 279
    - asexual, bradyzoite 184, 196, 228, 232, 303–304, 314
    - fecal-oral cycle 4, 147
    - heteroxenous 193
      - prevention 47, 70, 103–104, 487
      - sexual cycle 194, 228
      - transplacental, materno-fetal 69, 93–103
      - water borne 37, 38
  - TRAP 441
  - Treatment *see* chemotherapy
  - Triclosan 349, 428
  - Trimethoprim 37, 61, 64
    - immunocompromised treatment 45, 47
      - ocular disease 70
      - ocular disease 46, 59
  - Triose phosphate transporter 425
  - Tubulin 513–516
- ## U
- Ubiquinone biosynthesis 420
  - Ultrastructure 17–31
  - Uracil phosphoribosyltransferase (UPRT) 369
- ## V
- Vaccination 237
    - CD8+ cell response 96
    - maternal transmission 97
    - SAG1 96
      - soluble tachyzoite antigen (STAg) 95
  - Vaccines 95, 96, 237, 475, 477, 486–487
  - Vacuolar H<sup>+</sup> -ATPase 497
  - Vacuolar H<sup>+</sup> -pyrophosphatase 497
- ## W
- Wallabies 74
- ## Z
- Zidovudine
    - immunocompromised, ocular disease 70







