RHOMBOID PROTEASES AND SURFACE ADHESINS DURING *CRYPTOSPORIDIUM* DEVELOPMENT

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Ebot Sahidu Tabe

In Partial Fulfillment
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Molecular Pathogenesis

April 2013

Fargo, North Dakota

North Dakota State University
Graduate School

Title

RHOMBOID PROTEASES AND SURFACE ADHESINS DURING *CRYPTOSPORIDIUM* DEVELOPMENT

By

EBOT SAHIDU TABE

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. John McEvoy
Chair

Dr. Eugene Berry

Dr.Glenn Dorsam

Dr. Jane Schuh

Dr. Eakalak Khan

Approved:

04/26/2013
Date

Prof. Charlene Wolf-Hall
Department Chair

ABSTRACT

Cryptosporidium parvum, a primary cause of cryptosporidiosis in humans and livestock worldwide, has a complex life cycle that includes an environmental oocyst stage, and stages of merogony, gametogony, and sporogony that are completed in a single host. Development within the host takes place in a protected intracellular but extracytoplasmic niche at the apical surface of epithelial cells. The life cycle can be described as having alternating extracellular invasive and intracellular replicative stages. With no effective chemotherapeutics, understanding the mechanism of host cell invasion by this pathogen is paramount.

The first aim dissertation was to identify functions of sporozoite surface proteins and rhomboid proteases (CpROMs) during motility and invasion of host cells. We demonstrate that two CpROMs distinctively and collectively cleaved five thrombospondin-family proteins (TSPs) and a mucin-like glycoprotein in a heterologous assay. Further, there was differential co-expression and co-localization of the CpROMs and their substartes during in vitro life cycle development; CpROM4 and CpTSP10 proteins colocalized to the anterior, middle and posterior of sporozoites and in developing intracellular stages while CpROM5 and TRAP-C1 colocalized to intact and non-intact oocyst walls, the anterior of sporozoites, and intracellular stages as early as 2 h post infection. CpTSP7, also localized to the oocyst wall, the anterior and posterior of sporozoites and intracellular stages from 6 h post infection. Similar to CpTSP10, CpTSP9 was not present in the oocyst wall; however, it was expressed in sporozoites and intracellular stages from 6 h post infection. Short synthetic peptides derived from adhesive ectodomains in thrombospondins including a TRAP-C1 apple domain (TAAP), thrombospondin type I domains in CpTSP7 (7TS) and CpTSP9 (9TS), and a kringle domain in CpTSP10 (10K1) as well as their corresponding antibodies demonstrated competitive and neutralization inhibition

effect of *C. parvum* infection of host cells. Polyclonal antibodies against TAAP caused sporozoites to agglutinate in a concentration−dependent manner, suggesting a contribution to reduced infectivity. In conclusion, the specificity and expression profiles of CpROM4 and CpROM5 indicate that they have distinct functions in shedding surface adhesins during excystation, motility, invasion, and intracellular development.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to everyone who provided assistance to me during the completion of this project. I would like to express my sincere thanks and deep gratitude to my adviser, Dr. John McEvoy, for providing skillful guidance, encouragements and his constant support during the course of this project. I am also grateful to the members of my committee, Drs. Eakalak Khan, Eugene Berry, Jane Schuh, and Glenn Dorsam for their guidance and support during the course of this project.

I also recognize the assistance from the Center for Protease Research and the Advanced Imaging and Microscopy Laboratory, all at NDSU. I would like to thank the members of Dr. McEvoy's laboratory for their time spent in providing useful suggestions to improve my project especially the resousefull assistance from Mrs. Cathy Giddings. I am also indebted to the secretarial staff of Van Es Hall for their general assistance provided during the course of this project.

Finally, I acknowledge the unending support and encouragement from my family members: Nessie Tabe (my wife), Mrs. Regina Tabe, Mrs. Mary Mbu, Peptua Ebot (my mothers), Mr. Stephen Mbu (my Pa) as and friends who has been very inspiring.

This dissertation is dedicated to my late father (Mr. Ahmadou Tabe Tanyi) and my late Uncle (Justice Ebot Jacob Ebot).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

10K1……...Thrombospondin protein–10 Kringle domain–derived peptide.

7TS……....Thrombospondin protein–7 TSP–1–derived peptide.

9TS…….Thrombospondin protein–9 TSP–1–derived peptide.

CpROM……..*Cryptosporidium parvum* rhomboid protease.

iT…….Immature trophozoites.

mT……...Mature trophozoites.

p.e…….. .Post excystation.

p.i…….Post infection.

ROM……...Rhomboid protease.

S……..Sporozoites.

TAAP……Thrombospondin-related adhesive protein of *Cryptosporidium*–1– apple domain derive peptide.

TRAP-C1……Thrombospondin-related adhesive protein of *Cryptosporidium*–1.

TSP……Thrombospondin protein.

TSP-1……..Thrombospondin type 1 domain.

GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

Introduction

Cryptosporidium is a monoxenic apicomplexan parasite that causes the disease cryptosporidiosis [1]. Transmission occurs by ingestion of environmentally stable oocysts through direct fecal-oral transmission or via contaminated water or food [2]. Following ingestion, invasive sporozoites emerge from oocysts and invade the intestinal epithelium [3]. Proliferation of *Cryptosporidium* in the gastrointestinal tract results in diarrhea that is generally self-limiting, but which can become chronic in those with a compromised immune system. Chronic infection can lead to dehydration, malnutrition, malabsorption, wasting, and death [4]. In the United States, the incidence of cryptosporidiosis ranges from 1.0 and 1.3 per 100,000 persons per year [1], with most disease resulting from recreational and drinking water outbreaks [5,6]. In developing countries, and Sub-Saharan Africa in particular, cryptosporidiosis is inextricably linked to malnutrition and a high prevalence of HIV infection. Disease incidence in the region ranges from 15% to 28% of the adult population [7]. Complications of cryptosporidiosis can include biliary disease, hepatitis, pancreatitis, arthritis, and respiratory tract disease [8]. Despite the morbidity and mortality, cryptosporidiosis remains underreported worldwide due to inadequate surveillance and diagnostics [1]. As a consequence, *Cryptosporidium* is included in the World Health Organization (WHO) Neglected Diseases Initiative [9,10]. Neglected diseases often affect the poorest populations in rural areas, urban slums, and natural disaster regions [1]. The ultimate goal of biomedical research targeting *Cryptosporidium* must be an effective drug for the treatment of cryptosporidiosis, particularly in immunocompromised individuals. Given its impact on human health, it is remarkable that so little is known about *Cryptosporidium* pathogenesis. One of the early barriers to progress was a

lack of suitable models for studying *Cryptosporidium* infections *in vivo* and *in vitro*. Tzipori et al. [11] first demonstrated the usefulness of a mouse model in 1980, and Current and Haynes [12] later reported the complete development of *Cryptosporidium parvum* from sporozoite to oocyst *in vitro*. These key advances paved the way for ultra-structural studies of *Cryptosporidium* and a more complete understanding of its life cycle. *Cryptosporidium* is an obligate intracellular parasite with both asexual and sexual stages (Figure 1). It is an early branching member of the phylum apicomplexa, a phylum that includes *Toxoplasma* and *Plasmodium* [13]. Zoites, the invasive stage of apicomplexan development, are polarized cells with an apical complex composed of micronemes and rhoptries, storage vesicles that sequentially release proteins to the parasite surface to engage host cell receptors [14]. *Cryptosporidium* invades host cells using a combination of parasite and host cell factors and occupies a unique vacuole that is intracellular but extracytoplasmic [15]. This differs from the cytoplasmic location occupied by *Toxoplasma* and *Plasmodium*. Host cell factors that contribute to invasion include actin remodeling and the influx of water and glucose at the site of zoite attachment [15,16].

Parasite factors are less clearly understood but most likely involve the translocation of adhesins along the parasite surface. Understanding the parasite specific factors that contribute to *Cryptosporidium* invasion remains a key research gap. Progress in identifying *Cryptosporidium* invasion factors has been helped by the study of conserved apicomplexan genes. Indeed, much of what we know about the processes of motility, attachment, and invasion of host cells is derived from paradigms developed for *Plasmodium* and *Toxoplasma* [17,18]. Studies in these related parasites have shown that invasion is an active process that is linked to motility. Early studies in *Plasmodium* demonstrated that gliding motility involved the translocation of proteins in an anterior to posterior direction along the zoite surface, powered by an actomyosin motor [19-24].

An inability to propagate *Cryptosporidium* continuously *in vitro* has greatly impaired researchers' ability to obtain purified samples of the different developmental stages [25]. Also, since the parasite cannot be genetically manipulated, transformation methodologies are currently unavailable [25]. However, an increased research focus over the past twenty years has resulted in the identification of unique biochemical and metabolic pathways, and an understanding of pathogenesis and immunity that can be exploited for drug and vaccine development [26]. Research efforts have been facilitated by the sequencing of *C. parvum* [25], *C. hominis* [27], and *C. muris* genomes [28].

Aims of the dissertation project

The long-term objective of this research is to understand the mechanisms that underpin *Cryptosporidium* invasion of host cells in order to inform the development of effective, anti-cryptosporidial therapeutic agents. The specific objective pursued in this dissertation was to identify functions of sporozoite surface proteins and rhomboid proteases during motility and invasion of host cells. The central hypothesis is that *C. parvum* rhomboid proteases (CpROMs) and microneme adhesins function together to achieve adhesion site turnover during motility and invasion.

The specific aims of this dissertation are:

To determine the extent to which CpROMs function as sheddases during motility and invasion. The working hypothesis is that CpROMs cleave microneme adhesins, and are coexpressed with microneme adhesins in invasive stages.

To determine the contribution of adhesive domains on microneme proteins to host cell invasion. The working hypothesis is that neutralizing adhesive domains on thrombospondin-family microneme proteins will reduce the invasion of host cells by *C. parvum*.

Significance of the research

Cryptosporidium is an apicomplexan parasite that causes cryptosporidiosis, a disease reported in over forty countries on six continents [29]. There is no effective therapeutic treatment and chronic infection can develop in immunocompromised persons. In particular, *Cryptosporidium* is a cause of serious, life threatening complications in AIDS patients [30]. The importance of cryptosporidiosis was underscored in 2004 when it was included in the WHO Neglected Diseases Initiative, due to its global burden and impact on ability to thrive [9]. If we are to reduce the impact of this pathogen on society, then there is a critical need to understand its pathogenesis and to develop effective chemotherapeutics to treat the disease.

Although host cell invasion is critical for *Cryptosporidium* pathogenesis, specific invasion mechanisms are poorly understood. In *Toxoplasma*, a model of apicomplexan motility and invasion, microneme adhesins are secreted, translocated, and shed from the parasite surface in a continuous motion that propels the parasite forward along the host cell surface (resulting in motility) and into the cell (resulting in invasion) [18,31]. Recent studies have identified rhomboids as the proteases responsible for microneme adhesin shedding in *Toxoplasma* and *Plasmodium* [32,33]. The role of rhomboids in *Cryptosporidium* motility and invasion remains unknown, and may differ from that in model apicomplexans due to *Cryptosporidium*'s unique form of invasion that results in an intracellular but extracytoplasmic niche. This research project addresses the roles of rhomboid proteases and microneme adhesins during *Cryptosporidium* development. The data obtained from this research are significant because they contribute undamental knowledge that is necessary to understand *Cryptosporidium* pathogenesis and to develop effective chemotherapeutic interventions.

Organization of the dissertation

Chapter 1 provides a comprehensive introduction and review of the literature that is relevant to the thesis. The review covers aspects of parasite biology, epidemiology, and disease pathogenesis.

Experimental work is described in Chapters 2 to 4. Each chapter in this section is prepared in the format of a manuscript for submission to a journal. As such, each chapter is a stand–alone document with an abstract, introduction, methods, results, discussion, conclusion, and reference sections. There is an expected overlap in some of the materials presented such as in the introduction, materials and methods, and reference sections. In–text references to work in other chapters are included to enhance readability and understanding.

Chapter 5 is a general discussion that aims to provide context, perspective, and future directions for the body of work described in the experimental chapters.

LITERATURE REVIEW

Overview of the parasite and the disease

Discovered by Ernest Edward Tyzzer in 1907 in the stomach of a house mouse [34], *Cryptosporidium* is a ubiquitous parasite that infects all major vertebrate classes, and causes the diarrheal disease cryptosporidiosis. Twenty-five valid species have been described to date, based on molecular, morphological, and biological data [35-37]. However, this does not represent the true species diversity in the genus and it is likely that many more species will be named as data become available. Currently recognized species include *C. muris* and *C. tyzzeri* in rodents*; C. andersoni*, *C. bovis* and *C. ryanae* in cattle and sheep*; C. suis* and *C. scrofarum* in pigs*; C. parvum* in cattle, humans, and other mammals*; C. meleagridis* in birds and humans*; C. hominis* in humans*; C. viatorum* in humans; *C. ubiquitum* in multiple mammal species; *C. baileyi* and *C. galli* in birds*; C. serpentis* and *C. varanii* in snakes and lizards, respectively*; C. molnari* in fish*; C. wrairi* in guinea pigs*; C. felis* in cats*;* and *C. canis* in dogs [38]. Of these, *C. parvum* and *C. hominis* are of greatest public health concern as the primary causes of human cryptosporidiosis worldwide.

Cryptosporidium is a frequent cause of infectious diarrheal illness worldwide [39], with varied incidences in the US, Europe, Asia, Australia, Africa, and South America [40]. In the US, cryptosporidiosis is associated with 0.4 to 1% of all diarrhea [40]. Seroprevalence increases with age, particularly during adolescent years, due to increased exposure to the parasite [41]. Studies have shown a 32% prevalence rate among young adults joining the Peace Corps [42] and 36% seropositivity in a rural Wisconsin community [43]. The incidence of cryptosporidiosis in the population is likely to be underestimated because of inadequate testing by public health professionals and the failure of immunocompetent individuals to seek medical attention [44].

Cryptosporidium is of particular public health importance due to its capacity for widespread dissemination in water and its association with chronic, potentially fatal disease in immunocompromised individuals. This year (2013) marks the twentieth anniversary of the largest waterborne disease outbreak in the US, during which an estimated 403,000 persons in Milwaukee, WI contracted cryptosporidiosis from a contaminated municipal water supply [45]. Drinking and recreation water outbreaks of cryptosporidiosis continue to occur in the US [46,47] and worldwide [48-51]. In one outbreak in Nevada, the median weight loss in individuals with HIV was 13.5 kg (29.9 lb), and the median duration of diarrhea was 60 days [52]. Thirty-two of the 61 adults with AIDS who developed cryptosporidiosis died within 6 months of disease onset, and 20 of those who died had cryptosporidiosis listed as a contributing cause on their death certificate [53]. Several outbreaks associated with contaminated ground water have been reported in Canada [40]. From 1993 to 1996, outbreaks were reported in all regions of the country [54] particularly in Kitchener-Waterloo, Ontario where 1000 people were affected, Collingwood, Ontario and Cranbrook, British Columbia with 2000 cases, and Kelowna, British Columbia with 10,000–15,000 cases. Most cryptosporidiosis outbreaks occur in the US, Canada, Australia, and Europe (particularly in the UK and Ireland), and affect both adults and children [55].

Despite intensive research efforts, including the evaluation of more than 200 chemotherapeutic agents, adequate drug therapies to treat cryptosporidiosis and vaccines to prevent the disease are still lacking [56]. This failure has been attributed, in part, to the peculiar intracellular but extracytoplasmic niche occupied by *Cryptosporidium* following host cell invasion, and to the unique parasite biochemistry [53,56]. However, some therapies have shown promise. Monoclonal antibodies have been shown to neutralize *C. parvum* infection [57-59], and passive protection has been achieved by treating immunosuppressed mice with immune

colostrum generated against a recombinant surface protein from invasive stages [60]. Bovine immune colostrum, collected following immunization with *C. parvum* recombinant protein rC7, provided substantial protection against cryptosporidiosis in neonatal calves [61]. Most recently, a novel reverse vaccinology approach was employed to identify several new potential vaccine candidates. Three of these, Cp15, profilin, and a *Cryptosporidium* apyrase, were delivered in heterologous prime-boost regimens in fusion with Cytolysin A (ClyA) in a *Salmonella* live vaccine vector together with purified recombinant antigens, and were found to induce specific, potent, and protective humoral and cellular immune responses in mice, suggesting their potential as vaccines [62].

The *Cryptosporidium parvum* life cycle

The life cycles of relatively few *Cryptosoridium* species have been described, to date [12,63]. As a primary cause of human and animal disease, and one of the few species available for laboratory study, most is known about the *C. parvum* life cycle (Figure 1). The life cycle of *C. parvum* has both sexual and asexual stages taking place in a single host [56]. The multi-stage life cycle begins and ends with an environmentally resistant, sporulated oocyst. The 4–6 μm diameter oocyst contains four sporozoites into type II meronts, which produce four merozoites. Merozoites released from type II meronts give rise to the sexual developmental stages. Motile microgametes fertilize the macrogamont to produce a zygote; the only diploid stage in the life cycle [66]. Zygotes undergo sporogony to produce an oocyst with four, fully sporulated sporozoites while still within the parasitophorous membrane [67]. Oocysts are excreted fully sporulated in the feces of infected hosts [64,65] subsequently type I meronts, in which they undergo asexual replication to produce six or eight merozoites. enclosed in a two-layered oocyst wall. Unusually, however, it does not have a sporocyst. Both thin and thick–walled oocysts are

produced during an infection. Thin walled oocysts are thought to be important in perpetuating the infection through an autoinfective route. Thick-walled Merozoites released from type I meronts can reinvade cells and perpetuate the asexual cycle. Alternatively, and for reasons that remain unknown, some merozoites enter cells and develop Asexual cycling and the production of thin-walled, autoinfective and the production of thin−walled, autoinfective oocysts are critical to the perpetuation of the infection in those with a compromised immune system [63].

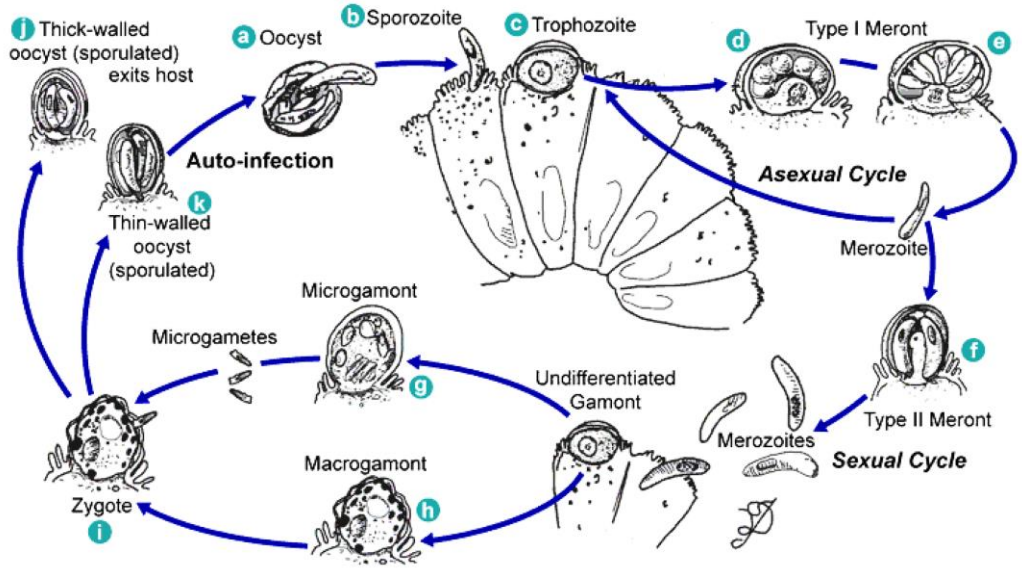

Figure 1. Life cycle of *Cryptosporidium* parvum. The cycle begins anew when oocysts are ingested by a new host. (a) Excystation of the orally ingested oocyst in the small bowel with release of the four sporozoites. (b) Invasion of intestinal epithelial cells via the differentiated apical end of the sporozoite within a vacuole formed by both host and parasite membranes and the initiation of the asexual intracellular multiplication stage. (c) Formation of trophozoites for nutrient sequestration from the host. (d) Asexual reproduction of type I merozoites. (e) Type I meronts harboring type I merozoites. (f) Type II meronts harboring type II merozoites. (g and h) Differentiation of microgametes and macrogametes. (i) Fertilization initiating sexual replication leading to zygote formation. (j and k) Development of oocysts which is then excreted in stool. Source: Center for Disease Control and Prevention, USA.

Following ingestion, oocysts travel through the stomach to the small intestine where they excyst, each releasing four sporozoites that invade epithelial cells and initiate asexual

development (merogony). The parasite has a predilection for certain sites within the host. In humans, the middle and lower small intestine are more often infected than the colon and upper small intestine [68,69] . Following invasion, sporozoites differentiate into rounded trophozoites, and oocysts are critical to the perpetuation of the infection in those with a compromised immune system current [70].

Clinical presentation of cryptosporidiosis

Human cryptosporidiosis is a severe diarrheal disease, often with a poor prognosis in malnourished children and immunocompromised individuals [71,72]. In immunocompetent humans, cryptosporidiosis typically manifests as a self–limiting diarrheal disease with a median duration of 9–15 days [73]. The infection is transmitted directly from animals or person to person, and indirectly via food or water [74]. The major symptom is watery diarrhea associated with abdominal cramps, anorexia, weight loss, nausea, vomiting, fatigue and low-grade fever [75]. Symptoms are similar in children and adults, although cryptosporidiosis acquired during infancy may have permanent effects on growth and development [76,77]. The clinical manifestation of *C. parvum* infection is directly related to the immune status of the host. Clinical symptoms range from acute, self limiting diarrhea in immunocompetent adults to chronic, debilitating, and potentially life-threatening diarrhea in immunosuppressed individuals such as AIDS patients [78], where the disease remains largely incurable and is associated with chronic, voluminous diarrhea [79,80]. Flanigan et al. reported that patients with CD4+ T-cell counts of greater than 180/ml cleared the infection spontaneously; whereas, 87% of patients with CD4+ T-cell counts less than 180/ml had persistent disease [81]. Despite the potential for severe, persistent diarrheal illness in this group of patients, there is actually marked variability in the clinical presentation [82-85]. Four general clinical categories of AIDS–related cryptosporidiosis

have been identified, which are: a cholera–like illness requiring intravenous rehydration therapy (33%), a chronic diarrheal illness (36%), an intermittent diarrheal illness (15%), and a transient diarrheal illness (15%) [86]. Although the intestinal tract is the primary site of cryptosporidiosis, infections have also been described in the lungs, middle ear, biliary tract, pancreas, and stomach [87-91]. It is suggested that these extraintestinal sites of infection represent luminal extension of a primary intestinal infection, rather than a primary infection [92]. Individuals with cryptosporidiosis can have chronic diarrhea that can last for more than two months, during which time they continue to shed oocysts. This chronic infection can cause severe dehydration, weight loss and malnutrition, extended hospitalizations, and mortality [93].

Cryptosporidium also frequently infects animals and cryptosporidiosis causes significant economic losses in production animals [73]. The common symptom of cryptosporidiosis in animals is yellow watery diarrhea, which leads to dehydration, weight loss, fever, and inappetence [94,95]. Most immunocompetent animals recover within 1–2 weeks of infection with supportive fluid therapy [96]. In ruminant livestock, cryptosporidiosis is typically symptomatic in pre-weaned animals [97] with prolonged shedding of oocysts [98]. However, infections in swine and horses are typically asymptomatic, even in young animals, provided animals are not under extreme stress or immunocompromised [99,100].

Epidemiology of cryptosporidiosis

The oocyst stage is of primary importance for the dispersal, survival, and infectivity of the *Cryptosporidium* and is of major importance for detection and identification [101]. Human infection with *Cryptosporidium*, first described in two cases in 1976, has now been reported from over 40 countries on six continents [102]. In the US, it is estimated 2% of all stools tested by health care providers are positive for *Cryptosporidium* [103]. Based on an estimate of 15 million

annual visits for diarrhea, 300,000 are expected to be due to *Cryptosporidium* [103]. Although, *C. hominis* and *C. parvum* are the major species responsible for human cryptosporidiosis worldwide, their relative contributions to disease vary in different regions [104]. A macro analysis of epidemiological data show that *C. hominis* is more prevalent in North and South America, Australia, and Africa; whereas, *C. parvum* causes more human infections in Europe, particularly in the UK [105].

Out of the 71 *Cryptosporidium*–associated outbreaks described in the last decade, 40 (56.3%) were waterborne [106]. Geographically, the outbreaks appear to be concentrated in the USA, Canada, Australia, and in Europe, especially in the UK and Ireland, and they appear to affect both adults and children [2]. *Cryptosoridium* has caused most (50.8%; 165/325) waterborne protozoan disease outbreaks worldwide [55]. It is followed by *Giardia duodenalis* (132 out of 325, 40.6%), *E. histolytica* (9 out of 325, 2.8%), *Cyclospora cayetanensis* (6 out of 325, 1.8%), *Toxoplasma gondii*, *Isospora belli* (3 out of 325, 0.9% each), *Blastocystis hominis* (2 out of 325, 0.6%), *Balantidium coli*, *Microsporidia*, *Acanthamoeba* and *Naegleria fowleri* (1 out of 325, 0.3% each) [55]. Worldwide, surveillance data show the presence of *Cryptosporidium* spp. in the entire water-treatment system, which represents an unacceptable health risk, particularly in sensitive (for example, pregnant women and children) and immunocompromised populations (for example, HIV-positive and transplant patients) [106]. Surveys in developing countries show a higher prevalence of infection than in industrialized countries [75]. Better sanitation and access to clean drinking water in industrialized countries probably account for most for this difference [77]. In developing countries, large populations and specific groups are at greater risk of infection, including children, the malnourished, the immunocompromised, and those who are institutionalized [77].

Cryptosporidium genomes

Cryptosporidium genomes are compact, and they reveal novel insights into apicomplexan biology and the general process of genome reduction in parasites [25,27,107,108]. Although *C. hominis* and *C. parvum* have differing host specificities [109], their genomes share 95-97% sequence identity, with no large insertions, deletions, or rearrangements evident [27], and essentially the same gene complement. Therefore, biological differences between these parasites are due to functionally significant polymorphisms in relevant protein-coding genes, and to subtle gene regulatory differences [110].

The *C. parvum* and *C. hominis* genomes include eight chromosomes ranging in size from 0.9 to 1.4 Mb [25,27]. At approximately 9.2 Mb with 3,800 predicted genes, the genomes are much smaller than the those of the related apicomplexans *P. falciparum* (23 Mb) and *T. gondii* (80 Mb) [111,112]. They do not have a mitochondrial genome, and they lack a TCA cycle and oxidative phosphorylation [110]. Therefore, they are largely dependent on glycolysis for energy production. Biosynthetic capabilities are limited and they are dependent on a host for essential nutrients such as amino acids, nucleotides, and simple sugars [113]. This explains the extensive array of transporters encoded in the genomes [113]. The genomes also have a few introns, approximately 5% of genes, and smaller intergenic regions relative to other apicomplexans [114]. It has been proposed that the low prevalence of introns is a consequence of ancient retrotransposon activity, where mRNAs were reverse-transcribed into cDNA and underwent homologous recombination with genomic regions [115].

Cryptosporidium motility and invasion

Progress towards an understanding of *Cryptosporidium* pathogenesis has been hindered, in part, by limited knowledge of its invasive machinery. However, with three genomes

[25,27,110], two proteomes [116,117], and a transcriptome [118] now available to researchers (www.cryptodb.org), molecules with putative roles in motility and invasion are increasingly being identified [119-123].

Sporozoites and merozoites, the invasive stage, are crescent shaped cells with a characteristic apical complex that includes micronemes and a rhoptry, which are storage vesicles that secrete protein onto the zoite surface during motility and invasion (Firgure 2). The parasite pellicle consists of the plasma membrane and a closely apposed inner membrane complex (IMC), which forms immediately subjacent to the plasma membrane of invasive zoites; susceptibility to competitive inhibition with recombinant and purified proteins, and neutralization with antibodies.

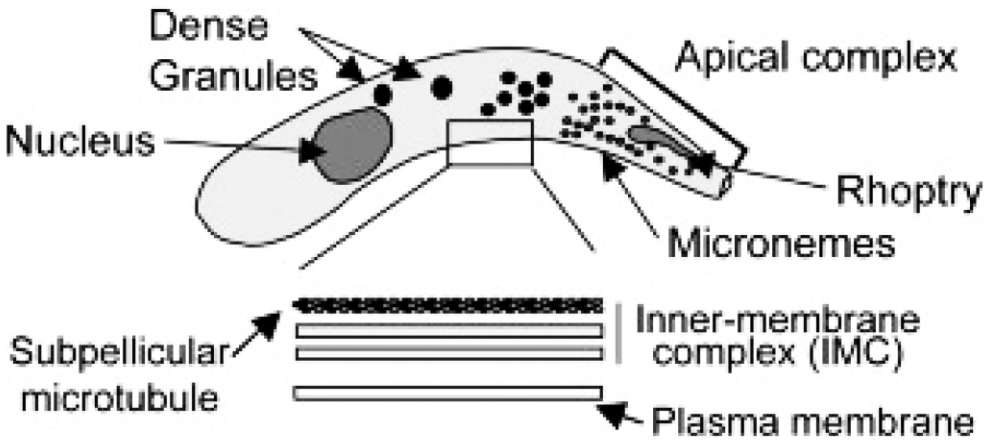

Figure 2. Cellular organization of Cryptosporidium sporozoite. From O'Hara and Chen (2011) The cell biology of Cryptosporidium. Microbes and Infection 13, 721 – 730.

Cryptosporidium sporozoites exhibit substrate–dependent gliding motility prior to host cell invasion (Figure 3). Gliding motility is conserved in apicomplexans and much of what we know about the mechanism is derived from studies in *T. gondii*. In *T. gondii*, motility and invasion are mechanistically similar, actomyosin–powered processes [124]. Gliding motility

involves the coordinated secretion of transmembrane adhesins from micronemes to the anterior parasite surface, where they bind to host cell receptors [33,125]. Receptor bound proteins undergo retrograde translocation, propelling the parasite forward, and are subsequently shed from the parasite surface by proteolytic cleavage. For invasion to take place the *T. gondii* zoite becomes apically oriented and forms a tight junction, known as a moving junction, with the host cell [126]. Posterior translocation of the moving junction allows the zoite to cross the cell membrane and establish an intracellular parasitophorous vacuole [127] . The moving junction is assembled through the interaction of RON2, a parasite derived protein injected into the host cell, and the parasite associated apical membrane antigen 1 (AMA1)[128].

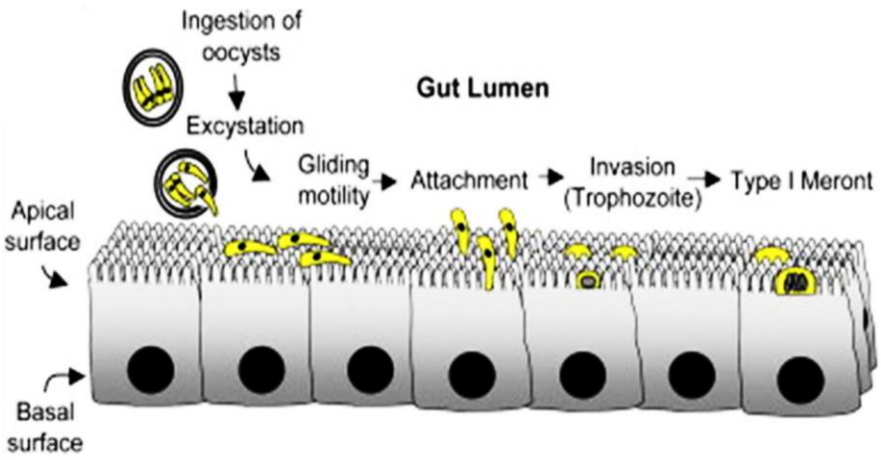

Figure 3. Cartoon depicting Cryptosporidium motility. From O'Hara and Chen (2011) The cell biology of *Cryptosporidium*. *Microbes and Infection* 13, 721 – 730.

There are three major limitations to using *T. gondii* as a model to understand *Cryptosporidium* invasion. First, in contrast to the cytoplasmic vacuole occupied by *Toxoplasma* in host cells, *Cryptosporidium* remains extracytoplasmic [129]. Second, *Cryptosporidium* is unusual among apicomplexans in that it does not encode homologues of AMA1 and RON2. Third, *Cryptosporidium* does not appear to require actomyosin to enter host cells [130], although

actomyosin-dependent motility is required prior to invasion [131,132]. The strategy that *Cryptosporidium* uses to invade a host cell is thus very different. However, we remain frustratingly in the dark about the specific *Cryptosporidium* invasion mechanisms, despite progress identifying parasite proteins that contribute to invasion.

Without a genetic system to interrogate function, evidence supporting a role for *Cryptosporidium* molecules in invasion tends to be circumstantial. Such evidence can include homology with known invasion proteins in other apicomplexa; coexpression with known invasion genes/proteins; immunolocalization to micronemes, rhoptries, or the plasma membrane of invasive zoites; susceptibility to competitive inhibition with recombinant and purified proteins, and neutralization with antibodies.

Thrombospondin family adhesins

Thrombospondin–family proteins have been identified in several apicomplexan genera [133-136], and they are necessary for gliding motility and invasion. Thrombospondins have multiple adhesive domains including one or more thrombospondin type I repeats, an epidermal growth factor (EGF)–like domain, an Apple domain, and a von-Willebrand Factor domain [137]. Thrombospondins localize to micronemes and to the surface of zoites [138,139]. A thrombospondin with an adhesive ectodomain, a transmembrane region, and a cytoplasmic tail that can engage the actomyosin motor complex, is known as a Thrombospndin Related Adhesive Protein (TRAP). This ability to link the intracellular motor to the extracellular adhesion site is critical in generating the force that drives gliding motility.

TRAP-C1, the first thrombospondin protein to be identified and characterized in *C. parvum*, has a typical TRAP protein domain architecture [140] . It is encoded by a single copy gene with no introns and is structurally related to TgMIC2 in *T. gondii* [140]. Although there is

limited data on the subcellular localization of TRAP-C1, its localization to the apical region, suggests an association with micronemes. Deng et al., [119] subsequently identified 11 thrombospondins (CpTSP2-CpTSP12) in addition to TRAP-C1 in the *C. parvum* genome. Six out of 11 have transmembrane regions (CpTSP2, CpTSP6, CpTSP7, CpTSP8, CpTSP9, and CpTSP10), and five do not (CpTSP3, CpTSP4, CpTSP5, CpTSP11, and CpTSP11). Deng et al. ,[141] erroneously reported that CpTSP2, a 3,530 amino acid protein, is missing a transmembrane domain, and that CpTSP3 has a transmembrane domain. An examination of the sequences published in CryptoDB (www.cryptodb.org) shows that the opposite is true: CpTSP2 has a transmembrane domain, and CpTSP3 is missing a transmembrane domain (unpublished observations). Similar to TRAP-C1, CpTSP8 is localized to the apical complex .

CpTSP8 was the second *Cryptosporidium* thrombospondin to be characterized [1] . The gene has an intron and it encodes a type I membrane protein of 614 amino acids, with three thrombospondin repeats and one EGF-like domain [84]. At the transcript level, CpTSP8 has a fully spliced mRNA, and two immature mRNAs with the intron totally or partially retained [137]. Similar to TRAP-C1, CpTSP8 is present in the apical complex of both sporozoites and type I merozoites, and it translocates to the parasite surface following exposure to host cells [137].

Glycoprotein adhesins

Unusually among the apicomplexans, the *Cryptosporidium* lineage shows a major expansion of glycoproteins [142], and many of these are known to play an important role in invasion. Arrowood [143] identified the 23 kDa glycoprotein P23 in *C. parvum*, which was later shown to have neutralization sensitive epitopes [144]. Petersen et al.,[145] identified and characterized GP900, a highly immunogenic mucin-like glycoprotein. They further

demonstrated that GP900 is surface exposed, localized to micronemes of invasive zoite stages, and it mediates invasion of host cells [146]. More recently, Chatterjee et al. [147] showed that GP900 localizes to the inside walls of oocysts and may tether sporozoites to the oocyst wall. Mucins such as GP900 have extensive O-linked glycosylation of serine and threonine residues and up to 80% of the molecular weight is attributable to O-linked carbohydrate[148]. GP40/15 is another major mucin-like glycoprotein that localizes to the apical complex surface of sporozoites, and oocyst wall [147,149], and it also has neutralization sensitive epitopes [149]. GP40, a proteolytically processed product of the gp40/15 gene that encodes both the 40 kDa mucin-like glycoprotein and the antigenically distinct 15 kDa protein GP15 [149]. It was recently shown that GP40/15 is processed at the parasite surface by the subtilase-like protease CpSUB1 [150].It was recently shown that GP900 forms an adhesive complex with P30, a Gal/GalNAc specific lectin, and GP40/15 at the sporozoite surface [120]. P30 is neutralized by antibodies and competing recombinant P30 protein [120]. The glycoprotein CP2 is as a membrane associated protein that is translocated to the host cell membrane during infection and has neutralization sensitive epitopes [115] . Of the 31 mucin-like genes identified in the *Cryptosporidium* database, seven genes were clustered on a single locus on chromosome 2, indicative of coordinated expression and/or biological function [123]. Two of these, CpMuc4 and CpMuc5, appear to be O-glycosylated and are expressed on the surface of sporozoites. CP47, a protein that localizes to the apical region of sporozoites and binds to a 57 kDa protein that is preferentially expressed on intestinal epithelial cells [151].

Rhomboid proteases – the sheddases

Rhomboid proteases (ROMs) were discovered through genetic analysis of *Drosophila* embryogenesis. They initiate *Drosophila* EGF signaling by releasing transmembrane EGF precursors Spitz, Keren, and Gurken from the membrane [152,153], and therefore play a critical role in signaling during development. ROMs are polytopic (6-7 transmembrane domains; Figure 4) intramembrane serine proteases [154] that are functionally conserved across all kingdoms of life [155-157], . Uniquely among serine proteases, the active site, a serine-histidine catalytic dyad, lies within the membrane. ROMs target helix destabilizing residues within the transmembrane domain of substrates [158,159], which they cleave by peptide bond hydrolysis [160].

Rhomboid-like genes are present in the genomes of all apicomplexan parasites sequenced to date, including *T. gondii, Plasmodium* spp., *Eimeria tenella, Cryptosporidium* spp. and *Theileria* spp [161-163]. Apicomplexan ROM nomenclature is based on a convention of using the initial letters of the genus and species and a number based on similarity to *T. gondii* and *P. falciparum* ROMs [164]. *Toxoplasma gondii* contains six rhomboid-like genes (TgROM1 through TgROM6) [161]. Discovering the diverse functions of apicomplexan ROMs can inform the development of novel therapeutic strategies to treat disease. In *Eimeria tenella*, vaccination of chickens with a modified virus containing a ROM sequence induced an immune response and offered partial protection against *E. tenella* challenge [165]. Although live vaccines are the predominant vaccines for coccidiosis control in pultry and have been used for many years,there has been limited use due their to thir potential pathogenicity, high cost, laborious immunization procedure and demanding operational and management requirements [165].

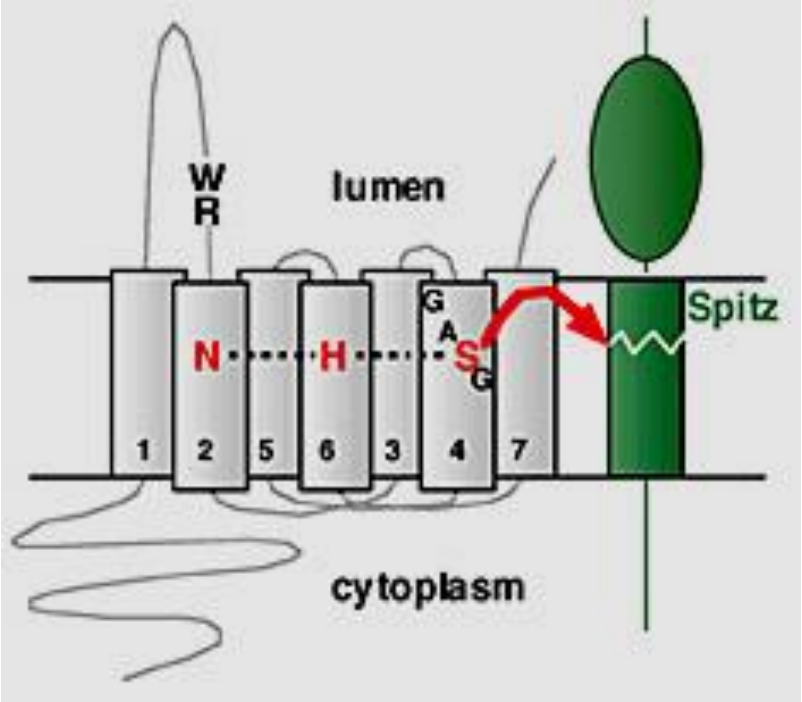

Figure 4. Cartoon of canonical rhomboid protease structure. A cartoon of a rhomboid (grey) cutting a substrate within the transmembrane domain. The proposed catalytic triad is highlighted in red. From Urban and Freeman (2003) Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. *Mol. Cell* 11, 1425–1434.

Inferred phylogentic trees constructed from apicomplexan ROM amino acid sequences show a *C. parvum* ROM (CpROM) that clusters with TgROM1 and TgROM2, and two CpROMs that cluster with TgROM4 and TgROM5 [162,163]. More recently, a ROM identified as CpROM6, but which was actually a ROM from another *Cryptosporidium* species, *C. muris* (CmROM6), was reported by Santos et al., [166]. ROM homologues from *T. gondii* (TgROMs) and *P. falciplarum* (PfROMs) show similar subcellular localizations [167] and may have similar functions. For example, TgROM1 and PfROM1 are catalytically active proteases localized to micronemes or microneme like vesicles called mononemes (PfROM1) [168-171]. The function or functions of ROM1 homologues remain largely unknown. Deletion of TgROM1 [172] or

PbROM1 (*P. berghei*) [173] caused no detectable defect; whereas, deletion of PyROM1 (*P.yoelii*), caused malformation of the PVC in some parasites [174]. ROM2 has been found only in the two closely related coccidian parasites, *Neospora caninum* and *T. gondii*, where it is localized to the Golgi apparatus. However, the function of ROM2 remains unknown [175] . ROM6 proteases, the only apicomplexan ROMs with homologues outside the phylum, are localiazed to mitochondrial membranes [176,177]. TgROM4 and PfROM4 are large proteins evenly distributed throughout the parasite plasma membrane [169,170,175,178]. TgROM5 also localizes to the plasma membrane of extracellular parasites, but in intracellular parasites its subcellular distribution is less well defined [33,175]. The initial insights into apicomplexan ROM functions came from studies on microneme protein protease 1 (MPP1) activity [167]. MPP1 activity is responsible for the proteolytic shedding of the adhesins from the parasite surface during motility and invasion [179]. Although the exact function of the ROM activity remains unknown, several hypotheses have been suggested [167] including 1) preventing the accumulation of parasite adhesins at the cell surface to avoid them becoming the target of neutralizing antibodies in the host [179] ; 2) contributing to the proper reorientation of the parasite preceding penetration of the host cell by generating a gradient of adhesins at the parasite surface; 3) disengagement of adhesion sites to facilitate productive motility and invasion.

In *T. gondii*, ROMs cleave TgMIC2, TgMIC6, TgMIC12 and TgAMA1, which have been implicated in distinct steps during invasion of host cells [180-183]. The ROM cleavage site was first mapped by mass spectrometry to the transmembrane domain (TMD) of TgMIC6 [182] and was found to be conserved in other transmembrane microneme proteins [162]. The first experimental evidence that a rhomboid protease was responsible for the MPP1 activity stemmed from the analysis of TgMICs in cell based cleavage assays [32,169,175]. Subsequently, *P.*

falciparum PfEBA–175 [170], PfAMA1 [181], PfRh1, PfRh4 [184] and PfTRAP (reviwed in [167]) were reported to be cleaved by a rhomboid-like activity in vivo, whereas PfCTRP, PfMTRAP and PfMAEBL were shown to be rhomboid substrates in vitro [32]. It is worth noting that, this group of substrates included critical *Plasmodium* adhesins implicated in each of the invasive stages of the parasite and in all of the alternative pathways of invasion (reviewed in[168]).

Although little is known about ROM function in apicomplexans other than *Toxoplasma* or *Plasmodium*, the broad range of putative substrates also extends to the other Apicomplexans [164]. TgROM4, which was considered to have a narrow substrate specificity in a cell–based heterologous assay [33], was demonstrated in conditional knockout studies to be essential for cleavage of TgMIC2, TgAMA1 and, possibly, TgMIC8 [185]. In addition, TgROM4-mediated cleavage of TgAMA1 in *Toxoplasma* has been shown to trigger the switch from an invasive to a replicative mode during *Toxoplasma* infection [186]. Thus TgAMA1 is an important player in invasion [187] since it is involved in the transition from invasion to replication upon entry into the host cell [186].

Host immune response to *Cryptosporidium* infection

The immune status of the host plays a critical role in determining susceptibility to infection, and the outcome and severity of cryptosporidiosis. In immunocompetent hosts, infection is often asymptomatic or mild to moderate and self-limiting [188]. However, in immunodeficient hosts such as patients with HIV/AIDS, congenital immunodeficiencies, and transplant recipients, infection can result in persistent, debilitating and possibly fatal diarrhea and wasting [189]. In areas where cryptosporidiosis is endemic, most symptomatic infections occur in early childhood and in the immunodeficient [190]. Although *Cryptosporidium* primarily

infects the distal small intestine, in severely immunodeficient patients this parasite can infect extraintestinal sites such as the lungs, the biliary tract and pancreas [190]. While the outcome and severity of infection is critically dependent on the immune status of the host, the nature of the immune response in cryptosporidiosis, particularly in humans, is poorly understood [191].

Most studies of the immune response to cryptosporidiosis in humans have focused on systemic antibody responses with a few addressing cell-mediated responses [191]. Studies of the cell-mediated response are challenging to perform since they involve invasive tissue sampling, as opposed to less invasive analysis of antibodies [191]. In vitro studies have been performed in tissue culture models using human cell lines , many of which are cancer–derived cell lines [192].

Studies in humans and human cell lines demonstrate the importance of specific innate immune responses in resistance to cryptosporidiosis [191]. Cryptosporidium parvum is known to induce recruitment of TLR2 and TLR4 to the site of infection leading to activation of downstream effectors in a human cholangiocyte model in vitro [193]. Knockdown of MyD88, TLR2 and TLR4 expression using dominant negative mutant and siRNA approaches resulted in inhibition of downstream signaling pathways and increased *C. parvum* infection in cells in which MyD88 expression was decreased [191]. TLR4 expression in this model appears to be regulated by the miRNA let–7 [194]. *Cryptosporidium parvum* infection resulted in MyD88 and NF–κB–dependent suppression of let–7 and consequent upregulation of TLR4 in these cells [194]. Although the aforementioned studies were performed in human cholangiocytes, which are not the primary site of infection, there have been no studies reported on the role of TLR–mediated pathways in *C. parvum* infection in intestinal epithelial cells, or other immune cells that the parasite or its antigens may come in contact with, such as dendritic cells and macrophages [191]. Although TLR ligands for *Cryptosporidium* have not been identified, the

parasite expresses surface−associated glycosylinositol phospholipids [195] and glycosylphosphatidylinositol anchors [196], which have been implicated as putative TLR ligands in other apicomplexan parasites such as *Plasmodium* [197].

Antimicrobial peptides (<100 amino acids), which are key components of the intestinal mucosal barrier, have antimicrobial and immunomodulatory properties and are evolutionarily conserved effectors of the innate immune system [198]. *In vitro* studies in human intestinal epithelial cell models have shown that *C. parvum* infection initially down regulates human β−defensin−1 (HBD−1) production, which may facilitate parasite survival [199]. HBD−1 is one of six known human β−defensins, and in contrast to HBD−2, −3 and −4, it is constitutively expressed during infection and/or inflammation. *Cryptosporidium parvum* infection also induces expression and secretion of HBD−2, which has antimicrobial activity against the parasite *in vitro* and may play a role in recruitment of T−cells and dendritic cells *in vivo*, facilitating clearance parasite clearance [191]. In addition, *C. parvum* has been shown to upregulate HBD−2 expression through TLR2− and TLR−mediated signaling and NF−κB activation in infected human cholangiocytes [193].

Chemokines such as the chemokine ligand CCL−5, a potent chemoattractant, were unregulated in a human intestinal epithelial cell model of *C. parvum* infection [200]. In another study of *C. parvum* infection in human intestinal epithelial cells and intestinal xenografts, there was increased expression of the CXC chemokine ligand CXCL−8 and growth−regulated oncogene (GRO) −α in infected cells [201]. It is perhaps worth noting that peripheral blood mononuclear cells of malnourished Haitian children with cryptosporidiosis expressed higher levels of CXCL−8 upon *ex vivo* stimulation with *C. parvum* [202]. This cytokine was also

detectable in stool samples of some Brazilian and Haitian children with cryptosporidiosis [203]. In addition, CXC chemokine CXCL−10 (IFN−γ inducible protein 10, which recruits IFN−γ−producing T cells), was expressed in higher levels in jejunal biopsies of AIDS patients with cryptosporidiosis compared with uninfected control AIDS patients [204].

Mannose-binding lectin (MBL) is a collagenous lectin found in serum that binds to specific carbohydrate residues on a variety of infectious organisms including *Cryptosporidium* [205]. Upon binding, MBL activates the lectin complement pathway in an antibody−dependent manner via mannose−binding lectin−associated serine proteases (MASPs), thereby promoting opsonization and phagocytosis. In a study of AIDS patients with cryptosporidiosis, patients homozygous for structural mutations in the *MBL2* gene, and consequently low serum MBL levels, were more susceptible to infection with *Cryptosporidium* [206]. A study of Haitian children with cryptosporidiosis, many of whom were malnourished, showed that children with MBL deficiency (MBL ≤ 70 ng/ml) were more likely to be infected with the parasite [207]. However, this study did not investigate polymorphisms in the *MBL2* gene in these children. In a recent study in Bangladeshi pre−school children, MBL deficiency and *MBL2* polymorphisms were strongly associated with *Cryptosporidium* infection, particularly in those with repeated infections [208].

Cryptosporidium parvum infection may downregulate IFN-γ by suppression of STAT1-α signaling, thus facilitating invasion [243]. In a study of experimentally infected humans, jejunal biopsies from previously uninfected (sero negative) individuals demonstrated no mucosal IFN-γ production, whereas mucosal IFN-γ production was detected in most of the seropositive individuals [209]. In the absence of IFN-γ production, seronegative individuals expressed IL-15, suggesting that other cytokines may play a role as well. Those volunteers that expressed higher

levels of IL-15 in their jejunal mucosa had symptomatic infection but shed fewer oocysts than seronegative volunteers that did not express IL-15 [210]. Subsequently, it was demonstrated that IL-15 activates natural killer cells and γδ-T cells and may assist in recruiting other cells that lead to parasite clearance [211]. IL-4 was also expressed in the jejunal mucosa from seropositive volunteers, but there was no association with presence of symptoms or oocyst shedding. Jejunal biopsies from some experimentally infected human volunteers also showed TGF-β expression [212].

Jejunal biopsies from experimental infections in humans showed expression of TNF-α and IL-1β, which can stimulate prostaglandin production; however, there was no association with symptoms [212]. Prostaglandins are potent lipid molecules that exert their effects via a wide array of receptors. *Cryptosporidium parvum* infection of human intestinal epithelial cells *in vitro* resulted in activation of prostaglandin H synthase 2 expression and increased production of prostaglandin-E2 and -F2-α [213]. Although prostaglandins may contribute to the pathogenesis of secretory diarrhea by altering chloride uptake and fluid secretion, they may also upregulate mucin production from epithelial cells, which can protect the mucosa from *C. parvum* infection by interfering with attachment [191]. In addition, prostaglandins may stimulate HBD production and down regulate expression of inflammatory cytokines [191]. Substance P, a neuropeptide in the GI tract [214], can cause chloride ion secretion and play a role in secretory diarrhea [214]. Jejunal biopsies from AIDS patients with cryptosporidiosis showed increased expression of substance P mRNA and protein in patients with diarrheal symptoms [215].

The criticall role of cell-mediated immunity and T-cell responses in protection from, and resolution of, cryptosporidiosis has been well established in both murine models and human studies [191,216]. The increased susceptibility of AIDS patients to *Cryptosporidium* infection

and resolution of cryptosporidiosis following immune reconstitution underscores the importance of CD4+ T cells [217]. Patients with CD4+ counts less than 50 cells/ml are more likely to have a fulminant form of the disease, while those with CD4+ counts of 180 cells/ml or more tend to have less severe, self-limited disease (reviewed in [190]). CD4+ cell responses are mediated in large part by the IFN-γ [64]. PBMCs from infected humans proliferate in response to recombinant and crude preparations of *Cryptosporidium* antigens [218]. The response is MHC class II-dependent and is characterized by increased production of IFN-γ [219]. T-cell clones derived from PBMCs of *Cryptosporidium*-exposed patients stimulated with native and recombinant antigens were predominantly CD4+ TCR-α/β $CD45RO^{+}$ (memory phenotype), and were characterized by hyper-production of IFN-γ [194]. Some of the T-cell clones exhibited a Th0 phenotype, secreting IL-4, IL-5 or IL-10 in addition to IFN-γ [194]. In a recent study, in which whole blood from seropositive and seronegative human volunteers was stimulated *ex vivo* with recombinant *C. hominis* GP15 antigen, both CD4+ and CD8+ cells from seropositive donors produced greater amounts of IFN-γ than those from seronegative donors [197]. In a human volunteer study of adults experimentally infected with *Cryptosporidium*, mucosal IFN-γ production correlated significantly with the presence of pre-existing anti-*Cryptosporidium* antibodies, and reduction in oocyst shedding, suggesting that prior exposure to *Cryptosporidium* may be important in developing protective IFN-γ-mediated memory responses in subsequent infections [198]. The role of CD8+ T cells in protection from, or clearance of infection is not clear. However, in the human study referred to above, both CD4+ and CD8+ T-cells secreted IFN-γ in response to *ex vivo* stimulation with recombinant GP15 [197].

While the critical role of T-cell-mediated responses in the control of cryptosporidiosis is undisputed, the specific role of humoral immunity remains unclear [220]. During infection, the invasive stages of the parasite are present in the intestinal lumen, and they are, therefore, the most likely stages to be targeted by specific antibodies, which may function by blocking host cell invasion by these stages [221]. Humans with X-linked hyper I-IgM syndrome present with selective IgA deficiencies to cryptosporidiosis [200,222] suggest that humoral immunity may play a role in clearing the parasite [221]. However, many of these individuals may have defects in both B- and T-cell responses [221]. A possible role for antibodies is also supported by the partial efficacy of some hyperimmune bovine colostrum preparations (derived from cows immunized with *C. parvum*) in cryptosporidial infections in healthy human volunteers and those with AIDS (reviewed in [53]). Sera from AIDS patients recognized fewer cryptosporidial antigens when compared with non-AIDS patients, suggesting that deficient CD4+ T-cell responses may additionally cause ineffective antibody responses [223]. A number of studies have reported the presence of *Cryptosporidium*- specific serum IgG, IgM and IgA, and fecal IgA antibodies following cryptosporidial infection in humans (reviewed in [206]. Many of these have been seroprevalence studies that have reported a wide range of seropositivity depending on age, geographic location, living and environmental conditions. A study of mucosal antibody responses in experimentally infected human volunteers found that anticryptosporidial fecal IgA was present in active infection [224] Studies in human volunteers and serological surveys suggest that individuals with pre-existing antibodies to *Cryptosporidium* may be partially protected and experience less diarrheal symptoms upon subsequent challenge [210,225]. However, it remains to be determined whether antibody responses are themselves protective or whether they are merely markers of a protective cell-mediated response [208].

References

1. Putignani L, Menichella D (2010) Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. Interdiscip Perspect Infect Dis 2010.

2. Aboytes RGD GDF, Abrams A, Rheinecker C, McElroy W, Shaw N, and LeChevallier MW Detection of infectious *Cryptosporidium* in filtered drinking water. J Am Water Works Assoc 2: 88-98.

3. Smith HV, Nichols RA, Grimason AM (2005) *Cryptosporidium* excystation and invasion: getting to the guts of the matter. Trends Parasitol 21: 133-142.

4. Rotterdam H, Tsang P (1994) Gastrointestinal disease in the immunocompromised patient. Hum Pathol 25: 1123-1140.

5. Hellard ME, Sinclair MI, Fairley CK, Andrews RM, Bailey M, et al. (2000) An outbreak of cryptosporidiosis in an urban swimming pool: why are such outbreaks difficult to detect? Aust N Z J Public Health 24: 272-275.

6. Mosier DA, Oberst RD (2000) Cryptosporidiosis. A global challenge. Ann N Y Acad Sci 916: 102-111.

7. UNAIDS (2009) AIDS epidemic update. Geneva: Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO).

8. J. A. Montero JTS, D. A. Holt, and C. Lloyd (2001) "Biliary cryptosporidiosis: current concepts,"Infections in Medicine. MMWR Surveill Summ 18: 312–316.

9. Savioli L, Smith H, Thompson A (2006) Giardia and *Cryptosporidium* join the 'Neglected Diseases Initiative'. Trends Parasitol 22: 203-208.

10. Organization WH (2010) Neglected Tropical Diseases Update. Geneva: World Health Organization.

11. Tzipori S, Angus KW, Campbell I, Gray EW (1980) *Cryptosporidium*: evidence for a single-species genus. Infect Immun 30: 884-886.

12. Current WL, Haynes TB (1984) Complete development of *Cryptosporidium* in cell culture. Science 224: 603-605.

13. Morrison LJ, Mallon ME, Smith HV, MacLeod A, Xiao L, et al. (2008) The population structure of the C*ryptosporidium parvum* population in Scotland: a complex picture. Infect Genet Evol 8: 121-129.

14. Tetley L, Brown SM, McDonald V, Coombs GH (1998) Ultrastructural analysis of the sporozoite of *Cryptosporidium parvum*. Microbiology 144 (Pt 12): 3249-3255.

15. O'Hara SP, Chen XM (2011) The cell biology of *Cryptosporidium* infection. Microbes Infect 13: 721-730.

16. Chen XM, O'Hara SP, Huang BQ, Splinter PL, Nelson JB, et al. (2005) Localized glucose and water influx facilitates *Cryptosporidium parvum* cellular invasion by means of modulation of host-cell membrane protrusion. Proc Natl Acad Sci U S A 102: 6338-6343.

17. Baum J, Richard D, Healer J, Rug M, Krnajski Z, et al. (2006) A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. J Biol Chem 281: 5197-5208.

18. Kappe S, Bruderer T, Gantt S, Fujioka H, Nussenzweig V, et al. (1999) Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. J Cell Biol 147: 937-944.

19. Montagna GN, Matuschewski K, Buscaglia CA (2012) *Plasmodium* sporozoite motility: an update. Front Biosci 17: 726-744.

20. Sattler JM, Ganter M, Hliscs M, Matuschewski K, Schüler H (2011) Actin regulation in the

malaria parasite. Eur J Cell Biol 90: 966-971.

21. Frénal K, Polonais V, Marq JB, Stratmann R, Limenitakis J, et al. (2010) Functional dissection of the apicomplexan glideosome molecular architecture. Cell Host Microbe 8: 343-357.

22. Schmitz S, Schaap IA, Kleinjung J, Harder S, Grainger M, et al. (2010) Malaria parasite actin polymerization and filament structure. J Biol Chem 285: 36577-36585.

23. King CA (1988) Cell motility of sporozoan protozoa. Parasitol Today 4: 315-319.

24. Vanderberg JP (1974) Studies on the motility of *Plasmodium* sporozoites. J Protozool 21: 527-537.

25. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, et al. (2004) Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304: 441-445.

26. Xiao L, Ryan UM (2004) Cryptosporidiosis: an update in molecular epidemiology. Curr Opin Infect Dis 17: 483-490.

27. Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM, et al. (2004) The genome of *Cryptosporidium hominis*. Nature 431: 1107-1112.

28. Carlton J, Lorenzi H, Silva J, Widmer G (2012) *Cryptosporidium Muris* Genome Project. 2012 ed. Washington DC: J. Craig Venter Institute.

29. Dillingham RA, Lima AA, Guerrant RL (2002) Cryptosporidiosis: epidemiology and impact. Microbes Infect 4: 1059-1066.

30. Abrahamsen MS, Lancto CA, Walcheck B, Layton W, Jutila MA (1997) Localization of alpha/beta and gamma/delta T lymphocytes in *Cryptosporidium parvum*-infected tissues in naive and immune calves. Infect Immun 65: 2428-2433.

31. Kappe SH, Buscaglia CA, Bergman LW, Coppens I, Nussenzweig V(2004) Apicomplexan

gliding motility and host cell invasion: overhauling the motor model. Trends Parasitol 20: 13-16.

32. Baker RP, Wijetilaka R, Urban S (2006) Two *Plasmodium* rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. PLoS Pathog 2: e113.

33. Brossier F, Jewett TJ, Sibley LD, Urban S (2005) A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by *Toxoplasma*. Proceedings of the National Academy of Sciences of the United States of America 102: 4146-4151.

34. Tyzzer EE (1907) A sporozoan found in the peptic glands of the common mouse Proceedings of The Society for Experimental Biology and Medicine 5: 12-13.

35. Fayer R (2010) Taxonomy and species delimitation in *Cryptosporidium*. Exp Parasitol 124: 90–97.

36. Kváč M, Kestřánová M, Pinková M, Květoňová D, Kalinová J, et al. (2013) *Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (*Sus scrofa*). Veterinary Parasitology 191: 218–227.

37. Elwin K, Hadfield SJ, Robinson G, Crouch ND, Chalmers RM (2012) *Cryptosporidium* viatorum n. sp. (Apicomplexa: Cryptosporidiidae) among travellers returning to Great Britain from the Indian subcontinent, 2007-2011. Int J Parasitol 42: 675-682.

38. Ryan UM, Monis P, Enemark HL, Sulaiman I, Samarasinghe B, et al. (2004) *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (Sus scrofa). J Parasitol 90: 769-773.

39. Fayer R, Leek RG (1984) The effects of reducing conditions, medium, pH, temperature, and time on in vitro excystation of *Cryptosporidium*. J Protozool 31: 567-569.

40. Rose JB, Slifko TR (1999) Giardia, *Cryptosporidium*, and Cyclospora and their impact on foods: a review. J Food Prot 62: 1059-1070.

41. Kuhls TL, Mosier DA, Crawford DL, Griffis J (1994) Seroprevalence of cryptosporidial antibodies during infancy, childhood, and adolescence. Clin Infect Dis 18: 731-735.

42. Ungar BL, Gilman RH, Lanata CF, Perez-Schael I (1988) Seroepidemiology of *Cryptosporidium* infection in two Latin American populations. J Infect Dis 157: 551-556.

43. Lengerich EJ, Addiss DG, Marx JJ, Ungar BL, Juranek DD (1993) Increased exposure to cryptosporidia among dairy farmers in Wisconsin. J Infect Dis 167: 1252-1255.

44. Boulter-Bitzer JI, Lee H, Trevors JT (2007) Molecular targets for detection and immunotherapy in *Cryptosporidium parvum*. Biotechnol Adv 25: 13-44.

45. (CDC) CfDCaP (1994) *Cryptosporidium* infections associated with swimming pools--Dane County, Wisconsin, 1993. MMWR Morb Mortal Wkly Rep 43: 561-563.

46. Hlavsa MC, Roberts VA, Anderson AR, Hill VR, Kahler AM, et al. (2011) Surveillance for waterborne disease outbreaks and other health events associated with recreational water--United States, 2007–2008. MMWR Surveill Summ 60: 1–32.

47. Yoder JS, Harral C, Beach MJ (2010) Cryptosporidiosis surveillance - United States, 2006–2008. MMWR Surveill Summ 59: 1–14.

48. Chalmers RM, Robinson G, Elwin K, Hadfield SJ, Thomas E, et al. (2010) Detection of *Cryptosporidium* species and sources of contamination with *Cryptosporidium hominis* during a waterborne outbreak in north west Wales. JWater Health 8: 311–325.

49. Mason BW, Chalmers RM, Carnicer-Pont D, Casemore DP (2010) A Cryptosporidium hominis outbreak in north-west Wales associated with low oocyst counts in treated drinking water. J Water Health 8: 299-310.

50. Mayne DJ, Ressler KA, Smith D, Hockey G, Botham SJ, et al. (2011) A community outbreak of cryptosporidiosis in sydney associated with a public swimming facility: a case-control study. Interdiscip Perspect Infect Dis 2011: 341065.

51. Waldron LS, Ferrari BC, Cheung-Kwok-Sang C, Beggs PJ, Stephens N, et al. (2011) Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak in Australia. Appl Environ Microbiol 77: 7766-7771.

52. Goldstein ST, Juranek DD, Ravenholt O, Hightower AW, Martin DG, et al. (1996) Cryptosporidiosis: an outbreak associated with drinking water despite state-of-the-art water treatment. Ann Intern Med 124: 459-468.

53. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, et al. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607-625.

54. Charron D, Thomas M, Waltner-Toews D, Aramini J, Edge T, et al. (2004) Vulnerability of waterborne diseases to climate change in Canada: a review. J Toxicol Environ Health A 67: 1667-1677.

55. Karanis P, Kourenti C, Smith H (2007) Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health 5: 1-38.

56. Mead JR (2002) Cryptosporidiosis and the challenges of chemotherapy. Drug Resist Updat 5: 47-57.

57. Riggs MW (2002) Recent advances in cryptosporidiosis: the immune response. Microbes Infect 4: 1067-1080.

58. Riggs MW, Schaefer DA, Kapil SJ, Barley-Maloney L, Perryman LE (2002) Efficacy of monoclonal antibodies against defined antigens for passive immunotherapy of chronic gastrointestinal cryptosporidiosis. Antimicrob Agents Chemother 46: 275-282.

59. Riggs MW, Stone AL, Yount PA, Langer RC, Arrowood MJ, et al. (1997) Protective monoclonal antibody defines a circumsporozoite-like glycoprotein exoantigen of *Cryptosporidium parvum* sporozoites and merozoites. J Immunol 158: 1787-1795.

60. Jenkins MC, O'Brien C, Trout J, Guidry A, Fayer R (1999) Hyperimmune bovine colostrum specific for recombinant *Cryptosporidium parvum* antigen confers partial protection against cryptosporidiosis in immunosuppressed adult mice. Vaccine 17: 2453-2460.

61. Perryman LE, Kapil SJ, Jones ML, Hunt EL (1999) Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a Cryptosporidium parvum recombinant protein. Vaccine 17: 2142-2149.

62. Manque PA, Tenjo F, Woehlbier U, Lara AM, Serrano MG, et al. (2011) Identification and Immunological Characterization of Three Potential Vaccinogens against *Cryptosporidium* Species. Clin Vaccine Immunol 18: 1796-1802.

63. Current WL, Upton SJ, Haynes TB (1986) The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. J Protozool 33: 289–296.

64. Chen XM, Keithly JS, Paya CV, LaRusso NF (2002) Cryptosporidiosis. N Engl J Med 346: 1723-1731.

65. Thompson RC, Olson ME, Zhu G, Enomoto S, Abrahamsen MS, et al. (2005) *Cryptosporidium* and cryptosporidiosis. Adv Parasitol 59: 77-158.

66. Arrowood MJ (2002) In vitro cultivation of *Cryptosporidium* species. Clin Microbiol Rev 15: 390-400.

67. Hijjawi NS, Meloni BP, Ng'anzo M, Ryan UM, Olson ME, et al. (2004) Complete development of *Cryptosporidium parvum* in host cell-free culture. International Journal for Parasitology 34: 769-777.

68. Lappin MR (2005) Enteric protozoal diseases. Vet Clin North Am Small Anim Pract 35: 81-88, vi.

69. Topouchian A, Huneau JF, Barbot L, Rome S, Gobert JG, et al. (2003) Evidence for the absence of an intestinal adaptive mechanism to compensate for *C. parvum*-induced amino acid malabsorption in suckling rats. Parasitol Res 91: 197-203.

70. Abubakar I, Aliyu SH, Arumugam C, Hunter PR, Usman NK (2007) Prevention and treatment of cryptosporidiosis in immunocompromised patients. Cochrane Database Syst Rev: CD004932.

71. Ayuo PO (2009) Human cryptosporidiosis: a review. East Afr Med J 86: 89-93.

72. Garrido Dávila JI, Ramírez Ronda CH (1991) Updates on AIDS cryptosporidiosis: a review. Bol Asoc Med P R 83: 65-68.

73. Ramirez NE, Ward LA, Sreevatsan S (2004) A review of the biology and epidemiology of cryptosporidiosis in humans and animals. Microbes Infect 6: 773-785.

74. Hunter PR, Nichols G (2002) Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. Clin Microbiol Rev 15: 145-154.

75. Marshall AT, LaMont JT (1997) Cryptosporidiosis and public health. Hosp Pract (Minneap) 32: 11, 15-16, 23.

76. Mølbak K, Højlyng N, Gottschau A, Sá JC, Ingholt L, et al. (1993) Cryptosporidiosis in infancy and childhood mortality in Guinea Bissau, west Africa. BMJ 307: 417-420.

77. Mølbak K, Andersen M, Aaby P, Højlyng N, Jakobsen M, et al. (1997) *Cryptosporidium* infection in infancy as a cause of malnutrition: a community study from Guinea-Bissau, west Africa. Am J Clin Nutr 65: 149-152.

78. Current WL, Garcia LS (1991) Cryptosporidiosis. Clin Microbiol Rev 4: 325-358.

79. Gellin BG, Soave R (1992) Coccidian infections in AIDS. Toxoplasmosis, cryptosporidiosis, and isosporiasis. Med Clin North Am 76: 205-234.

80. Petersen C (1992) Cryptosporidiosis in patients infected with the human immunodeficiency virus. Clin Infect Dis 15: 903-909.

81. Flanigan T, Whalen C, Turner J, Soave R, Toerner J, et al. (1992) *Cryptosporidium* infection and CD4 counts. Ann Intern Med 116: 840-842.

82. Blanshard C, Jackson AM, Shanson DC, Francis N, Gazzard BG (1992) Cryptosporidiosis in HIV-seropositive patients. Q J Med 85: 813-823.

83. Goodgame RW, Genta RM, White AC, Chappell CL (1993) Intensity of infection in AIDS-associated cryptosporidiosis. J Infect Dis 167: 704-709.

84. Janoff EN, Limas C, Gebhard RL, Penley KA (1990) Cryptosporidial carriage without symptoms in the acquired immunodeficiency syndrome (AIDS). Ann Intern Med 112: 75-76.

85. McGowan I, Hawkins AS, Weller IV (1993) The natural history of cryptosporidial diarrhoea in HIV-infected patients. AIDS 7: 349-354.

86. Manabe YC, Clark DP, Moore RD, Lumadue JA, Dahlman HR, et al. (1998) Cryptosporidiosis in patients with AIDS: correlates of disease and survival. Clin Infect Dis 27: 536-542.

87. Dunand VA, Hammer SM, Rossi R, Poulin M, Albrecht MA, et al. (1997) Parasitic sinusitis and otitis in patients infected with human immunodeficiency virus: report of five cases and review. Clin Infect Dis 25: 267-272.

88. French AL, Beaudet LM, Benator DA, Levy CS, Kass M, et al. (1995) Cholecystectomy in patients with AIDS: clinicopathologic correlations in 107 cases. Clin Infect Dis 21: 852-

858.

89. Lumadue JA, Manabe YC, Moore RD, Belitsos PC, Sears CL, et al. (1998) A clinicopathologic analysis of AIDS-related cryptosporidiosis. AIDS 12: 2459-2466.

90. Rossi P, Rivasi F, Codeluppi M, Catania A, Tamburrini A, et al. (1998) Gastric involvement in AIDS associated cryptosporidiosis. Gut 43: 476-477.

91. Ventura G, Cauda R, Larocca LM, Riccioni ME, Tumbarello M, et al. (1997) Gastric cryptosporidiosis complicating HIV infection: case report and review of the literature. Eur J Gastroenterol Hepatol 9: 307-310.

92. Clark DP (1999) New insights into human cryptosporidiosis. Clin Microbiol Rev 12: 554-563.

93. Farthing MJ (2000) Clinical aspects of human cryptosporidiosis. Contrib Microbiol 6: 50-74.

94. McGuigan CC, Steven K, Pollock KG (2010) Cryptosporidiosis associated with wildlife center, Scotland. Emerg Infect Dis 16: 895-896.

95. McGuigan C (2005) *Cryptosporidium* outbreak after a visit to a wildlife centre in northeast Scotland: 62 confirmed cases. Euro Surveill 10: E050428.050422.

96. O'Donoghue PJ (1995) *Cryptosporidium* and cryptosporidiosis in man and animals. Int J Parasitol 25: 139-195.

97. Xiao L, Herd RP (1994) Infection pattern of *Cryptosporidium* and *Giardia* in calves. Vet Parasitol 55: 257-262.

98. Sischo WM, Atwill ER, Lanyon LE, George J (2000) Cryptosporidia on dairy farms and the role these farms may have in contaminating surface water supplies in the northeastern United States. Prev Vet Med 43: 253-267.

99. Quílez J, Sánchez-Acedo C, Clavel A, del Cacho E, López-Bernad F (1996) Prevalence of

Cryptosporidium infections in pigs in Aragón (northeastern Spain). Vet Parasitol 67: 83-88.

100. Xiao L, Herd RP (1994) Review of equine *Cryptosporidium* infection. Equine Vet J 26: 9-13.

101. Webster KA, Smith HV, Giles M, Dawson L, Robertson LJ (1996) Detection of *Cryptosporidium parvum* oocysts in faeces: comparison of conventional coproscopical methods and the polymerase chain reaction. Veterinary Parasitology 61: 5-13.

102. Dillingham RA, Lima AA, Guerrant RL (2002) Cryptosporidiosis: epidemiology and impact. Microbes and Infection 4: 1059-1066.

103. Ungar BL, Ward DJ, Fayer R, Quinn CA (1990) Cessation of *Cryptosporidium*-associated diarrhea in an acquired immunodeficiency syndrome patient after treatment with hyperimmune bovine colostrum. Gastroenterology 98: 486-489.

104. Garrido Davila JI, Ramirez Ronda CH (1991) Updates on AIDS cryptosporidiosis: a review. Boletin - Asociacion Medica de Puerto Rico 83: 65-68.

105. Hunter PR (2000) Advice on the response from public and environmental health to the detection of cryptosporidial oocysts in treated drinking water. PHLS Advisory Committee on Water and the Environment. Commun Dis Public Health 3: 24-27.

106. Putignani L, Menichella D (2010) Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. Interdiscip Perspect Infect Dis 2010.

107. Widmer G, Lee Y, Hunt P, Martinelli A, Tolkoff M, et al. (2012) Comparative genome analysis of two *Cryptosporidium parvum* isolates with different host range. Infect Genet Evol 12: 1213-1221.

108. Heiges M, Wang H, Robinson E, Aurrecoechea C, Gao X, et al. (2006) CryptoDB: a

Cryptosporidium bioinformatics resource update. Nucleic Acids Research 34: D419-D422.

109. Xiao L (2010) Molecular epidemiology of cryptosporidiosis: an update. Experimental Parasitology 124: 80–89.

110. Widmer G, Sullivan S (2012) Genomics and population biology of *Cryptosporidium* species. Parasite Immunology 34: 61-71.

111. Kissinger JC, Gajria B, Li L, Paulsen IT, Roos DS (2003) ToxoDB: accessing the *Toxoplasma gondii* genome. Nucleic Acids Res 31: 234-236.

112. Aurrecoechea C, Heiges M, Wang H, Wang Z, Fischer S, et al. (2007) ApiDB: integrated resources for the apicomplexan bioinformatics resource center. Nucleic Acids Research 35: D427-D430.

113. Rider SD, Zhu G (2010) *Cryptosporidium*: genomic and biochemical features. Exp Parasitol 124: 2-9.

114. Bouzid M, Tyler KM, Christen R, Chalmers RM, Elwin K, et al. (2010) Multi-locus analysis of human infective *Cryptosporidium* species and subtypes using ten novel genetic loci. BMC Microbiol 10: 213.

115. O'Hara SP, Chen XM (2011) The cell biology of *Cryptosporidium* infection. MicrobesInfect 13: 721-730.

116. Snelling WJ, Lin Q, Moore JE, Millar BC, Tosini F, et al. (2007) Proteomics analysis and protein expression during sporozoite excystation of *Cryptosporidium parvum* (Coccidia, Apicomplexa). Molecular and Cellular Proteomics 6: 346-355.

117. Sanderson SJ, Xia D, Prieto H, Yates J, Heiges M, et al. (2008) Determining the protein repertoire of *Cryptosporidium parvum* sporozoites. Proteomics 8: 1398-1414.

118. Mauzy MJ, Enomoto S, Lancto CA, Abrahamsen MS, Rutherford MS (2012) The

Cryptosporidium parvum transcriptome during in vitro development. PLoS One 7: e31715.

119. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium* parvum genes containing thrombospondin type 1 domains. Infect Immun 70: 6987-6995.

120. Bhat N, Joe A, PereiraPerrin M, Ward HD (2007) *Cryptosporidium* p30, a galactose/N-acetylgalactosamine-specific lectin, mediates infection in vitro. Journal of Biological Chemistry 282: 34877-34887.

121. Tosini F, Drumo R, Elwin K, Chalmers RM, Pozio E, et al. (2010) The CpA135 gene as a marker to identify *Cryptosporidium* species infecting humans. Parasitol Int 59: 606-609.

122. Tosini F, Agnoli A, Mele R, Gomez Morales MA, Pozio E (2004) A new modular protein of *Cryptosporidium parvum*, with ricin B and LCCL domains, expressed in the sporozoite invasive stage. Molecular and Biochemical Parasitology 134: 137-147.

123. O'Connor RM, Burns PB, Ha-Ngoc T, Scarpato K, Khan W, et al. (2009) Polymorphic mucin antigens CpMuc4 and CpMuc5 are integral to *Cryptosporidium parvum* infection in vitro. Eukaryot Cell 8: 461-469.

124. Sibley LD, Hakansson S, Carruthers VB (1998) Gliding motility: an efficient mechanism for cell penetration. Current Biology 8: R12-R14.

125. Soldati D, Dubremetz JF, Lebrun M (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. International Journal for Parasitology 31: 1293-1302.

126. Besteiro S, Dubremetz JF, Lebrun M (2011) The moving junction of apicomplexan parasites: a key structure for invasion. Cellular Microbiology 13: 797-805.

127. Tyler JS, Treeck M, Boothroyd JC (2011) Focus on the ringleader: the role of AMA1 in apicomplexan invasion and replication. Trends Parasitol 27: 410-420.

128. Tonkin ML, Roques M, Lamarque MH, Pugniere M, Douguet D, et al. (2011) Host cell invasion by apicomplexan parasites: insights from the co-structure of AMA1 with a RON2 peptide. Science 333: 463-467.

129. O'Hara SP, Small AJ, Chen XM, Larusso NF (2008) Host cell actin remodeling in response to *Cryptosporidium*. Sub-Cellular Biochemistry 47: 92-100.

130. Chen XM, Larusso NF (2000) Mechanisms of attachment and internalization of *Cryptosporidium parvum* to biliary and intestinal epithelial cells. Gastroenterology 118: 368-379.

131. Wetzel DM, Schmidt J, Kuhlenschmidt MS, Dubey JP, Sibley LD (2005) Gliding motility leads to active cellular invasion by *Cryptosporidium parvum* sporozoites. Infection and Immunity 73: 5379-5387.

132. Wetzel DM, Hakansson S, Hu K, Roos D, Sibley LD (2003) Actin filament polymerization regulates gliding motility by apicomplexan parasites. Molecular Biology of the Cell 14: 396-406.

133. Robson KJ, Hall JR, Jennings MW, Harris TJ, Marsh K, et al. (1988) A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. Nature 335: 79-82.

134. Clarke LE, Tomley FM, Wisher MH, Foulds IJ, Boursnell ME (1990) Regions of an Eimeria tenella antigen contain sequences which are conserved in circumsporozoite proteins from Plasmodium spp. and which are related to the thrombospondin gene family. Mol Biochem Parasitol 41: 269-279.

135. Kawase O, Nishikawa Y, Bannai H, Igarashi M, Matsuo T, et al. (2010) Characterization of a novel thrombospondin-related protein in *Toxoplasma gondii*. Parasitol Int 59: 211-216.

136. Tomley FM, Soldati DS (2001) Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. Trends Parasitol 17: 81-88.

137. Putignani L, Possenti A, Cherchi S, Pozio E, Crisanti A, et al. (2008) The thrombospondin-related protein CpMIC1 (CpTSP8) belongs to the repertoire of micronemal proteins of *Cryptosporidium parvum*. Mol Biochem Parasitol 157: 98-101.

138. Soldati D, Dubremetz JF, Lebrun M (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite Toxoplasma gondii. Int J Parasitol 31: 1293-1302.

139. Opitz C, Soldati D (2002) 'The glideosome': a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*. Mol Microbiol 45: 597-604.

140. Spano F, Putignani L, Naitza S, Puri C, Wright S, et al. (1998) Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. Mol Biochem Parasitol 92: 147-162.

141. Deng M, Lancto CA, Abrahamsen MS (2004) *Cryptosporidium parvum* regulation of human epithelial cell gene expression. Int J Parasitol 34: 73-82.

142. Templeton TJ (2007) Whole-genome natural histories of apicomplexan surface proteins. Trends Parasitol 23: 205-212.

143. Arrowood MJ, Sterling CR, Healey MC (1991) Immunofluorescent microscopical visualization of trails left by gliding *Cryptosporidium parvum* sporozoites. Journal of Parasitology 77: 315-317.

144. Perryman LE, Bjorneby JM (1991) Immunotherapy of cryptosporidiosis in immunodeficient

animal models. J Protozool 38: 98S-100S.

145. Petersen C, Gut J, Doyle PS, Crabb JH, Nelson RG, et al. (1992) Characterization of a > 900,000-M(r) *Cryptosporidium parvum* sporozoite glycoprotein recognized by protective hyperimmune bovine colostral immunoglobulin. Infect Immun 60: 5132-5138.

146. Barnes DA, Bonnin A, Huang JX, Gousset L, Wu J, et al. (1998) A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. Molecular and Biochemical Parasitology 96: 93-110.

147. Chatterjee A, Banerjee S, Steffen M, O'Connor RM, Ward HD, et al. (2010) Evidence for mucin-like glycoproteins that tether sporozoites of *Cryptosporidium parvum* to the inner surface of the oocyst wall. EukaryotCell 9: 84-96.

148. Van Klinken BJ, Dekker J, Buller HA, Einerhand AW (1995) Mucin gene structure and expression: protection vs. adhesion. Am J Physiol 269: G613-627.

149. Cevallos AM, Bhat N, Verdon R, Hamer DH, Stein B, et al. (2000) Mediation of *Cryptosporidium parvum* infection in vitro by mucin-like glycoproteins defined by a neutralizing monoclonal antibody. Infection and Immunity 68: 5167–5175.

150. Wanyiri JW, Techasintana P, O'Connor RM, Blackman MJ, Kim K, et al. (2009) Role of CpSUB1, a subtilisin-like protease, in *Cryptosporidium parvum* infection in vitro. Eukaryot Cell 8: 470-477.

151. Nesterenko MV, Woods K, Upton SJ (1999) Receptor/ligand interactions between *Cryptosporidium parvum* and the surface of the host cell. Biochim Biophys Acta 1454: 165-173.

152. Urban S, Lee JR, Freeman M (2001) Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107: 173-182.

153. Urban S, Lee JR, Freeman M (2002) A family of Rhomboid intramembrane proteases activates all Drosophila membrane-tethered EGF ligands. EMBO J 21: 4277-4286.

154. Koonin EV, Makarova KS, Rogozin IB, Davidovic L, Letellier MC, et al. (2003) The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers. Genome Biol 4: R19.

155. Li H, Child MA, Bogyo M (2012) Proteases as regulators of pathogenesis: examples from the Apicomplexa. Biochim Biophys Acta 1824: 177-185.

156. Urban S, Freeman M (2002) Intramembrane proteolysis controls diverse signalling pathways throughout evolution. Curr Opin Genet Dev 12: 512-518.

157. Urban S, Schlieper D, Freeman M (2002) Conservation of intramembrane proteolytic activity and substrate specificity in prokaryotic and eukaryotic rhomboids. Curr Biol 12: 1507-1512.

158. Freeman M (2008) Rhomboid Proteases and their Biological Functions. Annual Review of Genetics 42: 191-210.

159. Urban S, Freeman M (2003) Substrate specificity of rhomboid intramembrane proteases is overned by helix-breaking residues in the substrate transmembrane domain. Mol Cell 11: 1425-1434.

160. Urban S, Dickey SW (2011) The rhomboid protease family: a decade of progress on function and mechanism. Genome Biol 12: 231.

161. Dowse T, Soldati D (2004) Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis. Curr Opin Microbiol 7: 388-396.

162. Dowse TJ, Soldati D (2005) Rhomboid-like proteins in Apicomplexa: phylogeny and nomenclature. Trends Parasitol 21: 254-258.

163. Dowse TJ, Koussis K, Blackman MJ, Soldati-Favre D (2008) Roles of proteases during invasion and egress by *Plasmodium* and *Toxoplasma*. Subcell Biochem 47: 121-139.

164. Dowse TJ, Soldati D (2005) Rhomboid-like proteins in apicomplexa: phylogeny and nomenclature. Trends in Parasitology 21: 254-258.

165. Yang G, Li J, Zhang X, Zhao Q, Liu Q, et al. (2008) Eimeria tenella: construction of a recombinant fowlpox virus expressing rhomboid gene and its protective efficacy against homologous infection. Exp Parasitol 119: 30-36.

166. Santos JM, Graindorge A, Soldati-Favre D (2012) New insights into parasite rhomboid proteases. Molecular and Biochemical Parasitology 182: 27-36.

167. M Santos J, Graindorge A, Soldati-Favre D (2011) New insights into parasite rhomboid proteases. Mol Biochem Parasitol.

168. Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. Cell 124: 755-766.

169. Brossier F, Jewett TJ, Sibley LD, Urban S (2005) A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by *Toxoplasma*. Proc Natl Acad Sci U S A 102: 4146-4151.

170. O'Donnell RA, Hackett F, Howell SA, Treeck M, Struck N, et al. (2006) Intramembrane proteolysis mediates shedding of a key adhesin during erythrocyte invasion by the malaria parasite. J Cell Biol 174: 1023-1033.

171. Singh S, Plassmeyer M, Gaur D, Miller LH (2007) Mononeme: a new secretory organelle in *Plasmodium falciparum* merozoites identified by localization of rhomboid-1 protease. Proc Natl Acad Sci U S A 104: 20043-20048.

172. Brossier F, Starnes GL, Beatty WL, Sibley LD (2008) Microneme rhomboid protease

TgROM1 is required for efficient intracellular growth of *Toxoplasma gondii*. Eukaryot Cell 7: 664-674.

173. Srinivasan P, Coppens I, Jacobs-Lorena M (2009) Distinct roles of *Plasmodium* rhomboid 1 in parasite development and malaria pathogenesis. PLoS Pathog 5: e1000262.

174. Vera IM, Beatty WL, Sinnis P, Kim K (2011) *Plasmodium* protease ROM1 is important for proper formation of the parasitophorous vacuole. PLoS Pathog 7: e1002197.

175. Dowse TJ, Pascall JC, Brown KD, Soldati D (2005) Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion. Int J Parasitol 35: 747-756.

176. Lemberg MK, Freeman M (2007) Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. Genome Res 17: 1634-1646.

177. McQuibban GA, Saurya S, Freeman M (2003) Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature 423: 537-541.

178. Sheiner L, Santos JM, Klages N, Parussini F, Jemmely N, et al. (2010) *Toxoplasma gondii* transmembrane microneme proteins and their modular design. Molecular Microbiology 77: 912-929.

179. Carruthers VB, Cotter PA, Kumamoto CA (2007) Microbial pathogenesis: mechanisms of infectious disease. Cell Host Microbe 2: 214-219.

180. Brossier F, Jewett TJ, Lovett JL, Sibley LD (2003) C-terminal processing of the toxoplasma protein MIC2 is essential for invasion into host cells. J Biol Chem 278: 6229-6234.

181. Howell SA, Hackett F, Jongco AM, Withers-Martinez C, Kim K, et al. (2005) Distinct mechanisms govern proteolytic shedding of a key invasion protein in apicomplexan pathogens. Mol Microbiol 57: 1342-1356.

182. Opitz C, Di Cristina M, Reiss M, Ruppert T, Crisanti A, et al. (2002) Intramembrane cleavage of microneme proteins at the surface of the apicomplexan parasite Toxoplasma gondii. EMBO J 21: 1577-1585.

183. Zhou XW, Blackman MJ, Howell SA, Carruthers VB (2004) Proteomic analysis of cleavage events reveals a dynamic two-step mechanism for proteolysis of a key parasite adhesive complex. Mol Cell Proteomics 3: 565-576.

184. Triglia T, Tham WH, Hodder A, Cowman AF (2009) Reticulocyte binding protein homologues are key adhesins during erythrocyte invasion by *Plasmodium falciparum*. Cell Microbiol 11: 1671-1687.

185. Buguliskis JS, Brossier F, Shuman J, Sibley LD (2010) Rhomboid 4 (ROM4) affects the processing of surface adhesins and facilitates host cell invasion by *Toxoplasma gondii*. PLoS Pathog 6: e1000858.

186. Santos JM, Ferguson DJ, Blackman MJ, Soldati-Favre D (2011) Intramembrane cleavage of AMA1 triggers *Toxoplasma* to switch from an invasive to a replicative mode. Science 331: 473-477.

187. Mital J, Meissner M, Soldati D, Ward GE (2005) Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. Mol Biol Cell 16: 4341-4349.

188. Chen XM, LaRusso NF (2002) Cryptosporidiosis and the pathogenesis of AIDS-cholangiopathy. Semin Liver Dis 22: 277-289.

189. Leav BA, Mackay M, Ward HD (2003) *Cryptosporidium* species: new insights and old challenges. Clin Infect Dis 36: 903-908.

190. Huang DB, White AC (2006) An updated review on *Cryptosporidium* and Giardia.

Gastroenterol Clin North Am 35: 291-314.

191. Borad A, Ward H (2010) Human immune responses in cryptosporidiosis. Future Microbiol 5: 507-519.

192. Pantenburg B, Dann SM, Wang HC, Robinson P, Castellanos-Gonzalez A, et al. (2008) Intestinal immune response to human *Cryptosporidium* sp. infection. Infect Immun 76: 23-29.

193. Chen XM, O'Hara SP, Nelson JB, Splinter PL, Small AJ, et al. (2005) Multiple TLRs are expressed in human cholangiocytes and mediate host epithelial defense responses to *Cryptosporidium parvum* via activation of NF-kappaB. J Immunol 175: 7447-7456.

194. Chen XM, Splinter PL, O'Hara SP, LaRusso NF (2007) A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against *Cryptosporidium parvum* infection. J Biol Chem 282: 28929-28938.

195. Priest JW, Mehlert A, Arrowood MJ, Riggs MW, Ferguson MA (2003) Characterization of a low molecular weight glycolipid antigen from *Cryptosporidium parvum*. J Biol Chem 278: 52212-52222.

196. Priest JW, Mehlert A, Moss DM, Arrowood MJ, Ferguson MA (2006) Characterization of the glycosylphosphatidylinositol anchor of the immunodominant *Cryptosporidium parvum* 17-kDa antigen. Mol Biochem Parasitol 149: 108-112.

197. Krishnegowda G, Hajjar AM, Zhu J, Douglass EJ, Uematsu S, et al. (2005) Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. J Biol Chem 280: 8606-8616.

198. Wehkamp J, Schauber J, Stange EF (2007) Defensins and cathelicidins in gastrointestinal

infections. Curr Opin Gastroenterol 23: 32-38.

199. Zaalouk TK, Bajaj-Elliott M, George JT, McDonald V (2004) Differential regulation of beta-defensin gene expression during *Cryptosporidium parvum* infection. Infect Immun 72: 2772-2779.

200. Maillot C, Gargala G, Delaunay A, Ducrotte P, Brasseur P, et al. (2000) *Cryptosporidium parvum* infection stimulates the secretion of TGF-beta, IL-8 and RANTES by Caco-2 cell line. Parasitol Res 86: 947-949.

201. Laurent F, Eckmann L, Savidge TC, Morgan G, Theodos C, et al. (1997) *Cryptosporidium parvum* infection of human intestinal epithelial cells induces the polarized secretion of C-X-C chemokines. Infect Immun 65: 5067-5073.

202. Kirkpatrick BD, Noel F, Rouzier PD, Powell JL, Pape JW, et al. (2006) Childhood cryptosporidiosis is associated with a persistent systemic inflammatory response. Clin Infect Dis 43: 604-608.

203. Alcantara CS, Yang CH, Steiner TS, Barrett LJ, Lima AA, et al. (2003) Interleukin-8, tumor necrosis factor-alpha, and lactoferrin in immunocompetent hosts with experimental and Brazilian children with acquired cryptosporidiosis. Am J Trop Med Hyg 68: 325-328.

204. Wang HC, Dann SM, Okhuysen PC, Lewis DE, Chappell CL, et al. (2007) High levels of CXCL10 are produced by intestinal epithelial cells in AIDS patients with active cryptosporidiosis but not after reconstitution of immunity. Infect Immun 75: 481-487.

205. Kelly P, Jack DL, Naeem A, Mandanda B, Pollok RC, et al. (2000) Mannose-binding lectin is a component of innate mucosal defense against *Cryptosporidium parvum* in AIDS. Gastroenterology 119: 1236-1242.

206. Takahashi K, Ip WE, Michelow IC, Ezekowitz RA (2006) The mannose-binding lectin: a

prototypic pattern recognition molecule. Curr Opin Immunol 18: 16-23.

207. Kirkpatrick BD, Huston CD, Wagner D, Noel F, Rouzier P, et al. (2006) Serum mannose-binding lectin deficiency is associated with cryptosporidiosis in young Haitian children. Clin Infect Dis 43: 289-294.

208. Carmolli M, Duggal P, Haque R, Lindow J, Mondal D, et al. (2009) Deficient serum mannose-binding lectin levels and MBL2 polymorphisms increase the risk of single and recurrent *Cryptosporidium* infections in young children. J Infect Dis 200: 1540-1547.

209. White AC, Robinson P, Okhuysen PC, Lewis DE, Shahab I, et al. (2000) Interferon-gamma expression in jejunal biopsies in experimental human cryptosporidiosis correlates with prior sensitization and control of oocyst excretion. J Infect Dis 181: 701-709.

210. Robinson P, Okhuysen PC, Chappell CL, Lewis DE, Shahab I, et al. (2001) Expression of IL-15 and IL-4 in IFN-gamma-independent control of experimental human *Cryptosporidium parvum* infection. Cytokine 15: 39-46.

211. Dann SM, Wang HC, Gambarin KJ, Actor JK, Robinson P, et al. (2005) Interleukin-15 activates human natural killer cells to clear the intestinal protozoan *Cryptosporidium*. J Infect Dis 192: 1294-1302.

212. Robinson P, Okhuysen PC, Chappell CL, Lewis DE, Shahab I, et al. (2001) Expression of tumor necrosis factor alpha and interleukin 1 beta in jejuna of volunteers after experimental challenge with *Cryptosporidium parvum* correlates with exposure but not with symptoms. Infect Immun 69: 1172-1174.

213. Laurent F, Kagnoff MF, Savidge TC, Naciri M, Eckmann L (1998) Human intestinal epithelial cells respond to *Cryptosporidium parvum* infection with increased prostaglandin H synthase 2 expression and prostaglandin E2 and F2 alpha production.

Infect Immun 66: 1787-1790.

214. Mazumdar S, Das KM (1992) Immunocytochemical localization of vasoactive intestinal peptide and substance P in the colon from normal subjects and patients with inflammatory bowel disease. Am J Gastroenterol 87: 176-181.

215. Robinson P, Okhuysen PC, Chappell CL, Weinstock JV, Lewis DE, et al. (2003) Substance P expression correlates with severity of diarrhea in cryptosporidiosis. J Infect Dis 188: 290-296.

216. McDonald V (2000) Host cell-mediated responses to infection with *Cryptosporidium*. Parasite Immunol 22: 597-604.

217. Schmidt W, Wahnschaffe U, Schäfer M, Zippel T, Arvand M, et al. (2001) Rapid increase of mucosal CD4 T cells followed by clearance of intestinal cryptosporidiosis in an AIDS patient receiving highly active antiretroviral therapy. Gastroenterology 120: 984-987.

218. Gomez Morales MA, Ausiello CM, Urbani F, Pozio E (1995) Crude extract and recombinant protein of *Cryptosporidium parvum* oocysts induce proliferation of human peripheral blood mononuclear cells in vitro. J Infect Dis 172: 211-216.

219. Gomez Morales MA, Mele R, Ludovisi A, Bruschi F, Tosini F, et al. (2004) *Cryptosporidium parvum*-specific CD4 Th1 cells from sensitized donors responding to both fractionated and recombinant antigenic proteins. Infect Immun 72: 1306-1310.

220. Bhat N, Joe A, PereiraPerrin M, Ward HD (2007) *Cryptosporidium* p30, a galactose/N-acetylgalactosamine-specific lectin, mediates infection in vitro. J Biol Chem 282: 34877-34887.

221. Geriletu, Xu R, Jia H, Terkawi MA, Xuan X, et al. (2011) Immunogenicity of orally administrated recombinant Lactobacillus casei Zhang expressing *Cryptosporidium*

parvum surface adhesion protein P23 in mice. Curr Microbiol 62: 1573-1580.

222. Kirkpatrick BD, Huston CD, Wagner D, Noel F, Rouzier P, et al. (2006) Serum mannose-binding lectin deficiency is associated with cryptosporidiosis in young Haitian children. Clin Infect Dis 43: 289-294.

223. Kirkpatrick BD, Daniels MM, Jean SS, Pape JW, Karp C, et al. (2002) Cryptosporidiosis stimulates an inflammatory intestinal response in malnourished Haitian children. J Infect Dis 186: 94-101.

224. Dann SM, Okhuysen PC, Salameh BM, DuPont HL, Chappell CL (2000) Fecal antibodies to *Cryptosporidium parvum* in healthy volunteers. Infection and Immunity 68: 5068-5074.

225. White AC, Robinson P, Okhuysen PC, Lewis DE, Shahab I, et al. (2000) Interferon-gamma expression in jejunal biopsies in experimental human cryptosporidiosis correlates with prior sensitization and control of oocyst excretion. J Infect Dis 181: 701-709.

PAPER 1. DIFFERING EXPRESSION PATTERNS AND SUBSTRATE SPECIFICITIES SUGGEST THAT TWO *CRYPTOSPORIDIUM PARVUM* RHOMBOID PROTEASES HAVE DISTINCT ROLES DURING MOTILITY AND INVASION

[1] The material in this chapter was co-authored by Ebot S. Tabe, Sinisa Urban and John M. McEvoy. Ebot had primary responsibility for designing and perforiming experiements as well as collecting data, analysis, interpretation, and manuscript preparation. Ebot also drafted and revised all versions of this chapter. Sinisa Urban performed the experiements on enzyme cleavage. Sinisa Urban and John M. McEvoy served as proofreaders and checked the the result analysis conducted by Ebot S. Tabe.

Abstract

Obligate parasites in the phylum Apicomplexa – particularly *Plasmodium, Toxoplasma,* and *Cryptosporidium* – contribute significantly to the global burden of human and animal disease. Apicomplexan zoites, the invasive stage, have conserved structural features, including anteriorly located micronemes and rhoptries that form part of the eponymous apical complex. Recent studies have identified rhomboid protease proteolysis of transmembrane micronemal proteins to be associated with cell and tissue invasion by apicomplexan parasites. Since proteolysis is irreversible and is necessary to disengage bound thrombospondin proteins to host cell receptors, it has been hypothesized that these adhesive interactions must ultimately be disengaged for continuous forward movement to occur during parasite motility and host cell invasion. Here, we show that two serine rhomboid proteases, CpROM4 and CpROM5 and two putative thrombospondin substrates, TRAP-C1 and CpTSP10, were distinctly expressed during motility (in sporozoites), invasion, and infection of host epithelial cells. We used enzyme cleavage assay, quantitative reverse transcriptase PCR (qRT-PCR), and indirect immunofluorescence laser confocal microscopy (LCM) to examine the temporal expression and spatial coloclization of rhomboid proteases and their putative thrombospondin substrates at various known stages of *C. parvum* life cycle. Our findings suggest that *C. parvum* rhomboid proteases could have distinct yet coordinated roles in the processing of thrombospondin adhesins during parasite development. Furthermore, the findings from this study mark the beginning of our understanding of the mechanisms essential for motility and invasion in *C. parvum*.

Introduction

The apicomplexan parasite *Cryptosporidium* is a leading cause of childhood diarrheal disease and associated mortality in developing countries [1], and it continues to be a major cause of waterborne disease outbreaks worldwide [2-8]. Drug treatments are limited, which is, in part, due to the protected, intracellular but extracytoplasmic vacuole occupied by *Cryptosporidium* following host cell invasion [9,10]. Therefore, an understanding of the mechanisms that underpin invasion and the formation of the unique epicellular niche can inform the development of much needed chemotherapeutics.

The genetic recalcitrance of *Cryptosporidium* has greatly hindered mechanistic studies; consequently, our understanding of invasion is largely predicated on studies of the genetically tractable apicomplexan *Toxoplasma*. Gliding motility and invasion are mechanistically similar processes in *Toxoplasma*. To achieve motility, invasive zoites secrete transmembrane adhesins from protein storage vesicles – primarily micronemes – to their anterior surface; the adhesins engage host cell receptors and undergo actomyosin-powered retrograde translocation along the zoite surface [11,12]. During invasion, motile zoites reorient, form a junction with the host cell membrane at their anterior surface [13], and undergo actomyosin-powered retrograde translocation of this junction to cross the membrane and enter an intracellular vacuole [14]

Among the microneme adhesins that function in apicomplexan motility and invasion, thrombospondin-family proteins play an important role in generating the necessary force for forward momentum [15-21]. These proteins have a general architecture characterized by thrombospondin type 1 repeats and other extracellular adhesive domains, a transmembrane region, and a cytoplasmic tail [22,23]. The cytoplasmic tail links with filamentous actin via an aldolase cross linker [24,25]. Filamentous actin, in turn, links to the parasite inner membrane

complex via myosin A [26,27] and moves in an anterior direction, displacing the actin bound thrombospondin protein in a posterior direction (retrograde translocation).

TRAP-C1, the first thrombospondin-family protein identified in *Cryptosporidium*, has multiple extracellular adhesive domains, is localized to the apical region of sporozoites, and has a cytoplasmic sequence that is predicted to interact with the actomyosin motor [28]. A further 11 thrombospondin-family genes (TSP2–TSP12) have been identified [29]; one of these, TSP8, encodes a protein that localizes to micronemes in sporozoites and merozoites, and is secreted to the surface of sporozoites during motility [30].

Cryptosporidium sporozoites express a number of other surface adhesins including P23 [31], CP2 [32], GP40/15 [33,34], GP900 [35,36], and P30 [37]. The mucin-like transmembrane glycoprotein GP900 localizes to micronemes, forms a complex with GP40/15 and P30 on the surface of sporozoites, and plays a critical role during attachment to and invasion of host cells [38].

The shedding of adhesins from the zoite surface is necessary for productive motility and successful host cell invasion, and rhomboid-family intramembrane serine proteases (ROMs) have been identified as likely sheddases in *Toxoplasma* and *Plasmodium* [12,39,40]. ROMs, which are found in all kingdoms of life [41,42], target helix-destabilizing residues [43-46] within the hydrophobic transmembrane domain of proteins [43]. *Toxoplasma gondii* encodes six ROMs (TgROM1-6), four of which have homologs in *Plasmodium falciparum* (PfROM1, 3, 4, and 6). In addition, *P. falciparum* has four ROMs (PfROM7-10) that are not found in *T. gondii*. The naming of other apicomplexan ROMs is based on their relatedness to *T. gondii* and *P. falciparum* ROMs in protein sequence phylogenies [47]. Related ROMs have similar subcellular localizations and may have similar functions. ROM1 localizes to micronemes or novel

microneme-like secretory bodies called mononemes in *T. gondii* and *Plasmodium*, respectively [48-50]. ROM2 localizes to the Golgi apparatus [51] and ROM6 localizes to the mitochondrion [52]. Based on their specificities for microneme adhesins and their localization to the plasma membrane of invasive zoites, the most likely candidates for the sheddase function are TgROM4 and TgROM5 in *T. gondii*, and PfROM4 in *P. falciparum* [12,53-55]. ROM4 and ROM5 group together in phylogenies, and the ROM4/5 cluster, which is conserved in apicomplexans, includes two *C. parvum* ROMs [52]. Trasarti et al. [56] reported that one of the CpROMs is expressed at posterior locations in sporozoites

We undertook a bioinformatic analysis of ROMs from three *Cryptosporidium* species, determined the substrate specificities of two CpROM4/5 proteases, and examined the temporospatial expression dynamics of CpROM4/5s and microneme adhesins in extracellular and intracellular life cycle stages. We showed that the two CpROM4/5s, which we name CpROM4 and CpROM5, have different substrate specificities and collectively cleave five thrombospondin-family proteins and mucin-like glycoprotein. Our findings suggest that CpROMs have distinct roles during excystation, motility, invasion, and intracellular development.

Materials and Methods

Bioinformatics. ROM protein sequences from T. gondii, P. falciparum, and other apicomplexans were used in a BLAST analysis of Cryptosporidium sequences deposited in CryptoDB (www.cryptodb.org) and GenBank. CryptoDB contains the complete genomes of C. parvum, C. hominis, and C. muris. Cryptosporidium parvum and C. hominis are genetically similar species that infect intestinal epithelial cells in humans (C. parvum and C. hominis) and neonatal cattle (C. parvum) [57]. These species are the primary cause of human cryptosporidiosis

worldwide [8,58,59]. Cryptosporidium muris infects the gastric epithelium of rodents, is not generally associated with human disease, and is relatively distantly related to C. parvum and C. hominis [60,61]. Protein sequences from Cryptosporidium rhomboid-like genes were examined for conserved rhomboid features including 7 transmembrane (TM) domains and a histidine-serine catalytic dyad. Sequences were aligned with ROM sequences from other apicomplexans using the ClustalX algorithm, and evolutionary relationships were inferred from a neighbor-joining tree constructed using the Poisson model. Bootstrap support for nodes was estimated by analyzing 1000 pseudoreplicates. Alignments and phylogenetic analyses were conducted using MEGA5 [62].

Oocyst source. Mouse passaged oocysts of the C. parvum Iowa isolate were purchased from Waterborne Inc. (New Orleans, LA). Oocysts were stored at 4°C in PBS *supplemented with* 100 IU/mL penicillin and 100 μg/mL streptomycin) until use. Oocysts were between 1 and 6 months old at the time of use.

Rhomboid-substrate cleavage assay. The cleavage activity of two C. parvum rhomboids from the ROM4/5 group (Cgd6_760 and Cgd7_3020; www.cryptodb.org), were examined using a heterologous cell-based cleavage assay as previously described [40]. For clarity, we have named the ROMs CpROM4 (Cgd6_760) and CpROM5 (Cgd7_3020). Seven thrombospondin-family adhesins with predicted transmembrane domains (TRAP-C1, CpTSP2, CpTSP6, CpTSP7, CpTSP8, CpTSP9, and CpTSP10) and the mucin-like glycoprotein GP900 from C. parvum were included as substrates. The canonical rhomboid protease substrate Spitz from Drosophila melanogaster [43,45,63] was also included. Hemagglutinin tagged CpROMs and N-terminal GFP tagged substrates were constructed and expressed in a pcDNA3.1 vector (Invitrogen) under the control of a CMV promoter [40]. Two hundred and fifty nanograms of GFP-adhesin or GFP-

Spitz pcDNA3.1 and pcDNA3.1-CpROM4 or pcDNA3.1-CpROM5 was used to transfect COS cells. The final amount of DNA in each transfection was adjusted to 1 μg with pBluescript, and transfection complexes were formed using FuGene6 (Roche) according to the manufacturer's instructions. Cells were washed 18 h after transfection, and serum-free DMEM was conditioned for the following 24 h. Metalloprotease inhibitors were included in the media to reduce background ectodomain shedding of the GFP-tagged substrates. Media and cell lysate samples were resolved by SDS-PAGE under denaturing conditions and probed with anti-GFP or anti-HA antibodies (Santa Cruz Biotechnologies). The resulting immune complexes were revealed with enhanced chemiluminescence (Amersham), detected with X-ray film, and digitized on an Umax scanner in transmissive mode, operating through Photoshop software (Adobe). For two-color detection, anti-HA and anti-CpROM4 and anti-CpROM5 complexes were detected using anti-rabbit IRDye-800 (Rockland) and anti-rat Alexa Fluor-680 (Invitrogen) secondary antibodies, respectively, and imaged using a LiCor Odyssey infrared scanner.

Polyclonal antibody production. Polyclonal antisera were produced to short peptide sequences from CpROM4, CpROM5, TRAP-C1, and CpTSP10 (Table 1). Peptides were synthesized *in silico* and used to immunize rabbits (ProSci Inc., Poway, CA). Polyclonal antibodies were affinity purified on peptide sepharose columns.

Gene expression during in vitro infection. Human colorectal adenocarcinoma (HCT-8) cells were seeded at a concentration of 10^6 cells/mL in a 6-well polystyrene plate (Lab-Tek™, Becton Dickinson, Franklin Lakes, N.J.). Cells were grown in RPMI1640 supplemented with 10% fetal bovine serum (RPMI-FBS), 1 mM sodium pyruvate, 50 U/mL penicillin G, 50 U/mL streptomycin, and 0.25 μg/mL amphotericin B (pH 7.4) (Sigma-Aldrich Co. St Louis, MO) at 37°C in 5% CO_2.

One million oocysts were treated with 10% (v/v) ice-cold sodium hypochlorite for 10 min at 4°C, washed three times with PBS at room temperature (16,000 x *g* for 3 min), and resuspended in infection media (RPMI-FBS, 10 mg streptomycin and 25 µg amphotericin B per mL, 1 mM sodium pyruvate, 50 mM glucose, 35 µg/mL ascorbic acid, 1.0 µg/mL folic acid, 4.0 µg/mL para-aminobenzoic acid, and 2.0 µg/mL calcium pantothenate). Resuspended oocysts were used to infect 80-90% confluent HCT-8 cells. Infections were stopped at 2, 6, 12, 24, 48, or 72 h by lysing the infected cells with Qiagen lysing reagent (600 µL per well; Qiagen, Hercules, CA) supplemented with 10 µL 2-β-Mercaptoethanol (Sigma Aldrich, St. Louis, MO). Total RNA was extracted using the Qiagen RNeasy extraction kit (Qiagen) in accordance with the manufacturer's instructions. Purified RNA was eluted in RNase-free water, quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and 2 µg was reverse transcribed to cDNA using SMART MMLV Reverse Transcriptase (Promega, Madison, WI) and random hexamer primers (500 µg/mL; Promega).

Transcripts of CpROM4, CpROM5, TRAP-C1, CpTSP2, CpTSP6, CpTSP7, CpTSP8, CpTSP9, CpTSP10, gp900, small subunit ribosomal RNA (SSU), and *Cryptosporidium* oocyst wall protein 8 (COWP8) were quantified using a TaqMan quantitative RT-PCR approach. Primers and probes are shown in Table 1. SSU was used as a normalizing gene and COWP8 was used as marker for sexual stages [64]. The qPCR reaction conditions consisted of an initial denaturation at 94°C for 1 min; followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. cDNA from HCT-8 cells mock infected with heat inactivated sporozoites (70°C for 1 h) were included as a negative control, and reactions without reverse transcriptase (RT) were included as a control for genomic DNA. All infections were carried out in duplicate.

Table 1. Primers and probes used for gene expression assay.

Primers	Direction/Probe	Nucleotide Sequence
18S	FOR_{qPCR}	5′-CAGCTTTAGACGGTAGGGTATTGG-3′
	REV_{qPCR}	5′-CGAACCCTAATTCCCCGTTAC-3′
	Probe	5′-CCGTGGCAATGAC-3′
CpROM4	FOR_{qPCR}	5′-TCCCAGGTGGTGGTACATCAG-3′
	REV_{qPCR}	5′-TCCACTAGTACCAGCGCCAAA-3′
	Probe	5′-ACATCACCAGGATCACCT-3′
CpROM5	FOR_{qPCR}	5′-GGACCATAATAATCCTGCAACAAA-3′
	REV_{qPCR}	5′-GGGATTTCCTTAAGTTTCAGTTCAA-3′
	Probe	5′-CCACCGATATGGGCC-3′
TRAP-C1	FOR_{qPCR}	5′-GGAGGAGGATGAGACAAATTATCAAT-3′
	REV_{qPCR}	5′-TTTCTTGAACATATTCTGAGTCTTGATCT-3′
	Probe	5′-CTTTGATCAATCTTCTGCTACT-3′
CpTSP2	FOR_{qPCR}	5′-AGCATGTGACCATTCGGTTTC-3′
	REV_{qPCR}	5′-TTTGCGCCGCTACTATTGTTATAC-3′
	Probe	5′-TCAGGACGACTGTTATACTA-3′
CpTSP6	FOR_{qPCR}	5′-GTGGTAAAGAGGCTCCTGGAATT-3′
	REV_{qPCR}	5′-TGAACATGGGTTTGTGTCGAA-3′
	Probe	5′-CAACAGGCCTAGCTGG-3′
CpTSP7	FOR_{qPCR}	5′-GCAGGATATATTTACTGATTCATTT-3′
	REV_{qPCR}	5′-TCCCTTTTTCCATGTAATCCATCT-3′
	Probe	5′-CTCAAGAATATGTATAGTTCC-3′
CpTSP8	FOR_{qPCR}	5′-CTCCTTGCTCAGCTTCTTGTGA-3′
	REV_{qPCR}	5′-GGAGCAGAGTGGGTCAATTCTC-3'
	Probe	5′-AGGGCTTACAATCAGGAC-3′
CpTSP9	FOR_{qPCR}	5′-TTTGGCCTTTAAATCAAGTCTTCTC-3′
	REV_{qPCR}	5′-GCAAACAATATTTGGAATCCATTG-3′
	Probe	5′-CGGTGGTAGTATTTCT-3′
CpTSP10	FOR_{qPCR}	5′-AAGGAGGGCCAAGCACAAC-3′
	REV_{qPCR}	5′-TGGTTCGCATGATTGGTTATTG-3′
	Probe	5′-AACGTGTAAAATGCC-3′
COWP8	FOR_{qPCR}	5′-AAGAATGTTGAGATCGACCCAGTT-3′
	REV_{qPCR}	5′-GCACATTTGACCGTCAGTTAAAGT-3′
	Probe	5-CTGTATAGCCCATCAGGAT-3′

FOR_{qPCR}= Forward primers, REV_{qPCR}=Reverse primers, 18S=Small ribosomal RNA, CpROM= *Cryptosporidium parvum* rhomboid protease, CpTSP = *Cryptosporidium parvum* thrombospondin proteins.

Protein immunolocalization in oocysts and sporozoites during in vitro excystation.

Protein immunolocalization was determined in oocyst walls and sporozoites before excystation (intact oocysts), following bleach treatment (10% v/v ice-cold sodium hypochlorite for 10 min at

4°C and three washes in PBS) to remove the outer glycocalyx layer (bleach treated oocysts), or following excystation (non-intact oocysts and free sporozoites). Fifty microliters of intact and bleach treated oocysts (approximately 10^4 oocyst/mL) were air-dried on pre-cleaned glass slides and fixed with equal volumes of ice-cold methanol (100%) and 4% formaldehyde for 10 min. Oocysts were excysted after bleach treatment by incubation in excystation media (RPMI-FBS with 0.075 % sodium taurocholate) in a humidified incubator at 37°C and 5% CO_2. Excystation was stopped at 60 min by transferring the suspensions to ice. Following excystation, the suspension was washed three times in PBS, air-dried on a pre-cleaned glass slide, and fixed with 4% formaldehyde.

Oocysts and sporozoites were permeabilized with 1% Triton X-100 in 1% bovine serum albumin (BSA) for 15 min; blocked with 1% BSA for 20 min, probed overnight at 4°C with biotinylated *Vicia villosa* lectin (VVL, a Gal/GalNAc specific lectin that labels both extracellular and intracellular parasite stages [65]; 1:2500 dilution in 1 % BSA; Vector Laboratories Inc, CA) and polyclonal antibodies specific for CpROM4, CpROM5, CpTSP10, or TRAP-C1 (1:100 dilution in 1 % BSA); and washed three times in PBS. A 1% BSA negative control was included with all treatments. Bound primary antibodies were detected with goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:1000 dilution in 1 % BSA, Life Technologies, NY) for 1 h at 37°C, followed by three washes in PBS. Bound VVL was detected using tetramethylrhodamine isothiocyanate (TRITC) conjugated streptavidin (1:1000 dilutions in 1 % BSA, Vector Laboratories Inc., CA), followed by three washes in PBS. Nuclei were labeled with 10 µg/mL 4', 6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif.) for 15 minutes at 4°C followed by five washes with PBS.

Slides were mounted with No-Fade™ mounting medium (Waterborne™, Inc., New Orleans, LA) and examined by confocal microscopy using a Zeiss Axio Observer Z1 inverted microscope powered by a Confocal Scanning Laser, LSM700 (Carl Zeiss Microscopy, Jena, Germany). An AxioCam MRc camera (Carl Zeiss Microscopy, Jena, Germany) was used to capture representative z-stack images with 0.33 µm spacing. Images captured at different wavelengths were merged using Zeiss ZEN 2011 Software (Carl Zeiss Microscopy, Jena, Germany).

Protein immunolocalization during in vitro infection. Infections were prepared as described for gene expression experiments with the exception that HCT-8 cells were seeded at a concentration of 10^5 cells/mL in a well of an 8-well chamber slide (Becton Dickinson Labware, Franklin Lakes, NJ), and infected with 100,000 oocysts per well. Infections were allowed to proceed for 2, 6, 12, 24, 48, or 72 h. Infections were performed in duplicate and a mock infection with heat-inactivated oocysts was included as a control. The procedure for the immunolocalization of CpROM4, CpROM5, TRAP-C1, and CpTSP10 was as described for oocysts and sporozoites with the exception that fixation was performed with 100% ice-cold methanol only.

Results

Bioinformatics . Predicted protein sequences from rhomboid-like genes in *C. parvum, C. hominis*, and *C. muris* were examined for conserved rhomboid motifs, and evolutionary relationships were inferred using a neighbor-joining approach. Each *Cryptosporidium* species had one ROM2 and two ROM4/5 homologues based on inferred phylogenies (Figure 5).

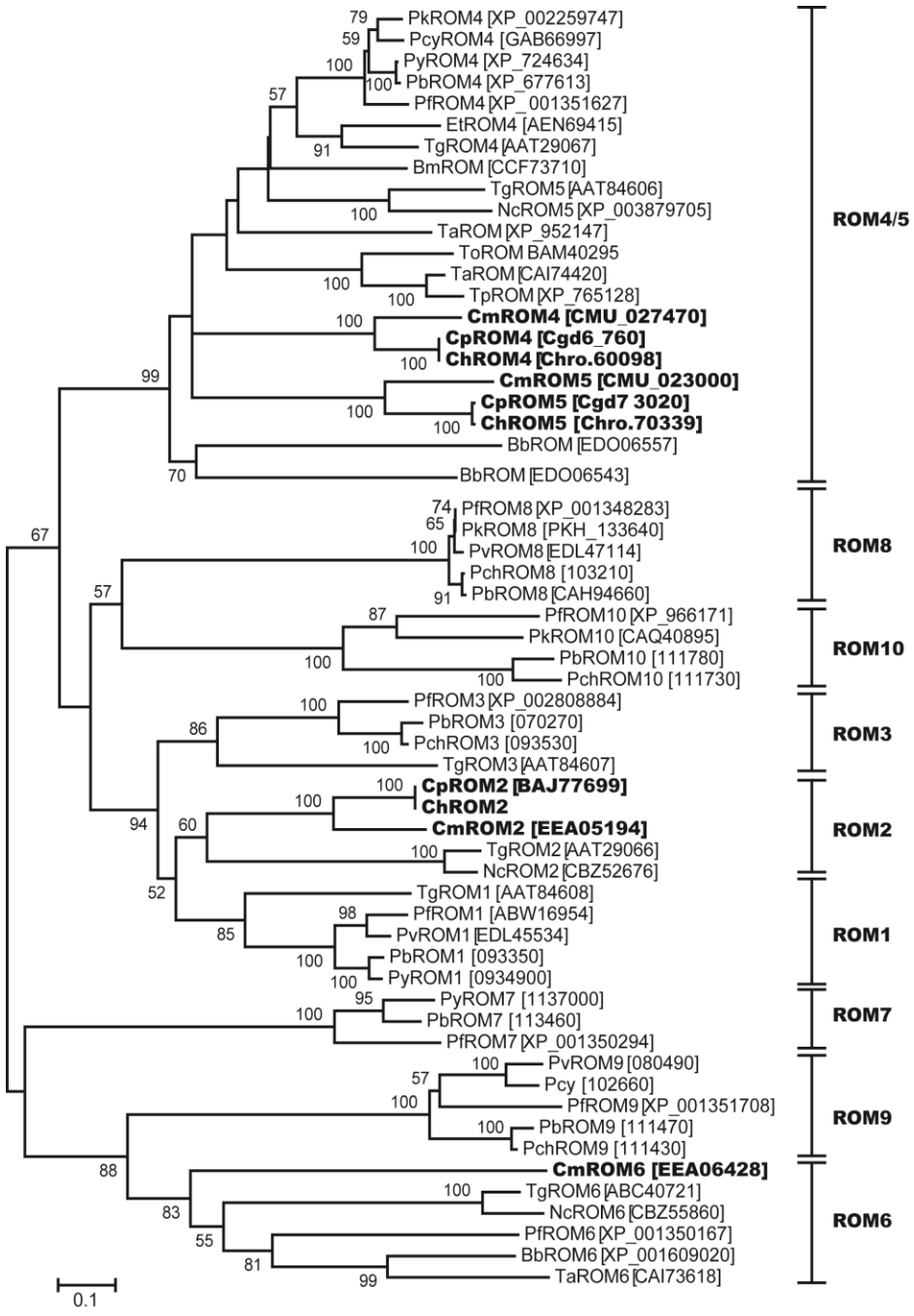

Figure 5. Phylogenetic tree of rhomboid protease from selected Apicomplexan. The tree is based on neighbor-joining phylogeny of apicomplexan rhomboid protein sequences from ApiDB and GenBank. Alignments and phylogenetic analyses were conducted using MEGA5 and bootstrap support for nodes was estimated by analyzing 1000 pseudoreplicates.

Cryptosporidium muris additionally had a ROM6 homologue that was not present in *C. parvum* or *C. hominis*. To avoid ambiguity, the *Cryptosporidium* ROM4/5 homologues were arbitrarily named Cp/Ch/CmROM4 (Cgd6_760, Chro.60098, and CMU_027470, respectively) and Cp/Ch/CmROM5 (Cgd7_3020, Chro.70339, and CMU_0273000, respectively). *Cryptosporidium* ROM2, ROM4, and ROM5 homologues had typical features of secretase rhomboids [66], including a conserved arginine motif in the L1 loop region, an HxxxxHxxxN motif in TM2, and a catalytic dyad consisting of the serine motif GxSx in TM4 and a histidine in TM6 (Figure 6). Similar to other PARL-like rhomboids, CmROM6 lacked the conserved arginine motif but retained the HxxxxHxxxN motif and the catalytic dyad. A single intron was detected in *Cryptosporidium* ROM2 homologues (Figure 7).

CpROM4 and CpROM5 have distinct substrate specificities. We used a heterologous assay to examine the specificity of CpROM4 and CpROM5 for substrates in *Cryptosporidium*, Toxoplasma, *Plasmodium*, and *Drosophila*. CpROM5 cleaved TRAP-C1, CpTSP6, CpTSP8, CpTSP9, CpTSP10, and GP900 from *C. parvum*, and the canonical rhomboid substrate Spitz. In contrast, CpROM4 had a relatively narrow specificity, cleaving only CpTSP10 and GP900 in *Cryptosporidium*.

Genes encoding CpROMs and their substrates are coexpressed during asexual development . The intracellular localization, asynchronous development, and paucity of biomarkers generally prevent the isolation of individual developmental stages. As a consequence, an assessment of gene expression dynamics during life cycle development is generally limited to temporal changes [67]. Our goal was to determine the extent to which genes encoding CpROMs and their substrates are temporally coexpressed during *C. parvum* development *in vitro*. We used COWP8 gene expression as a marker for sexual stages, as it is

associated with oocyst wall formation and is not significantly expressed during asexual development [64]. We examined gene expression in developmental stages from 2 to 72 h post infection (p.i.) of HCT-8 cells (Figure 8). COWP8 transcripts were largely absent before 48 h p.i., and increased sharply at 72 h p.i. (Figure 8A). All other genes, with the exception of CpTSP2, showed highest expression between 2 and 24 h p.i. (Figure 8B-K), suggesting an association with asexual stages. CpTSP2 (Figure 8J), which was not cleaved by CpROM4 or CpROM5 in a heterologous assay, showed a similar expression profile to COWP8, suggesting an association with late intracellular developmental stages. CpROM4 had a similar temporal expression profile to TRAP-C1, CpTSP6, CpTSP7, CpTSP9, and CpTSP10 with expression peaks at 6 and 24 h p.i., and relatively low expression at 2, 12, and 48 h p.i. The CpROM5 expression profile differed from this general pattern by its relatively high expression at 2 h p.i. CpTSP8 also showed relatively high expression at 2 h p.i., and had a less impressive expression peak at 24 h p.i., compared to other targets. gp900 had a single expression peak at 24 h p.i.

CpROMs and thrombospondin proteins colocalize in oocysts, sporozoites, and intracellular stages . We examined localization of CpROM4, CpROM5, TRAP-C1, and CpTSP10 in intact, bleach treated, and non-intact oocysts. All four proteins were expressed in sporozoites within intact oocysts but were not detected in the intact oocyst wall (Figure 9A). CpROM5 and TRAP-C1 showed punctate expression in non-intact oocyst walls following excystation (Figure 9B) suggesting either a] they were transported to the oocyst wall during excystation or b] they were present in the oocyst wall before excystation but epitopes were not accessible. In support of the latter, CpROM5 and TRAP-C1 were detected in oocyst walls following treatment with bleach treatment to remove the outer glycocalyx from oocysts (Figure 9C). CpROM4 and CpTSP10 were not detected in intact, bleach-treated, or non- intact oocysts.

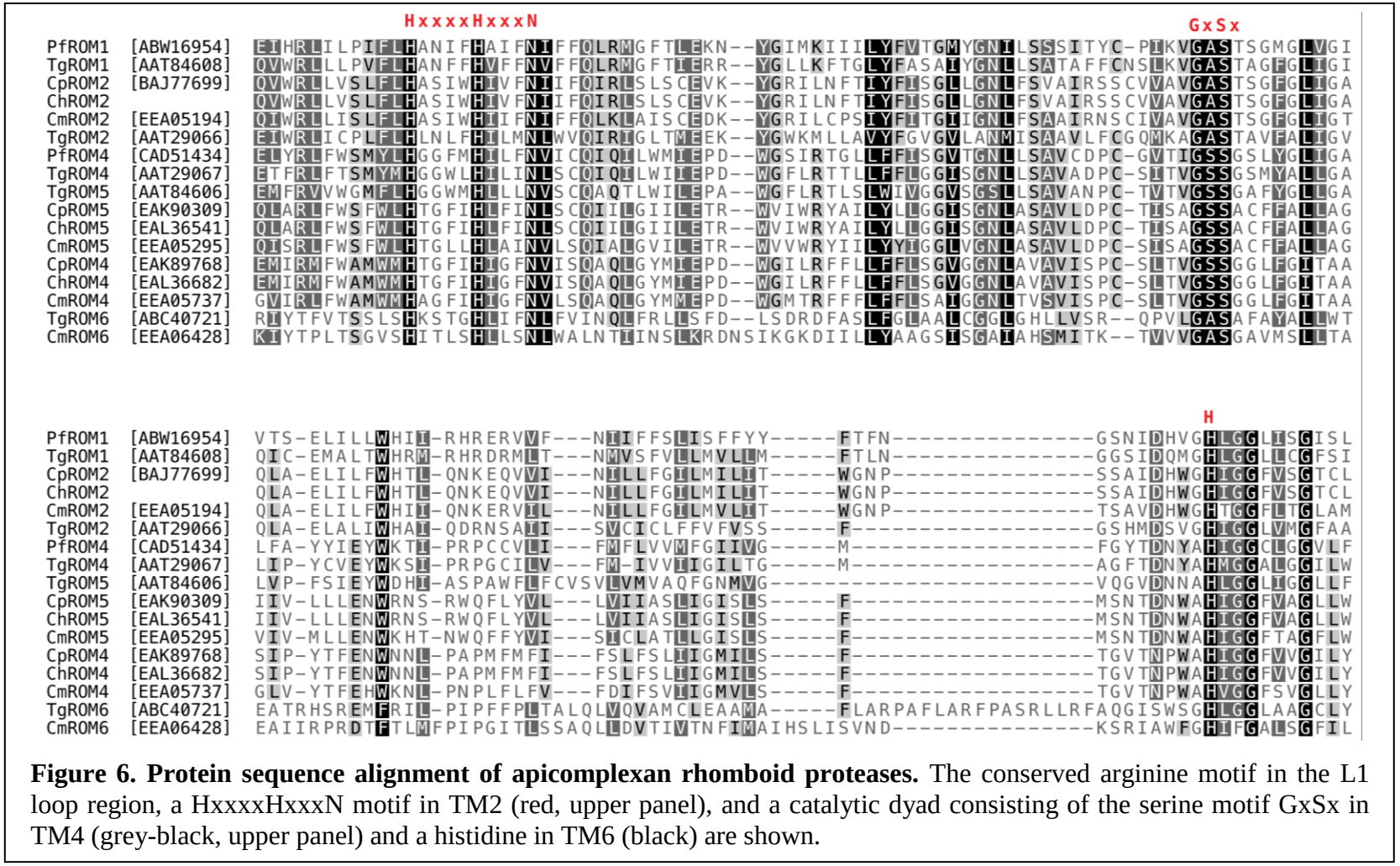

Figure 6. Protein sequence alignment of apicomplexan rhomboid proteases. The conserved arginine motif in the L1 loop region, a HxxxxHxxxN motif in TM2 (red, upper panel), and a catalytic dyad consisting of the serine motif GxSx in TM4 (grey-black, upper panel) and a histidine in TM6 (black) are shown.

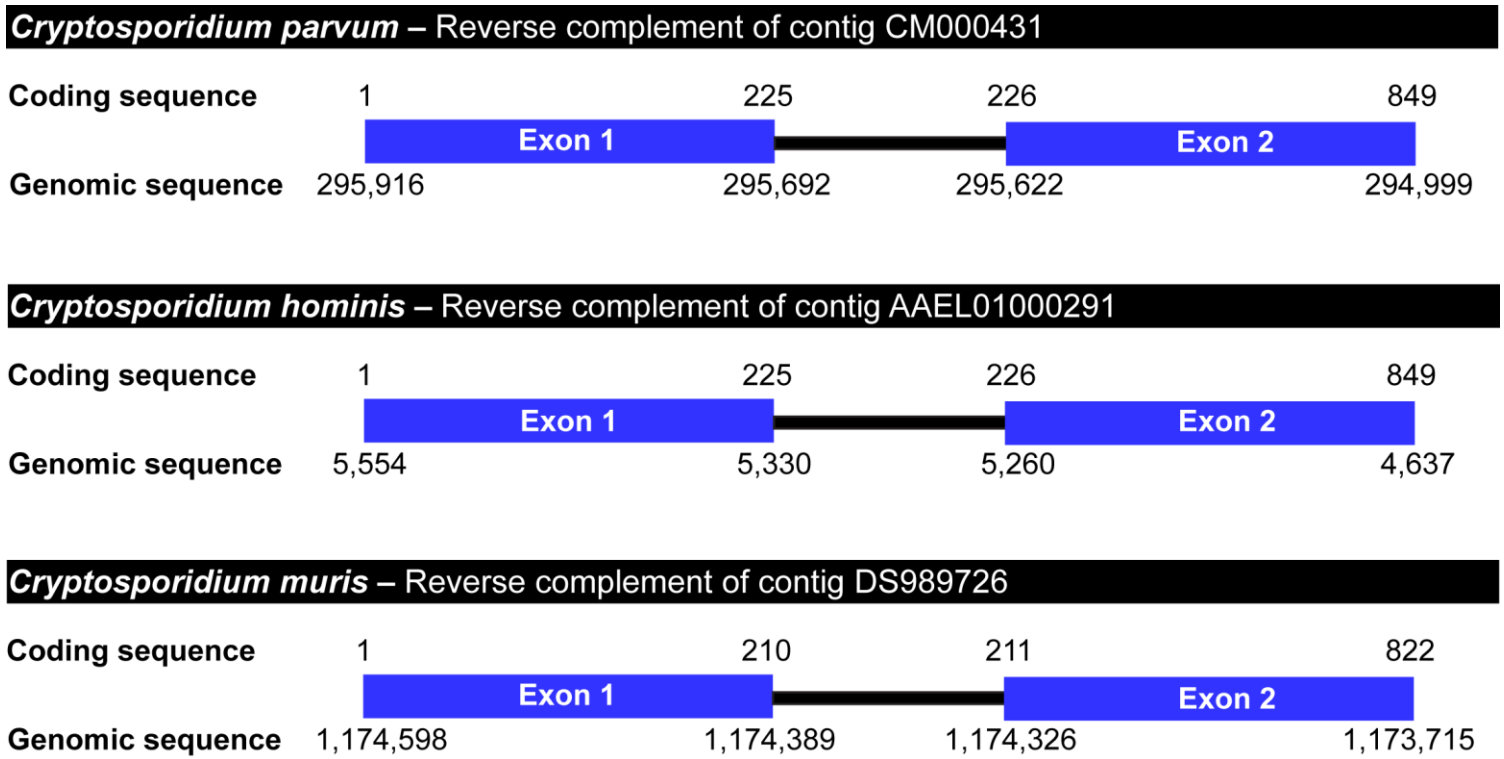

Figure 7. Intron-exon organization and chromosomal location of the ROM2 gene from *C. parvum*, *C. hominis*, and *C. muris*.

We examined the localization of CpROM4, CpROM5, TRAP-C1, and CpTSP10 in sporozoites following excystation in the absence of host cells (Figure 10). CpROM4 was localized to the anterior (apical) and posterior sporozoite poles, and in patches in a sub-apical region and a region immediately anterior to the nucleus. CpTSP10 was localized to the posterior pole and in patches on the sporozoite surface. CpROM5 and TRAP-C1 were predominantly localized to the apical and sub-apical region of sporozoites.

We examined the immunolocalization of CpROM4 (Figure 11), CpTSP10 (Figure 12), CpROM5 (Figure 13), and TRAP-C1 (Figure 14) during intracellular development *in vitro*. Although all proteins were detected in early trophozoites at 2 h p.i., CpROM5 and TRAP-C1 showed greater expression relative to CpROM4 and CpTSP10. By 6 h p.i., all proteins were localized to the periphery of trophozoites, giving a halo-like appearance. All proteins were detected in developing meronts at 12 and 24 h p.i., and were expressed in intracellular stages at 48 and 72 h p.i.

Discussion

Cryptosporidium encodes fewer ROMs (three or four) than *Toxoplasma* (six ROMs [12]) or *Plasmodium* (five to eight ROMs [68]). The intestinal species, *C. parvum and C. hominis*, appear not to have a mitochondrion-associated ROM6 homologue, which is consistent with the absence of true mitochondria, TCA cycle, and oxidative phosphorylation in these species. In contrast, *C. muris*, which has a ROM6 homologue, has retained a typical mitochondrion with a functional TCA cycle and oxidative phosphorylation [69,70]. This suggests that the intestinal cryptosporidia lost ROM6 and mitochondrial functions after they split from gastric species early in the evolution of the genus.

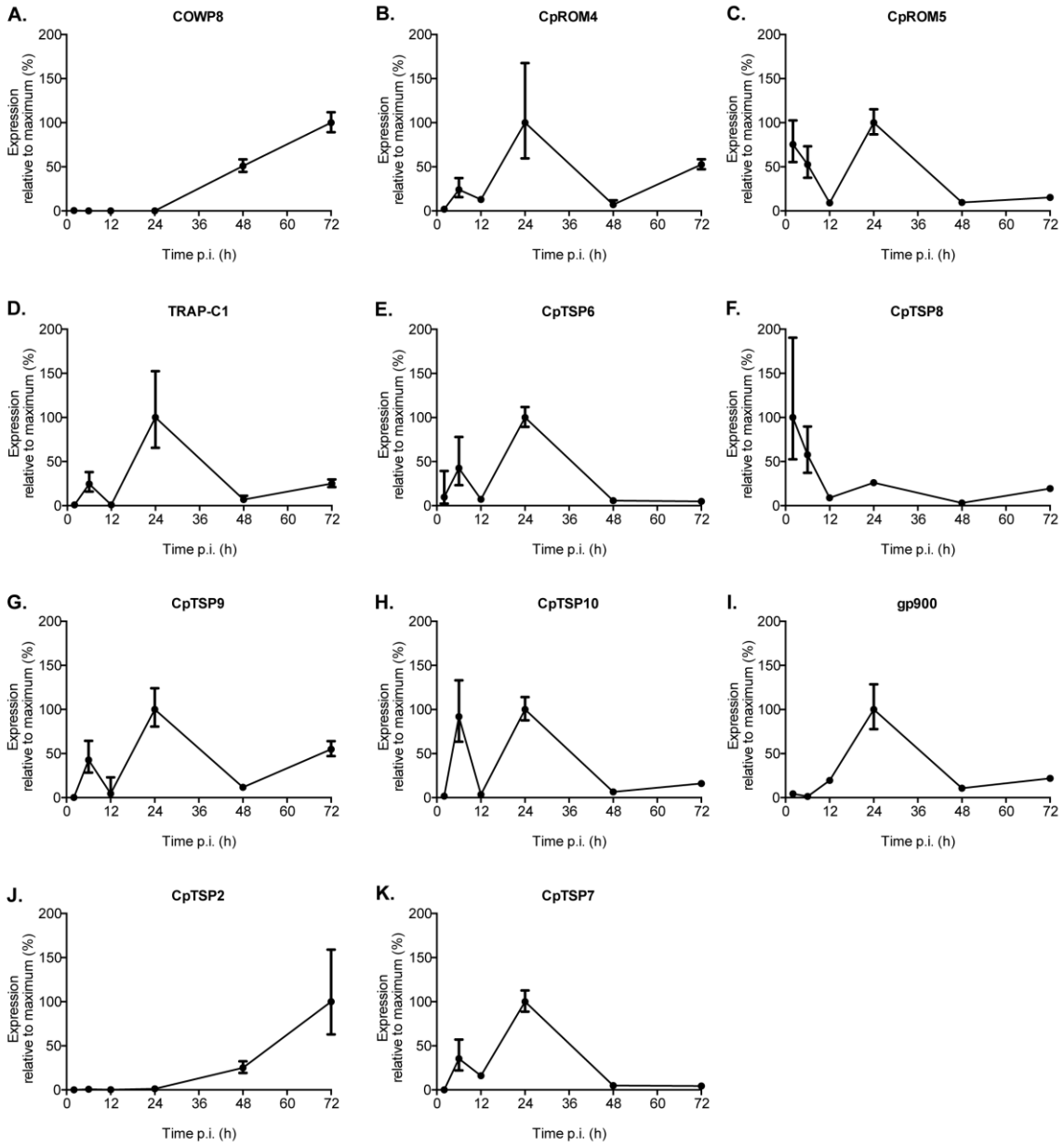

Figure 8. Expression of CpROMs and CpTSPs at time points post infection.COWP8 HCT-8 infection model was used to study temporal changes in mRNA levels of rhomboids (B and C) and their substrates during development. mRNA was quantified using qRT-PCR and normalized against 18SrRNA. COWP8 (A) is an oocyst wall protein that is a useful marker for sexual life cycle stages.COWP8 mRNA is barely detectable until 48 h p.i. CpROMs are expressed primarily during asexual stages with peak expression observed at 6 and 24 h p.i. A similar pattern of expression is observed for CpTSPs (D to H, J and K).

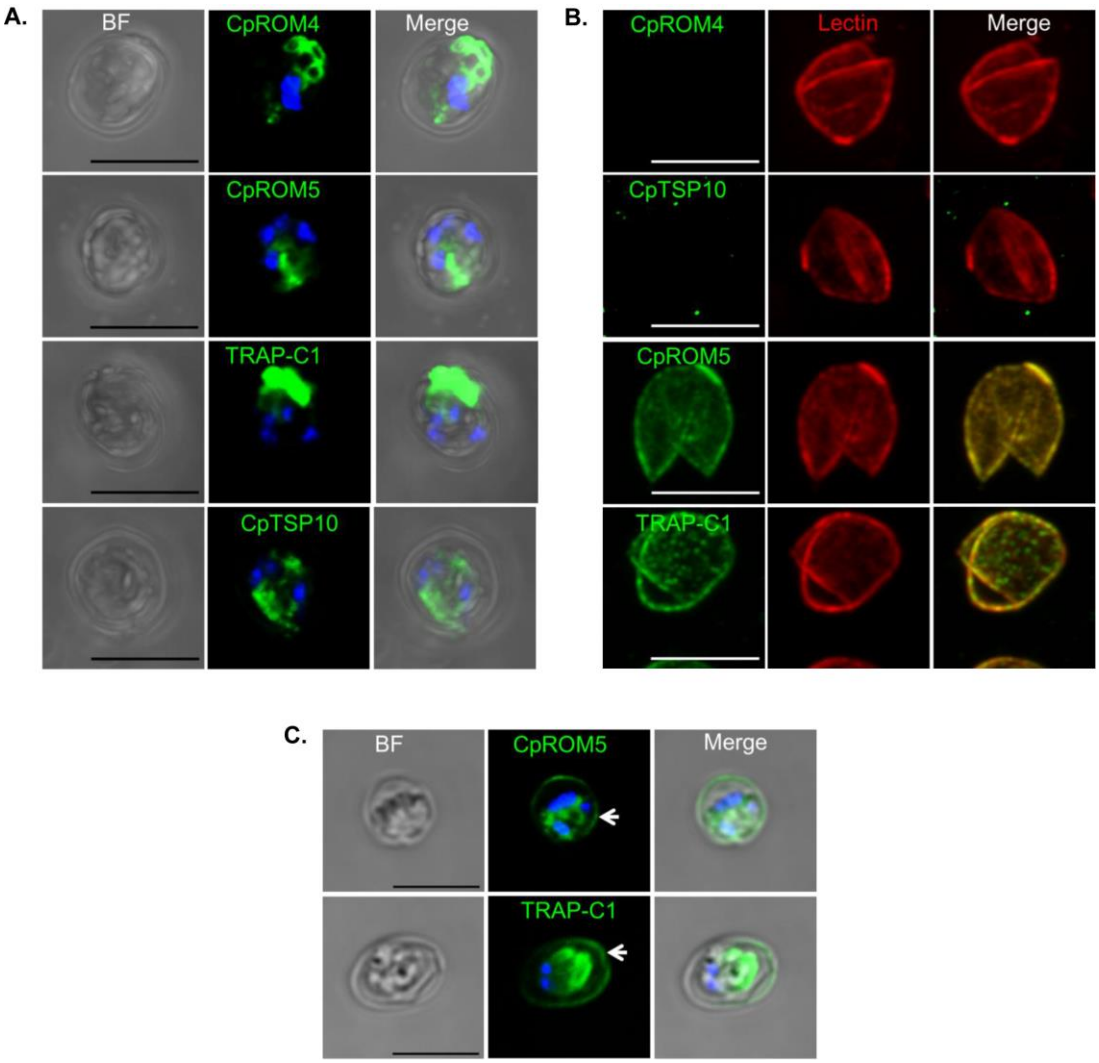

Figure 9. Immunolocalization of CpROM4, CpROM5, TRAP-C1, and CpTSP10 in the walls of intact (A and C) and non-intact oocysts (B). Following fixation with methanol, intact or non-intact oocysts were subjected to immunolocalization assay as described in methods. (Panel A) CpROM4, CpROM5, TRAP-C1, and CpTSP10 expressed and detected with FITC (green) conjugated anti-rabbit antibody on the surface of sporozoites within intact oocysts; (Panel B) CpROM5 and TRAP-C1 (bottom left) are expressed in the shells of excysted oocyst. (Panel C) CpROM5 and TRAP-C1 are indicated (white arrow) in the walls of intact oocysts; CpROM4 and cpTSP10 (top right panel) were not detected hence no FITC signal. Intact oocyst outline is shown in bright field (panels A and C). Lectin as indicated, binds to surface glycoproteins present on oocyst wall and revealed with Tetramethylrhodamine isothiocyanate (TRITC)-Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using a Zeiss AxioVision 4.8 image software. Scale bar = 5

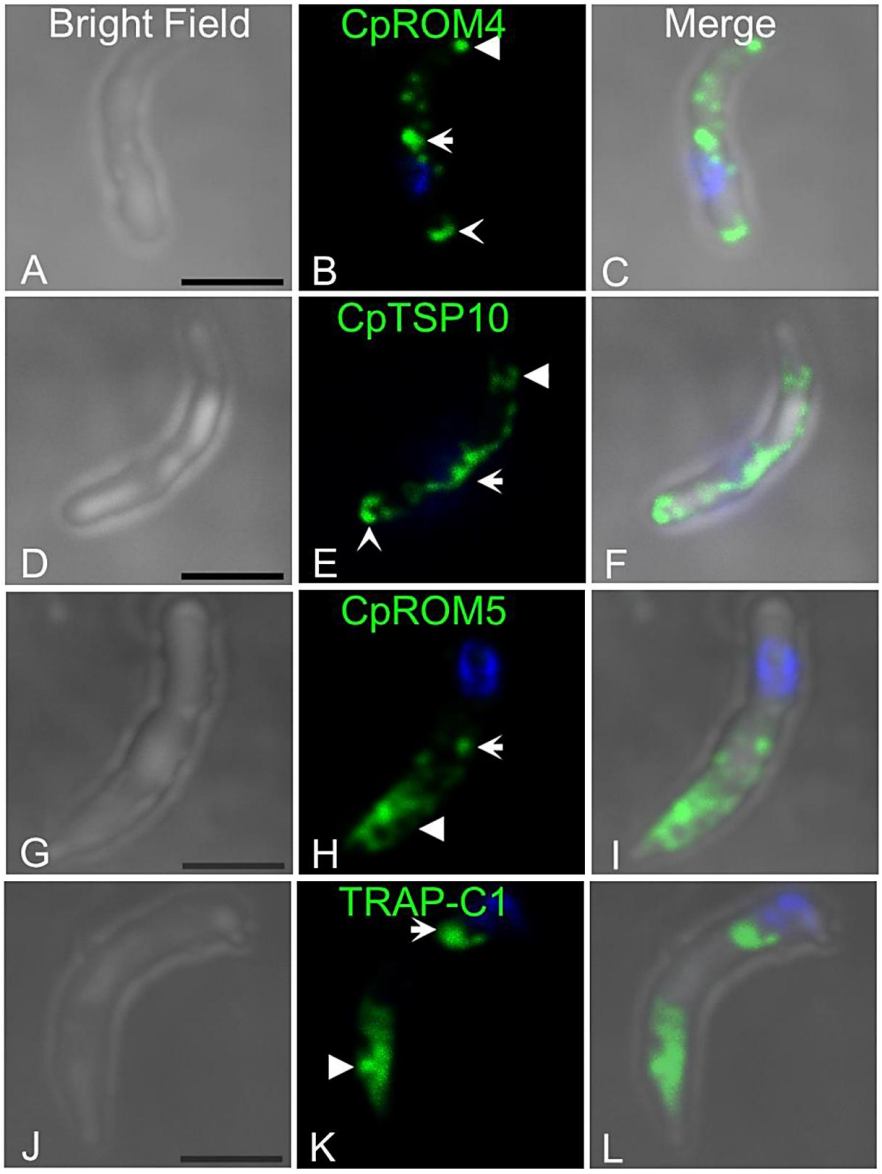

Figure 10. Subcellular localization of CpROM4, CpROM5, TRAP-C1, and CpTSP10 in C. parvum sporozoites during motility. Excysted sporozoites were fixed with 4 % formaldehyde, probed withantibodies to CpROM4 (B), CpROM5 (H), TRAP-C1 (K), and CpTSP10 (E) (FITC, green). CpROM4 (B) and CpTSP10 (E) was colocalized to three locations (green-FITC); the anterior (full arrow head), the medial (half-tail arrow head), and posterior (half arrow-head). Bright field is used to show the outline of the sporozoites (A and D). An overlay of FITC and fright field is also indicated (C, F, I, and L). CpROM5 (H) and TRAP-C1 (K) are colocalized to two locations (green-FITC); the anterior (full arrowhead) and the medial (half-tail arrow head). DAPI is used to stain zoite nuclei. Microscopy was performed as above. Scale bars = 2 μm.

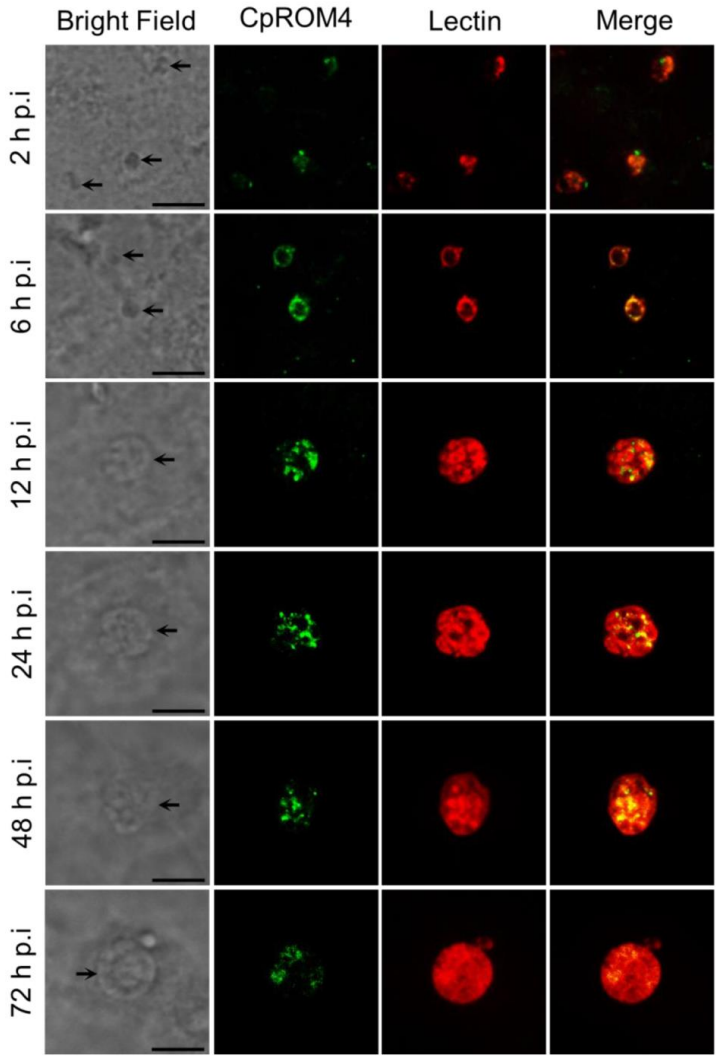

Figure 11. Intracellular localization of CpROM4 during a 2-72 h infection of HCT-8 cell line. Shown are representative intracellular stages of *C. parvum* following infection of HCT-8 cells during a 2 to 72 h infection time points. Following fixation with methanol, infected cells were subjected to immunolocalization assay as described in methods. (2 h p.i) CpROM4 (green) is expressed at the tip of semicircular invading sporozoites; (6 h p.i) CpROM4 becomes prominent in a 'halo-like' presentation on the surface of fully internalized structures-trophozoites. (12 to 24 h p.i) expression of CpROM4 is seen in punctate in meronts-like structures and also in unidentified structures, later in infection (48 to 72 h p.i). Lectin as indicated, binds to surface glycoproteins present on intracellular stages and revealed with Tetramethylrhodamine isothiocyanate (TRITC) - Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using a Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

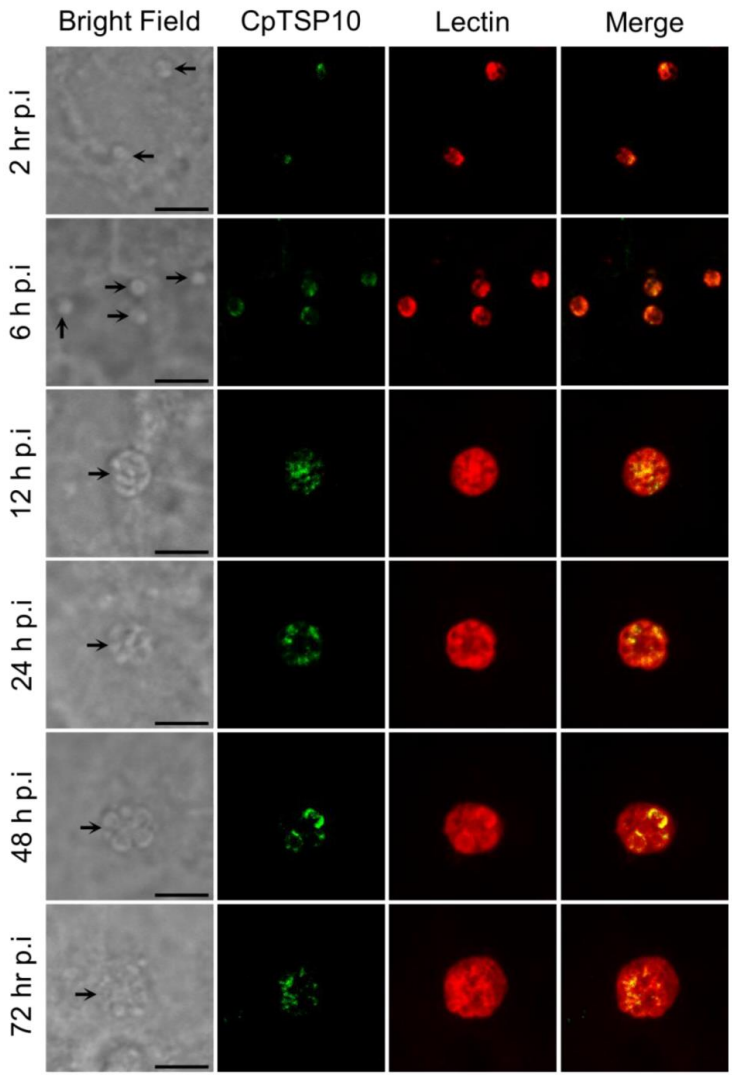

Figure 12. Intracellular localization of CpTSP10 during a 2-72 h infection of HCT-8 cell line. Shown are representative intracellular stages of *C. parvum* following infection of HCT-8 cells during a 2 to 72 h infection time points. Following fixation with methanol, infected cells were subjected to immunolocalization assay as described in methods. (2 h p.i) CpTSP10 (green) is barely expressed at the tip of invading sporozoites; (6 h p.i) CpTSP10 is mildly expressed in trophozoites. (12 to 24 h p.i) expression of CpTSP10 is seen in meronts-like structures concentrated in unidentified surface at the center of the structure; (48 to 72 h p.i) CpTSP10 is expressed at the periphery of the meronts-like structure. Lectin as indicated, binds to surface glycoproteins present on intracellular stages and revealed with Tetramethylrhodamine isothiocyanate (TRITC) - Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

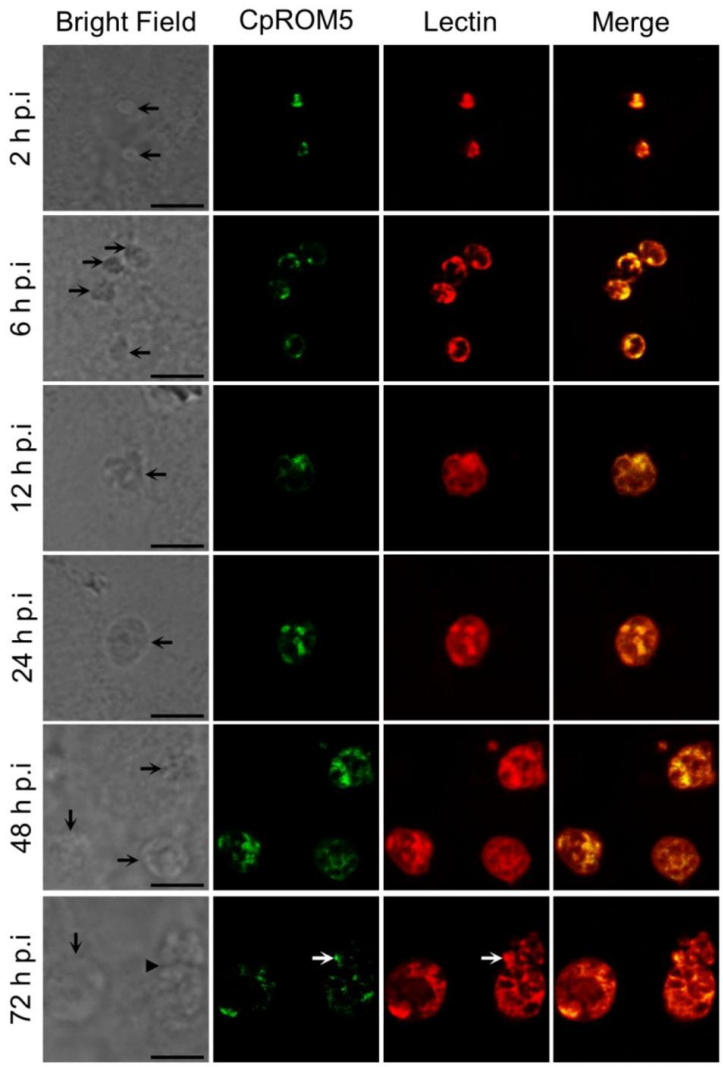

Figure 13. Intracellular localization of CpROM5 during a 2-72 h infection of HCT-8 cell line. Shown are representative intracellular stages of *C. parvum* following infection of HCT-8 cells during a 2 to 72 h infection time points. Following fixation with methanol, infected cells were subjected to immunolocalization assay as described in methods. (2 h p.i) CpROM5 (green) is expressed on the surface of invading sporozoites; (6 h p.i) CpROM5 becomes prominent in a 'halo-like' presentation on the surface similar to CpROM4 on fully internalized structures-trophozoites. (12 to 24 h p.i) expression of CpROM4 is seen in punctate in meronts-like structures and also in unidentified structures, later in infection (48 to 72 h p.i). Lectin as indicated, binds to surface glycoproteins present on intracellular stages and revealed with Tetramethylrhodamine isothiocyanate (TRITC) - Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

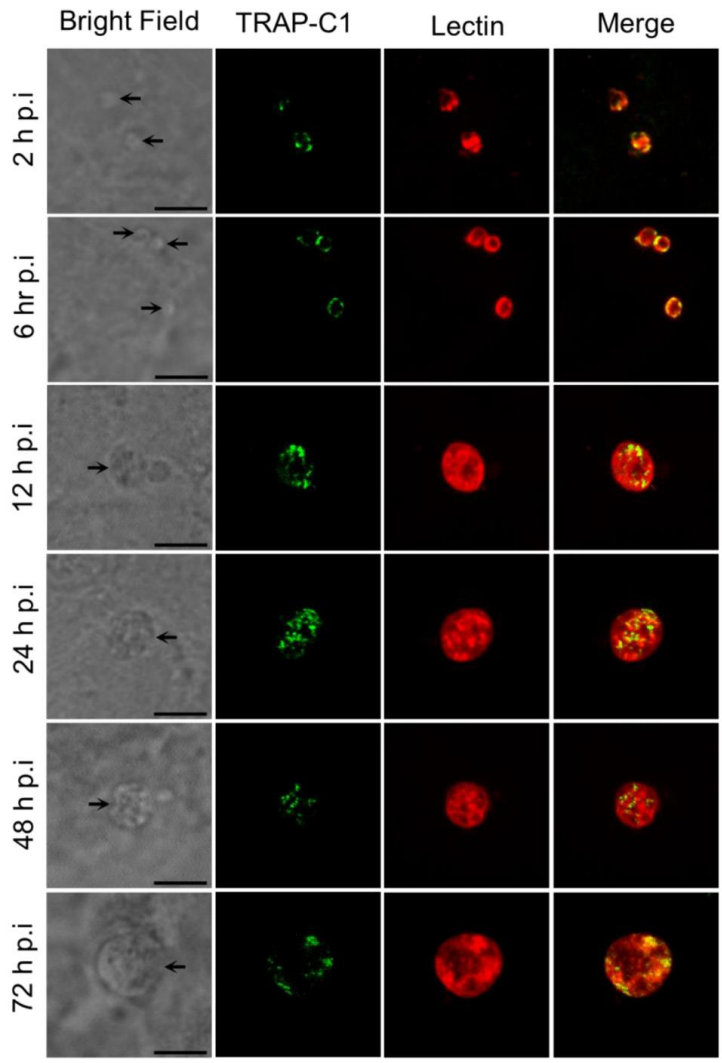

Figure 14. Intracellular localization of TRAP-C1 during a 2-72 h infection of HCT-8 cell line. Shown are representative intracellular stages of *C. parvum* following infection of HCT-8 cells during a 2 to 72 h infection time points. Following fixation with methanol, infected cells were subjected to immunolocalization assay as described in methods. (2 h p.i) TRAP-C1 (green) is expressed on the entire surface of invading sporozoites; (6 h p.i) TRAP-C1 is expressed in a 'halo-like' presentation on the surface of trophozoites. (12 to 24 h p.i) expression of TRAP-C1 is seen in punctate to a greater extent at the periphery of meronts-like structures. (48 to 72 h p.i). Mild expression of TRAP-C1 is observed later in infection in unidentified late stage structures. Lectin as indicated, binds to surface glycoproteins present on intracellular stages and revealed with Tetramethylrhodamine isothiocyanate (TRITC) - Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

Three secretase ROMs were common to the gastric and intestinal cryptosporidia. Consistent with their basal position in the phylum, *Cryptosporidium* ROMs have diverged considerably from homologues in other apicomplexans. We named *Cryptosporidium* ROM2s based on their relatedness to ROM2 homologues from the coccidian species *T. gondii* and *Neospora caninum*. Previous phylogenies placed CpROM2 (BAJ77699) within the ROM1 cluster [52], or as a basal member of a ROM1/2 cluster [68]. Identifying the subcellular localization and substrate specificity of *Cryptosporidium* ROM2s will help to clarify the nomenclature.

A major objective of our research was to better understand how CpROMs contribute to the shedding of microneme adhesins during *Cryptosporidium* motility and invasion. ROM4/5 proteases localize to the plasma membrane and cleave microneme adhesins in *T. gondii* (TgROM4 and TgROM5) and *P. falciparum* (PfROM4), so we undertook a focused biochemical and molecular analysis of two *C. parvum* ROM4/5 proteases, which we named CpROM4 (Cgd6_760) and CpROM5 (Cgd7_3020). CpROM4 and CpROM5 showed distinct but overlapping specificities in a heterologous assay. CpROM5 cleaved the canonical rhomboid substrate Spitz from *D. melanogaster*; CpTSP6; CpTSP8; CpTSP9; CpTSP10; TRAP-C1, which is predicted to engage the actomyosin motor; and GP900, a mucin-like glycoprotein that mediates attachment to and invasion of host cells [36,71,72]. In contrast, CpROM4, which did not cleave Spitz, cleaved only GP900 and CpTSP10 from *C. parvum*. With respect to specificity, CpROM4 and CpROM5 are somewhat analogous to TgROM4 and TgROM5 in *T. gondii* [12]. TgROM5 cleaved Spitz and multiple microneme adhesins, including the TRAP homologue TgMIC2 in a heterologous assay. TgROM4, in contrast, showed no activity in the heterologous assay, but was necessary for TgMIC2, TgMIC8, and AMA1 shedding by tachyzoites during an

in vitro infection [39,73]. We cannot exclude the possibility that CpROM4, or indeed CpROM5 cleave more substrates in their natural membrane environment than in the heterologous assay.

ROM4/5 protease activity is associated with motility, invasion, and early intracellular development in *T. gondii* and *P. falciparum*. Considering that sporozoite motility commences inside the oocyst and is essential for egress, we examined the expression of CpROMs and two thrombospondins, TRAP-C1 and CpTSP10, before and after excystation. Oocysts, which are largely dormant outside the host, are triggered to excyst by stimuli such as a 37°C temperature, pancreatic enzymes, and bile salts that are encountered inside the host. The sporozoite switch from inactivity to motility and egress from oocysts remains poorly characterized; however, it is known to coincide with selective increases in gene [74] and protein [75] expression. We detected CpROM4, CpROM5, TRAP-C1, and CpTSP10 in sporozoites within intact oocysts in the absence of excystation stimuli, suggesting that these proteins are synthesized and stored in preparation for excystation. This is consistent with an immediate role for CpROMs and thrombospondins during sporozoite egress from oocysts. Unexpectedly, we also detected CpROM5 and TRAP-C1 in the walls of non-intact oocysts and intact oocysts after removal of the outer glycocalyx, suggesting that these proteins are localized to the inside of the oocyst wall [64]. Although microneme adhesins are typically not associated with the oocyst wall, the *T. gondii* microneme protein TgMIC4 and an *Eimeria maxima* MIC4 homologue localize to the outer veil of oocysts [76,77]. TgMIC4 forms a complex with TgMIC1 and TgMIC6 during gliding motility and invasion, and it binds to carbohydrate epitopes on host cells via apple domains [78]. TRAP-C1 has two apple domains [29], though their binding specificity and function are not currently known. Chatterjee et al., [79] recently showed that GP900 is a component of carbohydrate-rich fibrils that tether *C. parvum* sporozoites to the oocyst wall. The

tethers detach from egressing sporozoites and GP900 can be observed in the globular fibrils that remain on the inner surface of the oocyst wall. Given that CpROM5 can cleave both TRAP-C1 and GP900, it is possible that all three proteins localize to fibrils, and CpROM5 disassembles the tethering during egress. Although previous studies have shown that serine protease activity is necessary for *Cryptosporidium* excystation [80,81], they failed to identify the protease or distinguish between activity in sporozoites and oocysts. CpROM5 is the first protease identified in the *Cryptosporidium* oocyst wall, and it is the first ROM identified in the oocyst wall of any apicomplexan.

CpROM4 and CpROM5 have distinct localization patterns in sporozoites: CpROM4 localizes with CpTSP10 at the anterior, middle, and posterior pole; and CpROM5 localizes with TRAP-C1 at the anterior. These findings suggest that despite overlapping specificities, CpROM4 and CpROM5 have distinct functions during motility. This is most evident in the colocalization of CpROM4 and CpTSP10 at the posterior pole. Although both CpROMs cleave CpTSP10, CpROM4 is apparently preferred at this posterior location. An early apicomplexan motility model placed the sheddase function towards the posterior of the zoite; a more anterior location was predicted to cause premature release of translocating adhesins. Because TgROM5 localizes to the posterior of extracellular tachyzoites, and cleaves a number of surface adhesins, it was considered the best candidate for sheddase function in *T. gondii* [12]. However, evidence that TgROM4 in *T. gondii* [39] and PfROM4 in *P. falciparum* [82] also have sheddase roles, and are distributed throughout the zoite plasma membrane, challenges the simplicity of the ‘lone posterior sheddase’ model. A more dynamic model of sheddase function is supported by studies of *Plasmodium* sporozoite interactions with surfaces during motility [83,84]. Using various microscopy techniques, adhesion-site turnover (adhesion and deadhesion) was observed to occur

at the anterior, middle, and posterior surfaces in a coordinated and sequential manner. Posterior deadhesion occurred last in the sequence, resulting in a period of sticking and the frequency of deadhesion at anterior and posterior surfaces was positively correlated with sporozoite speed [84].

Host cell invasion and intracellular development remain poorly understood in *Cryptosporidium*. Using an *in vitro* infection model, the presence of trophozoites at 2 h p.i., coincided with relatively high CpROM5 expression, and low or undetectable CpROM4 expression. Although the function of CpROM5 during early intracellular development is unknown, it may be necessary for the parasite switch to a replicative form. Santos et al [73] showed a mechanism by which *Toxoplasma* "senses" that the appropriate location for replication has been reached; i.e. the cytoplasmic vacuole within the host cell. TgROM4 cleaves AMA 1, releasing both its extracellular and cytoplasmic domains. Release of the cytoplasmic domain triggers replication [73]. The localization of CpROM4, CpROM5, CpTSP10, and TRAP-C1 in merozoites within developing meronts may be analogous to the expression of these proteins in sporozoites within oocysts. CpROMs may be stored in preparation for motility, or they may have a more direct function in egress. Treatment of *P. falciparum* infected cells with broad spectrum serine and cysteine protease inhibitors blocks egress leading to an accumulation of late stage schizonts [85].

Conclusion

We have shown that two *C. parvum* rhomboid proteases, CpROM4 and CpROM5, collectively cleave six microneme adhesins, including five thrombospondin family proteins and GP900. Although CpROM4 and CpROM5 have overlapping specificities, their expression and subcellular localization in oocysts, sporozoites and intracellular stages suggests that they have

distinct yet coordinated roles in *Cryptosporidium* development. Therefore, more studies will be required to evaluate and characterize their specific roles so as to inform the development of intervention strategies.

References

1. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, et al. (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. The Lancet.
2. Hlavsa MC, Watson JC, Beach MJ (2005) Cryptosporidiosis surveillance--United States 1999-2002. MMWRMorbidity and Mortality Weekly Report 54: 1-8.
3. Hlavsa MC, Roberts VA, Anderson AR, Hill VR, Kahler AM, et al. (2011) Surveillance for waterborne disease outbreaks and other health events associated with recreational water --- United States, 2007--2008. MMWR Surveill Summ 60: 1-32.
4. Reynolds KA, Mena KD, Gerba CP (2008) Risk of waterborne illness via drinking water in the United States. RevEnvironContam Toxicol 192: 117–158.
5. Yoder JS, Harral C, Beach MJ (2010) Cryptosporidiosis surveillance - United States, 2006-2008. MMWR Surveill Summ 59: 1-14.
6. Yoder JS, Beach MJ (2007) Cryptosporidiosis surveillance--United States, 2003-2005. MMWR CDC Surveillance Summaries 56: 1-10.
7. Chalmers RM, Robinson G, Elwin K, Hadfield SJ, Thomas E, et al. (2010) Detection of *Cryptosporidium* species and sources of contamination with *Cryptosporidium hominis* during a waterborne outbreak in north west Wales. JWater Health 8: 311–325.

8. Elwin K, Hadfield SJ, Robinson G, Chalmers RM (2011) The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000-2008. Epidemiology and Infection: 1-11.

9. Valigurova A, Jirku M, Koudela B, Gelnar M, Modry D, et al. (2008) Cryptosporidia: epicellular parasites embraced by the host cell membrane. International Journal for Parasitology 38: 913-922.

10. Valigurova A, Hofmannova L, Koudela B, Vavra J (2007) An ultrastructural comparison of the attachment sites between Gregarina steini and Cryptosporidium muris. Journal of Eukaryotic Microbiology 54: 495-510.

11. Soldati D, Dubremetz JF, Lebrun M (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite Toxoplasma gondii International Journal for Parasitology 31: 1293-1302.

12. Brossier F, Jewett TJ, Sibley LD, Urban S (2005) A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by *Toxoplasma*. Proceedings of the National Academy of Sciences of the United States of America 102: 4146-4151.

13. Besteiro S, Dubremetz JF, Lebrun M (2011) The moving junction of apicomplexan parasites: a key structure for invasion. Cell Microbiol 13: 797-805.

14. Tyler JS, Treeck M, Boothroyd JC (2011) Focus on the ringleader: the role of AMA1 in apicomplexan invasion and replication. Trends Parasitol 27: 410-420

15. Sultan AA, Thathy V, Frevert U, Robson KJ, Crisanti A, et al. (1997) TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. Cell 90: 511-522.

16. Morahan BJ, Wang L, Coppel RL (2009) No TRAP, no invasion. Trends Parasitol 25: 77-84.

17. Witcombe DM, Ferguson DJ, Belli SI, Wallach MG, Smith NC (2004) *Eimeria maxima* TRAP family protein EmTFP250: subcellular localisation and induction of immune responses by immunisation with a recombinant C-terminal derivative. International Journal for Parasitology 34: 861-872.

18. Ménard R (2000) The journey of the malaria sporozoite through its hosts: two parasite proteins lead the way. Microbes and Infection 2: 633-642.

19. Müller HM, Scarselli E, Crisanti A (1993) Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* in parasite-host cell interactions. Parassitologia 35 Suppl: 69-72.

20. Spaccapelo R, Naitza S, Robson KJ, Crisanti A (1997) Thrombospondin-related adhesive protein (TRAP) of *Plasmodium berghei* and parasite motility. Lancet 350: 335.

21. Carruthers VB, Tomley FM (2008) Microneme proteins in apicomplexans. Sub-Cellular Biochemistry 47: 33-45.

22. Kappe SH, Buscaglia CA, Bergman LW, Coppens I, Nussenzweig V (2004) Apicomplexan gliding motility and host cell invasion: overhauling the motor model. Trends in Parasitology 20: 13-16.

23. Akhouri RR, Bhattacharyya A, Pattnaik P, Malhotra P, Sharma A (2004) Structural and functional dissection of the adhesive domains of *Plasmodium falciparum* thrombospondin-related anonymous protein (TRAP). Biochemistry Journal 379: 815-822.

24. Buscaglia CA, Coppens I, Hol WG, Nussenzweig V (2003) Sites of interaction between aldolase and thrombospondin-related anonymous protein in *Plasmodium*. Molecular Biology of the Cell 14: 4947-4957.

25. Jewett TJ, Sibley LD (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. Molecular Cell 11: 885-894.

26. Dobrowolski JM, Carruthers VB, Sibley LD (1997) Participation of myosin in gliding motility and host cell invasion by Toxoplasma gondii. Molecular Microbiology 26: 163-173.

27. Meissner M, Schluter D, Soldati D (2002) Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. Science 298: 837-840.

28. Spano F, Putignani L, Naitza S, Puri C, Wright S, et al. (1998) Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. Molecular and Biochemical Parasitology 92: 147–162.

29. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium parvum* genes containing thrombospondin type 1 domains. Infection and Immunity 70: 6987-6995.

30. Putignani L, Possenti A, Cherchi S, Pozio E, Crisanti A, et al. (2008) The thrombospondin-related protein CpMIC1 (CpTSP8) belongs to the repertoire of micronemal proteins of *Cryptosporidium parvum*. Molecular & Biochemical Parasitology 157: 98-101.

31. Perryman LE, Jasmer DP, Riggs MW, Bohnet SG, McGuire TC, et al. (1996) A cloned gene of Cryptosporidium parvum encodes neutralization-sensitive epitopes. Molecular and Biochemical Parasitology 80: 137-147.

32. O'Hara SP, Yu JR, Lin JJ (2004) A novel Cryptosporidium parvum antigen, CP2, preferentially associates with membranous structures. Parasitology Research 92: 317-327.

33. Cevallos AM, Zhang X, Waldor MK, Jaison S, Zhou X, et al. (2000) Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15.

Infection and Immunity 68: 4108–4116.

34. Strong WB, Gut J, Nelson RG (2000) Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infection and Immunity 68: 4117–4134.

35. Petersen C, Gut J, Doyle PS, Crabb JH, Nelson RG, et al. (1992) Characterization of a > 900,000-M(r) Cryptosporidium parvum sporozoite glycoprotein recognized by protective hyperimmune bovine colostral immunoglobulin. Inf and Imm 60: 5132-5138.

36. Petersen CBDAGL (1997) *Cryptosporidium parvum* GP900 , a unique invasion protein. The Journal of Eukatyotic Microbiology 44: 89S-90S.

37. Bhat N, Joe A, PereiraPerrin M, Ward HD (2007) *Cryptosporidium* p30, a galactose/N-acetylgalactosamine-specific lectin, mediates nfection in vitro. iJournal of Biological Chemistry 282: 34877-34887.

38. Barnes DA, Bonnin A, Huang JX, Gousset L, Wu J, et al. (1998) A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. Molecular and Biochemical Parasitology 96: 93-110.

39. Buguliskis JS, Brossier F, Shuman J, Sibley LD (2010) Rhomboid 4 (ROM4) affects the processing of surface adhesins and facilitates host cell invasion by *Toxoplasma gondii*. PLoSPathog 6: e1000858.

40. Baker RP, Wijetilaka R, Urban S (2006) Two *Plasmodium* rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. PLoSPathog 2: e113.

41. Freeman M (2008) Rhomboid proteases and their biological functions. Annual Review of

Genetics 42: 191-210.

42. Freeman M (2009) Rhomboids: 7 years of a new protease family. Seminars in Cell and Developmental Biology 20: 231-239.

43. Urban S, Freeman M (2003) Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. Molecular Cell 11: 1425-1434.

44. Urban S, Schlieper D, Freeman M (2002) Conservation of intramembrane proteolytic activity and substrate specificity in prokaryotic and eukaryotic rhomboids. Current Biology 12: 1507-1512.

45. Urban S, Lee JR, Freeman M (2002) A family of Rhomboid intramembrane proteases activates all *Drosophila* membrane-tethered EGF ligands. EMBO Journal 21: 4277-4286.

46. Urban S, Lee JR, Freeman M (2001) *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107: 173-182.

47. Dowse TJ, Soldati D (2005) Rhomboid-like proteins in Apicomplexa: phylogeny and nomenclature. Trends Parasitol 21: 254-258.

48. Brossier F, Starnes GL, Beatty WL, Sibley LD (2008) Microneme rhomboid protease TgROM1 is required for efficient intracellular growth of *Toxoplasma gondii*. Eukaryotic Cell 7: 664-674.

49. Vera IM, Beatty WL, Sinnis P, Kim K (2011) *Plasmodium* protease ROM1 is important for proper formation of the parasitophorous vacuole. PLoS Pathog 7: e1002197.

50. Singh S, Plassmeyer M, Gaur D, Miller LH (2007) Mononeme: a new secretory organelle in *Plasmodium falciparum* merozoites identified by localization of rhomboid-1 protease. Proc Natl Acad Sci U S A 104: 20043-20048.

51. Dowse TJ, Pascall JC, Brown KD, Soldati D (2005) Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion. International Journal for Parasitology 35: 747-756.

52. Santos JM, Graindorge A, Soldati-Favre D (2012) New insights into parasite rhomboid proteases. Molecular and Biochemical Parasitology 182: 27-36.

53. O'Donnell RA, Hackett F, Howell SA, Treeck M, Struck N, et al. (2006) Intramembrane proteolysis mediates shedding of a key adhesin during erythrocyte invasion by the malaria parasite. J Cell Biol 174: 1023-1033.

54. Dowse TJ, Pascall JC, Brown KD, Soldati D (2005) Apicomplexan rhomboids have a potential role in microneme protein cleavage during host cell invasion. Int J Parasitol 35: 747-756.

55. Sheiner L, Santos JM, Klages N, Parussini F, Jemmely N, et al. (2010) *Toxoplasma gondii* transmembrane microneme proteins and their modular design. Molecular Microbiology 77: 912-929.

56. Trasarti E, Pizzi E, Pozio E, Tosini F (2007) The immunological selection of recombinant peptides from *Cryptosporidium parvum* reveals 14 proteins expressed at the sporozoite stage, 7 of which are conserved in other apicomplexa. Molecular & Biochemical Parasitology 152: 159-169.

57. Xiao L (2010) Molecular epidemiology of cryptosporidiosis: an update. Experimental Parasitology 124: 80–89.

58. Waldron LS, Dimeski B, Beggs PJ, Ferrari BC, Power ML (2011) Molecular epidemiology, spatiotemporal analysis, and ecology of sporadic human cryptosporidiosis in Australia. Appl Environ Microbiol 77: 7757-7765.

59. Feltus DC, Giddings CW, Schneck BL, Monson T, Warshauer D, et al. (2006) Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. Journal of Clinical Microbiology 44: 4303–4308.

60. Fayer R (2010) Taxonomy and species delimitation in *Cryptosporidium*. Exp Parasitol 124: 90–97.

61. Xiao L, Fayer R, Ryan U, Upton SJ (2004) *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev 17: 72-97.

62. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.

63. Baker RP, Young K, Feng L, Shi Y, Urban S (2007) Enzymatic analysis of a rhomboid intramembrane protease implicates transmembrane helix 5 as the lateral substrate gate. Proceedings of the National Academy of Sciences of the United States of America 104: 8257-8262.

64. Templeton TJ, Lancto CA, Vigdorovich V, Liu C, London NR, et al. (2004) The *Cryptosporidium* oocyst wall protein is a member of a multigene family and has a homolog in Toxoplasma. Infect Immun 72: 980-987.

65. Gut J, Nelson RG (1999) *Cryptosporidium parvum*: synchronized excystation in vitro and evaluation of sporozoite infectivity with a new lectin-based assay. J Eukaryot Microbiol 46: 56S-57S.

66. Lemberg MK, Freeman M (2007) Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. Genome Res 17: 1634-1646.

67. Mauzy MJ, Enomoto S, Lancto CA, Abrahamsen MS, Rutherford MS (2012) The *Cryptosporidium parvum* transcriptome during in vitro development. PLoS One 7: e31715.

68. Kuang R, Gu J, Cai H, Wang Y (2009) Improved prediction of malaria degradomes by supervised learning with SVM and profile kernel. Genetica 136: 189-209.

69. Uni S, Iseki M, Maekawa T, Moriya K, Takada S (1987) Ultrastructure of *Cryptosporidium muris* (strain RN 66) parasitizing the murine stomach. Parasitol Res 74: 123-132.

70. Mogi T, Kita K (2010) Diversity in mitochondrial metabolic pathways in parasitic protists *Plasmodium* and *Cryptosporidium*. Parasitol Int 59: 305-312.

71. Bonnin A, Ojcius DM, Souque P, Barnes DA, Doyle PS, et al. (2001) Characterization of a monoclonal antibody reacting with antigen-4 domain of gp900 in Cryptosporidium parvum invasive stages. Parasitol Res 87: 589-592.

72. Barnes DA, Bonnin A, Huang JX, Gousset L, Wu J, et al. (1998) A novel multi-domain mucin-like glycoprotein of Cryptosporidium parvum mediates invasion. Mol Biochem Parasitol 96: 93-110.

73. Santos JM, Ferguson DJ, Blackman MJ, Soldati-Favre D (2011) Intramembrane cleavage of AMA1 triggers *Toxoplasma* to switch from an invasive to a replicative mode. Science 331: 473-477.

74. Jenkins MC, O'Brien C, Miska K, Schwarz RS, Karns J, et al. (2011) Gene expression during excystation of *Cryptosporidium parvum* oocysts. Parasitology Research 109: 509-513.

75. Snelling WJ, Lin Q, Moore JE, Millar BC, Tosini F, et al. (2007) Proteomics analysis and protein expression during sporozoite excystation of *Cryptosporidium*

parvum (Coccidia, Apicomplexa). Molecular and Cellular Proteomics 6: 346-355.

76. Ferguson DJ, Belli SI, Smith NC, Wallach MG (2003) The development of the macrogamete and oocyst wall in *Eimeria maxima*: immuno-light and electron microscopy. Int J Parasitol 33: 1329-1340.

7. Ferguson DJ, Brecht S, Soldati D (2000) The microneme protein MIC4, or an MIC4-like 7 protein, is expressed within the macrogamete and associated with oocyst wall formation in *Toxoplasma gondii*. Int J Parasitol 30: 1203-1209.

78. Marchant J, Cowper B, Liu Y, Lai L, Pinzan C, et al. (2012) Galactose recognition by the apicomplexan parasite *Toxoplasma gondii*. J Biol Chem 287: 16720-16733.

79. Chatterjee A, Banerjee S, Steffen M, O'Connor RM, Ward HD, et al. (2010) Evidence for mucin-like glycoproteins that tether sporozoites of *Cryptosporidium parvum* to the inner surface of the oocyst wall. EukaryotCell 9: 84-96.

80. Forney JR, Yang S, Du C, Healey MC (1996) Efficacy of serine protease inhibitors against *C. parvum* infection in a bovine fallopian tube epithelial cell culture system. Journal of Parasitology 82: 638-640.

81. Forney JR, Yang S, Healey MC (1996) Protease activity associated with excystation of *C.parvum* oocysts. Journal of Parasitology 82: 889-892.

82. Ejigiri I, Ragheb DR, Pino P, Coppi A, Bennett BL, et al. (2012) Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity. PLoS Pathog 8: e1002725.

83. Hegge S, Münter S, Steinbuchel M, Heiss K, Engel U, et al. (2010) Multistep adhesion of *Plasmodium* sporozoites. FASEB Journal 24: 2222-2234.

84. Münter S, Sabass B, Selhuber-Unkel C, Kudryashev M, Hegge S, et al. (2009)

Plasmodium sporozoite motility is modulated by the turnover of discrete adhesion sites. Cell HostMicrobe 6: 551-562.

85. Lyon JA, Haynes JD (1986) *P. falciparum* antigens synthesized by schizonts and stabilized at the merozoite surface when schizonts mature in the presence of protease inhibitors. J.of Immunology 136: 2245-2251.

PAPER 2. CPTSP7 AND CPTSP9 EXPRESSION IN *CRYPTOSPORIDIUM PARVUM* LIFE CYCLE STAGES

[2] The material in this chapter was co-authored by Ebot S. Tabe, and John M. McEvoy. Ebot had primary responsibility for designing and perforiming experiements as well as collecting data, analysis, interpretation, and manuscript preparation . Ebot also drafted and revised all versions of this chapter. John M. McEvoy served as proofreader and checked the the result analysis conducted by Ebot S. Tabe.

Abstract

Thrombospondin-family proteins have key roles in apicomplexan motility and invasion of host cells. *Cryptosporidium parvum* encodes 12 thrombospondin-family proteins (TRAP-C1, CpTSP2 through CpTSP12) that remain poorly characterized. We used immunolocalization to determine the temporospatial expression of two *C. parvum* thrombospondins, CpTSP7 and CpTSP9. Polyclonal antibodies against peptides derived from CpTSP7 and CpTSP9 were used to localize proteins in oocysts, sporozoites, and intracellular development stages. Both CpTSP7 and CpTSP9 were localized in sporozoites within oocysts. CpTSP7 was also localized to the wall of bleach-treated intact and non-intact oocysts. Both proteins were expressed in sporozoites, and their expression patterns varied with time. Both proteins were expressed in trophozoites, in merozoites within developing meronts, and in merozoites exiting from meronts. The detection of these proteins in sporozoites and merozoites suggests roles in motility and invasion. Localization in the oocyst wall suggests a potentially novel function for CpTSP7 during excystation.

Introduction

Cryptosporidium parvum is an apicomplexan parasite that causes cryptosporidiosis, a waterborne diarrheal disease, which can be chronic and life threatening in those with a compromised immune system [1-3]. Cryptosporidiosis primarily affects the small intestine [4-6], but can affect extraintestinal sites immunocompromised individuals [7,8]. *Cryptosporidium parvum* is also a major cause of diarrheal disease in livestock, particularly pre−weaned dairy cattle [9-11]. There is currently no effective drug treatment or vaccine for cryptosporidiosis [12-15]; however, vaccines have shown some promise in animal models [16-18]. The discovery of effective therapeutics and vaccines has been hindered by a poor understanding of *C. parvum* pathogenesis [19].

Among the proteins known to function in apicomplexan attachment, motility, and invasion, thrombospondin family proteins have been shown to play an important role in generating the force required for forward momentum [20,21]. These proteins have a general architecture characterized by thrombospondin type 1 (TSP-1) repeats and other extracellular adhesive domains, a transmembrane domain and a cytoplasmic tail [22,23]. They are secreted from micronemes to the anterior surface of zoites where the adhesive ectodomain binds molecules on the host cell surface. The cytoplasmic tail links thrombospondin proteins with filamentous actin via an aldolase cross linker [24,25]. Myosin A links filamentous actin to the parasite inner membrane complex [26,27], and moves in an anterior direction, displacing the actin bound thrombospondin protein in a posterior direction. The retrograde translocation and subsequent shedding of thrombospondins creates the force that drives gliding motility.

TRAP-C1 was the first thrombospondin family protein identified in *C. parvum*, and genes encoding a further 11 putative thrombospondins (named CpTSP2 through CpTSP12) were later described [28]. Of these, TRAP-C1 [Chapter 2; 29,30], CpTSP8 [31] and CpTSP10 (Chapter 2) have been characterized to date. TRAP-C1 is localized to apical and sub-apical regions [Chapter 2] of sporozoites and the inner wall of oocysts [30], CpTSP8 is localized to the apical region of sporozoites [31], and CpTSP10 is localized to the posterior pole of sporozoites [Chapter 2].

In this study, we determine the localization of two thrombospondin proteins, CpTSP7 and CpTSP9, in oocysts, sporozoites, and intracellular stages of *C. parvum*.

Materials and Methods

Oocyst source **.** Mouse passaged oocysts of the *C. parvum* Iowa isolate were purchased from Waterborne Inc. (New Orleans, LA). Oocysts were stored at 4°C in PBS supplemented

with 100 IU/mL penicillin and 100 μg/mL streptomycin) until use. Oocysts were between 1 and 6 months old at the time of use.

Polyclonal antibody production. Polyclonal antisera were produced to short peptide sequences derived from the extracellular domain of CpTSP7 and CpTSP9. Peptides were synthesized in silico (see chapter 4) and used to immunize rabbits (ProSci Inc., Poway, CA). Antibodies were affinity purified on a peptide sepharose column.

*Protein immunolocalization in oocysts and sporozoites***.** Protein immunolocalization was determined in oocyst walls and sporozoites before excystation (intact oocysts), following bleach treatment (10% v/v ice-cold sodium hypochlorite for 10 min at 4°C and three washes in PBS) to remove the outer veil (bleach treated oocysts), or following excystation (non-intact oocysts and free sporozoites). Fifty microliters of intact and bleach treated oocysts (approximately 10^4 oocyst/mL) were air-dried on pre-cleaned glass slides and fixed with equal volumes of ice-cold methanol (100%) and 4% formaldehyde for 10 min. Oocysts were excysted after bleach treatment by incubation in excystation media (RPMI-FBS with 0.075 % sodium taurocholate) in a humidified incubator at 37°C and 5% CO_2. Excystation was stopped at 60 min by transferring the suspensions to ice. Following excystation, the suspension was washed three washes in PBS, air–dried on a pre–cleaned glass slide, and fixed with 4% formaldehyde.

Intact, bleach-treated, and non–intact oocysts and free sporozoites were permeabilized with 1% Triton X–100 in 1% bovine serum albumin (BSA) for 15 min, blocked with 1% BSA for 20 min, and probed overnight at 4°C with biotinylated *Vicia villosa* lectin (VVL; 1:2500 dilution in 1 % BSA; Vector Laboratories Inc., CA) and polyclonal antibodies specific for CpTSP7 and CpTSP9 (1:100 dilution in 1 % BSA). A 1% BSA negative control was included with all treatments. VVL is a Gal/GalNAc specific lectin that labels both extracellular and

intracellular parasite stages. Following three washes in PBS, bound primary antibodies were detected with goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:1000 dilution in 1 % BSA, Life Technologies, NY). Incubation of the secondary antibody was for 1 h at 37°C and was followed by three washes in PBS. Bound VVL was detected using tetramethylrhodamine isothiocyanate (TRITC) conjugated streptavidin (1:1000 dilutions in 1 % BSA, Vector Laboratories Inc., CA). Nuclei were labeled with 10 µg/mL 4', 6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif.) for 15 minutes at 4°C followed by five washes with chilled PBS.

Protein immunolocalization during in vitro infection. Human colorectal adenocarcinoma (HCT-8) cells were seeded at a concentration of 10^5 cells/mL in a well of an 8–well chamber slide (Becton Dickinson Labware, Franklin Lakes, NJ). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (RPMI–FBS), 1 mM sodium pyruvate, 50 U/mL penicillin G, 50 U/mL streptomycin, and 0.25 µg/mL amphotericin B (pH 7.4) (Sigma-Aldrich Co. St Louis, MO) at 37°C in 5% CO_2.

One hundred thousand (10^5) oocysts were treated with 10% (v/v) ice-cold sodium hypochlorite for 10 min at 4°C, washed three times with PBS at room temperature (16,000 x *g* for 3 min), and resuspended in infection media (RPMI-FBS, 10 mg streptomycin and 25 µg amphotericin B per ml, 1 mM sodium pyruvate, 50 mM glucose, 35 µg/mL ascorbic acid, 1.0 µg/mL folic acid, 4.0 µg/mL para-aminobenzoic acid, and 2.0 µg/mL calcium pantothenate). Resuspended oocysts were used to infect 80-90% confluent HCT-8 cells. Infections were stopped at 2, 6, 12, 24, 48, or 72 h. Infections were performed in duplicate and a mock infection with heat-inactivated oocysts was included as a control. The procedure for the

immunolocalization of CpTSP7 and CpTSP9 was as described for oocysts and sporozoites with the exception that fixation was performed with 100% ice-cold methanol only.

*Confocal microscopy***.** Slides were mounted with No–Fade™ mounting medium (Waterborne™, Inc., New Orleans, LA) and examined by confocal microscopy using a Zeiss Observer Z.1 inverted microscope powered by a Confocal Scanning Laser, LSM700 (Carl Zeiss Microscopy, Jena, Germany). An AxioCam MRc camera (Carl Zeiss Microscopy, Jena, Germany) was used to capture representative z–stack images with 0.33 µm spacing. Images captured at different wavelengths were merged using Carl Zeiss ZEN2011 Software (Carl Zeiss Microscopy, Jena, Germany).

Results

CpTSP7 and CpTSP9 are localized in sporozoites within intact oocysts, and CpTSP7 is localized in the oocyst wall. CpTSP7 and CpTSP9 were localized in sporozoites within intact oocysts (Figure 15, panel I and II). CpTSP7 was localized in the walls of intact oocysts following bleach treatment (Figure 15, panel I, C), and in the walls of excysted oocysts (Figure 15, panel II, IA). In non–intact oocysts, CpTSP7 was primarily localized in a region of the wall opposite the suture. In contrast, CpTSP9 was not detected in the walls of intact (Figure 15, panel II, G) or excysted oocysts (Figure 15, panel III, D).

*CpTSP7 and CpTSP9 are localized to distinct regions of sporozoites following excystation***.** We examined the localization of CpTSP7 and CpTSP9 in sporozoites at 5, 60, and 120 min post-excystation (Figure 16). After 5 min, CpTSP7 was detected to the apical and sub-apical regions of sporozoites (Figure 16, panel I, B). CpTSP9 was similarly detected at the apical and sub-apical regions, and a posterior region (Figure 16, panel II, B). At 60 min, more than 96% of oocysts had excysted and CpTSP7 (Figure 16, panel I, E) was detected at the apical region

only, while CpTS9 was localized in to the anterior two−thirds of the sporozoite and an area surrounding the nucleus (Figure 15, panel II, E). At 120 min post excystation, CpTSP7 (Figure 15, panel I, H) was detected at the middle and posterior of the sporozoite, and CpTSP9 (Figure 15, panel II h) was localized in a region surrounding the nucleus and unilaterally in a sub−apical region. CpTSP7 and CpTSP9 showed distinct expression patterns across the different excystation time points, which may reflect different functional roles for these proteins during motility.

*CpTSP7 and CpTSP9 are expressed during intracellular development.***.** We examined the subcellular location of CpTSP7 and CpTSP9 during an in vitro infection. At 2 h post infection (Figure 17, 2 h p.i.), CpTSP7 and CpTSP9 expression were relatively low. More prominent expression was detected in trophozoites at 6 h p.i. (Figure 17, 6 h p.i.) and meronts at 12 and 24 h p.i. (Figure 17, 12 and 24 h p.i.). Both proteins were also detected at later time points of infection (Figure 17, 48 and 72 h p.i.), including in merozoites near-ruptured meronts at 48 h p.i (Figure 17, 48 h).

Discussion

In previous work (Chapter 2), we showed that CpTSP7 and CpTSP9 have similar patterns of gene expression during *in vitro* infection, with highest expression during asexual stages. We further showed that CpTSP9 but not CpTSP7 is cleaved by a rhomboid protease, CpROM5, in a heterologous assay. The goal in the present study was to determine the immunolocalization of CpTSP7 and CpTSP9 in oocysts, sporozoites, and intracellular developmental stages of *C. parvum*. CpTSP7 and CpTSP9 were both detected in sporozoites within intact oocysts, suggesting that they are stored in readiness for the rapid, excystation-triggered switch to motility that is necessary for egress. The localization of CpTSP7 and CpTSP9 in sporozoites within oocysts is similar to the localization of other thrombospondins and ROMs studied (Chapter 2).

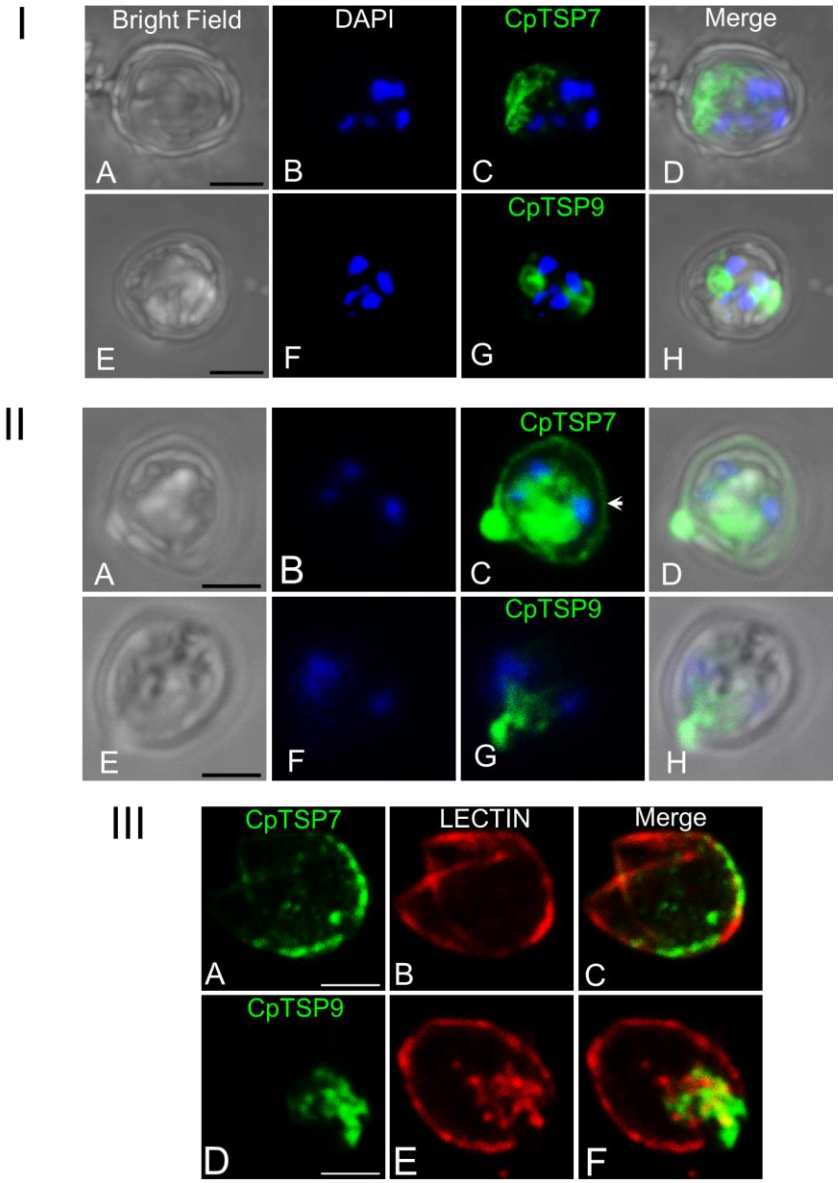

Figure 15. Immunolocalization of CpTSP7 and CpTSP9 in the walls of intact and non-intact oocysts. Following fixation with methanol, intact or non-intact oocyst were subjected to immunolocalization assay as described in methods. (Panel I) CpTSP7 and CpTSP9 expressed and detected with FITC (green) conjugated anti-rabbit antibody on the surface of sporozoites within intact oocysts; (Panel II) CpTSP7 and not CpTSP9 is expressed and detected in the walls of intact (Panel I) and non-intact oocysts (panel III) Lectin as indicated, binds to surface glycoproteins present on oocyst wall and revealed withTetramethylrhodamine isothiocyanate (TRITC) -Streptavidin (red). Images were captured using laser confocal scaning microscope (LSM700) and Z-stacked using a Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

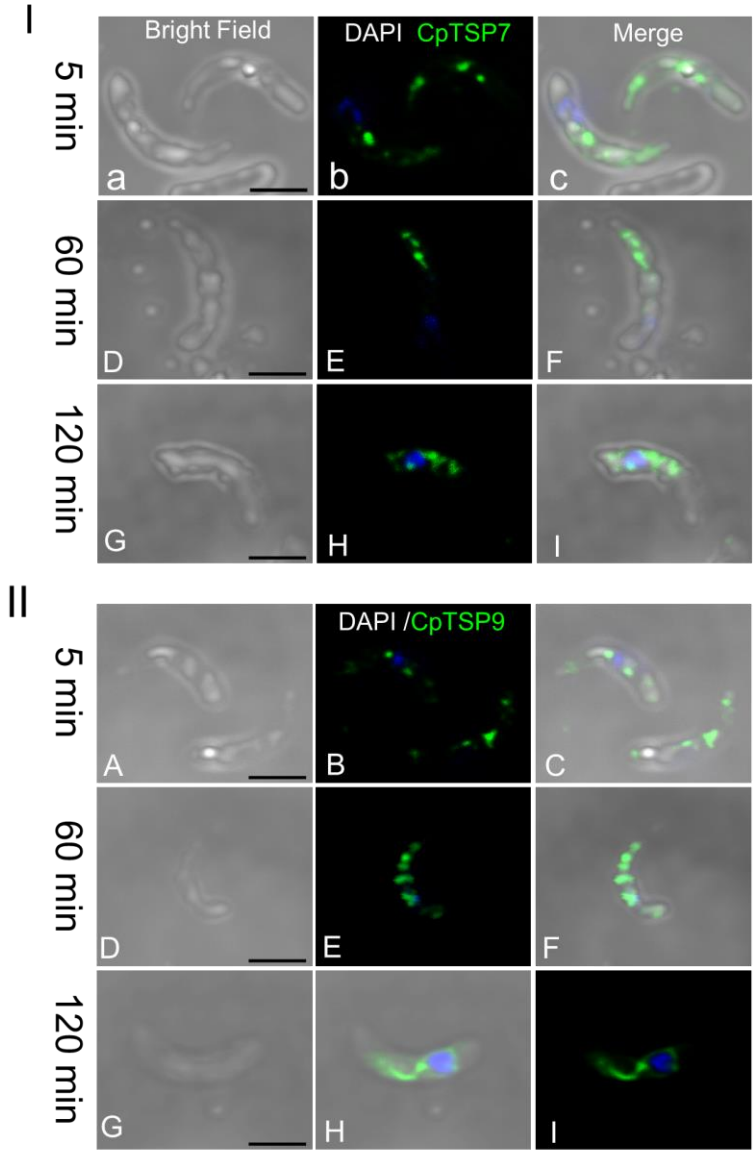

Figure 16. Subcellular localization of CpTSP7 and CpTSP9 sporozoites during motility. Excysted sporozoites were fixed with 4 % formaldehyde, probed with antibodies to CpTSP7 and CpTSP9. (Panel I) Early in excystation (5 min, B), CpTSP7 is localized predominantly to the apical and medial regions in sporozoites. At 60 min, CpTSP7 become localized only to the apical region (E), and subsequently within periphery of the nucleus (120 min, H). (Panel II) CpTSP9 is localized to the apical and medial regions (5 min, B). Expression is seen to intensify at 60 min (E) post excystation. (Panel II, H) CpTSP9 expression at 120 min is observed conspicuously around the nucleus of sporozoites (H) similar to that observed for CpTSP7. (Panel I and II- A, D, G) Bright field is used to show the outline of the zoites while an overlay of FITC and bright field is also indicated (Panel I and II: C, F, I). All images are Z stacked. Scale bars =2 μm.

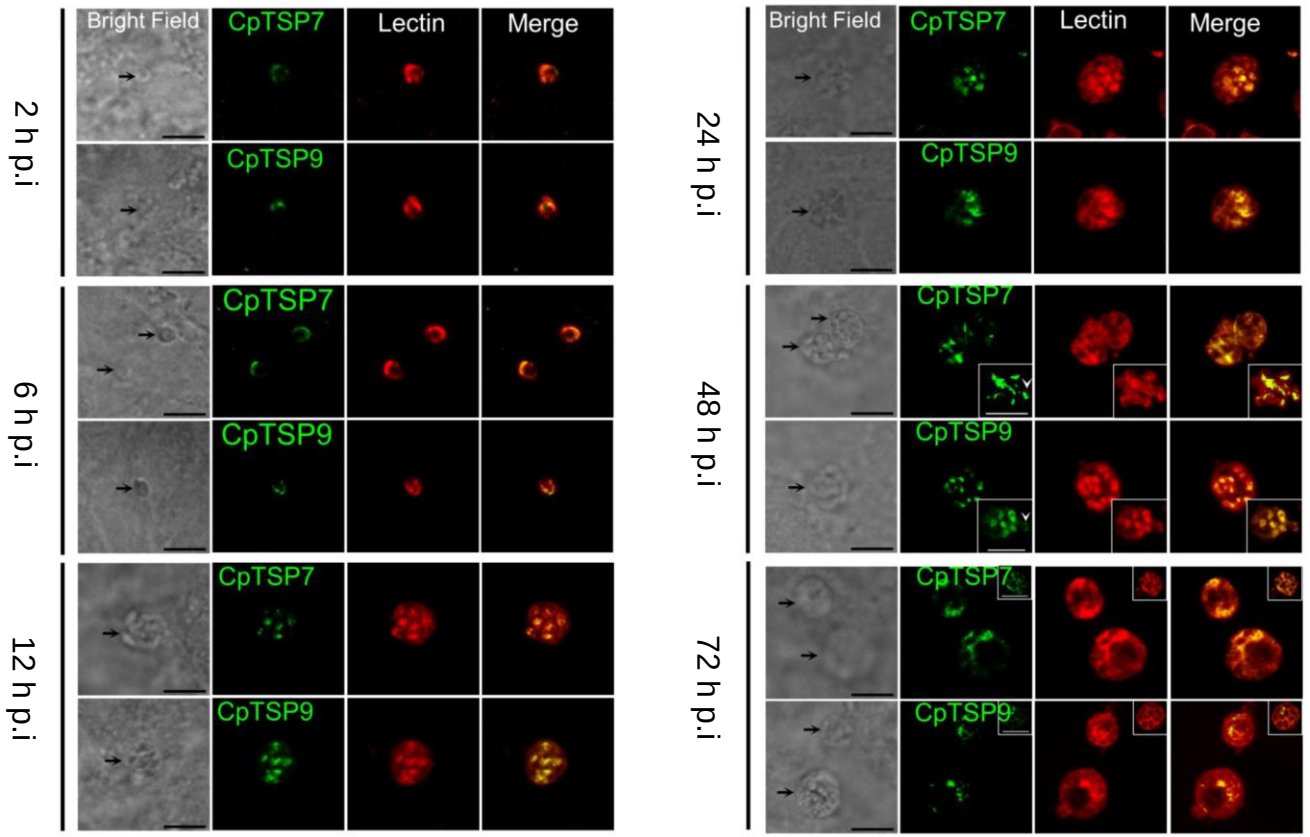

Figure 17. Intracellular localization of CpTSP7 and CpTS9 during a 2-72 h infection of HCT-8 cell line. Shown are representative intracellular stages of *C. parvum* following infection of HCT-8 cells during a 2 to 72 h infection time points. Following fixation with methanol, infected cells were subjected to immunolocalization assay as described in methods. (2 h p.i) Mild expression of both CpTSP7 and 9 (green) in invading sporozoites, and throughout intracellular stages; trophozoites (6 h p.i), in merozoites within meront-like structures (12 to 24 h p.i) and later stages of infection (48 to 72 h p.i). CpTSP7 and 9 are seen in what seem to be merozoites escaping a meront at 48 h p.i (white arrow, boxed lower right). Lectin as indicated, binds to surface glycoproteins present on intracellular stages and revealed with Tetramethylrhodamine isothiocyanate (TRITC) - Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using a Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

The detection of CpTSP7 in the walls of non-intact and bleach–treated intact oocysts is similar to previous findings for TRAP-C1 (Chapter 2); however, the localization patterns of the two proteins in non-intact oocysts differ. TRAP-C1 shows punctate expression throughout the oocyst wall, which is similar to the localization of CpROM5 (Chapter 2) and GP900 [32]. In contrast, CpTSP7 is primarily localized to regions opposite the suture. The differing localization of CpTSP7 and TRAP-C1 in the oocyst wall, and their differing susceptibilities to rhomboid protease activity (TRAP-C1 is cleaved by CpROM5; CpTSP7 is not cleaved), suggest that they may have different functions in the oocyst wall.

Thrombospondin proteins can contribute to the traction (through the formation of adhesion sites) and force (by connecting to the actomyosin motor) that is necessary for gliding motility and invasion of host cells [33]. We examined temporal changes in the localization of CpTSP7 and CpTSP9 in sporozoites post excystation. CpTSP7 was localized to the apical region shortly after excystation (5 min), but was localized to the posterior region of the sporozoite after 120 min. Similar to TRAP-C1, the cytoplasmic tail of CpTSP7 is predicted to interact with the actomyosin motor [28]; therefore, its posterior localization may have resulted from actomyosin-powered retrograde translocation on the sporozoite surface. In addition to a predominantly apical localization at 5 and 60 min post excystation, CpTSP9 was localized in an area surrounding the nucleus and unilaterally towards the anterior of the sporozoite at 120 min post excystation, which may reflect the synthesis and subsequent transport of the protein to the microneme vesicles in the apical region of the parasite [34].

CpTSP7 and CpTSP9 expression was low in trophozoites at 2 h p.i., relative to other stages. This is consistent with the low CpTSP7 and CpTSP9 mRNA transcript levels at this time point (Chapter 2). However, both proteins are clearly expressed in individual merozoites in

developing meronts at 12 and 24 h p.i. Merozoites have a similar cell structure to sporozoites and these thrombospondin proteins may have similar functions in both invasive stages.

Conclusion

In summary, we showed that both CpTSP7 and CpTSP9 were detected in oocysts, sporozoites, and intracellular stages of *C. parvum*. The different expression patterns of these proteins in oocysts and sporozoites suggests that they have distinct roles during motility and invasion. Therefore it is fundamental to characterize these proteins to further determine their role(s) in apicomplexan development which can serve as targets of protective antibody in order to develop rational strategies for therapy of cryptosporidiosis and for vaccine development.

References

1. Agarwal A, Ningthouja S, Sharma D, Mohen Y, Singh NB (1998) *Cryptosporidium* and HIV. J Indian Med Assoc 96: 276-277.

2. Ajjampur SS, Sankaran P, Kang G (2008) Cryptosporidium species in HIV-infected individuals in India: an overview. Natl Med J India 21: 178-184.

3. Anand L, Brajachand NG, Dhanachand CH (1996) Cryptosporidiosis in HIV infection. J Commun Dis 28: 241-244.

4. Kelly P, Makumbi FA, Carnaby S, Simjee AE, Farthing MJ (1998) Variable distribution of *Cryptosporidium parvum* in the intestine of AIDS patients revealed by polymerase chain reaction. Eur J Gastroenterol Hepatol 10: 855-858.

5. Laurent F, McCole D, Eckmann L, Kagnoff MF (1999) Pathogenesis of *Cryptosporidium parvum* infection. Microbes Infect 1: 141-148.

6. Pantenburg B, Dann SM, Wang HC, Robinson P, Castellanos-Gonzalez A, et al. (2008) Intestinal immune response to human *Cryptosporidium* sp. infection. Infect Immun 76: 23-29.

7. de Souza LoR, Rodrigues MA, Morceli J, Kemp R, Mendes RP (2004) Cryptosporidiosis of the biliary tract mimicking pancreatic cancer in an AIDS patient. Rev Soc Bras Med Trop 37: 182-185.

8. Palmieri F, Cicalini S, Froio N, Rizzi EB, Goletti D, et al. (2005) Pulmonary cryptosporidiosis in an AIDS patient: successful treatment with paromomycin plus azithromycin. Int J STD AIDS 16: 515-517.

9. Fayer R (2004) Cryptosporidium: a water-borne zoonotic parasite. Vet Parasitol 126: 37-56.

10. Meisel JL, Perera DR, Meligro C, Rubin CE (1976) Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. Gastroenterology 70: 1156-1160.

11. Nime FA, J. D. Burek, D. L. Page, M. A. Holscher, and J. H. Yardley. (1976) Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. Gastroenterology 70: 592-598.

12. Mead JR (2002) Cryptosporidiosis and the challenges of chemotherapy. Drug Resist Updat 5: 47-57.

13. Chalmers RM, Giles M (2010) Zoonotic cryptosporidiosis in the UK - challenges for control. J Appl Microbiol 109: 1487-1497.

14. Fayer R, Morgan U, Upton SJ (2000) Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int J Parasitol 30: 1305-1322.

15. O'Hara SP, Chen XM (2011) The cell biology of *Cryptosporidium* infection. Microbes Infect 13: 721-730.

16. Jenkins MC (2001) Advances and prospects for subunit vaccines against protozoa of

veterinary importance. Vet Parasitol 101: 291-310.

17. Jenkins M, Higgins J, Kniel K, Trout J, Fayer R (2004) Protection of calves against cryptosporiosis by oral inoculation with gamma-irradiated *Cryptosporidium parvum* oocysts. J Parasitol 90: 1178-1180.

18. Jenkins M, Kerr D, Fayer R, Wall R (1995) Serum and colostrum antibody responses induced by jet-injection of sheep with DNA encoding a *Cryptosporidium parvum* antigen. Vaccine 13: 1658-1664.

19. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium parvum* genes containing thrombospondin type 1 domains. Infect Immun 70: 6987-6995.

20. Sultan AA, Thathy V, Frevert U, Robson KJ, Crisanti A, et al. (1997) TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. Cell 90: 511-522.

21. Morahan BJ, Wang L, Coppel RL (2009) No TRAP, no invasion. Trends Parasitol 25: 77-84.

22. Kappe SH, Buscaglia CA, Bergman LW, Coppens I, Nussenzweig V (2004) Apicomplexan gliding motility and host cell invasion: overhauling the motor model. Trends in Parasitology 20: 13-16.

23. Akhouri RR, Bhattacharyya A, Pattnaik P, Malhotra P, Sharma A (2004) Structural and functional dissection of the adhesive domains of *Plasmodium falciparum* thrombospondin-related anonymous protein (TRAP). Biochemistry Journal 379: 815-822.

24. Jewett TJ, Sibley LD (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. Mol Cell 11: 885-894.

25. Buscaglia CA, Coppens I, Hol WG, Nussenzweig V (2003) Sites of interaction between aldolase and thrombospondin-related anonymous protein in *Plasmodium*. Molecular Biology of the Cell 14: 4947-4957.

26. Meissner M, Schluter D, Soldati D (2002) Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. Science 298: 837-840.

27. Dobrowolski JM, Carruthers VB, Sibley LD (1997) Participation of myosin in gliding motility and host cell invasion by Toxoplasma gondii. Molecular Microbiology 26: 163-173.

28. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium parvum* genes containing thrombospondin type 1 domains. Infection and Immunity 70: 6987-6995.

29. Spano F, Putignani L, Guida S, Crisanti A (1998) Cryptosporidium parvum: PCR-RFLP analysis of the TRAP-C1 (thrombospondin-related adhesive protein of *Cryptosporidium*-1) gene discriminates between two alleles differentially associated with parasite isolates of animal and human origin. Exp Parasitol 90: 195-198.

30. Spano F, Putignani L, Naitza S, Puri C, Wright S, et al. (1998) Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. Mol Biochem Parasitol 92: 147-162.

31. Putignani L, Possenti A, Cherchi S, Pozio E, Crisanti A, et al. (2008) The thrombospondin-related protein CpMIC1 (CpTSP8) belongs to the repertoire of micronemal proteins of *Cryptosporidium parvum*. Mol Biochem Parasitol 157: 98-101.

32. Chatterjee A, Banerjee S, Steffen M, O'Connor RM, Ward HD, et al. (2010) Evidence for mucin-like glycoproteins that tether sporozoites of *Cryptosporidium parvum* to the inner surface of the oocyst wall. EukaryotCell 9: 84-96.

33. Carruthers VB, Tomley FM (2008) Microneme proteins in apicomplexans. Subcell Biochem 47: 33-45.

34. Tetley L, Brown SM, McDonald V, Coombs GH (1998) Ultrastructural analysis of the sporozoite of *Cryptosporidium parvum*. Microbiology 144 (Pt 12): 3249-3255.

PAPER 3. SYNTHETIC PEPTIDE AND POLYCLONAL ANTIBODY INHIBITION OF *CRYPTOSPORIDIUM PARVUM* INVASION IN VITRO

[3] The material in this chapter was co-authored by Ebot S. Tabe, and John M. McEvoy. Ebot had primary responsibility for designing and perforiming experiements as well as collecting data, analysis, interpretation, and manuscript preparation . Ebot also drafted and revised all versions of this chapter. John M. McEvoy served as proofreader and checked the the result analysis conducted by Ebot S. Tabe.

Abstract

The apicomplexan parasite *Cryptosporidium parvum* causes diarrheal disease worldwide. As an intracellular parasite, attachment to and invasion of host intestinal epithelial cells by *C. parvum* sporozoites are critical steps during an infection. In this study, we examined the effects of short peptides and polyclonal antibodies in reducing *C. parvum* invasion of HCT–8 cells. Five peptides derived from conserved adhesive domains in four thrombospondin family proteins were selected for the study. The peptides were derived from an Apple domain in TRAP–C1 (TAAP), a thrombospondin type I repeat domain in CpTSP7 (7TS) and CpTSP9 (9TS), and a Kringle domain in CpTSP10 (10K1). Peptides were synthesized *in silico* and were used to immunize rabbits. Peptides and peptide-affinity purified rabbit polyclonal antibodies were examined for their effect on *C. parvum* infection in an in vitro infection model. TAAP, 7TS, 9TS, and 10K1 bound to unidentified receptors on HCT–8 cells. Individually and in combination, these peptides significantly reduced invasion of HCT–8 cells by *C. parvum* in a dose dependent manner. The greatest inhibitory effect was observed when peptides were used in combination. Polyclonal antibodies produced to TAAP, 7TS, 9TS, and 10K1 did not inhibit excystation but caused excysted sporozoites to agglutinate in a concentration–dependent manner. Polyclonal antibodies caused the greatest inhibition when used in combination. Collectively, the data presented in this study provide compelling evidence that conserved adhesive domains in thrombospondin family proteins are important for efficient host cell invasion, and may be effective targets for chemotherapeutic interventions.

Introduction

Since the discovery of *Cryptosporidium parvum* as a cause of the diarrheal disease cryptosporidiosis in humans, a major research focus has been the development of effective chemotherapeutic interventions. Unfortunately, efforts to date have been largely unsuccessful, particularly in the treatment of chronic, life-threatening cryptosporidiosis in immunocompromised individuals [2-6]. More than 200 antimicrobials have been tested in animals and humans and none have been found to be consistently effective against this parasite [7]. Paromomycin and nitazoxanide have shown some clinical efficacy in the treatment of biliary cryptosporidiosis [8] but neither drug is effective in the absence of a robust T-cell response. Nitazoxanide has been approved in the United States (U.S.) for treatment of cryptosporidiosis in immunocompetent children under 12 years of age [9]. Paromomycin has shown effectiveness against *C. parvum* infections in animals [10-12], cell cultured models [13], and AIDS patients [14,15]. Used in combination with azithromycin, paromomycin showed some effect in one uncontrolled trial involving AIDS patients with cryptosporidiosis [16]. However, there is not enough evidence to support the role of chemotherapeutic agents in the management of cryptosporidiosis among immunocompromised individuals as the results of several studies showed that nitazoxanide reduces the parasite load and may be useful in immunocompetent but not immunocompromised individuals [17]. Therefore, effective drug treatments are an urgent priority.

Animal and human studies have shown that antibodies can effectively reduce *C. parvum* infection [18,19]. Consequently, research efforts have been focused on identifying vaccine targets. Yet, despite considerable structural and immunological data generated from the characterization of sporozoite surface antigens, a vaccine is not yet available [20]. Given that

Cryptosporidium is refractory to genetic manipulation and difficult to propagate in the laboratory, *in silico* analyses based on the available genome sequence information are important in the pursuit of novel vaccine targets.

Apicomplexan microneme adhesins, particularly those belonging to the thrombospondin family, play an important role in substrate–dependent gliding motility and invasion of host cells [21-24]. Examples of apicomplexan thrombospondins include TRAP [25-27] and CTRP [28,29] in *Plasmodium*, Etp100 in *Eimeria* [30-32], TgMIC2 in *Toxoplasma* [33,34], NcMIC2 in *Neospora* [35], and TRAP-C1 in *Cryptosporidium*. TRAP–C1 is one of 12 thrombospondins identified in *C. parvum*. These proteins are developmentally regulated, with generally greatest expression during asexual stages [36, and Chapter 2 of this dissertation], and protein expression has been demonstrated in invasive sporozoites [37,38, and Chapters 2 and 3 of this dissertation]. Okhuysen et al., [39] showed that healthy adults produce antibodies to recombinant TRAP-C1 during a *C. parvum* infection, suggesting that these proteins may be useful targets for chemotherapies directed at the mucosal surfaces [40,41].

In the present study, synthetic peptides derived from the *C. parvum* thrombospondins CpTSP7, CpTSP9, CpTSP10, and TRAP-C1, and polyclonal antibodies to these peptides were examined for their ability to inhibit host cell invasion by *C. parvum* sporozoites.

Materials and Methods

Identification of peptide sequences. Predicted full-length protein sequences of C. parvum TRAP-C1, CpTSP7, CpTSP9, and CpTSP10 were obtained from the Cryptosporidium genome database CryptoDB (www.cryptodb.org). The domain architecture was predicted from protein sequences using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de). The locations of Apple domains in TRAP-C1 were obtained

from [1]. A schematic representation of as part of the Lasergene core suite (Lasergene, Madison, WI). Short antigenic sequences from predicted adhesive regions in the ectodomain of proteins were used in a BLAST analysis of proteins published in GenBank. Five sequences, showing no significant similarity to human proteins or Cryptosporidium proteins other than the target protein, were selected for peptide synthesis and polyclonal antibody production. The locations of the four peptides are shown in Figure 18. They are named TAAP (residues 298-312– SGNKNTRDADSDTGT in a TRAP-C1 Apple domain), 7TS (residues 376-392– STGSKARYRSVIMPS in a CpTSP7 thrombospondin type I repeat domain), 9TS (residues 316-328– GPGNKMRYRII in a CpTSP9 thrombospondin type I repeat domain), and 10K1 (residues 192-208– KFDAFDYLATLENSGIADH in the CpTSP10 Kringle domain) . The domain architecture with the predicted amino acid positions of each domain is shown in Figure 18. Antigenic regions were predicted from protein sequences using the Protean program.

Peptide and polyclonal antibody production. Synthetic peptide synthesis and polyclonal antibody production were carried out by ProSci Inc. (Poway, CA). Two rabbits were immunized in an eight-week schedule. Two pre-bleeds (week 0), approximately 5 mL per rabbit were collected followed by four immunizations (at weeks 0, 2, 4, and 6) with synthetic peptides (200 µg/rabbit in Complete Freund's Adjuvant at week 0 and 100 µg/ rabbit in Complete Freund's Adjuvant at weeks 2, 4, and 6). Post-immunization or production bleeds (20-25 mL serum) were collected at weeks 5, 7, and 8. Pre-and post-immunized rabbit sera were purified by immunoaffinity chromatography. Briefly, sera were loaded onto Profinity IMAC nickel-charged resin columns in buffer containing 20 mM imidazole. Five milliliters of IgG eluate was passed through the column 10 times at a slow flow rate (about 10 min/pass) for 1–1.5 h. This allowed antibodies in sera to bind to imidazole after which the bound antibody was eluted with buffer

containing 0.5 mL 1 M Tris, pH 8.0 and 1 mL of 50 mg/mL BSA and dialyzed overnight. Sodium azide was added to a final concentration of 0.02% and stored at 4°C or – 20°C. Purified antibodies were quantified by the Bradford method using Nanodrop 1000 (Thermo Fisher Scientific Inc, TX) spectrophotometer.

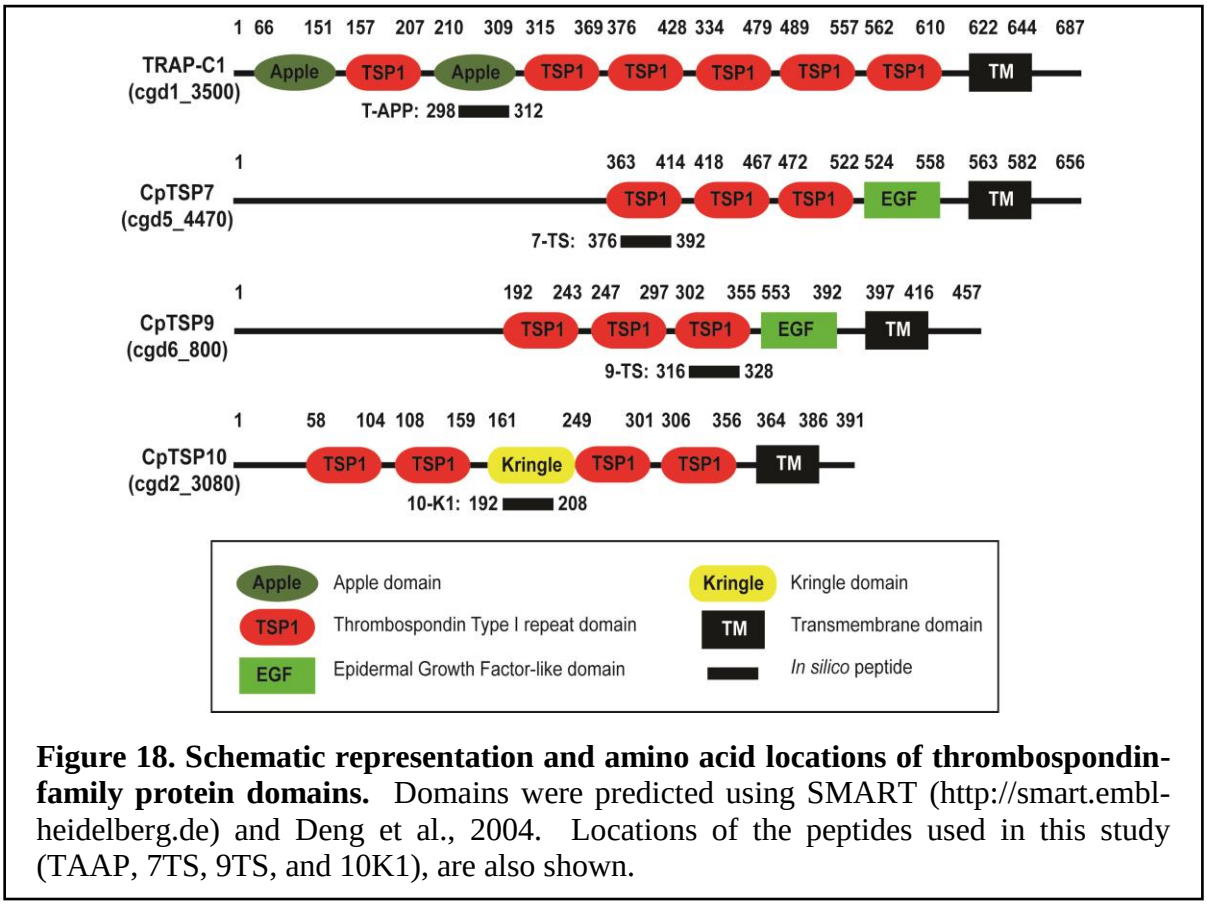

Figure 18. Schematic representation and amino acid locations of thrombospondin-family protein domains. Domains were predicted using SMART (http://smart.embl-heidelberg.de) and Deng et al., 2004. Locations of the peptides used in this study (TAAP, 7TS, 9TS, and 10K1), are also shown.

Quantification of peptide binding. HCT-8 cells were cultured on cover slips (Becton Dickinson) until 90- 95% confluent (approximately 24 h), and washed three times with ice-cold HEPES buffer (50 mM HEPES, 128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5% heat-inactivated fetal bovine serum). HEPES buffer containing 3% BSA (blocking buffer) was added for 2 h to block non-specific binding peptide.

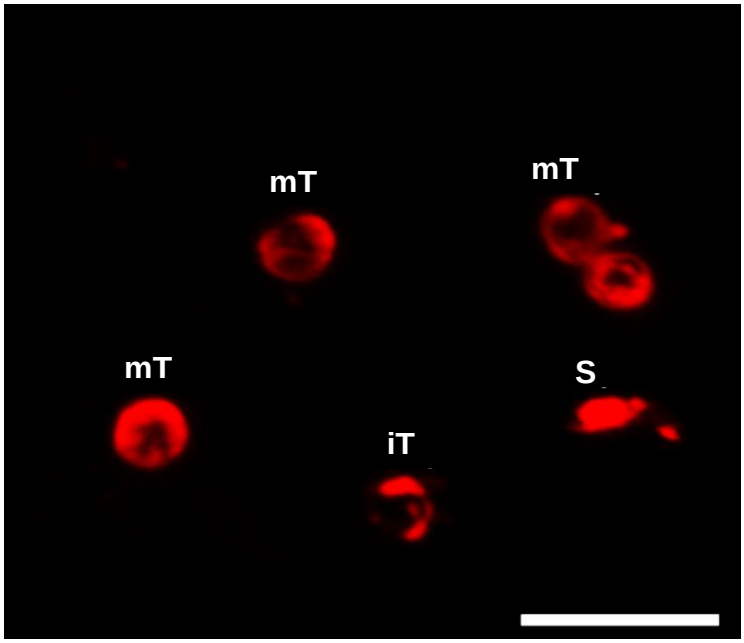

Figure 19. Multiple *C. parvum* stages used as outcome variables to measure peptide and antibody inhibition. Sporozoites (S), imature trophozoites (iT), and mature trophozoites (mT) were probed p.i with Lectin VVL and revealed with Tetramethylrhodamine isothiocyanate (TRITC) -Streptavidin (red). Images were captured using laser confocal scanning microscope (LSM700) and Z-stacked using Zeiss AxioVision 4.8 image software. Scale bar = 5 µm.

TAAP, 7TS, 9TS, and 10K1 were each suspended in HEPES buffer and added to the wells to achieve a final concentration of 100 or 200 µM. Peptides derived from the cytoplasmic domain of two C. parvum rhomboid proteases, CpROM4 and CpROM5, and cells incubated with HEPES buffer alone were used as negative controls. Cells were incubated at room temperature for 2 h, rinsed three times with HEPES buffer, fixed with methanol for 5 min and labeled with rabbit polyclonal antibodies specific for each peptide. DAPI was used to label cell nuclei. FITC-conjugated goat anti-rabbit antibody was used to detect primary antibody labeling. Localization of fluorescence labeling was determined microscopically using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany). The average fluorescence intensity for each peptide treatment was measured and recorded. Intensity of FITC fluorescence was

determined using the Zen 2010 analysis system (Carl Zeiss, Inc.) and reported as arbitrary units/μm^2.

Inhibition assay with synthetic peptide. In separate treatments, individual peptides were added to HCT-8 cells cultured on cover slips (VWR, Radnor, PA) to obtain a final concentration of 100 or 200 μM as described earlier. In an additional treatment, all peptides were combined in a cocktail, at a final concentration of 100 or 200 μM for each peptide. Following a 2 h incubation at 37°C, unbound peptide was rinsed from cells with warm HEPES buffer. Cryptosporidium parvum oocysts (10^4 oocysts/treatment) were treated with 10% (v/v) ice-cold sodium hypochlorite for 10 min at 4°C (bleach treated), washed three times in PBS, resuspended in infection media consisting of RPMI 1640 (Sigma Aldrich, St. Louis, MO) supplemented with glutamine, antibiotics and 5% fetal bovine serum (FBS), and allowed to excyst over 90-95% confluent HCT-8 cells. The infection was allowed to proceed at 37°C in a 5% CO_2 atmosphere for 2 or 6 h. Media was removed and cells were washed with chilled PBS before fixing with 100% ice-cold methanol (–20°C). All treatments were performed in duplicate.

Neutralization assay with polyclonal antibody. In separate treatments, polyclonal antibodies against TAAP, 7TS, 9TS, or 10K1 were added to bleach treated oocysts (10^3/100 μL) suspended in RPMI-FBS with 0.075% sodium taurocholate and incubated in a humidified incubator at 37°C and 5% CO_2. The final antibody concentration in treatments was 50, 100, 150, or 200 μM. In an additional treatment, antibodies were used in combination at a final concentration of 50, 100, 150, or 200 μM for each antibody. Excystation was terminated at 15, 30, 45, 60, or 120 min and the proportion of intact oocysts were calculated as previously described [42]. In separate experiments, antibodies were each added to 10^5 bleach treated oocysts in infection media to a final concentration 50, 100, 150, or 200 μM, or used in

combination at a final concentration of 50 or 100 or 150 or 200 µM for each antibody. Infection of HCT-8 cells was performed as described above. A control consisting of infection media without antibody was included as a negative control. A polyclonal antibody against the cytoplasmic tail of a rhomboid protease (anti-CpROM) was also included as a negative control. Following incubation, infection media was removed, cells were washed with chilled PBS, and fixed with 100% methanol (-20°C). All treatments were performed in duplicate.

*Immunofluorescence microscopy***.** The procedure for immunofluorescence microscopy was based on a protocol described previously [43], with some modifications. Cover slips from peptide and antibody treatments were rehydrated twice for 5 min in PBS. Non-specific binding sites were blocked with 1% (v/v) bovine serum (Jackson Immunoresearch Laboratory, Inc., West Grove, PA, USA) in PBS for 30 min. Cells were incubated with biotinylated Vicia villosa lectin (VVL, Vector Laboratory, San Mateo, CA) in PBS containing 1% BSA (w/v), incubated overnight in a humid chamber at 4°C, washed three times in PBS, incubated with streptavidin-TRITC (Vector Laboratories Inc., CA) in the dark for 1 h at room temperature, and washed three times in PBS. Slides were mounted with antifade medium (Waterborne Inc, LA), sealed with nail polish, and observed under an Olympus BX61 Motorized Microscope with epifluorescence attachments (Olympus Imaging America Inc., PA). Coverslips were mounted on slides with antifade medium (Waterborne™, Inc., New Orleans, LA).

Data analyses. The rate of excystation (percent excystation) was calculated using a previously reported formula [44]. The proportions of three separate invasion stages (sporozoites, immature trophozoites, and mature trophozoites, Figure 19) were calculated. Sporozoites are crescent shaped, motile, extracellular invasive stage. During invasion, the sporozoite initially becomes partially rounded (immature trophozoite; iTroph), and subsequently becomes fully

rounded (mature trophozoite; mTroph).The average number of agglutinated sporozoites in 30 high-power fields (HPF) was determined for each time point post-excystation (p.e.) and each antibody concentration. Agglutination was defined as two or more sporozoites in a clump. The significance of differences between treatments were tested using Student's t-test with equal variance (Microsoft Excel, 2007). A P value < 0.05 was considered statistically significant.

Results

Peptides bind to HCT-8 cells. We quantified the binding of four peptides, derived from the adhesive domains of C. parvum thrombospondin-family proteins, to the surface of HCT-8 cells. TAPP, 7TS, 9TS, and 10K1 were observed to bind to HCT-8 cell surfaces (Figure 20, A-D), suggesting that they are specific for surface receptors on host epithelial cells. In contrast, control peptides, derived from the cytoplasmic region of two C. parvum rhomboid proteases, showed no appreciable binding (data not shown). Immunofluorescence intensity, which was used as a surrogate measure of peptide binding, was greater at the 200 µM than the 100 µM concentration of 7TS (Figure 20, A), 9TS (Figure 20, B), and 10K1 (Figure 20, C) ($P < 0.05$). Fluorescence intensity of TAAP was not significantly different between the 100 and 200 µM concentrations (Figure 20, D).

Peptides inhibit C. parvum invasion of host cells. The attachment to and invasion of intestinal epithelial cells by C. parvum involves the interactions of specific host cell receptors and sporozoite surface adhesins (45). We therefore examined the extent to which synthetic peptides derived from thrombospondin-family protein adhesive domains competitively inhibited parasite attachment to and invasion of HCT-8 cells. The rate of invasion was determined by quantifying the proportions of sporozoites (S; pre-invasion), immature trophozoites (iT; early

invasion), and mature trophozoites (mT; late invasion) at 2 and 6 h post infection (p.i.). An increasing mT and decreasing sprozoite proportion over time indicates a productive infection. Proportions of each invasion stage in treatments are compared to no-peptide treated controls as shown on figure 21 which shows the inhibitory effects of peptides at 2 and 6 h p.i. At 2 h following infection, the sporozoite proportion (S) was significantly higher in all peptide treatments, with the exception of the 100 µM 7TS (51 %) and 100 µM 9TS (60 %) treatments versus the control (52 %) (Figure 21, 2 h infection, a and b). iT proportions were lower in treatments with 9TS (200 µM concentration, 29 %), 10K1 (100 (16 %) and 200 µM (21 %) concentrations), TAAP (100 (19 %) and 200 µM 21 %) concentrations) and the combined peptide cocktail (200 µM concentrations, 29 %) versus control which was 37 %. The inhibitory effect of peptides at 6 h p.i. showed a higher sporozoite proportion relative to mT proportion which was lower in all peptide treatments, with the exception of the 200 µM 10K1 treatment (70 %), in which the sporozotes proportion was not significantly different from the control (21 %, 6 h post infection, c). The iT proportion was significantly ($P < 0.05$) higher in the 7TS (100 µM concentration, 56 %) and 9TS (100 (50 %) and 200 µM (48 %) concentrations) treatments relative to the control (26 %).

Polyclonal antibodies do not reduce excystation but anti-TAAP causes sporozoites to agglutinate. We examined the neutralizing effect of polyclonal antibodies against 7TS, 9TS, 10K1, and TAAP during excystation. None of the polyclonal antibodies, used individually or in combination, reduced excystation relative to the control (data not shown for 7TS, 9TS, or 10K1; see Figure 23 for anti-TAAP data). Excystation increased over time in controls and antibody treatments at all concentrations, and approximately 90% of oocysts had excysted after 60 min.

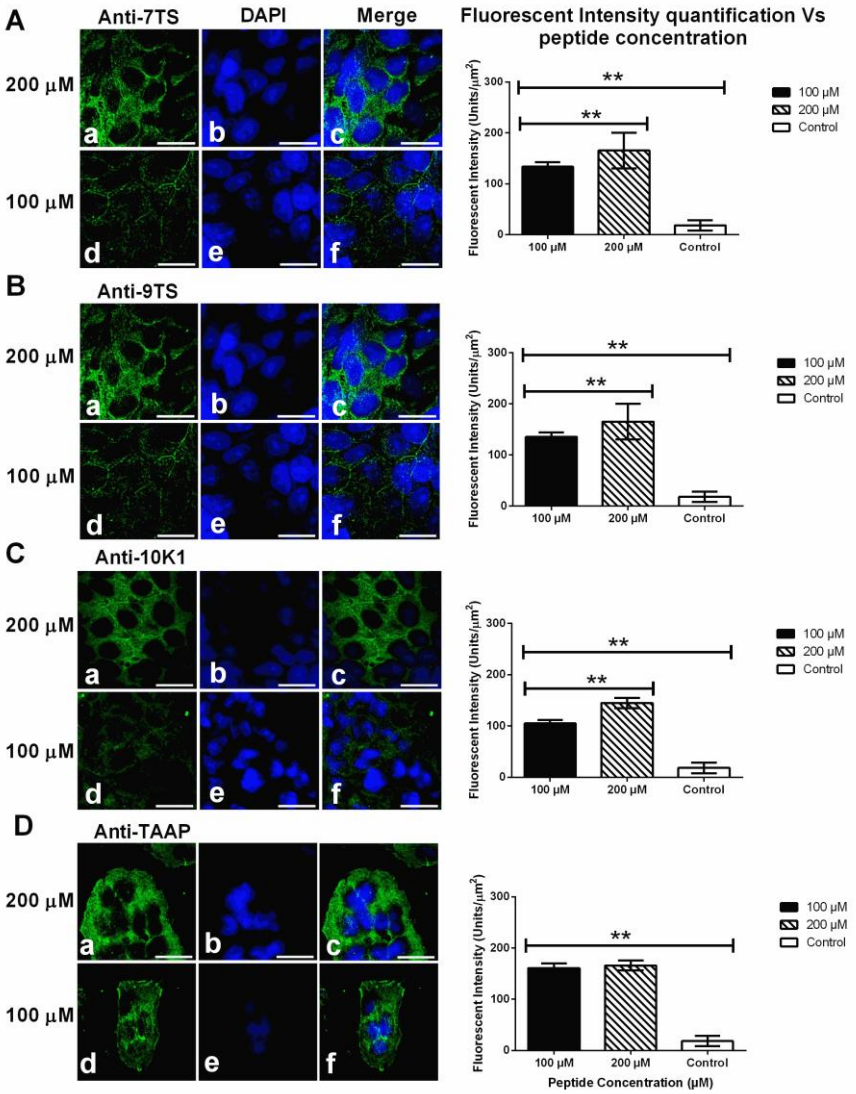

Figure 20. Peptide binding to HCT-8 cells during peptide and antibody inhibition assays. HCT-8 cells were cultured for 24 h and treated with 100 µM or 200 µM peptides (TAAP, 7TS, 9TS, and 10K1) and incubated for 2 h at 37°C. Cells are washed and fixed with methanol, blocked with 1% BSA, probed overnight at 4°C with corresponding anti-peptide antibodies. Peptides binding was detected with AlexaFluor 488 and then imaged. DAPI (blue) was used to stain the nuclei to provide contrast for the FITC (green). Peptide intensity between concentrations is measured in units/ µm2. (A through D, left panel) shows fluorescent labeling of peptide attached to host cell surface in a concentration dependent manner as indicated on the fluorescent intensity graphs (A through D, right panel). No fluorescence was seen on the control HCT-8 cells. Data (n=4) are presented as mean ± SD and significant at ** $P < 0.05$ when compared to controls. Scale bar = 20 µm.

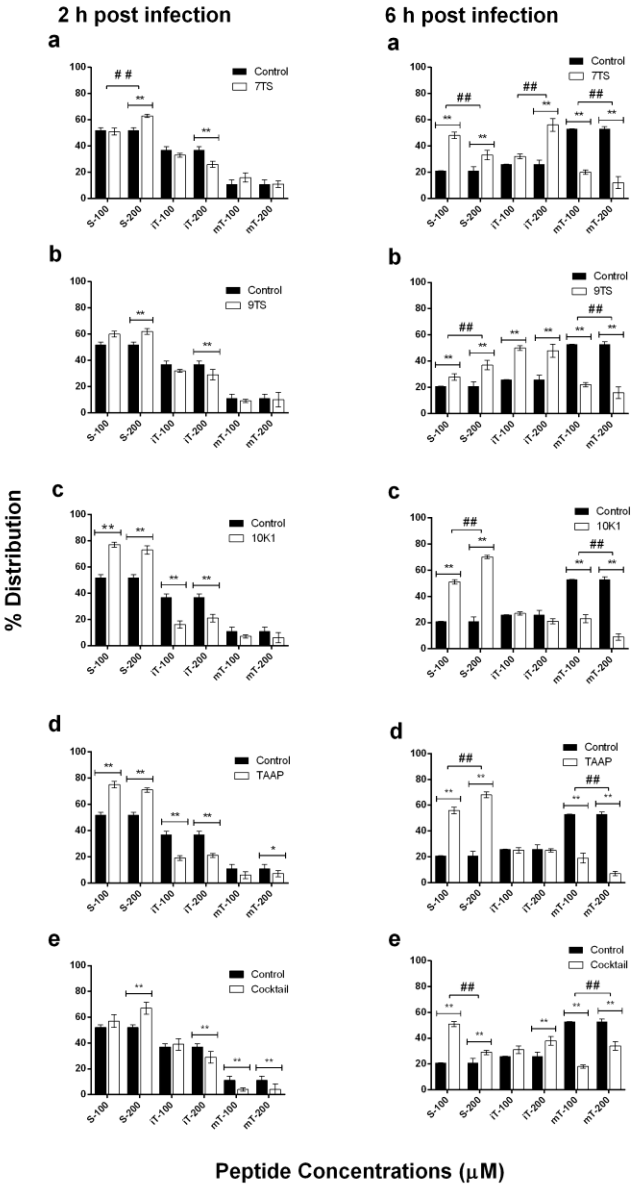

Figure 21. Effect of peptide (7TS, 9TS, 10K1 and TAAP) treatments on *C. parvum* infection of HCT-8 cells during a 2 and 6 h infection time points. HCT- 8 cells were treated with individual TSP-1 (a, b, c, and d) derived peptides or in combination (e) at each time points as described in methods. Following infection with *C. parvum* oocyst, proportion of sporozoites (S), immature (iT), and mature (mT) were recorded. White bars represent experimental peptide treatments (100 or 200μM) while black bars represent the control media treatment. Error bars represent the standard deviation of four independent counts. Student's two-tailed t–test (* # $P < 0.01$ and ** ## $P < 0.05$) was used to determine the level of significant difference between means.

Sporozoites excysting from oocysts treated with anti-TAAP were observed to agglutinate in an antibody concentration dependent manner (Figure 22). At all treatment times, there was a statistically significant positive correlation between antibody concentration and the average number of agglutinated sporozoite (15 min, R^2= 0.9256; 30 min, R^2=0.9404; 45 min, R^2= 0.9473; 60 min, R^2=0.9721; and 120 min, R^2=0.9472). Sporozoite agglutination was not observed in any other antibody treatment or control.

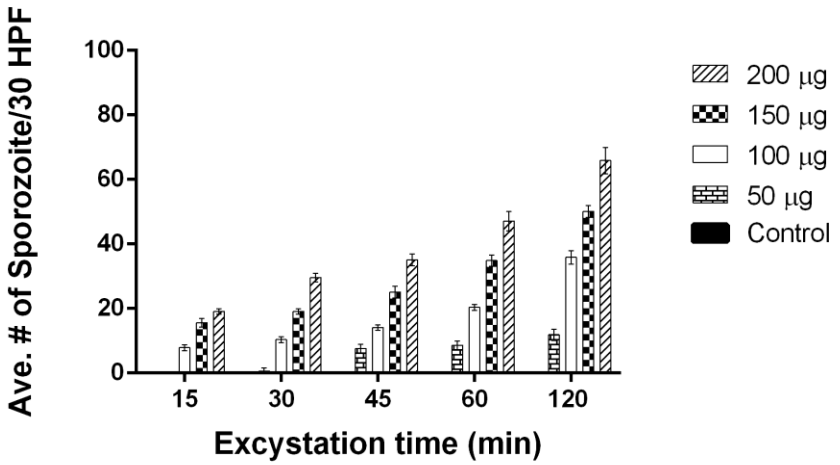

Figure 22. *C. parvum* sporozoites are agglutinated when treated with anti-TAAP antibody. Oocysts were bleach treated as described in methods, subjected to regular excystation medium or excystation medium with anti-TAAP antibody of increasing concentrations as indicated. Different bars denote average number of agglutinated sporozoites versus anti-TAAP concentration indicating the effect of anti-TAAP on *C. parvum* sporozoite agglutination.

Polyclonal antibodies neutralize infection. Using a similar approach to the peptide inhibition experiment, we examined the rate of host cell invasion in the presence or absence of antibodies against 7TS, 9TS, 10K1, and TAAP. Proportions of each invasion stage in treatments are compared to no-antibody controls. Figure 23 shows the effect of antibody treatments at 2 and 6 h p.i. For 2 h post infection, only anti-TAAP (67 and 69 % respectively for the 100 and 200 μM treatments) and the combined antibody cocktail (75 and 81 % respectively for the 100 and

200 µM treatments) had a significant ($P > 0.05$) effect on the S proportion: both treatments resulted in a low S proportion relative to the control (58 %). The mT proportion was lower in treatments with anti-10K1 (14 and 10 % respectively for 100 and 200 µM treatments), anti-TAAP (same as anti-10K1), and the antibody cocktail (10 and 5 % respectively), at both antibody concentrations (100 and 200 µM treatment). Control proportion for the mT was 19 %. The iT proportion with control proportion recorded at 23 %, was lower in treatments with anti-TAAP (100 µM, 14 %), anti-10K1 (100 µM, 14 %), and in the combined antibody cocktail (4 and 10 % respectively for 100 and 200 µM treatments) ($P<0.05$). In contrast, the iT proportion was higher in the 200 µM anti-10K1 (29 %) treatment ($P < 0.05$). At 6 h post infection, the S proportion was higher in treatments with anti-7TS (28 and 36 % respectively for 100 and 200 µM treatments), anti-10K-1 (100 µM concentration, 31 %), anti-TAAP (66 and 69 % respectively for 100 and 200 µM treatments), and the combined antibody cocktail (5 and 9 % respectively for 100 and 200 µM treatments). The mT proportion was significantly higher when treated with 7TS (200 µM concentration, 36 %), anti-TAAP (200 µM concentrations, 44 %), and the combined antibody cocktail (5 and 9 % respectively for 100 and 200 µM treatments). Control for the S, iT, and mT proportions were 22, 22, and 56 % respectively.

Discussion

Motile *Cryptosporidium* sporozoites invade host cells and establish an extracytoplasmic nich at the apical surface, undergo asexual replication, and release eight merozoites that can reinvade host cells to perpetuate the asexual cycle. Rapid and repeated cycling between extracellular invasive and intracellular replicative stages can massively increase apicomplexan numbers and contribute to pathogenesis. Therefore, immune or chemotherapeutic interventions that reduce the rate of transition between these stages will invariably reduce pathology.

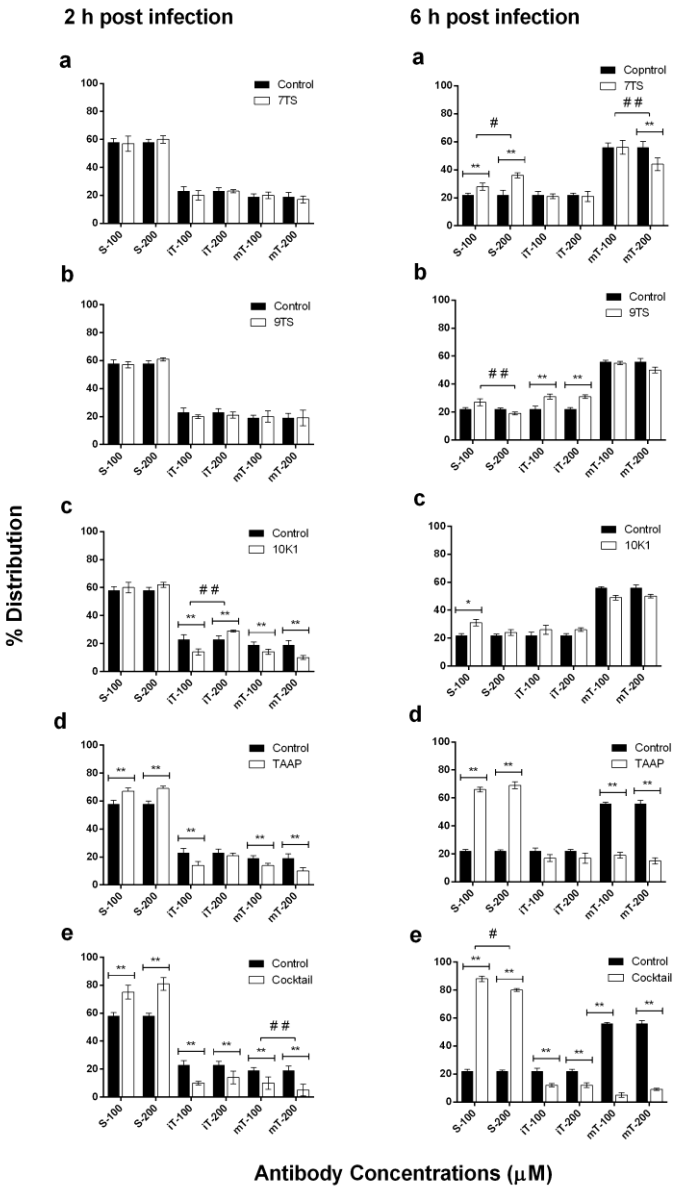

Figure 23. Effect of antibody (anti-7TS, -9TS, -10K1 and -TAAP) treatment to C. parvum infection of HCT-8 cells during a 2 and 6 h infection time point. Bleach treated oocyst were mixed with individual antibodies (a, b, c, and d) or in combination (e) as described in methods. Two antibody concentrations (100 or 200μM) were used. White bars represent experimental peptide treatments (100 or 200μM) while black bars represent the control (infection media) treatment. Following infection with antibody or treated media *C. parvum* oocysts, proportion of sporozoites (S), immature (iT), and mature (mT) were recorded. Error bars represent the standard deviation of four independent counts. Student's two-tailed t-test (* # $P < 0.01$ and ** ## $P < 0.05$) was used to determine the level of significant difference between means.

The availability of *Cryptosporidium* parasite genome sequences [46,47] has aided the identification of surface-exposed parasite proteins that may function during host cell invasion [48,49]. Without the ability to undertake gene knockout experiments, the interrogation of function in surface exposed proteins requires evidence from competitive inhibition and antibody neutralization experiments. Using these approaches, we examined the role in invasion of specific adhesive domains from thrombospondin family proteins. Collectively, the data from these studies show that CpTSP7, CpTSP9, CpTSP10, and TRAP-C1 contribute to invasion, and that the function of these proteins is achieved through thrombospondin type I repeat, apple, and kringle domains.

We showed that synthetic peptides derived from TSP-1 (7TS and 9TS), kringle (10K1), and apple (TAAP) bind to receptors on HCT-8 cells. In contrast, control peptides derived from cytoplasmic regions of two *C. parvum* proteins showed no appreciable binding to host cells. The receptors recognized by 7TS, 9TS, 10K1, and TRAP-C1 are not known. Receptor-ligand interaction studies have shown that the TSP-1 domain of *P. falciparum* TRAP interacts with sulfated glycoconjugates or glycans on hepatocytes. Similarly, the apple domain in TgMIC4 interacts with galactose and glycosaminoglycans on host cells [50,51]. Glycans are major components of glycolipids and glycoproteins, in particular mucins, on the surfaces of host cells [52,53]. Many enteropathogens have evolved strategies to exploit host glycans during intestinal colonization [54,55], and invasion by *C. parvum* is mediated by the interactions between sporozoite lectins and Gal/GalNAc epitopes on host cells [56-60]. The accumulation of host Gal/GalNAc glycoproteins at the site of sporozoite attachment triggers actin-dependent membrane protrusion from the host cell apical surface and encapsulation of the sporozoite in an extracytoplasmic vacuole [60,61]. Considering the demonstrated roles of apple and

thrombospondin domains in other organisms, it is possible that the peptides derived from these domains are exhibiting lectin activity. This hypothesis could be tested in future studies using approaches that include the selective deglycosylation of host cell surfaces and the competitive inhibition of peptide binding using specific lectins.

Among the individual treatments, the effect of peptide inhibition and antibody neutralization was most pronounced for TAAP, the peptide derived from the apple domain in TRAP-C1. After 6 h, the sporozoite proportion in infections treated with 200 µM TAAP or anti-TAAP was approximately 68%, compared to 21% in controls. In contrast, fewer than 7% of parasite stages were mature trophozoites following these treatments, compared to approximately 53% in controls. These data clearly show that parasites treated with TAAP or anti-TAAP are less successful at invading host cells. Two non-mutually exclusive explanations can be considered for these findings. The first is that the apple domain in TRAP-C1 plays a critical role during invasion, which makes it particularly sensitive to neutralization. This explanation is supported by the competitive inhibition data. The second explanation is that TRAP-C1 is relatively more sensitive to neutralization than the other thrombospondin proteins tested. Okhuysen et al. [39] showed that TRAP-C1 specific antibodies were produced in response to *C. parvum* infection in humans; although, the extent to which the antibodies were protective was not determined. Our observation that anti-TAAP causes sporozoites to clump in a concentration dependent manner suggests that antibodies targeting TRAP-C1 could neutralize sporozoites through the formation of antigen-antibody immune complexes.

Conclusion

There is a fundamental characteristic that is maintained among the apicomplexan obligate intracellular lifestyle which makes clearing infections extremely difficult, if not

impossible[63,70] . Along this line, the ultimate elimination of persistent infections in humans and animals is central to controlling their pathogenesis[22]. Since *C. parvum* thrombospondin family proteins CpTSP7, CpTSP9, CpTSP10, and TRAP-C1 contribute to host cell invasion, they may be effective targets for drug or vaccine development. Therefore, it is important to characterize those *C. parvum* thrombospondin-derived antigens which are targets of protective antibody in order to develop rational strategies for therapy of cryptosporidiosis and for vaccine development.

References

1. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium parvum* genes containing thrombospondin type 1 domains. Infection and Immunity 70: 6987-6995.
2. Boulanger MJ, Tonkin ML, Crawford J (2010) Apicomplexan parasite adhesins: novel strategies for targeting host cell carbohydrates. Curr Opin Struct Biol 20: 551-559.
3. Agarwal A, Ningthouja S, Sharma D, Mohen Y, Singh NB (1998) Cryptosporidium and HIV. J Indian Med Assoc 96: 276-277.
4. Ajjampur SS, Sankaran P, Kang G (2008) *Cryptosporidium* species in HIV-infected individuals in India: an overview. Natl Med J India 21: 178-184.
5. Anand A (1993) Cryptosporidiosis in patients with AIDS. Clin Infect Dis 17: 297-298.
6. Ananthasubramanian M, Ananthan S, Vennila R, Bhanu S (1997) *Cryptosporidium* in AIDS patients in south India: a laboratory investigation. J Commun Dis 29: 29-33.
7. Mead JR (2002) Cryptosporidiosis and the challenges of chemotherapy. Drug ResistUpdat 5: 47-57.
8. Griffiths JK (1998) Human cryptosporidiosis: epidemiology, transmission, clinical disease,

.treatment, and diagnosis. Adv Parasitol 40: 37-85.

9. Baishanbo A, Gargala G, Duclos C, François A, Rossignol JF, et al. (2006) Efficacy of nitazoxanide and paromomycin in biliary tract cryptosporidiosis in an immunosuppressed gerbil model. J Antimicrob Chemother
57: 353-355.

10. Rossignol JF, Ayoub A, Ayers MS (2001) Treatment of diarrhea caused by *Cryptosporidium parvum*: a prospective randomized, double-blind, placebo-controlled study of Nitazoxanide. J Infect Dis 184: 103-106.

11. Barbon AR, Forbes N (2007) Use of paromomycin in the treatment of a *Cryptosporidium* infection in two falcons. Falco 30: 22-24.

12. Barr SC, Jamrosz GF, Hornbuckle WE, Bowman DD, Fayer R (1994) Use of paromomycin for treatment of cryptosporidiosis in a cat. J Am Vet Med Assoc 205: 1742-1743.

13. Hahn NE, Capuano SV (2010) Successful treatment of cryptosporidiosis in 2 common marmosets (Callithrix jacchus) by using paromomycin. J Am Assoc Lab Anim Sci 49: 873-875.

14. Klein P, Cirioni O, Giacometti A, Scalise G (2008) In vitro and in vivo activity of aurintricarboxylic acid preparations against *Cryptosporidium parvum*. J Antimicrob Chemother 62: 1101-1104.

15. Palmieri F, Cicalini S, Froio N, Rizzi EB, Goletti D, et al. (2005) Pulmonary cryptosporidiosis in an AIDS patient: successful treatment with paromomycin plus azithromycin. Int J STD AIDS 16: 515-517.

16. Zardi EM, Picardi A, Afeltra A (2005) Treatment of cryptosporidiosis in immunocompromised hosts. Chemotherapy 51: 193-196.

17. Leav BA, Mackay M, Ward HD (2003) *Cryptosporidium* species: new insights and old challenges. Clinical Infectious Diseases 36: 903-908.

18. Abubakar I, Aliyu SH, Arumugam C, Usman NK, Hunter PR (2007) Treatment of cryptosporidiosis in immunocompromised individuals: systematic review and meta-analysis. Br J Clin Pharmacol 63: 387-393.

19. Tilley M, Eggleston MT, Upton SJ (1993) Multiple oral inoculations with *Cryptosporidium parvum* as a means of immunization for production of monoclonal antibodies. FEMS Microbiol Lett 113: 235-240.

20. Perryman LE, Kapil SJ, Jones ML, Hunt EL (1999) Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. Vaccine 17: 2142-2149.

21. Ehigiator HN, Romagnoli P, Priest JW, Secor WE, Mead JR (2007) Induction of murine immune responses by DNA encoding a 23-kDa antigen of *Cryptosporidium parvum*. Parasitol Res 101: 943-950.

22. Adams JC (2001) Thrombospondins: multifunctional regulators of cell interactions. Annu Rev Cell Dev Biol 17: 25-51.

23. Jewett TJ, Sibley LD (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. Mol Cell 11: 885-894.

24. Naitza S, Spano F, Robson KJ, Crisanti A (1998) The thrombospondin-related protein family of apicomplexan parasites: The gears of the cell invasion machinery. Parasitol Today 14: 479-484. 25. Robson KJ, Hall JR, Jennings MW, Harris TJ, Marsh K, et al. (1988) A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. Nature 335: 79-82.

26. Robson KJ, Naitza S, Barker G, Sinden RE, Crisanti A (1997) Cloning and expression of the thrombospondin related adhesive protein gene of Plasmodium berghei. Mol Biochem Parasitol 84: 1-12.

27. Schroeder AA, Brown AM, Abrahamsen MS (1998) Identification and cloning of a developmentally regulated *Cryptosporidium parvum* gene by differential mRNA display PCR. Gene 216: 327-334.

28. Dessens JT, Beetsma AL, Dimopoulos G, Wengelnik K, Crisanti A, et al. (1999) CTRP is essential for mosquito infection by malaria ookinetes. EMBO J 18: 6221-6227.

29. Yuda M, Sawai T, Chinzei Y (1999) Structure and expression of an adhesive protein-like molecule of mosquito invasive-stage malarial parasite. J Exp Med 189: 1947-1952.

30. Tomley FM, Soldati DS (2001) Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. Trends Parasitol 17: 81-88.

31. Tomley FM, Billington KJ, Bumstead JM, Clark JD, Monaghan P (2001) EtMIC4: a microneme protein from Eimeria tenella that contains tandem arrays of epidermal growth factor-like repeats and thrombospondin type-I repeats. Int J Parasitol 31: 1303-1310.

32. Brown PJ, Billington KJ, Bumstead JM, Clark JD, Tomley FM (2000) A microneme protein from *Eimeria tenella* with homology to the Apple domains of coagulation factor XI and plasma pre-kallikrein. Mol. Biochem. Mol Biochem Parasitol 107: 91-102.

33. Sheiner L, Santos JM, Klages N, Parussini F, Jemmely N, et al. (2010) *Toxoplasma gondii* transmembrane microneme proteins and their modular design. Molecular Microbiology 77: 912-929.

34. Achbarou A, Mercereau-Puijalon O, Autheman JM, Fortier B, Camus D, et al. (1991) Characterization of microneme proteins of Toxoplasma gondii. Mol Biochem Parasitol 47: 223-

233.

35. Lovett JL, Howe DK, Sibley LD (2000) Molecular characterization of a thrombospondin-related anonymous protein homologue in Neospora caninum. Mol Biochem Parasitol 107: 33-43.

36. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium parvum* genes containing thrombospondin type 1 domains. Infect Immun 70: 6987-6995.

37. Spano F, Putignani L, Naitza S, Puri C, Wright S, et al. (1998) Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. Molecular and Biochemical Parasitology 92: 147–162.

38. Putignani L, Possenti A, Cherchi S, Pozio E, Crisanti A, et al. (2008) The thrombospondin-related protein CpMIC1 (CpTSP8) belongs to the repertoire of micronemal proteins of *Cryptosporidium parvum*. Molecular & Biochemical Parasitology 157: 98-101.

39. Okhuysen PC, Rogers GA, Crisanti A, Spano F, Huang DB, et al. (2004) Antibody response of healthy adults to recombinant thrombospondin-related adhesive protein of *Cryptosporidium* 1 after experimental exposure to *Cryptosporidium* oocysts. Clinical and Diagnostic Laboratory Immunology 11: 235-238.

40. Doyle PS, Crabb J, Petersen C (1993) Anti-*Cryptosporidium parvum* antibodies inhibit infectivity in vitro and in vivo. Infect Immun 61: 4079-4084.

41. Belli SI, Walker RA, Flowers SA (2005) Global protein expression analysis in apicomplexan parasites: current status. Proteomics 5: 918-924.

42. Forney JR, Yang S, Healey MC (1996) Protease activity associated with excystation of *Cryptosporidium parvum* oocysts. J Parasitol 82: 889-892.

43. Mezzasoma L, Bacarese-Hamilton T, Di Cristina M, Rossi R, Bistoni F, et al. (2002) Antigen

microarrays for serodiagnosis of infectious diseases. Clin Chem 48: 121-130.

44. O'Hara SP, Chen XM (2011) The cell biology of cryptosporidium infection. Microbes Infect 13: 721-730.

45. Forney JR, Yang S, Healey MC (1996) Protease activity associated with excystation of *Cryptosporidium parvum* oocysts. Journal of Parasitology 82: 889-892.

46. Bengtsson DC, Sowa KM, Arnot DE (2008) Dual fluorescence labeling of surface-exposed and internal proteins in erythrocytes infected with the malaria parasite Plasmodium falciparum. Nat Protoc 3: 1990-1996.

47. Narum DL, Thomas AW (1994) Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of *Plasmodium falciparum* merozoites. Mol Biochem Parasitol 67: 59-68.

48. Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM, et al. (2004) The genome of Cryptosporidium hominis. Nature 431: 1107-1112.

49. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, et al. (2004) Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science 304: 441-445.

50. Marchant J, Cowper B, Liu Y, Lai L, Pinzan C, et al. (2012) Galactose recognition by the apicomplexan parasite *Toxoplasma gondii*. J Biol Chem 287: 16720-16733.

51. Gong H, Kobayashi K, Sugi T, Takemae H, Kurokawa H, et al. (2012) A novel PAN/Apple domain-containing protein from *Toxoplasma gondii*: characterization and receptor identification. PLoS ONE 7: e30169.

52. Brecht S, Carruthers VB, Ferguson DJ, Giddings OK, Wang G, et al. (2001) The *toxoplasma* micronemal protein MIC4 is an adhesin composed of six conserved apple domains. J Biol Chem 276: 4119-4127.

53. Carruthers VB (2002) Host cell invasion by the opportunistic pathogen Toxoplasma gondii. Acta Trop 81: 111-122.

54. McGuckin MA, Linden SK, Sutton P, Florin TH (2011) Mucin dynamics and enteric pathogens. Nat Rev Microbiol 9: 265-278.

55. Corfield AP, Carroll D, Myerscough N, Probert CS (2001) Mucins in the gastrointestinal tract in health and disease. Front Biosci 6: D1321-1357.

56. Joe A, Verdon R, Tzipori S, Keusch GT, Ward HD (1998) Attachment of *Cryptosporidium parvum* sporozoites to human intestinal epithelial cells. Infect Immun 66: 3429-3432.

57. Joe A, Hamer DH, Kelley MA, Pereira ME, Keusch GT, et al. (1994) Role of a Gal/GalNAc-specific sporozoite surface lectin in *Cryptosporidium parvum*-host cell interaction. J Eukaryot Microbiol 41: 44S.

58. Bhat N, Joe A, PereiraPerrin M, Ward HD (2007) *Cryptosporidium* p30, a galactose/N-acetylgalactosamine-specific lectin, mediates infection in vitro. Journal of Biological Chemistry 282: 34877-34887.

59. Chen XM, Larusso NF (2000) Mechanisms of attachment and internalization of *Cryptosporidium parvum* to biliary and intestinal epithelial cells. Gastroenterology 118: 368-379.

60. Nelson JB, O'Hara SP, Small AJ, Tietz PS, Choudhury AK, et al. (2006) *Cryptosporidium parvum* infects human cholangiocytes via sphingolipid-enriched membrane microdomains. Cell Microbiol 8: 1932-1945.

61. O'Hara SP, Small AJ, Chen XM, Larusso NF (2008) Host cell actin remodeling in response to *Cryptosporidium*. Sub-Cellular Biochemistry 47: 92-100.

GENERAL DISCUSSION

Apicomplexans, the most intensively studied protozoan parasites, are a diverse phylum with an obligate intracellular lifestyle. These parasites collectively infect more than one-third of the global population and exact a staggering toll on public health [2]. The apicomplexan *C. parvum* infects the intestinal mucosa of various mammals including humans. The disease caused by this parasite, cryptosporidiosis, can become chronic and life threatening in immunocompromised individuals such as those with AIDS [3-7], and can impair the physical and cognitive development of children [8]. There is no consistently effective drug treatment and there is no vaccine for the disease [9]. Therefore, the discovery of drug and vaccine candidates through the understanding of molecular mechanisms that underpin disease pathogenesis is an urgent priority. The specific objective pursued in this dissertation was to identify functions of sporozoite surface proteins and rhomboid proteases during motility and invasion of host cells. The hypothesis tested was that *C. parvum* rhomboid proteases (CpROMs) function with surface proteins to achieve the adhesion-site turnover (adhesion and de-adhesion) that is necessary for productive sporozoite motility and host cell invasion.

Cryptosporidium parvum sporozoites express a number of surface adhesins that engage host cell receptor molecules to mediate attachment, motility, and invasion. These molecules include CP2, Cpa135, CP47, GP40/15, GP900, P30, CP15, and thrombospondin-family proteins [10-15]. This dissertation focused on thrombospondin-family proteins and GP900 because they possess intramembrane helix destabilizing residues that are targeted by rhomboid proteases. The rhomboids are functionally conserved in prokaryotes and eukaryotes and have been implicated in cleavage of cell surface adhesins in apicomplexans [16,17]. We examined the cleavage of seven thrombospondin proteins (CpTSP2, CpTSP6, CpTSP7, CpTSP8, CpTSP9, CpTSP10, and

TRAP-C1) and a mucin-like glycoprotein (GP900) by two *C. parvum* rhomboids, which we named CpROM4 and CpROM5. CpROM4 showed a relatively narrow specificity for GP900 and CpTSP10, but not TRAP-C1 or other thrombospondins. In addition to CpTSP10 and GP900, CpROM5 cleaved TRAP-C1, CpTSP6, CpTSP8, and CpTSP9. Although these overlapping specificities suggest overlapping and redundant CpROM functions, such a conclusion is not supported by gene and protein expression studies. Instead, the following key observations are evidence that CpROM4 and CpROM5 have distinct functions during excystation, motility, and invasion:

CpROM5 and TRAP-C1 are localized to the inner oocyst wall and exhibit similar punctate labeling

These findings suggest that CpROM5 and TRAP-C1 function together in the oocyst wall. Their expression patterns are similar to that of GP900, which localizes to fibrils that tether sporozoites to the inside of the oocyst wall [1]. These tethers detach from sporozoites during excystation. The similar expression patterns together with the specificity of CpROM5 for GP900 in a heterologous assay lead us to hypothesize that CpROM5, TRAP-C1, and GP900 are molecular components of tethers, and the activity of CpROM5 breaks the tethers to facilitate sporozoite egress (Figure 26). The finding that CpTSP7 also localizes to the oocyst wall is interesting because neither CpROM4 nor CpROM5 cleaved this thrombospondin in a heterologous assay. Despite the lack of activity in the heterologous assay, CpROM5 may still cleave CpTSP7 in *Cryptosporidium*. TgROM4 showed no activity against *T. gondii* adhesins in the heterologous assay [16], but cleaved three adhesins in tachyzoites [18]. However, the transmembrane domain of CpTSP7 lacks the helix destabilizing residues that are normally targeted by rhomboid proteases, which suggests that CpTSP7 may be cleaved by a different

protease. The localization of CpTSP7 in a localized area opposite the rupture site, also appears to be different to that of CpROM5 and TRAP-C1. Immunoelectron microscopy will be required to determine the specific localization of CpROM5, TRAP-C1, and CpTSP7 in the oocyst wall.

CpROM4 and CpTSP10 colocalize at the anterior and posterior sporozoite poles; CpROM5 and TRAP-C1 colocalize in apical and sub-apical regions.

Although these data support distinct roles for CpROM4 and CpROM5 during gliding motility, it is not known why a specific CpROM/adhesin pairing would be preferred at a particular location on the sporozoite surface. Münter et al. [19] showed that *P. falciparum* sporozoites turnover discrete adhesion sites in a coordinated and sequential manner, and the rate of turnover at the anterior and posterior poles lag behind other surfaces, which results in a characteristic stick-slip motility. *Plasmodium falciparum* lacks a ROM5 homologue so sheddase activity appears limited to PfROM4, which is distributed throughout the plasma membrane with the exception of the anterior and posterior poles [20]. This leads us to hypothesize that the CpROM4/CpTSP10 pairing results in a different rate of adhesion site turnover than the CpROM5/TRAP-C1 pairing. This hypothesis could be tested using the microscopy approaches used by Münter et al. [19]. Figure 27A shows a cartoon depiction of the localization of CpROM4, CpROM5, and thrombospondin proteins in motile sporozoites.

CpROM5 and TRAP-C1 colocalize to early trophozoites at 2 h post infection.

During invasion, *C. parvum* sporozoites become progressively rounded and enveloped by host cell membrane [21]. The enveloping membrane intimately adheres to the sporozoite surface, probably via sporozoite surface adhesins. The finding that CpROM5 is upregulated relative to CpROM4 and most thrombospondin proteins in trophozoites at 2 h post infection suggests that this protease is necessary for early intracellular development. One explanation for the

colocalization of CpROM5 and TRAP-C1 in trophozoites is that TRAP-C1 mediates sporozoite binding to the host cell membrane during formation of the parasitophorous vacuole, and CpROM5 is subsequently required to disengage TRAP-C1 binding and free the parasite to undergo intracellular replication (Figure 27B). Thrombospondin proteins bind specifically to components of extracellular matrix and cell surface receptors via adhesive domains that are conserved among the different apicomplexan species [22]. For instance, thrombospondin type I repeat domains bind to highly variable sulfated glycoconjugates on the host cell surface that mediate cell-cell and/or cell-matrix interactions [23-25]. Although twelve thrombospondin family genes have been identified in *C. parvum* [26,27], their binding specificity and function remain unknown.

In *Plasmodium*, thrombospondin family proteins have been implicated in sporozoite motility and infection of mosquito salivary glands and human hepatocytes [22]. In this study, we showed that synthetic peptides derived from adhesive domains in TRAP-C1, CpTSP7, CpTSP9, and CpTSP10 bound to HCT-8 cells and inhibited *C. parvum* infection. Similarly, polyclonal antibodies produced against these peptides neutralized infection of HCT-8 cells. These outcomes will enhance the understanding of protein interactions that can be exploited as an effective target for drug or vaccine development. Collectively, this will improve our understanding of the importance of the thrombospondin domains in host cell attachment and their putative role in parasite infection. Monoclonal, polyclonal and recombinant antibodies have been used [28-30] to interrupt infection, and they represent useful immunotherapeutic means of combating infection [31]. Further studies to identify the specific receptors for thrombospondin adhesive domains could include synthetic glycoarray analyses with labeled peptides, or the selective deglycosyaltion of host cells. Studies to characterize the binding activities of these peptides and

their corresponding antibodies will be of importance in the development of the much needed therapy or vaccine against cryptosporidiosis.

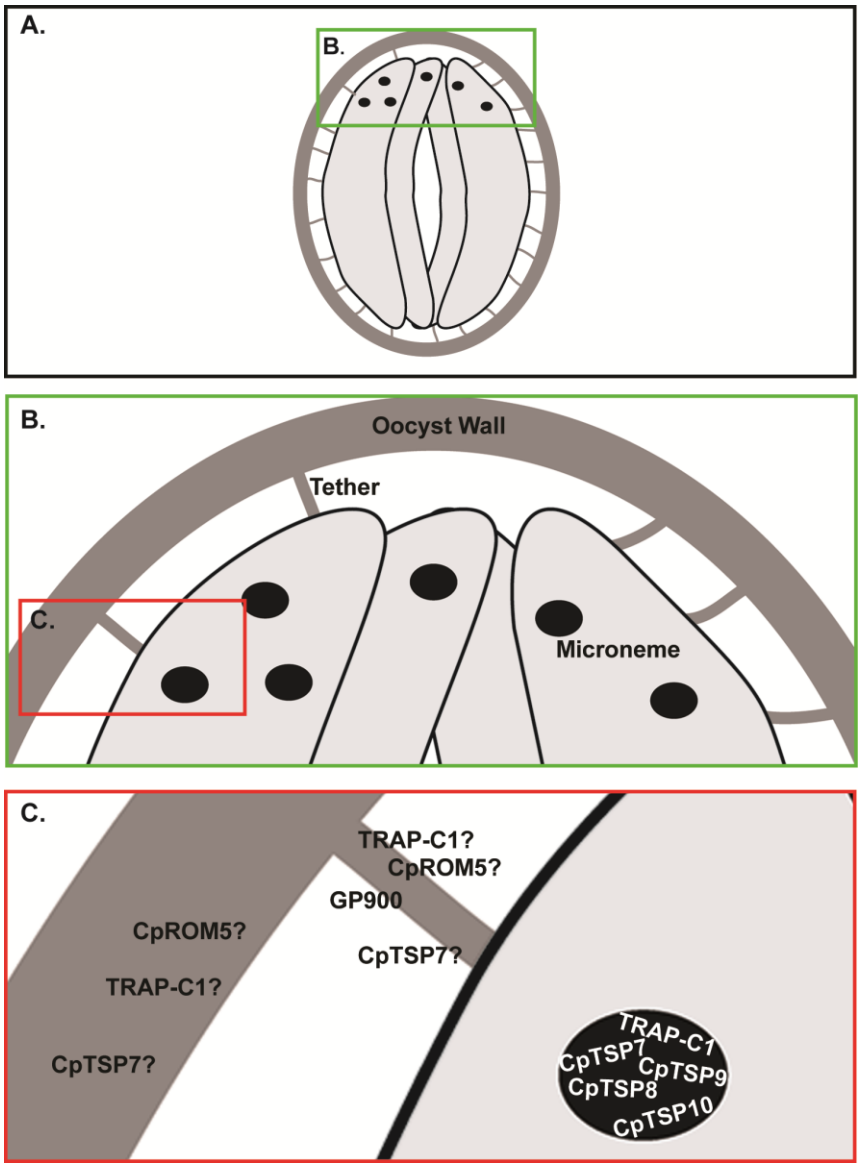

Figure 24. Cartoon depicting the locations of CpROM5, CpTSP7, TRAP-C1, and Gp900 in the walls of *C. parvum* oocyst. A) Four sporozoites (gray) are seen within an intact oocyst tethered to the internal surface of the oocyst. B) Enlarge one-quater of oocyst posterior region revealing in greater details glycoprotein tethers (C) and other putative *C. parvum* oocyst resident wall proteins (CpROM5, CpTSP7, and TRAP-C1) detected in this study for the first time.

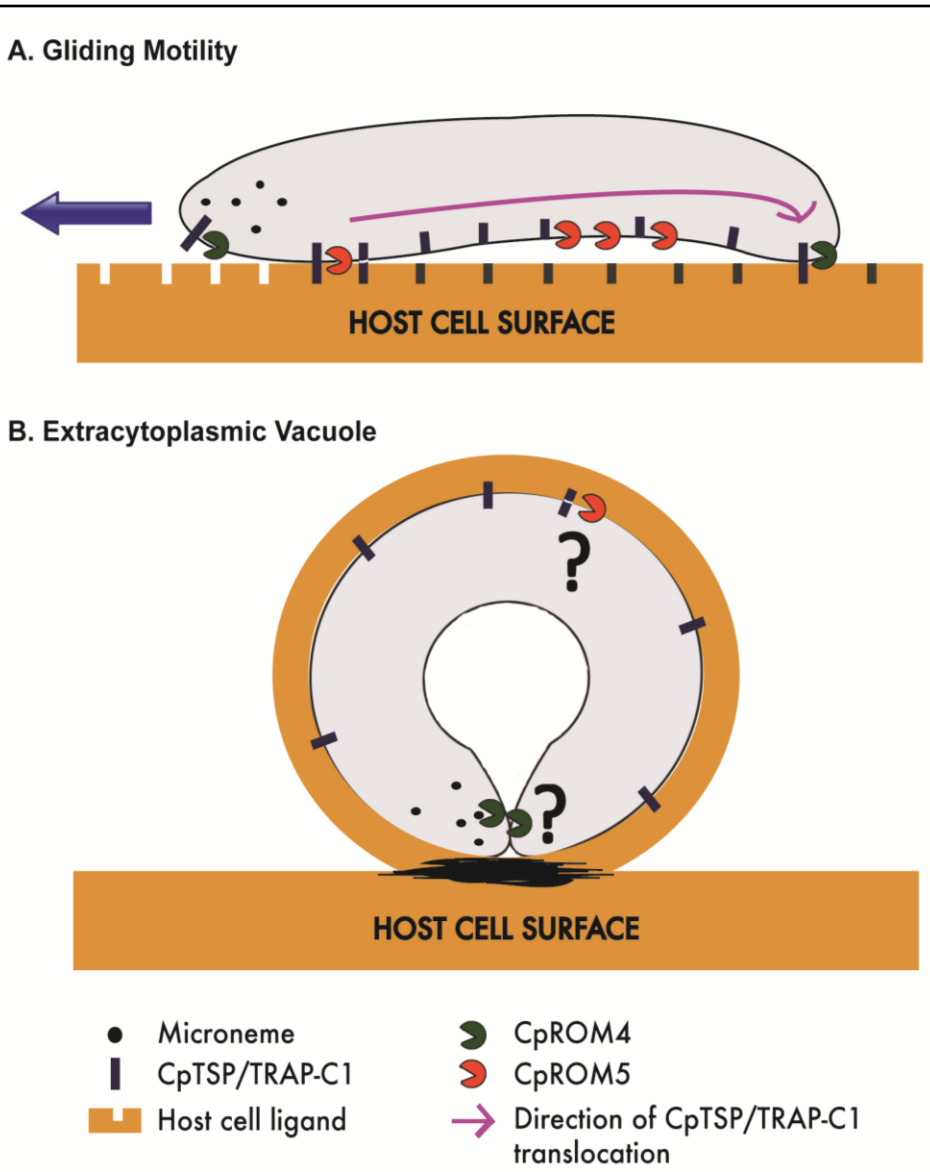

Figure 25. Cartoon depicting the location of CpROM4, CpROM5, TRAP-C1, and other TSPs in: A) sporozoites during motility and B) during invation. CpROM4 (green) is shown in a bipolar location colocalized with CpTSP10 (black bar) while CpROM5 (red) is shown colonized with TRAP-C1 (black bars-broken). B) extracytoplasmic vacuole showing CpROM4 (green) at the tips of circular intracellular zoite while CpROM5 (red) is spread on the entire surface of the invasive stage.

Conclusion

This dissertation identifies possible roles of rhomboid proteases and their putative substrates in *C. parvum* during excystation, motility, invasion, and intracellular development. The data support distinct yet coordinated roles for CpROM4 and CpROM5 during motility and invasion of host cells. Given an understanding of mechanisms of infection is key to intervention, we also provided data that that will help provide new avenues for research that seek to identify mechanisms that result in the surface adhesins-host ligand interaction in *Cryptosporidium* and, as a consequence, the development of effective chemotherapeutic agents or vaccines against the diseases caused by this pathogen.

It is important to emphasized that, since many proteins are heavily glycosylated, vaccine candidates may need to be generated in systems that can produce glycosylated proteins similar to or identical to native proteins [29]. This hold true for must eukaryotic organisms where peptides are the necessary component for antigen recognition and processing. However, glycosylation plays a role in antigen processing and presentation [8]. For example, evidence in yeast and certain eukaryotic microorganisms suggests that at least some glycosylation, in particular mannose linkages, may enhance immunogenicity [8].Thus, the formulation of an effective vaccine to prevent and control cryptosporidiosis is still a long way to go. This study has provided additional insights into potential targets of of *C. parvum* that can be further explore—especially in the intestinal tract—which should give us a better understanding of the role of the surface adhesins in immune responses needed to control and prevent infections. Advances in gycoarray will facilitate the identification critical cytoprotective glycoproteins and alterations of epithelial cells (host) necessary for Cryptosporidium attachement to host cells. In addition, techniques in vacinology, such as the use of DNA vaccines, the use of novel vacine vectors that can stimulate

mucosal immunity coupled with the use of CpGs oligonucleotides of putative adhsins as adjuvants may also facilitate the development of vaccines against *Cryptosporidium*.

References

1. Carruthers VB, Blackman MJ (2005) A new release on life: emerging concepts in proteolysis and parasite invasion. Mol Microbiol 55: 1617-1630.
2. Del Chierico F, Onori M, Di Bella S, Bordi E, Petrosillo N, et al. (2011) Cases of cryptosporidiosis co-infections in AIDS patients: a correlation between clinical presentation and GP60 subgenotype lineages from aged formalin-fixed stool samples. Ann Trop Med Parasitol 105: 339-349.
3. Kothavade RJ (2011) Challenges in understanding the immunopathogenesis of *Cryptosporidium* infections in humans. Eur J Clin Microbiol Infect Dis 30: 1461-1472.
4. Ayuo PO (2009) Human cryptosporidiosis: a review. East Afr Med J 86: 89-93.
5. Assefa S, Erko B, Medhin G, Assefa Z, Shimelis T (2009) Intestinal parasitic infections in relation to HIV/AIDS status, diarrhea and CD4 T-cell count. BMC Infect Dis 9: 155.
6. Corti M, Villafañe MF, Muzzio E, Bava J, Abuín JC, et al. (2008) [Pulmonary cryptosporidiosis in AIDS patients]. Rev Argent Microbiol 40: 106-108.
7. Guerrant DI, Moore SR, Lima AA, Patrick PD, Schorling JB, et al. (1999) Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. Am J Trop Med Hyg 61: 707-713.
8. Mead JR (2002) Cryptosporidiosis and the challenges of chemotherapy. Drug Resist Updat 5: 47-57.
9. Urban S, Dickey SW (2011) The rhomboid protease family: a decade of

progress on function and mechanism. Genome Biol 12: 231.

10. Urban S, Freeman M (2003) Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. Mol Cell 11: 1425-1434.

11. Brossier F, Jewett TJ, Sibley LD, Urban S (2005) A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by Toxoplasma. Proc Natl Acad Sci U S A 102: 4146-4151.

12. Baker RP, Wijetilaka R, Urban S (2006) Two *Plasmodium* rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. PLoSPathog 2: e113.

13. Ejigiri I, Ragheb DR, Pino P, Coppi A, Bennett BL, et al. (2012) Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity. PLoS Pathog 8: e1002725.

14. Forney JR, Yang S, Healey MC (1996) Protease activity associated with excystation of Cryptosporidium parvum oocysts. J Parasitol 82: 889-892.

15. Freeman M (2008) Rhomboid Proteases and their Biological Functions. Annual Review of Genetics 42: 191-210.

16. Chatterjee A, Banerjee S, Steffen M, O'Connor RM, Ward HD, et al. (2010) Evidence for mucin-like glycoproteins that tether sporozoites of *Cryptosporidium parvum* to the inner surface of the oocyst wall. EukaryotCell 9: 84-96.

17. Münter S, Sabass B, Selhuber-Unkel C, Kudryashev M, Hegge S, et al. (2009) *Plasmodium* sporozoite motility is modulated by the turnover of discrete adhesion sites. Cell HostMicrobe 6: 551-562.

18. Matuschewski K, Schüler H (2008) Actin/myosin-based gliding motility in apicomplexan parasites. Subcell Biochem 47: 110-120.

19. Kappe SH, Buscaglia CA, Bergman LW, Coppens I, Nussenzweig V (2004) Apicomplexan gliding motility and host cell invasion: overhauling the motor model. Trends in Parasitology 20: 13-16.

20. Mota MM (2006) Be in motion . Mol Microbiol 60: 1327-1328.

21. Harper JM, Huynh MH, Coppens I, Parussini F, Moreno S, et al. (2006) A cleavable propeptide influences *Toxoplasma* infection by facilitating the trafficking and secretion of the TgMIC2-M2AP invasion complex. Mol Biol Cell 17: 4551-4563.

22. Kappe S, Bruderer T, Gantt S, Fujioka H, Nussenzweig V, et al. (1999) Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. The Journal of Cell Biology 147: 937-944.

23. Moreno-Perez DA, Mongui A, Soler LN, Sanchez-Ladino M, Patarroyo MA (2011) Identifying and characterizing a member of the RhopH1/Clag family in Plasmodium vivax. Gene 481: 17-23.

24. Dubremetz JF, Garcia-Réguet N, Conseil V, Fourmaux MN (1998) Apical organelles and host-cell invasion by Apicomplexa. Int J Parasitol 28: 1007-1013.

25. Naitza S, Spano F, Robson KJ, Crisanti A (1998) The thrombospondin-related protein family of apicomplexan parasites: The gears of the cell invasion machinery. Parasitol Today 14: 479-484.

26. Tucker RP (2004) The thrombospondin type 1 repeat superfamily. International Journal of Biochemistry and Cell Biology 36: 969-974.

27. Deng M, Templeton TJ, London NR, Bauer C, Schroeder AA, et al. (2002) *Cryptosporidium*

parvum genes containing thrombospondin type 1 domains. Infection and Immunity 70: 6987-6995.

28. Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, et al. (2004) Complete genome sequence of the apicomplexan, Cryptosporidium parvum. Science 304: 441-445.

29. Boulter-Bitzer JI, Lee H, Trevors JT (2009) Single-chain variable fragment antibodies selected by phage display against the sporozoite surface antigen P23 Of *Cryptosporidium parvum*. J Parasitol 95: 75-81.

30. Pokorny NJ, Boulter-Bitzer JI, Hall JC, Trevors JT, Lee H (2008) Inhibition of *Cryptosporidium parvum* infection of a mammalian cell culture by recombinant scFv antibodies. Antonie Van Leeuwenhoek 94: 353-364.

31. Zheng J, Ren W, Pan Q, Wang Q, Elhag IA, et al. (2011) A recombinant DNA vaccine encoding C. andersoni oocyst wall protein induces immunity against experimental *C. parvum* infection. Vet Parasitol 179: 7-13.

32. Boulter-Bitzer JI, Lee H, Trevors JT (2007) Molecular targets for detection and immunotherapy in *Cryptosporidium parvum*. Biotechnology Advances 25: 13-44.

33. Bergman LW, Kaiser K, Fujioka H, Coppens I, Daly TM, et al. (2003) Myosin A tail domain interacting protein (MTIP) localizes to the inner membrane complex of Plasmodium sporozoites. J Cell Sci 116: 39-49.

34. Kappe SH, Kaiser K, Matuschewski K (2003) The *Plasmodium* sporozoite journey: a rite of passage. Trends in Parasitology 19: 135-143.

35. Donahue CG, Carruthers VB, Gilk SD, Ward GE (2000) The Toxoplasma homolog of Plasmodium apical membrane antigen-1 (AMA-1) is a microneme protein secreted in response to elevated intracellular calcium levels. Mol Biochem Parasitol 111: 15-30.

36. Hehl AB, Lekutis C, Grigg ME, Bradley PJ, Dubremetz JF, et al. (2000) Toxoplasma gondii homologue of plasmodium apical membrane antigen 1 is involved in invasion of host cells. Infect Immun 68: 7078-7086.

37. Okhuysen PC, Rogers GA, Crisanti A, Spano F, Huang DB, et al. (2004) Antibody response of healthy adults to recombinant thrombospondin-related adhesive protein of *Cryptosporidium* 1 after experimental exposure to *Cryptosporidium* oocysts. Clin Diagn Lab Immunol 11: 235-238.