FUNCTIONAL CHARACTERIZATION OF ACYL-CoA BINDING PROTEIN (ACBP) AND OXYSTEROL BINDING PROTEIN-RELATED PROTEINS (ORPS) FROM *CRYPTOSPORIDIUM PARVUM*

A Dissertation

by

BIN ZENG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee: Guan Zhu
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James Derr
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ABSTRACT

Functional Characterization of Acyl-CoA Binding Protein (ACBP) and Oxysterol Binding Protein-Related Proteins (ORPs) from C*ryptosporidium parvum.*

(December 2006)

Bin Zeng, B.S., Jiangxi Agricultural University;

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Chair of Advisory Committee: Dr. Guan Zhu

From opportunistic protist *Cryptosporidium parvum* we identified and functionally assayed a fatty acyl-CoA-binding protein (ACBP) gene. The CpACBP1 gene encodes a protein of 268 aa that is three times larger than typical ~10 KD ACBPs of humans and animals. Sequence analysis indicated that the CpACBP1 protein consists of an N-terminal ACBP domain (approximately 90 aa) and a C-terminal ankyrin repeat sequence (approximately 170 aa). The entire CpACBP1 open reading fragment (ORF) was engineered into a maltose-binding protein fusion system and expressed as a recombinant protein for functional analysis. Acyl-CoA-binding assays clearly revealed that the preferred binding substrate for CpACBP1 is palmitoyl-CoA. RT-PCR, Western blotting and immunolabelling analyses clearly showed that the CpACBP1 gene is mainly expressed during the intracellular developmental stages and that the level increases during parasite development. Immunofluorescence microscopy showed that CpACBP1 is associated with the parasitophorous vacuole membrane (PVM), which implies that this protein may be involved in lipid remodelling in the PVM, or in the transport of fatty acids across the membrane.

We also identified two distinct oxysterol binding protein (OSBP)-related proteins (ORPs) from this parasite (CpORP1 and CpORP2). The short-type CpOPR1 contains only a ligand binding (LB) domain, while the long-type CpORP2 contains Pleckstrin homology (PH) and LB domains. Lipid-protein overlay assays using recombinant proteins revealed that CpORP1 and CpORP2 could specifically bind to phosphatidic acid (PA), various phosphatidylinositol phosphates (PIPs), and sulfatide, but not to other types of lipids with simple heads. Cholesterol was not a ligand for these two proteins. CpOPR1 was found mainly on the parasitophorous vacuole membrane (PVM), suggesting that CpORP1 is probably involved in the lipid transport across this unique membrane barrier between parasites and host intestinal lumen. Although *Cryptosporidium* has two ORPs, other apicomplexans, including *Plasmodium, Toxoplasma,* and *Eimeria*, possess only a single long-type ORP, suggesting that this family of proteins may play different roles among apicomplexans.

DEDICATION

My father Xianzheng Zeng and in memory of my late mother Guilian Wang

For their love, support, patience, and friendship

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LIST OF ABBREVIATIONS

ACBP	acyl-CoA binding protein
ACP	acyl carrier protein
FAS	fatty acid synthase
GPI	glycosyl phosphatidylinositol
MBP	maltose binding protein
LB domain	ligand-binding domain
OSBP	oxysterol binding protein
ORP	OSBP-related protein
PA	phosphatidic acid
PH domain	pleckstrin homology domain
PIP	phosphatidylinositol phosphate
PI(n)P	phosphatidylinositol n-phosphate (n = 3, 4, and/or 5)
PVM	parasitophorous vacuole membrane
TMP	total membrane proteins

CHAPTER I

INTRODUCTION

Cryptosporidium is a genus of protozoan parasites belonging to the phylum Apicomplexa. At least 14 species within the genus have been reported from humans and a wide range of vertebrates including cattle, horses, dogs, cats and mice (Caccio, 2005; Chen *et al.*, 2002a; Thompson *et al.*, 2005; Tzipori & Widmer, 2000b)

Although *Cryptosporidium* typically causes only self-limited disease in immunocompetent patients, its infection in immunocompromised (such as AIDS patients) can be prolonged and life-threatening. *Cryptosporidium parvum* (zoonotic) and *C. hominis* (almost exclusively found in humans) are the two species commonly found in human patients, but a few other species have also been reported to infect humans, including *C. meleagridis, C. felis, C. canis, C. suis* and *C. muris*(Caccio, 2005; Thompson *et al.*, 2005; Xiao & Ryan, 2004).

Because the environmental stage, the oocyst, of *Cryptosporidium* spp. is highly resistant to all commonly used disinfectants including chlorine treatment, its contamination in a community water supply system can be a great threat to public health. Therefore, it is listed as a category B pathogen in the NIH Biodefense program.

This dissertation follows the style of *Microbiology*.

Today, nitozaxanide (NTZ) is the only drug recently approved by the FDA to treat cryptosporidiosis in immunocompetent patients. However, this drug is not 100% effective against *Cryptosporidium*, and can be easily shadowed by the emerging drug resistance. Therefore, there is an urgent need to further understand the critical metabolism in this parasite to facilitate the discovery and development of new anti-cryptosporidiosis compounds.

The present project has been focusing on the two types of lipid binding proteins (acyl-CoA binding protein [ACBP] and oxysterol-binding protein-related proteins [ORPs]) identified from the *C. parvum* genome. The aims of the study are to characterize the molecular and biochemical features of these parasite proteins as part of the lipid metabolic pathway, which may not only deepen our understanding on the basic biochemistry in *C. parvum*, but also provide the basis for exploring these proteins as potential therapeutic targets.

CHAPTER II

REVIEW OF LITERATURE

BACKGROUND

The history of *Cryptosporidium* spp.

In 1907, Ernest E. Tyzzer reported the first *Cryptosporidium* species, *C. muris,* that was isolated from a laboratory mouse (Tyzzer,1907). Tyzzer also reported the second species in this genus, *C. parvum,* that is zoonotic and commonly used as a model organism for studying cryptosporidiosis (Tyzzer, 1912). Fifty years later, the third species, *C. meleagridis*, was isolated from turkey, which was shown to be associated with the economic loss to the turkey industry. Cryptosporidiosis infections in humans were reported in 1976. Since the early 1980s, *Cryptosporidium* has been one of the important opportunistic pathogens among AIDS patients whose immune systems were comprised by HIV viruses. Since then, cryptosporidiosis-associated diarrhea was also reported among children in day care centers, and in other age groups whose immune systems were competent.

In the past few years, over 10,000 people in more than 31 locations have been reported to have acquired *Cryptosporidium* infection from recreational waters (Fayer *et al.*, 2000). The noteworthy symptom associated with *Cryptosporidium* infection in humans is diarrhea, usually voluminous and watery (Fayer, 2004). *C. parvum* usually causes

self-limiting diarrhea in immunocompetent individuals. However, immunocompromised individuals can develop a life-threatening infection (Chen et al., 2002; Chappell and Okhuysen, 2002). There have been two genotypes of *C. parvum:* type I was only isolated from humans, while type II could infect both humans and animals. More recently, genotype I *C. parvum* has been pronounced by some investigators as a new species, *C. hominis*, based on biological and molecular data (Morgan-Ryan *et al.*, 2002).

Life cycle and morphology of *Cryptosporidium* spp.

Cryptosporidium belongs to the phylum Apicomplexa, in which all members are parasites. Many apicomplexans are important human and/or animal pathogens, such as those cause malaria (*Plasmodium* spp.), Texas Fever (*Babesia* spp.), toxoplasmosis (*Toxoplasma gondii*), and coccidiosis (*Eimeria* spp.) (Fayer *et al.*, 2000). The life cycle of *C. parvum* resembles that of other coccidia, which consists of complex sexual and asexual stages (Fig. 2.1). It begins with the ingestion of infectious oocysts by humans or animals. Oocysts are an environmental stage with a highly chemical-resistant oocyst wall that contains 4 naked sporozoites. In the guts of humans or animals, the sporozoites emerge from oocysts by a process called excystation and invade the epithelial cells typically in the small intestine. Parasitophorous vacuoles (PV) form when the host cell plasma membrane engulfs and surrounds the invaded sporozoites. The sporozoites become trophozoites (“type I” meronts), and differentiate asexually into 8 merozoites after the first generation of merogony. Mature merozoites will rupture host cells and invade into new cells to form “type II” meronts by a second generation of merogony. Four merozoites are typically formed in a “type II” meront. Type II

merozoites may differentiate into microgamonts or macrogamonts. A zygote forms when a macrogamete is fertilized by a microgamete. Zygotes will first develop into premature oocysts with the formation of the oocyst wall, and then into mature oocysts, each containing 4 fully developed sporozoites. The formation of sporozoites is termed as sporulation. The sporulation of *Cryptosporidium* spp. takes place within the same host immediately after the formation of oocysts, whereas sporulation in typical coccidia (e.g., *Eimeria* or *Cyclospora*) takes place in the environment. There are two types of oocysts. The first type is thick-walled, while the other is thin-walled. There was an estimate that the ratio between thick- and thin-wall oocysts may be around 4:1 (Current & Garcia, 1991). Sporozoites in the thin-walled oocysts may excyst and infect the additional intestinal cells within the same host gut. Such a continuous recycling of infection is referred to as “auto-infection” or “auto-infective cycle”.

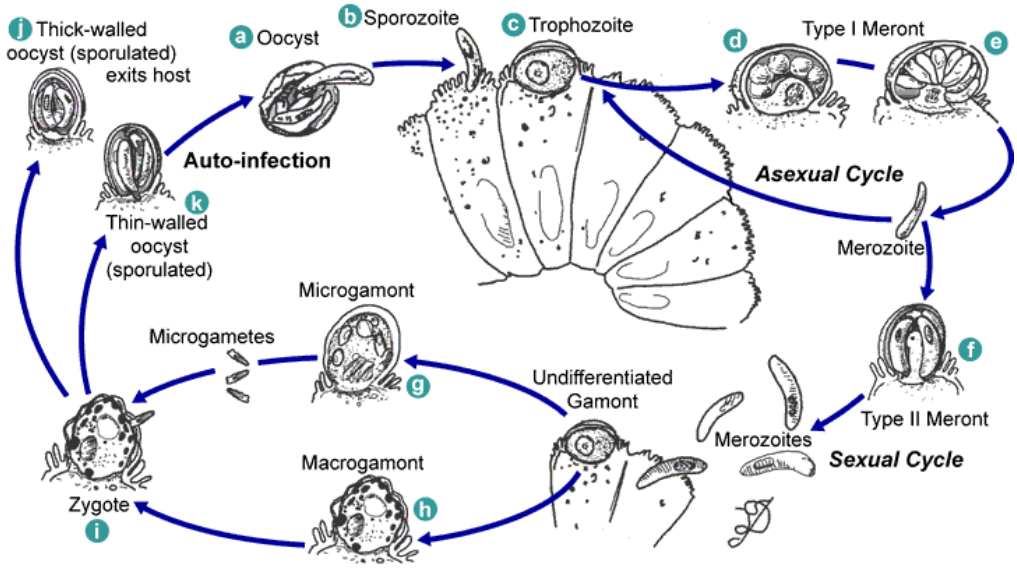

Fig. 2.1. Life Cycle of *Cryptosporidium parvum* and *C. hominis*. (Reproduced from http://www.dpd.cdc.gov/dpdx/HTML/Cryptosporidiosis.html)

In comparison to the oocysts of other intestinal coccidia, those of C. parvum are relatively small. The oocyst is subspherical, to most of the species in this genus. The typical size is 4.5–7.5 µm X 4.2–5.7 µm. The sizes of other life cycle stages are as follow: sporozoites, 4.5–7.5 µm X 1.2–1.8 µm; meronts, spherical 4.0–6.9 µm diameter; merozoites, 3.6–5.0 µm × 1.1 µm; microgamont, ~1.7 µm long (Harris *et al.*, 2003).

Cryptosporidium parasitizes the surface of the host cell. However, they are intracellular parasites residing in the parasitophorous vacuoles (PV) covered by host cell membranes referred to as PV membranes (PVM) (Fig. 2.2). Since intracellular *C. parvum* does not reside within the host cell cytosol, it is referred to as an "extracytoplasmic" parasite (Zhu, 2004). Another unique structure is the "feeder

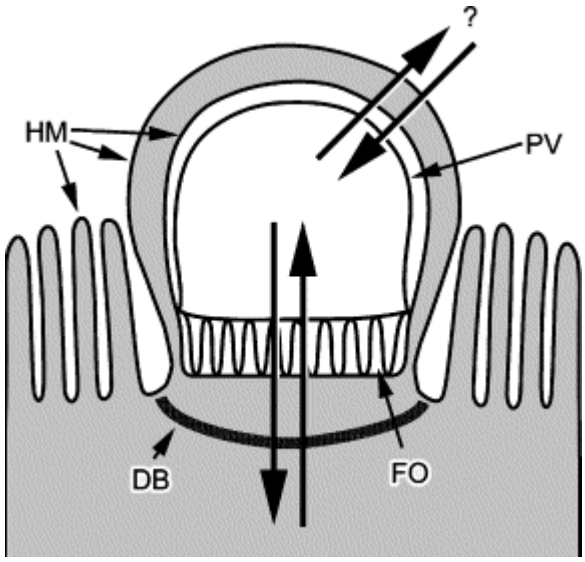

Fig. 2.2. A diagram of the parasitophorous vacuole (PV). HM: host membrane; FO: feeder organelle, DB; DB: electron dense band (Mead, 2002).

organelle (FO)", which is shown as a complex of folded membrane structure coupled with an electron-dense band at the parasite-host cell interface and contains a great amount of alpha-actin filaments at the host cell side (Beyer *et al.*, 2002; Elliott & Clark, 2000). Because of their specific locations, both the PVM and the "feeder organelle" are believed to play critical roles in the transport of nutrients from the intestinal lumen or host cells by the parasite.

Cryptosporidiosis

Both humans and animals acquire cryptosporidiosis by a fecal-oral transmission route (Egger *et al.*, 1990). However, the source of oocysts can be water, food, or direct contact between human or animal individuals. There was a study showing that 2% of the stool samples collected from health care providers in the United States were *Cryptosporidium* positive (Mead, 1999). In industrialized and developing countries, the prevalence for *Cryptosporidium* in human stools ranges from 2.1 to 6.1% (Martins & Guerrant, 1995). Several major waterborne outbreaks of cryptosporidiosis have been reported. The largest waterborne outbreak occurred in Milwaukee, Wisconsin, USA in 1993, which infected about 403,000 people and caused more than 100 deaths among immunocompromised individuals (MacKenzie *et al.*, 1995; Soave, 1995). The epidemiology of the *Cryptosporidium* infection has the following characteristics (Caccio & Pozio, 2006): 1) low infective dose; 2) immediate infectivity of excreted oocysts; 3) very high stability of oocysts (one month to more than 1 year when exposed to environment); 4) easy dispersal in environmental contamination (drinking water and food); and 5) great zoonotic potential.

Cryptosporidium can cause immunocompetent individuals self-limited diarrhea, which generally produce mild to severe diarrhea that may last for days or up to 2–3 weeks. The clinical signs manifest as a cholera-like diarrhea, vomiting nausea, abdominal pain, loss of appetite, fatigue, and weight loss. Among immunocompromised individuals, such as AIDS patients, cryptosporidiosis is chronic and life-threatening. During an outbreak in Nevada, the median weight loss of HIV-positive patients was 29.9 lb and the median duration of diarrhea was ~60 days (Goldstein *et al.*, 1996). Forty of the 61 AIDS patients infected with *Cryptosporidium* in this outbreak died within 6 months. *Cryptosporidium* in HIV-positive patients may also disseminate from the small intestine to other atypical places, such as the gall bladder, biliary tract, pancreas, or even respiratory system (Lopez-Velez *et al.*, 1995). For example, in the Milwaukee outbreak, Among 81 patients were diagnosed as cryptosporidiosis, 25% of them were found to have *Cryptosporidium* infection in their extraintestinal biliary tracts.

Today, there are still no completely effective drugs to treat cryptosporidiosis. Nitazoxanide (NTZ) is the only drug recently approved by FDA to treat cryptosporidiosis in immunocompetent patients (but not in immunocompromised individuals) in the United States. Paromomycin is another compound frequently mentioned in the literature, but in a recent clinic trial, it was found to be no more effective than placebo for treating cryptosporidiosis in patients with advanced HIV infection (Hewitt *et al.*, 2000). However, due to its efficacy against *Cryptosporidium* infection in vitro, paromomycin is commonly used as a standard positive control in drug studies (Mead, 2002; Meamar *et al.*, 2006; Rossignol, 2006).

Fatty acid metabolism in *Cryptosporidium*

Fatty acids are one of the major components of biomembranes. Despite of its importance, fatty acid metabolism in *Cryptosporidium* and other apicomplexans is a relative new area of research, only starting with the discovery of type II fatty acid synthases (FASs) in *Plasmodium falciparum* and *Toxoplasma gondii (Waller et al., 1998)*, and that of type I FAS in *Cryptosporidium* (CpFAS1) (Zhu *et al.*, 2000c). In type I system, all major enzymes involved in fatty acid synthesis are fused into one or two polypeptides, whereas in the type II system, all enzymes are discrete and monofunctional proteins. More recently, the apicomplexan genome projects have revealed that a type FAS is also present in *T. gondii*, but not in *Plasmodium* spp. (Zhu, 2004). In addition, a type I polyketide synthase (PKS) is also identified from the genomes of *C. parvum* and *T. gondii*, suggesting these parasites are capable of making more sophisticated fatty acid derivatives (Zhu *et al.*, 2002; Zhu *et al.*, 2004).

Besides type I FAS and PKS, *C. parvum* also possesses a long chain fatty acid elongase and its associated pathway for elongating long chain fatty acyl-CoAs. Because of the lack of type II FAS that is responsible for de novo synthesis of fatty acids (Zhu *et al.*, 2000b), together with the fact that CpFAS1 prefers the long chain fatty acids as its substrates (Zhu *et al.*, 2004), *C. parvum* appears to be incapable of synthesize fatty acids de novo, and has to scavenge fatty acids from host cells or the intestinal lumen.

In addition to the fatty acid synthetic enzymes, several lipid binding proteins are also present in the *C. parvum* genome, which include an ankyrin-containing acyl-CoA

binding protein (ACBP) and two oxysterol-binding protein-related proteins (ORPs). These lipid binding proteins are typically involved in the storage and transport of lipids (Im *et al.*, 2005; Knudsen *et al.*, 1993; Knudsen *et al.*, 1999; Lehto & Olkkonen, 2003; Rasmussen *et al.*, 1994). However, their roles in regulating lipid and sterol metabolism are also well documented (Choi *et al.*, 1996; Im *et al.*, 2005; Kerkhoff *et al.*, 1997; Knudsen *et al.*, 1999; Knudsen *et al.*, 2000; Olkkonen, 2004). The major players in the fatty acid metabolism in *C. parvum*, as well as their proposed roles are summarized by Zhu (Zhu *et al.*, 2004).

Fatty acid metabolism has served as a good drug target in bacteria. For example, enoyl reductase (ENR) has been identified as the true target both for diazaborines, including the front-line antituberculosis drug isoniazid (Baldock *et al.*, 1998; Mdluli *et al.*, 1998), and for triclosan and other 2-hydroxydipheyl ethers (Heath *et al.*, 1998; Heath *et al.*, 1999; McMurry *et al.*, 1999). This pathway is also being explored as rational drug target in apicomplexans. For example, thiolactomycin and triclosan have been found to potently inhibit the growth of *P. falciparum* in vitro and *P. berghei* in vivo (a mouse model) (Surolia & Surolia, 2001). Cerulenin, a metabolite of *Cephalosporium caerulens*, is a specific inhibitor to both types I and II β-keto-acyl-[ACP] synthases (Heath *et al.*, 2001; Mead, 2002). This compound has been shown to inhibit the growth of C*. parvum* in vitro without apparent toxicity to the host cells, indicating that fatty acid metabolism may also serve as a rational drug target in *Cryptosporidium* (Zhu et al., 2000b; Zhu 2004).

Acyl-CoA Binding Protein (ACBP)

Acyl-CoA binding protein (ACBP) was named after the protein's high ability to bind to medium to long chain acyl-CoA esters. ACBP was first isolated from bovine liver (Mikkelsen & Knudsen, 1987). It was also found that ACBP and diazepam-binding inhibitor (DBI) were the same protein (Knudsen, 1991), which could displace diazepam from the benzodiazepine recognition site of the gamma-aminobutyric acid receptor (Guidotti *et al.*, 1983; Kragelund *et al.*, 1999). ACBP homologues are present in almost all eukaryotes, including animals, plants, fungi and protists. ABCPs are typically short, ~10 kDa proteins. However, longer versions of ACBPs fused with other types of domains (e.g., ankyrin repeats) are also present. For example, *Arabidopsis thaliana* has at least 3 long type ACBPs, in which AtACBP2 contains an N-terminal ankyrin repeats probably responsible for interacting with other proteins (Li & Chye, 2004).

Although ACBPs can bind to a wide range of fatty acyl CoA esters, each ACBP may have different binding profile towards various fatty acyl chain lengths. For example, bovine intestinal ACBP (bACBP) prefers to long chain fatty acyl-CoAs (LCFA, C14 - C20), whereas *Trypanosoma brucei* ACBP (TbACBP) apparently has higher affinity towards medium chain acyl-CoAs (MCFA, C10 - C14) (Milne & Ferguson, 2000). On the other hand, *P. falciparum* ACBP (PfACBP) appears to have relatively similar binding affinity towards all medium to long chain acyl-CoAs (C14 – C22) (van Aalten *et al.*, 2001).

ACBPs may function as acyl-CoA transporters. In the parasite *T. brucei*, ACBP was found to be responsible for transport or delivery of the fatty acyl chains for synthesizing

the GPI anchors (Milne & Ferguson, 2000). ACBPs are also involved in the regulation and forming of the intracellular acyl-CoA pool. For example, overexpression of *S. cerevisiae* ACBP has increased the intracellular acyl-CoA pool size in the yeast (Knudsen *et al.*, 1994). In another experiment, transgenic mice expressing a higher level ACBP was studied. Although the transgenic mice didn't show the change to their body and liver weights, the pool size of liver LCFA-CoA was expanded by 69%, especially to the saturated and polyunsaturated, but not monounsaturated, LCFA-CoAs (Huang *et al.*, 2005). On the other hand, the liver acyl-CoA pool was significantly reduced in ACBP gene knockout mice (Martin *et al.*, 2003).

Cells may utilize the ACBP-associated acyl-CoA pools as one of the means in regulating fatty acid metabolism. For example, in human mononuclear phagocytes, the activity of acyl-CoA:cholesterol acyltransferase (ACAT) in rough endoplasmatic reticulum membranes could be inhibited by a molar ratio of ACBP/oleoyl-CoA in its complexes (Kerkhoff et al., 1997). However, ACAT activity was not affected by other lipid binding proteins such as bovine serum albumin (BSA) and the liver fatty acid binding protein (FABP).

Oxysterol Binding Protein-Related Protein (ORP)

Oxysterol-binding protein (OSBP) was first identified in humans by its ability to bind to oxysterols, 27-carbon products of cholesterol oxidation (Kandutsch *et al.*, 1977; Kandutsch & Shown, 1981). OSBPs were found to inhibit the cholesterol biosynthesis by repressing the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which

indicates that OSBP plays a role in the regulation of cholesterol homeostasis (Lehto & Olkkonen, 2003; Taylor *et al.*, 1984; Taylor & Kandutsch, 1985). Since then, numerous OSBP homologues were identified from humans, animals, fungi and many other organisms. Because not all OSBP homologues may bind to oxysterols, this group of proteins was then renamed to OSBP-related proteins (ORPs). It is currently known that all ORPs can bind to various types of phosphatidyl-inositol phosphates (PIPs) with different binding profiles (Lehto & Olkkonen, 2003; Olkkonen, 2004; Olkkonen *et al.*, 2006). The recently completed genome-sequencing projects have revealed at least 11 ORP homologues in humans and 7 in the budding yeast. Some of them are long type ORPs containing both pleckstrin homology (PH) and ligand binding (LB) domains, while others are short, only containing the LB domain (Im *et al.*, 2005; Lehto & Olkkonen, 2003). Although there has been an increasing interest to use yeast model to study ORPs, the ligand-binding features and biological roles of all yeast ORPs are not fully understood yet. Knockout of any single ORP gene is not lethal in yeast, but changes the sterol profile. However, yeast apparently cannot survive when all 7 ORP genes are knocked out, which suggesting some overlapping functions among these homologues (Beh & Rine, 2004; Lehto & Olkkonen, 2003; Lessmann *et al.*, 2006).

While all ORPs have the ligand binding (LB) domain, some of them may contain a pleckstrin homology (PH) domain. The human OSBP (hOSBP) contains both PH and LB domains. Upon binding to the ligand (i.e., 25-hydroxycholesterol [25-OHC]), hOSBP will be translocated to the Golgi apparatus, which facilitates the transport of oxysterols to the Golgi. PH domain appears to be responsible for the binding of hOSBP to the

Golgi, as the mutated, LB-lacking OSBP was still able to bind to this organelle in transfected cells (Ridgway *et al.*, 1992). The ability for PH domain to bind to various PIPs was reported in ORPs and other PH domain-containing proteins, such as the phospholipase C-δ_1. In the later case, the PH domain displays high affinity to PtdIns(4,5)P_2. On the other hand, the short-type ORPs were also shown to be able to bind to PIPs, suggesting that the PH domain is not the sole region responsible for the PIP-binding in ORPs (Beh & Rine, 2004).

The research data presented in this dissertation focuses on the molecular and biochemical characterizations of an ACBP and two ORPs in *C. parvum* as part of the fatty acid metabolism project running in the laboratory. Genes encoding the three lipid-binding proteins were cloned, sequenced and engineered into a maltose-binding protein (MBP)-fusion system. Recombinant proteins were expressed in bacteria and their biochemical features, including binding specificity and kinetics, were analyzed in detail. Specific polyclonal antibodies were also generated for protein detections by western blot analysis and immunofluorescence microscopy. Our results revealed that these lipid binding proteins differ from the counterparts in mammals, and two of them are localized in the unique PVM, suggesting that they are probably involved in the lipid uptake across, and/or the participation of membrane regeneration of the PVM by the parasite. (Chen *et al.*, 2002a)

CHAPTER III

CHARACTERIZATION OF A FATTY ACYL-CoA BINDING PROTEIN (ACBP) FROM THE APICOMPLEXAN CRYPTOSPORIDIUM PARVUM*

INTRODUCTION

Cryptosporidium parvum is a globally important parasitic protist that infects both humans and animals (Chappell & Okhuysen, 2002; Thompson *et al.*, 2005; Tzipori & Widmer, 2000a). *Cryptosporidium parvum* belongs to the Phylum Apicomplexa that contains many important human and animal parasites (e.g. *Plasmodium, Babesia, Toxoplasma* and *Eimeria*) (Zhu *et al.*, 2000a). This group of parasites shares some common biological features characteristic to the phylum. For example, all apicomplexans possess similar complex life cycle stages including oocyst formation, sporulation, merogony and gametogenesis. However, recent advancements in biochemistry and genome-sequencing have revealed that a number of metabolic pathways such as fatty acid biosynthesis are highly diverse within the Apicomplexa (Abrahamsen *et al.*, 2004; Zhu *et al.*, 2000a). Apicomplexans may possess either apicoplast-specific Type II fatty acid synthases (FASs) (e.g., *P. falciparum*), or Type I FAS (e.g., *C. parvum*), or both (e.g., *T. gondii)* (Roos *et al.*, 2002; Zhu, 2004b) (Zhu *et al.*, 2000e; Zhu *et al.*, 2002; Zhu *et al.*, 2004b). Although fatty acids are one of the major components in all cells, free fatty acids cannot enter any metabolic pathways unless

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they are activated by thioesterification with coenzyme A (CoA) to form an acyl-CoA or with acyl carrier protein (ACP) to form an acyl-ACP. Fatty acyl-CoA can immediately enter subsequent metabolic pathways, or may be stored/transported by a family of acyl-CoA binding proteins (ACBPs).

ACBPs are a group of highly conserved proteins and have been found in animals, plants, protists, and a number of pathogenic bacteria (Burton *et al.*, 2005). They are typically small, cytosolic molecules of ~10 kDa. However, a number of larger ACBP proteins (e.g. Mr >55-kDa) have also been identified in both animals and plants. Mammals possess multiple ACBP proteins that are differentially expressed in various tissues (e.g. T-ACBP in testis, L-ACBP in livers and I-ACBP in intestines) (Schroeder *et al.*, 1998). In *Trypanosoma brucei*, an ACBP protein was found to be involved in the synthesis of the glycosyl-phosphatidylinositol (GPI) anchor in variant surface glycoproteins (VSG) (Milne & Ferguson, 2000; Milne *et al.*, 2001). Although ACBPs are capable of binding medium to long chain fatty acyl-CoA esters, they may vary in their substrate preference and binding affinities. For example, the highest affinities of ACBP proteins from bovine (liver) and trypanosome (or *P. falciparum*) are C18 stearoyl- and C14 lauroyl-CoA, respectively (Milne & Ferguson, 2000; van Aalten *et al.*, 2001).
By data-mining the recently completed *C. parvum* genome sequences, we have identified a unique long-type ACBP (CpACBP1) that contains an N-terminal ACBP domain and a C-terminal ankyrin-repeat sequence, which is structurally similar to the membrane-associated ACBP1 and ACBP2 in *Arabidopsis thaliana*. In the present study, we have expressed CpACBP1 protein as a fusion protein and characterized its primary

biochemical features. We have also found that the *CpACBP1* gene is differentially expressed during the parasite life cycle, and its encoded protein is chiefly located on the parasitophorous vacuole membranes (PVM), suggesting that this protein may be involved in the formation of PVM and/or uptake of fatty acids by the parasite.

METHODS

Manipulation of parasite

Oocysts of *C. parvum* (Iowa strain) were purchased from Grass Bunch Farm (Deary, ID). The limited amount of bacterial contaminants and debris were removed with a Percoll gradient centrifugation technique and sterilized for 5 min in 10% Clorox® on ice. After washing in sterile water 5-8 times, the sterilized oocysts were excysted for 1-1.5 hr at 37˚C in phosphate buffered saline (PBS, pH 7.5) containing 0.25% trypsin and 0.75% taurodeoxycholic acid. Sporozoites were washed 3-5 times with sterile water and concentrated as previously described (Millership & Zhu, 2002; Zhu *et al.*, 2000d). Genomic DNA (gDNA) was isolated from the free sporozoites using the DNeasy isolation kit (Qiagen, Valencia, CA).

Identification of ACBP homologues from *C. parvum* and other apicomplexans

CpACBP1 was identified from the *C. parvum* genome as an intronless gene by a homology-search using characterized animal and plant ACBP proteins as queries. Similar searches were also performed to identify ACBP homologues from the

Plasmodium falciparum and *Toxoplasma gondii* genome databases (http://www.PlasmoDB.org and http://www.ToxoDB.org, respectively) for comparison. Domains in the apicomplexan ACBP homologues were identified by searching the Conserved Domain Database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and by comparing with other characterized proteins in the GenBank database.

Molecular cloning and engineering of *CpACBP1*

To facilitate biochemical analysis, we cloned *CpACBP1* and expressed CpACBP1 as a maltose-binding protein (MBP)-fusion as described below. Briefly, the entire open reading frame (ORF) of *CpACBP1* was amplified from *C. parvum* (Iowa strain) genomic DNA (gDNA) with the following pair of primers: 5'-ATG ACT GAT ATC TTA TCC ACG AAC-3' and 5'-atg gat ccT TAA CTG CTT TCG AGA ATT CTT-3' (Note: lower cases represent added *Bam*HI restriction site). A high-fidelity *Pfu* DNA polymerase (Stratagene) was used to minimize potential errors introduced by amplification. The PCR product was digested with *Bam*HI to produce a cohesive 3' end, but retained the blunt 5' end to facilitate unidirectional cloning. The 5' ends were phosphorylated by treating the amplicons with T4 polynucleotide kinase. After agarose gel electrophoresis, DNA fragments of the expected size were purified using a MinElute gel extraction kit (Qiagen), and ligated into an *Xmn*I and *Bam*HI double-digested pMAL-c2x vector (New England Biolabs) with a T4 DNA ligase. The ligated plasmids were transformed into the TOPO-10 strain of *Escherichia coli* cells (Invitrogen). The resulting colonies were first screened using a sense-stranded primer located upstream to the insert in the vector and the CpACBP1 antisense-stranded primer. Plasmids were isolated from PCR-positive colonies for sequencing to confirm their

identity and the sequence of the inserts. The resulting construct and encoded fusion protein were named pMAL-c2x-CpACBP1 and MBP-CpACBP1, respectively.

Similarly, we also constructed an MBP-fusion containing only the ACBP domain. Because of the presence of two *Eco*RI restriction sites flanking the ankyrin domain (i.e., nucleotides from 298 to 788), we removed the entire C-terminal ankyrin repeats by digesting pMAL-c2x-CpACBP1 plasmid with *Eco*RI and agarose gel separation. The remaining plasmid fragment was re-ligated back, resulting a construct (pMAL-c2x-CpACBP1-S) that encodes only the N-terminal 100 amino acids (aa) plus 7 extra aa derived from the remaining nucleotides at the 3' end of insert and the vector's multiple cloning site (MBP-CpACBP1-S). We predict that the extra 7 aa (RKQLRIL) will not alter the function of the ACBP domain as they do not resemble any known functional sequences in the databases.

For each construct, a plasmid containing the correct insert was transformed into *E. coli* Rosetta cells (Novagen) for protein expression. Briefly, a single clone of the *E. coli* transformants was inoculated in 10 ml LB broth containing 100 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol for growing overnight at 37 °C. On the second day, the bacterial suspension was transferred into 1 L fresh medium containing the two antibiotics and grown at 30 °C until the OD_{495} reached to 0.3-0.5. After adding IPTG to the broth (100 mg ml^{-1} final concentration), bacteria were further incubated for 4 hours at 30 °C and collected by centrifugation for 10 min at 8,000 g. Bacterial pellets were suspended in 50 ml of column buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM EDTA) containing a protease inhibitor cocktail for bacteria (Sigma-Aldrich), disrupted by sonication, and centrifuged (8,000 g, 10 min) to remove cell debris. Supernatants were applied to an

amylose-resin column (New England Biolabs), washed with column buffer (>10 X bed vol.), and the MBP-CpACBP1 fusion proteins were eluted from the column with elution buffer (10 mM maltose, 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM EDTA) according to the manufacturer's protocol. The size and purity of the recombinant proteins were analyzed by SDS-PAGE. Protein concentrations were determined by the Bradford method using a commercial kit and bovine serum albumin (BSA) as standard. The aliquots of each protein sample were either used immediately or stored at -80 °C.

Semi-quantitative RT-PCR

The entire semi-quantitative RT-PCR (semi-qRT-PCR) procedure, including the isolation of parasite total RNA from various intracellular stages including asexual and sexual life cycles, the normalization of parasite RNA contents in intracellular samples, reverse transcription, amplification and analysis, has been previously described in detail (Millership *et al.*, 2004a; Millership *et al.*, 2004b). In this study, the following pair of primers was used to amplify CpACBP1 transcripts: CpACBP1-F334 (5'-CCT TTA TTA GAA TCA AAC CTG G-3') and CpACBP1-R334 (5'-TTG GAT AGG AGT CAA ACC ATC-3'). Another pair of previously reported primers (995F, 5'-TAG AGA TTG GAG GTT GTT CCT-3' and 1206R, 5'-CTC CAC CAA CTA AGA ACG GCC-3') was used to amplify 18S rRNA as a control for normalization (Abrahamsen & Schroeder, 1999). Each semi-qRT-PCR reaction contained a comparable amount of parasite RNA, and was subjected to 45-min reverse transcription and 23 (for CpACBP1) or 20 (for 18S rRNA) thermal cycles of PCR amplification. After agarose gel electrophoresis, the intensity of each product was measured using GENETOOLS program v.3 (Hitachi Software Engineering) and the

relative level of CpACBP1 transcripts was determined as the signal ratio between the CpACBP1 and rRNA amplicons.

Production of polyclonal antibodies to CpACBP

Polyclonal antibodies to recombinant CpACBP1 protein were raised in a pathogen-free rabbit. Initial immunization used 0.2 mg of affinity-purified MBP-CpACBP1 protein emulsified in an equal 2 ml of complete Freund's adjuvant. Two subsequent booster immunizations (0.1 mg) were injected on 30 and 60 days, after the primary immunization. Rabbit sera were collected prior to and after the immunization protocol. The anti-MBP portion of the polyclonal antibodies was removed by absorbing antiserum with equal volume of amylose-resin conjugated with MBP. The antibody titer and specificity were evaluated by western blot analysis.

Western blot analysis

Western blot analysis was performed to test whether CpACBP1 protein is present in various parasite life cycle stages. Oocysts ($5x10^6$/lane), free sporozoites ($2x10^7$/lane), and HCT-8 cells ($1x10^6$/lane) infected with *C. parvum* oocysts (oocysts : host cell ratio = 1:1) for 24, 48 and 72 hr were lysed in loading buffer containing a protease inhibitor cocktail for mammalian cells (Sigma-Aldrich) at 95 °C for 8 min. Uninfected HCT-8 cells cultured for the same periods of time were also lysed and used as controls. After centrifugation for 5 min, soluble materials were fractionated in a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was first blocked with

5% BSA in TBS (20 mM Tris pH 7.5, 50 mM NaCl) for 1 h, and then incubated with rabbit anti-CpACBP antibodies and a monoclonal anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma-Aldrich) in 1% BSA in TBST (TBS with 0.05% Tween-20). The blot was washed 3 times with TBST after each incubation step, and all procedures were performed at room temperature. Finally, the labeled proteins were developed using 5'bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma-Aldrich).

Immunofluorescence microscopy

Intracellular parasites were prepared by infecting HCT-8 cells grown on poly-L-lysine-treated glass coverslips for 24, 48 or 72 hr. Cells were fixed with 10% formalin, rinsed with PBS, extracted with cold methanol (-20 °C for 5 min), blocked in 0.5 % BSA-PBS (10 min), labeled with primary antibodies (1 hr in 0.5 % BSA-PBS), and incubated with secondary antibodies conjugated with FITC or TRITC (Sigma-Aldrich, 1/20 and 1/400 dilution respectively) (60 min in 0.5 % BSA-PBS). Samples were washed after each incubation step (3 times, 5 min each) using PBS. Free sporozoites were fixed in suspension, directly applied onto poly-L-lysine-treated coverslips, extracted, and air-dried prior to the incubations with antibodies. Co-localization of CpACBP1 with total membrane proteins (TMP) and an SFP-type phosphopantetheinyl transferase (CpSFP-PPT) immunolocalization was similarly performed, except that the respective rabbit antibodies were directly labeled with Alexa Fluor 488 or Alexa Fluor 546 using the appropriate fluorophore-labeling kits (Invitrogen) prior to the immuno-labeling experiments. No secondary antibodies were used in co-localization experiments. The TMP antibody has previously been shown to mainly label the PVM and feeder organ in intracellular parasites (Chen *et al.*, 2003), while cytosolic localization of CpSFP-PPT

has also been previously reported (Cai *et al.*, 2005). All samples were mounted using a SlowFade Light Antifade medium containing 4', 6'-diamidino-2-phenylindole (DAPI) for DNA counter-staining (Invitrogen) and examined with an Olympus BX51 Epi-Fluorescence microscope equipped with Differential Interference Contrast (DIC) and FITC/TRITC/DAPI filters.

Acyl-CoA binding assay

The binding affinity of CpACBP1 with fatty acyl-CoA was measured by Lipidex 1000 assay as previously described (Rasmussen *et al.*, 1990; Rosendal *et al.*, 1993). Briefly, 40 pmol of recombinant CpACBP1 was mixed with [^{14}C] palmitoyl-CoA (0 – 8 mM) in 100 ml of binding buffer (10 mM potassium phosphate, pH 7.4) and incubated at 37 °C for 30 min. The mixture was then chilled on ice for 10 min, mixed with 0.6 ml of cold Lipidex 1000 (hydroxyalkoxypropyl dextran, Type VI from Sigma-Aldrich) (50% slurry, v/v in binding buffer) with gentle rotation at 4 °C for 1 hr, and centrifuged for 5 min at 12,000 *g* at 4 °C to remove free acyl-CoA esters. An aliquot of 200 ml supernatant was taken from each sample for counting radioactivity in a Beckman LS6500 scintillation counter. Each experiment included negative controls using MBP-tag only for background subtraction. At least three replicates were performed for each experimental condition. The dissociation constant (K_d) was determined by plotting the amount of bound substrate against the total concentration of substrate using nonlinear regression and Prism v4.0 (GraphPad Software).

The substrate preference for CpACBP1 was determined by a competition-binding assay. It was performed in 100 ml of binding buffer containing 40 pmol of recombinant CpACBP1 (or MBP in control groups) and 80 pmol of [^{14}C] palmitoyl-CoA in the absence or presence of 80 pmol of non-radioactive fatty acyl-CoA esters of various chain lengths (i.e., ranging from 4 to 20 carbons). All samples were incubated, extracted with Lipidex 1000, and counted for radioactivity as described above.

In addition, we also tested whether CpACBP1 could specifically bind to long chain fatty acid. In this assay, 80 pmol recombinant CpACBP1 protein was incubated with 80 pmol [^{3}H] palmitic acid or [^{14}C] palmitoyl-CoA in 100 ml buffer. After extraction with Lipidex 1000, the supernatants were counted for radioactivity as described above.

Autoradiography

To visualize the binding of CpACBP1 with acyl-CoA, 80 pmol of fusion protein was incubated with 80 pmol of [^{14}C] palmitoyl-CoA in the presence or absence of 80 pmol of non-radioactive palmitoyl-CoA in 100 ml of binding buffer for 30 min at 37 °C. Subsequently, 20 ml of the reaction was mixed with 5 ml of 5X native loading buffer, and fractionated in a 10% native PAGE gel. The gel was dried on a heated gel-drier and radioactive protein bands were visualized with X-ray film in a BioMax TranScreen LE intensifying system (Kodak).

RESULTS

CpACBP1 is a "long-type" ACBP containing ankyrin repeats

CpACBP1 (nucleotide sequence data are available in the GenBank database under the accession number DQ406676) is the only ABCP homologue that can be identified from the *C. parvum* genome by repeated BLAST searches using several animal, plant and protist ABCP proteins as queries. This intronless gene encodes 268 aa that constitute an N-terminal ACBP domain (~ 90 aa) and a C-terminal sequence (~178 aa) containing two ankyrin repeats (Fig. 3.1). Other apicomplexans appear to possess more ACBP homologues in their genomes. For example, *P. falciparum* has 4 ACBP homologues (PfACBPs, 3 short and one long), and *T. gondii* has two (TgACBPs, one short and one long). The small ACBP domains in all apicomplexan proteins share many conserved residues with their homologues found in animals and plants that are characteristic to this group of proteins (Fig. 3.2). Amino acids that are critical to the ligand binding activity are all present in CpACBP1 and other apicomplexan ACBPs (Fig. 3.2, indicated by solid dots) (Burton *et al.*, 2005). Similar to CpACBP1, the long-type ACBP in *T. gondii* contains two ankyrin repeats (Fig. 3.1). However, it also possesses an N-terminal signal peptide, possibly for secretion. On the other hand, the *P. falciparum* long-type ACBP contains no ankyrin repeats (Fig. 3.1). These observations indicate that apicomplexans may differ from each other by possessing different numbers and types of ACBP proteins.

CpACBP1 has a highest binding affinity to C16:0 palmitoyl-CoA

In order to investigate the binding features of CpACBP1, we have expressed the full-length protein as well as the ACBP domain as MBP-fusion proteins (i.e., MBP-

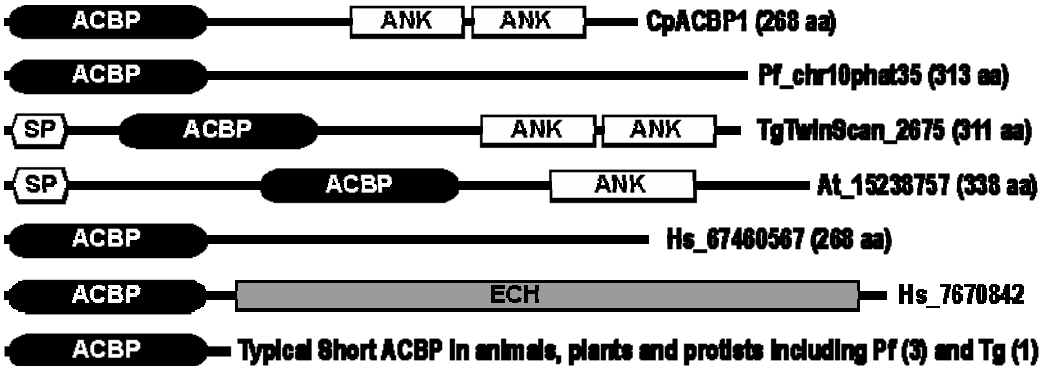

Fig. 3.1. Domain organization of CpACBP1 in comparison with those from other representative eukaryotic acyl-CoA binding proteins (ACBPs). Pf = *Plasmodium falciparum*. Tg = *Toxoplasma gondii*. At = *Arabidopsis thaliana*. Hs = *Homo sapiens*.

Fig. 3.2. Multiple alignment of conserved region in the ACBP domain between CpACBP1 and other representative eukaryotic ACBP proteins. Amino acids shared between CpACBP1 and other sequences are shaded, while residues conserved among all listed sequences are boxed. Pf = *Plasmodium falciparum*. Tg = *Toxoplasma gondii*. At = *Arabidopsis thaliana*. Hs = *Homo sapiens*. Numbers following the species abbreviations are the accession numbers in GenBank or the corresponding apicomplexan genome databases (http://www.PlasmoDB.org or http://www.ToxoDB.org). Solid dots indicate amino acids critical to the acyl-CoA binding activity.

CpACBP1 and MBP-CpACBP1-S). Both fusion proteins were purified to homogeneity using amylose resin-based affinity chromatography (Fig. 3.3). Although the fusion proteins expressed using pMAL-c2x vector contain a factor Xa cleavage site, attempts to effectively remove the MBP-tag using factor Xa were not successful. It is possible that the MBP-CpACBP1 fusion protein folded in a way that interfered with the access of factor Xa to the cleavage site. The difficult in removing the MBP-tag was also observed for the recombinant *C. parvum* malate and lactate dehydrogenases, in which their activities were assayed with the presence of MBP-tag (Madern *et al.*, 2004).

Using the Lipidex 1000 assay, we first confirmed that the intact MBP-CpACBP1 was able to specifically bind to palmitoyl-CoA, while MBP had no or little affinity to the same ligand (Fig. 3.4A). With both ligand and protein concentrations at 80 mM (near the highest concentration of 100 mM in the kinetics assay), the nonspecific binding by MBP-tag was ~ 5% of the specific binding observed for MBP-CpACBP1. In another assay using both palmitic acid and palmitoyl-CoA as substrates, both MBP-tag and MBP-CpACBP1 displayed almost the same radioactivity in binding to the palmitic acid (Fig. 3.4B), indicating that fatty acid is not a favorite ligand for CpACBP1. Based on these observations, we decided to use the uncleaved fusion proteins in all subsequent analyses.

We first studied the specific binding between CpACBP1 and palmitoyl-CoA and determined that the K_d for CpACBP1 to bind to palmitoyl-CoA was at 407 nM (Fig. 3.5). This value is significantly higher than those reported for many other ACBPs that are typically at 1 – 10 nM ranges (Burton *et al.*, 2005). This observation suggests that, although Lipidex assay is a reliable assay for determining the acyl-CoA binding profile

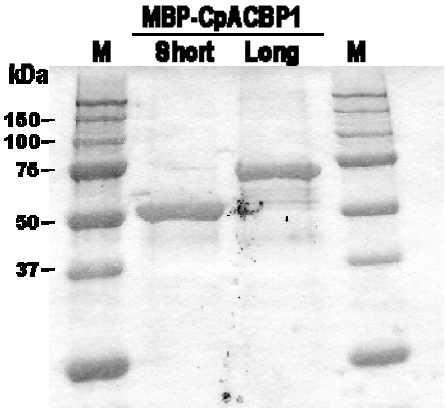

Fig. 3.3. SDS-PAGE analysis of purified MBP-fused CpACBP1 proteins. Short = MBP-fusion protein containing ACBP domain only. Long = MBP-fusion protein containing the entire CpACBP1 sequence. M = protein molecular marker.

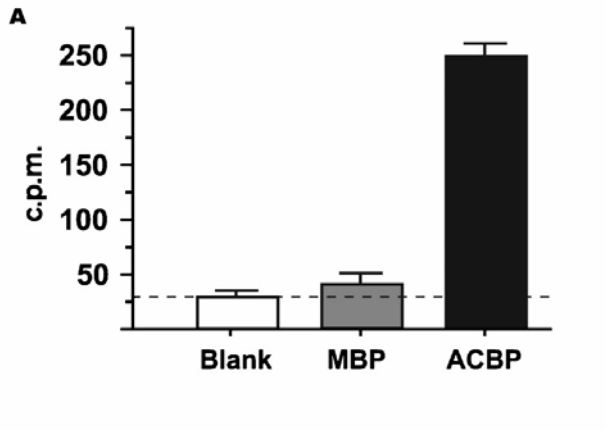

Fig. 3.4A. Specific and nonspecific binding of [^{14}C] palmitoyl-CoA (80 mM) by MBP-fused CpACP1 (40 mM) and MBP-tag (40 mM) by the Lipidex 1000 extraction assay.

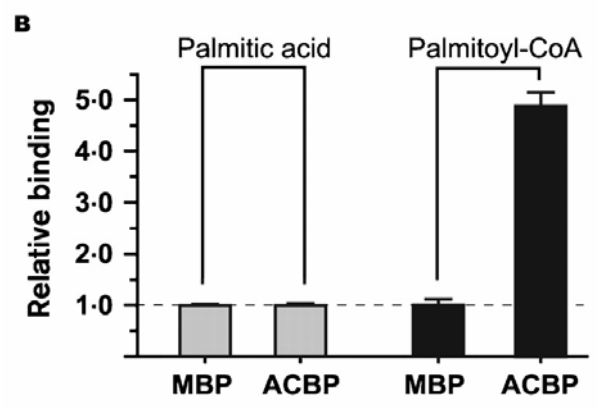

Fig. 3.4B. Relative binding between CpACBP1 and [^{14}C] palmitoyl-CoA (80 mM) or [^{3}H] palmitic acid as determined by Lipidex 1000 assay. Radioactivity was normalized using MBP-tag as control.

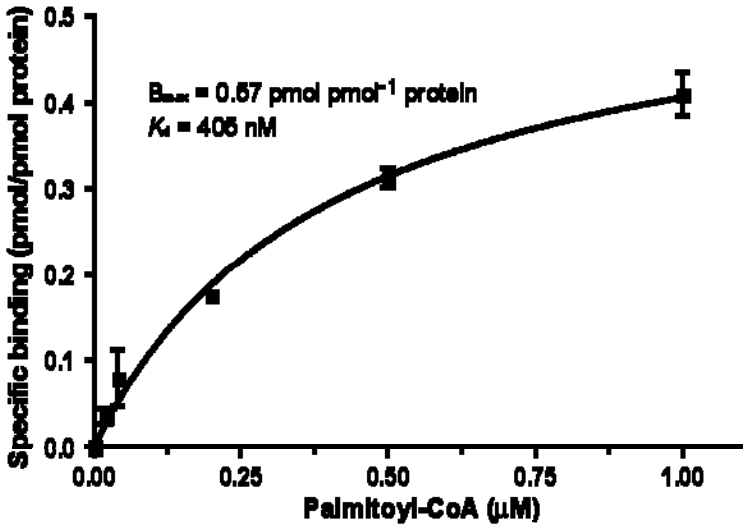

Fig. 3.5. Binding kinetics of recombinant CpACBP1 with palmitoyl-CoA as determined by Lipidex 1000 assay.

for an ACBP, it is probably not a sensitive method for determining the binding kinetics due to the binding competition between ACBP and Lipidex 1000 during the extraction step (Rasmussen *et al.*, 1994). It is also possible that a significant portion of the CpACBP1 fusion protein was inactive and incapable of binding to acyl-CoA, and/or the N-terminal 42-kDa MBP-tag might physically interfere the binding kinetics of the recombinant proteins.

We also tested the specific binding of recombinant CpACBP1-S to palmitoyl-CoA in comparison with that of full-length CpACBP1 using the same Lipidex assay. Under the condition of using 0.4 mM of protein and 0.8 mM of palmitoyl-CoA, both fusion proteins displayed similar specific binding activities (i.e., 0.180 and 0.206 pmol $pmol^{-1}$ protein for CpACBP1 and CpACBP1-S, respectively), thus confirming that the ACBP domain was responsible for the acyl-CoA binding.

The specific binding of CpACBP1 to fatty acyl-CoAs was further confirmed by autoradiography. When [^{14}C] palmitoyl-CoA was incubated with various fusion proteins, only recombinant CpACPB1 (full-length) or CpACBP1-S (ACBP domain only), but not MBP-tag, displays radioactivity (Fig. 3.6). The radioactive intensity associated with the fractionated CpACBP1 was reduced when an equal molar amount of non-radioactive fatty acyl-CoA was added into the reaction. It is also noticeable that multiple radioactive bands were observed in lanes containing CpACBP1. Since protein fractionation was performed in native PAGE gels, this observation suggests that CpACBP1 may also function as dimers or tetramers. However, it is also possible that protein aggregation might occur under the experimental conditions used.

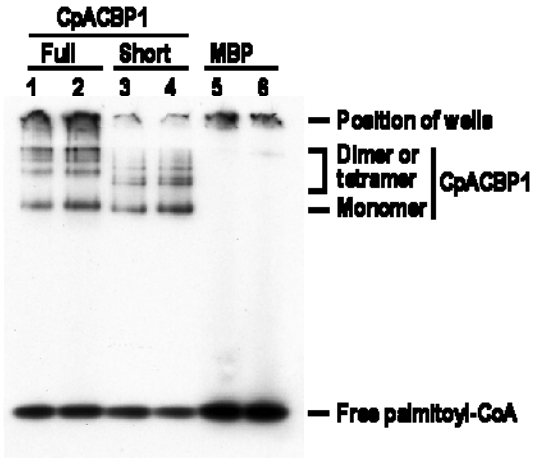

Fig. 3.6. Autoradiography showing the binding of [^{14}C]-palmitoyl-CoA by full-length (lanes 1 and 2) and short ACBP-domain only (lanes 3 and 4) fusion proteins after native PAGE fractionation. MBP-tag only (lanes 5 and 6) was used as a control. In lanes 1, 3 and 5, the same molar amount of non-radioactive palmitoyl-CoA was also included to compete with the radioactive acyl-CoA, which resulted in the reduced intensity of radioactivity in these lanes. Positions of CpACBP1 monomers, dimers and polymers are indicated. Some acyl-CoA molecules were retained in the loading wells as indicated.

Based on the specific binding data, we decided to test the substrate preference for CpACBP1 using 0.8 mM of various fatty acyl-CoAs to compete with the same molar concentration of palmitoyl-CoA. This concentration is ~ 2X of the K_d value for palmitoyl-CoA, so that the protein occupancy by the ligands was neither too low or too high. The results show that CpACBP1 can bind to the medium and long chain acyl-CoAs (Fig. 3.7).

CpACBP1 gene is differentially expressed and its encoded protein is likely localized to the parasitophorous vacuole membranes (PVM)

Semi-quantitative RT-PCR showed that the *CpACBP1* gene is expressed differentially in the complex parasite life cycle (Fig. 3.8). *CpACBP1* transcripts were barely detectable in free sporozoites, but started to appear after parasites' invasion into host cells. The level of *CpACBP1* transcripts was relatively low during early intracellular development (i.e., from 3 to 24 hr post-infection, P.I.), but gradually increased with the time of infection (Fig. 3.9).

More surprisingly, immunofluorescence microscopy indicates that CpACBP1 is probably located on the PVM (Fig. 3.10A,B,C). Rabbit polyclonal antibodies clearly labeled the surface of meronts with a homogeneous pattern of distribution, but failed to label the merozoites within the meronts (Fig. 3.10A). In a dual-labeling experiment using a rabbit polyclonal antibody mainly against PVM(the total membrane proteins' antibody was made by our lab, which is localized on the membrane on the parasite) and the electron dense connection between host cell and parasite, we co-localized CpACBP1 and PVM proteins (Fig. 3.10B). On the other hand, a polyclonal antibody against CpSFP-PPT

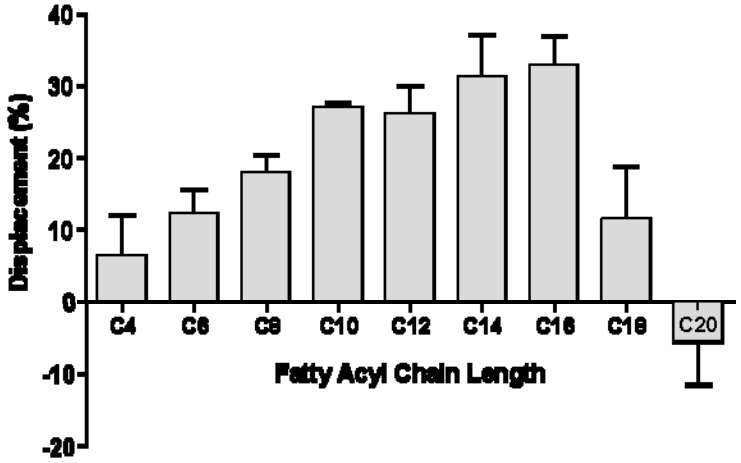

Fig. 3.7. Acyl-CoA binding specificity of CpACBP1 determined by Lipidex 1000 competition binding assay. In each reaction, [^{14}C]-palmitoyl-CoA was mixed with the same molar amount of non-radioactive acyl-CoA with specified carbon chain length. The binding affinity was presented as the percent displacement of radioactive palmitoyl-CoA by non-radioactive acyl-CoA. SEM values were determined from at least three individual samples.

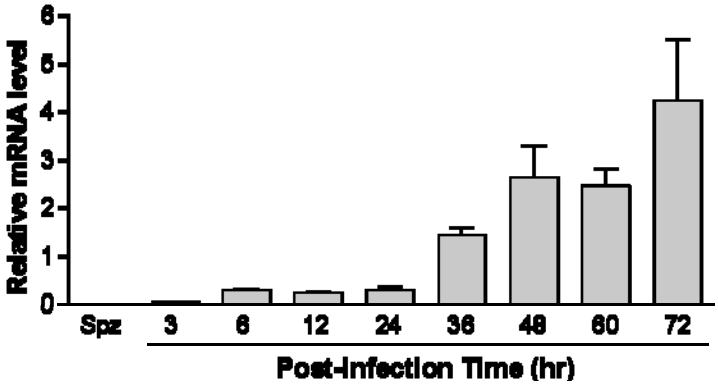

Fig. 3.8. Relative levels of CpACBP1 gene transcripts in various *Cryptosporidium parvum* life cycle stages as determined by semi-quantitative RT-PCR. The level of transcripts is normalized using that of the parasite 18S rRNA as a control. Spz = excysted free sporozoites.

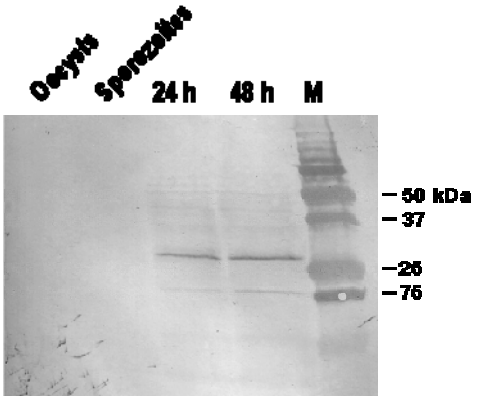

Fig. 3.9. Western blot detection of CpACBP1 protein in *Cryptosporidium parvum* oocysts, excysted free sporozoites, and intracellular parasites grown for 24 and 48 hr. CpACBP1 was only detected in the intracellular parasites, but not in oocysts and free sporozoites.

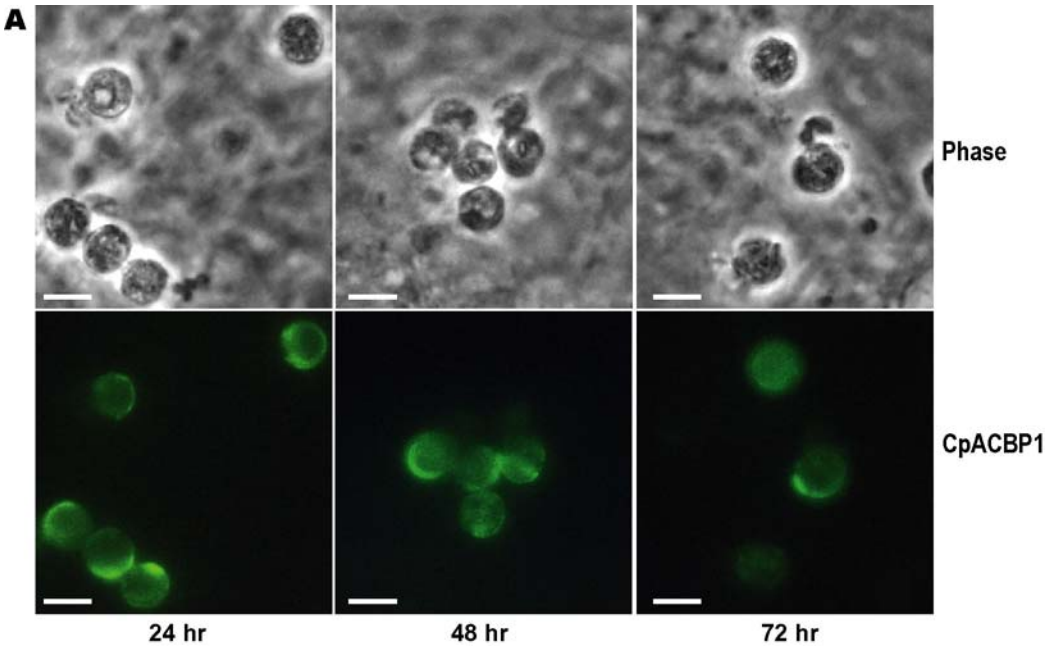

Fig. 3.10A. Indirect immuno-labeling of intracellular *Cryptosporidium parvum* grown for 24, 48 and 72 hr using a rabbit polyclonal antibody against CpACBP1 and a secondary antibody conjugated with FITC.

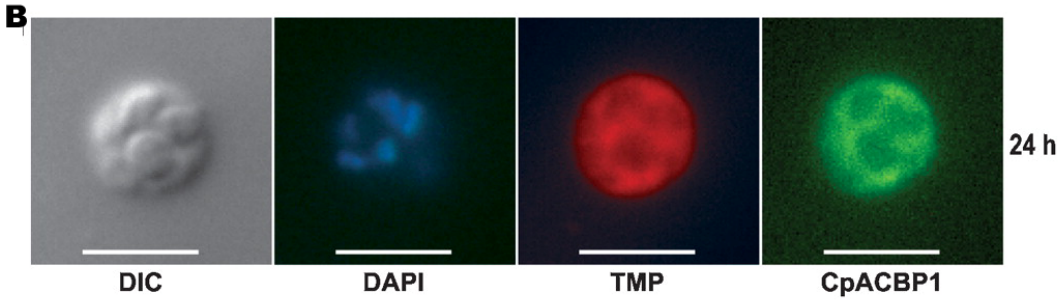

Fig. 3.10B. Direct dual-labeling of intracellular parasites grown for 24 hr using an Alexa Fluor 546-conjugated antibody against CpACBP1 and an Alexa Fluor 488-conjugated antibody against parasite total membrane protein (TMP) that mainly labels parasitophorous vacuole membrane and feeder organ. Both antibodies displayed the same labeling pattern on the surface of a meront.

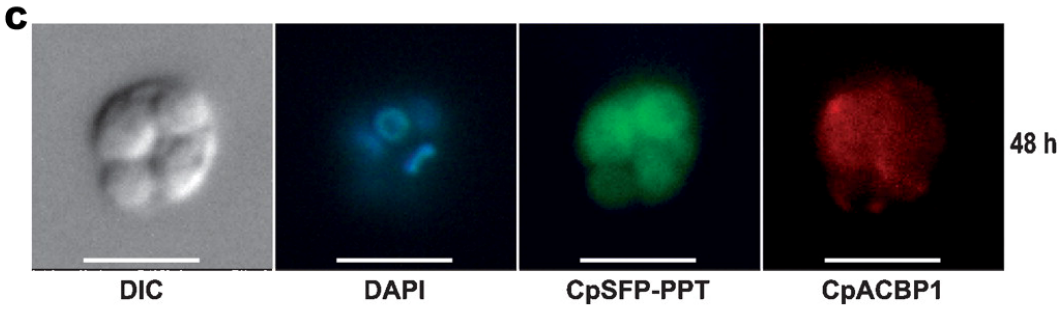

Fig. 3.10C. Dual-labeling of intracellular parasites grown for 48 hr using an Alexa Fluor 488-conjugated antibody against CpACBP1 and an Alexa Fluor 546-conjugated antibody against cytosolic CpSFP-PPT, showing CpACBP1 mainly on the surface of meronts, rather than in merozoites. DIC = differential interference contrast. DAPI = 4',6-diamidino-2-phenylindole for counterstaining nuclei. Bar = 5 mm.

clearly labeled the merozoites, but not on the surface of meronts in another dual-labeling experiment (Fig. 3.10C).

DISCUSSION

ACBP was originally identified as a mammalian diazepam binding inhibitor – a neuropeptide that could inhibit diazepam binding to the γ-aminobutyric acid (GABA) receptor (Guidotti *et al.*, 1983). Typical ACBPs are small, ~10 kDa cytosolic proteins (Burton *et al.*, 2005). However, there are a number of long-type ACBPs that are fused with ankyrin repeats, such as the ACBP1 and ACBP2 in *Arabidopsis thaliana* (Chye *et al.*, 1999; Li & Chye, 2003), or with other functional domains, such as the human peroxisomal D3, D2-enoyl-CoA isomerase (Geisbrecht *et al.*, 1999). ACBP mainly functions as an intracellular acyl-CoA transporter and pool former, and is critical to lipid metabolism in cells (Gossett *et al.*, 1996; Knudsen *et al.*, 2000; Schroeder *et al.*, 1998). However, ACBP has only been found in eukaryotes, but not in prokaryotes except for a few pathogenic eubacteria that might have acquired ACBP from eukaryotic hosts via lateral gene transfer (Burton *et al.*, 2005).

Among apicomplexans, *C. parvum* only possesses a single, long-type ACBP that is fused with an ankyrin repeats domain. However, other apicomplexans may have multiple ACBP proteins of various types (i.e., short-type, long-types fused with ankyrin repeats or with uncharacterized sequences) (Fig. 3.1), indicating that the ACBP-mediated metabolic pathways may be highly divergent in the Apicomplexa. *Arabidopsis* ACBP1 and ACBP2 are membrane proteins that differ from typical cytosolic ACBPs

(Chye *et al.*, 1999; Leung *et al.*, 2005). The ankyrin repeats in these proteins are responsible for docking these proteins to the membrane by interacting with an ethylene-responsive element-binding protein. Our immuno-labeling data indicate that CpACBP1 is also a membrane protein (i.e., mainly associated with PVM) (Fig. 3.10). Such a membrane association is likely mediated by the interaction of ankyrin repeats with a yet unknown protein(s) in the PVM.

Although *C. parvum* is an intracellular parasite, it does not reside within the host cytoplasm. Instead, this parasite is extracytoplasmic, covered by PVM on the surface of intestinal epithelial cells (Chen *et al.*, 2002b). Therefore, the PVM is the only barrier separating parasites from the intestinal lumen. The localization of CpACBP1 to the PVM is thus intriguing, although it is currently uncertain whether CpACBP1 is also associated with the feeder organ at the host cell-parasite interface. It implies that CpACBP1 may be involved in the formation of the PVM or uptake of fatty acids across the PVM. However, since CpACBP1 mRNA and protein are undetectable (or barely detectable) in sporozoites and in the invasion stages (i.e., first 3 hr of infection), it seems less likely that CpACBP1 is associated with the early stage of PVM formation. On the other hand, it is known that *C. parvum* may have to import fatty acids from host cells or the intestinal lumen since it is likely to be incapable of synthesizing fatty acids de novo, although it is capable of elongating long chain fatty acids (Zhu, 2004b). Therefore, it is possible that CpACBP1 may function as a fatty acyl-CoA scavenger in conjunction with an acyl-CoA synthetase on or around the PVM (or the feeder organ) to facilitate the fatty acid uptake by the parasite.

Another possibility is that CpACBP1 may be involved in the synthesis of a glycosyl-phosphatidylinositol (GPI) anchor. In African trypanosomes, ACBP has been found to be responsible for supplying myristoyl-CoA to the fatty acid remodeling machinery during GPI synthesis (Milne & Ferguson, 2000; Milne *et al.*, 2001). Although it is yet unclear whether GPI anchored molecules are present in the PVM, a recent comprehensive chemical analysis has clearly revealed the presence of complex glycosylinositol phospholipids in the *C. parvum* sporozoites (Priest *et al.*, 2003). In addition, a number of enzymes involved in the biosynthesis of GPI anchors are also present in the *C. parvum* genome, which includes phosphatidylinositol N-acetylglucosaminyltransferases (e.g., Genbank accession numbers XP_628152, XP_627129 and XP_626317). Nonetheless, further investigations are necessary to test these hypotheses.

CpACBP1 has highest affinity for palmitoyl-CoA (C16:0) with a gradually decreased binding affinity for other acyl-CoA esters. However, CpACBP1 is incapable of binding CoA esters with an acyl chain of 20 carbons or longer. This feature makes CpACBP1 different from many other ACBP proteins, including those from bovines and trypanosomes that can bind to CoA esters with an acyl chain of 24 or more carbons (Milne & Ferguson, 2000; van Aalten *et al.*, 2001).

The expression pattern of CpACBP1 differs from that of many other *C. parvum* genes, such as the replication protein A (RPA) subunits, CpSFP-PPT and b-tubulin, but is similar to those of oocyst wall proteins in this parasite (Abrahamsen & Schroeder, 1999; Cai *et al.*, 2005; Millership *et al.*, 2004a; Rider *et al.*, 2005). Such a differential expression pattern is also supported by western blot and immunofluorescence

microscopic analyses that only detected CpACBP1 protein in the intracellular parasites, but not in oocysts or free sporozoites (Figs. 3.9 and 3.10A,B,C).

Our biochemical data show that, although CpACBP1 can bind to medium to long chain fatty acids with chain lengths up to 18-carbons, it displays highest binding affinity towards to the C16:0 palmitoyl-CoA. The K_d value at 407 nM was obtained by Lipid 1000 assay, which is comparable to those of other ACBPs determined by the same assay (Rasmussen *et al.*, 1994). However, this value does not represent the true acyl-CoA binding affinity. Rather, it reflects the competitive binding between ACBP and Lipidex 1000 (Rasmussen *et al.*, 1994). The K_d values determined by fluorescence or dialyzer-based methods are typically at lower nM range (i.e., 1 – 10 nM) (Chao *et al.*, 2002; Frolov & Schroeder, 1998; Milne & Ferguson, 2000; van Aalten *et al.*, 2001; Wadum *et al.*, 2002). On the other hand, although Lipidex 1000 cannot be used to assess the true binding affinity for ACBPs, this method can be used as a qualitative assessment, such as the ligand competition assay. Autoradiography indicates that both the full-length CpACBP1 protein and its ACBP domain may form dimers or even polymers (Fig. 3.5). However, it is unclear whether CpACBP1 is truly present as dimers or polymers in the parasites, or the observed multiple bands in autoradiography are only an artifact induced by the experimental conditions.

Fatty acids are essential to all organisms. Recently, fatty acid metabolism has been considered as a promising target for the drug development against cryptosporidiosis and other important apicomplexans (Gornicki, 2003; Kuo *et al.*, 2003; Ralph *et al.*, 2001; Roberts *et al.*, 2003; Waller *et al.*, 2003; Zhu, 2004b). Because ACBP plays a critical role in fatty acid metabolism, it is reasonable to speculate that CpACBP1 and other

apicomplexan ACBPs may be explored as new drug targets for the control of cryptosporidiosis or other apicomplexan-based diseases. This notion is further supported by the targeted disruption of ACBP in *tryponosoma brucei* causing the lethality (Milne *et al.*, 2001). A more detailed investigation on the structure of parasites' ACBPs may contribute to possible therapeutic targets.

CHAPTER IV

TWO DISTINCT OXYSTEROL BINDING PROTEIN-RELATED PROTEINS FROM APICOMPLEXAN *CRYPTOSPORIDIUM PARVUM* *

INTRODUCTION

Cryptosporidium parvum is a parasitic protist belonging to the Phylum Apicomplexa, in which all members are parasites and many of them are important pathogens in humans and/or animals (e.g., *Plasmodium, Babesia, Toxoplasma, Cyclospora,* and *Eimeria*) (Thompson *et al.*, 2005; Tzipori & Widmer, 2000a; Zhu *et al.*, 2000a). This parasite infects both humans and animals, causing various degrees of watery diarrhea that is typically self-limited in immunocompetent individuals, but prolonged and life-threatening in immunocompromised patients (Chappell & Okhuysen, 2002; Chen *et al.*, 2002b). Unlike other apicomplexan parasites, *C. parvum* parasitizes on the surface of epithelial cells, rather than within the host cell cytosol. However, it is also covered by a layer of parasitophorous vacuole membrane (PVM) that is mostly derived from host cell membranes, which makes *C. parvum* an intracellular, but extracytoplasmic parasite (Chen *et al.*, 2002b; Tzipori & Widmer, 2000a).

In comparison to other apicomplexans, *C. parvum* apparently has the most streamlined

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and simplified metabolism encoded by its genome (Abrahamsen *et al.*, 2004). It differs from other apicomplexans in a number of key metabolic pathways. For example, this protist lacks an apicoplast and associated metabolic pathways including the Type II fatty acid synthase (FAS), but uses a multifunctional Type I FAS (CpFAS1), a Type I polyketide synthase (CpPKS1), and a long chain fatty acid elongase (CpLCE1)-mediated pathway for elongating fatty acids (Abrahamsen *et al.*, 2004; Zhu *et al.*, 2000e; Zhu *et al.*, 2002; Zhu, 2004b; Zhu *et al.*, 2004b). *Cryptosporidium* also lacks most (if not all) de novo synthetic pathways, thus likely relying solely on the update of nutrients (such as lipids, sugars, amino acids and nucleosides) from host cells or the intestinal lumen. Genes encoding sugar, amino acid and nucleoside transporters have been identified from the *C. parvum* genome, some of which are being characterized (Abrahamsen *et al.*, 2004). However, typical fatty acid transporters are absent in the parasite, and it is yet unclear how this parasite uptakes fatty acids and lipids from the host environment. Recently, we have characterized a unique, long-type acyl-CoA binding protein from *C. parvum* (CpACBP1) that is capable of binding long chain fatty acyl-CoA esters (Zeng *et al.*, 2006a). CpACBP1 has been chiefly localized to the PVM, implying that this membrane protein is involved in the import of activated fatty acids from the intestinal lumens, or lipid remodeling within the PVM, or both. Since CpACBP1 is undetectable in the invasive sporozoites and in the entry stage during the parasite intracellular development, this protein is less likely essential to the formation of PVM. These observations indicate that the PVM may play an important role in nutrient uptake by the parasite.

In the present study, we report the identification and preliminary characterization of two oxysterol binding protein (OSBP)-related proteins (ORPs) from *C. parvum* (CpORP1

and CpORP2). OSBPs and ORPs have been found to be involved in the transport and metabolism of cholesterol and related lipids in eukaryotes (Beh & Rine, 2004; Hynynen *et al.*, 2005; Im *et al.*, 2005; Lehto & Olkkonen, 2003; Olkkonen, 2004; Olkkonen & Levine, 2004; Perry & Ridgway, 2005; Wang *et al.*, 2005a; Wang *et al.*, 2005b; Wang *et al.*, 2005c). Although OBSP was originally identified from humans by its capacity to bind to oxysterols, not all ORPs share the same binding capacity. However, all OSBPs and ORPs have been found to be able to bind to different phosphatydinositol phosphates (PIPs) with various affinities (Lehto & Olkkonen, 2003; Olkkonen, 2004). Here we have expressed both CpORP1 and CpORP2 as recombinant proteins and determined their substrate-binding profiles towards various PIPs. We have also localized one of the parasite proteins (CpORP1) to the PVM, suggesting that this protein may be involved in the lipid-trafficking and/or remodeling within the PVM.

METHODS

Identification of ORPs from *C. parvum* and other apicomplexans

By data-mining the newly sequenced *C. parvum* genome, our laboratory has identified two genes that encode ORP homologues. Both gene products have been annotated by the *C. parvum* genome consortium as “oxysterol binding protein” (Genbank accession No. **XP_627892**) and “oxysterol binding protein 1A-like pleckstrin homology (PH) domain containing protein” (**EAK87842**). For clarification, we have named these two proteins as CpORP1 and CpORP2, and their genes as *CpORP1* and *CpORP2*.

Similarly, two ORP homologues were also identified from the C. hominis genome that were referred to as *ChORP1* and *ChORP2*.

We also data-mined the completely (or near completely) sequenced genomes from other distinct groups of apicomplexans, i.e., *Plasmodium falciparum, P. vivax* and *P. yoelii* at http://PlasmoDB.org (release 4.4), *Toxoplasma gondii* at http://ToxoDB.org (release 3.0), as well as *Eimeria tenella* at http://www.sanger.ac.uk/Projects/E_tenella (assembled contigs from 8X coverage of the whole genome, last access on May 10, 2006). Among them, the two *C. parvum* ORPs proteins were used as queries to BLAST searches against the genome and predicted proteins (available for at PlasmoDB and ToxoDB). The resulting hits were retrieved and subjected to further analysis, including homology searches and identification of domains to confirm their identity as members of the ORP family.

Molecular cloning and engineering of CpORP1 and CpORP2 gene

To confirm the function of these apicomplexan ORP homologues, we engineered and expressed the two *C. parvum* ORPs for ligand binding studies. Both *CpORP1* and *CpORP2* are intronless genes, which permits the amplification of their entire open reading frames (ORFs) directly from genomic DNA (gDNA isolated from the same the source and DNA extraction method as the chapter III). PCR amplification employed a mixture of *Pfu* and *Taq* DNA polymerases (1 unit each per 50 ml reaction) and the following two primer pairs: CpORP1-F1 (5'- gcg gatc cAT AAT AGT GTG GAG GTA AAT CAT ATG -3') and CpORP1-R1 (5'- gcg gat ccT TAT TTA TCG ATA TTA AAA ATA TCA AT -3'); CpORP2-F1 (5'- gcg gat ccA TGT TGG TTA ATG AGC AAT CAA

AAA AA -3) and CpORP2-R1 (5'- gcg gat ccT CAA AAA ATA TTT GGT AAA TCT GTA AA -3)' (**Note**: lower cases represent added *Bam*HI restriction site). The PCR amplicons were first cloned into pCR4Blunt-TOPO vector with the use of TOPO cloning kit (Invitrogen). After sequencing to confirm the identity, the *CpORF1* and *CpORF2* inserts were released by *Bam*HI restriction digestion and ligated into linearized pMAL-c2x vector (New England Biolabs). The resulting plasmids were again sequenced to confirm the identity and orientation of the inserts, and the correct clones were used for expression of maltose-binding protein (MBP)-fusion proteins. The Rosetta strain of *Escherichia coli* (Novagen) containing extra set of tRNA for correcting the codon use bias between bacterial and eukaryotic genes was used to host the expression of the fusion proteins.

The entire expression procedure started with the inoculation of a single colony of bacterial transformants into 10 ml LB broth containing 100 mg/ml ampicillin and 34 mg/ml chloramphenicol, which was allowed to grow at 37 °C overnight. The bacterial suspension was then mixed into 1 L fresh broth containing the two antibiotics. After incubation at room temperature until the OD_{495} reached to 0.3-0.5, IPTG was added into the broth at a final concentration of 0.1 mM, followed by an 8-hr expression at 16 °C. The bacteria were centrifuged at 8000 *g* for 10 min at 4 °C, and the pellet was suspended in 50 ml of column buffer (20 mM Tris-HCl, 20 mM NaCl, 2 mM EDTA, pH 7.5) with a protease inhibitor cocktail formulated for bacteria (Sigma-Aldrich). Bacterial cells were ruptured by a mild sonication on ice and cell debris was removed by centrifugation at 8,000 *g* for 10 min. The supernatant was loaded to an amylose-resin column (New England Biolabs). After washing with more than 10 volumes of column

buffer, the MBP-fused proteins (MBP-CpORP1 and MBP-CpORP2) were eluted by an elution buffer (i.e., column buffer containing 10 mM maltose). Ten percent SDS-PAGE was used to analyze the size and purity of fusion proteins. The Bradford method was used to determine the protein concentrations (ref). Aliquots of each protein were either used immediately or stored at -80 °C for future use.

Lipid-protein overlay assay

The nitrocellulose membrane blots spotted with various phosphoinositides (PIP strips) and sphingolipids (SphingoStrips) were purchased from Molecular Probes. To determine the binding specificity of recombinant CpORP1 and CpORP2 with phosphoinositides and sphingolipids, membrane blots were first treated with a blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 2 mg/ml fatty acid-free bovine serum albumin [BSA]) for 1 hr. The blots were then incubated overnight at 4°C with gentle rotation in a fresh blocking buffer containing 5 nM of the MBP-fusion proteins and washed for 10 times (5 min each) in a TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). The detection of bound proteins was facilitated by a typical Western blot analysis procedure. Briefly, the blots were incubated with a rabbit anti-MBP polyclonal antibody (New England Biolabs) in blocking buffer for 1 hr, washed with TBST for 6 times (5 min each) to remove free antibodies, incubated with an alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Alderich) in TBST for 1 hr, and washed again with TBST for 12 times (5 min each). The proteins were then visualized using 5’ bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (substrate source) as substrates for AP. In all experiments,

separate PIP strips and SphingoStrips were also incubated with MBP-tag alone as controls.

Production of rabbit polyclonal antibodies to CpORP1

The antigen used in antibody production was a stretch of MBP-fused short peptide (^{134}LNTTK TCVEN EDIEK EETCN ITRRR NLPVP KTSAK FSLLS MMRQV FGKDL SRISM^{188}) in CpORP1. This sequence is unique to CpORP1 and reasonably antigenic as determined by various antigenicity indexes using MacVector v.8.1.1 program. The engineering, expression and purification of fusion proteins followed similar procedure as described above for the full-length CpORP1 and CpORP2 proteins. Polyclonal antibodies to the recombinant CpORP1 antigen were raised in two pathogen-free rabbits in a commercial site (Lampire Biological Laboratories, Pipersville, PA). Rabbit preimmune sera were pre-screened by dot blot test using parasite oocyst protein extracts, and only the rabbits with no reactive sera were used for antibody production. The following immunization protocol was used. On day 1, preimmune rabbit sera were collected and initial immunization was performed using 0.2 mg of antigen emulsified with 0.5 ml of complete Freund's adjuvant. Two booster shots (0.1 mg of antigen each) with 0.5 ml of incomplete adjuvant were performed on day 21 and day 42. Rabbit antisera were collected on day 50. The titers and specificity of the rabbit polyclonal antibodies were evaluated by dot and western blot analysis with recombinant proteins and protein extracts of parasites and host cells.

Immunofluorescence microscopy for detecting CpORP1 during the parasite development

The expression and localization of CpORP1 in the invasive stage (sporozoites) and intracellular parasites were studied by immuno-labeling. Free *C. parvum* sporozoites (IOWA strain) were prepared by incubating oocysts in an excystation medium containing 0.25% trypsin and 0.5% TDC in PBS (pH 7.2) for 90 min at 37 C. Excystation was stopped by adding an equal volume of 20% BSA (chilled on ice) into the medium, followed by washes with PBS for 5 times by centrifugation. Intracellular parasites were prepared by infecting HCT-8 cells with *C. parvum* for various time periods. Host cells were seeded on poly-L-lysine-treated glass coverslips and grown for 24 hr. Parasite oocysts were sterilized by a 10-min treatment with 10% Clorox on ice, washed 8 times with distilled water by centrifugation, resuspended in culture medium, and added to the 24-well plates containing cultured cells in a ratio of 1 oocyst per 5 host cells. Parasite excystation and invasion into host cells was allowed to take place for 3 hr at 37 °C. Parasites that failed in excystation and invasion were removed from the culture by a medium exchange. Infected cells were then allowed to grow for 12, 24, 48 and 72 hr, and fixed in 10% formalin solution balanced with PBS. Fixed cells were rinsed with PBS and water respectively, and extracted with methanol/acetone (v/v = 1:1) for 5 min at –20 °C. Samples were rinsed with water and then PBS, blocked in 0.5 % BSA in PBS (BSA-PBS) for 10 minutes, labeled with 1:500 dilution primary antibodies in PBS containing 0.5 % BSA for 1 h, and treated with a TRITC-conjugated monoclonal antibody against rabbit IgG (Sigma-Aldrich ,1/400 dilution). Samples were washed with PBS containing 0.05% Tween-20 for 3 times after each incubation step. Slides were mounted by a SlowFade Light Antifade mounting medium (Molecular Probes) containing 4',6'-

diamidino-2-phenylindole (DAPI) for counterstaining nuclei and examined using an Olympus BX51 Epi-Fluorescence microscope system equipped with differential interference contrast (DIC) apparatus and FITC/TRITC/DAPI filters.

RESULTS

Sequence characterization of ORPs in *Cryptosporidium* and other apicomplexans

OSBP and ORPs are a family of proteins only found in eukaryotes (Lehto & Olkkonen, 2003). Indeed, extensive BLAST searches using ORPs from animals, plants, yeast and protists failed to identify any significant homologues from the other two domains of life (i.e. eubacteria and archaea). Although ORPs have been found to be critical in the lipid metabolism, most recent investigations have mainly focused on human and yeast homologues (Lehto & Olkkonen, 2003). No ORPs were previously reported or characterized from protists – the most divergent group of unicellular organisms. Therefore, the apicomplexan ORPs reported here represent the first ones characterized from protists.

The *C. parvum* genome contains two ORPs, which differs significantly from human host that possess at least 12 ORPs, as well as from yeast that has 7 ORP homologues (Jaworski *et al.*, 2001; Lehto & Olkkonen, 2003). Sequence analysis clearly separated these two CpORPs into two distinct categories: the 70-kDa CpORP1 belongs to the short-type family that contains only a ligand binding (LB) domain, while the 90-kDa CpORP2 is a long-type protein that contains an N-terminal Pleckstrin homology (PH)

domain and a C-terminal LB domain (Fig. 4.1). Both ORPs possess the highly conserved “fingerprint” sequence found in all human and yeast ORPs (i.e., EQVSHHPP) with only one amino acid difference (i.e., the Ser residue is replaced by Met in CpORP1 and by Gly in CpORP2) (Fig. 4.1, indicated by ovals within the LB domains) (Lehto & Olkkonen, 2003). However, CpORP1 and CpORP2 appear more divergent from each other than expected, as these two proteins only share ~20% identity at the amino acid level. This suggests a possible very early split between these two proteins, probably before the separation of the *Cryptosporidium* lineage from other apicomplexans.

In addition, both types of ORPs are also present in the genome of *C. hominis* (ChORP1 and ChORP2), a species that is mostly found to infect humans and was previously known as the Type 1 genotype of *C. parvum*. These two *Cryptosporidium* species virtually share identical amino acid sequences for the two ORPs. There are only 8 amino acid substitutions between CpORP1 and ChORP1 (1.4%), and 13 between CpORP2 and ChORP2 (1.6%), respectively.

More surprisingly, other apicomplexans (including the three *Plasmodium* species, *T. gondii*, and *E. tenella*) possess only a single ORP homologue in each of their genomes. The predicted protein sequences for *Plasmodium* and *Toxoplasma* ORPs were available in their corresponding databases (Fig. 4.1), while only the genomic contig containing the ORP homologue was available for *E. tenella*.

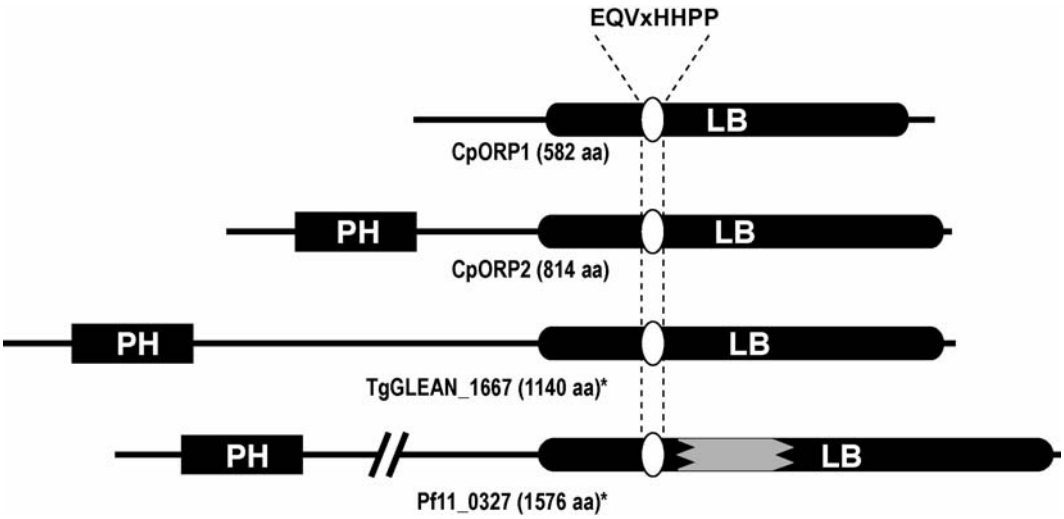


Fig. 4.1. Domain organization of ORPs from *Cryptosporidium parvum* (CpORP1 and CpORP2) and other two apicomplexans, *Toxoplasma gondii* (TgGLEAN_1667) and *Plasmodium falciparum* (Pf11_0327). PH and LB represent Pleckstrin homology domain and ligand binding domain, respectively. Oval circles indicate ORP "fingerprint" sequences within the LB domains. Gray region within the *P. falciparum* protein indicates a stretch of inserted amino acids within the LB domain. Asterisks indicate sequences that were retrieved from corresponding genome databases (i.e., http://ToxoDB.org and http://PlasmoDB.org).

Ligand binding property of CpORP1 and CpORP2

Both CpORP1 and CpORP2 were successfully expressed as MBP-fusion proteins at expected sizes and purified to homogeneity using amylose-based affinity chromatography (Fig. 4.2). The expression levels of these fusion proteins were generally low (i.e., < 1 mg/L), but sufficient protein could be purified from several liters of culture for biochemical analysis.

Lipid-protein overlay assays using PIP strips clearly showed that CpORP1 and CpORP2 could bind to phosphatidic acid (PA) and various phosphatidylinositol phosphates (PIPs) (Fig. 4.3A). Both fusion proteins displayed almost identical binding patterns, despite their sequence divergence, and showed the highest binding affinity to PA, the three phosphatidylinositol mono-phosphates [i.e., PI(3)P, PI(4)P, and PI(5)P], and slightly reduced affinities to phosphatidylinositol bi- and tri-phosphates [i.e., $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$]. Both proteins displayed little or no affinity to other phospholipids included in the same blots, including phosphatidylserine (PS), sphingosine 1-phosphate [Sp(1)P], phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PIP), lysophosphatidylcholine (LPC), and lysophosphatidic acid (LPA) (Fig. 4.3B and Fig. 4.3C). The binding between MBP-fused proteins and PA/PIPs was specifically contributed by the parasite ORP portion, since the MBP-tag alone displayed no affinity at all towards to any of the lipids on the membranes (Fig. 4.3B and Fig. 4.3C).

In another lipid-protein overlay assay using SphingoStrips, CpORP1 and CpORP2 showed no affinity to any of the lipids with small head groups, but only strongly bound to

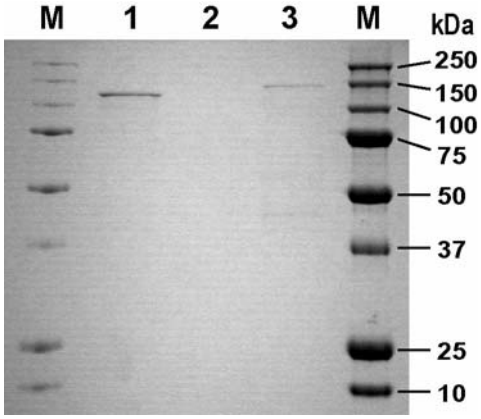


Fig. 4.2. SDS-PAGE analysis of affinity chromatography purified maltose-binding protein (MBP) fused CpORP1 and CpORP2 proteins. Lane 1 = MBP-CpORP1; Lane 2 = blank; Lane 3 = MBP-CpORP2; M = Protein molecular weight markers.

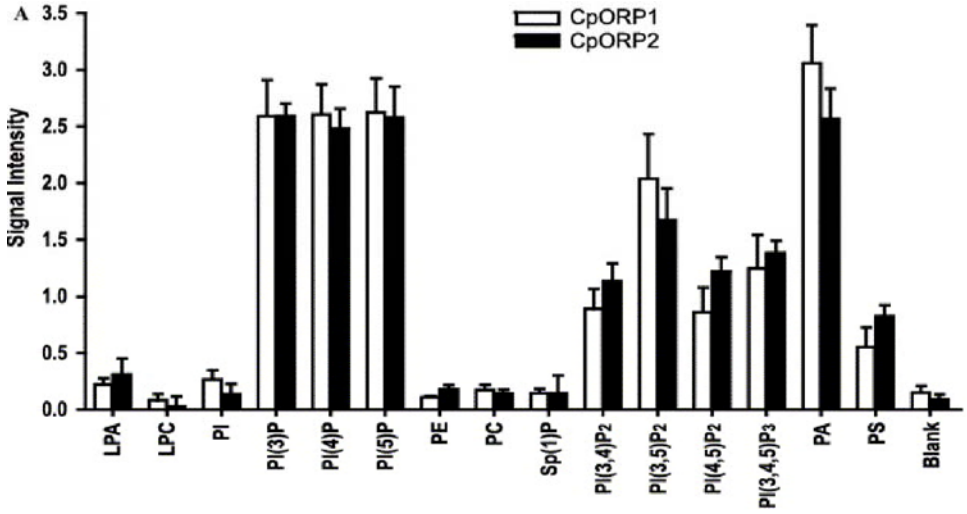


Fig. 4.3A. Binding affinity of CpORP1 and CpORP2 towards various phosphatidylinositol phosphates (PIPs) as determined by measuring the signal intensities (integrated densities) generated in lipid-protein overlay assay using ImageJ v1.3.7 program (available at http://rsb.info.nih.gov/ij/). Bars represent SEM values from three PIP membrane blots.

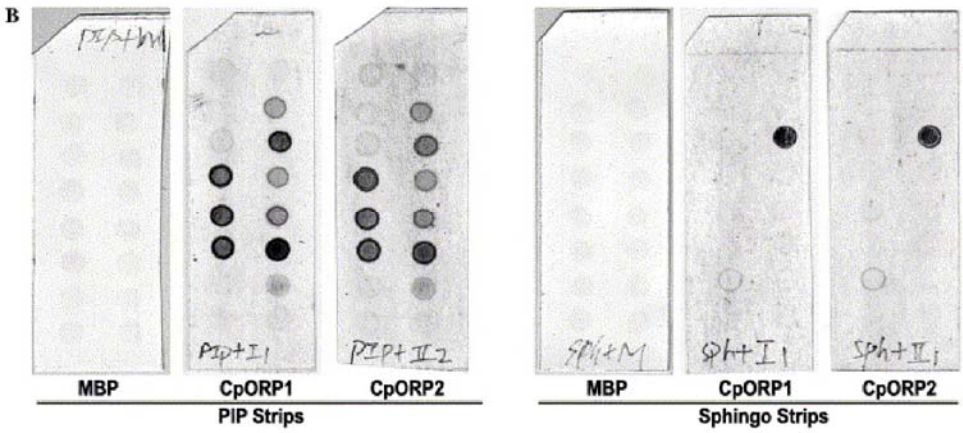
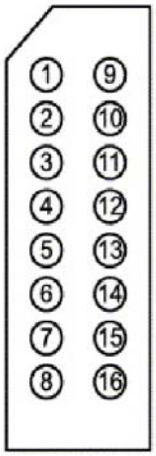


Fig. 4.3B. Representative membrane blots (PIP strips and Sphingo Strips) resulting from lipid-protein overlay assay using recombinant CpORP1 and CpORP2 proteins. MBP-tag alone was used as controls.

C



	PIP Strips		Sphingo Strips
1	Lysophosphatidic acid [LPA]	1	Sphingosine [Sp]
2	Lysophosphatidylcholine [LPC]	2	Sphingosine 1-phosphate [Sp(1)P]
3	Phosphatidylinositol [PI]	3	Phytosphingosine [PhSp]
4	**PI(3)P**	4	Ceraminde [Cer]
5	**PI(4)P**	5	Sphingomyelin [SpMy]
6	**PI(5)P**	6	Sphingosylphosphocholine [SpPC]
7	Phosphatidylethanolamine [PE]	**7**	Lysophosphatidic acid [LPA]
8	Phosphatidylcholine [PC]	8	Myriocin [Myr]
9	Sphingosine 1-phosphate [Sp(1)P]	9	Monosialoganglioside [GM1]
10	**PI(3,4)P_2**	10	Disialoganglioside [GD3]
11	**PI(3,5)P_2**	**11**	**Sulfatide [Sulf]**
12	**PI(4,5)P_2**	12	Sphingosylgalactoside (pyschosine)
13	**PI(3,4,5)P_3**	13	Cholesterol [Chol]
14	**Phosphatidic acid [PA]**	14	Lysophosphatidylcholine [LPC]
15	Phosphatidylserine [PS]	15	Phosphatidylcholine [PC]
16	Blank	16	Blank

Fig. 4.3C. Legends for the PIPs and sphinogolipids spotted on the blots. Preferred ligands are shown in bold.

sulfatide, a 3-sulfate ester of galactosylcerebrosides (Fig. 4.3B). In addition, both proteins showed no binding affinity to cholesterol that was found to be a ligand for some ORPs (such as human OSBP and yeast Osh4 (Im *et al.*, 2005), which suggests that both parasite ORPs are unlikely (or at least not directly) to be involved in cholesterol-sensing in the parasite.

Localization of CpORP1 in intracellular parasites

To facilitate the study of localization of CpORPs, we developed a rabbit polyclonal antibody to CpORP1 by immunizing rabbits with a recombinant protein containing 55 amino acids unique to CpORP1. The production of antibodies to CpORP2 is currently ongoing, as our initial attempts to raise antibodies to CpORP2 were unsuccessful. Using the mono-specific polyclonal antibody, we first found that CpORP1 was not present (or present at an undetectable level) in the environmental stages including intact oocysts and the excysted invasive sporozoites, both by western blot analysis and by immunofluorescence microscopy (data not shown). However, CpORP1 proteins could be clearly detected among intracellular parasites developed for various times (Fig. 4.4A). In general, the fluorescence signal resulting from the immuno-labeling covered homogenously the entire surface of meronts (both 1st and 2nd generations of meronts) with more intensive signals along the edges. These features of labeling are typical to proteins present on the PVM – a host cell membrane-derived barrier between parasites and the intestinal lumen (Chen *et al.*, 2003; Zeng *et al.*, 2006a; Zeng *et al.*, 2006b), although one can not completely rule out the possibility of some minor distribution of CpORP1 in the merozoites and/or the electron-dense feeder organelle connecting parasites and host cells.

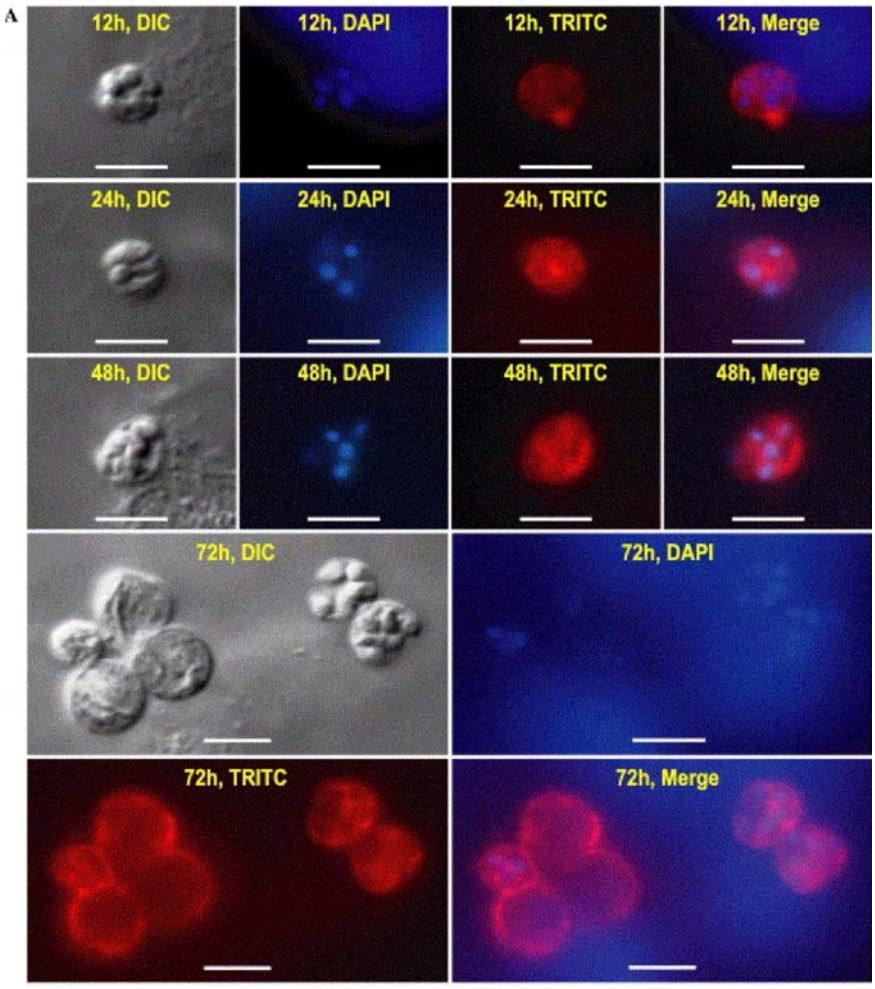


Fig. 4.4A. Immunofluorescence microscopic detection of CpOPR1 proteins in intracellular *Cryptosporidium parvum* cultured in vitro for 12, 24, 48 and 72 hr, respectively. DIC = differential interference contrast. DAPI = 4',6'-diamidino-2-phenylindole for counterstaining nuclei. TRITC = labeled CpORP1 proteins. Merge = DAPI-stained nuclei + TRITC-stained CpORP1. **Note:** In some DAPI stained slides, host cell nuclei were also stained and shown as large blue backgrounds. Bar = 5 mm.

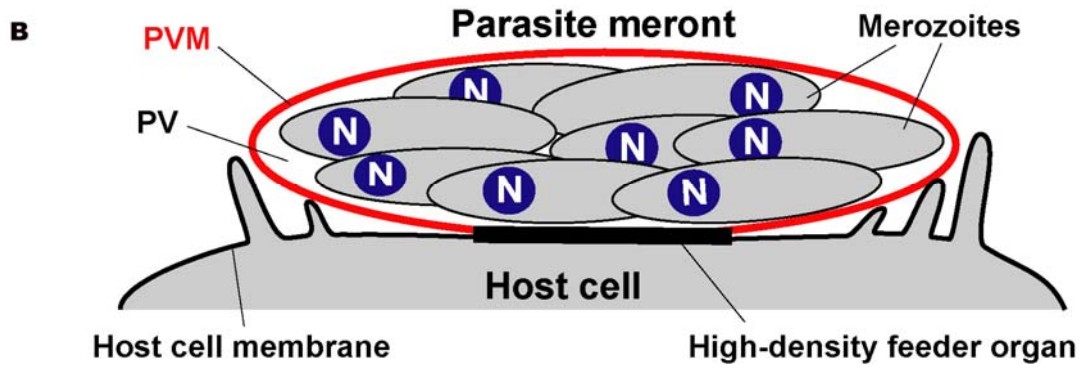


Fig. 4.4B. Diagram of a parasite meronts containing 8 merozoites. Parasitophorous vacuole membrane (PVM) labeled by anti-CpORP1 antibody is shown in red. N = nucleus.

DISCUSSION

In contrast to CpORP1 and CpORP2 that are intronless, all other apicomplexan ORP genes are heavily interrupted by introns and encode the long-type ORPs containing an N-terminal PH domain. These ORPs are generally much larger than their counterpart in *C. parvum* (CpORP2) by containing extra amino acids between the PH and LB domains. The ORPs from various *Plasmodium* species also containing in large insertion within the LB domain, shortly after the “fingerprint” sequence (Fig. 4.1, marked in gray). There is also a single amino acid substitution at the “fingerprint” sequence, in which the Ser is replaced by Gly in *T. gondii* and by Val in *P. falciparum*, respectively. These observations suggest that the function of PH domain-containing ORPs may be shared among all apicomplexans, but that of short CpORP1 is probably unique to *Cryptosporidium*.

Low temperature expression at 16 °C could enhance the expression level and the amount of soluble protein. Fresh bacterial transformants (< 1 week old) generally performed much better than older ones. The use of Rosetta strain of bacteria was also critical to expression of the AT-rich genes from *C. parvum*.

Similar patterns of CpORP1 present on the PVM were recently reported for an acyl-CoA binding protein (CpACBP1) that binds to various long chain fatty acyl-CoAs (Zeng *et al.*, 2006a). Because *C. parvum* lacks apparent *de novo* synthetic pathways for fatty acids and sterols, and likely has to uptake these nutrients from either host cells or the nutrient-rich intestinal lumens (Zhu, 2004b), it has been speculated that CpACBP1 may

be involved in the transport of activated fatty acids across the PVM and/or the lipid remodeling within the PVM. The involvement of ORPs in intracellular lipid metabolism and trafficking has been well documented in yeast and human cells (Hynynen *et al.*, 2005; Im *et al.*, 2005; Lehto & Olkkonen, 2003; Olkkonen, 2004; Perry & Ridgway, 2005). It is reasonable to assume that CpORP1 may also be involved in the transport and/or regulation of lipid, particularly sterols, across the PVM, although such a hypothesis remains to be tested in the future.

On the other hand, although CpACBP1 and CpORP1 are likely critical to the parasite intracellular development, both proteins are not present in the intact oocysts and excysted free sporozoites, suggesting they may play little or no role in the parasite environmental (extracellular) stages, or that their functions are not essential (or important) for the parasite survival in the environment, for the excystation and movement of sporozoites in the gut, and for the early stage of invasion of parasites into host cells.

Although CpORP1 has been chiefly localized to the PVM, the subcellular location of CpORP2 is yet undetermined. We are currently in a process to raise antibodies to CpORP2 and will report related findings in the future. Since the PH domain-containing ORPs are also present in other apicomplexans of divergent lineages, further studies on CpORP2 will potentially reveal some common features shared among all apicomplexans.

In summary, we have identified, cloned and expressed two distinct ORPs from the parasitic protist *C. parvum*. This feature indicates *Cryptosporidium* differs from other

apicomplexans including the Haematozoa and the Coccidia that all possess only one PH domain containing ORP in their genomes. Using the recombinant proteins, we have performed the lipid-protein overlay assay and confirmed that CpORP1 and CpORP2 could specifically bind to PA and various PIPs, a feature that is characteristic to this family of proteins. Immuno-staining has chiefly localized CpORP1 to the PVM, suggesting that this protein may be involved in the lipid transport across the membrane barrier between intracellular parasites and host intestinal lumen. If the two ORPs are essential to intracellular parasite development, it is possible to explore these PVM proteins as chemotherapeutic and/or immunotherapeutic targets in this parasite for which fully effective treatment is yet unavailable.

CHAPTER V

SUMMARY AND CONCLUSIONS

There are two major areas of research reported in this dissertation, which concern the unique long-type ACBP and two distinct ORP homologues in *Cryptosporidium parvum*, and can be summarized as follows.

First, we have identified and conducted functional analysis of a fatty acyl-CoA binding protein (ACBP) gene from the opportunistic protist *C. parvum*. The CpACBP1 gene encodes a protein of 268 aa that is 3X larger than the typical ACBP proteins (i.e., ~90 aa) of humans and animals. Sequence analysis indicated that CpACBP1 consists of an N-terminal ACBP domain (~ 90 aa) and a C-terminal ankyrin repeat sequence (~ 170 aa). The entire CpACBP1 ORF was engineered into a maltose-binding protein fusion system and expressed as a recombinant protein for functional analysis. Acyl CoA-binding assays clearly revealed that the preferred binding substrate for CpACBP1 was palmitoyl-CoA. RT-PCR, Western blotting and immuno-labeling analyses clearly showed that the CpACBP1 gene was mainly expressed in the intracellular developmental stages and the level increases during the parasite development. Immunofluorescence microscopy shows that CpACBP1 is associated with the parasitophorous vacuole membrane (PVM), which implies that this protein may be involved in the lipid remodeling in the PVM or the transport of fatty acids across the membrane.

Second, we have identified two distinct oxysterol binding protein (OSBP)–related proteins (ORPs) from the parasitic protist *C. parvum* (CpORP1 and CpORP2). The short-type CpOPR1 contains only a ligand binding (LB) domain, while the long-type CpORP2 contains Pleckstrin homology (PH) and LB domains. Lipid-protein overlay assays using recombinant proteins revealed that CpORP1 and CpORP2 could specifically bind to phosphatidic acid (PA), various phosphatidylinositol phosphates (PIPs), and sulfatide, but not to other types of lipids with simple heads. Cholesterol was not a ligand for these two proteins. CpOPR1 was found mainly on the parasitophorous vacuole membrane (PVM), suggesting that CpORP1 is probably involved in the lipid transport across this unique membrane barrier between parasites and host intestinal lumen. Although *Cryptosporidium* has two ORPs, other apicomplexans including *Plasmodium*, *Toxoplasma* and *Eimeria* possess only a single long-type ORP, suggesting that this family of proteins may play different roles among apicomplexans.

It is intriguing that both CpACBP1 and CpORP1 proteins were found to be associated with the PVM, which clearly displays the importance of this unique membrane structure in the transport and/or metabolism of lipids in *C. parvum*.

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