Functional characterization of a fatty acyl-CoA-binding protein (ACBP) from the apicomplexan *Cryptosporidium parvum*

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In this paper, the identification and functional analysis of a fatty acyl-CoA-binding protein (ACBP) gene from the opportunistic protist *Cryptosporidium parvum* are described. The *CpACBP1* gene encodes a protein of 268 aa that is three times larger than typical ACBPs (i.e. ~90 aa) of humans and animals. Sequence analysis indicated that the *CpACBP1* protein 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* 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.

INTRODUCTION

Cryptosporidium parvum is a globally important parasitic protist that infects both humans and animals (Chappell & Okhuysen, 2002; Thompson *et al.*, 2005; Tzipori & Widmer, 2000). *C. 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 advances 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 apicoplast-specific Type II fatty acid synthases (e.g. *Plasmodium falciparum*), Type I fatty acid synthase (e.g. *C. parvum*) or both (e.g. *Toxoplasma gondii*) (Roos *et al.*, 2002; Zhu, 2004; Zhu *et al.*, 2000b, 2002, 2004). Although fatty acids are one of the major components in all cells, free

fatty acids cannot enter any metabolic pathways unless they are activated by thioesterification with coenzyme A (CoA), to form an acyl-CoA ester, or with acyl carrier protein. 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 ACBPs (e.g. >55 kDa) have also been identified in both animals and plants. Mammals possess multiple ACBPs that are differentially expressed in various tissues (e.g. T-ACBP in testis, L-ACBP in liver and I-ACBP in intestine) (Schroeder *et al.*, 1998). In *Trypanosoma brucei*, an ACBP was found to be involved in the synthesis of the glycosylphosphatidylinositol (GPI) anchor in variant surface glycoproteins (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 ACBPs from bovines (liver) and trypanosomes (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 sequence, we have identified a unique long-type ACBP

Abbreviations: ACBP, acyl-CoA-binding protein; PPT, phosphopantetheinyl transferase; GPI, glycosylphosphatidylinositol; MBP, maltose-binding protein; PVM, parasitophorous vacuole membrane; SFP, surfactin production element; TMP, total membrane proteins.

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(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 the protein it encodes is chiefly located on the parasitophorous vacuole membrane (PVM), suggesting that this protein may be involved in the formation of PVM and/or the uptake of fatty acids by the parasite.

METHODS

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 ACBPs as query sequences. Similar searches were also performed to identify ACBP homologues from the *Plasmodium falciparum* and *Toxoplasma gondii* genome databases (www.PlasmoDB.org and 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, www.ncbi.nlm.nih.gov) and by comparison 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 ORF of *CpACBP1* was amplified from *C. parvum* (Iowa strain) genomic DNA with the following primer pair: 5'-ATGACTGATATCTTATCCACGAAC-3' and 5'-atggatcTTAACTGCTTTCGAGAATTCTT-3' (lower case letters represent an 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 T4 DNA ligase. The ligated plasmids were transformed into *Escherichia coli* TOPO-10 (Invitrogen). The resulting colonies were first screened using a sense-stranded primer located upstream from the insert in the vector and the *CpACBP1* antisense strand 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. nt 298–788), we removed the entire C-terminal ankyrin repeats by digesting pMAL-c2x-CpACBP1 with *Eco*RI followed by agarose gel separation. The remaining plasmid fragment was religated back, resulting in a construct (pMAL-c2x-CpACBP1-S) encoding only the N-terminal 100 aa plus 7 extra aa derived from the remaining nucleotides at the 3' end of the insert and the multiple cloning site of the vector (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 into 10 ml LB broth containing 100 µg ampicillin ml⁻¹ and 34 µg chloramphenicol ml⁻¹ and grown 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 OD₄₉₅ reached 0.3–0.5. After adding IPTG to the broth (100 µg ml⁻¹ final concn), bacteria were further incubated for 4 h at 30 °C and collected by centrifugation for 10 min at 8000 g. Bacterial pellets were resuspended in 50 ml column buffer (20 mM Tris/HCl, pH 7.5, 20 mM NaCl, 2 mM EDTA) containing a protease inhibitor cocktail (Sigma-Aldrich), disrupted by sonication and centrifuged (8000 g, 10 min) to remove cell debris. Supernatants were applied to an amylose-resin column (New England Biolabs), washed with column buffer (> 10 × 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 analysed by SDS-PAGE. Protein concentrations were determined by the Bradford (1976) method using a commercial kit and bovine serum albumin as standard. Aliquots of each protein sample were either used immediately or stored at –80 °C.

Semi-quantitative RT-PCR. The entire semi-quantitative RT-PCR procedure, including the isolation of parasite total RNA from various parasite life-cycle stages, the normalization of parasite RNA contents in intracellular samples, reverse transcription, amplification and analysis, has been described in detail by Millership *et al.* (2004a, b). In this study, the following primer pair was used to amplify CpACBP1 transcripts: CpACBP1-F334 (5'-CCTTTATTAGAATCAACCTGG-3') and CpACBP1-R334 (5'-TTGGATAGGAGTCAAAACCATC-3'). Another pair of previously reported primers (995F, 5'-TAGAGATTGGAGGTTGTTCT-3' and 1206R, 5'-CTCCACCACTAAGAACGGCC-3') was used to amplify 18S rRNA as a control for normalization (Abrahamsen & Schroeder, 1999). Each semi-quantitative RT-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 the 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 affinity-purified MBP-CpACBP1 protein emulsified in an equal volume of complete Freund's adjuvant. Two subsequent booster immunizations (0.1 mg) were injected at 30 and 60 days, respectively, 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 an equal volume of amylose-resin conjugated with MBP. The antibody titre and specificity were evaluated by Western blot analysis.

Western blot analysis. Western blot analysis was performed to test for the presence of CpACBP1 protein at various parasite life-cycle stages. Oocysts (5 × 10⁶ per lane), free sporozoites (2 × 10⁷ per lane) and HCT-8 cells (1 × 10⁶ per lane) infected with *C. parvum* oocysts (oocysts/host cell ratio = 1 : 1) for 24, 48 and 72 h were lysed in loading buffer containing a protease inhibitor cocktail for mammalian cells (Sigma-Aldrich) at 95 °C for 8 min. 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 in 1 % BSA in TTBS (TBS with 0.05 % Tween-20). The blot was washed three times with TTBS after each incubation step and all procedures were performed at room temperature. Finally, the labelled proteins were developed using 5'-bromo-4-chloro-3-indolyl phosphate (BCIP).

Immunofluorescence microscopy. Intracellular parasites were prepared by infecting HCT-8 cells grown on poly-L-lysine-treated glass coverslips for 24, 48 or 72 h. 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), labelled with primary antibodies (1 h in 0.5 % BSA-PBS) and incubated with secondary antibodies conjugated with FITC or TRITC (60 min in 0.5 % BSA-PBS). Samples were washed after each incubation step (three 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 incubation with antibodies. Co-localization of CpACBP1 with total membrane proteins (TMPs) and with a surfactin production element (SFP)-type phosphopantetheinyl transferase (CpSFP-PPT) immunolocalization was similarly performed, except that the respective rabbit antibodies were directly labelled with Alexa Fluor 488 or Alexa Fluor 546 using the appropriate fluorophore-labelling kits (Invitrogen) prior to the immunolabelling experiments. No secondary antibodies were used in co-localization experiments. The TMP antibody has been shown previously to mainly label the PVM and feeder organ in intracellular parasites (Chen *et al.*, 2003), while cytosolic localization of CpSFP-PPT has also been reported previously (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 and FITC/TRITC/DAPI filters.

Acyl-CoA binding assay. The binding affinity of CpACBP1 with fatty acyl-CoA was measured by a Lipidex 1000 assay as previously described (Rasmussen *et al.*, 1990; Rosendal *et al.*, 1993). Briefly, 40 pmol recombinant CpACBP1 was mixed with [^{14}C]palmitoyl-CoA (0–8 μM) in 100 μl 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 cold Lipidex 1000 (hydroxyalkoxypropyl dextran, Type VI; Sigma-Aldrich) (50 %, v/v, slurry in binding buffer) with gentle rotation at 4°C for 1 h and centrifuged for 5 min at 12000 g at 4°C to remove free acyl-CoA esters. An aliquot of 200 μl 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 non-linear regression and Prism v4.0 (GraphPad Software).

The substrate preference for CpACBP1 was determined by a competition-binding assay. It was performed in 100 μl binding buffer containing 40 pmol recombinant CpACBP1 (or MBP in control groups) and 80 pmol [^{14}C]palmitoyl-CoA in the absence or presence of 80 pmol non-radioactive fatty acyl-CoA esters of various chain lengths (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 acids. In this assay, 80 pmol recombinant CpACBP1 protein was incubated with 80 pmol [^3H]palmitic acid or [^{14}C]palmitoyl-CoA in 100 μl 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 fusion protein was incubated with 80 pmol [^{14}C]palmitoyl-CoA in the presence or absence of 80 pmol non-radioactive palmitoyl-CoA in 100 μl binding buffer for 30 min at 37°C . Subsequently, 20 μl of the reaction was mixed with 5 μl 5 $\times$ 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 is the only ACBP homologue that can be identified from the *C. parvum* genome by repeated BLAST searches using several animal, plant and protist ACBP 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. 1). Other apicomplexans appear to possess more ACBP homologues in their genomes. For example, *P. falciparum* has four ACBP homologues (three short and one long), and *T. gondii* has two (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. 2). Amino acids that are critical to ligand-binding activity are all present in CpACBP1 and other apicomplexan ACBPs (Fig. 2, indicated by solid dots) (Burton *et al.*, 2005). Similar to CpACBP1, the long-type ACBP in *T. gondii* contains two ankyrin repeats (Fig. 1). However, it also possesses an N-terminal signal peptide, possibly for secretion. On the other hand, the *P. falciparum*

Fig. 1. Domain organization of CpACBP1 in comparison to those from other representative eukaryotic ACBPs.

Fig. 2. Multiple alignment of the conserved region in the ACBP domain between CpACBP1 and other representative eukaryotic ACBPs. 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 (www.PlasmoDB.org or www.ToxoDB.org). Solid dots indicate amino acids critical to acyl-CoA-binding activity.

long-type ACBP contains no ankyrin repeats (Fig. 1). These observations indicate that apicomplexans may differ from each other by possessing different numbers and types of ACBPs.

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

To investigate the binding features of CpACBP1, we expressed the full-length protein as well as the ACBP domain as MBP-fusion proteins (MBP-CpACBP1 and MBP-CpACBP1-S). Both fusion proteins were purified to homogeneity using amylose-resin-based affinity chromatography (Fig. 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 folds in a way that interferes with the access of

factor Xa to the cleavage site. Difficulties in removing the MBP-tag have also been found in studies using recombinant *C. parvum* malate and lactate dehydrogenases in which their activities were assayed in 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 for the same ligand (Fig. 4a). With both ligand and protein concentrations at 80 μ M (near the highest concentration of 100 μ M in the kinetics assay), the non-specific binding of MBP-tag accounted for $\sim 5\%$ 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 amount of radioactivity when binding to palmitic acid (Fig. 4b), indicating that fatty acids are not the favourite ligands for CpACBP1. Based on these observations, we decided to use uncleaved fusion proteins in all subsequent analyses.

Fig. 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.

We first studied the specific binding between CpACBP1 and palmitoyl-CoA, and determined that the K_d for CpACBP1 binding to palmitoyl-CoA was 407 nM (Fig. 4c). This value is significantly higher than those reported for many other ACBPs which are typically in the 1–10 nM range (Burton *et al.*, 2005). This observation suggests that, although the Lipidex assay is a reliable assay for determining the acyl-CoA-binding profile for an ACBP, it is probably not a sensitive method for determining 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 with the binding kinetics of the recombinant proteins.

We also tested the specific binding of recombinant CpACBP1-S to palmitoyl-CoA in comparison to full-length CpACBP1 using the same Lipidex assay. With

Fig. 4. (a) Specific and non-specific binding of [14 C]palmitoyl-CoA (80 μ M) by MBP-fused CpACBP1 (40 μ M) and MBP-tag (40 μ M) as determined by the Lipidex 1000 extraction assay. (b) Relative binding between CpACBP1 and [14 C]palmitoyl-CoA (80 μ M) or [3 H]palmitic acid as determined by the Lipidex 1000 assay. Radioactivity was normalized using MBP-tag as control. (c) Binding kinetics of recombinant CpACBP1 with palmitoyl-CoA as determined by the Lipidex 1000 assay (see Methods).

0.4 μ M protein and 0.8 μ M palmitoyl-CoA, both fusion proteins displayed similar specific-binding activities [0.180 and 0.206 pmol (pmol protein) $^{-1}$ for CpACBP1 and CpACBP1-S, respectively], thus confirming that the ACBP domain was responsible for 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,

Fig. 5. 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 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, resulting 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.

only recombinant CpACBP1 (full-length) or CpACBP1-S (ACBP domain only), but not MBP-tag, displayed radioactivity (Fig. 5). The intensity of radioactivity associated with fractionated CpACBP1 was reduced when an equimolar amount of non-radioactive fatty acyl-CoA was added to the reaction. It was also observed that multiple radioactive bands appeared in lanes containing CpACBP1. Since protein fractionation was performed in native polyacrylamide gels, this observation suggests that CpACBP1 may also function as a dimer or tetramer. However, it is also possible that protein aggregation might occur under the experimental conditions used.

Based on the specific-binding data, we decided to test the substrate preference for CpACBP1 using 0.8 μ M of various fatty acyl-CoAs in competition with equimolar concentrations of palmitoyl-CoA. This concentration is approximately twice that of the K_d value for palmitoyl-CoA, so that the protein occupancy by the ligands was neither too low nor too high. The results show that CpACBP1 can bind to medium- and long-chain acyl-CoAs (Fig. 6). This protein has highest affinity for palmitoyl-CoA (C16:0), but decreased binding affinity for other acyl-CoA esters. However, CpACBP1 is incapable of binding acyl-CoA esters with an acyl chain of 20 carbons or longer. This feature makes CpACBP1 different to many other ACBPs, including those from bovines and trypanosomes that can bind to acyl-CoA esters with an acyl chain of 24 or more carbons (Milne & Ferguson, 2000; van Aalten *et al.*, 2001).

Fig. 6. Acyl-CoA binding specificity of CpACBP1 determined by the Lipidex 1000 competition binding assay. In each reaction, [14 C]-palmitoyl-CoA was mixed with an equimolar amount of non-radioactive acyl-CoA with specified carbon chain length. Binding affinity is presented as the percentage displacement of radioactive palmitoyl-CoA by non-radioactive acyl-CoA. SEM values were determined from at least three individual samples.

CpACBP1 is differentially expressed and its encoded protein is probably localized to the PVM

Semi-quantitative RT-PCR showed that *CpACBP1* is expressed differentially in the complex parasite life cycle (Fig. 7). *CpACBP1* transcripts were barely detectable in free sporozoites, but started to appear after parasite invasion into host cells. The level of *CpACBP1* transcripts was relatively low during early intracellular development (from 3 to 24 h post-infection), but gradually increased with the time of infection. This expression pattern differs from that of many other *C. parvum* genes, such as the replication protein A subunits, CpSFP-PPT and β -tubulin, but is similar to those of oocyst wall proteins in this parasite (Abrahamsen & Schroeder, 1999; Cai *et al.*, 2005; Millership *et al.*, 2004a;

Fig. 7. Relative levels of *CpACBP1* gene transcripts in various *C. 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.

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 8 and 9).

More surprisingly, immunofluorescence microscopy indicates that CpACBP1 is probably located on the PVM (Fig. 9). Rabbit polyclonal antibodies clearly labelled the surface of meronts with a homogeneous pattern of distribution, but failed to label the merozoites within the meronts (Fig. 9a). In a dual-labelling experiment using a rabbit polyclonal antibody mainly against PVM and the electron-dense connection between host cell and parasite, we co-localized CpACBP1 and PVM proteins (Fig. 9b). On the other hand, a polyclonal antibody against CpSFP-PPT clearly labelled the merozoites, but not on the surface of meronts in another dual-labelling experiment (Fig. 9c).

DISCUSSION

ACBP was originally identified as a mammalian diazepam-binding inhibitor – a neuropeptide that could inhibit diazepam binding to the γ -aminobutyric acid 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 ACBP1 and ACBP2 in *A. 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, 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).

Fig. 8. Western blot detection of CpACBP1 protein in *C. parvum* oocysts (O), excysted free sporozoites (S) and intracellular parasites grown for 24 and 48 h. CpACBP1 was only detected in the intracellular parasites, but not in oocysts and free sporozoites.

Fig. 9. Immunofluorescence microscopy of the CpACBP1 protein in intracellular *C. parvum*. (a) Indirect immunolabelling of intracellular parasites grown for 24, 48 and 72 h using a rabbit polyclonal antibody against CpACBP1 and a secondary antibody conjugated with FITC. (b) Direct dual-labelling of intracellular parasites grown for 24 h using an Alexa Fluor 488-conjugated antibody against CpACBP1 and an Alexa Fluor 546-conjugated antibody against parasite TMPs that mainly labels PVM and feeder organ. Both antibodies displayed the same labelling pattern on the surface of a meront. (c) Dual-labelling of intracellular parasites grown for 48 h using an Alexa Fluor 546-conjugated antibody against CpACBP1 and an Alexa Fluor 488-conjugated antibody against cytosolic CpSFP-PPT, showing CpACBP1 mainly on the surface of meronts, rather than in merozoites. Phase, Phase-contrast; DIC, differential interference contrast; DAPI was used for counterstaining nuclei. Bars, 5 µm.

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 ACBPs of various types (short-type, long-types fused with ankyrin repeats or with uncharacterized sequences) (Fig. 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 immunolabelling data indicate that CpACBP1 is also a membrane protein (i.e. mainly associated with PVM) (Fig. 9). Such a membrane association is probably 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.*, 2002). 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. the first 3 h 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, 2004). 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 fatty acid uptake by the parasite.

Another possibility is that CpACBP1 may be involved in the synthesis of GPI anchor. In African trypanosomes, ACBP has been found to be responsible for supplying myristoyl-CoA to the fatty acid remodelling machinery during GPI synthesis (Milne & Ferguson, 2000; Milne *et al.*, 2001). Although it is as 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 *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, including phosphatidylinositol *N*-acetylglucosaminyltransferases (e.g. GenBank accession numbers XP_628152, XP_627129 and XP_626317). Nonetheless, further investigations are necessary to test these hypotheses.

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 the highest binding affinity towards the C16:0 palmitoyl-CoA. The K_d value of 407 nM obtained by the Lipidex 1000 assay 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 dialyser-based methods are typically lower, in the range of 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 of 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. 4c). However, it is unclear whether CpACBP1 is truly present as a dimer or polymers *in vivo*, or whether the observed multiple bands in autoradiography are only artefacts induced by the experimental conditions.

Fatty acids are essential to all organisms. Recently, fatty acid metabolism has been considered as a promising target for drug development against cryptosporidiosis and other important pathogenic apicomplexans (Gornicki, 2003; Kuo *et al.*, 2003; Ralph *et al.*, 2001; Roberts *et al.*, 2003; Waller *et al.*, 2003; Zhu, 2004). 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.

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