cloned and expressed a recombinant trypanosome acyl-CoA-binding protein that has a preference for binding relatively short chain acyl-CoAs and that has a high affinity for binding myristoyl-CoA (Kd = 3.5 × 10^-10 M). This protein enhances fatty acid remodeling of glycosylphosphatidylinositol precursors in the trypanosome cell-free system. We speculate that the trypanosome acyl-CoA-binding protein plays an active role in supplying myristoyl-CoA to the fatty acid remodeling machinery in the parasite.

The protozoan parasite Trypanosoma brucei is responsible for causing African sleeping sickness in humans and Nagana in cattle. The parasite is spread between hosts by the tsetse fly vector and, in the bloodstream, possesses a dense surface coat of 10 million copies of variant surface glycoprotein (VSG) (1). This coat is responsible for evading the host's defenses by a coat of variant surface glycoprotein molecules, each of which is attached to the plasma membrane by a glycosylphosphatidylinositol anchor (4). The C-terminal amino acid of the VSG protein is attached, via an ethanolamine-phosphate bridge, to a conserved moiety of Man3GlcN-PI (1–4GlcN-1–2Man3GlcN-PI, Man3GlcN-PI). The T. brucei GPI biosynthetic pathway was elucidated using a cell-free system containing washed trypanosome membranes (5, 6). The pathway involves assembly of ethanolamine-P-Man3GlcN-PI (glycolipid A') from PI followed by fatty acid remodeling whereby both fatty acids of the PI moiety are remodeled to myristate (C14:0) through the sequential decaylation and reacylation of the glycerol backbone (Fig. 1). The product, a dimyristoylated GPI known as glycolipid A (8), is linked to newly synthesized VSG in the endoplasmic reticulum by a transamidation reaction (10–12). Experiments using the cell-free system also showed that the donor for the myristoylation reactions of fatty acid remodeling was myristoyl-CoA (8). In most, possibly all, eukaryotes it is known that acyl-CoAs are bound to acyl-CoA-binding proteins (ACBPs) that prevent their metabolism and allow the efficient shuttling of acyl-CoA to the cell acylation machinery (13). In this study we have expressed, purified, and characterized a recombinant trypanosome ACBP and studied the effect of this molecule on the fatty acid remodeling machinery of GPI biosynthesis.

**Experimental Procedures**

**Organisms and Reagents—**Genomic DNA from T. brucei MIT1.4 was isolated as described previously (14). Routine DNA manipulations were performed in Escherichia coli strain DH5α. All chemicals were of the highest grade available from BDH or Sigma, and restriction enzymes were from Promega. DNA sequencing of double-stranded DNA was accomplished by the dideoxynucleotide chain termination method (15) by automated cycle sequencing using the dye terminator method (ABI PRISM big dye terminator kit, Perkin-Elmer). Trypanosome EST (accession No. W40086) was a gift from P. Majiwa (International Live- stock Research Institute, Kenya) and recombinant bovine ACBP was a gift from J. Knudsen (Institute of Biochemistry, Denmark).

Cloning of the T. brucei acyl-CoA-binding Protein—BLASTn search of the GenBank database was performed using the gene encoding human ACBP (accession No. P07108). A DNA fragment corresponding to the identified EST sequence (accession No. W40086) was amplified by polymerase chain reaction (PCR). Primers for amplification were 5′-gctaatgagaaagctg-3′ (sense) and 5′-cggtagctacagtctgg-3′ (antisense). The sense primer contains a NdeI site and the antisense primer contains a BamHI site for cloning. The cDNA plasmid encoding the EST sequence was used as template. Amplification conditions were 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1 min for 30 cycles. The PCR product was separated on an agarose gel, and the DNA of the expected size was isolated (Qiaex II kit, Qiagen). The PCR product was labeled with fluorescein-dUTP by random priming (Gene Images kit, Amersham Pharmacia Biotech) and used as a probe in a Southern blot of restriction-digested genomic T. brucei DNA. This probe was also used to screen a 5.5-kilobase EcoRI restriction-digested genomic T. brucei size selected library. The filters were washed at high stringency (0.5× SSC, 0.1% SDS, 60 °C), and one positive colony from the size selected library was confirmed by PCR. This bacterial colony, containing a genomic clone of the T. brucei ACBP gene, was replated and screened twice more prior to sequencing.

Overexpression of T. brucei ACBP—The ACBP gene was amplified by PCR using the sequenced genomic clone as template. The sense strand primer 5′-gctaatgagaaagctg-3′ contained a NeoI site (underlined) and an ATG initiation codon, and the antisense strand primer 5′-cggtagctacagtctgg-3′ contained a XhoI site (underlined) and a stop codon. The PCR product was cloned using the NeoI and XhoI into pUC18 vector (Sureclone kit, Amersham Pharmacia Biotech). The insert was subsequently excised by digestion with NeoI and XhoI, and the gel-purified insert was ligated into the NeoI and XhoI cloning site of the pfFastBac HTa expression vector (Life Technologies, Inc.). The pfFastBac-ACBP plasmid was used in the Bac-to-Bac™ Bacu-
lovirus Expression System (Life Technologies, Inc.). Briefly, the plasmid was transformed into competent DH10Bac E. coli cells, and recombinant bacmid DNA recovered after transposition. Sf9 insect cells were transfected with the bacmid DNA in the presence of CellFECTIN reagent and recombinant baculovirus particles recovered as stock virus. Sf9 cells were grown in suspension culture in SF-900 II medium (Life Technologies, Inc.) containing 50 units/ml penicillin, 50 μg/ml streptomycin, 5% fetal calf serum at 27 °C. Optimal expression of recombinant protein was achieved using 4 × 10^5 pfu/ml virus to infect 2 liters of Sf9 cells at 2 × 10^6 cells/ml for 3 days at 27 °C. Sf9 cells were pelleted (500 × g, 5 min, 4 °C) and resuspended in 200 ml of lysis buffer (20 mM Tris-HCl, pH 8, 100 mM KCl, 1 mM phenylmethanesulfonyl fluoride), and cells were disrupted by sonication (6 × 45 s pulses interrupted with cooling on ice). Cell debris was removed by centrifugation at 10,000 × g for 10 min prior to applying the supernatant to a Ni^2+ -resin column (1.6 × 12 cm chelating Sepharose fast flow, Amersham Pharmacia Biotech). The histidine-tagged recombinant protein was eluted from the column with a linear gradient of imidazole from 10 mM to 1 M in 20 mM Bis-Tris propane, 20 mM Tris-HCl, pH 7.5, 300 mM NaCl at 3 ml/min. Fractions were concentrated and desalted with a Centricon plus-20 (Amicon) filtration unit, and the histidine tag was removed with rTEV protease (Life Technologies, Inc.) containing 50 units/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml streptavidin, 1 μg/ml tunicamycin, 1 mM dithiothreitol) supplemented with 0.2 mM CoA, 1 mM UDP-GlcNAc, and 1 mM MgCl_2. 5 mM MnCl_2, 0.1 mM N-omega-tosyl-L-lysine chloromethyl ketone, 1 μg/ml leupeptin, 0.4 μg/ml tunicamycin, 1 mM dithiothreitol) supplemented with 0.2 mM CoA, 1 mM UDP-GlcNAc, and 1 mM GDP-Man for 5 min at 37 °C. The suspension was then added to 50,000 cpm or 250,000 cpm for 1 or 5 pmol of [3H]myristoyl-CoA, respectively, which had been dried in the tube and incubated in 5 μl of incorporation buffer in the presence or absence of trypanosome ACBP (1 or 5 pmol) for 5 min at 37 °C. After 5 min, GPs were extracted with chloroform/methanol/water (10:10:3, v/v), dried, and partitioned between 0.5 ml of butan-1-ol/water (1:1, v/v); the labeled lipids in the butan-1-ol phase were concentrated, boiled for 8 min in 50 μl of 1 M butan-1-ol, sprayed the HPTLC plates with EN3HANCE (NEN Life Science Products), and visualized by fluorography after spraying the HPTLC plates with ENHANCE (NEN Life Science Products).

For GDP-[3,4-3H]Man (24.24 Ci/mmol; NEN Life Science Products) labeling, cell membranes were resuspended at 10^6 cell equivalents/ml in incorporation buffer supplemented with 14 μCi/ml GDP-[3H]Man and 1 μM UDP-GlcNAc and incubated for 10 min at 30 °C. Thereafter, 1 μM GDP-Man was added, and the incubation continued for a further 5 min.
Identification and Cloning of Trypanosome ACBP Gene—We identified a cDNA fragment (GenBank™ accession number W40086) encoding a putative trypanosome ACBP by BLASTn search using the human ACBP gene as a query. The putative ACBP open reading frame present in this EST was amplifed by PCR, gel-purified, labeled by random priming, and used as a probe.

A Southern blot of T. brucei genomic DNA digested with several restriction enzymes and hybridized with the ACBP probe indicated a single copy ACBP gene/haploid genome (Fig. 2). The identification of two restriction fragments when the genomic DNA was digested with MboI or BglII (Fig. 2, lanes 6 and 7) was consistent with the EST DNA sequence that predicts single MboI and BglII restriction sites within the putative ACBP open reading frame.

Based on the Southern blot (Fig. 2, lane 3), gel-purified 5.5-kilobase EcoRI fragments of genomic DNA were ligated into a pUC vector to produce a size selected library. The library was screened for the ACBP gene (19), and a clone containing a 279-base pair gene encoding a putative 93-amino acid trypanosome ACBP (predicted molecular mass of 10.69 kDa) was isolated. Alignment of the predicted amino acid sequence of the putative trypanosome ACBP with the human, bovine, and yeast ACBP sequences (20–22) using Clustal V is shown (Fig. 3). The predicted trypanosome sequence showed 14% identity and 41% similarity to these other ACBP proteins. The putative trypanosome ACBP was 7 amino acids longer than the other homologs with a 6-amino acid extension close to the N terminus.

Overexpression and Purification of Recombinant Trypanosome ACBP—The putative trypanosome ACBP gene was cloned into the pFastBac plasmid that was used to produce a baculovirus encoding the recombinant his-tagged protein. This virus was used to infect Sf9 cells, and after optimization of virus titer levels and exposure times, expression of the recombinant protein at 1.5 mg/l was achieved (Fig. 4, lane 1). The presence of a N-terminal histidine tag sequence on our recombinant putative trypanosome ACBP in Sf9 cells, lane 2, soluble proteins from Sf9 cells overexpressing trypanosome ACBP, lane 3, nickel chelating column elute; lane 4, cleavage of N-terminal histidine tag with rTEV protease; lanes 5 and 6, gel filtration purified recombinant ACBP (0.1 and 1 μg).

Identification and Cloning of Trypanosome ACBP—Sequence alignment of the putative trypanosome acyl-CoA-binding protein with acyl-CoA-binding proteins of human (20), bovine (21), and yeast (22) origin using the Clustal V alignment program. * and + indicate sequence identity and similarity, respectively.

at 30 °C. This reaction mix was then mixed with an equal volume of incorporation buffer containing 2 mM ATP, 2 μM CoA, and 2 μM ACBP, and the incubation continued at 30 °C. Aliquots (4 × 10⁷ cell equivalents) were removed, and the reaction was terminated by adding 275 μl of chloroform/methanol (1:1, v/v). GPs were extracted as before and resolved on HPTLC plates developed in chloroform, methanol, 1 M ammonium acetate, 13 mM NH₃, water (180:140:9:9:23, v/v). Radioactive products were visualized by fluorography of the HPTLC plates.

RESULTS

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myristoyl-CoA binding to trypanosome ACBP. The values plotted on a Scatchard plot of the trypanosome ACBP with myristoyl-CoA. Trypanosome ACBP was incubated in the absence or presence of myristoyl-CoA prior to analysis. B absence (.) or presence (.) of myristoyl-CoA.2

To determine the specificity of trypanosome ACBP for chain length acyl-CoA and compare this with the specificity of bovine ACBP, we studied the competition for binding [3H]myristoyl-CoA by various unlabeled acyl-CoAs. Bovine ACBP showed broad specificity with the greatest relative affinity for stearoyl-CoA (Fig. 7). This is similar to the previously reported finding using a similar assay and an epr spectroscopy assay that measured the displacement of spin-labeled acyl-CoA by various acyl-CoAs (17). However, the trypanosome ACBP showed a very different acyl-CoA specificity with the greatest relative affinity for shorter chain acyl-CoAs such as lauroyl-CoA (Fig. 7).

The Effects of Trypanosome ACBP on GPI Fatty Acid Remodeling in the Cell-free System—Trypanosome membranes were incubated with UDP-GlcNAc and GDP-Man to generate in situ GPI substrates for fatty acid remodeling and subsequently with [3H]myristoyl-CoA in the presence or absence of equimolar trypanosome ACBP. In the absence of trypanosome ACBP, incorporation of [3H]myristate into lipids, including into mature GPI precursors glycolipid A and glycolipid C, was observed (Fig. 8A, lanes 1 and 2) as described previously (8). Incorporation was significantly greater at 100 nM than at 20 nM [3H]myristoyl-CoA. The incorporation of [3H]myristate into neutral lipids and phospholipids was unaffected by the presence of ACBP, but the incorporation of [3H]myristate into glycolipids A and C was increased about 3-fold, as determined by densitometry (Fig. 8A, lanes 3 and 4). These data gave the first indication that trypanosome ACBP can facilitate the fatty acid remodeling and/or fatty acid exchange reactions. To study this further and to look specifically at the fatty acid remodeling reactions, the cell free system was pulse-labeled with GDP-[3H]Man in the presence of UDP-GlcNAc to produce prelabeled glycolipid A’ and θ (Fig. 8B, lane 1). In our hands, we find that fatty acid remodeling of these species to glycolipid A’ and glycolipid A can be achieved over 15 min (though somewhat inefficiently) by the addition of ATP and CoA alone (Fig. 8B, lanes 2–4). Presumably, endogenous acyl-CoA activity can generate myristoyl-CoA in situ from some membrane-bound store of myristate. This conversion of glycolipids A’ and θ to A’ and A was greatly stimulated by the inclusion of recombinant trypanosome ACBP (Fig. 8B, lanes 5–7). Furthermore, the glycolipid A that was produced was further processed to glycolipid C, the inositol-acylated version of glycolipid A, and the final product of the GPI biosynthetic pathway. These results show that trypanosome ACBP greatly facilitates the fatty acid remodeling and inositol-acylation of GPI intermediates in the trypanosome cell-free system and suggests that trypanosome ACBP is likely to be involved in supplying myristoyl-CoA to the fatty acid remodeling machinery in vivo. To access whether this affect was specific to trypanosome ACBP, the affect of recombinant trypanosome ACBP on fatty acid remodeling was directly compared with that of recombinant bovine ACBP (Fig. 8B, lanes 8 and 9). The bovine ACBP appears to be just as efficient as the trypanosome protein in stimulating fatty acid remodeling and inositol acylation.

**DISCUSSION**

We were interested in studying the fatty acid remodeling (8) and fatty acid exchange reactions (18, 23) responsible for the exclusivity of myristic acid in the formation of the GPI anchor of trypanosome VSG (4). Studies using myristic acid analogs, such as O-11, suggest that their incorporation into the GPI anchor of VSG ultimately leads to the death of the parasites in culture (24, 25). Previous studies using trypanosome cell-free system experiments have shown that the donor of myristate for fatty acid remodeling (8) and exchange (18, 23) is myristoyl-CoA. However, other studies also suggested that the parasite was able to utilize myristoyl-lyso-phosphatidylcholine as a source of myristate (26–28). This has been further investigated, and it appears that myristoyl-lyso-phosphatidylcholine

**FIG. 5.** Positive ion electrospray mass spectrometry analysis of trypanosome ACBP. Trypanosome ACBP was incubated in the absence (A) or presence (B) of myristoyl-CoA prior to analysis.

**FIG. 6.** Equilibrium dialysis measurement of the interaction of the trypanosome ACBP with myristoyl-CoA. Scatchard plot of [3H]myristoyl-CoA binding to trypanosome ACBP. The values plotted are mean ± S.E. (n = 3). The graph is representative of three separate experiments that produced similar Kₐ values.
is converted to myristoyl-CoA, which can be subsequently used in the fatty acid remodeling reactions (29). Thus, the consensus view is that myristoyl-CoA is the donor for myristic acid incorporation into trypanosome GPI anchors.

Based on the work of Knudsen and others (13, 30–32), it is clear that the levels of free acyl-CoAs in the cytoplasm of most eukaryotic cells are very low because of the presence of ACBPs with high affinity for long chain acyl-CoAs. We wished to find the trypanosome ACBP homolog and to assess the effect of including this protein in the in vitro fatty acid remodeling assays. These assays use washed membrane preparations (the so-called cell-free system) that are likely to be devoid of endogenous (soluble) ACBP.

We searched GenBank™ for potential trypanosome ACBP homologs with the gene encoding human ACBP and were able to detect a trypanosome EST sequence (accession No. W40086). We subsequently cloned the full-length genomic sequence, which showed significant similarity to other ACBP homologs (Fig. 2). However, sequence conservation was lower for the putative trypanosome ACBP than that previously observed between the other ACBP homologs (13). Previous studies identified 13 conserved amino acids found in all ACBP homologs and suggested that all of these residues might be necessary for binding acyl-CoAs (13). In bovine ACBP these amino acids are Phe-5, Leu-15, Tyr-28, Lys-31, Gln-33, Gly-37, Pro-44, Lys-54, Trp-58, Gly-63, Ala-69, Tyr-73, and Leu-80. However, only six of those residues are absolutely conserved in trypanosome ACBP (Tyr-35, Gln-40, Gly-44, Pro-51, Lys-61, Ala-76) together with four conservative substitutions (Leu-6, Arg-65, Phe-80, Val-87) and three nonconservative substitutions (Thr-22, His-39, Lys-70). We considered that these differences from the other eukaryotic ACBPs studied to date might result in an altered specificity for binding acyl-CoAs. Having demonstrated that recombinant putative trypanosome ACBP was indeed a high affinity ACBP (Figs. 5 and 6), we tested the specificity of the trypanosome ACBP for acyl-CoA chain length using a competition assay (Fig. 7). These data showed that trypanosome ACBP had a distinct preference for shorter chain acyl-CoAs when compared directly to the bovine ACBP (Fig. 7).

We utilized the previously resolved three-dimensional NMR structure of the complex between bovine ACBP and palmitoyl-CoA (33) to predict the structure of trypanosome ACBP using the Collaborative Computational Project (34). This model predicts that the amino acids in the trypanosome ACBP at positions Thr-16, Lys-20, Ile-28, Lys-31, Leu-32, Tyr-35, Trp-38, Val-57, and Lys-61 are within 0.3 nm of the bound palmitoyl-CoA.
CoA. Of these, Leu-32, Tyr-35, and Lys-61 are conserved; Lys-20 and Trp-38 are similar in bovine ACBP, whereas Thr-16, Ile-28, Lys-31, and Val-57 are not conserved between the two structures. These differences may have a role in defining the chain length specificity of the two ACBPs. We intend to investigate these issues using site-directed mutagenesis.

Previous studies have investigated the binding affinity of acyl-CoAs for ACBP using epr spectroscopy (17) and titration microcalorimetry (22, 35–37) ranging from 4 × 10^{-10} to 4.5 × 10^{-14} M depending on acyl chain length and the method used. Measurements made by direct titration microcalorimetry range from 2.4 × 10^{-7} and 1.7 × 10^{-8} M for octanoyl-CoA and doco-ycanoyl-CoA with bovine ACBP (35, 36) to 8.5 × 10^{-11} M for palmitoyl-CoA with yeast ACBP (22). In our studies, we used equilibrium dialysis to directly measure the K_d value of trypanosome ACBP for myristoyl-CoA and obtained a figure of 3.5 ± 0.4 × 10^{-10} M (Fig. 6). This K_d value is consistent with the generally high affinity of acyl-CoAs for ACBPs. We were unable to obtain an accurate K_d value for bovine ACBP and myristoyl-CoA, which was out of the range of our assay and must have a K_d value of < 1 × 10^{-10} M. Previous titration studies showed that bovine ACBP has a preference for longer chain acyl-CoAs, with an optimal chain length of C18–20 (17, 35, 36). The competition experiment (Fig. 7) is consistent with these data and shows that whereas bovine ACBP has a relatively low affinity for shorter acyl-CoAs (<C12), the trypanosome homolog has a preference for shorter acyl-CoAs (<C20) and a remarkably high affinity for C10 and C12 acyl-CoAs.

Finally, we investigated the effect of trypanosome ACBP on the trypanosome cell-free system that has previously been used to study GPI biosynthesis. We anticipated that we might observe an effect because the washed membranes of the cell free system should be depleted of endogenous ACBP, whereas in vivo the majority of cellular myristoyl-CoA would be bound to ACBP. Initially, we investigated the direct effect on fatty acid remodeling and/or fatty acid exchange by adding exogenous [3H]myristoyl-CoA ± ACBP to the cell-free system. This showed that the presence of trypanosome ACBP substantially enhanced the labeling of glycolipids A and C with [3H]myristoyl-CoA (Fig. 8A). We then further investigated the effect of ACBP on fatty acid remodeling by prelabeling glycolipids A' and A with [3H]myristoyl-CoA in situ by the addition of ATP and CoA. When ACBP was included, the efficiency of fatty acid remodeling and inositol-acetylation was significantly increased. This strongly suggests that myristoyl-CoA is usually supplied to the fatty acid remodeling machinery via ACBP. This is consistent with the roles of ACBP in other eukaryotes where the ACBP is responsible for the transport of acyl-CoA directly to cell acylation machinery (22, 30–32). Indeed, despite the limited similarity between the trypanosome and bovine ACBP, the latter seems to be able to supply myristoyl-CoA to the fatty acid remodeling machinery in the trypanosome cell-free system (Fig. 8B).

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