ACBD6 protein controls acyl chain availability and specificity of the N-myristoylation modification of proteins

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Abstract Members of the human acyl-CoA binding domain-containing (ACBD) family regulate processes as diverse as viral replication, stem-cell self-renewal, organelle organization, and protein acylation. These functions are defined by nonconserved motifs present downstream of the ACBD. The human ankyrin-repeat-containing ACBD6 protein supports the reaction catalyzed by the human and Plasmodium N-myristoyltransferase (NMT) enzymes. Likewise, the newly identified Plasmodium ACBD6 homologue regulates the activity of the NMT enzymes. The relatively low abundance of myristoyl-CoA in the cell limits myristoylation. Binding of myristoyl-CoA to NMT is competed by more abundant acyl-CoA species such as palmitoyl-CoA. ACBD6 also protects the Plasmodium NMT enzyme from lauryl-CoA and forces the utilization of the myristoyl-CoA substrate. The phosphorylation of two serine residues of the acyl-CoA binding domain of human ACBD6 improves ligand binding capacity, prevents competition by unbound acyl-CoAs, and further enhances the activity of NMT. Thus, ACBD6 proteins promote N-myristoylation in mammalian cells and in one of their intracellular parasites under unfavorable substrate-limiting conditions.—Soupene, E., and F. A. Kuypers. ACBD6 protein controls acyl chain availability and specificity of the N-myristoylation modification of proteins. J. Lipid Res. 2019. 60: 624–635.

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The N-myristoylation modification of specific proteins at an N-terminal glycine residue affects the activity of the myristoylated proteins, modulates their association with membranes, and mediates oligomeric assembly and the interaction with other proteins (1–4). N-Myristoylation occurs mainly during the translation of nascent peptides after the removal of the initiator methionine by methionyl aminopeptidase and the exposure of a glycine. The activity of N-myristoyltransferase (NMT) enzymes 1 and 2 is essential for human protein function. Likewise, N-myristoylation is of crucial importance for the intracellular development of pathogens such as parasitic protozoa, fungi, and viruses (5–12). N-Myristoylation is a multistep process initiated by the binding of myristoyl-CoA to the N-terminal domain of apo-NMT, covalent linkage of the myristoyl chain to the glycine residue, and release of CoA, followed by the final release of the acyl peptide. In retinal photoreceptor cells, C14:1 and C14:2 rather than C14:0 are the fatty acids used to modify proteins such as the α-subunit of the G-protein photoreceptor (13–15). While the 14-carbon chain is the preferred substrate, NMT proteins lack binding specificity for C14:0-CoA. This renders NMT incapable of preventing the unwarranted occupation of the site by acyl-CoA species other than C14:0-CoA (4–6, 10, 13). Because the transfer rate of acyl chains with a length other than C14 is extremely slow, binding of the “wrong” acyl-CoA blocks the myristoyltransferase cycle for NMT enzymes. In the cell, the greater abundance of acyl-CoAs such as C16:0-CoA compared with C14:0-CoA would therefore prevent protein myristoylation. It follows that mechanisms that prevent binding or trigger the release of these nontransferable competitors from the myristoyl-CoA binding site are essential in supporting NMT enzyme activity.

Acyl-CoA binding domain-containing (ACBD) proteins are involved in the maintenance of diverse cellular functions. They interact with a multitude of proteins and can be located in the cytosol, organelles, or nucleus; bound to membranes; or secreted (16, 17). This protein family regulates processes as diverse as neural stem-cell self-renewal, protein and lipid acylation, lipid homeostasis, intracellular vesicle trafficking, organelle formation, viral replication, and apoptotic response (18–35). Plant ACBD proteins are also implicated in essential functions such as embryogenesis and resistance to various stresses (36–38). Acyl-CoAs

Abbreviations: ACB, acyl-CoA binding; ACBD, acyl-CoA binding domain-containing; ACN, acetonitrile; Glu, glutamate; hACBD6, human ACBD6; hNMT2, human NMT2; NMT, N-myristoyltransferase; P-Ser, phosphoserine; αH, α helix.

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bind to the N-terminal acyl-CoA binding (ACB) domain, and various motifs present at the carboxyl terminal of the different members of this family appear to define independent and nonredundant physiological functions of the ACB proteins. The requirement and role of the acyl-CoA ligand bound to the ACB domain in the interaction with other proteins is poorly understood. The phosphorylation of two serine residues of α-helix 4 of the ACB domain of ACBD6 was detected in vivo, but the effect on its activity has not been determined (39, 40).

We previously showed that ACBD6 stimulated the activity of NMT2 and prevented the competition of the NMT reaction by C16-CoA (26). An interaction between ACBD6 and NMT2 is required, and ligand binding to ACBD6 further enhanced its stimulatory effect on NMT2. Mutants of ACBD6 deficient in ligand binding did not stimulate the activity of NMT2. They also could not protect this enzyme from the competitor C16-CoA. In the NMT2/ACBD6 complex, the acyl-CoA carrier appears to prevent the access of C16-CoA and allows C14-CoA to reach the acyl-transferase binding site in the presence of more abundant acyl-CoA. ACBD6 also interacts with NMT1 and likely regulates its activity.

The effect of the phosphorylation of Ser106 and Ser108 on the acyl-CoA binding property of ACBD6 and on the myristoyltransferase reaction was determined. We established that human ACBD6 (hACBD6) enhanced the activity of the malaria parasite Plasmodium falciparum NMT (PfNMT) enzyme. Although C14-CoA was a stronger competitor of C14-CoA than C16-CoA for the parasite myristoyltransferase enzyme, hACBD6 also protected PfNMT from inhibition by the shorter acyl-CoA species. An ankyrin-repeat-containing acyl-CoA binding protein of Plasmodium, PfACBD6, was identified. Like the human homologue, PfACBD6, was an acyl-CoA binding protein of Plasmodium. The shorter acyl-CoA species. An ankyrin-repeat-containing enzyme, hACBD6 also protected PfNMT from inhibition by 200 ng of the constructs and the reporter pLuc at a pACT-pBIND-pLuc ratio of 2.25:0.25:0.5. Measurements were performed after 48 h of transfection with the Dual-Glo Luciferase system (Promega).

### Protein expression and purification

Human ACBD1, ACBD3, ACBD6, and NMT2 were produced as previously described (26–28, 43). Human ACBD4 and ACBD5 were cloned in pETM41 and produced as maltose binding protein fusion forms (gift of Joseph Costello) (19) in RosettaDE3 cells (Novagen). The production of ACBD6 forms phosphorylated on Ser106, Ser108, or on both of these serine residues was performed in E. coli grown in LB medium supplemented with 2 mM phosphoserine (41, 42). PfNMT and PfACBD6 were expressed in RosettaDE3. Although a large portion of PfNMT formed aggregates, the addition of 1% CHAPS during lysis of the cells and the soluble fraction throughout the purification procedure was successful in maintaining PfNMT in solution. PfACBD6 was maintained in solution by the addition of 1% Triton X-100 in all buffers during the extraction and purification steps. Human ACBD1, ACBD3, ACBD6, NMT2, PfNMT, and PfACBD6 were purified by affinity metal chromatography. Human ACBD4 and ACBD5 were purified with an N-terminal acyl-CoA carrier (New England Biolabs, Inc.). Purified ACBD1–6 proteins were stored at −80°C in 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM EDTA, and 10% glycerol (v/v). Human NMT2 was stored in the same buffer in the presence of 0.2% Triton X-100. PfNMT was stored in 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM EDTA, and 1% CHAPS, and 20% glycerol to prevent the loss of activity. PfACBD6 was stored in 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM EDTA, 10% glycerol, and 1% Triton X-100.

### Binding, competition, and displacement assays

[14C]C18:1-CoA binding by ACBD proteins was performed as previously described (27). For competition experiments, unlabeled acyl-CoA was mixed with [14C]C18:1-CoA prior to the addition of the protein. Concentrations of ligands, competing acyl-CoA species, and proteins are indicated in the legend of each figure. The displacement of ACBD6-bound [14C]C18:1-CoA by unlabeled acyl-CoA was performed by purifying the [14C]C18:1-CoA/ACBD6 complex, removing unbound [14C]C18:1-CoA, and mixing the complex with competing acyl-CoA (see below). ABCD6 (1 μM) was incubated with 5 μM [14C]C18:1-CoA in 500 μL for 20 min at 37°C. The reaction was chilled on ice, and ABCD6 was pulled down with 50 μL NTA 50% slurry at 4°C for 10 min. The resin was washed 3 times in binding buffer and eluted with 50 μL 6 M imidazole. Ten microliters of the eluate were then added to 240 μL buffer containing 0–10 μM C18:1-CoA. The displacement experiments were performed with 5 μM [14C]C18:1-CoA and 0.5, 1,
2, and 4 μM ACBD6 in 500 μl. The purified complex was then exposed to 2.5 μM C14-CoA. Following incubation for 20 min at 37°C, ACBD6 was then pulled down a second time with 60 μl NTA slurry and washed as above. The amount of [14C]C14-CoA left in the ACBD6 bound-resin fraction was quantified with a scintillation counter.

**N-Myristoyltransferase activity measurements**

Real-time measurements of released CoA from acyl-CoAs by purified NMT forms were performed at 30°C in a Cary Varian Cary 50 UV-Vis spectrophotometer in the presence of 200 μM Ellman’s reagent as previously described (26). Detection and quantification of the formation of the acyl peptide in the presence of acyl-CoAs and the peptide GLYVSRLF was performed by monitoring of the absorbance of the tyrosine residues at 274 nm. Reactions were performed in 20 mM sodium phosphate (pH 8.0), 1 mM EDTA, 1% CHAPS, and 200 μM peptide residues at 274 nm. Reactions were performed in 20 mM sodium phosphate (pH 8.0), 1 mM EDTA, 1% CHAPS, and 200 μM C14-CoA with concentrations of C14-CoA from 5 to 50 μM. The volume of the reactions, the concentrations of C14-CoA and competing acyl-CoA, the NMT enzyme, and the ACBD proteins are indicated in the legend of each figure. Reactions were performed at 37°C and were stopped at various times by adding 1.08 vol ice-cold methanol/TFA 8%. Samples were vortexed and precipitated on ice for 20 min. Proteins were removed by centrifugation at 8,000 g for 10 min at 4°C. The supernatants containing the peptide and acyl peptide were collected and dried down under vacuum overnight at 20°C. The pellets were suspended in 50% methanol/TFA 4% (v/v) from 30 to 80 μl. A 10 μl sample was injected on the C18 column, and separations were performed as described previously (10, 44, 45). An acetonitrile (ACN) gradient was generated in the presence of 0.1% TFA and HPLC-grade water. Samples were loaded onto the column equilibrated with 10% ACN/TFA 0.1%. The ACN concentration was increased to 68% in 30 min at a flow rate of 1 ml per minute and to 100% in 1 min at 1.5 ml/min. After 3 min at 100% ACN/TFA 0.1%, the ACN concentration was decreased to 0%, and the column was equilibrated for 10 min at 1 ml/min. The percentage of product formed, detected by absorbance of the peptide at 274 nm, is reported as the relative volume of the myristoyl peptide peak (34.5 min) relative to the sum of the volume of the peptide peak (18 min) and of the acyl peptide detected in each injected sample. In one experiment, the reaction was performed in the absence of C14-CoA but in the presence of the bound C14-CoA/ACBD6 complex. ACBD6 (20 μM) was incubated with 100 μM C14-CoA for 20 min at 37°C. The bound complex was pulled down as described above, and the 50 μl eluate was incubated with 60 μg PINMT and 200 μM peptide for 2 h at 37°C in 1 ml buffer. A control reaction was performed with the eluate obtained from the pull down of C14-CoA incubated in the absence of ACBD6. Calculations and statistical analysis were performed with GraphPad Prism 7.

**Cell culture and transfection**

HeLa229 cells, obtained from ATCC (CCL-2.1), were maintained in MEM α (Invitrogen) containing 10% FBS and 2 mM glutamine. For microscopy studies, cells were grown and transfected with Turbofect (Thermo Fisher Scientific) on 12 mm round coverslips (Electron Microscopy Sciences) in 24-well plates (46). Imaging was performed with a Keyence microscope equipped with a 40x objective.

![Fig. 1](image-url)  
**Fig. 1.** Phosphorylated ACBD6 serine residues are not conserved in ACBD members. A: The amino acid alignment of the α-helices 3 and 4 (H3, H4) of the ACB domain of the ACBD members of the human, P. falciparum, and Arabidopsis thaliana family is shown. Some of the highly conserved residues are shown in red. A cartoon representation of the four α-helices of the ACB domain of hACBD6 is presented above the alignment. The phosphorylated residues Ser106 and Ser108, shown in blue, are indicated with a blue arrow. Substitution of Ser108 to a Glu residue in some forms is also shown in blue. The position of α-helices 3 and 4 of hACBD members is highlighted in gray and was obtained from the deposited structures in the Protein Data Bank (www.rcsb.org) for ACBD1 (DB1; PDB:2FJ9), ACBD2 (ECI2; PDB:4U18), ACBD4 (PDB:2WH5), ACBD5 (PDB:3FLV), ACBD6 (PDB:2COP), and ACBD7 (PDB:3EPY). B: NMR structure resolution of the ACB domain of hACBD6 (20 μM) was incubated with 100 μM C14-CoA for 20 min at 37°C. The bound complex was pulled down as described above, and the 50 μl eluate was incubated with 60 μg PINMT and 200 μM peptide for 2 h at 37°C in 1 ml buffer. A control reaction was performed with the eluate obtained from the pull down of C14-CoA incubated in the absence of ACBD6. Calculations and statistical analysis were performed with GraphPad Prism 7.
RESULTS

Ser^{106} and Ser^{108} of hACBD6 are not essential for ligand binding

Two serine residues located in the acyl-CoA binding domain of hACBD6, Ser^{106} and Ser^{108}, are phosphorylated in vivo (26, 39, 40). Ser^{106}, near the fourth α helix (αH4; S108 to K120), is present in some ACBD forms of humans and plants but not in the Plasmodium proteins, including the homologue PfACBD6 (Fig. 1A, B). Ser^{108}, at the beginning of αH4, is not conserved, but most ACBD proteins carry a glutamate (Glu) residue at this position. Glu can structurally mimic the presence of a phosphoserine, which might indicate that the activity of unphosphorylated ACBD6 could be different from phospho-ACBD6. The acyl chain of the acyl-CoA ligand is bound in the cavity formed by αH1, αH2, and αH4, and the CoA head group faces αH2 in the proximity of αH3 (Fig. 1C). Site-directed mutagenesis of conserved residues in the four α helices of ACBD6 identified two aromatic residues, Phe46 of αH1 and Tyr114 of αH4, whose substitution to alanine had the most influence in acyl-CoA binding (27). Based on the structure, these two residues protrude in the acyl-chain binding cavity, and Ser^{106} and Ser^{108} are located at the top of the pocket.

The substitution of the two serine residues to alanine generated ACBD6 forms with similar binding affinity compared with the native ACBD6 (Fig. 2A). However, a significant decrease in binding capacity was detected, which indicates that although neither Ser^{106} norSer^{108} are essential, they are implicated in binding. The substitution and truncation of the serine residues did not appear to affect the cellular location of GFP-tagged forms (Fig. 2B). Deleting an entire α helix, αH4, also had no apparent effect. Thus, the phosphorylation of Ser^{106} and Ser^{108}, as well as ligand binding, do not appear to be essential for the cytosolic and nuclear localization of hACBD6 in the cell.

Serine phosphorylation enhances ligand binding capacity of hACBD6

Phospho-Ser^{106} (P-Ser^{106}), P-Ser^{108}, and P-Ser^{106}/P-Ser^{108} ACBD6 forms were produced in E. coli and purified (see Materials and Methods). The binding activity of the phosphorylated forms was determined by competition experiments in the presence of radiolabeled [1^4]C_{18:1}-CoA (i.e., ligand) and unlabeled acyl-CoAs (i.e., competitors). Competition depends on the binding affinity of the protein for its ligand and competitor. As expected, the decreased binding of the radiolabeled acyl-CoA was a function of the relative concentration of ligand to competitor at a given protein concentration (Fig. 3). At ligand concentrations close to the observed K_d of ~10 μM (Fig. 2A), the binding of the ligand was sensitive to the presence of the competitor, and the addition of acyl-CoA of various chain lengths resulted in lower binding values (Fig. 3A). Competition was observed with assays performed with the unphosphorylated form hACBD6 and the double-mutant Ser^{106}Ser^{108}-Ala. However, with the double-phosphorylated P-Ser^{106} P-Ser^{108} form, binding of the ligand [1^4]C_{18:1}-CoA was higher in the presence of the competitor (Fig. 3A). This phenomenon was observed for all five acyl-CoAs tested, even when unlabeled C_{18:1}-CoA was added to [1^4]C_{18:1}-CoA. In addition, each of the single phosphorylated forms, P-Ser^{106} and P-Ser^{108}, also showed increased ligand binding in the presence of competitors (Fig. 3B). To render ligand binding competition of P-Ser^{106}/P-Ser^{108} dependent on the total concentration of acyl-CoAs present in the reaction, titration experiments were performed in the presence of a fixed concentration of the competitor, resulting in the strongest effect on binding, C_{24}-CoA (Fig. 3A), and with increasing concentrations of the ligand [1^4]C_{18:1}-CoA. Limited amounts of protein were also used, and under such conditions no binding could be detected at the lowest ligand concentrations in the absence of the competitor (Fig. 3C, D).
inset). However, the addition of the competitor increased binding of the ligand from the lowest concentration (0.5 μM) to concentrations closest to the $K_d$ (Fig. 3C). Competition did occur when the concentration of ligands reached a $K_d$ of 10 μM. To a lesser degree, this behavior was also observed with the unphosphorylated form under the limiting ligand concentration of 2.5 μM (Fig. 3D).

**Acyltransferase activity of NMT enzymes is stimulated by hACBD6**

The interaction of NMT2 with hACBD6 enhances the myristoyl-CoA esterase (26) and myristoyltransferase activity of the enzyme (Fig. 4). hACBD6 stimulation of myristoyl peptide formation occurs even under very limiting conditions (Fig. 4B) and in the presence of the competitor palmitoyl-CoA (C16-CoA) (Fig. 4C). hACBD6 increases both the rate and yield of production of the acyl peptide and protects binding of the substrate myristoyl-CoA (C14-CoA) to NMT from competition. To determine whether the role of hACBD6 was limited to the human myristoyltransferase enzyme, the activity of PNMT was determined (Fig. 5). As observed for NMT2, hACBD6 stimulates PNMT activity, and the increased formation of the myristoyl peptide in its presence was greater under substrate-limiting conditions (up to 40-fold) (Fig. 5C, D). The interaction was significantly weaker than with NMT2, but an hACBD6/PNMT complex appears to have formed in the cells (supplemental Fig. S1A). An hACBD6 form with truncated ankyrin-repeat motifs (ACBD6ΔC-ter) did not associate with either PNMT or NMT2. A mutant form affected in ligand binding and carrying the 4 substitution in the ACB domain (FFKY-to-A) (27) also fails to interact with PNMT. This mutant protein interacted with hNMT2, suggesting that complex formation with the *Plasmodium* enzyme might require the acyl-CoA-bound form of hACBD6.

**ACBD6 compensates for lack of NMT specificity for the C14 acyl chain**

The abundant C16-CoA is a competitor of the myristoyltransferase reaction. hNMT2 cannot prevent binding of an acyl chain two carbon atoms longer than C14-CoA, but the palmitoyl chain is not a substrate of the acyl-transferase reaction, and the enzyme cannot release it (4–6, 10). hACBD6 protects hNMT2 from C16-CoA (Fig. 4C) (26). Although myristoyl peptide formation by PNMT was also reduced in the presence of C16-CoA, the -2 carbon species lauroyl-CoA (C12-CoA) was far more inhibitory (Fig. 6A). Under a low C16-CoA concentration (10 μM), a 2-fold C12-CoA excess reduced the production of the myristoyl peptide by half, whereas a 12-fold excess of C16-CoA was required (Fig. 6B, C). Measurements of the esterase activity of the enzyme confirmed that the two competitors bind and were processed...
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by PfNMT (supplemental Fig. S1B). The presence of hACBD6 in the reaction protected the enzyme from the usage of an acyl chain other than C₁₄. hACBD6 successfully relieved the inhibition by C₁₆-CoA and further increased product formation (Fig. 6B). C₁₄-CoA processing in the presence of C₁₂-CoA was also increased by hACBD6 (Fig. 6C).

A new acyl peptide peak was detected in the presence of C₁₂-CoA, and its formation increased as the levels of the C₁₄ peptide peak dropped (Fig. 6A, Fig. 7A). The formation of the lauroyl peptide was differently affected by hACBD6 depending on the presence of the correct substrate C₁₄-CoA. In the absence of C₁₄-CoA, hACBD6 could not prevent PfNMT from processing C₁₂-CoA, and the reaction was nearly independent of its presence (Fig. 7B). In the presence of C₁₄-CoA, a 2-fold concentration excess of C₁₂-CoA resulted in a 2-fold reduction of myristoyl peptide, but in

Fig. 4. Activity of NMT2 in the presence of hACBD6. Measurements of the formation of the myristoyl peptide were performed in the absence (open symbols) or presence (closed symbols) of 6 µM hACBD6 from 0 to 10 min at 37°C. Reactions were performed in 600 µl, and at the indicated time points 100 µl were removed and transferred into a solution of 50% methanol/TCA 0.1%. The peptide and myristoyl peptide were extracted and quantified by HPLC on a C18 column (see Materials and Methods). hNMT2 was added at a concentration of 250 nM (circle) and 50 nM (triangle) in the presence of 5 µM C₁₄-CoA (A) and at 50 nM in the presence of 1 µM C₁₆-CoA (B). The competitor C₁₆-CoA (50 µM) was added to the reaction mixtures containing 5 µM C₁₄-CoA and 50 nM NMT2 in the absence or presence of 6 µM hACBD6, as indicated (C). Activity rate values are presented relative to the value obtained in the absence of hACBD6 and C₁₆-CoA. Error bars in all three plots represent the standard deviations of values obtained from three reactions. **P < 0.005 and ***P < 0.001.

Fig. 5. hACBD6 supports PfNMT activity under substrate-limiting concentrations. A: Measurements of the formation of the myristoyl peptide were performed in the absence (open symbols) or presence (closed symbols) of 6 µM hACBD6 from 0 to 16 min at 37°C. PfNMT was added at a concentration of 250 nM in the presence of 10 µM C₁₄-CoA in 500 µl. At the indicated time points, 80 µl were removed and analyzed. B: Measurements were performed with 300 nM PfNMT in the presence of 10 µM C₁₄-CoA and increasing concentrations of hACBD6 (0–6 µM). Reactions were performed in 120 µl at 37°C for 2 h. C: Measurements were performed with 250 nM PfNMT in the absence or presence of 6 µM hACBD6 with increasing concentrations of C₁₄-CoA (0–40 µM). Reactions were performed in 120 µl at 37°C for 2 h. The plot of the low values obtained in the absence of hACBD6 is presented at a different scale in the inset. D: Values obtained in the presence of hACBD6 at the different C₁₄-CoA concentrations shown in panel C are presented relative to the values obtained in its absence. Note the higher fold change in activity at a low C₁₄-CoA concentration (~10µM) compared with the saturating concentration (40 µM). Error bars in the four plots represent the standard deviations of values obtained from three reactions.

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the presence of hACBD6, a 4-fold excess of C_{12}-CoA was required to achieve a 50% reduction (Fig. 7C). In the presence of hABCD6, the increased preference of PfNMT for the C_{14} chain over the C_{12} chain was also confirmed by the decrease in C_{12} processing when the enzyme had the choice of acyl chains (Fig. 7C). Although when challenged with nonphysiological conditions (high concentration of C_{12}-CoA in the absence of C_{14}-CoA), hACBD6 had little to no detectable effect, it could slow the usage of the C_{12} chain by the PfNMT enzyme in a mixture containing C_{14}-CoA. These data established that ACBD6 can provide substrate specificity for the myristoyltransferase reaction and prevent the enzyme from processing the “wrong” but more abundant acyl-chain species under conditions that are likely encountered in vivo.

Plasmodium ankyrin-containing ACBD6 protein stimulates PfNMT activity

A search of PlasmoDB, a genome database for *Plasmodium* (plasmodb.org), retrieved five genes encoding putative acyl-CoA binding proteins (Fig. 8A). Three genes produced the shortest of the forms, homologous to the human ACBD1 (DBI) protein. The other two genes carry different motifs at the extended carboxy-terminal end. PfACBP1 carries two conserved motifs implicated in export through the parasite membrane and localization in the erythrocyte: *Plasmodium* helical interspersed subtelomeric and *Plasmodium* ring-infected erythrocyte antigen N-terminal (47). On the basis of the presence of two ankyrin-repeat motifs downstream of the ACB domain, the unnamed product of the fifth gene was referred to as PfACBD6. A transmembrane-spanning segment motif was predicted at the N-terminal end and had to be removed for successful expression in *E. coli* (see Materials and Methods). A 60-residue-long motif, rich in lysine (12) and asparagine (26), is inserted between the second and third α-helix of the ACB domain (Fig. 8A). These poly-KN tracks are often found in *Plasmodium* proteins and reflect the AT-rich genome of the parasite (48, 49).

PfACBD6 is a functional protein and binds acyl-CoA (Fig. 8B, C). In addition, it stimulates the NMT activity of PfNMT and counters inhibition by C_{16}-CoA (Fig. 8D, supplemental Fig. 1B). Thus, the ankyrin-repeat containing acyl-CoA binding proteins of humans and *Plasmodium* regulate the NMT reaction.

Myristoyl-CoA bound to hACBD6 is a substrate of NMT

The ability of different members of the human acyl-CoA binding protein family to stimulate the myristoyltransferase reaction was determined. Of the five ACBD forms tested, hACBD6 was the most efficient in enhancing the production of the myristoyl peptide by PfNMT (Fig. 9A, B). ACBD1, ACBD3, ACBD4, and ACBD5 were able to stimulate PfNMT but to levels similar or lower than that of the ACBD6 mutant altered in acyl-CoA binding (FFKY-A form). The preference for the ACBD protein that interacts with the enzyme confirmed that complex formation improves
the ability of ACBD6 to enhance the myristoyltransferase reaction. The role of the binding activity of hACBD6 in the mechanism leading to increased NMT activity was investigated. The substrate C14-CoA was incubated with hACBD6, and the C14-CoA-bound hACBD6 complex was isolated from the unbound C14-CoA left in the reaction. The purified C14-CoA/ACBD6 was then fed to PfNMT in a buffer mixture lacking C14-CoA. The formation of the myristoyl peptide under such reaction conditions established that the C14-CoA bound to hACBD6 was available to the NMT enzyme, displace those unfavorable cellular conditions strongly suggests that these two acyl modifications are not equivalent. In fact, some proteins are palmitoylated and myristoylated, on the ability of ACBD6 to enhance the myristoyltransferase reaction to proceed efficiently, even at concentrations below the binding affinity of the enzyme. This would account for the greater stimulatory effect of hACBD6 observed under substrate-limiting concentrations compared with saturating conditions (Fig. 5D).

**Phosphorylation-dependent stimulation of myristoylation**

To further investigate the ability of hACBD6 to increase the availability of the substrate to the NMT enzyme, displacement experiments of ACBD6-bound ligand by acyl-CoA competitors were performed. The complex hACBD6/[14C]C18:1-CoA was isolated, and the amount of [14C]C18:1-CoA still bound to the protein following incubation with increasing concentrations of the competitor C24:CoA was quantified (Fig. 10A). Compared with the hACBD6 form, phosphorylation of Ser106 and Ser108 residues protected the bound ligand, and a higher concentration of competitor was required to achieve 50% displacement. This observation was confirmed when increasing amounts of the ACBD6-ligand complex were exposed to a fixed concentration of competitor (Fig. 10B). Although the removal of the two serine residues did not influence the dissociation of the ligand, their phosphorylation resulted in significant resistance of ligand bound to the P-Ser106/P-Ser108 ACBD6 form (Fig. 10C). The phosphorylated forms also further increased the activity of the PfNMT enzyme compared with ACBD6 (Fig. 11). Substitution of the two serines to glutamate residues (Ser106/Ser108Glu) did not appear to produce a form that was as active as the phosphorylated serine forms.

Thus, the improved binding capacity of the phosphorylated hACBD6 form enhanced its ability to hold the bound ligand in a mixture of competing acyl-CoAs under similar conditions encountered in vivo. This property of hACBD6 gives C14-CoA a competitive advantage in the presence of more abundant acyl-CoAs and provides the NMT enzyme with the correct substrate in the unfavorable cellular environment.

**DISCUSSION**

The NMT enzymes lack selectivity for myristoyl-CoA and display a binding affinity (~600 nM) that is significantly higher than the cellular concentration of this acyl-CoA, ranging from 5 to 200 nM. These findings imply that mechanisms exist to support the NMT reactions in the cell (2, 3, 50, 51). The use of a relatively low abundant acyl chain under those unfavorable cellular conditions strongly suggests that the covalent linkage of a myristate to an N-terminal glycine residue confers properties to the acylated proteins that the more abundant palmitate chain cannot provide. In fact, some proteins are palmitoylated and myristoylated, on N-terminal cysteine and glycine, respectively, which indicate that these two acyl modifications are not equivalent (52). In vitro, acyl-CoA species that are either two carbon atoms longer or shorter (C12–C16) bind with similar affinity (52). In vitro, acyl-CoA species that are either two carbon atoms longer or shorter (C12–C16) bind with similar affinity (52).
The transfer of acyl chains from the enzyme to the protein other than C14 is either blocked or very slow, which favors the addition of a myristate. However, occupation of the binding site by these acyl-CoA species would lead to the progressive inactivation of the enzyme because they could not be released once bound. The acyl-chain species bound to the enzyme also affect the interaction of NMT with the target proteins. The binding of acyl chains other than C14 can significantly increase the $K_m$ binding affinity of NMT for the protein substrate (10). It has been established that, in vivo, the enzyme can preferentially transfer species other than C14:0. In the retina, the unsaturated C14:1 chain is the major species detected in myristoylated proteins. In addition, the polyunsaturated C14:2 chain is the major species detected in myristoylated proteins. In addition, the polyunsaturated C14:2 chain is the major species detected in myristoylated proteins.

**Fig. 9.** C14-CoA bound to hACBD6 is a substrate for the PfNMT enzyme. A: Measurements of the formation of the myristoyl peptide were performed in the absence or presence of 20 µM human ACBD1, ACBD3, ACBD4, ACBD5, and ACBD6 and the ACBD6 mutant FKYK-to-A. FKYK-to-A carries a substitution of the residues Phe, Lys, Lys, and Tyr to alanine and displayed low binding capacity (27). The chromatogram traces obtained at 274 nm at the time of the elution of the C14-peptide (blue peak; ~34.5 min) from the C18 column are shown. Reactions were performed in 120 µl at 37°C for 2 h with 700 nM PfNMT in the presence of 10 µM C14-CoA. B: The amount of the myristoyl peptide obtained in the experiments shown in panel A was quantified. Error bars represent the standard deviations of at least three reactions. C: hACBD6 (20 µM) was incubated with 100 µM C14-CoA for 20 min at 37°C and then purified to remove the unbound C14-CoA. The hACBD6/C14-CoA complex was incubated with 1.25 µM PfNMT and 200 µM peptide in a 1 ml reaction for 2 h at 37°C (bottom chromatogram). A control reaction was performed with the pull-down sample obtained from C14-CoA incubated in the absence of hACBD6 (top). The chromatogram traces obtained at 274 nm are shown, and the C14-peptide is shown as a blue peak.

**Fig. 10.** Phosphorylation of hACBD6 prevents ligand displacement by competitor. Displacement of [14C] C18:1-CoA bound to the proteins was performed as described in the Materials and Methods section. A: hACBD6, P-Ser106, P-Ser106, and P-Ser106/P-Ser108 proteins (1 µM) were incubated with 5 µM [14C]C18:1-CoA, and the ligand-protein complexes were isolated and incubated in the presence of increasing concentrations of C24-CoA (0–10 µM). Amounts of [14C]C18:1-CoA still bound to the proteins in the presence of the competitor are presented in percentages relative to values obtained in its absence. The calculated concentration of C24-CoA required to achieve 50% displacement of prebound [14C]C18:1-CoA to hACBD6 and P-Ser106/P-Ser108 is shown in the inset. B: Displacement assays were performed at a fixed concentration of C24-CoA (2.5 µM) with complexes isolated from the incubation of 5 µM [14C]C18:1-CoA with increasing concentrations of hACBD6 (0.5–4 µM). C: Displacement values obtained with 2 and 4 µM hACBD6, mutant Ser106/108-A, and phosphorylated P-Ser106/P-Ser108 are presented in percentages relative to values obtained in the absence of C24-CoA. Error bars in the three plots represent the standard deviations of values obtained from four reactions. ****P = 0.0001.
which carries the substitution of the residues Ser106 and Ser108 to Glu also suggests that motifs that are located downstream from man and Plasmodium ACBD6 proteins stimulated PfNMT with the other ACBD members. The fact that both the hu-
account for its greater effect on myristoylation compared
interact directly with NMT, and this unique property could
activity. ACBD6 appears to be the only member to also
the conserved ACB domain are unique to the ankyrin-
repeat motif-containing member of the ACBD family. Phosphorylation of the two serine residues of the ACB domain of ACBD6 has a significant positive effect on binding capacity and myristoyl peptide formation. Other ACBD proteins are phosphorylated in vivo, but although ACBD3 and ACBD5 have six and nine phosphorylated residues, respectively, none are located in the ACB domain (39, 40). The only two ACBD6 residues known to be phosphorylated are near and part of the fourth α-helix of the ACB domain. These serine residues are not conserved in the ACBD family, and Ser108 appears to be substituted by the phospho-
mimic Glu residue in other members. The effect of the phosphorylation of these serine residues on the properties of ACBD6 suggests that the activity of this member of the family is regulated in vivo. Cell-cycle global profiling of human phosphoproteins detected a specific upregulation of Ser106 and Ser108 phosphorylation during the M phase (39, 40). This finding strongly suggests that the function of the phosphorylated form of ACBD6 is increased during mitosis. Given the significant difference in NMT activity in the presence of phospho-ACBD6 compared with the unphosphorylated form, an increased need for protein myristoylation during that phase of the cell cycle could be possible.

Mystroiylation of proteins occurs during translation after the removal of the initiator methionine on the nascent polypeptide. However, during apoptotic events, NMT en-
zymes can also modify proteolytically cleaved proteins after the exposure of an N-terminal glycine and a cryptic myris-
toylation motif (50, 51, 53). ACBD6 phosphorylation could also be increased during apoptosis to support the addi-
tional need for NMT activity. A variety of pathogens rely on protein acylation processes. The development of P. falci-
parum in erythroid cells is blocked by several chemicals that inhibit the action of the PINMT enzyme (11, 12, 54, 55). The host and parasite ACBD6 regulate PINMT activity, and the disruption of this process may provide an opportunity to block the parasite growth. In conclusion, our results pro-
vide evidence that ACBD6 proteins have an important role in the myristoylation of proteins in eukaryotic cells and support the substrate availability and selectivity of the NMT enzymes.

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