Identification of a palmitoyl acyltransferase required for protein sorting to the flagellar membrane

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Summary
Protein palmitoylation has diverse effects in regulating protein membrane affinity, localization, binding partner interactions, turnover and function. Here, we show that palmitoylation also contributes to the sorting of proteins to the eukaryotic flagellum. African trypanosomes are protozoan pathogens that express a family of unique Ca\(^{2+}\)-binding proteins, the calflagins, which undergo N-terminal myristoylation and palmitoylation. The localization of calflagins depends on their acylation status. Myristoylation alone is sufficient for membrane association, but, in the absence of palmitoylation, the calflagins localize to the pellicular (cell body) membrane. Palmitoylation, which is mediated by a specific palmitoyl acyltransferase, is then required for subsequent trafficking of calflagin to the flagellar membrane. Coincident with the redistribution of calflagin from the pellicular to the flagellar membrane is their association with lipid rafts, which are highly enriched in the flagellar membrane. Screening of candidate palmitoyl acyltransferases identified a single enzyme, TbpAT7, that is necessary for calflagin palmitoylation and flagellar membrane targeting. Our results implicate protein palmitoylation in flagellar trafficking and demonstrate the conservation and specificity of palmitoyl acyltransferase activity by DHHC-CRD proteins across kingdoms.

Key words: Flagellum, Palmitoylation, Membrane microdomains, Acylation, Trypanosoma

Introduction
Protein palmitoylation is the thioester linkage of long chain fatty acids, most commonly palmitic acid, to cysteine residues of protein substrates. Palmitoylation was first described nearly four decades ago (Braun and Radin, 1969), but only recently have the discoveries of the first protein palmitoyl acyltransferases (PATs) in yeast (Lobo et al., 2002; Roth et al., 2002) led to rapid progress in understanding the nature and functions of this important protein modification (Linder and Deschenes, 2007). PAT activity is found in proteins with a signature aspartate-histidine-histidine-cysteine cysteine-rich domain, the DHHC-CRD, which mediates enzymatic activity through the formation of a palmitoyl-enzyme intermediate (Mitchell et al., 2006). Palmitoylation is remarkable among protein lipid modifications in that the modification often takes place on proteins already localized to a membrane. In addition to its ability to strengthen membrane association, palmitoylation can regulate broad aspects of protein localization and function. In particular, protein sorting between different membrane domains can be modulated by palmitoylation (Greaves and Chamberlain, 2007).

The kinetoplastid parasite Trypanosoma brucei is the etiologic agent of African sleeping sickness, a blood-borne infection that is fatal if untreated. Aside from the tremendous global health burden it causes, T. brucei is an excellent model organism for studying the eukaryotic flagellum/cilium (Kohl and Bastin, 2005). T. brucei cells are amenable to genetic manipulation and possess a single polarized flagellum that drives cellular motility and compartmentalizes signaling proteins. We and others have shown the trypanosome pellicular (cell body) membrane and flagellar membrane to have distinct lipid compositions (Kaneshiro, 1990; Tetley, 1986). Specifically, we have found the flagellar membrane to be enriched in lipid rafts: regions of increased membrane order that may serve as signaling or recruitment platforms for associated molecules (Tyler et al., 2009). Association with lipid rafts in the flagellar membrane might therefore provide a general mechanism by which specific proteins traffic to the flagellar/ciliary membrane.

A family of proteins that might be expected to use such a mechanism is the calflagin family of Ca\(^{2+}\)-binding proteins. These proteins localize to the flagellar membrane but not to the pellicular membrane. They were discovered as the predominant molecules for which a dedicated palmitoyl acyltransferase has been identified. Single polarized flagellum/cilium

In this study, we sought to determine the impact of palmitoylation on calflagin biology, and to identify the molecular components involved in this modification. Our results demonstrate that palmitoylation of calflagins by a single DHHC-CRD enzyme, TbpAT7, promotes association with lipid raft microdomains and redirects the calflagins from the pellicular to the flagellar membrane. These findings add flagellar trafficking to the list of functions ascribed to protein palmitoylation and the calflagins to the list of proteins for which a dedicated palmitoyl acyltransferase has been identified.
Results
Detection of palmitoylation by acyl-biotin exchange chemistry
To test for protein palmitoylation, T. brucei procyclic stage extracts were subjected to an acyl-biotin exchange reaction. In this assay, cell lysates are treated with hydroxylamine to cleave protein-palmitoyl linkages and generate reactive thiol groups, which are then labeled with N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). Biotinylated proteins are then purified by streptavidin-agarose chromatography. Non-specific labeling of free thiols is blocked by pre-incubation of the extract with N-ethylmaleimide before hydroxylamine treatment, and residual background labeling and purification is monitored by treating samples in parallel with Tris-HCl in place of hydroxylamine. This technique was recently used in yeast to establish the first palmitoylproteome (Roth et al., 2006).

Application of this approach to T. brucei demonstrated hydroxylamine-dependent purification of the positive control CAP5.5 protein, which was previously shown to be palmitoylated by 3H-palmitate radiolabeling (Hertz-Fowler et al., 2001), but none of the control proteins from the cytoskeleton (β-tubulin, WCB (Woods et al., 1992)), endoplasmic reticulum (BiP (Bangs et al., 1993)), plasma membrane (procyclin) or cytoplasm (Hsp70) (Fig. 1). Like CAP5.5, all three calflagin proteins were biotinylated and purified by streptavidin under experimental but not control conditions. The calflagin-specific antiserum also detected a cross-reactive protein of 38 kDa in acyl-biotin exchange samples, but this protein was not purified by streptavidin under any conditions. The hydroxylamine-dependent purification of calflagins Tb44, Tb17 and Tb24 by acyl-biotin exchange and streptavidin chromatography indicates that these proteins are in fact palmitoylated in procyclic stage T. brucei cells.

Distinct roles of myristoylation and palmitoylation in calflagin trafficking
To identify the specific residues modified by acylation and to investigate the consequences of disrupted acylation on calflagin trafficking, we performed site-directed mutagenesis of individual calflagin amino acids. The N-terminus has a high degree of sequence identity among the calflagins that is shared to a lesser extent with the T. cruzi flagellar Ca²⁺-binding protein (FcaBP) (Fig. 2A), which is also dually acylated and exhibits acylation-dependent flagellar membrane targeting (Godsel and Engman, 1999). We engineered constructs for expression of wild-type and mutant calflagin Tb44 in T. brucei. A C-terminal myc tag was included to distinguish the transprotein from endogenous calflagins. The myristoylation state of the tagged protein was monitored by 3H-myristate labeling and the palmitoylation state by acetyl-biotin exchange. Substitution of the glycine residue in the 2nd position with alanine prevented both acyl modifications, while substitution of the cysteine residue in the 3rd position with alanine abrogated palmitoylation but left myristoylation intact, as expected (Fig. 2B).

Immunofluorescence microscopy revealed the wild-type protein to be flagellar (Fig. 3), identical to what is seen with calflagin antiserum staining of uninfected cells. The G2A mutant, which is neither myristoylated nor palmitoylated, was found diffusely throughout the cytoplasm. The C3A mutant, which is myristoylated but not palmitoylated, localized to the pellicular membrane but not the flagellar membrane. Myristoylation, therefore, which occurs cotranslationally in the endoplasmic reticulum, promotes plasma membrane association for the calflagins. Palmitoylation, which occurs post-translationally at an unknown site and requires prior myristoylation, then provides a secondary signal necessary for calflagin sorting to the flagellar membrane.

Because of the different membrane localizations of dually-acylated and myristoylated-only calflagin, and because of the enrichment of lipid rafts in the flagellar membrane, we next tested for the dependence of calflagin lipid raft association on acylation. Two independent assays were used to test for raft association. First, parasites were treated with 1% Triton X-100 and fractionated into supernatant and pellet fractions at 4°C and 37°C. Lipid raft-associated proteins exhibit a temperature dependence in this assay, fractionating in the pellet and supernatant at 4°C but in the supernatant only at 37°C. Second, parasite lysates were analyzed by discontinuous sucrose density gradient centrifugation. Lipid raft-associated proteins are more buoyant, and are found in the low density interface fraction (fraction 2 in this assay). By both analyses, wild-type calflagin, but neither G2A nor C3A mutant calflagins, exhibited properties that indicated lipid raft association (Fig. 4). Myristoylation, therefore, although sufficient for plasma membrane localization, is insufficient for partitioning into lipid rafts. Only the mature protein with both acyl modifications is found in lipid rafts.

Screening of T. brucei DHHC-CRD containing genes uncovers a single enzyme necessary for calflagin palmitoylation in vivo
Given the importance of palmitoylation in conferring flagellar localization to the calflagins, we set out to identify the enzyme catalyzing this reaction. The availability of the T. brucei genome sequence (www.genedb.org) allowed us to identify all of the genes containing the DHHC-CRD characteristic of palmitoyl acyltransferases in yeast and mammalian cells. Twelve such genes are found in the T. brucei genome (Table 1). These genes are predicted to encode proteins ranging in size between 30 and 90 kDa, all with multiple transmembrane domains and conserved in other kinetoplastid parasites.
Inhibition of candidate PATs was performed by generating individual parasite strains inducible for RNA interference of each protein. Target sequences were selected using RNAit software, which scans the open reading frame to identify regions with minimal similarity to off-target genes (Redmond et al., 2003). The target regions were amplified from T. brucei genomic DNA and subcloned into the pZJM vector, which provides flanking opposing T7 promoters and sequences for integration into a rDNA spacer region (Wang et al., 2000). Constructs for each candidate PAT were then transfected into a T. brucei procyclic line expressing both bacterioophoge T7 RNA polymerase and tet repressor, enabling drug-inducible production of dsRNA (Wirtz et al., 1999). Inducible RNAi cell lines were generated for each of the twelve candidate PATs, and semi-quantitative RT-PCR verified inhibition of each candidate PAT (data not shown). None of the targeted genes proved to be essential, as parasites continued to divide in culture in all cases. The gross morphology and flagellar motility furthermore appeared unaffected in each mutant. These findings suggest either that no single DHHC-CRD protein is required for parasite viability, proliferation or motility in procyclic stage trypanosomes, or that residual amounts of protein remaining after RNA interference are sufficient to prevent a discernible phenotype.

We screened these twelve mutants for calflagin localization by immunofluorescence microscopy. Eleven of the 12 mutants showed clear flagellar localization for calflagin, indistinguishable from wild-type cells. However, for a single RNAi target, TbPAT7, drug induction resulted in mislocalization of calflagin to the pellicular membrane (Fig. 5), identical to our calflagin C3A mutant (Fig. 3). The effect was highly specific, as ~95% of TbPAT7 RNAi cells mislocalized calflagin after 48 hours, whereas none of the other 11 mutants lines yielded >5% of cells with mislocalized calflagin (Fig. 6A). Analysis of the kinetics of calflagin mislocalization showed a t1/2 of ~24 hours (Fig. 6B). Mislocalization of calflagin by TbPAT7 depletion was reversible, as removal of tetracycline at 96 hours allowed calflagin to localize properly to the flagellar membrane.

As our screen relied on calflagin mislocalization as a surrogate for calflagin palmitoylation, it remained possible that, rather than affecting calflagin palmitoylation, TbPAT7 RNAi interfered with another aspect of protein trafficking. To test for a direct effect of
Our results implicate palmitoylation in another cellular function: the trafficking of proteins to the eukaryotic flagellar membrane. The pellicular membrane localization of calflagins upon inhibition of IFT, discovered in *Chlamydomonas reinhardtii* (Kozinski et al., 1993) but seemingly functional in nearly all kinetoplastid parasites, this signal can be superseded by additional cues.

Recently, great strides have been made in understanding the molecular basis of flagellar protein trafficking. Unlike proteins specific for organelles such as the endoplasmic reticulum and lysosome, there does not appear to be a single signal shared by a majority of flagellar proteins. Rather, a process called intraflagellar transport (IFT), discovered in *Chlamydomonas reinhardtii* (Kozinski et al., 1993) but seemingly functional in nearly all ciliated and flagellated cells, remains to be seen. In the related parasite *Leishmania major*, SMP-1 also localizes to the flagellar membrane in a manner that requires N-terminal myristoylation and palmitoylation (Tull et al., 2004). Another *L. major* protein, LmPPEF, also contains an N-terminal peptide whose dual acylation is sufficient for flagellar membrane localization of a fusion protein (Mills et al., 2007). However, the full-length protein localizes to intracellular membranes by virtue of additional protein-protein interactions. Similarly, the *T. brucei* protein CAP5.5 (Hertz-Fowler et al., 2001) and the *T. cruzi* protein TePI-PLC (Furuya et al., 2000; Okura et al., 2005) are dually acylated, yet localize to the pellicular membrane. For CAP5.5, this is believed to be mediated by a biochemical association with subpellicular microtubules. Therefore, although dual acylation in itself appears to elicit flagellar membrane association in kinetoplastid parasites, this signal can be superseded by additional cues.
Palmitoylation-dependent flagellar membrane targeting

reinhardtii, at least two transmembrane proteins have been shown to be transported by IFT (Huang et al., 2007; Qin et al., 2005). However, multiple groups have observed the presence of a ‘flagellar sleeve’, an extension of nascent flagellar membrane arising from the basal body, even in the absence of axoneme assembly (Absalon et al., 2008; Davidge et al., 2006). This has led to the suggestion that membrane delivery to the flagellum may be independent of intraflagellar transport.

The simultaneous loss of calflagin lipid raft association and flagellar localization upon inhibition of palmitoylation is suggestive, but not proof, of lipid raft recruitment serving as a mechanism for palmitoylation-dependent calflagin trafficking. Palmitoylation of mammalian H-Ras on Cys181 similarly promotes an association with lipid raft microdomains (Roy et al., 2005). It is important to note that our results, which indicate a crucial role for palmitoylation in sorting to the flagellar membrane, do not exclude a role for palmitoylation in IFT. Palmitoylation, rather than simply redistributing protein along the membrane, might confer association of calflagins onto IFT barges. IFT barges appear to have a tight association with membrane components, as is evident in transmission electron micrographs (Kozminski et al., 1995). However, the detergent treatments involved in purification of IFT barges have precluded identification of their membrane components.

Recent evidence from our laboratory suggests that membrane associated with IFT barges might be enriched in lipid raft components (Tyler et al., 2009). Detergent extraction of T. brucei cells revealed distinct patches of detergent-resistant membrane along the flagellum. Intriguingly, these patches were regularly spaced along the axoneme, but not found anywhere along the adjacent paraflagellar rod. The size and distribution of these patches were consistent with their being the membrane components of IFT barges. These findings suggest that palmitoylation of calflagins might confer flagellar membrane targeting by facilitating their loading onto IFT barges.

A number of other possible pathways, however, are consistent with the notion that palmitoylation promotes calflagin targeting to the flagellar membrane. Human and yeast PATs are distributed at multiple sites throughout the cell, including the plasma membrane, Golgi apparatus and endoplasmic reticulum (Ohno et al., 2006). Calflagin palmitoylation might occur directly at the pellicular membrane, upon which calflagin would actively sort out of this domain into the flagellum, where the distinct lipid environment might be more permissive to dually acylated proteins. Alternatively, myristoylated proteins might, at a low rate, diffuse from the pellicular membrane into the flagellum independently of this modification. Palmitoylation might then occur in the flagellar

Fig. 5. Calflagins localize to the pellicular membrane upon inhibition of TbPAT7. RNAi against each candidate PAT was induced by the addition of tetracycline to cultured procyclic cells. Calflagin immunofluorescence microscopy was obtained 48 hours post-induction. (A) Representative cells of each mutant are shown, with the number in the top left corner of each box indicating the target TbPAT. Inhibition of TbPATs 1-6 and 8-12 had no effect on the flagellar localization of calflagin, whereas TbPAT7 RNAi resulted in calflagin localization to the pellicular membrane. Bar, 5 μm. (B) The TbPAT7 RNAi cell is shown at higher magnification together with DIC microscopy. Close inspection reveals that fluorescence is restricted to the pelliculum, with no fluorescence in the flagellum (outlined, lower right).
membrane itself, where it would promote anchoring or retention of protein in this organelle. Finally, the pellicular membrane localization of non-palmitoylated calflagin might represent not an intermediate step of trafficking, but rather an alternative destination realized only upon inhibition of normal protein maturation. In this case, palmitoylation could occur at an earlier stage of the trafficking pathway, such as the Golgi apparatus or endoplasmic reticulum. Distinguishing among these possible pathways will be a focus of future investigation.

Finally, calflagin palmitoylation is remarkable for its degree of specificity. In yeast, palmitoylation of many proteins can be mediated by multiple PATs, such that prevention of individual protein palmitoylation requires inhibition of multiple PATs (Roth et al., 2006). Calflagins, by contrast, appear to be modified by a single PAT, as indicated by the inability of other enzymes to compensate upon TbPAT7 knockdown. Elucidation of the specific pathway, such as the Golgi apparatus or endoplasmic reticulum. Related to this issue is whether pellicular membrane calflagin, which is mislocalized by TbPAT7 RNAi, might be able to be palmitoylated and re-localize to the flagellum upon tetracycline washout, or whether it would remain inaccessible to PAT7. The in vivo specificity of calflagin palmitoylation by TbPAT7 makes this an optimal system for further investigations of the enzymology and molecular basis of PAT-substrate recognition and activity.

Materials and Methods
Parasites and antibodies
All T. brucei strains described in this study are derivatives of the procyclic 29-13 strain, a 427 strain engineered to co-express bacteriophage T7 RNA polymerase and the tetr repressor (Wirtz et al., 1999). Antibodies were obtained from the following sources: anti-procyclin (IF 1:1000) from Cedarlane Laboratories (Ontario, Canada); rabbit anti-myc (IF 1:100) from Rockland Immunochemicals (Gilbertsville, PA); mouse monoclonal anti-myc 9E10 (Woods et al., 1989) and anti-tubulin E7 ascites (IB 1:1000) from the Developmental Studies Hybridoma Bank (Iowa City, Iowa); anti-CAP5.5 (IB 1:500), anti-ROD-1 (IF neat) and anti-WCB (IB 1:1000) from the Developmental Studies Hybridoma Bank (Iowa City, Iowa); anti-myc (IF 1:100) from Cedarlane Laboratories (Ontario, Canada); rabbit anti-myc (IF 1:1000) from Cedarlane Laboratories (Ontario, Canada); rabbit anti-procyclin (IB 1:1000) from Cedarlane Laboratories (Ontario, Canada); rabbit anti-myc (IF 1:100) from Rockland Immunochemicals (Gilbertsville, PA); mouse monoclonal anti-myc 9E10 supernatant (IB 1:100) and anti-dsRNA (IB 1:1000) from the Developmental Studies Hybridoma Bank (Iowa City, Iowa); anti-CAP5.5 (IB 1:500), anti-ROD-1 (IF neat) and anti-WCB (IB 1:1000) kindly provided by Keith Gull (Oxford University, Oxford, UK); anti-Hsp70 (IB 1:5000) generated in our laboratory and described previously (Olsson et al., 1994); and anti-BiP (IB 1:1000) kindly provided by James D. Bangs (University of Wisconsin, Madison, WI). Calflagin-specific mouse antiserum (IB 1:1000, IF 1:1000) was generated in our laboratory (Tyler et al., 2008).

Plasmids and transgenic parasite engineering
All RNAi cell lines were created by cloning of the target sequence into the pZJM vector (Wang et al., 2000). Genes predicted to encode proteins with the DHHC-CRD motif (Pfam ID: PF01529) were identified in the T. brucei genome database (www.genedb.org). A 400-600 bp region of each gene was selected on the basis of its lack of similarity to other genes using the RNAi software (Redmond et al., 2003). Oligonucleotides (Integrated DNA Technologies, Coralville, IA) were designed to amplify this target region and provide flanking HindIII and XhoI sites. Template DNA was purified as described previously (Medina-Acosta and Cross, 1993) and PCR was conducted with Taq polymerase (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The amplicon was gel purified (Quagen, Gaithersberg, MD) and ligated into pCR-2.1-TOPO (Invitrogen). The insert was digested out of this plasmid and ligated into pZJM for the generation of the RNAi construct.

To express epitope-tagged parasite proteins, primers were designed to amplify the desired ORF and provide flanking 5’ HindIII and 3’ XhoI sites. Amplicons were gel purified and subcloned into pCR-BluntII-TOPO before digestion and ligation into pLEW79-Myc, which was generated by the removal of the NOG1 insert from the
plasmid pLEW79-NOG1-Myc (the kind gift of Marilyn Parsons) with HindIII and XbaI. Site-directed mutagenesis of the N terminus was performed with 40-50 bp, gel-purified primers (Integrated DNA Technologies, Coralville, IA) containing the desired mutation and the QuikChange kit (Strategene, La Jolla, CA).

To generate transgenic parasites, pZJM and pLEW79-Myc plasmids were linearized by digestion with NotI and 100-1000 μg of DNA in 100 μl of sterile water were mixed with 2.25×1010 parasites in 450 μl of electroporation medium (120 mM KCl, 0.15 mM CaCl2, 9.2 mM KH2PO4, 25 mM HEPES, 2 mM EDTA, 4.75 mM MgCl2, 69 mM sucrose, pH 7.6). Samples were transferred to a 0.4 cm gap cuvette, electroporated for 10-20 seconds, washed for 2 minutes with ultrapure water and mounted with peptide inhibitor for 1 hour at 30°C to starve cells of exogenous lipid. Radiolabeled [3H]-myristic acid was also tested by flotation of lystate from 2×106 parasites in labeling medium (SDM-79 containing 7.5 mg/ml hemin and 0.5 mg/ml Gammabind G Sepharose beads and 1 ml Gammabind G Sepharose beads and 1 ml of lysis buffer containing 0.2% Triton X-100, protease inhibitors). Samples were incubated at room temperature for 1 hour with end-over-end rotation. The reactions were stopped and the labeling reagent was removed by three sequential rounds of chloroform-methanol precipitation. Resuspension following the first two precipitations was performed as for NEM removal. Following the final precipitation, pelleted samples were resuspended in 600 μl of solubilization buffer (50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 4% SDS) and 30 μl was collected to represent the input fraction. To the remaining 120 μl was added 2.28 ml of lysis buffer to dilute out SDS that would interfere with the streptavidin-biotin interaction. Samples were then added batchwise in 1.2 ml aliquots to tubes containing 60 μl streptavidin-agarose beads that had been pre-washed with pulldown wash buffer (lysis buffer containing 0.1% SDS, 0.1% 100 μg/ml NEM). Input and elution fractions were mixed with 2× Laemmli sample buffer, boiled for 5 minutes, and analyzed by immunoblotting.

References


