Molecular Determinants of Ciliary Membrane Localization of
Trypanosoma cruzi Flagellar Calcium-binding Protein*

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The flagellar calcium-binding protein (FCaBP) of Trypanosoma cruzi is localized to the flagellar membrane in all life cycle stages of the parasite. Myristoylation and palmitoylation of the N terminus of FCaBP are necessary for flagellar membrane targeting. Not all dually acylated proteins in T. cruzi are flagellar, however. Other determinants of FCaBP therefore likely contribute to flagellar specificity. We generated T. cruzi transfectants expressing the N-terminal 24 or 12 amino acids of FCaBP fused to GFP. Analysis of these mutants revealed that although amino acids 1–12 are sufficient for dual acylation and membrane binding, amino acids 13–24 are required for flagellar specificity and lipid raft association. Finally, FCaBP was expressed in the protozoan Leishmania amazonensis, which lacks FCaBP. The flagellar localization and membrane association of FCaBP in L. amazonensis suggest that the mechanisms for flagellar targeting, including a specific palmitoyl acyltransferase, are conserved in this organism.

Eukaryotic cilia and flagella are membranous organelles that project from the cell surface and have multiple functions, including powering cell motility, the movement of extracellular fluid across the cell surface, and cell signaling (1). A number of human disorders, collectively known as the ciliopathies, have been linked to mutations in genes involved in ciliary biogenesis and function (2). Because cilia lack the machinery for protein synthesis, ciliary proteins must be synthesized outside of this organelle and then imported (3). Kinetoplastid protozoa, besides being of interest for their global health burden, serve as excellent model organisms for the elucidation of mechanisms underlying ciliary trafficking.

The surface of trypanosomes contains three distinct membrane domains: the cell body (pellicular) membrane, the flagellar membrane, and the flagellar pocket membrane (4–6). The flagellar membrane is structurally and functionally distinct from the other surface membranes (3). There is an asymmetric distribution of proteins across these membrane domains, and several proteins have been identified in kinetoplastids that are heavily enriched or restricted to the flagellar membrane (7–13). Likewise, the kinetoplast flagellar membrane has a unique lipid composition from the rest of the cell surface. The flagellar membrane is enriched in sterols (14–16), glycolipids (17), and sphingolipids (18, 19), all which are components of canonical lipid raft microdomains. Due to their composition, lipid rafts are relatively resistant to solubilization with cold non-ionic detergents such as Triton X-100 and hence are often experimentally defined as detergent-resistant membranes (DRMs).5 Many dually acylated proteins coalesce into lipid rafts, and raft disruption leads to mislocalization of DRM-associated flagellar proteins (20). Therefore, it has been proposed that protein association with lipid rafts might serve to recruit and/or retain flagellar membrane proteins (20).

One dually acylated protein that is highly enriched in the flagellar membrane of the protozoan Trypanosoma cruzi is a 24-kDa flagellar calcium-binding protein (FCaBP). The N terminus of FCaBP, which is dually acylated with myristate and palmitate, localizes to the internal face of the flagellar membrane, and both modifications are necessary for this localization (12). The N terminus of FCaBP is also sufficient to direct the cytoplasmic GFP to the flagellum (12). Although dual acylation is required for localization of FCaBP, it is not clear whether it is sufficient or whether other properties of the N terminus contribute, perhaps by promoting an association with lipid rafts.

Although a variety of membrane proteins with specific localization to the flagellum have been identified in protozoan parasites, the pathways of membrane targeting and sorting among pellicular and flagellar pocket and flagellar membranes are poorly understood. The studies presented herein further define the requirements for flagellar membrane targeting and lipid raft association, and elucidate the molecular mechanisms that are likely conserved in trypanosomes and other ciliated cells.

MATERIALS AND METHODS

Parasites—Epimastigotes of the Y strain of T. cruzi were used throughout this study and were cultured in liver digest-neutral-

5 The abbreviations used are: DRM, detergent-resistant membrane; FCaBP, flagellar calcium-binding protein; N24-GFP, N-terminal 24 amino acids of FCaBP fused to GFP; biotin-HPDP, N-(6-[biotinamido]hexyl)-3’-(2’-pyridyl)dithio)propionamide; M199, medium 199.

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ized tryptose medium supplemented with hemin and 10% heat-inactivated FBS, as described (21). L. amazonensis (LV79) was grown in medium 199 (M199) (Invitrogen) supplemented with 10% heat-inactivated FBS.

Generation of FCAβP Expression Constructs—The T. cruzi epimastigote expression vector pTEX-9E10 (22) was used for all studies. DNA inserts in pTEX-9E10 were expressed in transfected T. cruzi epimastigotes as proteins containing a C-terminal 10-amino acid c-Myc epitope tag. Sequences for cloning were generated by PCR amplification of a FCAβP cDNA template (23) and directionally cloned into pTEX-9E10 using 5′ XbaI and 3′ EcoRV sites. GFP sequences were PCR-amplified from Aequorea victoria pS65T plasmid as a template (24). Oligonucleotides used for PCR were as follows (5′-3′): WT FCAβP-Myc, (sense) TCTAGAATGGGTGCTTGTGGGTC and (antisense) GATAATTCCATAAAGTGGAGAATGTC; G2A (sense) ATGGCTGCTTTGTTGGGTCAG and (antisense) GGCGCCTCTCCGGCAGCT; C4A (sense) ATGGGTGCTCTGGGTTGCAG and (antisense) CGCGCCTCTCCGGCAGCT; N12 (sense) GATCTAGATGGGTGCTTGTGGGTC and (antisense) TGATCTAGATGGGTGCTTGTGGGTC; N24-GFP (sense) GATCCTGCCCGAGCT and (antisense) CGCGCCTCTCCGGCAGCT; C4A (sense) ATGGGTGCTGCTGGGTCAG and (antisense) AGCTTTCTGCTCGAGCCCTTCCACGACGACAGC and (antisense) CGCGCCTCTCCGGCAGCT; and GFP (sense) TCTAGAATGGGTGCTTGTGGGTC and (antisense) GATAATTCCATAAAGTGGAGAATGTC. All plasmids were purified using Qiagen kits (Qiagen, Gaithersburg, MD), and the relevant regions of all constructs were sequenced to assure accuracy.

Parasite Transfections—For transfection experiments, T. cruzi epimastigotes were grown to a density of 1–2 × 10^7 cells/ml at 26 °C in liver digest-neutralized tryptose, washed twice with 1000 mg/liter d-glucose, 36 mg/liter sodium pyruvate (PBSG buffer, Invitrogen) and suspended in ice-cold transfection buffer (0.5 mM MgOAc, 0.1 mM CaCl_2 in PBS) at a density of 1 × 10^8 cells/ml. 400 μl of the cell suspension was placed into a 0.2-cm electrode gap cuvette with 10 μg of supercoiled plasmid DNA and pulsed once at 0.45 kV (all incubations at room temperature), quenched with 50 mM glycine in PBS for 15 min, and washed with PBS for 30 min, exchanging wash buffer approximately every 5 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 min and incubated in blocking buffer (2% normal goat serum, 1% BSA in PBS) for 1 h. Cells were incubated with primary antibodies diluted into blocking buffer overnight at 4 °C and washed with PBS over 30–60 min with replacement of wash buffer every 5–10 min. Cells were then incubated with Alexa Fluor-conjugated secondary antibodies diluted 1:400 in blocking buffer for 1 h at room temperature and washed as after primary incubation. Cells were incubated with 1 μg/ml DAPI for 10–20 s, washed for 2 min with ultrapure water, and mounted with Gelvatol containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane. Imaging was performed on a Zeiss AxioImager microscope with a 63× objective. Image analysis was performed with Axiovision software (Zeiss, Thornwood, NY).

Cell Fractionation—A total of 2 × 10^7 T. cruzi epimastigotes were washed with PBS and incubated on ice in hypotonic lysis buffer (10 mM Tris-Cl buffer, pH 7.4) for 10 min. Lysates were sonicated at 50% maximum output for two 5-s pulses with a 1-min cooling on ice between bursts using a Branson Digital Sonifier (Model S-450D, Danbury, CT). Samples were pre-diluted at 10,000 × g for 10 min to remove unbroken cells and nuclei. Clarified supernatants were then centrifuged at 100,000 × g at 4 °C for 1 h using a Beckman TLA100.3 rotor. Supernatants were precipitated with acetone. All samples were prepared by addition of prewarmed sample buffer and heated for 10 min at 95 °C. Approximately 10^6 cell equivalents/lane were analyzed by Western blotting.

Western Blot Analysis—Parasite protein lysates were analyzed by SDS-PAGE. Resolved proteins were electrophoretically transferred to 0.45-μm nitrocellulose membranes (Schleicher and Schuell, Keene, NH) for Western blot analysis. The antibodies used in these studies included the following: (i) mouse polyclonal anti-FCAβP (1:1000) (12); (ii) mouse anti-Myc 9E10 supernatant (1:10) (Developmental Studies Hybridoma Bank, Iowa City, Iowa); (iii) mouse anti-tubulin (1:1000) (Developmental Studies Hybridoma Bank, Iowa City, Iowa); (iv) rabbit polyclonal anti-green fluorescent protein antiserum (1:2000) (Caltag, Burlingame, CA); (v) mouse polyclonal anti-Hsp70 (1:5000) (25); (vi) rabbit polyclonal anti-phosphoinositide-specific phospholipase C (1:2500) (26); and (vii) rabbit polyclonal anti-p36 (1:2500) (27) against the stable cytoplasmic oxidoreductase of L. amazonensis. The secondary antibodies for Western blotting used were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:2000) (Invitrogen). Blots were developed with the ECL reagent (Amersham Biosciences).

Immunofluorescence Microscopy—T. cruzi epimastigote transfectants were centrifuged for 7 min at 1000 × g, washed twice with PBS, and resuspended in 4% paraformaldehyde. Fixed parasites were settled onto poly-l-lysine-coated slides for 30 min (all incubations at room temperature), quenched with 50 mM glycine in PBS for 15 min, and washed with PBS for 30 min, exchanging wash buffer approximately every 5 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 min and incubated in blocking buffer (2% normal goat serum, 1% BSA in PBS) for 1 h. Cells were incubated with primary antibodies diluted into blocking buffer overnight at 4 °C and washed with PBS over 30–60 min with replacement of wash buffer every 5–10 min. Cells were then incubated with Alexa Fluor-conjugated secondary antibodies diluted 1:400 in blocking buffer for 1 h at room temperature and washed as after primary incubation. Cells were incubated with 1 μg/ml DAPI for 10–20 s, washed for 2 min with ultrapure water, and mounted with Gelvatol containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane. Imaging was performed on a Zeiss AxiosImager microscope with a 63× objective. Image analysis was performed with Axiovision software (Zeiss, Thornwood, NY).

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Density Gradient Centrifugation and Analysis—T. cruzi epimastigotes were grown to mid-log phase, washed in PBS and resuspended at 2 × 10^7 cells/ml in 1 ml of lysis buffer (1% Triton X-100, 25 mM Tris- HCl, 150 mM NaCl, EDTA-free protease inhibitor (Roche Applied Sciences)). Cells were lysed at 4 °C for 30 min while rotating. This lysate was adjusted to 40% density with the addition of OptiPrep (Axis-Shield, Oslo, Norway) and then layered with 5 μl of a 35% OptiPrep solution and 1.5 ml of a 5% OptiPrep solution. All OptiPrep solutions were made using the lysis buffer. The samples were centrifuged for 5 h at 35,000 rpm
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RESULTS

Localization and Detergent-resistant Membrane Association of FCaBP—We examined the cellular localization of endogenous FCaBP and WT FCaBP\textsubscript{myc} in \emph{T. cruzi} epimastigotes by immunofluorescence microscopy with FCaBP- or Myc-specific antisera, respectively (Fig. 1A). Both the endogenous FCaBP and WT FCaBP\textsubscript{myc} proteins are enriched in the \emph{T. cruzi} flagellum, indicating that the C-terminal Myc tag does not alter protein localization. To examine the association of FCaBP with DRMs, we performed discontinuous density gradient centrifugation. In this assay, DRMs and associated proteins are found in the low density interface fractions (fractions 2–3) due to their greater buoyancy. This analysis revealed that both the endogenous FCaBP and FCaBP\textsubscript{myc} are DRM-associated (Fig. 1B). This is in contrast to the plasma membrane protein Gp63, which is distributed across the cell surface and is known not to associate with DRMs in \emph{T. cruzi} (30). These results confirm that endogenous FCaBP and FCaBP\textsubscript{myc} both localize to the flagellum and associate with DRMs in \emph{T. cruzi} epimastigotes.

Cellular Localization and Biochemical Characterization of FCaBP Acylation Mutants—To analyze the contribution of acylation to FCaBP localization, immunofluorescence microscopy was performed on acylation point mutants. This revealed that neither the non-acylated FCaBP\textsubscript{G2A-Myc} protein nor the myristoylated-only FCaBP\textsubscript{C4A-Myc} protein exhibited flagellar localization, instead appearing cytoplasmic (Fig. 2A). However, the FCaBP\textsubscript{G2A-Myc} and FCaBP\textsubscript{C4A-Myc} transfectants were analyzed by discontinuous (5–35% to 40%) OptiPrep gradient centrifugation and Western bloting using FCaBP- or Gp63-specific antisera. Neither FCaBP\textsubscript{G2A-Myc} (non-acylated) nor FCaBP\textsubscript{C4A-Myc} (myristoylated-only) localize to the flagellum. Both transfectants were lysed by hypotonic sonication and fractionated into cytosolic (C) and membrane (M) fractions by centrifugation. Fractions were analyzed by SDS-PAGE and Western bloting using antisera specific for FCaBP, Hsp70 (both fractions), or phosphoinositide-specific phospholipase C (PI-PLC) (membrane control). Although some endogenous FCaBP (E) partitioned with the membrane fraction, the Myc-tagged mutant FCaBP\textsubscript{G2A-Myc} and FCaBP\textsubscript{C4A-Myc} proteins (M) were found only in the cytosolic fraction, indicating that the C-terminal Myc tag does not alter protein localization. To examine the association of FCaBP with DRMs, we performed discontinuous density gradient centrifugation. In this assay, DRMs and associated proteins are found in the low density interface fractions (fractions 2–3) due to their greater buoyancy. This analysis revealed that both the endogenous FCaBP and FCaBP\textsubscript{myc} are DRM-associated (Fig. 1B). This is in contrast to the plasma membrane protein Gp63, which is distributed across the cell surface and is known not to associate with DRMs in \emph{T. cruzi} (30). These results confirm that endogenous FCaBP and FCaBP\textsubscript{myc} both localize to the flagellum and associate with DRMs in \emph{T. cruzi} epimastigotes.

and then for 12 h at 25,000 rpm using a Beckman SW41Ti rotor (Beckman Coulter, Brea, CA). Fractions were collected from the top and were added to 2× Laemmli sample buffer, heated at 95 °C for 10 min, and analyzed by Western bloting.

Temperature-dependent Detergent Extraction—\emph{T. cruzi} epimastigotes were grown to mid-log phase, washed twice in PBS, and resuspended at 1×10^8 cells/ml in 500 μl of 1% Triton X-100 in PBS, pre-equilibrated to either 4 or 37 °C. Cells were incubated at 4 or 37 °C for 10 min and centrifuged at 1,000 × g for 7 min. Supernatants were removed from the pellets and transferred to new tubes, and the pellets were resuspended in 500 μl of 1% Triton X-100 in PBS at temperature. Supernatants were centrifuged again at 20,000 × g for 7 min at either 4 °C or 37 °C. Soluble (supernatant) and insoluble (pellet) fractions were added to 2× Laemmli sample buffer, heated at 95 °C for 10 min, and analyzed by Western bloting.

Acyl-biotin Exchange Chemistry—To detect palmitate incorporation, \emph{T. cruzi} epimastigotes were analyzed according to the protocol used for purification of the \emph{Trypanosoma brucei} palmitoyl proteome (28), originally developed for yeast (29).

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A

| N24-GFP | N12-GFP |
|---------|---------|
| MGAGCSKGSTSDKGLASDKDGKNA | MGAGCSKGSTSD |

GFP

B

Western

| N24-GFP | N12-GFP | GFP | vector |
|---------|---------|-----|-------|
| Tubulin |

C

| WCL | I | HA | E | Tris | E | Tris |
|-----|---|----|---|------|---|------|
| GFP |

Transfectant

| N24-GFP | N12-GFP |
|---------|---------|
| GFP |

Merge

FIGURE 3. Dual acylation is maintained in truncations of N terminus 1–12 and 1–24 FCaBP fused to GFP. A, the amino acid sequence for T. cruzi epimastigote transfectants expressing the N-terminal 24 or 12 amino acids of FCaBP fused to GFP, GFP alone, and vector alone (not shown) were generated using standard methods. The sites of myristoylation (G2) and palmitoylation (C4) in FCaBP are indicated. B, transfectants were analyzed by Western blotting with antisera specific for GFP or tubulin. C, the palmitoylation states of N24-GFP and N12-GFP transfectants were assessed by acyl-biotin exchange followed by Western blotting of streptavidin-purified proteins. Palmitoylated proteins in T. cruzi lysates are labeled in this assay by cleavage of fatty acyl-thioester bonds with hydroxylamine (HA) but not by Tris, conjugation of the newly liberated thiols to biotin-HPDP and affinity chromatography and elution on streptavidin. Samples from each input (I) and eluate (E) fraction from the streptavidin purification were analyzed by Western blotting with antisera specific for FCaBP, GFP, and tubulin. Endogenous FCaBP (positive control), N24-GFP, and N12-GFP were all purified by streptavidin in a hydroxylamine-specific manner (palmitoylated), whereas tubulin (negative control) was not. A whole cell lysate (WCL) is also included in each panel.

A fraction, the Myc-tagged mutants FCaBP<sub>G2A-Myc</sub> and FCaBP<sub>C4A-Myc</sub> were both found exclusively in the cytosolic fraction. As expected, the cytosolic Hsp70 and membranous phosphoinositide-specific phospholipase C proteins associated predominantly with cytosolic and membrane fractions, respectively (Fig. 2B). Analysis of the acylation mutants by discontinuous density gradient centrifugation and Western blotting revealed that neither FCaBP<sub>G2A-Myc</sub> nor FCaBP<sub>C4A-Myc</sub> retained an association with the buoyant DRM like the endogenous FCaBP (Fig. 2C).

Cellular Localization and Biochemical Characterization of FCaBP-GFP Fusion Proteins—The N terminus of FCaBP contains the determinants for acylation and is sufficient to direct cytoplasmic GFP to the flagellar membrane (12). Although prevention of acylation disrupts FCaBP flagellar localization, it is not clear whether the presence of fatty acids alone is sufficient for localization or whether other domains within the N terminus are also important. To further analyze the FCaBP N terminus, we designed a truncated fusion construct expressing either the N-terminal 24 or 12 amino acids of FCaBP fused to GFP (Fig. 3A). In both truncation mutants, sufficient sequence was left intact to preserve the N terminus myristoylation motif (31). Mutant cells were generated by transfection with either N24-GFP, N12-GFP, or GFP alone, and expression of fusion proteins was confirmed (Fig. 3B).

Because acylation of FCaBP is critical for its association with the flagellar membrane, we tested whether serial truncations within the N terminus of FCaBP led to alterations in acylation. Transfectants were analyzed by acyl-biotin exchange and Western blotting. In this assay, cell lysates were treated with hydroxylamine to cleave protein-palmitoyl linkages and generate reactive thiol groups, which were then labeled with N-[6-(biotinamido)hexyl]-3′,3′,3′-(2′-pyridyldithio)propionamide (biotin-HPDP). Biotinylated proteins were then purified by streptavidin-agarose chromatography. Nonspecific labeling of free thiols was blocked by preincubation of the extract with N-ethylmaleimide before hydroxylamine treatment, and residual background labeling and purification were monitored by treating samples in parallel with Tris-HCl in place of hydroxylamine. Endogenous FCaBP (positive control) demonstrated hydroxylamine-dependent purification, whereas tubulin (negative control) did not. Like FCaBP, N24-GFP and N12-GFP proteins were both biotinylated and purified by streptavidin under experimental but not control conditions (Fig. 3C). Because myristoylation was a requirement for palmitoylation of FCaBP and other dually acylated proteins, incorporation of palmitate in this assay was an indicator of dual acylation.

To determine the cellular localization of either N24-GFP, N12-GFP, or GFP alone, T. cruzi transfectants expressing these proteins were analyzed by immunofluorescence microscopy using antisera specific for FCaBP or GFP (Fig. 4). This showed clear differences in the localization of the fusion proteins. N24-GFP colocalized with endogenous FCaBP, confirming that the N-terminal 24 amino acids are sufficient to direct cytosolic GFP to the flagellum. On the other hand, N12-GFP failed to localize to the flagellum but instead remained associated with the cell body. This suggested that amino acids 13–24 of FCaBP are also
required for flagellar targeting. Because dual acylation of N12-GFP is intact (Fig. 3C), the contribution of N13–24 to flagellar localization is not attributable to its requirement for acylation.

As with the acylation mutants, we next tested whether N12-GFP mislocalized into the cytoplasm or retained an association with the pellicular membrane. T. cruzi transfectants expressing N24-GFP, N12-GFP, or GFP alone were subjected to hypotonic sonication and fractionated into cytosolic (C) or membrane (M) fractions by centrifugation. Fractions were analyzed by Western blotting using antisera specific to GFP. Both N24-GFP and N12-GFP are found in the membrane fraction, whereas GFP partitions in the cytosolic fraction. Controls for a cytosolic Hsp70 and membrane-bound FCaBP are included.

FIGURE 5. Both N24-GFP and N12-GFP associate with a membrane fraction. T. cruzi transfectants expressing N24-GFP, N12-GFP, or GFP alone were subjected to hypotonic sonication and fractionated into cytosolic (C) or membrane (M) fractions by centrifugation. Fractions were analyzed by Western blotting using antisera specific to GFP. Both N24-GFP and N12-GFP are found in the membrane fraction, whereas GFP partitions in the cytosolic fraction. Controls for a cytosolic Hsp70 and membrane-bound FCaBP are included.

Role of Conserved N-terminal Lysine Residues in Flagellar Membrane Targeting of FCaBP—Analysis of the FCaBP N terminus revealed that, in addition to the conserved acylation sites, several positively charged lysine residues are conserved between FCaBP and the calflagins, the T. brucei homologues (Fig. 7A). Structural analysis of FCaBP shows that the side chains of these N-terminal lysines form a cluster of positive charge on one face of the protein surface (32). To test the possibility that these lysines underlie the requirement for N13–24 in flagellar targeting, perhaps by promoting or stabilizing interaction with flagellar membrane phospholipids, we replaced lysine residues 13, 19, and 22 with alanines by site-directed mutagenesis. We confirmed that the alanine mutant did not affect the palmitoylation state of this protein by acyl-biotin exchange (Fig. 7B). Immunofluorescence microscopy revealed that N24K13A,K19A,K22A-GFP localized to the cell body, rather than the flagellum (Fig. 7C). Thus, the positively charged lysine residues within domain N13–24 are essential for flagellar specificity. Finally, N24K13A,K19A,K22A-GFP transfectants were treated with 1% Triton X-100 and fractionated into supernatant and pellet fractions at 4 and 37 °C. N24K13A,K19A,K22A-GFP remained completely soluble at both temperatures, indicating that the loss of the lysines had abolished the DRM association of N24-GFP (Fig. 7D).

Expression and Characterization of FCaBP in Leishmania—To investigate whether the mechanism underlying FCaBP targeting to the flagellum is conserved in other trypanosomatids, we used the related protozoan Leishmania amazonensis, which lacks an FCaBP homologue, as a “living test tube” for flagellar targeting. We transfected L. amazonensis promastigotes with full-length FCaBP using pTEX:FCaBP MYC or vector alone and analyzed these transfectants by Western blotting. FCaBP MYC was stably expressed in L. amazonensis (Fig. 8A) and localized to the non-DRM fraction.

FIGURE 6. N24-GFP, but not N12-GFP, associates with detergent-resistant membranes. A, T. cruzi transfectants expressing N24-GFP, N12-GFP, or GFP alone were analyzed by discontinuous (5–35% to 40%) OptiPrep gradient centrifugation and Western blotting using GFP- or FCaBP-specific antisera. N24-GFP is associated with the DRM fraction (2), whereas N12-GFP and GFP are not. B, the same transfectants were analyzed by temperature-dependent Triton X-100 solubility, together with wild-type epimastigotes (WT). Cells were solubilized in 1% Triton X-100 for 30 min at 4 or 37 °C, separated into soluble (S) and pellet (P) fractions by centrifugation, and analyzed by Western blotting with FCaBP-specific or GFP-specific antisera. FCaBP partly partitions in the pellet fraction at 4 °C but is solubilized at 37 °C, a property of DRM-associated proteins. Like FCaBP, N24-GFP exhibits temperature-dependent DRM association, whereas N12-GFP and GFP do not. WCL, whole cell lysate.
to the flagellum by immunofluorescence microscopy (Fig. 8B), similar to the localization of FCaBP in T. cruzi. To test whether FCaBP associates with DRMs in L. amazonensis, we analyzed L. amazonensis expressing FCaBP<sub>MyC</sub> by temperature-dependent Triton X-100 solubility and Western blotting. Like FCaBP in T. cruzi, FCaBP<sub>MyC</sub> partly partitions in the pellet fraction at 4 °C but is solubilized at 37 °C, a property of DRM-associated proteins (Fig. 8C). These results suggest that the molecular mechanisms for the targeting of FCaBP and perhaps other DRM-associated proteins is conserved among kinetoplastid protozoans.

**DISCUSSION**

The plasma membrane of kinetoplastids (Trypanosoma and Leishmania species) has three distinct domains: the cell body (or pellicular) membrane, the flagellar membrane, and the flagellar pocket membrane. The flagellar membrane has a distinct lipid and protein composition in comparison with the other membrane domains. It is enriched in sterols (14–16) and sphingolipids (18, 19), both of which are known components of the lipid raft microdomains that have hypothesized roles in protein sorting and signaling. In addition to a distinct lipid composition, the flagellar membrane is characterized by asymmetric distribution of some proteins (33). Research from various laboratories has demonstrated that certain proteins are found exclusively or are highly concentrated in the flagellar membrane. Well studied flagellar membrane proteins across the kinetoplastids include calcium-binding proteins in T. cruzi (FCaBP) (12) and T. brucei (calflagins) (28), receptor adenylate cyclases (ESAG4) (8), LDL receptor (7), glucose transporter isoform 1 (9), and the small myristoylated protein (10). Despite the identification of proteins having specific localization to certain membrane domains in protozoans, no common targeting sequences or motifs have yet been identified. The cellular mechanisms for flagellar membrane targeting and the degree to
which they are conserved across species are now being elucidated (35).

In this study, we examined the requirements for flagellar membrane localization of FCaBP in T. cruzi epimastigotes. Previously, we found that FCaBP is dually acylated at the N terminus, with a myristoyl moiety covalently attached to a glycine at position 2 and a palmitate attached to the cysteine residue at position 4 (12). Many dually acylated proteins associate with lipid rafts (36), and disruption of lipid rafts, by cholesterol depletion or by fluidization of the membrane, abolished localization of FCaBP homologues to the flagellar membrane (20). Therefore, it was proposed that protein association with lipid rafts might serve to recruit and/or retain some flagellar membrane, or some other aspect of the N terminus confers flagellar specificity of which would therefore be dictated by the target membrane, or some other aspect of the N terminus confers flagellar specificity. The first possibility seems unlikely as there are numerous proteins that do not localize to the flagellum. Some examples include CAP5.5, a cytoskeleton-associated protein in T. brucei, which associates with the pellicular membrane and subpellicular cytoskeleton, (47), the HASPB protein of Leishmania, which is an extracellular protein (48, 49), and the phosphoinositide-specific phospholipase C of T. cruzi, which localizes to the plasma membrane in amastigotes and to the cytoplasm in epimastigotes (26, 50). All of these proteins have the same myristate and palmitate modifications as FCaBP and yet do not localize to the flagellum. Thus, although acyl modifications could explain membrane localization, other domains within the N terminus of FCaBP are likely responsible for flagellar specificity.

To test whether other domains within the 24 amino acid N-terminal region of FCaBP are responsible for flagellar membrane specificity, we generated a truncation mutant missing amino acids 13–24 (N12-GFP), thus preserving the sites for acylation. Analysis of this truncated fusion protein revealed clear differences in localization. N24-GFP localized predominantly to the flagellum and associated with lipid rafts, whereas N12-GFP, did not, despite being dually acylated (Figs. 4 and 6). Thus, although N1–12 supports dual acylation and membrane association, additional elements contained within N13–24 are required for flagellar localization and lipid raft association.

To gain insight into the contribution that N13–24 may provide for flagellar membrane specificity, we examined the recently completed crystal structure of FCaBP (32). The crystal structure revealed multiple charged residues unevenly distributed on one face of the protein surface (32). Most noticeable was the arrangement of several lysine residues whose side chains form a cluster of positive charges (32). Alignment of the FCaBP N terminus to the N termini of its homologues of in T. brucei revealed that all of the lysine residues in the N terminus domain are conserved, suggesting that they may be important for flagellar specificity and lipid raft association. Because we had already established that the N13–24 domain contributed to flagellar localization, we replaced these lysines with alanines (N13, 19, and 22), which resulted in mislocalization of N24-GFP and abolished DRM association, despite preserved palmitoylation (Fig. 7). These lysine residues may promote electrostatic interactions with negatively charged head groups on the inner leaflet of the flagellar membrane. A similar electrostatic membrane interaction has been observed in the myristoylated alanine-rich C kinase substrate, Src kinases, ADP-ribosylation factor, and human immunodeficiency virus-1 matrix proteins (51–54). Electrostatic interaction through these basic residues, in conjunction with dual acylation, is likely to augment membrane and DRM association.

Related trypanosomes T. brucei and Trypanosoma rangeli have FCaBP homologues (55–57) in syntenic regions of the genome, both of which are also enriched in the flagellum (57). Interestingly, the Leishmania amazonensis genome does not contain an obvious FCaBP homologue, and the syntenic region is missing (58). To test whether the mechanism of flagellar localization is operative in L. amazonensis, we transfected L. amazonensis promastigotes with an FCaBP expression construct. These transfecteds showed that FCaBP, when expressed in L. amazonensis, has the same cellular and biochemical properties as it did in T. cruzi (Fig. 8). These findings...
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imply that the molecular components of flagellar targeting of FCaBP, including myristoylation by N-myristoyltransferase, palmitoylation by a palmitoyl acyltransferase, and lipid raft association are conserved in L. amazonensis.

Taken together with the work of others, our results suggest that flagellar membrane localization of a protein requires an association with lipid raft microdomains. For N24-GFP, several elements, myristoylation, palmitoylation, and a cluster of nearby basic amino acids, are each required to collectively support this protein-lipid interaction. The failure of other dually acylated proteins to localize to the flagellar membrane can thus be explained by the insufficiency of dual acylation alone to target lipid rafts. Alternatively, a protein containing additional domains that interact with another cellular protein (34) might possibly override flagellar membrane targeting. In either case, we are not aware of any dually acylated flagellar membrane proteins that do not also associate with lipid raft microdomains, supporting the intricate and possibly causal link between these properties (10, 28).

An understanding of the human ciliopathies has been much advanced through exploration of the choreography of intraflagellar transport in model organisms. In addition to identifying the common domains contributing to flagellar or ciliary membrane association, a dissection of the determinants for protein and lipid sorting from the Golgi to target organelles will be fundamental step toward understanding cell polarization. Such polarization, whether of epithelial or endothelial barriers, immune cells, neurons, and possibly host cells undergoing invasion by pathogens, is a hallmark of complex organisms. Unraveling the mechanisms in protozoa may contribute greatly to our understanding of human biology.

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