The flagellar calcium-binding protein (FCaBP) of the flagellated protozoan *Trypanosoma cruzi* associates with the flagellar membrane via its N-terminal myristate and palmitate moieties in a calcium-modulated, conformation-dependent manner. This mechanism of localization is similar to that described for neuronal calcium sensors, which undergo calcium-dependent changes in conformation, which modulate the availability of the acyl groups for membrane interaction and partner association. To test whether FCaBP undergoes a calcium-dependent conformational change and to explore the role of such a change in flagellar targeting, we first introduced point mutations into each of the two EF-hand calcium-binding sites of FCaBP to define their affinities. Analysis of recombinant EF-3 mutant (E151Q), EF-4 mutant (E188Q), and double mutant proteins showed EF-3 to be the high affinity site \((K_d \sim 9 \mu M)\) and EF-4 the low affinity site \((K_d \sim 120 \mu M)\). These assignments also correlated with partial \((E188Q)\), nearly complete \((E151Q)\), and complete \((E151Q,E188Q)\) disruption of calcium-induced conformational changes determined by NMR spectrometry. We next expressed the FCaBP E151Q mutant and the double mutant in *T. cruzi* epimastigotes. These transproteins localized to the flagellum, suggesting the existence of a calcium-dependent interaction of FCaBP that is independent of its intrinsic calcium binding capacity. Several proteins were identified by FCaBP affinity chromatography that interact with FCaBP in a calcium-dependent manner, but with differential dependence on calcium-binding by FCaBP. These findings may have broader implications for the mechanisms that modulate the availability of the acyl groups for membrane association, and calcium-dependent localization of FCaBP, we concluded that the protein is a calcium acyl switch protein. These proteins undergo calcium-dependent membrane association by virtue of calcium-regulated extrusion or sequestration of a myristate moiety that mediates membrane binding (4).

The best studied calcium acyl switch protein is recoverin (Rv), a myristoylated calcium-binding protein of the mammalian retina (5) that inhibits rhodopsin kinase (RK) (6) and regulates recovery of the photo-receptor cell after photoexcitation (7). Rv regulates RK via the calcium myristoyl switch mechanism (4). In the resting state of the cell, Rv binds two calcium ions and associates with the retinal outer segment (ROS) membrane through its exposed myristoyl group (5, 8). When localized at the ROS membrane, Rv then binds to rhodopsin kinase (RK) and inhibits it from acting upon its target protein, rhodopsin (5, 7, 9). Phototocitation of the receptor cell results in an efflux of calcium. As the intracellular calcium level drops, Rv loses its calcium and adopts a conformation in which the myristic acid is sequestered within a hydrophobic cleft in the protein (10). Unable to associate with the ROS membrane, Rv dissociates from RK, allowing RK to phosphorylate rhodopsin. Phosphorylation of rhodopsin leads to a rise in the level of intracellular calcium as the cell recovers from excitation. Rv binds calcium and adopts the open conformation and resumes the membrane-associated, RK-inhibiting position. This unique calcium-myristoyl switch mechanism allows calcium regulation of two proteins (RK and rhodopsin) that do not themselves bind calcium (4, 5, 8).

Numerous calcium sensor proteins have been identified in kinetoplastids. FCaBP-like proteins are found not only in *T. cruzi* but also in the trypanosomes *Trypanosoma rangeli*, *Trypanosoma lewisi*, and *Trypanosoma brucei*. A flagellar calmodulin has also been characterized in *T. brucei* (11). These proteins all localize to the flagellum, a unique organelle that has many functions, including motility, chemotaxis, and cell signaling. In addition to the traditional "9 + 2" microtubule structure of the axoneme, there is a structure known as the paraflagellar rod that runs alongside the axoneme. The axoneme, paraflagellar rod, and flagelloplasm are encased by the flagellar membrane. It has been shown by freeze-fracture analysis that the flagellar membrane contains a higher concentration of sterols than does the pellicular (cell body) membrane (12), and current work in our laboratory demonstrates that the flagellum of *T. brucei* is highly enriched in lipid rafts. It is especially interesting, then, to study the behavior of acylated calcium sensor proteins as they interact with this unique membrane.

FCaBP appears to undergo calcium-acyl switching, where association with the flagellar plasma membrane is mediated by calcium and the two N-terminal acyl groups (3). However, a calcium-dependent conformational change in FCaBP has not been demonstrated. c29, a sequence variant of the FCaBP described here (13), was previously shown to bind
calcium and undergo calcium-dependent conformational changes using circular dichroism and tryptophan intrinsic fluorescence spectrometry (14). We wanted to investigate the role of calcium binding in the acyl switch mechanism by introducing point mutations in EF-3 and EF-4 to disrupt calcium binding and generated these FCaBP mutant proteins and _T. cruzi_ epimastigotes expressing these proteins (i) to delineate the high and low affinity binding sites in FCaB, (ii) to assess calcium-dependent conformational changes in these proteins, and (iii) to directly determine the role of calcium in the flagellar localization of FCaB.

**EXPERIMENTAL PROCEDURES**

**Parasites**—The Y strain of _T. cruzi_ was used for all experiments, except for experiments involving the transfectants expressing FCaB-Myc, which were previously generated in the Silvio X-10/4 clone (3). _T. cruzi_ epimastigotes were maintained at 26 °C in supplemented liver digest neutralized tryptose medium (15).

**Engineering of FCaB Point Mutants**—Point mutations were introduced into the third and fourth EF hands of FCaB by PCR amplification using an FCaB cDNA as a template. For mutagenesis of the third EF hand, sense and antisense primers (GAGGAGCAGTTCAAGCG and TGAACCTGCTCCTGTCG, respectively) were used to substitute glutamate at position 151 with glutamine (E151Q). For mutagenesis of the fourth EF hand, sense and antisense primers (TCCGACCTTTTGCTGCAG and AACCTGTACGTAGCACAG, respectively) were used to substitute glutamate at position 188 with glutamine (E188Q). The E151Q, E188Q double mutant (DM) was generated using E188Q cDNA as a template for PCR with the sense and antisense primers used to generate the E151Q substitution. These primers also introduced 5′ EcoRI and 3′ XbaI restriction sites. Coding regions for the three mutant proteins were cloned into pBluescript S/K (Stratagene) using EcoRI and XbaI and subcloned directionally into pcDNA3.1 (Invitrogen) to add a C-terminal Myc (9E10) and hexahistidine epitope tag. These coding regions were further subcloned directionally into the _T. cruzi_ epimastigote expression vector pTEX-9E10 (16) using PmeI and SmaI. Mutant proteins and FCaB were expressed in _Escherichia coli_ BL21 (DE3) cells developed and were available for study after cloning in pET23b (Novagen) and were expressed in _Escherichia coli_ BL21(DE3) cells (Stratagene). After induction with 1 mM isopropyl-β-D-thiogalactopyranoside, transformants were incubated for 3 h at 37 °C, pelleted, and lysed by heat shock and sonication. Lysates were cleared by two rounds of centrifugation at 20,000 × _g_ for 15 min, and recombinant proteins were purified using a Ni²⁺-charged His-Bind chromatography column (Amersham Biosciences). Eluted samples were dialyzed with a solution of 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and concentrated using Centricon Centriplus YM filters (Amicon), molecular mass cut-off 3,000 Da. 5 μg of each sample was separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by ⁴⁵Ca²⁺ overlay analysis (17).

**Calculus Titrations by Trypspin Intrinsic Fluorescence Spectrometry**—Experiments were performed as described in Ref. 14. Steady-state measurements were performed in an ISS K2 spectrophotometer (ISS Fluorescence, Analytical, and Biomedical Instrumentation). The temperature of the solution was maintained at 18 °C using a circulating water bath (Fisher). Protein solutions were measured in a 1-cm rectangular quartz cuvette. For trypspin fluorescence calcium titration experiments, the excitation wavelength was 295 nm, and the emission spectra were recorded from 305 to 450 nm. Fluorescence spectra of 2.7 mM protein solution in 20 mM Tris-HCl buffer, pH 7.9, containing 150 mM NaCl were titrated with increasing amounts of calcium to saturation. Intensity at each point was corrected for dilution. To eliminate background the spectra of buffers, containing or not containing calcium, were measured and subtracted from the samples measured spectra.

**Isothermal Titration Calorimetry**—Calorimetry was performed using a VP-ITC microcalorimeter (MicroCal, Inc.), essentially as described (18). In each titration, 40 × 10⁻⁵-μl aliquots of 15 mM CaCl₂ were added to 180 μM protein.

**NMR Spectroscopy**—Samples for NMR spectroscopy were prepared by dissolving ¹⁵N-labeled FCaB proteins (0.5 mM) in 0.5 M of a 95% H₂O, 5% ²H₂O solution containing 10 mM ²H₄ imidazole (pH 7.0), 1 mM ²H₂ dithiothreitol, 50 mM KCl, and either 1 mM EDTA (Ca⁺²-free) or 2 mM CaCl₂ (Ca⁺²-bound). All NMR experiments were performed at 35 °C on a Bruker DRX-600 spectrometer equipped with a four-channel interface and a triple resonance probe with triple axis pulsed field gradients. The ¹⁵N-¹H HSQC spectra were recorded on samples of ¹⁵N-labeled FCaB (in 95% H₂O, 5% ²H₂O). The two-dimensional spectra were processed and analyzed as described previously (19).

**Localization of FCaB Mutant Transproteins by Immunofluorescence Microscopy**—_T. cruzi_ transfectants expressing the various FCaB mutant proteins were isolated by centrifugation, washed in PBS, and resuspended in PBS at a density of 1 × 10⁶ cells/ml. 20 μl of this suspension was added to an amino-alkylsilane-coated Silane-Prep slide (Sigma), and the cells were allowed to settle to the slide. The following solutions contained neither calcium nor calcium chelator. Cells were fixed by treatment with 1% paraformaldehyde in PBS for 5 min and incubated at −20 °C in anhydrous methanol overnight. For immunofluorescence microscopy, slides were incubated in blocking buffer (1% bovine serum albumin, 2% normal goat serum in PBS) for 1 h, followed by incubation with either His-specific monoclonal antibody (Ab-1 OB05; Oncogene), c-Myc-specific 9E10 monoclonal antibody hybridoma supernatant (ATCC CRL1729, Myc 1—9E10.2), or FCaB-specific mouse serum overnight at 4 °C. Parasites were washed with PBS and incubated for 2 h with a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM (Caltag Laboratories). Slides were then mounted in Permaflour aqueous mounting medium (Immunotech) and viewed by fluorescence microscopy. Microscopy was performed using an Leica DM IR2 inverted system microscope, and images were captured using Open Lab 4.0.2 (Improvement).

**Immunoblot Analysis**—Parasite protein lysates were boiled and analyzed by one-dimensional 12% SDS-PAGE. Gels were either stained with Coomassie Blue or transferred to nitrocellulose (20) and incubated with FCAB-specific (1) mouse serum, hsp70-specific mouse serum, or
c-Myc-specific, 9E10 monoclonal antibody hybridoma supernatant overnight. Blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG + IgM (Caltag Laboratories) and developed using ECL reagents (Amersham Biosciences).

**Metabolic Labeling and Immunoprecipitation—*T. cruzi* transfectants expressing WT, E151Q, or DM mutant proteins were grown to midlog phase (5–7 x 10⁶ cells/ml), and 2 x 10⁶ cells of each line were suspended in 6 ml of liver digest neutralized tryptophan medium containing 1 mM of [9,10-²H]myristate (30–50 Ci/mmol) or Dulbecco’s minimal essential medium containing 1 mM of [9,10-²H]palmitate (30–60 Ci/mmol). Cultures were incubated at room temperature, shaking slowly for 4 h (myristate labeling) or 1 h (palmitate labeling) and then washed several times each with 20 ml of PBS to remove excess label. Cells were then lysed in 1 ml of radioimmune precipitation buffer (1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 0.02% NaN₃, EDTA-free protease inhibitor (Roche Applied Science)). The lysate was clarified by centrifugation 14,000 x g for 10 min, divided into 250-µl aliquots, and stored at −20 °C until use. Aliquots of the labeled radioimmuno precipitation lysates were precleared over protein G beads for 4 h at 4 °C and then incubated with FCaBP-specific mouse serum cross-linked to protein G beads and rotated overnight at 4 °C. Immunoprecipitates were collected by centrifugation and washed several times with radioimmune precipitation buffer. [²H]Myristate-labeled precipitates were suspended in 2 x Laemmli sample buffer and boiled for 8 min. [²H]Palmitate-labeled samples were suspended in nondenaturing sample buffer (2 x Laemmli sample buffer without dithiotreitol or β-mercaptoethanol) and boiled for 2 min to avoid cleavage of the labile thioester bond. Samples were analyzed on one-dimensional, 12% Tris-HCl precast gels (Bio-Rad). Gels were examined by immunoblotting or staining with Coomassie Blue, destained with acetic acid and methanol, treated for fluorography (Amplify), dried, and exposed to BioMax MR film (Eastman Kodak Co.) film for both 72 h and 1 week.

**GST Pull-down Assays—*Recombinant FCaBP was engineered to contain an N-terminal GST tag by cloning in pGEX-2T (Amersham Biosciences) and was expressed in *E. coli* DH5α cells (Stratagene). After induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, transformants were incubated for 4 h at 37°C, pelleted and lyzed with lysosome, sonicated, and incubated with 1% Triton X-100. Lysates were cleared by centrifugation at 20,000 x g for 30 min; the recombinant protein was purified using a GST chromatography column (Amersham Biosciences); and eluted samples were dialyzed into PBS. Midlog *T. cruzi* epimastigotes were lysed in 1% Nonidet P-40, 100 mM NaCl, 20 mM HEPES containing either 1 mM CaCl₂ or 1 mM EGTA and resuspended at 3.5 x 10⁶ cells/ml. Lysates were clarified by centrifugation at 2800 x g for 7 min at 4°C and divided into 500-µl aliquots and stored at −20 °C until use. Lysates were precleared using glutathione-agarose beads (Sigma) and then incubated with 50 µg of GST-FCaBP or GST and rotated overnight at 4°C. For experiments using the cross-linking agent dithiobis(sulfosuccinimidyl propionate) (Pierce), dithiobis(sulfosuccinimidyl propionate) was dissolved in Me₂SO and added to a final concentration of 1 mM. Samples were rotated for 2 h at 4°C; Tris HCl, pH 7.5, was added to a final concentration of 20 mM; and the samples were incubated on ice for 15 min. Bound proteins were collected by centrifugation and washed several times with phosphate-buffered saline. Precipitates were suspended in 2 x Laemmli sample buffer, boiled for 8 min, separated by SDS-PAGE, and analyzed by SYPRO Ruby staining (Bio-Rad).

**RESULTS**

**Determination of High and Low Affinity Calcium-binding Sites—**FCaBP contains four canonical EF hand calcium-binding sequences, the third and fourth of which bind calcium (2). To assign the individual binding affinities to their respective binding sites, we introduced a point mutation into each EF hand sequence to disrupt calcium binding while maintaining the overall structure of the domain. The calcium acid residue at position 12 of the EF hand is critical for conjugating the calcium ion and substitution of this residue with glutamine dramatically lowers the affinity of calcium-binding at that site (21). The mutant proteins were generated using the following substitutions: E151Q (in EF-3), E188Q (in EF-4), and E151Q,E188Q (DM) (Fig. 1). The calcium dissociation constants of these proteins were determined by tryptophan fluorescence spectrometry. The change in the quantum yield of tryptophan fluorescence during the titration of calcium in recombinant wild type (WT) FCaBP and DM proteins is shown (Fig. 2A). Scatchard analysis of the curve of binding (not shown) revealed two binding sites, with association constants (Kₛ) 9.0 ± 0.9 and 122 ± 1.9 µM and no evidence of cooperativity. This curve also shows that the complete saturation of binding sites is reached at 350 µM calcium. These results are similar to calcium binding properties previously determined for another isoform of FCaBP, rCC9, which binds with affinities of 3.3 ± 0.5 and 190 ± 0.2 µM (14).

To assign the high and low affinity sites, recombinant FCaBP proteins were examined by [⁴⁰Ca]⁺ overlay analysis (17) (Fig. 2B). The filters were stained with Ponceau stain to visualize the amount of protein in each lane. At low calcium concentration (3 µM CaCl₂), calcium binding was significantly less in the E151Q and DM proteins compared with that of the WT protein. The E188Q protein showed an intermediate level of binding. Binding to all proteins increased with increasing concentration of [⁴⁰Ca]⁺. It should be noted that the calcium overlay assay has high background at calcium concentrations exceeding 10 µM, and protein molecular weight standards also bound [⁴⁰Ca]⁺ at this and higher concentrations (data not shown). Calcium binding of the proteins decreases in the order WT > E188Q > E151Q > DM, demonstrating that EF-3 is the high affinity site and EF-4 is the low affinity site. This conclusion was confirmed by the performance of isothermal titration calorimetry on the mutant proteins (Fig. 2C). Titration of CaCl₂ into wild type FCaBP resulted in a biphasic calorimetric calcium binding isotherm that could be fit by the binding of two or more calcium ions. The wild type isotherm exhibits an initial exothermic phase representing stoichiometric
binding of one calcium to the protein \((K_d \approx 10 \, \text{μM} \text{ and } \Delta H = -9.8 \text{ kcal/mol})\), followed by an endothermic phase, representing lower affinity binding of one or more calcium ions \((K_d \approx 100 \, \text{μM} \text{ and } \Delta H = 1.1 \text{ kcal/mol})\). The E188Q isotherm also contains an initial exothermic phase that represents high affinity calcium binding to EF-3 \((K_d \approx 10 \, \text{μM} \text{ and } \Delta H = -10 \text{ kcal/mol})\) followed by an endothermic phase that represents very low affinity and nonspecific binding to EF-4 \((K_d > 1 \, \text{mM} \text{ and } \Delta H = 2 \text{ kcal/mol})\). The E188Q mutation does not completely abolish calcium binding to EF-4 but rather lowers its binding affinity by more than 10-fold. The E151Q isotherm lacks the high affinity phase seen in wild type and E188Q \((K_d \approx 10 \, \text{μM})\), indicating that EF-3 is the high affinity site. Again, the E151Q mutation does not completely abolish calcium binding to EF-3 but rather lowers its binding affinity by more than 10-fold.
Effect of Calcium Binding on the Conformation of FCaBP—We previously discovered that FCaBP displays calcium-dependent flagellar membrane association, reminiscent of calcium-acyl switching (3, 4). To directly test whether the switch mechanism involves a calcium-modulated change in protein conformation, we used NMR spectroscopy to assess structural differences in the calcium-free and calcium-bound states of FCaBP and its calcium-binding mutants. Two-dimensional NMR spectra (1H-15N HSQC) of uniformly 15N-labeled calcium-free and calcium-bound forms of the various FCaBP proteins were determined (Fig. 3). Peaks in each spectrum represent main chain and side chain amide protons that serve as fingerprints of the overall conformation of the protein. The NMR spectra of calcium-free and calcium-bound WT FCaBP exhibit clear differences (Fig. 3, A and B), indicating that FCaBP changes conformation in response to calcium binding. In particular, the spectrum of calcium-free WT FCaBP exhibits many strong and clearly resolved peaks, suggesting that the protein is monomeric and adopts a stable and well defined tertiary structure. In contrast, the spectrum of calcium-bound WT FCaBP exhibits very broad and poorly resolved peaks, suggesting that the calcium-bound protein may be structurally heterogeneous and may exist as a dimer or other oligomeric species. Indeed, dynamic light scattering analysis of FCaBP reveals that the calcium-bound protein exhibits a polydisperse light scattering distribution with an average molecular mass of around 60 kDa (data not shown). In contrast, the calcium-free protein exhibits monodisperse light scattering that corresponds to an average molecular mass of a monomeric species (25 kDa). In summary, the binding of calcium to FCaBP leads to very large overall conformational changes that cause the calcium-bound protein to multimerize in solution, perhaps due to calcium-induced exposure of hydrophobic residues, as seen previously in many other EF-hand proteins (22–24).

The NMR spectra of calcium-free forms of the E151Q, E188Q, and DM mutant proteins (Fig. 3, C, E, and G) are nearly identical to that of calcium-free WT FCaBP (Fig. 3A), indicating that the mutations do not perturb the overall structure and stability of the calcium-free protein. The NMR spectrum of the calcium-bound E151Q mutant (Fig. 3D) is different from that of calcium-bound WT FCaBP (Fig. 3B) and more closely resembles the spectrum of the calcium-free FCaBP protein. Therefore, E151Q mutant undergoes only a slight conformational change due to the binding of calcium to the low affinity EF-4 site. In contrast, the E188Q mutant displays a clear calcium-induced spectral change (Fig. 3, E and F), similar, although not identical, to that of the WT FCaBP protein. Thus, the binding of calcium to EF-3 appears to be responsible for the vast majority of the calcium-modulated conformational change in FCaBP. Finally, the DM protein (Fig. 3, G and H) did not exhibit any calcium-induced change in its NMR spectrum, confirming that the mutagenesis strategy successfully ablated calcium-binding of this mutant protein.

Acylation of Point Mutants—FCaBP is modified at its N terminus by myristate and palmitate, both of which are required for association with the flagellar membrane (3). We hypothesized that the calcium-binding mutants, when expressed in the trypanosome, would fail to localize to the flagellar membrane, as observed for FCaBP upon calcium chelation (3). We generated T. cruzi epimastigote transfectants expressing WT FCaBP as well as the E151Q and DM proteins, all containing C-terminal Myc (WT) or Myc + His (E151Q and DM) epitope tags. For reasons that are still unclear, we have not yet been able to produce an E188Q transfectant despite many attempts with several independently generated plasmid constructs. While determining the localization of these transproteins, we tested whether they were properly myristoylated and palmitoylated, since acylation is necessary for flagellar localization. The E151Q- and DM-expressing transfectants, as well as untransfected control cells, were metabolically labeled with either [3H]myristate or [3H]palmitate, and cell lysates were immunoprecipitated using FCaBP-specific antiserum and examined by SDS-PAGE and autoradiography (Fig. 4). Immunoprecipitates were also analyzed by Western blotting using anti-FCaBP specific antiserum. The control cells showed a single acylated FCaBP protein, whereas the transfectants each produced two radiolabeled FCaBP proteins, indicating that the endogenous protein and transproteins are myristoylated and palmitoylated in both lines.

Localization of Point Mutants in Vivo—FCaBP localizes to the flagellar membrane of T. cruzi epimastigotes, but this localization is lost upon calcium chelation (3). We examined the localization of the mutant proteins to determine how disruption of calcium binding may affect flagellar localization. Untransfected T. cruzi control cells (Ctrl) and transfectants expressing Myc-tagged WT FCaBP (WT) or Myc + His-tagged
E151Q and DM proteins were analyzed by immunofluorescence microscopy (Fig. 5). In cells expressing WT FCaBP, flagellar localization of FCaBP is clearly visible by anti-Myc staining of the transfected cells. To our surprise, the cells expressing E151Q and DM also showed flagellar localization of the transproteins, suggesting that flagellar localization is independent of the calcium binding ability of FCaBP.

Partner Protein Association of FCaBP—NCS proteins have been well defined as regulatory proteins that transduce calcium signals to their binding partners. To investigate whether FCaBP also functions in this manner, we attempted to identify FCaBP-binding proteins by affinity chromatography using glutathione S-transferase (GST) FCaBP fusion proteins. T. cruzi epimastigotes were lysed in buffers containing either CaCl₂ or EGTA and incubated with GST-FCaBP, GST-DM, or GST alone coupled to glutathione-agarose beads. Because EGTA could also potentially affect the potential binding partners as well as FCaBP, we included a GST-DM fusion protein in the assay as well. The GST fusion proteins were also incubated with lysis buffer alone to identify proteins in the bacterial lysate or fusion protein degradation products that might bind to the glutathione column. After incubation with the cell lysate, protein complexes were collected by glutathione chromatography, washed, and eluted by boiling in SDS sample buffer; separated by SDS-PAGE; and visualized using SYPRO Ruby staining (Fig. 6). Three proteins were identified by this approach. A 120-kDa protein was purified only by GST-FCaBP in the presence of calcium, indicating that its interaction with FCaBP depended on the presence of active FCaBP calcium-binding sites (white circles). Proteins of 80 and 30 kDa were purified both by GST-FCaBP and by GST-DM in a calcium-dependent manner (black circles). These are proteins whose association with FCaBP requires calcium but not calcium binding by FCaBP. The inclusion of a cross-linking reagent (dithiobis(sulfosuccinimidyl propionate)) did not result in the purification of additional proteins.

**DISCUSSION**

Our previous model of FCaBP posited that it is a calcium acyl switch protein that localizes to the flagellar membrane upon binding calcium and dissociates from the membrane when in the calcium-unbound state (2). Like Rv, this would involve calcium-dependent sequestration of the acyl group(s), leading to membrane dissociation. By introducing a single point mutation into each calcium-binding site, we were able to ablate calcium-binding by that site (Figs. 1 and 2). Whereas the calcium-binding affinities of FCaBP had previously been determined, it was not known to which EF hand these values corresponded. Using 

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\text{FIGURE 5.} \text{ Cellular localization of FCaBP calcium-binding mutants.} \quad T. \text{ cruzi epimastigotes expressing WT, E151Q, DM, or no transprotein (control) were analyzed by immunofluorescence (IFA) and brightfield (BF) microscopy using FCaBP-specific antiserum, c-Myc-specific 9E10 monoclonal antibody, or a His-specific monoclonal antibody. Untransfected control cells were also stained with αFCaBP as a positive control.}
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a mutation in the high affinity calcium-binding site of Rv disrupts its calcium-dependent conformational shift (25). As predicted by the switch model, FCaBP undergoes a substantial conformational change upon binding calcium. This is due primarily to binding through EF-3, the high affinity site. The calcium-induced NMR spectral change of the E151Q mutant is less extensive than that of the wild type protein, similar to what had been seen in Rv. Whereas the high affinity site of Rv is responsible for the bulk of the calcium-induced conformational change, mutation of the low affinity site of Rv results in a conformational intermediate (26). Subsequently, this low affinity site was found to control the residence time of Rv within the retinal outer segment membrane (27). Calcium binding to the low affinity EF-4 site of FCaBP (E151Q) does induce a modest conformational change, which is eliminated by mutation of that site (DM). The NMR spectra of the calcium-binding point mutants provide insight into the mechanism of calcium-induced conformational changes of FCaBP and indicate that FCaBP probably forms homodimers in the presence of calcium. Some NCS proteins have been shown to homodimerize under different calcium conditions, a property that is directly related to their functions (22, 24).

We examined the localization of the mutant FCaBP proteins in vivo. Unexpectedly, the calcium-binding mutants also localize to the flagellum, just like the wild type protein (Fig. 5). Given our previous finding that FCaBP can be washed out of permeabilized cells if calcium is removed by EGTA chelation (3), we expected that at least the DM would have a diffusely cytoplasmic localization. We presumed that calcium chelation had a direct effect on the flagellar localization of FCaBP by inducing the protein to adopt a conformation in which the acyl groups are sequestered. The fact that the E151Q and DM proteins are flagellar suggests that calcium-binding by FCaBP is not required for its localization and that myristate and palmitate may be sufficient for flagellar membrane targeting. Indeed, the 24-amino acid N terminus of FCaBP, which contains the two acylation states, is sufficient to direct green fluorescent protein to the flagellum (3).

To date, a flagellar localization peptide has not been identified, and there is much speculation about the mechanisms by which proteins are targeted to the flagellum in kinetoplastids. Rosenbaum et al. have elucidated the process of flagellar targeting in Chlamydomonas known as intraflagellar transport. According to that model, a protein that is destined for the flagellum forms a complex, or "barge," with other flagellar-bound intraflagellar transport proteins as well as dyneins and kinesins. These motor proteins are responsible for retrograde and anterograde transport of flagellar components. It has been shown that as these barges negotiate the flagellum they are in contact with both the axoneme and the flagellar plasma membrane (reviewed in Ref. 28).

Recently, a family of small myristoylated proteins (SMPs) was identified in Leishmania (29). SMP-1 is found in the flagellum and is myristoylated and palmitylated at the N terminus. As with FCaBP, dual acylation is required for flagellar membrane localization. In addition, SMP-1 is also associated with detergent-resistant membranes. We recently found that the flagellar membrane of T. brucei contains high concentrations of sterols and sphingolipids, molecules found in detergent-resistant membranes, or lipid rafts (30). The same appears to be true in T. cruzi, where filipin-cholesterol complexes were more abundant (31). The unique composition of this membrane could direct dually acylated proteins to the flagellum, an idea proposed by Simons and Ikonen in 1997 (32). They proposed that there may be two post-Golgi routes for delivery of proteins to the plasma membrane. One route transports proteins with sorting signals in their cytoplasmic domains, whereas the other route traffics sphingolipid-cholesterol complexes and...
the proteins associated with them (32). FcαBP associates with lipid rafts, and this raft interaction is independent of calcium.4 Up to this point, we had modeled FcαBP after Rv. Given the calcium-dependent structural changes in FcαBP, it seemed counterintuitive that the acyl groups would be accessible to the lipid rafts in the absence of calcium. It is possible that the acyl groups of FcαBP are constitutively exposed, as is the case for some NCS proteins (33), or that FcαBP has a strong interaction with a partner protein that allows for raft association in low calcium.

It should be noted that, in two separate studies, calcium-free Rv was found to interact with ROS membranes. In the first, a myristoylated calcium-binding mutant still associated weakly with reconstituted ROS membranes and even exhibited limited inhibition of RK (34). In the second, Rv was found in lipid rafts even in the presence of EGTA (35). These latter results, considered with our finding that the DM protein is flagellar despite not being able to bind calcium, suggest that these proteins may in fact always remain associated with the membrane in vitro and simply function as calcium sensors, associating and dissociating from their binding partners depending on their calcium-dependent conformation.

Whereas the localization of FcαBP appears to be independent of its calcium binding ability (Fig. 5), FcαBP function is certainly modulated by calcium. The GST pull-down assay identified putative partner proteins of FcαBP, which are currently under investigation. FcαBP interacts with two classes of protein in a calcium-dependent manner (Fig. 6), one only of which depends on active calcium binding by FcαBP itself. The other does not and may be responsible for flagellar retention of the FcαBP DM protein. Based on these results, we revisited our model of FcαBP regulation. A model of FcαBP activity patterned after Rv is shown in Fig. 7A. In this model, the flagellar membrane association and partner protein association of FcαBP are directly determined by calcium-binding by FcαBP. This model predicted that the FcαBP DM protein would not be localized to the flagellum, since the protein would sequester the acyl groups and lose interactions with both the flagellar membrane and the binding partner. In view of the results presented here, it seems likely that FcαBP acts as a simple calcium sensor (Fig. 7B). In this model, FcαBP associates with the flagellar plasma membrane regardless of its ability to bind calcium. It associates with two types of proteins in a calcium-dependent manner, one association dependent on active calcium binding by FcαBP and the other independent of calcium binding.

FcαBP is a novel member of the NCS protein family, because it is both myristoylated and palmitoylated (3). Equally important, FcαBP is a prototype of a growing group of flagellar calcium-binding proteins of parasites. Similar proteins have been identified in T. lewisi (13), T. brucei, the agent of African sleeping sickness (36, 37), T. rangeli (38), and Trypanosoma cruzi (39). Some of these proteins have up to eight potential EF-hand calcium-binding motifs, sites for N-terminal myristoylation and palmitoylation, and even C-terminal acylation. Characterization of these proteins will serve to expand our understanding of NCS-like proteins and may also illuminate possible drug targets.

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