Structural Insights into Membrane-targeting by the Flagellar Calcium-binding Protein (FCaBP), a Myristoylated and Palmitoylated Calcium Sensor in T. cruzi*

Jennifer N. Wingard,1# Jane Ladner,1 Murugendra Vanarotti,2 Andrew Fisher,2 Howard Robinson,3 Kathryn T. Buchanan,4 David M. Engman,4 and James B. Ames2||

1Center for Advanced Research in Biotechnology, University of Maryland, National Institute of Standards and Technology, Rockville, MD 20850,

2Department of Chemistry, University of California, Davis, CA 95616,

3Biology Department, Brookhaven National Laboratory, Upton, NY 11973-5000,

4Departments of Pathology and Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611.

Running title: Crystal structure of FCaBP.

#Current address: Department of Molecular Physiology & Biological Physics, University of Virginia, Charlottesville, VA 22908.

||To whom correspondence should be addressed: Department of Chemistry, One Shields Avenue, University of California, Davis, CA 95616, Tel (530) 752-6358, FAX (530) 752-8995, E-mail: ames@chem.ucdavis.edu

http://www.jbc.org/cgi/doi/10.1074/jbc.M803178200
The latest version is at JBC Papers in Press. Published on June 17, 2008 as Manuscript M803178200
Copyright 2008 by The American Society for Biochemistry and Molecular Biology, Inc.
The flagellar calcium-binding protein (FCaBP) of the protozoan Trypanosoma cruzi is targeted to the flagellar membrane where it regulates flagellar function and assembly. As a first step toward understanding the Ca$^{2+}$-induced conformational changes important for membrane-targeting, we report here the x-ray crystal structure of FCaBP in the Ca$^{2+}$-free state determined at 2.2 Å resolution. The first 17 residues from the N-terminus appear unstructured and solvent exposed. Residues implicated in membrane-targeting (K19, K22 and K25) are flanked by an exposed N-terminal helix (residues 26 – 37), forming a patch of positive charge on the protein surface that may interact electrostatically with flagellar membrane targets. The four EF-hands in FCaBP each adopt a “closed conformation” similar to that seen in Ca$^{2+}$-free calmodulin. The overall fold of FCaBP is closest to that of grancalcin and other members of the penta EF-hand superfamily. Unlike the dimeric penta EF-hand proteins, FCaBP lacks a fifth EF-hand and is monomeric. The unstructured N-terminal region of FCaBP suggests that its covalently attached myristoyl group at the N-terminus may be solvent exposed, in contrast to the highly sequestered myristoyl group seen in recoverin and GCAP1. NMR analysis demonstrates that the myristoyl group attached to FCaBP is indeed solvent exposed in both the Ca$^{2+}$-free and Ca$^{2+}$-bound states, and myristoylation has no effect on protein structure and folding stability. We propose that exposed acyl groups at the N-terminus may anchor FCaBP to the flagellar membrane and that Ca$^{2+}$-induced conformational changes may control its binding to membrane-bound protein targets.

Flagellar calcium-binding protein (FCaBP) is a 24-kDa highly immunogenic protein found in the flagellum of the protozoan parasite Trypanosoma cruzi (1). FCaBP contains four EF-hand calcium-binding motifs (2,3) (Fig. 1), the third and fourth (EF-3 and EF-4) of which bind calcium (4). The protein is modified at the N terminus by covalent attachment of myristate at Gly2 and palmitate at Cys4, both of which are required for association with the inner leaflet of the flagellar membrane (5). Calcium is required for stable flagellar localization as well, since FCaBP can be washed out of detergent-permeabilized trypanosomes if calcium chelators are included in the wash solutions. The N-terminal acylation and calcium-dependent membrane localization of FCaBP suggested that the protein may possess a functional calcium-acyl switch, similar to the Ca$^{2+}$-myristoyl switch observed previously for recoverin (6,7) and other members of the neuronal calcium sensor (NCS) family (8). Acyl switch proteins undergo calcium-dependent membrane association by virtue of calcium-regulated extrusion or sequestration of a myristate moiety that mediates membrane binding (9). However, an FCaBP mutant unable to bind calcium still maintains its flagellar localization, suggesting that FCaBP may not cycle on and off the membrane like some calcium acyl switch proteins (4).

The best studied calcium myristoyl switch protein is recoverin, a calcium-binding protein in retinal rod cells that inhibits rhodopsin kinase (RK) only at high Ca$^{2+}$ levels (10,11) and regulates the recovery phase of phototransduction (12,13). In the resting dark-state, recoverin binds two calcium ions and associates with retinal rod outer segment (ROS) membranes through its exposed myristoyl group (6,7). Photoexcitation of the rod cell results in a lowering of cytosolic calcium (14), causing recoverin to lose its bound calcium and adopt a conformation in which the myristoyl group becomes sequestered within a hydrophobic cleft in the protein (9). Ca$^{2+}$-free recoverin then dissociates from RK at the membrane, allowing RK to phosphorylate light-excited rhodopsin and promote receptor inactivation.
Crystal Structure of FCaBP

The calcium-myristoyl switch mechanism allows calcium regulation of two proteins (RK and rhodopsin) that do not themselves bind calcium. One major difference between FCaBP and recoverin is the presence of palmitate in FCaBP, which may or may not participate in a potential switch mechanism.

A variety of FCaBP-like proteins are found in other trypanosomes: Trypanosoma rangeli, Trypanosoma lewisi, and Trypanosoma brucei (Fig. 1). A flagellar calmodulin has also been characterized in T. brucei (15). These Ca^{2+}-binding 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 (16), and the flagellum of T. brucei is highly enriched in lipid rafts. Flagellar membrane stability and function is also controlled by the recruitment of FCaBP to the membrane surface, where it is believed to interact with a variety of membrane-bound protein targets (4).

We propose that the exposed acyl groups and N-terminal positively charged residues (K19, K22, and K25) may promote the binding of FCaBP to the flagellar membrane and that Ca^{2+}-induced protein conformational changes may control its binding to membrane-bound protein targets.

**EXPERIMENTAL PROCEDURES**

Protein expression and purification. To prepare recombinant unmyristoylated FCaBP (and Se-methionine labeled FCaBP), FCaBP tagged with a C-terminal (His)_6 tract was expressed in Escherichia coli strain BL21(DE3) carrying a derivative of the pET23d vector (Novagen) as previously described (4) grown in M9 minimal medium supplemented with or without Se-methionine, according to well-established procedures (19-21).

Recombinant unmyristoylated FCaBP for the native crystal was expressed as above in LB media and was initially purified via Ni-affinity chromatography. Peak fractions were pooled and diluted three-fold with buffer containing 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT and applied to a Q-HP HiTrap column (Amersham) at 5 ml/min. FCaBP was eluted with a 200 ml linear KCl gradient to a final concentration of 250 mM KCl. Peak fractions were pooled and purified FCaBP was dialyzed and concentrated to 10 mg/ml in 20 mM Tris, pH 7.5, 1 mM DTT in a centrifugal filter device with a 10,000 molecular weight cutoff (Amicon).

Recombinant myristoylated FCaBP was generated by co-expressing FCaBP and N-myristoyl CoA transferase (pBB131-NMT) in BL21(DE3) cells grown on M9 medium supplemented with myristic acid (10 mg/L). Myristoylated and non-myristoylated FCaBP was purified from the soluble fraction of bacterial cell lysates using Ni^{2+}-chelate affinity chromatography on a nitrioltriacetate-resin (Qiagen), according the manufacturer’s instructions. Peak fractions were then applied to an anion-exchange column (Hi-Trap DEAE-FF, Pharmacia) equilibrated in buffer A (1 mM
EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.4) and eluted with a linear salt gradient (0 to 0.2 M KCl) at flow rate of 5 ml min\(^{-1}\) over the course of 150 min. Peak fractions were concentrated to 5 ml and subjected to size-exclusion chromatography (Sephacryl S-100, Pharmacia) in buffer B (1 mM dithiothreitol, 2 mM CaCl\(_2\), 50 mM HEPES, pH 7.4). Final purity was greater than 98%, as judged by SDS-PAGE.

**Protein crystallization.** Two different native crystals were grown by the hanging drop method (22). Crystal form #1 was obtained by combining 900 µl of original well solution (3.2 M ammonium sulfate and 0.1 M MES pH 6.0) with 100 µl of OptiSalts (Qiagen) optimization solution (0.75 M magnesium chloride and 0.1 M sodium acetate pH 4.6). Crystal form #2 was obtained using a well solution containing solely 3.2 M ammonium sulfate and 0.1 M MES pH 6.0. The selenomethionine protein crystal was grown in a sitting drop with the well solution containing 3.2 M ammonium sulfate, 0.1 M MES pH 5.7. The drop was made with 6 microliters of protein (20 mg/mL) and 3 microliters of well solution. All crystallizations were at room temperature.

**X-ray crystallography.** Diffraction data for the native structure (from crystal #1) were collected using a Rigaku Micro Max 007 rotating anode generator (Rigaku/MSC, The Woodlands, TX). The crystal was cooled to 100 K with a Cryocool low temperature probe (Cryo Industries of America) and was cryoprotected by pulling the loop mounted crystal through immersion oil. The diffraction data were processed with CrystalClear/d*Trek (23). Statistics are shown in Table 1 (crystal #1). SAD (single wavelength anomalous dispersion) data for the selenomethionine protein were collected at Brookhaven National Laboratory on beamline X29 using the wavelength 0.9790 Å and processed with HKL2000 (24). When the anomalous data were processed in space group P2\(_1\), 15,413 measurements were rejected. With space group P1, only 442 reflections were rejected and the 33 Å axis was identified as the screw axis. When processed in space group P2\(_1\), 504 reflections were rejected. The anomalous signal was weak but usable to 2.6 Å. The software program, SHELXC/D/E (25,26) was used to find eight selenium sites and examine the hand of the sites. A reasonable value for the Matthews coefficient (1.8) indicated that there were two molecules in the asymmetric unit for space group P2\(_1\) or one for P2\(_1\)2\(_1\)2\(_1\). The selenium sites were used in SOLVE/RESOLVE (27,28) to produce an electron density map, and the map was examined in COOT (29) and looked good. Using the iterative script (www.solve.lanl.gov) which includes cycles of density modification and automated model building by RESOLVE and molecular refinement by REFMAC5 (30), 271 of the 422 residues were built and 104 side chains were placed. The model was completed by iteratively building in COOT and refining in REFMAC5. The model was transferred to the native data and refined in both space group P2\(_1\) and P2\(_1\)2\(_1\)2\(_1\). The model and the statistics were essentially the same. There was no compelling reason to use P2\(_1\) for the native data so the final statistics for the P2\(_1\)2\(_1\)2\(_1\) space group are shown in Table 2. The final model includes residues 17-208. The side-chains of 11 residues were modeled with double conformations and C66 was modeled as s-oxy cysteine. The sequence has four methionines, but the selenium sites seen were 122M, 145M, and a double conformation for 135M. The loop region including residues 102-113 has minimal density but the path is clear. In a Ramachandran plot, 97.8% of the residues fall into the preferred regions and 2.2% fall into the allowed regions as analyzed by COOT.

**NMR spectroscopy.** Samples for NMR analysis consisted of \(^{15}\)N-labeled FCaBP with and without a covalently attached, \(^{13}\)C-labeled myristoyl group. FCaBP protein for NMR studies (1.0 mM) was dissolved in 0.3 ml of a 95% \(\mathrm{H}_2\mathrm{O}/5\%\{\mathrm{D}\}\mathrm{H}_2\mathrm{O}\) solution containing 10 mM Tris-HCl (pH 7.4) and 2 mM EDTA (Ca\(^{2+}\)-free) or 2 mM CaCl\(_2\) (Ca\(^{2+}\)-bound). All
NMR experiments were performed at 25°C on a Bruker DRX-600 spectrometer equipped with a four-channel interface and triple-resonance probe with triple-axis pulsed field gradients; and DRX-600 spectrometer equipped with an Ultrashield Bruker magnet, a three-channel interface, and cryo-probe with Z-axis pulsed field gradients. The $^{15}$N-$^1$H HSQC spectra (see Fig. 4) were recorded on a sample of $^{15}$N-labeled FCaBP (in 95% H$_2$O, 5% D$_2$O). The number of complex points and acquisition times were: 256, 180 ms ($^{15}$N (F$_1$)); and, 512, 64 ms ($^1$H (F$_2$)). The $^{13}$C-$^1$H HMQC spectra (Fig. 5) and $^{13}$C(F1)-edited, $^{13}$C(F3)-filtered NOESY-HMQC spectra were recorded on a sample of unlabeled FCaBP protein attached to $^{13}$C-labeled myristate (31) as well as $^{13}$C-labeled FCaBP bound to unlabeled myristate (data not shown). Intermolecular NOESY experiments were performed as described previously (32).

$^{15}$N-$^1$H NOE data were measured using 2D $[^{15}$N,$^1$H]-HSQC based experiments as described previously (33). Saturation was carried out with a series of 120° $^1$H pulses separated by 5 ms delays applied during the interscan delay (3 s).

## RESULTS AND DISCUSSION

### Protein crystallization

Recombinant FCaBP crystallized in the orthorhombic space group, P2$_1$2$_1$2$_1$, with one molecule of FCaBP in the asymmetric unit. It is seen to be monomeric in the crystal, as measured in solution by NMR and dynamic light scattering analysis. The polypeptide structure derived from diffraction data on crystal #1 (in the presence of saturating Mg$^{2+}$) and crystal #2 (apo-form) appear virtually identical except for very minor differences in the EF2 loop structure (residues, 102-113), suggesting that Mg$^{2+}$ has almost no effect on the overall structure. In each crystal form, the first 17 residues from the N-terminus appear disordered. Amino acid sequencing of the protein from dissolved crystals showed that all 17 N-terminal residues remained intact in both crystal forms and therefore were not cleaved by proteolysis during crystallization.

### Three-dimensional structure

The x-ray crystal structure of Ca$^{2+}$-free FCaBP was solved at 2.2 Å-resolution, with a final R-factor of 16.95% and R-free of 21.97% (Fig. 2). The entire polypeptide chain has been traced except for the first 17 residues from the N-terminus. High temperature factors for the N-terminal residues suggest they may be dynamically disordered. NMR relaxation studies and heteronuclear $^{15}$N NOE analysis on FCaBP also confirms that the first 17 N-terminal residues are indeed unstructured (data not shown) and the N-terminal myristoyl group is solvent exposed (see below). The overall main chain structure for residues 17-208 of FCaBP determined by x-ray crystallography was consistent with and nearly identical to a much lower resolution NMR solution structure (not shown).

The protein structure visible in the crystal has a globular fold with overall dimensions of 32 Å x 23 Å x 21 Å (Fig. 2). The first structured residue in the crystal begins at A18 that marks the start of an exposed, 5-residue conformation (residues, 18-23), resembling a hook that forms a right angle with an adjacent $\alpha$-helix (residues, 24-37). Following this N-terminal helix is a compact arrangement of four EF-hands: EF1 (residues 49-77, green); EF2 (residues 98-126, red); EF3 (residues 131-159, cyan); and EF4 (residues 168-196, yellow). Overall, FCaBP contains a total of eight $\alpha$-helices and four $\beta$-strands: $\alpha$1 (residues 24-37), $\alpha$2 (residues 44-57), $\alpha$3 (residues 67-76), $\alpha$4 (residues 87-105), $\alpha$5 (residues 116-139), $\alpha$6 (149-162), $\alpha$7 (residues 169-176), $\alpha$8 (residues 186-201), $\beta$1 (residues 64-66), $\beta$2 (residues 113-115), $\beta$3 (residues 146-148) and $\beta$4 (residues 183-185) (Fig. 1). The four EF-hands of FCaBP associate into two pairs through a characteristic, short, two-stranded $\beta$-sheet arrangement: EF1 (green) pairs with EF2 (red), while EF3 (cyan) pairs with EF4 (yellow). The two pairs of EF-hands are
Crystal Structure of FCaBP

connected by a long, central helix (α5) formed by merging the exiting helix of EF2 into the entering helix of EF3. This topology causes three long, central helices (α4, α5 and α8) to interact closely as a twisted, vertical bundle. As a consequence, EF2 and EF4 make close contact with one another. This globular arrangement of EF-hands is most similar to that seen for the first four EF-hands in the penta-EF-hand proteins, grancalcin (37) and domain IV of calpain (38). However, unlike penta-EF-hand proteins, FCaBP lacks a fifth EF-hand and is not dimeric.

**EF-hands and Ca\(^{2+}\)-binding.** The individual metal-free EF-hands in FCaBP each consist of a helix-turn-helix structure similar to the “closed conformation” of EF-hands seen in previous Ca\(^{2+}\)-free structures of calmodulin (39,40), troponin C (41) and grancalcin (37). The interhelical angles for the “closed” EF-hands in FCaBP are 119º (EF1), 128º (EF2), 122º (EF3) and 120º (EF4). The binding of Ca\(^{2+}\) to each EF-hand in calmodulin causes a marked decrease in interhelical angle that promotes formation of an open conformation, leading to the exposure of many hydrophobic residues that interact with protein targets (42,43). Functional Ca\(^{2+}\) binding to EF3 and EF4 in FCaBP (4) may cause a similar Ca\(^{2+}\)-induced exposure of hydrophobic residues and might explain the observed Ca\(^{2+}\)-bound structure of FCaBP to protein phosphatase-2A (D. Engman, unpublished results) and various other protein targets (4). Indeed, Ca\(^{2+}\)-dependent membrane binding by FCaBP might arise in part by its Ca\(^{2+}\)-induced binding to protein targets localized on the flagellar membrane surface.

The 12-residue Ca\(^{2+}\)-binding loop of EF2 in FCaBP appears loosely structured in crystal form #1 and adopts an unusual, distorted conformation in crystal form #2, which may explain why EF2 is not able to bind Ca\(^{2+}\) (4). The instability and/or structural distortion of the EF2 loop may be due in part to the presence of G109 at position-3 in the binding loop, which lacks an acidic side-chain required for Ca\(^{2+}\) chelation at this key position.

The lack of Ca\(^{2+}\) binding at EF2 may also explain why Ca\(^{2+}\) is not able to bind to EF1, as the helices of EF1 and EF2 interlock to form a four-helix bundle, causing both EF-hands to be cooperatively locked in the Ca\(^{2+}\)-free closed conformation. In addition, C66 at position-9 in the EF1 loop is not able to form a hydrogen-bond with E69, which may destabilize the binding-loop structure and prevent Ca\(^{2+}\) binding at EF1.

By contrast, EF3 and EF4 both adopt a more favorable conformation for binding Ca\(^{2+}\), consistent with functional Ca\(^{2+}\)-binding measured at these sites (4). In the Ca\(^{2+}\)-binding loop of EF3, side-chain oxygen atoms from residues D140, S142, N144 and E151 are held in a cage-like arrangement surrounding a bound H\(_2\)O molecule (Fig. 2A) that resembles the binding of Ca\(^{2+}\) with the familiar pentagonal bipyramid geometry (3). For EF4, side-chain oxygen atoms from residues D177, N179, T181 and E188 are also pre-arranged with a similar geometry that surrounds a bound H\(_2\)O. In addition, the main-chain nitrogen of the sixth residue in the Ca\(^{2+}\)-binding loops of EF3 (M145) and EF4 (G182) both form hydrogen bonds with aspartate at the one-position, forming a loop conformation characteristic of Ca\(^{2+}\)-bound calmodulin and troponin C. Therefore, the Ca\(^{2+}\)-binding loops in EF3 and EF4 both adopt a favorable, pre-formed local conformation that should promote rapid and functional Ca\(^{2+}\) binding.

**Surface Properties of FCaBP.** A space-filling representation of FCaBP reveals multiple charged residues in FCaBP unevenly distributed on one side of the protein surface (Fig. 3A) with an exposed hydrophobic patch on the opposite side (Fig. 3B). Most striking is the arrangement of N-terminal Arg and Lys residues (K19, K22, K25, K28, R33, and R35) whose side-chains form a cluster of positive charge on the protein surface (highlighted blue in Fig. 3A). Some of these N-terminal basic residues (K19, K22, and K25) have been implicated previously in membrane binding (D. Engman, unpublished results). We propose that the exposed patch of N-terminal
Arg and Lys residues may promote electrostatic interactions with negatively charged head groups on the surface of the flagellar membrane. A similar electrostatic membrane interaction has also been proposed for the myristoyl switch proteins, recoverin (44) and the Src protein (45). The additional electrostatic attraction helps augment membrane anchoring by the N-terminal myristoyl group and can increase the membrane binding affinity by more than 10-fold (45,46).

The protein surface on the opposite face (Fig. 3B) contains an exposed patch of hydrophobic residues near the C-terminus (A170, A171, L172, L176, A191, W192, A195, V196, L198, A200 and highlighted yellow in Fig. 3B). The exposed hydrophobic residues are primarily located on the helices of EF4 (α7 and α8). Ca^{2+}-induced conformational changes expected in EF4 therefore might alter the arrangement and environment of these hydrophobic residues. We propose that the exposed hydrophobic patch in Fig. 3B may represent a binding site for the interaction with membrane-bound protein targets.

**NMR analysis of myristoylation.** The absence of detectable electron density from the first 17 residues of FCaBP suggested that the N-terminal region and myristoyl group may be structurally disordered. To more directly examine the structural role of the myristoyl group, we recorded two-dimensional (^{15}N-^1H HSQC) NMR spectra of both myristoylated and unmyristoylated FCaBP, demonstrating that both spectra are nearly identical (Fig. 4). Hence, the presence of the myristoyl group does not appear to affect the overall structure of Ca^{2+}-free FCaBP, in contrast to the large structural changes in Ca^{2+}-free recoverin that result from N-terminal myristoylation (17,31). The similarity in the spectra of FCaBP and myr-FCaBP suggests that the fatty acyl chain may be exposed to solvent and, hence, not interacting significantly with the protein.

NMR experiments were also performed on FCaBP containing a ^{13}C-labeled myristoyl group to directly probe the structural environment and disposition of the N-terminal myristoyl group. Previously, two-dimensional (^{1}H-^{13}C HMQC) and three-dimensional (^{13}C-filtered NOESY-HMQC) NMR experiments on samples of recoverin that contained a ^{13}C-labeled myristoyl group were used to selectively probe the chemical environment around the amino-terminal myristoyl group (31,34). These studies revealed that the covalently-attached fatty acyl chain in recoverin is sequestered in a hydrophobic pocket in the Ca^{2+}-free protein and that binding of Ca^{2+} leads to conformational changes that extrude the N-myristoyl group into solvent.

Similar NMR experiments were performed on samples of FCaBP that contained a ^{13}C-labeled myristoyl group (Fig. 5). Because the HMQC experiment selectively probes protons that are covalently attached to ^{13}C, only the methylene and methyl proton resonances of the fatty acyl chain appear in these spectra. Weak extraneous peaks near 0.9, 1.4 and 1.7 ppm (^{1}H dimension) are due to natural abundance ^{13}C signals from the protein. The spectrum of the N-myristoyl group in Ca^{2+}-free FCaBP (Fig. 5B) looks quite similar to the spectrum of free myristic acid in solution (Fig. 5A) and the resonance frequencies of corresponding peaks are nearly identical. Assignments of the myristoyl group resonances for Ca^{2+}-free FCaBP (Fig. 5B) were therefore derived from assignments of those for free myristic acid, which were determined previously (31). The similarity in the spectrum of the myristoyl group in Ca^{2+}-free FCaBP to that of free myristic acid in solution indicates that, in Ca^{2+}-free FCaBP, the fatty acyl chain is solvent-exposed, in contrast to Ca^{2+}-free recoverin where the myristoyl chain is deeply buried inside the core of the protein (9).

To further test whether the myristoyl group of FCaBP is solvent-exposed, three-dimensional (^{13}C/F) edited and (^{13}C/F) filtered NOESY experiments (31) were performed on unlabeled FCaBP protein.
containing a $^{13}$C-labeled myristate. These spectra selectively probed atoms of residues in the protein that lie within 5 Å of the labeled CH$_3$-group of the myristoyl chain. Nuclear Overhauser effect (NOE) dipolar interactions between the myristate methyl group and the protein could not be detected (data not shown). The lack of observable NOEs in this experiment suggests that the methyl group of the fatty acyl chain is more than 5 Å away from atoms in the protein, providing additional support for the conclusion that the N-myristoyl group of FCaBP is solvent-exposed and does not interact intimately with the protein.

The addition of Ca$^{2+}$ to myr-FCaBP caused no discernable change in the NMR spectrum of the myristoyl group (Fig. 5C). In addition, NOE interactions between the myristate and protein could not be detected (data not shown) in three-dimensional ($^{13}$C/$^{1}$F$_1$)-edited and ($^{13}$C/$^{1}$F$_3$)-filtered NOESY spectra of Ca$^{2+}$-bound $^{13}$C-myr-FCaBP, suggesting that the methyl group of the fatty acyl chain does not interact closely with the Ca$^{2+}$-bound protein. Hence, the myristoyl group of Ca$^{2+}$-bound FCaBP appears solvent-exposed and does not undergo a calcium-induced change in environment, in contrast to what has been observed previously in recoverin (31).

**Structural relationship with PEF proteins.** The N-terminal myristoylation and four EF-hands in FCaBP would suggest that it might be structurally similar to recoverin (9,47) and related Ca$^{2+}$-myristoyl switch proteins (48,49). However, the overall three-dimensional structures of FCaBP and recoverin are surprisingly quite different and unrelated. Instead, the overall main chain fold of Ca$^{2+}$-free FCaBP is most similar to that of apo-grancalcin (37) and related proteins that belong to the penta EF-hand (PEF) family (50). Interestingly, FCaBP bears very little sequence homology to the PEF proteins (< 20% identity). The root-mean-square deviation (RMSD) is 3.5 Å when comparing the main chain atoms of the four EF-hands of FCaBP with those of the first four EF-hands of grancalcin (Fig. 6). The structural similarity is even more striking when comparing the main chain atoms from EF1, EF3 and EF4 (RMSD = 2.9 Å), whereas the structure of EF2 is much more divergent between the two (RMSD = 4.5 Å). The second helix of EF2 and first helix of EF3 in FCaBP are merged into a long, 23-residue helix ($\alpha$5) that is flanked in an anti-parallel fashion by helices, $\alpha$4 and $\alpha$8, forming a twisted bundle. A similar helical bundle and overall topology is also seen in grancalcin and seems to be a structural hallmark of all PEF proteins. The similarity of the main chain conformations between FCaBP and PEF proteins suggests that their functions might be related. Indeed, grancalcin and PEF proteins exhibit Ca$^{2+}$-induced membrane binding (51-53) and interact with target proteins such as L-plastin (54), annexins (55) and integrins (56) implicated in cytoskeletal dynamics. FCaBP also binds to membranes and has been shown recently to interact with several candidate target proteins (4). Future studies are needed to determine whether any of the FCaBP target proteins might be related to L-plastin or other PEF protein targets that regulate cytoskeletal adhesion and migration.

An important structural difference between FCaBP and PEF proteins is that FCaBP is monomeric in solution and does not have a fifth EF-hand like the dimeric PEF proteins. The fifth EF-hand of FCaBP may have been deleted during evolution perhaps to allow for the exposure of hydrophobic residues in EF4 (Fig. 3B) that otherwise would be inaccessible if the fifth EF-hand were present. Another difference is the presence of the lipophilic N-terminal extension (LNT) region in PEFs that have variable Gly-Pro-Ala sequences implicated in membrane targeting (57). By contrast, FCaBP has conserved basic residues (K19, K22, K25) in the N-terminal region that we suggest might be important for membrane-binding in addition to N-terminal myristoylation and palmitoylation sites (Figs. 1 and 7). We propose that the four EF-hands of FCaBPs are evolutionarily related to the first four EF-hands of PEF proteins, and that
FCaBPs may represent a sub-branch of the PEF family that lack a fifth EF-hand and have N-terminal myristoylation and palmitoylation membrane-targeting motifs instead. It will be interesting to look for other examples of truncated PEF proteins that are structurally related to FCaBP and perhaps serve as myristoylated, membrane-targeting calcium sensors.

Membrane-targeting mechanism. The crystal structure of FCaBP (Fig. 2) has implications for its Ca\textsuperscript{2+}-induced membrane-targeting mechanism (Fig. 7). The solvent exposed myristoylation (and presumably palmitoylation (5)) sites at the N-terminus suggest that the covalently attached fatty acyl groups serve as membrane anchors that insert inside the lipid bilayer. In addition, exposed basic residues in the N-terminal region (Fig. 3A) are suggested to provide additional binding energy by interacting electrostatically with negatively charged headgroups on the membrane surface. A similar combination of electrostatic effects and myristoylation was seen previously for membrane binding by the myristoyl-electrostatic switch of Src and MARCKS protein (45).

Our NMR analyses (Figs. 4-5) indicate that the fatty acyl group is exposed regardless of Ca\textsuperscript{2+} concentration, suggesting that both the Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound forms of FCaBP may be anchored to the flagellar membrane (Fig. 7). But how then is membrane binding by FCaBP regulated by calcium? We propose that the Ca\textsuperscript{2+} sensitive localization of FCaBP at the flagellar membrane could be controlled by Ca\textsuperscript{2+}-dependent binding of FCaBP to various target proteins. Previous studies have identified candidate target proteins that differentially bind to either the Ca\textsuperscript{2+}-free or Ca\textsuperscript{2+}-bound forms of FCaBP (4). The binding of Ca\textsuperscript{2+} to calmodulin and other EF-hand proteins generally leads to the exposure of hydrophobic residues that promotes their binding to protein targets (42,43). A similar Ca\textsuperscript{2+}-dependent exposure of hydrophobic residues in EF4 (Fig. 3B) might promote association of Ca\textsuperscript{2+}-bound FCaBP with a membrane-bound target protein and preferentially localize FCaBP at the flagellar membrane at high Ca\textsuperscript{2+} levels (Fig. 7B). Alternatively, the Ca\textsuperscript{2+}-free state of FCaBP might preferentially bind to a cytosolic target protein that would localize FCaBP in the cytosol at low Ca\textsuperscript{2+} levels (Fig. 7A). The binding of a cytosolic target protein to Ca\textsuperscript{2+}-free FCaBP might help to sequester the N-terminal fatty acyl groups inside a protein environment (perhaps involving exposed hydrophobic residues in EF4 (Fig. 3B)) like that seen for Ca\textsuperscript{2+}-free recoverin (17) or GCAP1 (18). Sequestration of the myristoyl group would stabilize cytosolic FCaBP and therefore promote its membrane dissociation only at low Ca\textsuperscript{2+} levels. Lastly, reversible cleavage of the labile palmitoyl group attached to FCaBP (by a thioester linkage) may further modulate its membrane anchoring capacity. The enzyme catalyzed cleavage of palmitate may also be regulated by Ca\textsuperscript{2+}.

In short, both the Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound FCaBP have exposed myristoyl groups (Fig. 5) that we propose anchor the protein to the flagellar membrane. Thus, FCaBP does NOT exhibit Ca\textsuperscript{2+}-induced extrusion of the myristoyl group to control membrane localization like what is seen for recoverin (6) and related Ca\textsuperscript{2+}-myristoyl switch proteins (58). Instead, we propose that Ca\textsuperscript{2+}-induced protein conformational changes in FCaBP (4) may modulate its interaction with target proteins that control membrane localization as depicted in Fig. 7. Future structural studies on Ca\textsuperscript{2+}-bound FCaBP bound to various target proteins are needed to further test and refine the proposed membrane-targeting mechanism.
REFERENCES

Crystal Structure of FCAbP

FOOTNOTES

*This work was supported by grants EY012347 and NS045909 (J.B.A.), RR11973 (UC Davis NMR Facility) and AI46781 (D.M.E.) from the National Institutes of Health.

The atomic coordinates and structure factors (code 3CS1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

1The abbreviations used are: FCaBP, flagellar calcium binding protein; HSQC, heteronuclear single quantum coherence; HMQC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; RMSD, root-mean-squared deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ACKNOWLEDGMENTS

We thank Jeff de Ropp for help with NMR experiments, Dr. Frits Abildgaard for providing NMR pulse-sequence programs, John Cieslak for producing the E. coli strains coexpressing FCaBP and yeast N-myristoyltransferase, and Frank Delaglio and Dan Garrett for writing computer software for NMR data processing and analysis. Certain commercial equipment, instruments, and materials are identified in this paper in order to specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.
Table 1.
Data Statistics Table.

<table>
<thead>
<tr>
<th></th>
<th>native</th>
<th>SeMet</th>
</tr>
</thead>
<tbody>
<tr>
<td>space group</td>
<td>P2,2,1</td>
<td>P2_1</td>
</tr>
<tr>
<td>cell parameters (a,b,c) (Å)</td>
<td>32.91,37.67,141.33</td>
<td>37.71,32.91,140.88</td>
</tr>
<tr>
<td>cell parameters (α,β,γ) (°)</td>
<td>90.0,90.0,90.0</td>
<td>90.0,90.0,90.0</td>
</tr>
<tr>
<td>wavelength of data collection (Å)</td>
<td>1.541</td>
<td>0.979</td>
</tr>
<tr>
<td>no. of measured intensities</td>
<td>53,305</td>
<td>52,133</td>
</tr>
<tr>
<td>no. of unique reflections</td>
<td>12,558</td>
<td>18,011</td>
</tr>
<tr>
<td>resolution of data (Å)</td>
<td>29.8-2.0</td>
<td>30.0-2.1</td>
</tr>
<tr>
<td>highest resolution shell (Å)</td>
<td>2.07-2.0</td>
<td>2.18-2.10</td>
</tr>
<tr>
<td>R_sym (overall/high resolution shell)</td>
<td>0.070/0.308</td>
<td>0.059/0.261</td>
</tr>
<tr>
<td>completeness (%) (overall/high resolution shell)</td>
<td>99.8/97.9</td>
<td>87.3/45.6</td>
</tr>
<tr>
<td>redundancy (overall/high resolution shell)</td>
<td>4.2/4.1</td>
<td>5.9/2.4</td>
</tr>
<tr>
<td>mean I/σ (overall/high resolution shell)</td>
<td>10.9/4.1</td>
<td>11.1/2.5</td>
</tr>
</tbody>
</table>

Table 2.
Refinement Statistics Table

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>resolution limits (Å)</td>
<td>20.0-2.0</td>
</tr>
<tr>
<td>number of reflections used/for R_free</td>
<td>11,896/600</td>
</tr>
<tr>
<td>R-factor (overall/high resolution shell)</td>
<td>0.211/0.251</td>
</tr>
<tr>
<td>R_free (overall/high resolution shell)</td>
<td>0.289/0.339</td>
</tr>
<tr>
<td>number of water molecules</td>
<td>65</td>
</tr>
<tr>
<td>rms deviation bond length (Å)</td>
<td>0.019</td>
</tr>
<tr>
<td>rms deviation angle (°)</td>
<td>1.73</td>
</tr>
<tr>
<td>average B main chain/side chain/water (Å²)</td>
<td>30.3/31.7/32.4</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Primary and secondary structure features of FCaBP.
Alignment of the primary sequence of T. cruzi FCaBP with that of T. rangeli and T. brucei. Secondary structural elements indicated schematically were derived from the xray structure and analysis of NMR data ($^3J_{HNH}$, chemical shift index (59), and sequential NOE patterns). The four EF-hands (EF1, EF2, EF3 and EF4) are highlighted green, salmon, cyan, and yellow, respectively. Residues in the 12 residue Ca$^{2+}$-binding loops are underlined and chelating residues are highlighted bold. Invariant basic residues on the protein surface and implicated in membrane binding are colored blue.

Figure 2. X-ray crystal structure of FCaBP (PDB accession no., 3CS1).
(A) Representative electron density map of FCaBP, showing the Ca$^{2+}$-binding loop of EF3. A bound water molecule (red sphere) is present in the EF3 Ca$^{2+}$-binding site of apo FCaBP. (B) Ribbon diagrams depicting the main chain structure of FCaBP viewed from the membrane-binding interface (top) and rotated 180$^\circ$ (bottom). The EF-hands are colored as in Fig. 1.

Figure 3. Surface representation of FCaBP protein structure.
Space-filling models of FCaBP depicting (A) positively charged, membrane-binding interface, and (B) exposed hydrophobic surface. The orientations are similar to those in Fig. 2B. Exposed hydrophobic, basic and acidic residues are highlighted in yellow, blue and red, respectively.

Figure 4. NMR spectral analysis of myristoylated and unmyristoylated FCaBP.
Two-dimensional ($^{15}$N-$^1$H HSQC) NMR spectra of $^{15}$N-labeled unmyristoylated (black) and myristoylated FCaBP (red) in the Ca$^{2+}$-free state. Peaks corresponding to the -NH$_2$ groups of the side-chain amides of Gln and Asn residues are connected by dotted lines. Sequence-specific assignments are indicated.

Figure 5. The N-terminal myristoyl group of FCaBP appears to be solvent-exposed.
Two-dimensional ($^1$H-$^{13}$C HMQC) NMR spectra of free myristic acid dissolved in chloroform (A) and the myristoyl group of Ca$^{2+}$-free (B) and Ca$^{2+}$-bound (C) FCaBP. The myristoyl group was labeled with carbon-13 and the protein was unlabeled. The peaks in the spectrum represent protons attached to $^{13}$C-labeled fatty acyl chain.

Figure 6. Structural comparison of FCaBP with penta EF-hand proteins.
The main chain atoms of the EF-hand regions of FCaBP (red, 3CS1.pdb) and first 4 EF-hands of apo-grancalcin (blue, 1F4Q.pdb) are superimposed (3.5 Å RMSD).

Figure 7. Model of membrane-target recognition by FCaBP.
Schematic diagram of FCaBP binding to membrane and protein targets at low (A) and high (B) Ca$^{2+}$ levels. Myristoyl and palmitoyl groups highlighted magenta; EF-hands colored as in Fig. 1; N-terminal Arg and Lys residues colored blue; and cytosolic and membrane-bound target proteins shown in gray.
T. cruzi  MGACGSKGS--SDKGRKKAKDRKEAWERIQAIPEKTAEEKQRRLOLFKFP
T. rangeli  MGACGSKGS-----AGNKDGKSATVRKVAWERIRQVIPREKTAERERRIDLFKFP
T. brucei MGCSGSKNASPKDGAASKGGKDGKTADAHRKVAWERIRCAIPRDKDAEKSRRRIELFKQP

T. cruzi  DKTGKLCADEVHGCEVLKLEFTRVDRITKRAFDKARALSGKLENKGSEDFVEFL
T. rangeli  DKNLGKLSEDEVYSGCEVLKLDEFTPRVH-ITKRAFNKAKDGKSKLENKGSEDFVEFL
T. brucei  DLTGKLGFREVLDCYSILKLDEFTTHLPDIVQKAFDKAKLGKVNKVGVEEDLVEFL

T. cruzi  EFRLMLCYIYDFELTVMFDEIADSLMLVDEEFELKRAVPKLEAWGAKVDDPAALFKELD
T. rangeli  EFRLMLCYLDFELTVMFDEIDTSNLMLDEAFVEKAVPKLEQWGAIELEDPEAVKELD
T. brucei  EFRLMLCYIYDFELTVIFDTMDKGSLLLEGFKEALPKLKYGVIDTDAEVTNEID

T. cruzi  KNGTSVFPEFAAWASAVKLDAGPADNVPESA 211
T. rangeli  RNSGSVFPEFAAWASARKLDVGDPDNVPESA 205
T. brucei  TNGSVVFPEFSCHWAVTTLQGSDPDDENEGANEDGANAGDGVPAAEAS 233

Fig. 1
Fig. 2A.
Fig. 2B
Fig. 3
Fig. 4

Crystal Structure of FCaBP
Fig. 5
Fig. 6
Fig. 7