Identification of calcium binding sites in the trypanosome flagellar calcium-acyl switch protein

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Abstract

The 24 kDa flagellar calcium binding protein (FCaBP) of the protozoan Trypanosoma cruzi is a calcium-acyl switch protein. FCaBP is modified by the addition of myristate and palmitate at its amino terminal segment and both modifications are required for calcium-modulated flagellar membrane association. FCaBP has four sequence motifs for potential calcium binding, and comparison to other calcium-acyl switch proteins, such as recoverin, suggested that only two of these sites are functional. Because it is not possible to predict with certainty the calcium binding affinity or selectivity based on motif analysis alone, we determined the quantitative calcium binding activity of FCaBP by direct ligand binding using the flow dialysis method. The results demonstrated the presence of two calcium binding sites in the full length FCaBP and in a mutant (FCaBP D12) lacking the amino terminal pair of sites. FCaBP D12 retains its ability to localize to the flagellum. A mutant FCaBP lacking the two carboxyl-terminal sites (FCaBP D34), did not bind calcium with high affinity and selectivity under the conditions used. The calcium binding properties of FCaBP are therefore distinct from other myristoyl switch proteins such as recoverin. The results add to a growing body of knowledge about the correlation of sequence motifs with calcium binding activity. Moreover, they demonstrate the need to determine the apparently novel mechanism by which FCaBP undergoes calcium modulated flagellar membrane association and its relation to calcium signal transduction. © 1999 Elsevier Science B.V. All rights reserved.

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Abbreviations: CaBP, calcium-binding protein; FCaBP, 24-kDa flagellar calcium-binding protein.
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1. Introduction

The central role of calcium as a regulator of cellular activity is well established [1,2]. Cell division, motility, gene expression and secretion are regulated by calcium ions and the intracellular calcium concentration must be precisely controlled during these processes. In the protozoan Trypanosoma cruzi, parasite proliferation and differentiation [3] and host cell invasion [4,5] are regulated by calcium, and calcium-modulated cell signaling pathways similar to those of mammalian cells are present [6]. Furthermore, T. cruzi possesses a highly specialized calcium-containing organelle, the acidocalcisome [7], which is believed to permit the amastigote stage of the parasite to survive in the low calcium environment of the mammalian cell cytoplasm in which it replicates [8]. Calcium is clearly an important modulator of parasite metabolism.

Intracellular calcium signals are transduced into biological responses through their interaction with calcium binding proteins (CaBPs) or calcium binding domains in multidomain proteins. T. cruzi has a variety of proteins in this class of signal transducing molecules. For example, calmodulin [9] and its associated calmodulin-binding proteins [10] and calmodulin regulated enzymes [11,12] have been reported. A flagellar calcium binding protein (FCaBP) with a computed mass of 24 kDa, is found in the flagellum of T. cruzi [13] and other trypanosomatids [14–16], and is highly immunogenic [17–19]. FCaBP specifically localizes to the flagellar plasma membrane via amino terminal myristoyl and palmitoyl modifications [20]. These acyl modifications are necessary, but not sufficient, for localization. Calcium binding is also required. For example, when calcium is chelated with EGTA in detergent-permeabilized trypanosomes, FCaBP loses its flagellar membrane association. Thus, FCaBP is a calcium-acyl switch protein: a lipoprotein whose membrane association is modulated by its calcium binding state [21].

The best studied of the calcium-acyl switch proteins is recoverin, a myristoylated calcium sensor in the retinal rod cells of vertebrates that functions in cellular recovery from photoexcitation [22]. Recoverin has four calcium binding sequence motifs, but binds only two calcium ions [23]. In the 3-D structure of recoverin determined by X-ray crystallography [24], a single calcium is bound in the third calcium binding motif from the amino terminus. A more thorough NMR analysis showed that both motifs two and three bind calcium [25]. Recoverin associates in a calcium-dependent manner via its amino terminal myristoyl group with the plasma membrane, where it binds to and inhibits the activity of rhodopsin kinase [26]. When the intracellular calcium levels drop upon retinal cell photoexcitation, the calcium dissociation from recoverin results in a conformational change and sequestration of the myristoyl group in a hydrophobic cleft [27]. Unable to associate with the membrane, recoverin moves into the cytoplasm, leaving rhodopsin kinase to phosphorylate and inactivate rhodopsin, the first step in the cellular recovery phase. When the intracellular calcium increases, recoverin assumes its calcium-bound form and returns to the membrane, where it again inactivates rhodopsin kinase, completing the cycle. Both recoverin and FCaBP: (i) possess four calcium binding motifs but only bind two molecules of calcium; (ii) are myristoylated at the amino terminus; and (iii) display calcium-modulated membrane association (calcium-acyl switch). The present study was undertaken to determine which of the four putative sites in FCaBP bind calcium. This is a critical first step in our understanding of the protein’s calcium-acyl switch mechanism and how it compares with that of recoverin.

2. Materials and methods

2.1. Parasites

Epimastigotes of the Silvio X-10/4 clone of T. cruzi were used for all studies and maintained at 26°C in supplemented liver digest neutralized tryptose medium (LDNT + ) as described [28].
2.2. Engineering of recombinant FCaBP and FCaBP deletion mutants

The FCaBP coding region was produced by DNA amplification from a previously described cDNA clone [29] using a sense primer containing an NdeI restriction site, 5’-CATATGGGT-GCTTGTTGGGTCG-3’, and an antisense primer containing an XhoI restriction site, 5’-GTC-GAGCCCGCTCTCCGGCACG-3’. The PCR product was cloned into the pT7 Blue T vector (Novagen, Madison, WI) and directionally subcloned into NdeI + XhoI-digested pET23b (Novagen). The termination codon was eliminated during amplification, allowing expression of an FCaBP fusion protein containing a carboxyl terminal hexahistidine tag. Two FCaBP deletion mutants were engineered in a similar manner. The Δ12 mutant was constructed from two PCR products, one containing the 5’ end of the coding sequence up to the beginning of EF-1 (sense primer containing an NdeI site as described above and antisense primer containing an EcoRI site, 5’-GAATTCCTGTTTTGCGGTG-3’) and another extending from the beginning of EF-3 through the end of the coding region (sense primer containing an EcoRI site, 5’-GAATTCAGCTGACGGTGATGTTCG-3’), and XhoI-containing antisense primer described above. The Δ34 mutant was constructed from two PCR products, one containing the 5’ end of the coding sequence up to the end of EF-2 (NdeI-containing sense primer described above and antisense primer containing an SalI site, 5’-GTCGACGTGACAGCATCACGAGCG-3’) and another beginning directly downstream of EF-3 and extending through the end of the coding region (sense primer containing a SalI site, 5’-GTCGACAACTGGACGCGCACGGC-3’, and XhoI-containing antisense primer described above). All PCR products were cloned into the pT7 Blue T vector and directionally subcloned into NdeI + XhoI-digested pET23b. DNA constructs were sequenced on both strands using the dye-deoxy chain termination method [30].

2.3. Expression and purification of recombinant proteins

pET23b constructs were introduced into the Escherichia coli strain BL21 (DE3) (Novagen) for expression. Bacterial cultures were grown in LB medium containing 100 µg/ml ampicillin at 37°C in a shaking incubator overnight. Cultures were diluted 1:5 in LB + ampicillin and grown at 30°C to an OD_{600} of 0.6, at which time α-1,4-β-D-isopropylthiogalactopyranoside was added to a final concentration of 0.6 mM. After an additional 3 h of growth at 30°C, the cultures were placed on ice for 5 min and bacterial cells were harvested by centrifugation at 5000 x g for 5 min at 4°C. The cells were suspended in 0.2 pellet vol. of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9, 0.1 mM phenylmethylsulfonylfluoride) and sonicated in 5 s pulses, on ice, for 10 min using an ultrasonic processor. Another 0.2 vol. of binding buffer was added to the sonicate and the solution was clarified by centrifugation at 18 000 x g for 20 min at 4°C and passed through a 0.22 µm Millipore filter. Recombinant proteins were isolated by affinity chromatography on His-Bind™ resin (Novagen) according to the manufacturer’s instructions, and dialyzed for ≈ 20 h against 2 x 1 l changes of 20 mM Tris–HCl, pH 7.5, 1 mM DTT. Proteins were analysed by denaturing SDS-PAGE [31] and immunoblotting [32] using standard methods.

2.4. Calcium binding assays

Protein samples were prepared and analysed by 45Ca²⁺ flow dialysis essentially as described [33]. Briefly, the amino terminal sequences of purified, decalcified recombinant proteins were determined to assure polypeptide chain integrity and concentrations were assessed by amino acid composition analysis. This is the most accurate method of protein determination available and is the key to accurate determination of stoichiometry in such studies. The sample in the upper chamber of the apparatus had a total volume of 400 µl, and contained 20–50 µM protein in Buffer A (50 mM HEPES, pH 7.5, 5 mM MgCl₂, 150 mM KCl, 15 mM NaCl). At the start of each experiment, 2–3
μCi of radioactive calcium was added (ICN Pharmaceuticals, Costa Mesa, CA; specific activity 0.62 Ci/mmol). Unlabeled CaCl$_2$ was added to the flow dialysis chamber every 1.5 min (made up from calibrated 100 mM CaCl$_2$ solution, Radiometer, Copenhagen, Denmark). The lower chamber was perfused at a flow rate of 3 ml/min and 1 ml fractions were collected. Radioactivity was determined, after addition of 10 ml of Ecoscint A (National Diagnostics, Atlanta, GA), using a Beckman 6500 liquid scintillation counter. No quenching corrections were applied. For each given total calcium concentration, free and bound calcium was estimated, and mol calcium bound per mol protein was plotted versus free calcium concentration.

### 2.5. Generation of T. cruzi transfectants expressing myc-tagged FCaBP proteins

The T. cruzi episomal expression vector pTEX-9E10 [34] was used for all studies. DNA fragments encoding FCaBP and FCaBP deletion mutants were directionally cloned into pTEX-9E10 for expression in transfected parasites as fusion proteins containing carboxyl terminal c-myc epitope tags. T. cruzi epimastigotes were washed once with 132 mM NaCl, 8 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 0.5 mM Mg(C$_2$H$_3$O$_2$)$_2$·4H$_2$O, 0.1 mM CaCl$_2$, pH 7.0, and resuspended in this buffer at a density of 1 × 10$^8$ ml. A 400 µl volume of the cell suspension was placed into a 0.2 cm electrode gap cuvette (BioRad, Hercules, CA) with 15–25 µg of supercoiled plasmid DNA and pulsed 4 times at 0.3 kV and 500 µF using a BioRad Gene Pulser. Electroporated cells were placed in 5 ml LDNT$^+$ and G418 (0.5 mg/ml) was added 48 h later. Drug-resistant lines developed subsequently and were available for study after approximately 6 weeks. Proteins were fractionated by electrophoresis on reducing 12% SDS polyacrylamide gels [31] and analysed by Coomassie blue staining and western blotting [32] using FCaBP-specific antiserum [17]. Blots were developed using alkaline phosphatase-conjugated goat antimouse Ig (G + M) (Caltag Laboratories, San Francisco, CA) and 5-bromo-4-chloro-3-indolyl phosphate (United States Biochemical, Cleveland, OH).

### 2.6. Immunofluorescence microscopy

T. cruzi transfectants expressing the various FCaBP proteins were isolated by centrifugation, washed in PBS and resuspended in PBS at a density of 1 × 10$^6$ cells/ml. Twenty microliters of this suspension was added to each well of a printed slide (Cel-Tek, Glenview, IL), air dried, fixed at −20°C in anhydrous methanol for 30 min, and incubated in blocking buffer (1% bovine serum albumin, 2% normal goat serum in PBS) for 30 min. Slides were incubated with c-myc specific, 9E10 monoclonal antibody hybridoma supernatant (ATCC # CRL1729, myc 1-9E10.2) overnight at 4°C. Cells were washed with PBS and incubated for 3–4 h with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat-antimouse Ig (G + M) (Caltag Laboratories, San Francisco, CA). Slides were then mounted in medium containing 10% polyvinyl alcohol (Air Products and Chemicals, Allentown, PA), 25% glycerol, 0.1 M Tris–HCl (pH 8.5), and 2.5% 1,4-diazobicyclo[2.2.2]octane (Sigma, St Louis, MO) and viewed by fluorescence microscopy.

### 3. Results

#### 3.1. Determination of the calcium binding activity of wild type and mutant FCaBPs

A previous calcium binding study using a relatively crude $^{45}$Ca$^{2+}$ overlay method indicated that FCaBP binds 2 mol of calcium per mol protein [13]. To predict which of the four putative binding sites binds calcium with comparative selectivity and high affinity, we analyzed the sequence of FCaBP using the recently updated EF-hand sequence motif in the PROSITE database [35] (Fig. 1). EF-1 and EF-4 conform to the binding consensus. EF-2 is missing the expected calcium ligating side chains, but contains a methionine residue at position 6 [36,37]. To test the hypothesis that FCaBP has only one calcium binding site in each half of the molecule, we
produced recombinant FCaBP and two FCaBP deletion mutants and analysed them using a direct calcium binding method. Pairs of sites were deleted because the prevailing helix-loop-helix motifs generally demonstrate high affinity and selective calcium binding activity only when they occur in pairs [38,39]. The unique amino and carboxyl terminal regions were preserved in both deletion mutants (Fig. 2A) and carboxyl terminal hexahistidine tags were added to facilitate purification. Production of the full-length protein (WT) and each of the deletion mutants (Δ34 and Δ12) in E. coli and purification by nickel affinity chromatography yielded protein of 85–95% purity that reacted with FCaBP-specific antiserum (Fig. 2B). The molecular masses of the proteins were predicted to be 25, 18 and 16 kDa, for the WT, Δ34 and Δ12 proteins, respectively; each migrates with a slightly larger mass on an SDS gel, as observed previously for native FCaBP [17].

FCaBP has two sites which bind calcium with high affinity and selectivity in the presence of physiological concentrations of magnesium (Fig. 3A). Surprisingly, the binding curves for the wild type and Δ12 mutant FCaBP proteins are very similar, indicating that FCaBP binds calcium through EF-3 and EF-4. Both proteins bind two mols of calcium per mol of protein, while no binding activity was detected for the Δ34 mutant under these conditions. Therefore, the experimental results do not bear out the predictions of the most current EF-hand motif and emphasize the need to test experimentally the predictions based solely on motif analyses of open reading frames. In addition, since recoverin binds calcium through sites EF-2 and EF-3, the results suggest that

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**Fig. 1.** Putative calcium-binding sites in FCaBP and recoverin. The amino acid sequence of FCaBP is shown with its four EF-hand sequence motifs (shaded) aligned with the four EF-hand motifs of recoverin below. Consensus amino acids for selected positions in the EF-hand motif are: D (position 1); D, N, or S (position 3); D, E, N, S, T, or G (position 5); any amino acid except G or P (position 7); E or D (position 12); I, L, V, M, F, Y, or W (position 13). Calcium ligating residues in the calcium binding loop are indicated. Amino acid substitutions (black highlights) at any of these critical positions may eliminate calcium binding. The Δ12 protein contains FCaBP amino acids M1-F57, EF (derived from an EcoRI cloning site), E141-A211, and hexahistidine tag. The Δ34 sequence contains FCaBP amino acids M1-F140, VE (derived from a SalI cloning site), K197-A211, and hexahistidine tag.
possible that the structural alteration in FCaBP resulting from deletion of the amino terminal sequence motifs might alter myristoyl/palmitoyl sequestration either positively (i.e. prevent membrane localization) or negatively (i.e. lead to constitutive membrane localization). Staining with FCaBP and recoverin differ fundamentally in their calcium-acyl switch mechanisms.

3.2. Localization of the Δ12 mutant FCaBP protein

To determine whether the calcium binding Δ12 mutant protein displays the same flagellum-specific localization in the parasite as does the native protein, *T. cruzi* epimastigotes were transfected with constructs encoding myc-tagged full-length (WT-Myc) and mutant (Δ12-Myc) proteins and analysed by immunofluorescence microscopy (Fig. 3B). Although we have previously shown that the amino terminal 24 amino acids are necessary and sufficient for flagellar localization [20], it remained
the tag-specific monoclonal antibody showed that both proteins localize to the flagellum, indicating that deletion of the amino terminal set of sequence motifs does not result in constitutive acyl sequestration. Upon calcium chelation, the wild type protein loses flagellar membrane localization [20], the defining feature of a calcium switch. A similar analysis of the Δ12 mutant gave ambiguous results, with some cells showing flagellar staining upon calcium chelation and others showing a lack of staining (not shown). More conclusive determination of the role for each sequence motif awaits analysis of FCaBP point mutants, especially those in motifs three and four.

4. Discussion

Despite the fact that the first sequence motif of FCaBP conforms to the binding consensus, calcium binding could not be detected to the Δ34 mutant that contains this site. Direct $^{45}\text{Ca}^{2+}$ binding data of full-length and Δ12 mutant proteins demonstrated that only FCaBP proteins containing motifs three and four have calcium binding activity. Based on precedents with calmodulin, it is possible that the calcium binding activity of motif one in FCaBP is suppressed by defects in sequence motif two. Specifically, it was shown that a point mutation in one calcium binding site of calmodulin can suppress the calcium binding activity of the neighboring site [37,40]. Regardless, it is clear from the data presented here that the calcium sites used by FCaBP and recoverin are distinct and only a subset of the those identified by amino acid sequence motif analysis. Even if both proteins evolved from a common ancestor, the sites used for the calcium-acyl switching differ. Like recoverin, FCaBP is likely to transduce calcium signals through differential engagement of partner proteins. In the case of recoverin, rhodopsin kinase has been identified as one potential target protein which is negatively regulated by interaction with the acyl switch protein [26]. Such a partner has yet to be found for FCaBP, but likely candidates are protein kinases or other proteins that need to be regulated in a calcium regulated fashion. If a binding part-

ner for FCaBP that is unique to the parasite can be found, then it or FCaBP might be novel drug discovery targets.

Considered together with our recent study [20], the results presented in this paper suggest a model for calcium-regulated flagellar membrane localization of FCaBP (Fig. 4). FCaBP possesses at its amino terminus a consensus sequence for coupled myristoyl/palmitoyl modification, the former occurring cotranslationally and the latter at a membrane site where the palmitoyltransferase enzyme is believed to reside. Calcium binding to EF-3 and EF-4 triggers a switch to an open conformation in which the myristate is extruded from a hydrophobic cleft, thereby permitting transient membrane association. Palmitoylation then provides the additional hydrophobic group that stabilizes membrane localization and, possibly, calcium binding [41]. Thus, FCaBP can be regulated by two distinct, but complementary mechanisms—calcium-acyl switching and dynamic palmitoylation. In this regard it is unique among all acylated CaBPs described to date. It is possible that both fatty acids are sequestered in the calcium-free state, although this may be difficult to demonstrate since the lability of the thioester linkage of palmitate hinders in vitro study. Regardless, if FCaBP functions in a manner similar to that of recoverin, we would predict that it binds to a flagellar membrane protein in the ‘resting’, calcium-bound state and dissociates from that protein under conditions of low intracellular calcium. Where and how this may occur during the T. cruzi life cycle or cell cycle are not known. Although the host cell cytoplasm provides a calcium-poor environment for the T. cruzi amastigote, the parasite’s acidocalcisome maintains calcium homeostasis [7]. Indeed, FCaBP is expressed in the amastigote, where it is localized to the small flagellar remnant [20]. It is also possible that FCaBP regulation does not occur in a life cycle or cell cycle stage-specific manner, but that it occurs on a much smaller time frame, such as the flagellar beat cycle.

One of the most interesting aspects about FCaBP is its flagellar membrane localization and apparent exclusion from the pellicular (cell body) membrane. This may occur because the chemical composition of the flagellar membrane is uniquely
suitable for insertion of FCaBP’s acyl groups; i.e. the flagellar membrane constitutes a unique domain or functional ‘raft’, as has been described in mammalian cells [42]. Alternatively, the palmitoyltransferase may be associated only with the flagellar membrane. Finally, localization may be mediated by association with a flagellum-specific binding partner, which may be mediated by a hexapeptide identified common to a variety of flagellar membrane proteins in trypanosomatids, including FCaBP (R/K S/T A/G S/T S/T S/T, FCaBP amino acids 6–11; E.L. Snapp and S.M. Landfear, personal communication). Whether the latter possibility is true remains to be determined, but the cumulative work on recoverin and other CaBPs strongly suggests that FCaBP functions as a calcium sensor, regulating the function of its target at its flagellar membrane or nonmembrane location.

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References


