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Flagellar protein localization mediated by a calcium–myristoyl/palmitoyl switch mechanism

Lisa M.Godsel and David M.Engman[1]

Departments of Microbiology–Immunology and Pathology, Northwestern University Medical School, Chicago, IL 60611, USA

[1]Corresponding author
e-mail: d-engman@nwu.edu

The mechanisms by which proteins are targeted to flagella and cilia are poorly understood. We set out to determine the basis for the specific localization of a 24 kDa flagellar calcium-binding protein (FCaBP) expressed in all life cycle stages of *Trypanosoma cruzi*. Through the study of trypanosome transfectants expressing various FCaBP deletion mutants, we found that the N-terminal 24 amino acids of the protein are necessary and sufficient for flagellar localization. Subsequent experiments revealed that FCaBP is myristoylated and palmitoylated and, in fact, is one of very few proteins in the cell possessing these acyl modifications. Both fatty acids are required for flagellar localization, suggesting that FCaBP localization may be mediated through association with the flagellar plasma membrane. Indeed, FCaBP associates with the flagellar membrane in a calcium-dependent manner, reminiscent of the recoverin family of calcium–myristoyl switch proteins. Thus, FCaBP is a novel member of the calcium–acyl switch protein family and is the only member described to date that requires two fatty acid modifications for specific membrane association. Its unique localization mechanism is the first described for any flagellar protein. The existence of such a protein in this protozoan suggests that acylation and calcium switch mechanisms for regulated membrane association are conserved among eukaryotes.

Keywords: calcium/flagellum/myristoyl switch/recoverin/trypanosome

Introduction

Trypanosoma cruzi, a unicellular eukaryotic parasite, is the etiologic agent of Chagas' disease, a significant cause of morbidity and mortality in Central and South America (Brener, 1991). Currently, there are 19 countries in which the disease is endemic, with 16 million people infected with *T.cruzi* and 90 million more at risk (Molyneux, 1997). A 24 kDa flagellar calcium-binding protein (FCaBP) is found exclusively in the flagellum of the parasite (Engman *et al*., 1989). FCaBP is a member of the EF-hand calcium-binding protein superfamily which possesses four putative binding motifs comprising 75% of the molecule (Engman *et al*., 1989; Wong *et al*., 1992; Godsel *et al*., 1995). FCaBP binds two calcium ions through the third and fourth EF-hand domains (Maldonado *et al*., 1999). The calcium-binding affinities of these sites place FCaBP in the calcium sensor group of EF-hand proteins, which regulate the activities of other proteins in a calcium-dependent manner (da Silva and Reinach, 1991).

Myristoylation and palmitoylation are fatty acid modifications found at the N-termini of some mammalian proteins. Myristate is a 14 carbon fatty acid covalently attached through an amide bond to the N-terminal glycine residue of a protein during its synthesis (Han and Martinage, 1992). Palmitate is a 16 carbon fatty acid attached to a protein post-translationally through a thioester linkage, most commonly to a cysteine residue. There is no known sequence requirement for palmitoylation; however, in many instances, this modification occurs when the cysteine is located near a previously acylated amino acid residue (Hallak *et al*., 1994; Mumby *et al*., 1994; Shenoy-Scaria *et al*., 1993, 1994; Robbins *et al*., 1995). Both myristoylation and palmitoylation are involved in the membrane associations of some proteins, such as G protein α-subunits and src tyrosine kinases (Resh, 1993; Milligan *et al*., 1995). Palmitoylation may also be involved in protein–protein interactions, as well as in the stabilization of membrane associations (Shenoy-Scaria *et al*., 1993; Wedegaertner *et al*., 1993).

The associations of some myristoylated proteins with membranes are regulated by a variety of factors in addition to fatty acid modification; these are known as myristoyl switch proteins. Factors that may modulate the associations of myristoyl switch proteins with membranes include pH (Hanakam *et al*., 1996a,b), phosphorylation (Thelen *et al*., 1991), the binding of GTP (Randazzo *et al*., 1995) or the binding of calcium (Zozulya and Stryer, 1992). This fourth type of switch characterizes a subfamily of EF-hand calcium-binding proteins—the recoverin family—whose involvement in signal transduction is regulated by the calcium–myristoyl switch mechanism (Zozulya and Stryer, 1992). In the calcium-bound state, recoverin associates with the plasma membrane of retinal rod cells, where it binds to and inhibits the activity of rhodopsin kinase (Calvert *et al*., 1995). When the intracellular calcium level drops upon photoexcitation, the conformation of recoverin changes and its myristoyl group becomes sequestered in a hydrophobic cleft (Tanaka *et al*., 1995). Recoverin loses its membrane association and its interaction with rhodopsin kinase. In this way, the binding of recoverin to its partner protein is modulated by the membrane accessibility of its fatty acid, which is itself regulated by the calcium-binding state of the protein.

The results presented here demonstrate that N-terminal myristoylation and palmitoylation are necessary for the association of FCaBP with the flagellar membrane. This association is regulated further by the protein's binding of calcium, reminiscent of the recoverin family of calcium–myristoyl switch proteins. However, FCaBP is

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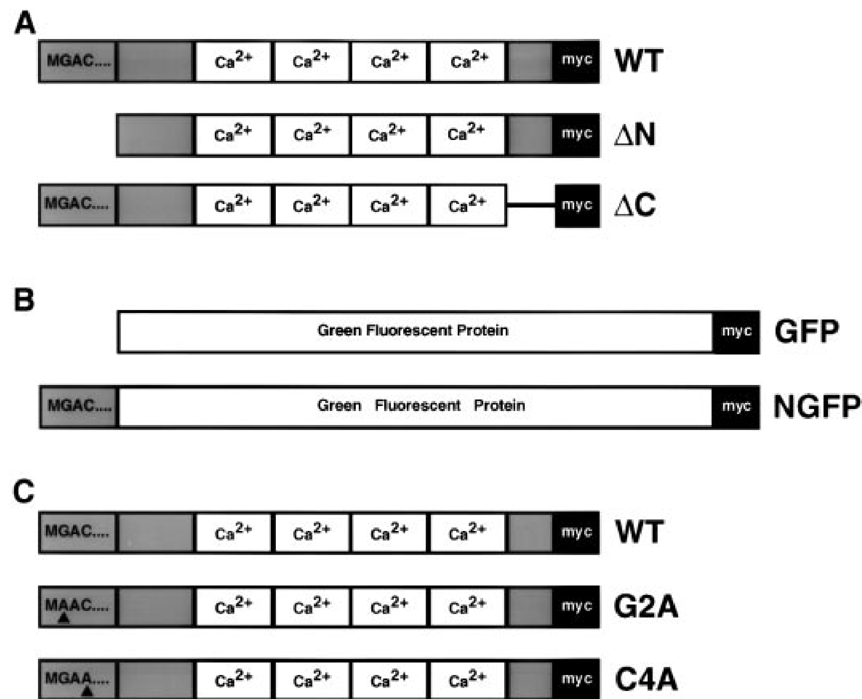

Fig. 1. Schematic representation of FCaBP mutants and fusion proteins used in this study. Domains: MGAC...., N-terminal 24 amino acids (MGACGSKGSTSDKGLASDKDGKNA); Ca^{2+}, EF-hand calcium-binding domain; COOH, C-terminal 15 amino acids (KLDADGDPDNVPESA); myc, 10 amino acid epitope tag of the human c-myc oncoprotein (EQKLISEEDL). (**A**) Deletion mutants: WT, full-length FCaBP; ΔN, mutant in which the first 24 amino acids of FCaBP were replaced by a single methionine; ΔC, mutant lacking the C-terminal 15 amino acids of FCaBP. (**B**) Green fluorescent protein fusions: GFP, the *A.victoria* green fluorescent protein (Chalfie *et al*., 1994); NGFP, GFP fusion containing the first 24 amino acids of FCaBP at the N-terminus. (**C**) Substitution mutants: G2A, FCaBP point mutant containing an alanine substitution of the position 2 glycine; C4A, FCaBP point mutant containing an alanine substitution of the position 4 cysteine. *Trypanosoma cruzi* epimastigotes were transfected with the various constructs as described in Materials and methods.

different from the recoverins in that it is modified with both myristate and palmitate; therefore, it is a calcium–myristoyl/palmitoyl switch protein. The implications of these findings for the function of FCaBP and for membrane organization in the trypanosome are discussed.

Results and discussion

The N-terminus of FCaBP is necessary for flagellar localization

FCaBP is a calcium-binding protein that localizes to the flagellum of the parasite (Engman *et al*., 1989). To our knowledge, no flagellar targeting sequence has been identified in any organism, and an alignment of the sequences of trypanosome flagellar proteins did not reveal any obvious consensus. Therefore, we set out to determine the molecular basis for the flagellar localization of FCaBP. FCaBP has six distinct domains: a 48 amino acid N-terminus, four EF-hand domains and a 15 amino acid C-terminus (Figure 1A). Because there are other EF-hand calcium-binding proteins that are not located specifically in the flagellum, it seemed unlikely that the centrally located EF-hand domains participate in the localization of FCaBP. Therefore, we generated two mutants of FCaBP, one lacking the N-terminal 24 amino acids (ΔN) and one lacking the C-terminal 15 amino acids (ΔC). Epimastigotes were transfected with episomes encoding ΔN, ΔC and full-length (WT) proteins (Figure 1A). These transfectants were examined by indirect immunofluorescence microscopy using a monoclonal antibody specific for a myc epitope tag present at the C-terminus of each protein (Figure 2A). The WT protein is concentrated in the flagellum, indicating that the epitope tag does not affect protein localization (Engman *et al*., 1989). ΔC also localizes to the flagellum, indicating that the C-terminal 15 amino acids are not necessary for localization. By contrast, ΔN does not display flagellar localization and is distributed throughout the cell. Thus, the N-terminus of FCaBP is necessary for the protein's specific flagellar localization.

The N-terminus of FCaBP is sufficient for flagellar localization

To determine whether the N-terminus is sufficient for flagellar localization, transfectants were generated that express the green fluorescent protein of *Aequorea victoria* either containing (NGFP) or lacking (GFP) the N-terminus of FCaBP (Figure 1B). Transfectants were analyzed by direct fluorescence microscopy (Figure 2B), since GFP fluorescence is stimulated by UV light (Chalfie *et al*., 1994). NGFP localizes to the flagellum, while GFP is distributed throughout the cell body. Therefore, the N-terminus of FCaBP is sufficient for flagellar localization.

FCaBP is myristoylated and palmitoylated at its N-terminus

Inspection of the N-terminal sequence of FCaBP revealed a myristoylation motif within the first eight amino acids (Towler *et al*., 1988). The myristoyl moiety would

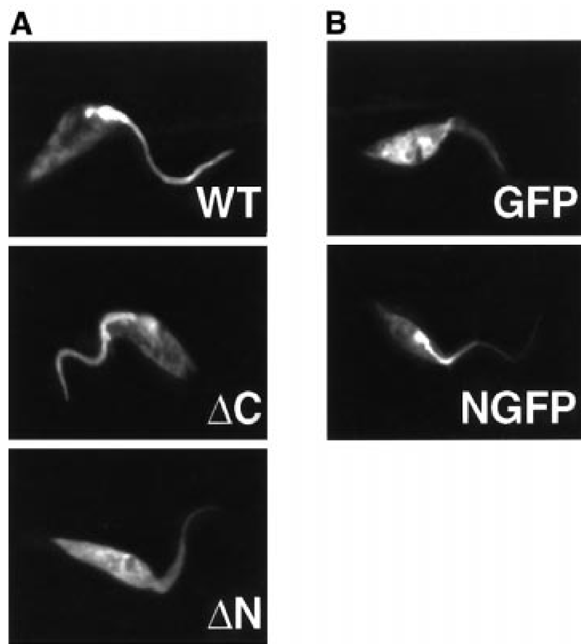

Fig. 2. The N-terminus of FCaBP is necessary and sufficient for flagellar localization. *Trypanosoma cruzi* transfectants expressing various mutant fusion proteins (Figure 1A and B) were analyzed by immunofluorescence (A) or direct fluorescence microscopy (B). (**A**) Localization of FCaBP deletion mutants. The WT and ΔC proteins localize to the flagellum, as does endogenous FCaBP (Engman *et al.*, 1989; and Figure 6), while the ΔN protein is found throughout the cell. (**B**) Localization of GFP fusion proteins. GFP is distributed throughout the cell, while NGFP displays flagellar localization.

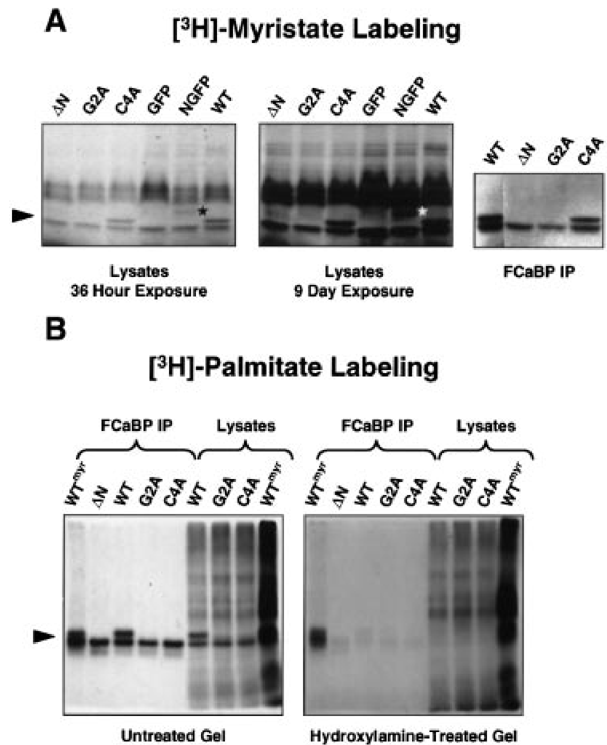

Fig. 3. FCaBP is myristoylated and palmitoylated *in vivo*. *Trypanosoma cruzi* transfectants expressing various FCaBP fusion proteins (see Figure 1) were labeled with [^{3}H]myristate (A) or [^{3}H]palmitate (B). Cell lysates were prepared and analyzed by SDS–PAGE and fluorography before or after immunoprecipitation with FCaBP-specific antiserum. The arrowhead in both panels marks the position of the endogenous and epitope-tagged FCaBP proteins. (**A**) Myristate labeling. Immunoprecipitations are presented at the far right to confirm the identities of the proteins. Fluorograms were exposed for 36 h or 9 days to demonstrate both abundant and rare myristoylated proteins in the cell. Endogenous FCaBP (all lanes), the WT protein, the C4A protein and the NGFP protein (star) are labeled with myristate, while the ΔN and G2A proteins display neither acyl modification. (**B**) Palmitate labeling. After electrophoresis, gels were treated with buffer containing or lacking hydroxylamine, which cleaves labile thioester palmitate linkages. A [^{3}H]myristate-labeled lysate was included in the immunoprecipitation (WT^{myr}) as a control. Endogenous FCaBP (all lanes) and the WT protein are labeled with palmitate, while the ΔN, G2A and C4A proteins are not. The G2A protein is not palmitoylated because myristoylation at the position 2 glycine is required for subsequent palmitoylation, as described previously (Alland *et al.*, 1994; Hallak *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995).

be covalently attached to the glycine residue in the second position (G2) of the coding sequence, which is the N-terminal residue of the native protein. In addition, the cysteine residue in the fourth position (C4) is a potential site for palmitoylation. Protein acylation is important for the subcellular localization of a variety of proteins, but has not yet been implicated in flagellar targeting (Resh, 1993; Alland *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995; Wedegaertner *et al.*, 1995). We generated two FCaBP mutants (Figure 1C) containing alanine substitutions at either glycine (G2A) or cysteine (C4A) to determine whether these residues are acylated *in vivo* and, if so, to examine the importance of the acylation in flagellar localization. Transfectants expressing these and other proteins were metabolically labeled with either [^{3}H]myristate (Figure 3A) or [^{3}H]palmitate (Figure 3B) and lysates and immunoprecipitates were analyzed by SDS–PAGE and fluorography. For the palmitoylation experiment, gels were treated with buffer containing or lacking hydroxylamine to confirm that the labeled proteins are indeed palmitoylated. Endogenous FCaBP (all lanes) was both myristoylated and palmitoylated, as was the full-length, myc-tagged protein (WT), which is slightly larger due to the presence of the tag (arrowheads). Not only is FCaBP modified by these two fatty acids, but also it is the major palmitoylated protein and one of the few myristoylated proteins in the cell (see lysates, Figure 3A and B). ΔN was neither myristoylated nor palmitoylated, as expected, due to the deletion of the N-terminus of the protein. NGFP, which contains the N-terminus of FCaBP, was myristoylated, as expected, while GFP was not. As predicted, C4A was myristoylated, but not palmitoylated, while G2A was not acylated, indicating that the glycine is required for these two modifications. The failure of the G2A protein to be palmitoylated is consistent with the finding that the palmitoylation step requires the presence of a nearby myristoyl moiety, as has been described for some G protein α-subunits (Hallak *et al.*, 1994; Mumby *et al.*, 1994), src family protein tyrosine kinases (Shenoy-Scaria *et al.*, 1993, 1994; Robbins *et al.*, 1995) and other proteins (Robinson *et al.*, 1995; Garcia-Cardena *et al.*, 1996). It is believed that myristoylation is necessary either for translocation of the protein to the site of the palmitoylation machinery or for recognition of the protein by that machinery (Alland *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995).

Fig. 4. The acyl modifications are required for the flagellar localization of FCaBP. Transfectants expressing WT, G2A and C4A proteins were analyzed by immunofluorescence microscopy as described. Neither the G2A mutant protein, which lacks both acyl modifications, nor the C4A protein, which lacks palmitate, show flagellar localization. This indicates that both modifications are required for flagellar targeting, although it is formally possible that palmitate alone is sufficient.

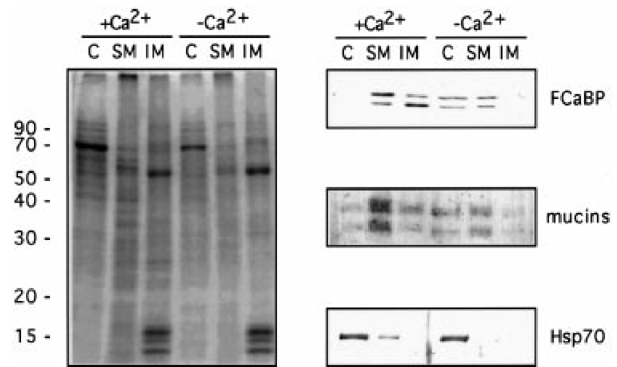

Fig. 5. FCaBP associates with *T.cruzi* membrane fractions in a calcium-dependent manner. Cell fractionation was performed using the *T.cruzi* transfectant expressing WT FCaBP, in the presence (+Ca^{2+}) or absence (–Ca^{2+}) of calcium. Cytosolic proteins (C), Triton X-100-soluble membrane proteins (SM) and Triton X-100-insoluble membrane proteins (IM) were analyzed by SDS–PAGE and staining with GelCode (left panel) or immunoblotting (right panels) using antisera specific for FCaBP, Hsp70 and *T.cruzi* surface mucins (gp35/gp50). The positions of protein molecular weight markers are indicated on the left (kDa). FCaBP (both endogenous and epitope-tagged transprotein) is found predominately in the membrane fractions (both soluble and insoluble) in the presence of calcium, and shifts to the cytoplasmic fraction in the absence of calcium. Hsp70 and the mucins are found primarily in the cytosolic and membrane fractions, respectively. These results strongly suggest that FCaBP is associated primarily with the cell membrane in the presence of calcium and becomes cytoplasmic upon removal of calcium.

The acyl modifications are necessary for the flagellar localization of FCaBP

The role of acylation in the localization of FCaBP was determined through indirect immunofluorescence analyses of the substitution mutants (Figure 4). Neither the G2A protein nor the C4A protein localized to the flagellum, indicating that both myristoyl and palmitoyl moieties are necessary for flagellar localization. It is possible that palmitoylation is the only requirement; however, as mentioned above, palmitoylation does not occur in the absence of a nearby acyl group. The importance of protein acylation in membrane association has been well documented for members of the src family of protein tyrosine kinases and G protein α-subunits, which are dually acylated with myristate and palmitate (Resh, 1993; Alland *et al*., 1994; Shenoy-Scaria *et al*., 1994; Robbins *et al*., 1995; Wedegaertner *et al*., 1995). Both modifications are necessary for membrane association, and members of these protein families have been localized to the plasma membrane, caveolar membranes and the endoplasmic reticulum (Resh, 1993; Milligan *et al*., 1995). In addition to mediating membrane association, the acyl moieties of some of these proteins are thought to be involved in protein–protein interactions (Shenoy-Scaria *et al*., 1993; Wedegaertner *et al*., 1993).

FCaBP associates with the cell membrane in a calcium-dependent manner

The presence of acyl modifications at the N-terminus of FCaBP suggested that the protein associates with a cell membrane. To investigate this possibility and the potential role of calcium in modulating membrane association, cytosolic and membrane fractions were prepared from epimastigotes expressing full-length tagged FCaBP (WT) in the presence (+Ca^{2+}) or absence (–Ca^{2+}) of calcium and analyzed by SDS–PAGE and immunoblotting (Figure 5). Cells were first lightly permeabilized with digitonin to release cytoplasmic proteins (lanes C). The pellet was then treated with Triton X-100 to separate soluble membrane proteins (lanes SM) from insoluble ones (lanes IM). Immunoblots were probed with antisera specific for FCaBP, Hsp70 or surface mucins. FCaBP (endogenous protein and epitope-tagged transprotein) is found predominantly in the membrane fractions when calcium is present during the fractionation, as are the glycosyl-phosphatidylinositol (GPI)-anchored gp35/gp50 surface mucins, as expected. However, when calcium is removed using EGTA, there is a clear shift of FCaBP to the cytosolic fraction. The partitioning of the mucins also changes upon calcium chelation, as is true of other GPI-anchored surface proteins. The mechanism of calcium-regulated localization of these proteins involves association with annexins (Parkin *et al*., 1996). Hsp70, a cytoplasmic protein, is found in the cytosolic fraction. FCaBP is found not only in the Triton X-100-soluble membrane fraction (SM), but in the insoluble fraction (IM) as well. The latter suggests that FCaBP is associated with membrane rafts (Simons and Ikonen, 1997), specialized regions of cell membranes rich in sphingolipids and sterols where certain membrane proteins are concentrated. These results strongly suggest that FCaBP associates with the

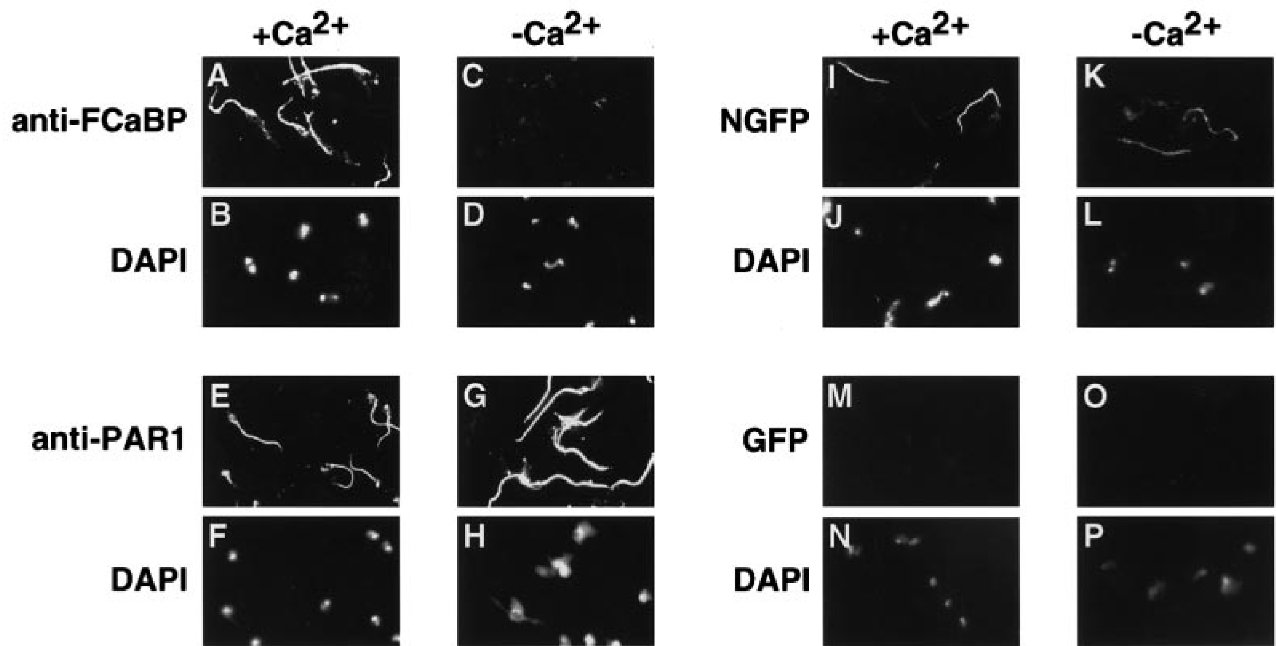

Fig. 6. The flagellar localization of FCaBP is calcium dependent. Untransfected epimastigotes (**A**–**H**) as well as NGFP (**I**–**L**) and GFP (**M**–**P**) transfectants were placed on coverslips, permeabilized by light detergent extraction and treated with buffers lacking ($+Ca^{2+}$) or containing ($-Ca^{2+}$) EDTA (or EGTA, data not shown). Treated cells were analyzed by immunofluorescence microscopy (A–H) or direct fluorescence microscopy (I–P); cellular DNA was stained with DAPI before mounting to mark the cells. FCaBP is present in the flagellum when calcium is present (A), but dissociates and is washed away upon calcium chelation (C). The calcium-binding domains are required for this calcium-modulated localization, since NGFP, which contains the flagellar targeting region but lacks calcium-binding domains, is found in the flagellum whether or not calcium is present (I–L); GFP without the flagellar targeting sequence is not retained after detergent treatment (M–P). As a final control, the PAR1 protein, a major constituent of the polymeric paraflagellar rod, was studied and found to be present under both conditions (E–H). These results indicate that FCaBP is a calcium–acyl switch protein which associates with the flagellum only in the calcium-bound state and that a targeted protein lacking calcium-binding domains is flagellar under all circumstances.

flagellar membrane in a calcium-dependent manner and is, therefore, a calcium–acyl switch protein.

FCaBP is a calcium switch protein

The most thoroughly studied member of the calcium–acyl switch protein family is recoverin, a protein of the retinal rod cell (Dizhoor *et al.*, 1989, 1991). In the calcium-bound state, recoverin associates with the lipid bilayer through its myristoyl group, where it associates with and inhibits the activity of rhodopsin kinase (Calvert *et al.*, 1995). When the calcium level in the rod cell drops upon photoexcitation, the calcium dissociation from recoverin results in a conformational change and sequestration of the myristoyl group in a hydrophobic cleft (Tanaka *et al.*, 1995). 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. Like recoverin, FCaBP is acylated at the N-terminus, contains EF-hand calcium-binding motifs and associates with the membrane fraction in a calcium-regulated manner. We performed a calcium chelation study to test the hypothesis that FCaBP is a calcium switch protein (Figure 6). Untransfected *T.cruzi* epimastigotes or epimastigotes expressing GFP or NGFP were allowed to adhere to coverslips and permeabilized lightly with Triton X-100 to release cytoplasmic proteins. Coverslips were incubated with phosphate-buffered saline (PBS) or PBS containing EDTA to chelate calcium, and fixed with paraformaldehyde. EGTA was used in subsequent studies with identical results (not shown). The locations of FCaBP, GFP and NGFP were assessed by immunofluorescence (Figure 6A–H) or direct UV microscopy (Figure 6I–P). The flagellar association of FCaBP is indeed regulated by calcium, as FCaBP is only present in the flagella of cells treated with PBS (Figure 6A and B). FCaBP is no longer found in the flagella of cells treated with PBS/EDTA (Figure 6C and D). Because FCaBP no longer associates with the membrane and is therefore soluble under these conditions, it is washed from the permeabilized cells. The chelation of calcium did not affect the localization of paraflagellar rod protein 1 (PAR1), a protein of the flagellar cytoskeleton that is not affected by detergent treatment (Saborio *et al.*, 1989) (Figure 6E–H). To confirm that the calcium-regulated localization of FCaBP directly involves the EF-hand calcium-binding domains, cells expressing GFP and NGFP were analyzed in the same manner. GFP is cytoplasmic and therefore not retained after detergent treatment (Figure 6M–P), whereas NGFP associates with the flagellar membrane whether calcium is present or absent (Figure 6I–L). These results indicate that binding of calcium by the EF-hand domains modulates the association of FCaBP with the flagellar membrane. Moreover, they suggest that FCaBP may regulate the activity of a flagellar membrane protein through calcium-modulated membrane association and concomitant protein–protein interaction.

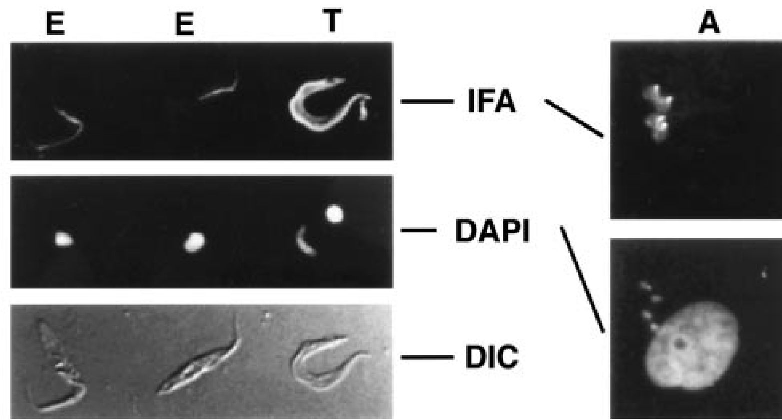

Fig. 7. Expression of FCaBP in different life cycle stages of *T.cruzi*. *Trypanosoma cruzi* epimastigotes (E), culture-derived metacyclic trypomastigotes (T) and amastigotes within a mammalian cell (A) were analyzed by immunofluorescence microscopy using an FCaBP-specific mouse serum (IFA). Cells were also visualized by DAPI staining and differential interference microscopy (DIC). In epimastigotes and amastigotes, the nuclear and mitochondrial (kinetoplast) nucleoids are close together, while they are separated in the trypomastigote (DAPI frames). The host cell nucleus is the large DAPI-staining structure in the right lower panel and the amastigotes are the small structures to the top and left of the host nucleus. In the epimastigote, FCaBP staining is restricted to the flagellum, while in the trypomastigote and amastigote, intense staining in the flagellum (T) or flagellar 'remnant' (A) is accompanied by diffuse staining throughout the cell.

FCaBP is expressed in all life cycle stages of T.cruzi

If FCaBP is involved in the transduction of a flagellum-specific calcium-modulated signal, it is possible that it is involved in flagellar biogenesis or movement. In this case, the protein may exhibit differential expression and/or localization in life cycle stages of the parasite that differ in flagellar structure and motility. Two motile forms of *T.cruzi*, the epimastigote (E) and the trypomastigote (T), and the non-motile amastigote (A), found within the mammalian cell cytoplasm, were analyzed by immunofluorescence microscopy using an FCaBP-specific mouse serum (Figure 7). FCaBP is expressed in all three life cycle stages of the parasite, although the level of expression and specific localization in these stages differ. As previously reported (Engman *et al*., 1989), FCaBP is restricted to the flagellum in the epimastigote, while it is found at high levels in the trypomastigote flagellum and the flagellar 'remnant' of the amastigote. Interestingly, the protein is also readily detectable in the cell bodies of these latter forms, although the precise localization is not yet known. It is possible that this staining reflects pellicular (somatic) membrane localization or cytoplasmic localization. The differences in the localization of FCaBP among the three life cycle stages may result from differences in (i) acylation, (ii) level of expression, (iii) partner protein expression and localization or (iv) membrane composition (see below). In any case, expression of FCaBP in the non-motile amastigote suggests that the protein may play a role in a calcium-dependent sensory/signaling process.

FCaBP is the first calcium–acyl switch protein found in cells other than those of the mammalian nervous system and is the first protein of this type that is both myristoylated and palmitoylated. That trypanosomes use N-terminal acylation, and calcium-modulated protein–membrane association indicates that these mechanisms are ancient. It has been suggested that the palmitoyl moiety is involved in protein–protein interactions and not simply in stabilizing protein–membrane associations (Shenoy-Scaria *et al*., 1993; Wedegaertner *et al*., 1993). Thus, the localization of FCaBP may result in part from an association with the plasma membrane through its myristoyl group and also from an association with a partner protein through palmitate. The pellicular membrane composition may differ among the life cycle stages of the parasite, explaining the presence of non-flagellar FCaBP in the trypomastigote and amastigote. Since the pellicular and flagellar membranes of the epimastigote may differ in composition (da Cunha e Silva *et al*., 1989; de Souza, 1995), it is also possible that the acyl moieties of FCaBP may only be able to interact with the flagellar membrane in this life cycle stage. In this case, the entire flagellar membrane might constitute a functional membrane raft (Simons and Ikonen, 1997) containing specific sterols and sphingolipids that allow the attachment of specifically modified proteins, FCaBP among them. In polarized mammalian cells, these microdomains have been hypothesized to be involved in the sorting of proteins to apical or basal surfaces (Brown and Rose, 1992; Weimbs *et al*., 1997). It is therefore possible that the trypanosome may sort proteins not to a basal or apical surface, but to pellicular or flagellar membranes. Membrane rafts also contain a number of signal transduction molecules (Arreaza *et al*., 1994; Rodgers *et al*., 1994) and, in this regard, FCaBP may have a role in a flagellum-specific calcium signaling pathway.

Palmitoylation is required for the specific flagellar localization of FCaBP, a finding unique among calcium–acyl switch proteins described to date. It is possible that both myristoyl and palmitoyl moieties associate with a hydrophobic pocket in the protein in the absence of calcium. Alternatively, FCaBP may be a simple calcium–myristoyl switch protein that is regulated further by reversible palmitoylation, as is true of signaling proteins such as $p21^{ras}$ (Magee *et al*., 1987) and G protein α-subunits (Degtyarev *et al*., 1993; Wedegaertner and Bourne, 1994). One might predict that FCaBP is a molecular sensor that transduces a regulatory signal in a calcium-dependent manner. Finally, the similarities between recoverin and FCaBP provide another molecular link among sensory and motile cilia, and suggest that similar proteins will be found in the flagella and cilia of

other organisms. Future studies of the calcium–acyl switch mechanism, life cycle-specific expression and localization of the protein and the basis for flagellum-specific membrane association should illuminate the function of this protein in the trypanosome.

Materials and methods

Parasites

The Silvio X-10/4 clone of *T.cruzi* was used for all experiments. *Trypanosoma cruzi* epimastigotes were grown at 26°C in supplemented liver digest neutralized tryptose medium ($LDNT^+$) as described (Kirchhoff and Neva, 1985). Trypomastigotes arose spontaneously in high density cultures. Amastigotes were derived after infection of Vero cells with *T.cruzi* trypomastigotes.

Generation of FCaBP expression constructs

The *T.cruzi* episomal expression vector pTEX-9E10 (Tibbetts *et al*., 1995) was used for all studies. DNA inserts in pTEX-9E10 were expressed in transfected *T.cruzi* epimastigotes as proteins containing C-terminal, 10 amino acid c-myc epitope tags. Sequences for expression (see Figure 1 for schematic representation) were generated by PCR using an FCaBP cDNA template (Godsel *et al*., 1995) and directionally cloned into pTEX-9E10 using 5′ *Xba*I (or *Eco*RI) and 3′ *Eco*RV sites. GFP sequences were generated in a similar manner, using the *A.victoria* pS65T plasmid as a template (Chalfie *et al*., 1994). NGFP was produced by ligating an *Xba*I-flanked PCR product encoding the N-terminal 24 amino acids of FCaBP (MGACGSKGSTSDKGLASDKDGKNA) into the *Xba*I-digested GFP construct. The G2A and C4A mutants were produced in a manner similar to that used to produce wild-type tagged FCaBP. Oligonucleotides used for PCR: FCaBP-myc (WT), sense TCTAGAATGGGTGCTTGTGGGTC and antisense GATATCCATAAAGTGGAGAATGTGC; ΔN, sense ATGAAGGACCGCAAGGAAG and antisense CGCGCTCTCCGGCACGT; ΔC, sense ATGGGTGCTTGTGGGTCG and antisense GACTGCAGAAGCCCACGC; GFP, sense TCTAGAATGAGTAAAGGAGAAGAACTTTTC and antisense GATATCTTTGTATAGTTCATCCATGCCATG; NGFP, sense TCTAGAATGGGTGCTTGTGGGTC and antisense TCTAGAGGCGTTCTTGCCGTCCTT; G2A, sense ATGGCTGCTTGTGGGTCGAAG and antisense CGCGCTCTCCGGCACGT; C4A, sense ATGGGTGCTGCTGGGTCGAAG and antisense CGCGCTCTCCGGCACGT.

Transfection of T.cruzi

Trypanosoma cruzi epimastigotes were grown to a density of $1–2\times10^7$/ml in $LDNT^+$, washed once with electroporation buffer [132 mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $Mg(C_2H_3O_2)_2 \cdot 4H_2O$, 0.1 mM $CaCl_2$, pH 7.0 with 0.5 M acetic acid] and resuspended in this buffer at a density of 1×10^8/ml. Then 400 μl of the cell suspension was placed into a 0.2 cm electrode gap cuvette with 15–25 μg of supercoiled DNA and pulsed four times at 0.3 kV and 500 μF using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). Electroporated cells were placed in 5 ml of $LDNT^+$, and G418 (0.5 mg/ml) was added 48 h later. Drug-resistant lines developed subsequently and were available for study after ~6 weeks.

Fluorescence microscopy

Trypanosoma cruzi epimastigotes and trypomastigotes were isolated by centrifugation, washed in PBS and resuspended in PBS at a density of 1×10^6 cells/ml. A 20 μl aliquot 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 (BSA), 2% normal goat serum in PBS] for 30 min. Infected Vero cells were grown in chamber slides fixed and blocked in a similar manner. Slides were incubated with c-myc-specific, 9E10 monoclonal antibody hybridoma supernatant (ATCC #CRL1729, myc 1-9E10.2) or FCaBP-specific mouse serum (Engman *et al*., 1989) overnight at 4°C. Parasites were washed with PBS and incubated for 3–4 h with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM (Caltag Laboratories, South 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 (gelvatol; Sigma, St Louis, MO) and viewed by fluorescence microscopy. Epimastigotes expressing GFP fusions were washed in ice-cold PBS and resuspended at a density of 1×10^6 cells/ml. A 20 μl aliquot of the suspension was added to wells of a printed slide, air dried, fixed at –20°C in anhydrous methanol for 30 min and rehydrated with PBS. Slides were mounted in gelvatol and cells were visualized by fluorescence microscopy.

Metabolic labeling and immunoprecipitation

Trypanosoma cruzi transfectants were grown to a density of $5–7\times10^6$/ml, and 2×10^8 cells of each line were suspended in 5 ml of $LDNT^+$ containing 1 mCi of [9,10-^{3}H]myristate (30–50 Ci/mmol, DuPont-New England Nuclear) or Dulbecco's modified Eagle's medium containing [9,10-^{3}H]palmitate (30–60 Ci/mmol, DuPont-New England Nuclear). 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 RIPA buffer [150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris base (pH 8.0), 0.02% NaN_3, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)], and the lysate clarified by centrifugation, divided into 200 μl aliquots and stored at –20°C until use. Aliquots of the labeled RIPA lysates were incubated with FCaBP-specific mouse serum overnight at 4°C with gentle agitation. Immunoprecipitates were collected on protein G–agarose for 1–3 h at 4°C and washed five times with RIPA buffer. Myristate-labeled precipitates were suspended in 2× Laemmli sample buffer and boiled for 5 min. Palmitate-labeled samples were suspended in non-denaturing sample buffer [2× Laemmli sample buffer without dithiothreitol (DTT) or β-mercaptoethanol)] and boiled for only 1–2 min to avoid breakage of the labile thioester bond. Samples were analyzed by one-dimensional, 14% SDS–PAGE. Palmitate gels were treated with two 30 min washes with 1 M hydroxylamine, pH 7.5 or 1 M Tris–HCl, pH 7.5 and rinsed three times for 5 min each with water. Hydroxylamine treatment cleaves thioester linkages, and palmitate, but not covalently linked myristate, diffuses from the gel. Gels were stained with Coomassie blue, destained with acetic acid and methanol, treated for fluorography (Amplify, Amersham, Arlington Heights, IL), dried and exposed to Hyperfilm-MP (Amersham, Arlington Heights, IL) film for 36 h or 9 days for the myristate labeling experiment and for 120 h for the palmitate labeling experiment.

Preparation and analysis of cytosolic and membrane fractions of T.cruzi

A total of 2×10^8 *T.cruzi* epimastigotes were isolated by centrifugation and suspended in 150 μl of MS buffer (20 mM MOPS, pH 7.0, 0.25% sucrose; ~1 ml of buffer per 500 mg cell pellet) containing either 1 mM $CaCl_2$ or 5 mM EGTA. Then 150 μl of 6 mg/ml digitonin in MS buffer (for a final concentration of 3 mg/ml digitonin) was added and the suspension was vortexed briefly every 15 s for 5 min at room temperature. The cells were centrifuged at 5220 *g* for 10 min at room temperature and the supernatant was transferred into a tube containing 75 μl of 5× Laemmli sample buffer (cytosolic fraction). The pellet was washed once with 500 μl of MS + $CaCl_2$ or the MS + EGTA, and solubilized by vortexing for 1.5 min at room temperature in 300 μl of 1% Triton X-100, 140 mM NaCl, 10 mM Tris–HCl (pH 7.4). The solution was centrifuged at 11 750 *g* for 10 min at room temperature and the supernatant was transferred into a tube containing 75 μl of 5× Laemmli sample buffer (Triton X-100-soluble membrane fraction). The final pellet was suspended in 300 μl of 1× PBS and 75 μl of sample buffer (Triton X-100-insoluble fraction). Samples were boiled and approximately equal amounts of each protein fraction were analyzed by one-dimensional 12% SDS–PAGE (Laemmli, 1970). Gels were either stained with GelCode (Pierce, Rockford, IL) or transferred to nitrocellulose (Towbin *et al*., 1979) and probed with FCaBP-specific (Godsel *et al*., 1995), Hsp70-specific (Olson *et al*., 1994) or gp35/gp50 surface mucin-specific (Yoshida *et al*., 1989) mouse sera and alkaline phosphatase-conjugated goat anti-mouse IgG + IgM (Caltag Laboratories, San Francisco, CA), and blots were developed using BCIP/NBT (Life Technologies, Gaithersburg, MD).

Calcium chelation assay

A total of 5×10^6 *T.cruzi* epimastigotes were allowed to adhere to poly-D-lysine-coated coverslips in 24-well plates at 4°C for 15 min and non-adherent cells were removed by washing with PBS. Cells were permeabilized with 1% Triton X-100 in 50 mM PIPES (pH 7.4), 1 mM NaCl, 25 mM $CaCl_2$, at room temperature for 1–3 min. Cells were washed four times with PBS in the absence (+Ca^{2+}) or in the presence (–Ca^{2+}) of the divalent cation chelator EDTA (25 mM) and fixed in 4% paraformaldehyde at 4°C for 1 h. GFP-expressing cells were then incubated for 10 min with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) to visualize cellular DNA in the absence of antibody staining.

Coverslips were mounted in gelvatol and cells were visualized directly by fluorescence. For immunofluorescence assays, cells were fixed as described above and were blocked with 1% BSA in PBS for 1 h. Slides were incubated with FCaBP-specific mouse serum (1:5000) or PAR1-specific rabbit serum (1:1500) at 4°C, overnight, and then incubated with FITC-conjugated goat anti-mouse (1:200) or goat anti-rabbit (1:800) secondary antibodies at room temperature for 3 h. Cells were then incubated for 10 min with DAPI and coverslips were mounted in gelvatol and visualized by fluorescence microscopy.

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