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## Calcium-dependent membrane association of a flagellar calcium sensor does not require calcium binding

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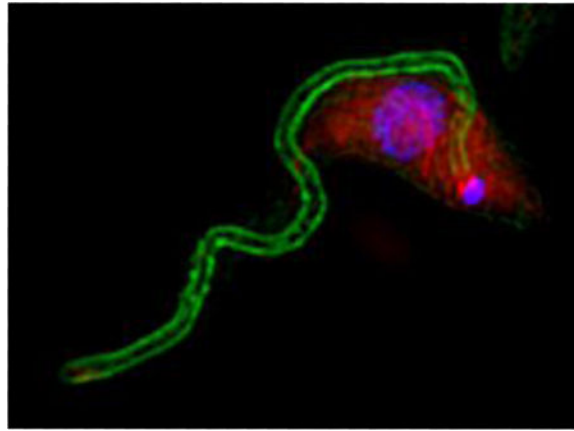
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### Abstract

Flagellar calcium-binding protein (FCaBP) is a dually acylated Ca<sup>2+</sup> sensor in the *Trypanosoma cruzi* flagellar membrane that undergoes a massive conformational change upon Ca<sup>2+</sup> binding. It is similar to neuronal Ca<sup>2+</sup> sensors, like recoverin, which regulate their binding partners through a calcium acyl switch mechanism. FCaBP is washed out of permeabilized cells with buffers containing EDTA, indicating Ca<sup>2+</sup>-dependent flagellar membrane association. We hypothesized that, like recoverin, FCaBP projects its acyl groups in the presence of Ca<sup>2+</sup>, permitting flagellar membrane and binding partner association and that it sequesters the acyl groups in low Ca<sup>2+</sup>, disassociating from the membrane and releasing its binding partner to perform a presumed enzymatic function. The x-ray crystal structure of FCaBP suggests that the acyl groups are always exposed, so we set out to test our hypothesis directly. We generated *T. cruzi* transfectants expressing FCaBP or Ca<sup>2+</sup>-binding mutant FCaBP<sup>E151Q/E188Q</sup> and recombinant wildtype and mutant proteins as well. Both FCaBP and FCaBP<sup>E151Q/E188Q</sup> were found to associate with lipid rafts, indicating the Ca<sup>2+</sup>-independence of this association. To our initial surprise, FCaBP<sup>E151Q/E188Q</sup>, like wildtype FCaBP, exhibited Ca<sup>2+</sup>-dependent flagellar membrane association, even though this protein does not bind Ca<sup>2+</sup> itself [16]. One possible explanation for this is that FCaBP<sup>E151Q/E188Q</sup>, like some other Ca<sup>2+</sup> sensors, may form dimers and that dimerization of FCaBP<sup>E151Q/E188Q</sup> with endogenous wildtype FCaBP might explain its Ca<sup>2+</sup>-dependent localization. Indeed both proteins are able to form dimers in the presence and absence of Ca<sup>2+</sup>. These results suggest that FCaBP possesses two distinct Ca<sup>2+</sup>-dependent interactions—one involving a Ca<sup>2+</sup>-induced change in conformation and another perhaps involving binding partner association.

### Graphical abstract

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### Keywords

Calcium; flagellum; dimer; trypanosome

The central role of  $\text{Ca}^{2+}$  as a regulator of cellular activity is well established [1, 2]. Cell division, motility, gene expression and secretion are regulated by  $\text{Ca}^{2+}$  ions and the intracellular  $\text{Ca}^{2+}$  concentration must be precisely controlled during these processes. In *T. cruzi*, parasite proliferation and differentiation [3] and host cell invasion [4, 5] are regulated by  $\text{Ca}^{2+}$ , and  $\text{Ca}^{2+}$ -modulated cell signaling pathways similar to those in mammalian cells are present [6]. Moreover, *T. cruzi* possesses a highly specialized  $\text{Ca}^{2+}$ -containing organelle, the acidocalcisome [7], which is believed to permit the amastigote stage of the parasite to survive in the low  $\text{Ca}^{2+}$  environment of the mammalian cell cytoplasm in which it replicates [8]. Intracellular  $\text{Ca}^{2+}$  signals are transduced into biological responses through their interaction with  $\text{Ca}^{2+}$ -binding proteins (CaBPs) or  $\text{Ca}^{2+}$ -binding domains in multidomain proteins. *T. cruzi* has a variety of proteins in this class of signal transducing molecules, including calmodulin [9], calmodulin-binding proteins [10] and calmodulin regulated enzymes [11, 12]. Despite the identification of several  $\text{Ca}^{2+}$ -regulated processes in *T. cruzi*, the molecular mechanisms by which specific CaBPs transduce the  $\text{Ca}^{2+}$  signals remain to be elucidated.

Flagellar  $\text{Ca}^{2+}$  binding protein (FCaBP, TritypID TcCLB.507491.151) is a 24 kDa highly immunogenic protein found in the flagellum of the protozoan parasite *T. cruzi* [13]. FCaBP specifically localizes to the flagellar plasma membrane via amino terminal myristoyl and palmitoyl modifications [14] and positively charged lysine residues [15]. FCaBP interacts with lipid raft microdomains [16], which are specifically enriched within the flagellar membrane of *T. cruzi* [17]. It has been hypothesized that interaction with lipid raft domains is responsible for either targeting or retention of some dually acylated proteins to the flagellar membrane. Additionally, FCaBP contains four EF hand  $\text{Ca}^{2+}$ -binding motifs, the third and fourth of which bind  $\text{Ca}^{2+}$  [16, 18]. Based on its size, acylation,  $\text{Ca}^{2+}$  binding and membrane association, we hypothesized that FCaBP is a  $\text{Ca}^{2+}$  acyl switch protein. Such proteins undergo  $\text{Ca}^{2+}$ -dependent membrane association by virtue of  $\text{Ca}^{2+}$ -regulated extrusion or sequestration of a myristate moiety that mediates membrane binding [19].

The prototypical  $\text{Ca}^{2+}$ -acyl switch protein is Recoverin (Rv), a myristoylated  $\text{Ca}^{2+}$  sensor in retinal rod cells that functions in cellular recovery from photoexcitation [20]. Rv has four EF hands but binds two  $\text{Ca}^{2+}$  ions [21] through the second and third EF hand domains [22]. Rv associates in a  $\text{Ca}^{2+}$  dependent manner via its amino terminal myristoyl group with the plasma membrane, where it binds to and inhibits the activity of rhodopsin kinase (RK) [23]. When the intracellular  $\text{Ca}^{2+}$  level drops upon retinal cell photoexcitation,  $\text{Ca}^{2+}$  dissociation from Rv leads to a conformational change and sequestration of the myristoyl group in a hydrophobic cleft [24]. Unable to associate with the membrane, Rv moves off the rod outer segment membrane, freeing RK to phosphorylate and inactivate rhodopsin, the first step in the cellular recovery phase. When the intracellular  $\text{Ca}^{2+}$  rises, Rv assumes its  $\text{Ca}^{2+}$ -bound conformation and returns to the membrane, once again inactivating RK and completing the cycle.

The flagellar membrane localization of FCaBP requires several elements: dual N-terminal acylation with myristate and palmitate [14] and a cluster of nearby basic amino acids [15]. If FCaBP were in fact a protein like Rv that cycles on and off the flagellar membrane, we would expect its membrane association to be directly dependent on  $\text{Ca}^{2+}$  binding as well, as it is in Rv. To test this hypothesis, we employed *T. cruzi* transfectants expressing myc/his-tagged FCaBP or myc-tagged  $\text{Ca}^{2+}$  binding mutant FCaBP<sup>E151Q/E188Q</sup>, which is absolutely devoid of  $\text{Ca}^{2+}$  binding. FCaBP binds two molecules of  $\text{Ca}^{2+}$  and the E151Q and E188Q mutations disrupt binding at EF-hand domains 3 and 4, respectively [16]. The epitope tags are slightly different (myc and myc/his) because the transfectants were produced previously at different times for separate studies. Since protein localization is not affected by the tags, we did not generate a new transfectant so as to have matched tags. Immunofluorescence microscopy of these cell lines revealed that, like FCaBP-myc/his, the  $\text{Ca}^{2+}$  binding mutant FCaBP<sup>E151Q/E188Q</sup>-myc properly localizes to the flagellum (Fig. 1A), indicating that binding of  $\text{Ca}^{2+}$  via the high affinity sites is not required for localization.

A major factor determining the localization of FCaBP to the flagellar membrane is its association with lipid rafts [15], which are highly enriched in the flagellar membrane [17]. Flagellar membrane association requires dual acylation by myristate and palmitate [14], and these two modifications are also required for lipid raft association [15]. To test whether  $\text{Ca}^{2+}$  binding is also required for lipid raft association, we tested whether FCaBP<sup>E151Q/E188Q</sup>-myc would float on a sucrose gradient. The FCaBP<sup>E151Q/E188Q</sup>-myc expressing cell line from Fig. 1A was lysed in ice-cold Triton X-100 and analyzed by a discontinuous sucrose density step gradient. Gradient fractions were collected, separated by SDS-PAGE and examined by western blotting using FCaBP-specific antiserum (Fig. 1B). Both the FCaBP<sup>E151Q/E188Q</sup>-myc transprotein and endogenous FCaBP floated to the 5%–35% interface, indicating that they associate with detergent-resistant membranes (lipid rafts).

One of the defining biophysical properties of FCaBP is that its flagellar membrane association is dependent on  $\text{Ca}^{2+}$ . This can be assessed *in vivo* via a  $\text{Ca}^{2+}$  chelation slide assay, in which permeabilized trypanosomes are washed in buffers containing or lacking  $\text{Ca}^{2+}$  [14]. That FCaBP<sup>E151Q/E188Q</sup> localizes normally to the flagellar membrane and associates with lipid rafts does not mean that it would exhibit  $\text{Ca}^{2+}$ -dependent membrane association. To test this, we analyzed FCaBP<sup>E151Q/E188Q</sup>-myc-expressing cells by the  $\text{Ca}^{2+}$

chelation slide assay (Fig. 2A). As was observed with endogenous FCaBP (left panels), FCaBP<sup>E151Q/E188Q</sup>-myc washed out of the cell upon Ca<sup>2+</sup> chelation (right panels), indicating that direct Ca<sup>2+</sup> binding by FCaBP is not necessary for the Ca<sup>2+</sup>-dependent membrane association. Paraflagellar rod (PFR) and FCaBP<sup>1-24</sup>-GFP (N-terminal acylated peptide fused to GFP) served as controls (middle panels). Despite its inability to bind Ca<sup>2+</sup>, FCaBP Ca<sup>2+</sup>-binding mutant surprisingly demonstrated Ca<sup>2+</sup>-dependent flagellar membrane localization, indicating a second level of Ca<sup>2+</sup>-modulation of protein localization and possible function. One possible explanation for the behavior of FCaBP<sup>E151Q/E188Q</sup> in the Ca<sup>2+</sup> chelation slide assay is that this Ca<sup>2+</sup> binding mutant might form dimers with endogenous wildtype FCaBP. A number of neuronal Ca<sup>2+</sup> sensor proteins, (including Rv [25], GCAP2 [26], and DREAM [27, 28]) form dimers and even higher order oligomers. We analyzed both FCaBP and FCaBP<sup>E151Q/E188Q</sup> by size exclusion chromatography in the presence or absence of Ca<sup>2+</sup> (Fig. 2B). Both proteins formed dimers independent of Ca<sup>2+</sup>.

Our previous model of FCaBP regulation was largely based on Rv and other neuronal calcium sensors [16]. This model postulated that the flagellar membrane association and partner protein association of FCaBP are directly determined by Ca<sup>2+</sup>-binding by FCaBP. It further predicted that the FCaBP Ca<sup>2+</sup>-binding null mutant would not localize to the flagellum, since, like Rv, the protein might sequester the acyl groups and lose interactions with both the flagellar membrane and the binding partner. X-ray crystallography indicated that the acylated N-terminus of FCaBP is always exposed [29] and NMR studies of calflagin Tb24, a closely related flagellar Ca<sup>2+</sup> sensor in the African trypanosome *T. brucei*, corroborated those findings [30, 31]. Dual acylation with myristate and palmitate [14], an N-terminal polybasic region [15], and lipid raft association [15, 17] are required for flagellar membrane association of FCaBP. Ca<sup>2+</sup> is also required, but direct binding via the high affinity Ca<sup>2+</sup> binding sites is not. This result may be explained by dimerization of FCaBP<sup>E151Q/E188Q</sup> to endogenous FCaBP, a possibility that can best be tested by analysis of FCaBP<sup>E151Q/E188Q</sup> behavior in a cell lacking endogenous FCaBP. This can now be explored experimentally with the advent of CRISPR-Cas9-mediated genome editing in *T. cruzi* [32]. Another intriguing possibility is that FCaBP participates in Ca<sup>2+</sup>-dependent partner protein association. While the association of Rv with RK is depending on Ca<sup>2+</sup>-binding by the high affinity sites in Rv, this may not be true of FCaBP. We found that both FCaBP and FCaBP<sup>E151Q/E188Q</sup> associate with proteins of 80-kDa and 30-kDa in a Ca<sup>2+</sup>-dependent manner [16]. Identification of these binding partners will permit this possibility to be tested directly.

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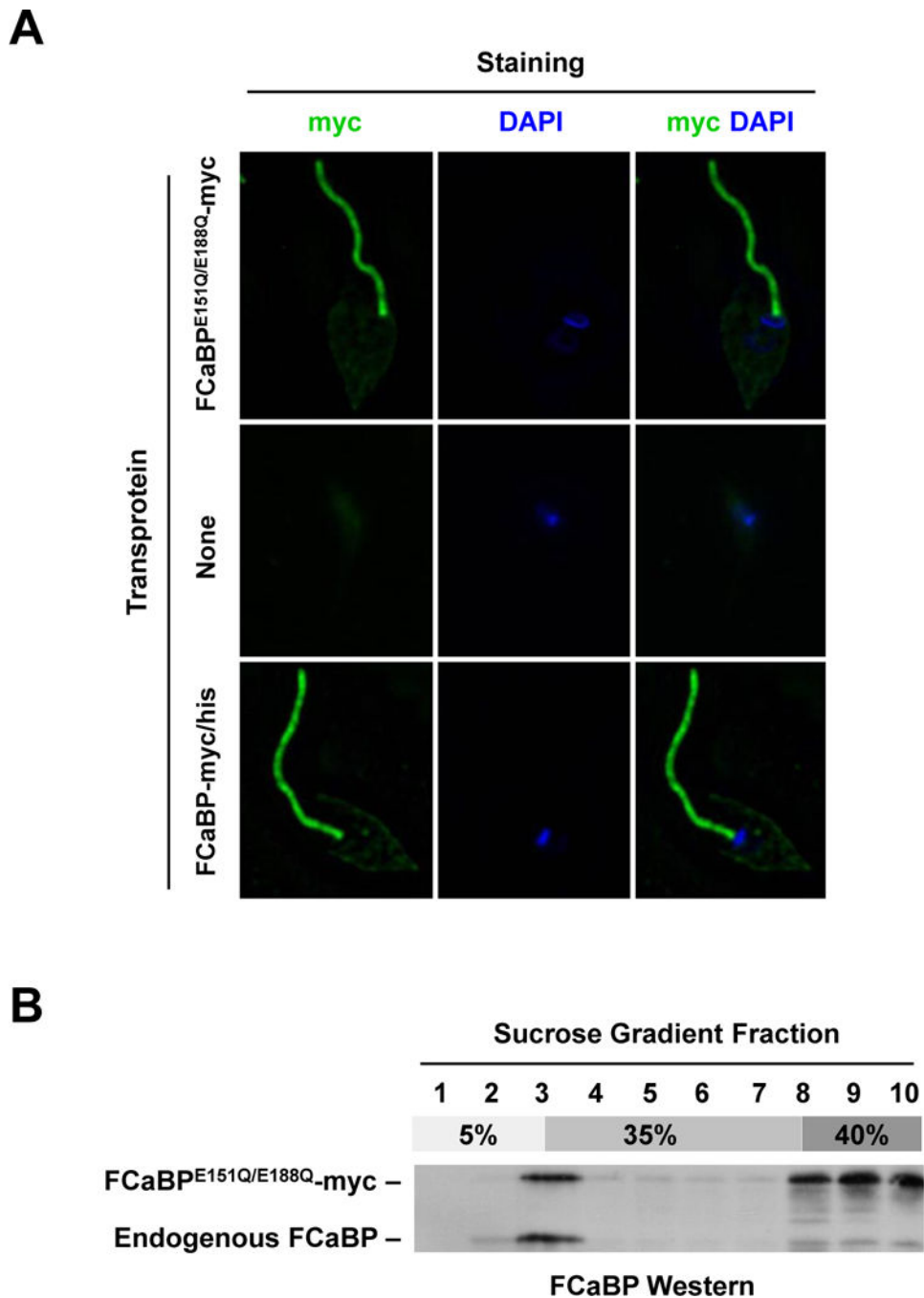
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### Highlights

- ▶ FCaBP is a flagellar calcium sensor of *Trypanosoma cruzi* that displays calcium-dependent membrane association.
- ▶ An FCaBP mutant that does not bind calcium still shows calcium-dependent membrane association.
- ▶ FCaBP can form dimers, so the FCaBP calcium-binding mutant is still flagellar because it can dimerize with resident FCaBP.





**Fig. 1.** Mutation of the Ca<sup>2+</sup> binding sites in FCaBP does not affect flagellar membrane localization or lipid raft association. (A) The flagellar localization of FCaBP is independent of Ca<sup>2+</sup> binding. *T. cruzi* epimastigotes expressing a myc-tagged Ca<sup>2+</sup>-binding mutant of FCaBP (FCaBP<sup>E151Q/E188Q</sup>-myc), no transprotein (None) or wildtype myc/his-tagged FCaBP (FCaBP-myc/his) were analyzed by immunofluorescence microscopy using the myc-specific 9E10 monoclonal antibody. Parasite DNA was stained with DAPI. Both FCaBP-myc/his and FCaBP<sup>E151Q/E188Q</sup>-myc are flagellar. (B) FCaBP associates with lipid rafts independent of

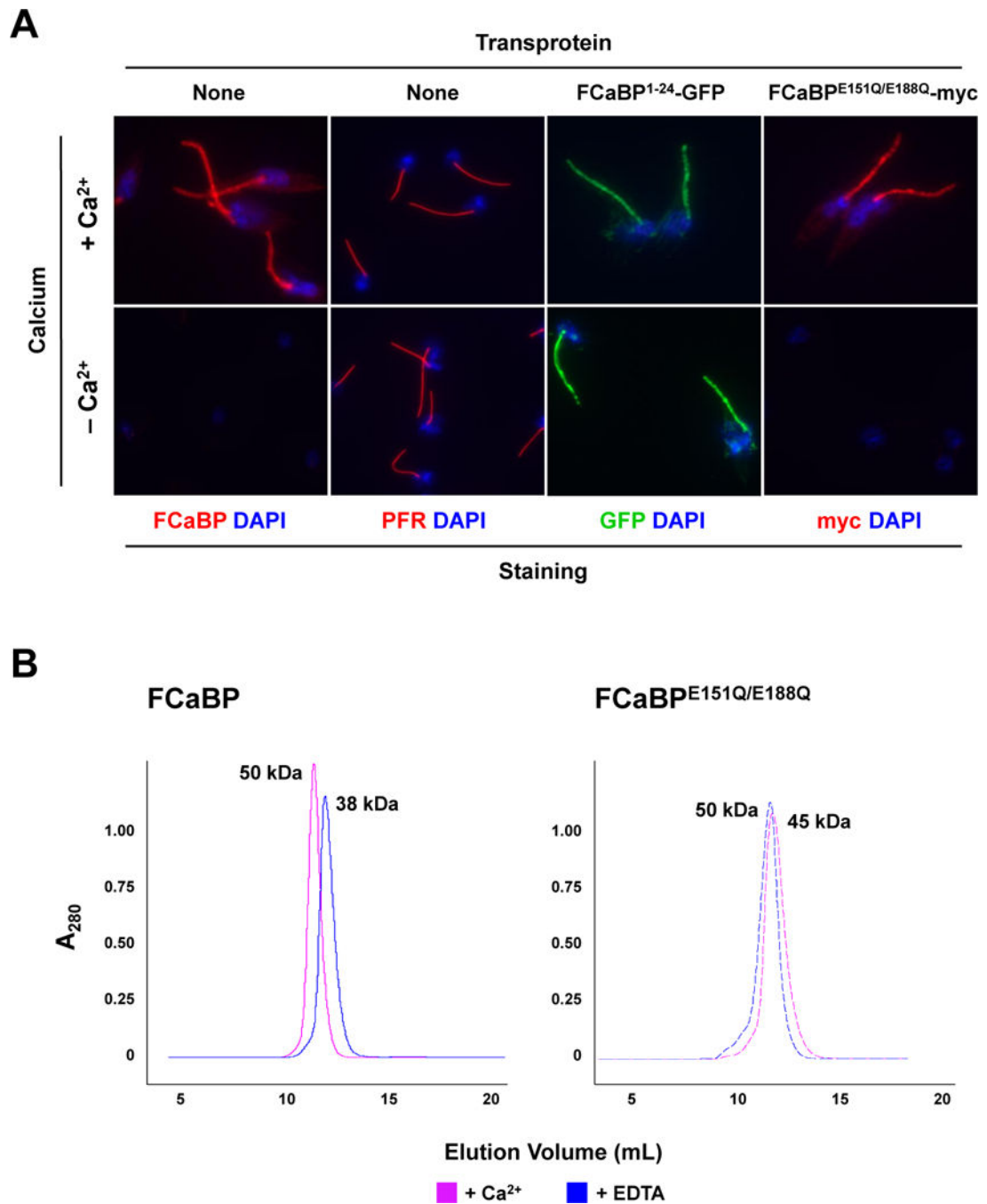
Ca<sup>2+</sup> binding. *T. cruzi* epimastigotes expressing the FCaBP Ca<sup>2+</sup>-binding mutant (FCaBP<sup>E151Q/E188Q</sup>-myc) were lysed in ice-cold Triton X-100 and analyzed by discontinuous (5% – 35% – 40%) sucrose (Optiprep) gradient centrifugation and western blotting using FCaBP-specific antiserum. Both FCaBP<sup>E151Q/E188Q</sup>-myc and endogenous FCaBP float to fraction 3, which contains detergent-resistant membranes.

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**Fig. 2.** Mutation of the Ca<sup>2+</sup> binding sites in FCaBP does not affect Ca<sup>2+</sup>-dependent flagellar membrane localization, possibly due to dimerization with endogenous wildtype FCaBP. (A) The flagellar retention of FCaBP<sup>E151Q/E188Q</sup> is Ca<sup>2+</sup>-dependent. Untransfected epimastigotes and transfectants expressing the N-terminal 24 amino acids of FCaBP fused to GFP (FCaBP<sup>1-24</sup>-GFP) or the FCaBP Ca<sup>2+</sup>-binding mutant FCaBP<sup>E151Q/E188Q</sup>-myc were permeabilized and washed with buffers containing or lacking (EDTA) Ca<sup>2+</sup>. Cells were then analyzed by direct fluorescence (GFP) or immunofluorescence microscopy with

paraflagellar rod [PFR (*T. cruzi* PAR1)]- or FCaBP-specific antisera. DNA was stained with DAPI. Both endogenous FCaBP and FCaBP<sup>E151Q/E188Q</sup>-myc were washed out of the flagellum upon Ca<sup>2+</sup> chelation. (B) Recombinant wildtype FCaBP and the Ca<sup>2+</sup>-binding mutant FCaBP<sup>E151Q/E188Q</sup> form dimers in a Ca<sup>2+</sup>-independent manner. Size exclusion chromatography of recombinant wildtype FCaBP and Ca<sup>2+</sup> binding mutant FCaBP<sup>E151Q/E188Q</sup> were conducted in the presence of 2 mM CaCl<sub>2</sub> (solid line) or 2 mM EDTA (dashed line). The absorbance at 280 nm is plotted vs elution volume. Both wildtype FCaBP and FCaBP<sup>E151Q/E188Q</sup> elute as dimers in both Ca<sup>2+</sup>-bound and Ca<sup>2+</sup>-free states. Multi-angle light scattering confirmed that FCaBP is a dimer in both Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-bound states (not shown).