Calcium binding protein 1 of the protozoan parasite *Entamoeba histolytica* interacts with actin and is involved in cytoskeleton dynamics

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Accepted 8 March 2004
Journal of Cell Science 117, 3625-3634 Published by The Company of Biologists 2004
doi:10.1242/jcs.01198

Summary

Blocking expression of EhCaBP1, a calmodulin-like, four EF-hand protein from the protozoan parasite *Entamoeba histolytica*, resulted in inhibition of cellular proliferation. In this paper we report that EhCaBP1 is involved in dynamic changes of the actin cytoskeleton. Both endocytosis and phagocytosis were severely impaired in cells where EhCaBP1 expression was blocked by inducible expression of the antisense RNA. In wild-type cells both actin and EhCaBP1 were found to co-localize in phagocytic cups and in pseudopods. However, in antisense-blocked cells the phagocytic cup formation is affected. Analysis of the staining patterns in the presence and absence of actin dynamics inhibitors, jasplakinolide and cytochalasin D suggested that EhCaBP1 and polymerized F-actin co-localize on membrane protrusions. Direct interaction between soluble EhCaBP1 and F-actin was further demonstrated by a co-sedimentation assay. A variant of EhCaBP1 did not bind F-actin showing the specificity of the interaction between EhCaBP1 and actin. There is no significant change in the kinetics of in vitro polymerization of actin in presence of EhCaBP1, indicating that EhCaBP1 does not affect filament treadmilling. In addition, using atomic force microscopy; it was found that filaments of F-actin, polymerized in presence of EhCaBP1, were thinner. These results indicate that EhCaBP1 may be involved in dynamic membrane restructuring at the time of cell pseudopod formation, phagocytosis and endocytosis in a process mediated by direct binding of EhCaBP1 to actin, affecting the bundling of actin filaments.

Key words: Phagocytosis, Antisense, Actin, Atomic force microscopy, Cytoskeleton

Introduction

Entamoeba histolytica, is the etiological agent for human amoebic colitis and liver abscess and causes a high level of morbidity and mortality worldwide particularly in the developing countries. It is the third highest cause of death among all the parasitic infections (WHO Report, 1997). Pathogenesis involves penetration into human tissues, attachment of *E. histolytica* to the host cells followed by their cytolysis and phagocytosis. *E. histolytica* not only phagocytoses epithelial cells, but also red blood cells (Tsutsumi et al., 1992), bacteria (Bracha et al., 1982) and cells from the immune system (Ravdin, 1988). Motility and phagocytosis of the parasite require dynamic cytoskeletal organization based on the activity of microfilaments and actin-binding proteins (Voigt and Guillén, 1999). Like eukaryotic cells, regulation of the cytoskeleton in protozoans is triggered by calcium and calcium binding proteins that are critical for the progression and completion of phagocytosis, growth and host-parasite interaction (Camacho, 2003; Burleigh and Andrews, 1998). In *Histoplasma capsulatum*, CBP, a calcium binding protein aids the yeast's survival when they are in a low-calcium environment, such as the phagolysosomal compartment within macrophages (Batanghari et al., 1998). Calmodulin (CaM) has been shown to be involved in regulation of cell growth in *Trypanosoma cruzi* and deletion of the CaM gene slows the procyclic parasite growth by about 50% (Eid and Sollner-Web, 1991). In *Toxoplasma gondii*, depletion of calcium, addition of channel blockers and use of CaM inhibitors blocked the cell invasion process. CaM was found to concentrate at the apical end of *Toxoplasma* and may be involved in cytoskeletal rearrangements for cell entry (Pettzella et al., 1997). Centrin, a Ca^{2+}-dependent cytoskeletal protein is essential for overcoming the G_2/M check point, thus implicating calcium in *Leishmania* growth (Selvapandiyan et al., 2001). Free calcium concentration is acutely elevated upon the engagement of phagocytic receptors of human neutrophils (Stendahl et al., 1994). The calcium/CaM signal is known to be involved in vacuole fusion, trafficking and exocytosis (Peters and Mayer, 1998; Colombo et al., 1997).

Calcium activation events have been less studied in *E. histolytica*. Fibronectin-mediated adhesion in *E. histolytica* can modify cytosolic calcium concentration. This induces the formation of actin adhesion plates and focal contacts, which is a link between calcium signaling and cytoskeletal structures (Carbajal et al., 1996). Also it has been shown that protein kinase C relocates from the cytosol to the membrane fraction

in actively phagocytosing trophozoites (De Meester et al., 1990). The ubiquitous calcium binding protein calmodulin has been shown biochemically to be present in *E. histolytica* (Munoz et al., 1991). CaM has been implicated in many functions, such as channel activation, electron dense granule release, cell proliferation and pathogenic activity of *E. histolytica* (Ravdin et al., 1982; Makioka et al., 2001). However, the mechanism of CaM action is not known, as the corresponding gene has not yet been characterized. A number of other calcium binding proteins have been identified in *E. histolytica*. Two EF-hand calcium binding proteins, grainin 1 and grainin 2 were purified from the granules of *E. histolytica* and are thought to be involved in endocytosis (Nickel et al., 2000). *E. histolytica* also has a functionally diverse calmodulin-like calcium binding protein (EhCaBP1) with no known homologue in the database (Prasad et al., 1992). Though this protein is similar to calmodulins in many structural properties, it has been shown to be functionally different (Yadava et al., 1997). Inhibition of the expression of the EhCaBP1 gene by regulatable antisense RNA expression results in loss of cell growth, suggesting that the gene is essential for *E. histolytica* (Sahoo et al., 2003). In this paper, we show the involvement of EhCaBP1 in actin cytoskeleton-dependent events such as erythrophagocytosis and endocytosis. A detailed analysis suggests that EhCaBP1 may participate in cytoskeleton dynamics through direct interaction with actin.

Materials and Methods

Cell culture

E. histolytica strain HM-1: IMSS clone 6 was maintained and grown in TYI-S-33 medium containing 125 μl of 250 U/ml benzyl penicillin and 0.25 mg/ml streptomycin per 100 ml of medium. Hygromycin (Sigma) was added at 10 μg/ml during maintenance of transgenic cell-lines EhCaBP1-S and EhCaBP1-AS (Sahoo et al., 2003). For experiments, the transfected cells were grown with 20 μg/ml hygromycin and transgenes were induced in the presence of 5 μg/ml tetracycline.

Immunoprecipitation

The cell lysate was prepared in 1 mM Tris-HCl, pH 7.5, 1% SDS containing 2 mM p-hydroxymercuribenzoic acid (PHMB), 1 mM phenylmethylsulfonyl fluoride (PMSF), 6 mM leupeptin and 1 mM *N*-ethyl-maleimide, and was centrifuged at 15,000 rpm to remove the cellular debris. The lysate (500 μg) pre-absorbed on protein A-Sepharose beads, was incubated with the EhCaBP1 antibody (Prasad et al., 1993) at 1:20 dilution for 2 hours at 4°C in a reaction volume of 200 μl. Immune complexes were separated by using protein A beads (50 μl suspension; Sigma, USA) followed by three washes with buffer 1 [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% ovalbumin (w/v), 0.1% Triton X-100 (w/v), 0.05% sodium azide (w/v)] followed by buffer 2 [10 mM Tris-HCl, pH 7.5, 150 mM NaCl] and buffer 3 [0.06 M Tris-HCl, pH 6.8]. The pellet was resuspended in 50 μl of SDS-PAGE buffer (125 mM Tris-HCl 6.8, 2% SDS, 0.1 M DTT, 30% glycerol, 5% β-mercaptoethanol, and Bromophenol Blue) and boiled for 5 minutes. The bound proteins were separated from beads by brief centrifugation and the supernatant was analyzed by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris-glycine buffer. Western blotting was performed as described previously (Vargas et al., 1997). Western blots were developed with monoclonal anti-actin (ICN Biomedicals) at 1:1000 dilution.

Immunofluorescence labeling

E. histolytica cells resuspended in incomplete TYI-S-33 medium at 37°C were transferred onto acetone-cleaned coverslips placed in a Petri dish and allowed to adhere for 10 minutes at 37°C. The culture medium was removed and cells were fixed with 3.7% pre-warmed paraformaldehyde (PFA) for 30 minutes. After fixation, the cells were permeabilized with 0.1% Triton X-100/PBS for 1 minute. Additional permeabilization for 3 minutes with –20°C methanol was needed for myosin II and myosin IB staining. Cells were then washed with PBS and quenched for 30 minutes in PBS containing 50 mM NH_4Cl. The coverslips were blocked with 1% BSA/PBS for 30 minutes, followed by incubation with primary antibody at 37°C for 1 hour. The coverslips were washed with PBS followed by 1% BSA/PBS before incubation with secondary antibody of 30 minutes at 37°C. When F-actin was labeled with phalloidin, the methanol step was omitted. Antibody dilutions used were: EhCaBP1 at 1:50 (Prasad et al., 1993), phalloidin (Sigma; 1 mg/ml) at 1:500, myosin IB at 1:30 (Voigt et al., 1999), PAK at 1:30 (Labruyère et al., 2003), anti-rabbit Alexa 488 (Molecular Probes) at 1:200, anti-rabbit Alexa 594 (Molecular Probes) at 1:300. The preparations were further washed with PBS and mounted on a glass slide using DABCO [1,4-diazbicyclo (2,2,2) octane (Sigma) 10 mg/ml in 80% glycerol]. The edges of the coverslip were sealed with nail-paint to avoid drying.

Confocal laser scanning microscopy

Fluorescent samples were examined on an LSM 510 confocal laser scanning microscope (Zeiss, Germany) equipped with a 63× objective. Alexa-red-labeled samples were visualized after excitation at 543 nm using a He/Ne laser, and Alexa-green-labeled samples after excitation at 488 nm using an argon laser. Focal sections of 0.8 μm with a shift of objective by 1 μm, were captured for 20-30 planes from the bottom to the top of each cell. Images were processed using LSM 510 software, Zeiss, Germany.

FITC-dextran uptake analysis

The endocytosis of *E. histolytica* was studied by observing the uptake of FITC-dextran. Mid-log phase cells were harvested, washed and resuspended in fresh medium. Cells were incubated with FITC-dextran (2 mg/ml, FD-40; Sigma) for 30 minutes at 36°C followed by harvesting and washing with PBS. The slides were prepared in the presence of 70% glycerol in PBS containing 0.1% 2,5-diphenyl-1,3,4-oxadiazole (PPD). The uptake was observed in the presence or absence of tetracycline (5 μg/ml) for the EhCaBP1-S and EhCaBP1-AS cell lines under a microscope with a fluorescence attachment (Axiovert 25, Zeiss). The total number of fluorescent vesicles engulfed by a cell was counted for 10 cells randomly from each slide at 100× magnification, and for each sample five such slides were counted. The amount of endocytosed material was also determined by measurement of total fluorescence in a cell using a fluorescence microscope (Varion, Cary).

Phagocytosis of red blood cells by trophozoites

To quantify the red blood cells (RBC) ingested by amoebae, the colorimetric method of estimation with some modifications was followed (Rabinovitch and Stefano, 1971). Briefly, 1×10^8 RBCs were washed with PBS followed by TYI-S-33 and then incubated with 1×10^6 amoebae for 10 minutes or, as indicated, at 37°C in 0.2 ml culture medium. The amoebae and erythrocytes were pelleted and non-engulfed RBCs were lysed with cold distilled water and centrifuged at 1000 ***g*** for 2 minutes. This step was repeated twice, followed by resuspension in 1 ml formic acid to burst amoebae containing engulfed RBCs. The optical density of the samples was determined by a spectrophotometry at 400 nm using formic acid as the blank.

Actin and EhCaBP1 co-sedimentation assay

A co-sedimentation assay was carried out following essentially the published conditions (Vargas et al., 1997). Briefly, 5 μM rabbit muscle G-actin (Sigma) per reaction was polymerized for 60 minutes in polymerization buffer containing 100 mM KCl and 2 mM $MgCl_2$ at room temperature. After polymerization, actin was mixed with 0.2 mM ATP and the appropriate target protein (5 μM) in a total volume of 150 μl of G buffer (10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 2.5 mM β-mercaptoethanol, 0.5 M KCl, 10 mM $MgCl_2$) and incubated for 2 hours at room temperature. The samples were centrifuged at 100,000 ***g*** for 45 minutes at 4°C. The supernatant (one fourth of total) and pellet fractions (total) were analyzed by 15% SDS-PAGE followed by Coomassie Blue staining. To calculate the binding affinity, increasing concentrations of EhCaBP1 (1.6 μM, 6 μM and 17 μM) were incubated with 2.5 μM actin in polymerization buffer. The supernatant and pellet fractions were collected after ultracentrifugation and analyzed by SDS-PAGE, followed by western blotting for EhCaBP1 at 1:2000 dilution. The band intensity was determined by densitometry to estimate the ratio of bound to free EhCaBP1 and calculate the binding affinity.

Solid phase assay

Different wells of a 96-well plate were coated with 5 μM G-actin in PBS overnight at 4°C and were blocked with 3% BSA in PBS for an additional 24 hours. After washing with PBS-T (Tween 20, 0.05% w/v), EhCaBP1 was added to the wells, in duplicate, at concentrations ranging from 1.7 μM to 10 μM. Bound EhCaBP1 was detected with anti-EhCaBP1 antibody followed by Alkaline phosphatase-linked anti-rabbit IgG using the colorimetric substrate p-nitro-phenylphosphate (PNPP; Sigma). The absorbance was monitored at 405 nm with a microplate reader (Bio-Rad, USA). The concentrations of EhCaBP1 were calibrated to allow reading of the absorbance under a linear range of detection.

Actin polymerization assay

Polymerization of actin was monitored by the increase in fluorescence of pyrene-labeled actin with excitation at 366 nm and emission at 407 nm. The assays were carried out at 20°C in a Safas flx spectrofluorimeter. Samples (80 μl) contained typically 2.5 μM MgATP-G-actin (10% pyrene-labeled), 6 μM EhCaBP1 and other reagents as indicated in polymerization buffer (5 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.01% NaN_3, 0.1 M KCl and 1 mM $MgCl_2$).

Atomic force microscopy (AFM)

The AFM data was collected employing contact mode AFM using the CP-Research model of Thermomicroscopes, USA. The cantilevers employed for this purpose had a force constant of 0.2 nN/m. Images were obtained at a scan rate of 1.5 Hz. The set force in contact mode applied by the cantilever was kept below 5 nN. Topographical dimensions of actin strands were analyzed using the IP 2.1 software supplied with the instrument by the manufacturer. Before each experiment fresh F-actin were prepared by polymerization in presence or absence of EhCaBP1 as described above with some modifications. Briefly, 5 μM rabbit muscle G-actin (Sigma) per reaction was polymerized for 1 hour in polymerization buffer containing 100 mM KCl and 2 mM $MgCl_2$ at room temperature. After polymerization, actin was mixed with 0.2 mM ATP and appropriate target protein (5 μM) in a total volume of 150 μl of buffer (10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 2.5 mM β-mercaptoethanol, 0.5 M KCl, 10 mM $MgCl_2$) and incubated for 3 hours at room temperature. The sample was diluted 1:5000 in double distilled water before laying on freshly cleaved mica and air dried for 30 minutes at room temperature. The specimen was then mounted on a metal disc for imaging by AFM.

Results

EhCaBP1 is enriched on the phagocytic cup of *E. histolytica*

We have previously described EhCaBP1, a calmodulin-like four EF-hand containing Ca^{+2} binding protein (Prasad et al., 1992). The genomic sequence of *E. histolytica* is currently available as part of an effort to sequence the genome in the form of GSS and contig databases. These databases were used to determine putative copy number and presence of EhCaBP1 homologues, if any. This analysis led us to the identification of an isoform of EhCaBP1 sharing 79% identity at the amino acid level. This isoform has been named EhCaBP2 (accession number: AZ542766). EhCaBP2 is a paralogue of EhCaBP1, differing from the latter mainly in the central linker region. Since antibodies against EhCaBP1 are likely to cross react with EhCaBP2 it was important to generate an antibody that only recognizes the EhCaBP1 isoform. The polyclonal antibody against recombinant EhCaBP1 (Prasad et al., 1993) was purified by passing through an EhCaBP2-Sepharose column in order to remove cross-reacting molecules. The column-purified antibody recognized EhCaBP1 and did not recognize EhCaBP2 protein in western blots suggesting that the affinity purified antibody was specific for EhCaBP1 (data not shown).

Trophozoites in culture or in the presence of red blood cells (RBCs) were fixed, immunostained with anti-EhCaBP1-purified antibody and analyzed by laser scanning confocal microscopy (LSCM). The cellular compartments found to contain EhCaBP1 included cytoplasm, vacuoles and membrane extensions. EhCaBP1 was particularly enriched within the phagocytic cup (Fig. 1). To examine whether EhCaBP1 co-localizes with proteins that are known to be associated with the *E. histolytica* cytoskeleton during phagocytosis, we performed double staining of trophozoites with antibody against EhCaBP1 and one of the following: phalloidin, an F-actin cross-linker (Fig. 1A); an antibody against myosin IB (Fig. 1B), or an antibody against PAK (Fig. 1C). Actin molecules present in the RBCs were also decorated and revealed with phalloidin during these experiments. The cells undergoing erythrophagocytosis showed enrichment of F-actin (Fig. 1A), myosin IB (Fig. 1B) and PAK (Fig. 1C) around phagocytic cups and early phagosomes. EhCaBP1 was found to co-localize with F-actin and myosin IB around the phagocytic cups (Fig. 1A,B) and within membrane extensions with PAK (Fig. 1C).

Depletion of EhCaBP1 in *E. histolytica* inhibits cytoskeleton-related functions such as endocytosis and phagocytosis

Membrane deformation leading to pseudopod protrusion is important for phagocytosis and cell motility in *E. histolytica*. The fact that EhCaBP1 localizes in protruding pseudopods prompted us to investigate the role of EhCaBP1 in these two phenomena. We took advantage of parasite strains depleted of EhCaBP1 by antisense RNA technology. In *E. histolytica* cells expressing EhCaBP1 antisense RNA, the level of EhCaBP1 protein is about 40% that of control parasites (Sahoo et al., 2003). The tetracycline-inducible antisense expression system was used and the cell lines carrying the two chimeric plasmids, pEhCaBP1-S (sense) and pEhCaBP1-AS (antisense) were referred to as EhCaBP1-S and EhCaBP1-AS, respectively. The

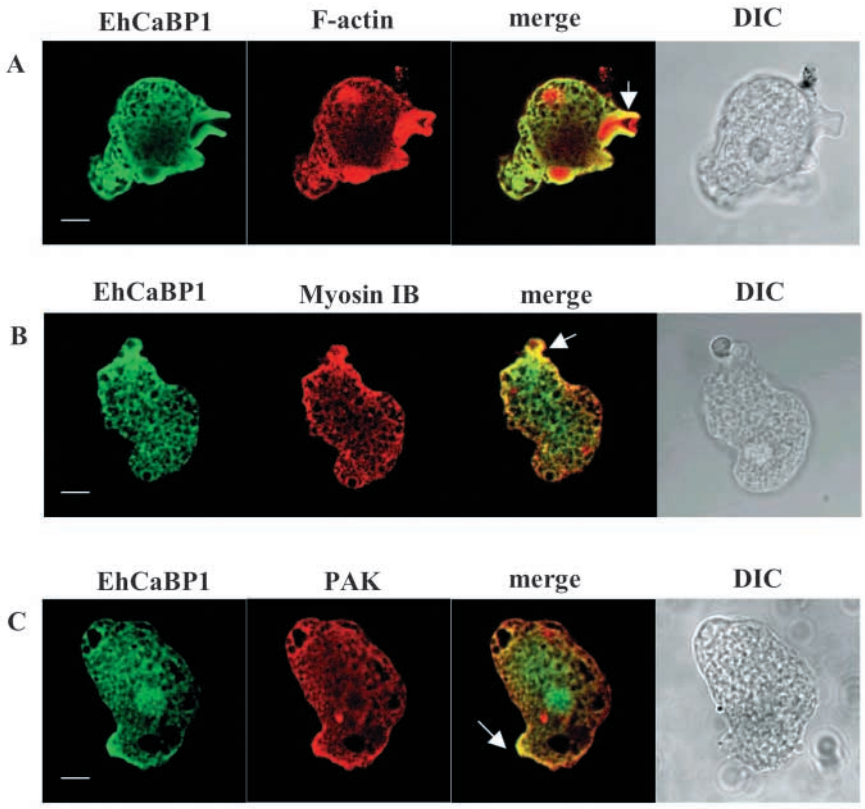

Fig. 1. Immunolocalization of EhCaBP1 in erythrophagocytosing *E. histolytica*. Trophozoites grown for 48 hours were transferred to pre-warmed, acetone-washed cover-slips for 10 minutes at 37°C. The attached cells were further incubated with RBC for 7 minutes. The cells were then fixed with paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS followed by immunostaining with various antibodies mentioned below. Amoebae were double labeled for EhCaBP1 and (A) F-actin (Phalloidin Red), (B) myosin IB and (C) PAK. Myosin IB and PAK were labeled with CY3-conjugated secondary antibody and EhCaBP1 with Alexa-green secondary antibody. Scale bar: 5 μm. Arrow indicates the region of enriched co-localization. Notice co-localization (yellow) of EhCaBP1 with F-actin and myosin IB around the phagocytic cup and with PAK in the pseudopod.

EhCaBP1-AS cells showed a defect in cellular proliferation (Sahoo et al., 2003). Endocytosis constitutes an important physiological property of *E. histolytica* (Batista et al., 2000). Since amoebae ingest as much as 30% of their volume per hour and endocytosis is the major source of food and nutrients, a defect in the endocytic pathway would affect the growth of these organisms.

Fluid-phase endocytosis was inferred by determining the level of uptake of the fluorescent marker FITC-dextran in EhCaBP1-S and EhCaBP1-AS cells in the presence and absence of tetracycline. The level of endocytosis was determined by the number of vesicles containing the fluorescent marker. In EhCaBP1-expression-blocked cells uptake was reduced by 70% compared to un-induced cells (Fig. 2A). There was no significant difference in the uptake of FITC-dextran in EhCaBP1-S cells in presence or absence of tetracycline.

Phagocytosis of human cells is a major indicator of *E. histolytica* pathogenesis. Erythrophagocytosis was measured by a spectrophotometric assay where the quantity of haemoglobin contained in the amoebae as a result of engulfment of RBCs was determined. The amount of erythrophagocytosis by EhCaBP1-AS cells in absence of tetracycline and EhCaBP1-S cells in presence and absence of tetracycline was found to be comparable (Fig. 2B). In contrast, EhCaBP1-AS cells displayed a reduction of 60% in phagocytosis of RBC. The decline in erythrophagocytosis activity was visible within ten minutes of addition of RBCs. The number of RBCs taken up in 10 minutes was estimated as described in Materials and Methods. EhCaBP1-AS cells engulfed about 5 RBCs in 10 minutes in comparison to 10-15 RBCs for control cells (Fig. 2C). The results show that both endocytosis and phagocytosis are inhibited in *E. histolytica* cells where expression of EhCaBP1 is blocked, suggesting the involvement of EhCaBP1 in these processes.

Altered actin organization in EhCaBP1-AS cells

We examined whether the reduction in EhCaBP1 could affect actin localization as phagocytosis of RBCs was inhibited in EhCaBP1 antisense-blocked cells and EhCaBP1 and F-actin co-localized around phagocytic cups. EhCaBP1-AS and EhCaBP1-AS+tet cells incubated with RBCs were fixed, stained with phalloidin and anti-EhCaBP1-purified antibody and analyzed by LCSM. The distribution of EhCaBP1 and actin in EhCaBP1-AS cells was equivalent to the distribution observed in the wild-type cells (Fig. 3A). EhCaBP1 along with actin localized around the phagocytic cups. In contrast, in tetracycline-treated EhCaBP1-AS cells, where the level of EhCaBP1 was reduced, the recruitment of actin to phagocytic cups was largely impaired (Fig. 3B), indicating that EhCaBP1 is necessary for changes in the actin-rich cytoskeleton during phagocytosis.

Localization of EhCaBP1 and actin in the presence of inhibitors of actin dynamics

To investigate the dynamic of actin and EhCaBP1 interaction, we incubated parasites with agents that affect actin dynamics and then stained them for EhCaBP1 and F-actin. We utilized jasplakinolide, which inhibits depolymerization of F-actin, and cytochalasin D (CD), which prevents addition of G actin by capping barbed end of F-actin. In cells treated with jasplakinolide, staining of EhCaBP1 and F-actin showed co-

localization of both EhCaBP1 and F-actin (Fig. 4). In CD-treated parasites, bright patches containing actin were seen and there was no co-localization of EhCaBP1 with these actin patches (Fig. 4). Both these inhibitors did not affect the cytoplasmic distribution of EhCaBP1 in *E. histolytica* cells. The data suggests that co-localization may involve interaction of EhCaBP1 with F-actin.

EhCaBP1 interacts with F-actin

The cellular analysis by confocal microscopy indicated that EhCaBP1 co-localizes with F-actin in cell protrusions and this may have a role in endocytosis and phagocytosis. We addressed the question of direct interaction of EhCaBP1 with actin filaments using an in vitro test involving immunoprecipitation with specific EhCaBP1 antibody. The proteins collected along with the immune complex were analyzed by electrophoresis and immunoblotting. Antibodies against proteins related to actin cytoskeleton dynamics were used to identify the proteins co-immunoprecipitated with EhCaBP1. These included antibodies against actin, myosin IB, PAK and profilin. Of these, only actin was detected in the western blot. Thus EhCaBP1

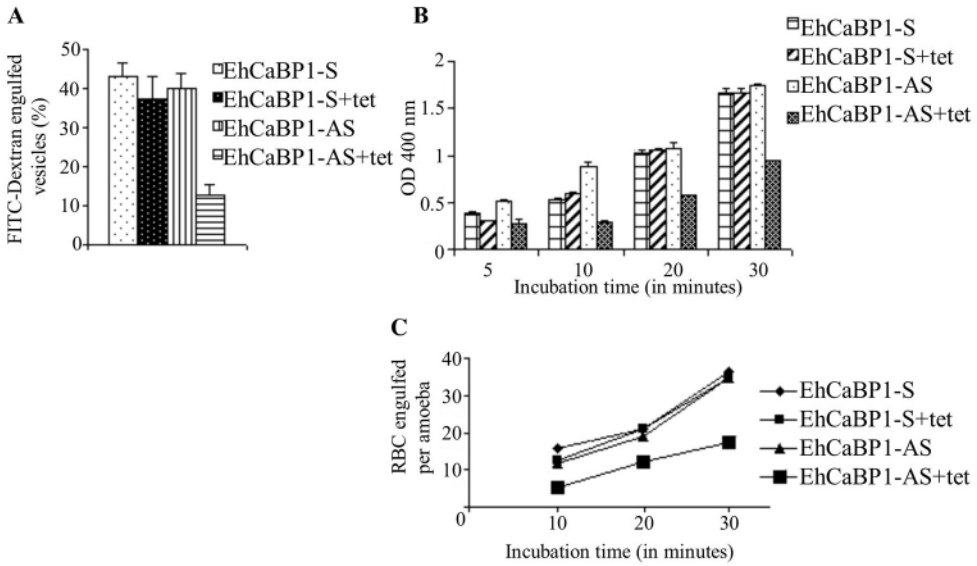

Fig. 2. Phagocytosis and endocytosis are affected by down regulation of EhCaBP1. (A) Fluid-phase endocytosis in EhCaBP1-AS and EhCaBP1-S cells. Transformed cells were grown in 20 μg/ml hygromycin with or without 5 μg/ml tetracycline for 48 hours. For the FITC-dextran uptake assay, 2×10^5 cells were incubated with 1 mg/ml FITC-dextran in PBS for 30 minutes followed by thorough washing. Quantitative analysis of the fluorescent images was carried out by counting the number of vesicles containing fluorescent FITC-dextran in each cell. The data represent percentage mean (±s.d.) of fluorescent-labeled vesicles per cell. Ten cells were randomly selected per slide and counting of engulfed beads was done for five separate slides for each cell type. (B) Erythrophagocytosis in EhCaBP1-AS and EhCaBP1-S cells. Transformed cells were grown in 20 μg/ml hygromycin with or without 5 μg/ml tetracycline for 48 hours. The cells (2×10^6) were incubated with red blood cells (RBCs: 2×10^8) for the indicated times followed by washing with water to remove adhering cells. The amoebae with engulfed RBCs were then lysed with formic acid. The amount of heme was determined by measuring the optical density at 400 nm for the different cell types indicated. Values are mean ± s.d. (C) The number of RBCs internalized for each amoeba cell-type. The data from sector B was used to compute the number of RBCs internalized as described in Materials and Methods.

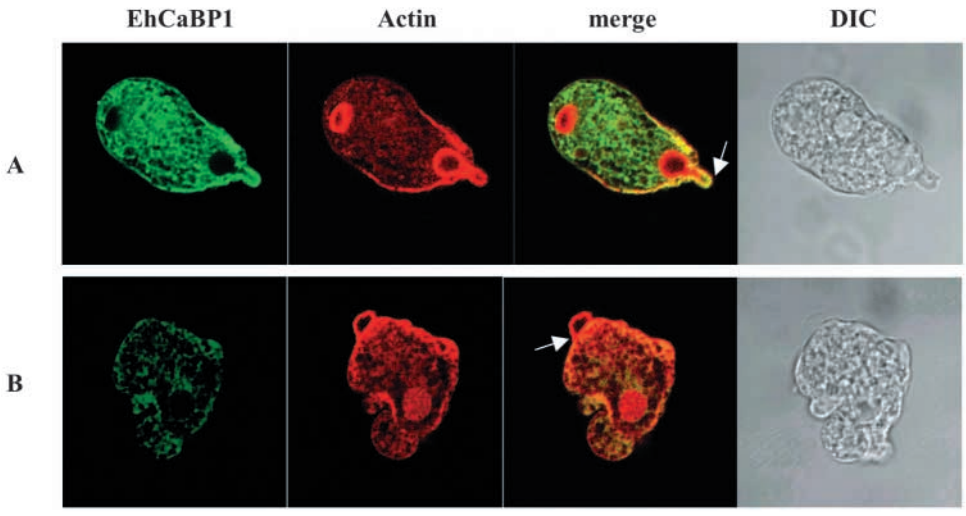

Fig. 3. Distribution of F-actin is affected in tetracycline-induced EhCaBP1-AS cells during phagocytosis. The attached EhCaBP1-AS (A) and EhCaBP1-AS+tet (B) cells were incubated with RBCs for 7 minutes prior to fixing and immunostaining. Amoebae were double labeled for EhCaBP1 and F-actin (Phalloidin red). EhCaBP1 was labeled with Alexa-green secondary antibody. Notice the actin rich phagocytic cup in the EhCaBP1-AS cell (arrow), absence of a cup around the RBC (arrow) and reduced expression of EhCaBP1 in EhCaBP1-AS+tet cell.

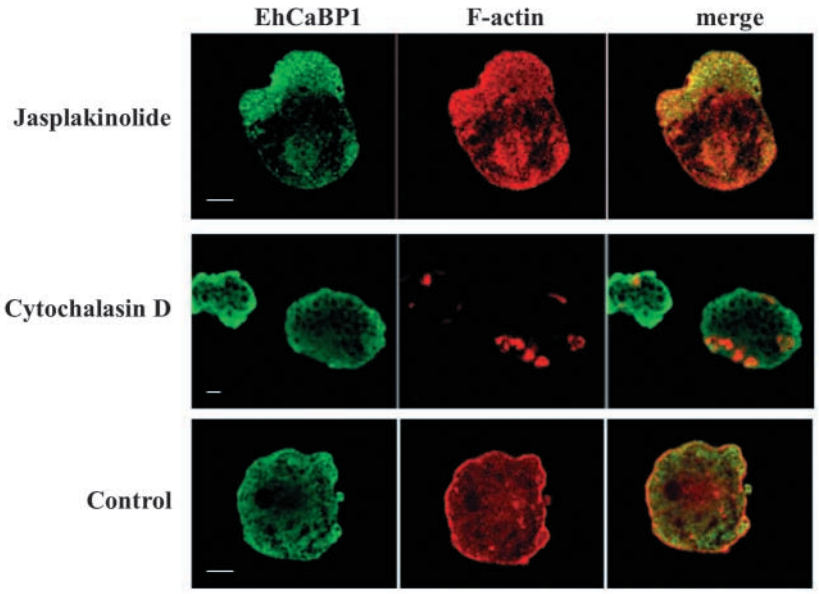

Fig. 4. Distribution of F-actin and EhCaBP1 in jasplakinolide- and cytochalasin D-treated cells. The HM-1: IMSS cells grown for 48 hours were incubated at 37°C with jasplakinolide at 10 μM for 30 minutes or cytochalasin D at 10 μM in a reaction volume of 1 ml for 15 minutes or with DMSO alone. The cells were pelleted and fixed before immunostaining with rhodamine-phalloidin (red) and anti-EhCaBP1 antibody. EhCaBP1 was labeled with Alexa-green secondary antibody. Finally the cell-pellet was diluted in DABCO for laying on glass slides. Notice enrichment of EhCaBP1 in areas where F-actin was fixed by jasplakinolide. Scale bar: 5 μm.

may interact directly with actin despite the absence of any bonafide actin-binding site in this protein (Fig. 5A).

The ability of EhCaBP1 to bind F-actin was also examined by a co-sedimentation assay. Actin filaments were incubated with purified EhCaBP1 and assessed for co-sedimentation at high speed. A known actin-binding protein α-actinin, used as a positive control, co-sedimented with actin in the pellet fraction as expected (Fig. 5B, lane 8). EhCaBP1 by itself was not observed in the pellet fraction (Fig. 5B, lane 4). However, upon incubation with actin, EhCaBP1 was readily detected in the pellet fraction (Fig. 5B, lane 6). In contrast, BSA, a negative control could not be pelleted down with actin (Fig. 5B, lanes 1 and 2). This result suggests a direct interaction between F-actin and EhCaBP1. By using the co-sedimentation assay, the affinity constant of EhCaBP1 for actin was determined (Fig. 5C). The apparent K_d for EhCaBP1 binding to F-actin was 2±0.1 μM, indicating that EhCaBP1 is a potential binder of F-actin. The binding affinity was also determined by a solid phase method where binding of EhCaBP1 to G-actin was determined by an ELISA-based assay. From a standard curve of known values of EhCaBP1, the bound and free values were calculated (Fig. 5D). The K_d value of 2.6±0.3 μM was found to be similar to that obtained by sedimentation assay. The role of calcium ions in this binding was investigated by carrying out the solid phase binding reaction in presence of EGTA. There was 40-50% reduction in the amount of EhCaBP1 bound in presence of EGTA (Fig. 5F) suggesting that calcium ions play an important role, as expected.

The specificity of binding of EhCaBP1 was further tested by studying binding of EhCaBP2 to actin by co-sedimentation (Fig. 5E). The major difference between EhCaBP1 and EhCaBP2 proteins is in the central linker (Chakrabarty et al., 2004). The results showed that there was no significant binding of EhCaBP2 with actin. Since the central linker region showed maximum diversity between the two proteins the role of the central linker region in actin binding was further checked by using delcenEhCaBP1, a central-linker deletion mutant of EhCaBP1. This mutant did not bind to actin as seen from absence of the molecule in the pellet. Moreover, no significant binding was observed when EhCaBP2 was used instead of EhCaBP1 in the solid phase assay (data not shown here). The results suggested that EhCaBP1 specifically binds actin and that the interaction is probably through the central linker region.

EhCaBP1 does not affect the rate of polymerization of G-actin

It is probable that changes seen in the organization of F-actin in EhCaBP1-expression-blocked cells may be due to changes in the kinetics of actin polymerization. In order to check this hypothesis, actin polymerization assay in the presence of EhCaBP1 was carried out in vitro. The result is shown in Fig. 6. There was no significant difference in rates of nucleation and polymerization of actin in the presence or absence of EhCaBP1, suggesting that filament treadmilling is not affected by EhCaBP1.

EhCaBP1 affects F-actin bundle thickness

Actin interacts with a very wide variety of proteins, including molecular motors such as myosins and other anchoring, sequestering and cross-linking molecules. We decided to explore whether EhCaBP1 could induce changes in the actin filaments. Electron microscopy (negative staining with uranyl acetate and rotary shadowing) of pelleted samples obtained in co-sedimentation experiments was not able to reveal any change in the organization of actin filaments recovered in the presence of EhCaBP1 (data not shown here). We decided to utilize atomic force microscopy (AFM) because of its high signal-to-noise ratio and the fact that AFM allows the observation of macromolecules in hydrated environment. Reproducible images were obtained under different ratios of F-actin to EhCaBP1 (1:1 and 1:2) and incubation periods of polymerization (1-3 hours). F-actin was seen to have varied lengths and branching patterns. Two representative images of actin filaments (polymerized for 3 hours) are shown in Fig.

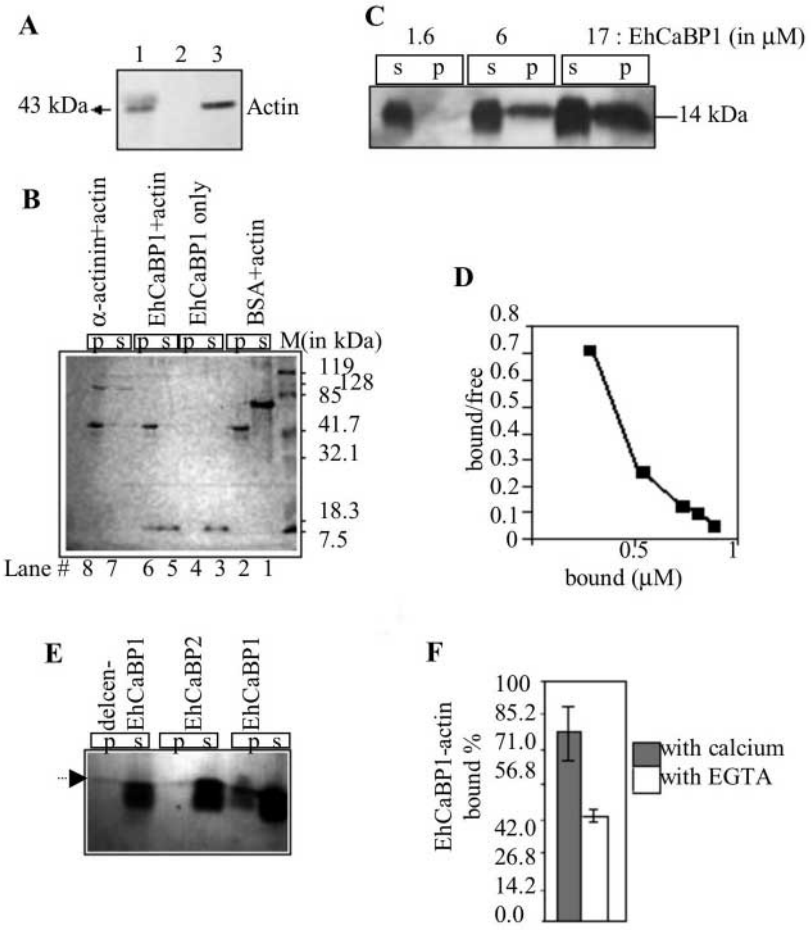

Fig. 5. Binding of EhCaBP1 to actin.
(A) Immunoprecipitation of amoebic proteins with anti-EhCaBP1 antibody and western analysis with anti-actin antibody. *E. histolytica* cell lysate was used for immunoprecipitation using anti-EhCaBP1 antibody and protein A beads. The immunoprecipitate was eluted from beads in SDS-PAGE buffer and separated on a 10% SDS-PAGE gel. The proteins were immobilized on nitrocellulose by electrophoretic transfer for immunostaining with anti-actin antibody and anti- mouse IgG linked to peroxidase. The bound antibody was detected using the ECL system. Lanes 1: immunoprecipitated amoebic proteins; lane 2: control precipitation with only protein A beads; lane 3: total lysate (10 μg protein). Molecular mass is indicated.
(B) Co-sedimentation of EhCaBP1 with F-actin. Purified recombinant EhCaBP1 (5 μM), α-actinin (5 μM) or bovine serum albumin (5 μM) were incubated with F-actin (5 μM), in polymerization buffer containing salts, followed by ultracentrifugation to separate the soluble and pellet fractions. Pellet (p) and supernatant (s) were resolved in 15% SDS-PAGE followed by Coomassie Blue staining. Total pellet fraction and one fourth of total supernatant was loaded for gel analysis.
(C) Immunoblotting of EhCaBP1 co-sedimented with F-actin. Different amount of EhCaBP1 (1.6 μM, 6 μM and 17 μM) was incubated with F-actin in polymerization buffer containing salt as described in B. The pellet and supernatant fraction after ultracentrifugation were resolved in 15% SDS-PAGE followed by western analysis with anti-EhCaBP1 antibody as described in Materials and Methods. The amount of EhCaBP1 co-sedimented was determined by densitometry. The binding affinity was computed using a Scatchard plot.
(D) Scatchard plot for EhCaBP1-actin interaction. Different wells of a microtiter plate were coated with 50 μl of 5 μM actin overnight at 4°C. After blocking with BSA, EhCaBP1 was added at the indicated concentrations ranging from 10 μM to 0.1 μM, followed by incubation with anti-EhCaBP1 antibody. The amount of bound EhCaBP1 was determined using anti-rabbit alkaline phosphatase-linked IgG. The amount of product formed was detected at 405 nm. (E) Co-sedimentation of EhCaBP1, delcenEhCaBP1 and EhCaBP2 with actin. Purified EhCaBP1, delcenEhCaBP1 or EhCaBP2 (5 μM), were incubated with F-actin (5 μM) in sedimentation buffer, followed by ultracentrifugation to separate the soluble and pellet fractions as described in Materials and Methods. Pellet and supernatant fractions were resolved in 15% SDS-PAGE followed by Coomassie Blue staining. Arrow indicates the position of soluble EhCaBP1 (14 kDa).
(F) Calcium requirement for binding of EhCaBP1 to actin. Binding was carried out by solid phase assay in the presence of excess calcium (5 mM) or EGTA (2 mM). The plot shows the relative mean intensity (as the percentage ±s.d.) obtained from three independent experiments.

7A,B. The line plot of the actin strand gives the thickness of the filaments. Images showed that F-actin appeared as a well defined fiber containing several parallel actin filaments. The average full-width of actin strands at half height calculated from ten independent points was found to be 45 nm. In the presence of EhCaBP1, the fiber consistently had thinner strands with an average width of 34 nm. Two representative images of F-actin in presence of EhCaBP1 are shown in Fig. 7C,D. The contribution of EhCaBP1 to the F-actin bundle thickness was ruled out because of its small size and inability to form complexes with self molecules. EhCaBP1 may be affecting the arrangement of F-actin filaments such that in the presence of EhCaBP1 fewer parallel strands of single filaments constitute the actin fiber.

Discussion

We have previously identified the EhCaBP1 gene in *E. histolytica*, which encodes a calcium-binding protein containing four EF hands. In this study, the function of EhCaBP1 was deciphered using biochemical, cell biological and molecular approaches.

Confocal microscopy of cells immunolabeled with EhCaBP1 antibody showed cytoplasmic localization of the protein, excluding the vesicles and the nucleus, but present also on pseudopods and beneath the plasma membrane. The association of EhCaBP1 with different components of the cytoskeleton was also visualized by co-localization studies. Amoebic pseudopods are not only enriched with cytoskeletal proteins, such as myosin IB and actin, but also with EhCaBP1. The enrichment of EhCaBP1 on pseudopods suggests its association with areas rich in actin cytoskeleton and its associated proteins. However, this enrichment pattern was not seen over the portion of the membrane not undergoing any ruffle. Therefore it appears that presence of actin in some of the areas underneath the plasma membrane ensures recruitment of EhCaBP1 or vice versa. When amoebic cells are incubated with RBCs, phagocytic cups can be seen prior to engulfment

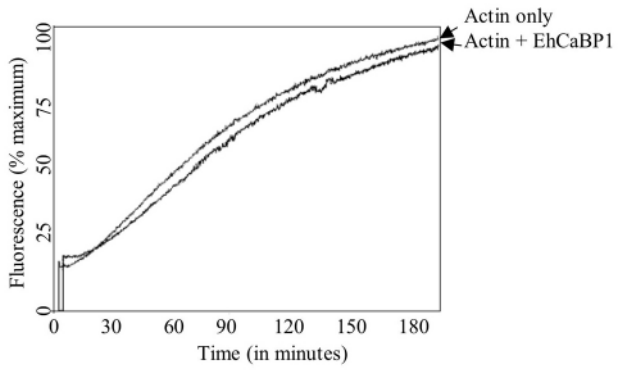

Fig. 6. Effect of EhCaBP1 on actin polymerization. Actin-labeled with pyrene (2.5 μM) with or without EhCaBP1 (6 μM) was polymerized as described in Materials and Methods. The fluorescence of pyrene actin was observed as percentage maximum over a period of 3 hours.

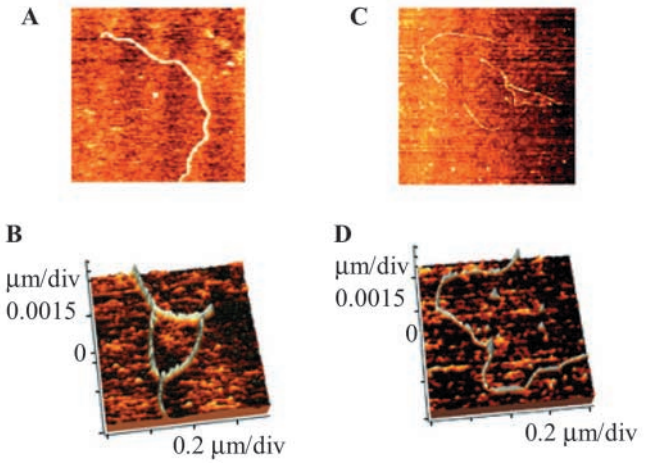

Fig. 7. Atomic force micrographs of F-actin. Actin (5 μM) was incubated in polymerization buffer in absence (A,B) or presence of EhCaBP1 (C,D). The samples were diluted 1:5000 times before mounting on freshly cleaved mica for atomic force microscopy. (A,C) two-dimensional view; 2 μm scan. (B,D) Three-dimensional top view; 0.8 μm scan.

of the RBCs. EhCaBP1 is observed to be present around the cup along with actin. It is probable that EhCaBP1 is recruited early in the initiation of phagocytosis and is lost after phagocytic vesicles are formed, although association of actin with the vesicles continues. During phagocytosis of RBCs in EhCaBP1-expression-blocked cells, neither actin nor EhCaBP1 were efficiently recruited to form a phagocytic cup and this altered actin dynamics inhibiting membrane deformation and RBC uptake. Jasplakinolide treatment of cells freezes the actin filaments by inhibiting depolymerization. In these cells clear co-localization and enrichment of EhCaBP1 and F-actin was seen. The association of EhCaBP1 with F-actin in jasplakinolide-treated cells may be due to a high affinity of EhCaBP1 for actin bundles. In contrast, when the cells lost these filaments; for instance, in the presence of cytochalasin D, EhCaBP1 dissociates from the remaining array of filaments. This suggests that EhCaBP1 may not play a direct role in actin filament production but rather in the higher organization of these filaments. It is difficult to speculate on the detailed mechanism from these results at present; however, it is clear that EhCaBP1 in association with actin may be involved in dynamic changes of the actin cytoskeleton.

The *E. histolytica* cells blocked for EhCaBP1 expression using antisense technology were used in many experiments such as FITC-dextran uptake and erythrophagocytosis. Incubation of these expression-blocked cells with human RBCs showed that these amoebae were deficient in erythrophagocytosis, suggesting a role for EhCaBP1 in the pathogenic activity of a human parasite. The level of uptake of RBCs remained low even on prolonged incubation (30 minutes), indicating that attachment and/or intake may be affected in these cells. Fluorescent staining of the erythrophagocytosis-impaired cells failed to show active phagocytic cups around RBCs, as seen for the uninduced, transformed cells and wild-type cells.

The result with EhCaBP1 was found to be similar to the results obtained by overexpression of myosin IB (Voigt et al., 1999) that led to a modification of phagocytosis. A detailed pathway implicating these two molecules at the molecular level needs further exploration.

Whole cell imaging studies have clearly shown an association of EhCaBP1 with actin in the form of co-localization within a specific region of the cell. For in vitro status, interaction of EhCaBP1 with the cytoskeletal fraction was also demonstrated using a co-sedimentation assay and immunodetection of actin in the immunoprecipitated fraction with anti-EhCaBP1 antibody. The presence of EhCaBP1 along with F-actin in the pellet after co-sedimentation suggests their direct interaction in vitro.

The binding affinities of actin binding proteins were found to vary from 0.1 to about 1.0 μM. EhCaBP1 binds actin with an affinity constant of around 2.0 μM, which is somewhat lower than other actin binding molecules. Therefore EhCaBP1 may be required in higher amounts for cellular activity. Large phenotypic changes observed in expression-blocked cells may be caused by reduction of EhCaBP1 concentration below a threshold. There are however, proteins that bind actin with even lower affinity, such as calponin with 6.0 μM (Lu et al., 1995). The calcium-induced conformational change involves a central linker in target binding. A 50% reduction in binding to actin in the presence of EGTA again confirms calcium-dependent functionality of EhCaBP1. EhCaBP1 is also likely to be involved in different processes/pathways as it is known to bind a number of cytoplasmic proteins (Yadava et al., 1997). The binding to the cytoskeleton may be through either actin or other molecules that are also associated with it. The binding of EhCaBP1 to actin was also visualized by AFM using an indirect gold-labeled immunological staining. EhCaBP1 appeared to be uniformly distributed on actin with no preferential site, unlike other actin binding proteins, such as N-RAP and nebulin which are found exclusively in sarcomeres and at the end of actin bundles, respectively (data not shown) (Herrera et al., 2000). Its distribution is similar to actinin, which is found homogenously all over the stress fibers as well as in filaments (Langanger et al., 1984).

From the data presented here it is clear that in the absence EhCaBP1 there are major changes in actin organization in *E. histolytica*. This may be the result of EhCaBP1 binding to actin

either directly or through other proteins that interact with actin and or EhCaBP1. In general, the changes in F-actin based on activity of binders may be caused by changes in the rates of polymerization and depolymerization of filaments and in bundling of different F-actin molecules. The data presented here showed that in vitro actin polymerization and/or nucleation processes are not altered by EhCaBP1. However, changes were observed in bundling properties as seen from the AFM experiments. This indicates that the association of filaments in a bundle may be regulated by EhCaBP1. Since these experiments were carried out with pure actin and EhCaBP1 the role of other molecules in these processes may have been overlooked.

The detailed mechanism by which EhCaBP1 may be functioning needs further investigation. It may also be involved in the localization of the cytoskeletal complex at the base of the membrane in response to a stimulus signaled by calcium that initiates phagocytosis and/or endocytosis. A number of calcium binding proteins are known to exert influence on the functioning of cytoskeleton. DAP-kinase a calcium-regulated death-promoting kinase is known to bind actin filaments. One of the substrates of DAPk was identified as myosin light chain (MLC), the phosphorylation of which mediates membrane blebbing (Shohat et al., 2002). The neuronal calcium sensors are a family of EF-hand-containing Ca^{2+}-binding proteins. Neurocalcin a member of this family is an *N*-myristoylated calcium-binding protein that directly interacts with actin in a calcium-dependent manner (Mornet and Bonet-Kerrache, 2001). There is a possibility that neurocalcin delta may be involved in the control of clathrin-coated vesicle traffic (Ivings et al., 2002). CaM is known to participate in endocytosis and actin cytoskeleton organization (Geli et al., 1998; Ohya and Botstein, 1994). Regulation of cytoskeletal organization by CaM is through modulation of PtdIns(4,5)P_2 levels and subsequently phospholipase D activity (Desrivieres et al., 2002). The cytoplasm of *E. histolytica* has numerous granules. Calcium binding proteins have been identified in these granules that may have important role in granule discharge during cell killing (Nickel et al., 2000). Therefore it is probable that EhCaBP1 modulates actin-mediated processes through the participation of other proteins in a complex network.

It is also possible that EhCaBP1 may be able to pull the membrane-attached actin filaments away from the membrane or denucleate these into shorter filaments, thereby allowing ingestion of RBC as phagosomes, similar to some of the actin depolymerization factors, such as ADF/cofilins.

This study suggests that EhCaBP1 might be a novel addition to the already known list of essential cytoskeleton effectors. Studies on the correlation between the already characterized factors and this novel protein, which has so far been seen only in the early branching eukaryote *E. histolytica*, will further help us to understand regulation of some of the fundamental processes such as phagocytosis and endocytosis in this primitive organism.

This work was supported by the Department of Science and Technology and the Department of Biotechnology, India. Special thanks to C. Zimmer and M. F. Carlier for encouraging discussions. N. Sahoo acknowledges receipt of a senior research fellowship from the University Grants Commission, India and thanks the French Embassy, New Delhi for the travel grant and fellowship.

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