

# Calcium regulation of actin crosslinking is important for function of the actin cytoskeleton in *Dictyostelium*

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## Summary

The actin cytoskeleton is sensitive to changes in calcium, which affect contractility, actin-severing proteins, actin-crosslinking proteins and calmodulin-regulated enzymes. To dissect the role of calcium control on the activity of individual proteins from effects of calcium on other processes, calcium-insensitive forms of these proteins were prepared and introduced into living cells to replace a calcium-sensitive form of the same protein. Crosslinking and bundling of actin filaments by the *Dictyostelium* 34 kDa protein is inhibited in the presence of micromolar free calcium. A modified form of the 34 kDa protein with mutations in the calcium binding EF hand (34 kDa  $\Delta$ EF2) was prepared using site-directed mutagenesis and expressed in *E. coli*. Equilibrium dialysis using [ $^{45}$ Ca]CaCl<sub>2</sub> revealed that the wild-type protein is able to bind one calcium ion with a K<sub>d</sub> of 2.4  $\mu$ M. This calcium binding is absent in the 34 kDa  $\Delta$ EF2 protein. The actin-binding

activity of the 34 kDa  $\Delta$ EF2 protein was equivalent to wildtype but calcium insensitive *in vitro*. The wild-type and 34 kDa  $\Delta$ EF2 proteins were expressed in 34-kDa-null and 34 kDa/ $\alpha$ -actinin double null mutant *Dictyostelium* strains to test the hypothesis that calcium regulation of actin crosslinking is important *in vivo*. The 34 kDa  $\Delta$ EF2 failed to supply function of the 34 kDa protein important for control of cell size and for normal growth to either of these 34-kDa-null strains. Furthermore, the distribution of the 34 kDa protein and actin were abnormal in cells expressing 34 kDa  $\Delta$ EF2. Thus, calcium regulation of the formation and/or dissolution of crosslinked actin structures is required for dynamic behavior of the actin cytoskeleton important for cell structure and growth.

Key words: Cytoskeleton, Motility, EF hands, Calcium regulation, Actin-binding protein, *Dictyostelium*

## Introduction

The actin cytoskeleton makes essential contributions to diverse processes, including endocytosis, morphogenesis, growth and division. Precise spatial and temporal control of the assembly, disassembly and contractility of the actin cytoskeleton is key to production of coordinated cell movements. Calcium is a prominent regulator that can exert multiple effects on the structure and dynamics of the actin cytoskeleton. Such effects of calcium are supported by direct measurement of calcium transients during normal cell movements, and effects of experimentally induced calcium transients on the structure and contractility of cells and cell extracts (Condeelis and Taylor, 1977; Cubitt et al., 1995; Hellewell and Taylor, 1979; Janson et al., 1991; Nebl and Fisher, 1997; Taylor and Fechheimer, 1982; Taylor et al., 1976; Taylor et al., 1980b; Unterweger and Schlatterer, 1995; Yumura et al., 1996). Such effects of calcium on cytoplasmic consistency and contractility may occur through effects of calcium on a wide variety of known calcium-binding cytoskeletal proteins. For example, calcium can affect biological processes by stimulation of myosin contractility (Tan and Boss, 1992), activation of actin filament severing (Yamamoto et al., 1982), inhibition of actin crosslinking by  $\alpha$ -actinin (Fechheimer et al., 1982; Witke et al., 1993) or the 34 kDa actin-crosslinking protein (Fechheimer and Taylor, 1984), binding to annexins (Doring et al., 1995), calmodulin (Clarke

et al., 1980; Lydan et al., 1994; Zhu and Clarke, 1992; Zhu et al., 1993) or other low molecular weight calcium-binding proteins including CBP1-4 (Andre et al., 1996; Dharamsi et al., 2000), calpain (Huttenlocher et al., 1997) or other potential targets. This paper tests the hypothesis that control of actin filament crosslinking by calcium is critical to normal cell structure and function.

The calcium-regulated 34 kDa protein has been localized to the cell cortex, filopodia and pseudopodia, phagocytic cup and cell-to-cell contact sites (Fechheimer, 1987; Fechheimer et al., 1994; Furukawa et al., 1992; Furukawa and Fechheimer, 1994; Johns et al., 1988; Okazaki and Yumura, 1995). The 34 kDa protein has been proposed to play a role in regulating the viscoelastic properties of the actin cytoskeleton. *In vitro* studies show that the 34 kDa protein is able to crosslink F-actin into bundles in a calcium-regulated manner. The 34 kDa protein bundles actin at low calcium ( $1 \times 10^{-8}$  M), but at elevated calcium levels ( $1 \times 10^{-6}$  M) the protein is unable to bundle F actin (Fechheimer, 1987; Fechheimer and Taylor, 1984; Lim and Fechheimer, 1997). The cDNA sequence of the 34 kDa protein indicates that the protein has 295 amino acids, with two putative EF hand regions (Fechheimer et al., 1991). Mutants lacking the 34 kDa protein grow and develop normally at 20°C but have abnormal filopodia, decreased persistence of motility and are cold-sensitive for growth (Rivero et al., 1996;

Rivero et al., 1999). Cells lacking two calcium-sensitive actin-crosslinking proteins,  $\alpha$ -actinin and the 34 kDa protein, grow slowly at 15°C and 20°C, endocytose fluid phase slowly, produce small cells and undergo morphogenesis to produce aberrant fruiting bodies (Rivero et al., 1999).

To test the hypothesis that calcium regulation of actin crosslinking is important, we first investigated the basis of calcium binding to the 34 kDa protein. The EF hand regions of the 34 kDa protein were modified by site-directed mutagenesis to change critical charged amino acids at the coordinating X, Y, Z positions to uncharged alanines (Fig. 1A). In vitro studies of these proteins show that the 34 kDa protein has one high-affinity calcium-binding site, which resides in the putative second EF hand. However, the actin binding and crosslinking of the 34 kDa  $\Delta$ EF2 protein is not regulated by calcium. We have expressed the 34 kDa wild-type and 34 kDa  $\Delta$ EF2 proteins in mutant *Dictyostelium* cell lines lacking the 34 kDa protein or both 34 kDa protein and  $\alpha$ -actinin (Rivero et al., 1996; Rivero et al., 1999). The results reveal that expression of the 34 kDa  $\Delta$ EF2 protein did not restore function to the 34-kDa-null or 34 kDa/ $\alpha$ -actinin-null cell lines. These results show that calcium regulation is necessary for 34 kDa protein function and that calcium regulation of actin filament crosslinking is essential for normal function and dynamics of the actin cytoskeleton.

## Materials and Methods

Preparation of wild-type and mutant forms of the 34 kDa protein in *E. coli*

The wild-type 34 kDa protein was expressed from the plasmid pET 15b-F18 in *E. coli* as described previously (Lim and Fehheimer, 1997). Mutations in the second putative EF hand of the 34 kDa protein were produced in plasmid pET15b-F18 using the Chameleon Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. The notation 34 kDa  $\Delta$ EF2 refers to mutations in the second EF hand as shown in Fig. 1A. Mutagenesis primers were made for the anti-sense strand of the 34 kDa protein nucleotide sequence with nucleotides numbered as described previously (Fehheimer et al., 1991). Primer 34 kDa  $\Delta$ EF2 has the sequence 5'GAGACACGACCAGCGAAAGCGACAGCGACTTTT-TCTCTTAATTC 3', corresponding to nucleotides 455-412, and was used to create the mutations in the second putative EF hand. Selection primer AccIpET15 5'CGATAGCGGAGTGGTACCTGGCTTAATC-ATGCG 3' was used to convert the *AccI* site in pET 15b-F18 to a *KpnI* site for selection of mutated plasmids. All primers were synthesized and 5' phosphorylated at the Molecular Genetics Instrumentation Facility at the University of Georgia. All plasmids were sequenced to confirm that changes in the coding region were restricted to those intentionally introduced by the mutagenesis procedure. Automated sequencing was performed at the Molecular Genetics Instrumentation Facility at the University of Georgia. The wild-type 34 kDa protein was purified from *E. coli* as described previously (Lim and Fehheimer, 1997), and modified 34 kDa  $\Delta$ EF2 protein was purified essentially by the same procedure with minor modifications.

## Analytical methods and antibodies

Protein concentration was determined according to the bicinchoninic acid (BCA; Pierce Chemical Co., St. Louis, MO) method (Smith et al., 1985) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a protein standard. Monoclonal anti 34 kDa antibody B2C (Furukawa et al., 1992) and alkaline phosphatase-conjugated goat anti-mouse antibodies (Promega Corp., Madison, WI) were used

for western blot analysis as described previously (Towbin et al., 1979).

## Measurement of calcium binding by equilibrium dialysis

Ultra pure water (Continental Water Systems Corporation, San Antonio, TX) and buffers were passed over a 2x30 cm Chelex 100 (BioRAD, Hercules, CA) resin to remove any traces of calcium. All plastic ware was rinsed in calcium-free water before use. The proteins were first dialyzed against storage buffer (10 mM Tris pH 7.0, 50 mM KCl, 0.2 mM DTT, 0.1 mM EDTA) for 24 hours at 4°C. To remove EDTA, the proteins were dialyzed in storage buffer as above but without the EDTA for 24 hours with one buffer change at 4°C. For calcium-binding measurements, the proteins were dialyzed in 40 ml of storage buffer without EDTA containing different concentrations of  $^{45}\text{Ca}[\text{CaCl}_2]$  for 48 hours at 25°C. To achieve different concentrations of calcium in the buffer, calcium was added from a 200  $\mu\text{M}$  calcium stock solution that contained approximately 10  $\mu\text{Ci}/\mu\text{mole}$   $^{45}\text{Ca}$  (DuPont NEN, Boston, MA). Protein concentrations of samples were determined after dialysis. After dialysis, 100  $\mu\text{l}$  of buffer from the outside and 100  $\mu\text{l}$  of sample from inside the dialysis bag was sampled in triplicate, and radioactivity was determined by liquid scintillation counting. To determine the amount of calcium bound, the number of counts outside was averaged and subtracted from the averaged counts inside and divided by the specific activity of  $^{45}\text{Ca}$ .

## Actin

G-actin was isolated from rabbit skeletal muscle acetone powder and gel-filtered as described previously (MacLean-Fletcher and Pollard, 1980; Spudich and Watt, 1971). The actin was stored dialyzing in G-actin buffer for one week with fresh buffer changes every day.

## Measurement of binding by co-sedimentation with actin

High-speed actin co-sedimentation assays were performed as outlined previously (Fehheimer, 1987; Fehheimer and Taylor, 1984; Lim and Fehheimer, 1997). 3  $\mu\text{M}$  of G-actin was mixed with 3  $\mu\text{M}$  of wild-type 34 kDa or 34 kDa  $\Delta$ EF2 proteins in 20 mM PIPES, pH 7.0, 50 mM KCl, 50  $\mu\text{M}$   $\text{MgCl}_2$ , 1 mM ATP, 0.2 mM DTT, 5 mM EGTA plus or minus 4.5 mM  $\text{CaCl}_2$ , in a final volume of 130  $\mu\text{l}$ . Supernatant and pellet fractions were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970), visualized with Coomassie brilliant blue. The amount of protein in the pellet and supernatant was quantified by scanning densitometry (Molecular Dynamics, Sunnyvale, CA). Control experiments verified that none of the 34 kDa proteins showed any significant sedimentation in the absence of actin in either the presence or absence of calcium (data not shown). To determine the stoichiometry of binding of the 34 kDa proteins, high-speed F-actin co-sedimentation was performed as described above as a function of the 34 kDa proteins at low free calcium concentrations.

## Electron microscopy

Negative staining of mixtures of actin and 34 kDa proteins was performed as previously described (Fehheimer and Furukawa, 1993). Briefly, 5  $\mu\text{M}$  G-actin was mixed with 2.5  $\mu\text{M}$  34 kDa protein or 34 kDa  $\Delta$ EF2 protein in 20 mM PIPES, pH 7.0, 50 mM KCl, 50  $\mu\text{M}$   $\text{MgCl}_2$ , 1 mM ATP, 0.2 mM DTT, 5 mM EGTA plus or minus 4.5 mM  $\text{CaCl}_2$  and incubated overnight at 4°C. The mixture was applied to a 300-mesh copper grid coated with 0.3% Formvar and carbon for 30 seconds, washed with the above buffer for 1 minute, stained with 2% uranyl acetate for 30 seconds and visualized using a Phillips 400 transmission electron microscope.

### Expression of mutant 34 kDa proteins in *Dictyostelium*

Wild-type or mutant 34 kDa or GFP-fused proteins were expressed in *Dictyostelium* after subcloning into either the *Bam*HI site of the vector pBORP (Ostrow et al., 1994) or the *Hind*III/*Kpn*I site of pDXA-GFP (Levi et al., 2000). The coding sequences were taken from the plasmids encoding full-length 34 kDa pET 15b-F18 (Lim and Fechheimer, 1997) and p34 kDa  $\Delta$ EF2 by PCR using primers to modify the coding sequences with either *Bam*HI, *Hind*III or *Kpn*I restriction enzyme sites for the purpose of subcloning. The 34 kDa proteins were expressed in 34-kDa-null and 34 kDa/ $\alpha$ -actinin double null strains of *Dictyostelium* described previously (Rivero et al., 1996; Rivero et al., 1999). Electroporation and growth of cells was performed as described previously (Maselli et al., 2002). The 34-kDa/ $\alpha$ -actinin-deficient cells were co-transformed with pBSK-BSR, derived from pBsR479 (Putka and Zeng, 1998), to impart blasticidin resistance for selection purposes as the parental cells and pBORP both utilize G418 resistance cassettes. 24 hours after transformation the media was removed and replaced with media containing antibiotics required for selection. The cells were cloned, and expression of the 34 kDa proteins was assessed by western blotting and immunofluorescence. Cells were fixed and stained as described previously (Fechheimer, 1987). Rhodamine-labeled or Oregon-green-labeled phalloidin (Molecular Probes, Eugene, OR) was utilized to localize F-actin, and monoclonal antibody B2C (Furukawa et al., 1992), followed by a rhodamine-labeled secondary antibody was utilized to localize the 34 kDa protein.

### Growth

*Dictyostelium* AX-2 and derivatives described below were routinely maintained in axenic shaking cultures at 150 rpm in HL-5 media (Loomis, 1971) at 20°C. To assess growth rates, cells were inoculated at a starting concentration of  $4 \times 10^4$  cells/ml at 20°C or 15°C in axenic HL-5 culture media with shaking at 150 or 120 rpm, respectively. Each data point represents the average of three flasks, all of which were sampled daily in duplicate and counted on a standard hemocytometer. Growth rates are expressed as doubling time in hours and were calculated from the daily counts from inoculation until the first day that plateau was approached.

### Cell size

Cells were induced to assume a spherical shape as described previously (Rivero et al., 1996). The cells were then allowed to settle in a Bio-unique chamber (Bionique Laboratories, Saranac Lake, NY), and images were recorded using a Nikon inverted microscope equipped with a Mighty Max Cooled CCD camera (Princeton, Trenton, NJ). The major and minor axis of each cell was measured using IP lab image analysis software (Scanalytics, Fairfax, VA). The diameter of the cells was determined by averaging the major and minor axis.

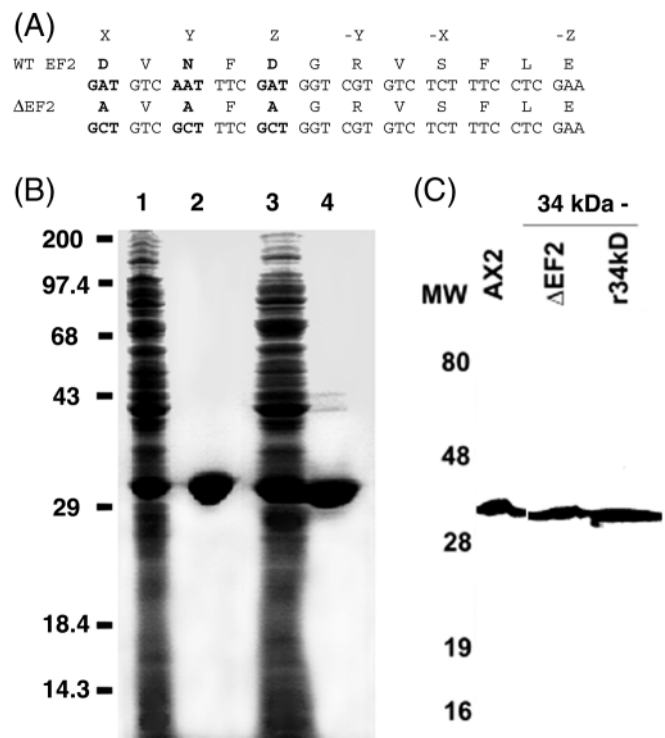
### Ratio imaging and analysis

Images of vegetative cells fixed and stained as described above were recorded using a Nikon inverted microscope equipped with a Mighty Max Cooled CCD camera (Princeton, Trenton, NJ) with the IP lab image analysis software interface (Scanalytics, Fairfax, VA). Care was taken not to saturate any pixels in the image. The leading and trailing edges of the amoebae were identified in the images of either the actin or the 34 kDa protein. These regions were outlined manually using NIH image. The background noise was sampled in three rectangular regions close to each of the edges. The average pixel value of the background was subtracted from the maps. The subsequent values were averaged over all pixels in the region to obtain the average intensity value. The ratio of actin to the 34 kDa protein in the trailing and leading edges was obtained by simple division.

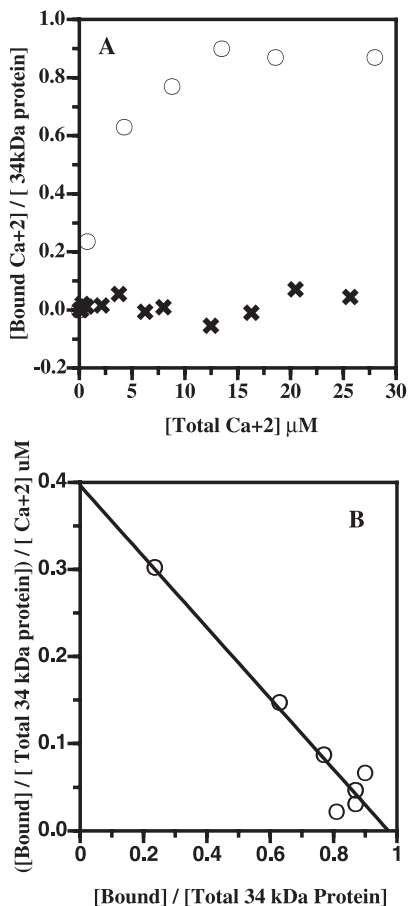
## Results

### Production of a 34 kDa protein deficient in calcium binding

The sequence of the *Dictyostelium* 34 kDa protein (Fechheimer et al., 1991) contains two putative calcium-binding EF hands, structural motifs involved in coordination of calcium in a number of calcium-binding proteins (Strynadka and James, 1989). We produced an altered form of the protein by conversion of the aspartate residues at the X, Y and Z positions of the second EF hand to alanines (Fig. 1A) and termed this protein 34 kDa  $\Delta$ EF2. We predicted that these changes would abrogate high-affinity calcium binding (Strynadka and James, 1989), since the first putative EF hand is a poor match to the consensus calcium-binding loop and is not expected to bind calcium. In addition, the full-length protein binds only one mole of calcium (see below). The wild-type 34 kDa and 34 kDa  $\Delta$ EF2 proteins were expressed and purified from *E. coli* (Fig. 1B) and were employed for studies of calcium binding and actin binding in vitro.



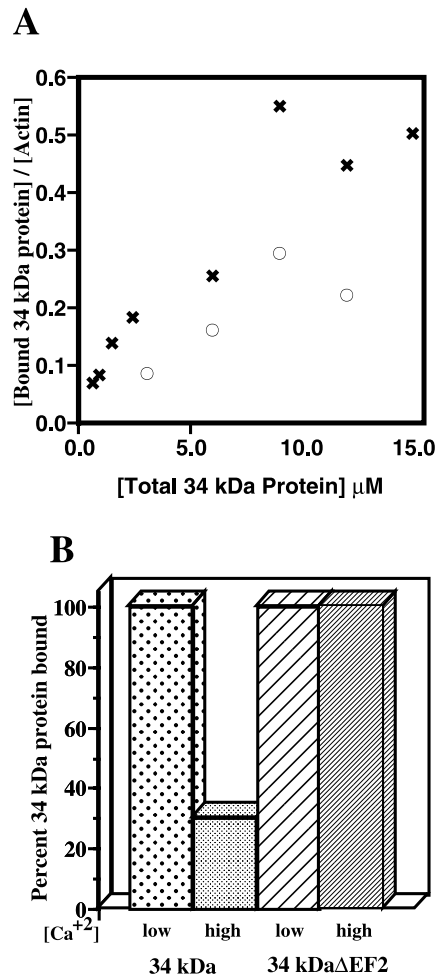
**Fig. 1.** Wild-type and calcium-insensitive forms of the 34 kDa protein. (A) Sequence of wild-type 34 kDa and 34 kDa  $\Delta$ EF2 proteins. Nucleotide and amino-acid sequences of the wild-type and modified EF hand 34 kDa proteins are shown. The altered nucleotides and amino acids are shown in bold. (B) Expression and purification of the 34 kDa and 34 kDa  $\Delta$ EF2 proteins. SDS-PAGE gel stained with Coomassie blue. Lanes 1 and 3 were loaded with 100  $\mu$ g of BL21(DE3) expressing 34 kDa and 34 kDa  $\Delta$ EF2 proteins, respectively. Lanes 2 and 4 were loaded with 25  $\mu$ g of purified 34 kDa and 34 kDa  $\Delta$ EF2 proteins, respectively. (C) Western blot showing *Dictyostelium* AX2 and 34-kDa-null cells expressing the wild-type 34 kDa protein and 34 kDa  $\Delta$ EF2 proteins. Proteins from cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal antibody B2C reactive to the 34 kDa protein.



**Fig. 2.** Calcium binding to 34 kDa and 34 kDa  $\Delta\text{EF2}$  proteins measured by equilibrium dialysis. (A) Calcium binding. Approximately 10  $\mu\text{M}$  34 kDa and 34 kDa  $\Delta\text{EF2}$  proteins were dialyzed as a function of  $[\text{Ca}^{45}]\text{CaCl}_2$  concentration in buffer as described in Materials and Methods. The moles of calcium bound per mole of 34 kDa protein are plotted versus the free calcium concentration for each protein. 34 kDa protein (○); 34 kDa  $\Delta\text{EF2}$  protein (✱). (B) Scatchard plot from equilibrium dialysis. The moles of calcium bound per mole of 34 kDa protein divided by the free calcium concentration are plotted versus moles of calcium bound per mole of 34 kDa protein. The straight line indicates the best fit line yielding  $K_d$  of 2.4  $\mu\text{M}$ .

Prior studies of calcium binding to the 34 kDa protein employed a qualitative blot overlay approach (Fechheimer and Furukawa, 1993; Lim and Fechheimer, 1997). Therefore,  $^{45}\text{Ca}[\text{CaCl}_2]$  and equilibrium dialysis were used to determine the number and affinity of calcium-binding sites in the 34 kDa protein (Fig. 2). The results reveal that calcium binds saturably to a single site on the 34 kDa protein with an affinity of 2.4  $\mu\text{M}$ . By contrast, essentially no calcium binding was observed using the 34 kDa  $\Delta\text{EF2}$  protein (Fig. 2), demonstrating that the second EF hand is the site of high-affinity calcium binding to the wild-type 34 kDa protein.

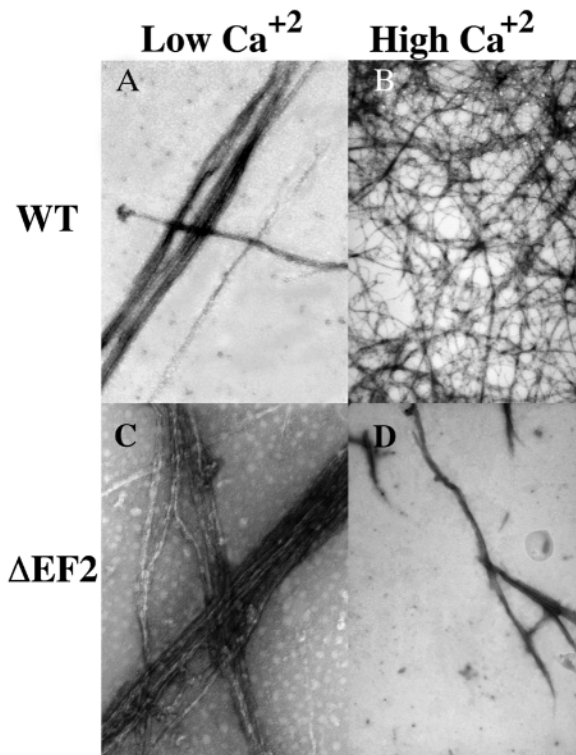
The actin binding and crosslinking activities of the purified 34 kDa  $\Delta\text{EF2}$  protein was compared to wildtype using the F-actin co-sedimentation assay to assess actin binding. All assays were performed in the presence or absence of micromolar free calcium to investigate calcium regulation of actin binding. The wild-type 34 kDa protein bound to F-actin



**Fig. 3.** Binding of the 34 kDa and 34 kDa  $\Delta\text{EF2}$  proteins to F-actin measured by co-sedimentation. (A) Moles of 34 kDa protein bound per mole of actin subunits in filaments is plotted as a function of the total 34 kDa protein added. 34 kDa protein (○); 34 kDa  $\Delta\text{EF2}$  protein (✱). The 34 kDa  $\Delta\text{EF2}$  protein binds F-actin moderately with a slightly greater affinity than the wild-type protein. (B) The relative amount of 34 kDa protein bound to F-actin in the presence and absence of micromolar concentrations of calcium. The amount of wild-type 34 kDa protein bound to F-actin decreases by 70% in the presence of micromolar calcium whereas the amount of 34 kDa  $\Delta\text{EF2}$  protein bound to F-actin is calcium insensitive.

substoichiometrically and with moderate affinity in the co-sedimentation assay at low calcium (Fig. 3A). In the presence of micromolar free calcium, the amount of the wild-type 34 kDa protein bound to F-actin decreased by 70% (Fig. 3B). The wild-type protein forms tight bundles with F-actin in the absence, but not in the presence, of micromolar calcium (Fig. 4A,B), in agreement with previous reports of calcium-regulated actin binding by the native and recombinant 34 kDa protein (Fechheimer and Taylor, 1984; Furukawa and Fechheimer, 1996; Lim and Fechheimer, 1997).

The actin-binding activity of the 34 kDa  $\Delta\text{EF2}$  protein was similar to that of the wild-type 34 kDa protein at low calcium ion concentrations (Fig. 3A). However, the 34 kDa  $\Delta\text{EF2}$  protein failed to show calcium-sensitive actin binding, as expected from the absence of calcium binding to this protein



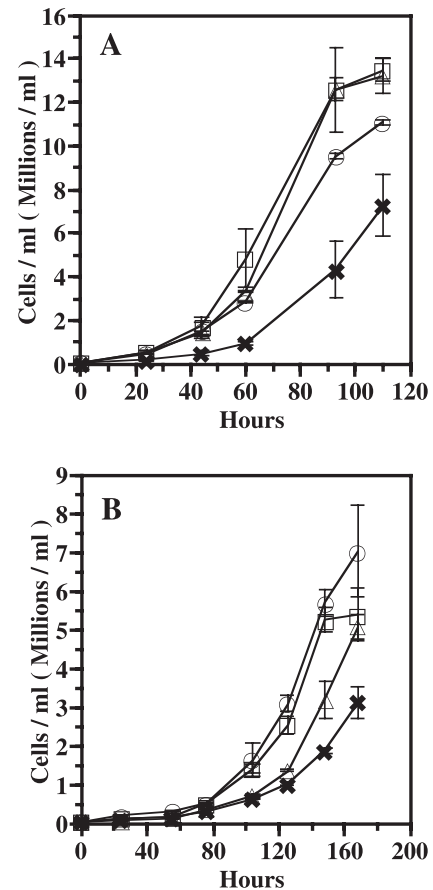
**Fig. 4.** Bundling of F-actin filaments by the 34 kDa  $\Delta$ EF2 protein is not inhibited by calcium. Mixtures of F-actin and the 34 kDa proteins in the presence of low and high concentrations of calcium were viewed by transmission electron microscopy using negative staining. Top row, wild-type 34 kDa protein and F-actin at low (A) and high (B) concentrations of calcium. Bottom row, 34 kDa  $\Delta$ EF2 protein and F-actin at low (C) and high (D) concentrations of calcium. Mixtures of the wild-type 34 kDa protein and F-actin form bundles in the presence of low but not high concentrations of calcium. By contrast, mixtures of 34 kDa  $\Delta$ EF2 protein and F-actin form bundles that are not calcium regulated. Magnification 14,000 $\times$ .

(Fig. 2). In the presence of micromolar free calcium, the amount of 34 kDa  $\Delta$ EF2 protein bound to F-actin was virtually identical to that bound at low calcium concentrations (Fig. 3B). Similarly, the 34 kDa  $\Delta$ EF2 protein induced formation of actin bundles similar to those formed with the wild-type protein, and actin bundling by the 34 kDa  $\Delta$ EF2 protein was not inhibited in the presence of micromolar calcium (Fig. 4C,D). Thus, actin binding by the 34 kDa  $\Delta$ EF2 protein is similar to that of the wild-type protein except for the absence of regulation of the interaction by micromolar calcium.

#### Expression of wild-type and mutant 34 kDa proteins in *Dictyostelium*

The wild-type and 34 kDa  $\Delta$ EF2 proteins were expressed in *Dictyostelium* 34-kDa-null cells and 34 kDa/ $\alpha$ -actinin-null cells, and the level of expression of these proteins was examined by western blot with mouse monoclonal antibody B2C to the 34 kDa protein. Expression levels were similar to those observed for wild-type protein in AX2 cells (Fig. 1C).

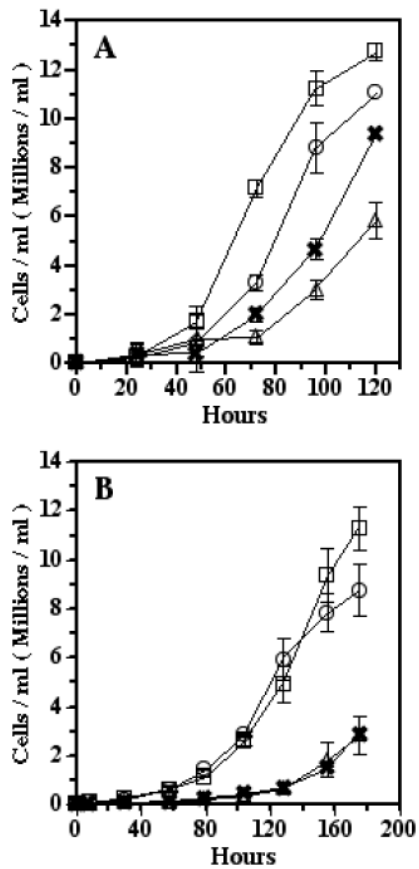
Growth and division are the result of the culmination of a number of processes involving the actin cytoskeleton. To probe



**Fig. 5.** Growth curves in 34-kDa-null amoebae expressing the 34 kDa and 34 kDa  $\Delta$ EF2 proteins. (A) 20°C, (B) 15°C, AX2 ( $\square$ ), 34-kDa-null ( $\triangle$ ), r34 kDa ( $\circ$ ), r34kDa  $\Delta$ EF2 ( $\times$ ). Cells were grown axenically in shaking cultures, and cell density was measured as a function of time after inoculation. The 34-kDa-null cells grow as well as wild-type (AX-2) at 20°C but more slowly than wild-type at 15°C. Expression of the 34 kDa  $\Delta$ EF2 slows the growth of 34-kDa-null cells at 20°C (A). Expression of the r34 kDa protein restores the normal growth rate to that of 34-kDa-null cells at 15°C. By contrast, 34 kDa  $\Delta$ EF2 fails to rescue the slow growth phenotype (B).

the potential significance of the EF hands for the function of the 34 kDa protein, the growth rates of cells expressing the wild-type or 34 kDa  $\Delta$ EF2 proteins were examined (Fig. 5A). Under standard laboratory growth conditions at 20°C, 34-kDa-null cells grow at a rate similar to that for wild-type AX2 cells (12.1 hours per division) as reported previously (Rivero et al., 1996; Rivero et al., 1999). The 34-kDa-null cells and 34-kDa-null cells expressing the wild-type 34 kDa protein grow at similar rates (12.1 and 13.3 hours per division, respectively). By contrast, 34-kDa-null cells expressing 34 kDa  $\Delta$ EF2 protein grow more slowly (15.3 hours per division).

The 34-kDa/ $\alpha$ -actinin-null cells grow slowly at 20°C compared with AX2 cells (16.5 compared to 12.7 hours per division) as reported previously (Rivero et al., 1996). When the wild-type 34 kDa protein is expressed in these cells, the wild-type growth rate is restored (12.3 hours per division). However, expression of the 34 kDa  $\Delta$ EF2 protein does not restore the growth rate (15.0 hours per division) to that observed for AX2 (Fig. 6A).



**Fig. 6.** Growth curves in 34 kDa/ $\alpha$ -actinin double null amoebae rescued with 34 kDa and 34 kDa  $\Delta$ EF2 proteins. Cells were grown axenically in shaking cultures, and cell density was measured as a function of time after inoculation. (A) 20°C, (B) 15°C, AX2 ( $\square$ ), 34 kDa/ $\alpha$ -actinin double null ( $\triangle$ ), r34 kDa ( $\circ$ ), r34 kDa  $\Delta$ EF2 ( $\ast$ ). The 34 kDa/ $\alpha$ -actinin double null cells grow more slowly than wild-type at both 20°C and 15°C. Expression of the wild-type 34 kDa protein restores wild-type growth. By contrast, expression of 34 kDa  $\Delta$ EF2 protein stimulates growth only slightly at 20°C (A) and has no restorative effect on growth of the 34 kDa/ $\alpha$ -actinin double mutant at 15°C (B).

In its natural environment, *Dictyostelium* must grow in conditions of varying temperature, osmolarity and nutrient supply that are optimal and invariant in standard laboratory culture conditions. To provide an environmental challenge, we grew the cells in shaking culture at 15°C. The 34-kDa-null cells grow slowly (Rivero et al., 1999) (23.2 hours per division) when compared with wild-type AX2 (20.1 hours per division) at 15°C. When the wild-type 34 kDa protein is expressed, the wild-type growth rate is nearly restored (21.5 hours per division) under these conditions. By contrast, expression of 34 kDa  $\Delta$ EF2 protein fails to stimulate the rate of growth of 34-kDa-null cells at 15°C. These cells expressing the 34 kDa  $\Delta$ EF2 grow more slowly than the 34-kDa-null cells (27.7 hours per division) (Fig. 5B). The effect of reduced temperature on growth is more pronounced in 34 kDa/ $\alpha$ -actinin double null cells, which grow extremely slowly at 15°C (28.7 hours per division). Expression of the wild-type 34 kDa protein restores wild-type growth rate to 21.4 hours. Expression of 34 kDa  $\Delta$ EF2 protein has no restorative effect on growth of the 34

**Table 1.** Cell diameter of *Dictyostelium* amoebae expressing wild-type and altered forms of the 34 kDa protein

Cell type	Cell diameter (Mean $\pm$ s.d.)
AX-2	12.8 $\pm$ 1.9 $\mu$ m
34 kDa/ $\alpha$ -actinin $^{-}$	9.3 $\pm$ 1.9 $\mu$ m*
34 kDa/ $\alpha$ -actinin $^{-}$ + r34 kDa	11.2 $\pm$ 2.6 $\mu$ m $^{\dagger}$
34 kDa/ $\alpha$ -actinin $^{-}$ + 34 kDa $\Delta$ EF2	9.8 $\pm$ 1.9 $\mu$ m*
34 kDa/ $\alpha$ -actinin $^{-}$ + GFP	10.0 $\pm$ 3.6 $\mu$ m*
34 kDa/ $\alpha$ -actinin $^{-}$ + 34 kDa GFP	12.9 $\pm$ 3.17 $\mu$ m $^{\dagger}$

The Mann-Whitney-Wilcoxon test was used to assess statistical significance of differences.  
\*Statistically different from AX-2 at  $\alpha=0.01$ .  
 $^{\dagger}$ Statistically different from 34 kDa/ $\alpha$ -actinin $^{-}$  at  $\alpha=0.01$ .

kDa/ $\alpha$ -actinin double mutant at 15°C (27.5 hours per division) (Fig. 6B).

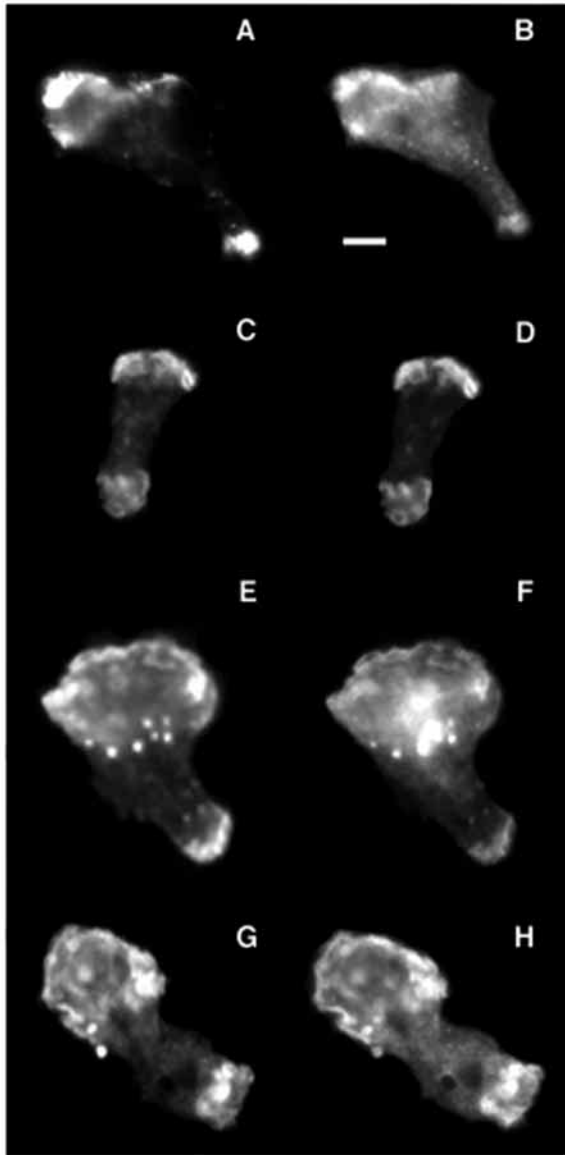
The ability of a calcium-insensitive form of the 34 kDa protein to function in vivo was also tested by measurements of cell diameter. The 34-kDa/ $\alpha$ -actinin-null cells grow to reduced size in shaking culture at 20°C, attaining an average size of 9.3  $\mu$ m compared with 12.8  $\mu$ m for AX2 (Rivero et al., 1999). Expression of wild-type 34 kDa restores wild-type cell size to 11.2  $\mu$ m. However, cells expressing the 34 kDa  $\Delta$ EF2 protein have an average size of only 9.8  $\mu$ m (Table 1). Thus, expression of 34 kDa  $\Delta$ EF2 fails to restore wild-type cell size and does not supply function to the 34-kDa/ $\alpha$ -actinin-null cells

#### Localization of the 34 kDa and F-actin

Failure of the 34 kDa  $\Delta$ EF2 protein to support proper function of the actin cytoskeleton in 34-kDa-null and 34 kDa/ $\alpha$ -actinin double null cells suggests that its localization and/or dynamics in the cells may be abnormal. For example, calcium regulation might be required for proper localization of the 34 kDa protein to the cortex, the cell-to-cell contact regions or the phagocytic cup. Alternatively, the dynamics of the actin cytoskeleton might be impeded by the absence of calcium regulation. To address these questions, the localization of the 34 kDa protein and F-actin were investigated in 34-kDa-null cells expressing wild-type 34 kDa protein, wild-type 34 kDa protein fused to GFP or the calcium-insensitive 34 kDa  $\Delta$ EF2 protein.

A default hypothesis to explain the localization of the 34 kDa protein with respect to F-actin is that the localization of the 34 kDa protein simply reflects the distribution of actin filaments in the cell. To test this hypothesis, a ratiometric approach was employed to assess quantitatively the distributions of F-actin and the 34 kDa protein in the leading and trailing edges of cells. The ratio of labeling of two fluorophores labeling F-actin and the 34 kDa protein in both the front and back of the cell was measured. A ratio of these two values indicates whether the relative amount of F-actin compared with the 34 kDa protein in the front of the cell differed from that in the trailing region.

To confirm the validity and absence of technical artifacts with this approach, a control experiment was performed in which the staining of F-actin labeled with both Oregon green 488 and TRITC-labeled phalloidins was measured in the front and back of the cells (Fig. 7A,B). The ratio of Oregon-green-



**Fig. 7.** Fluorescence images of amoebae used in ratio mapping. Wild-type AX2 cells were stained with Oregon-green-labeled (A) and TRITC-labeled phalloidin (B) for the F-actin/F-actin ratio map. 34-kDa-null cells were rescued with either wild-type 34 kDa protein (C,D), 34 kDa GFP (E,F) or 34 kDa  $\Delta$ EF2 (G,H). Cells were stained with Oregon-green-labeled phalloidin (C,E,G) and monoclonal antibody B2C against 34 kDa protein followed by a TRITC anti-mouse secondary antibody (D,H) or viewed directly for fluorescence from 34 kDa GFP (F). (C) F-Actin, (D) 34 kDa protein, (E) F-Actin, (F) 34 kDa GFP, (G) F-Actin, (H) 34 kDa  $\Delta$ EF2. Bar, 5  $\mu$ M.

labeled phalloidin to TRITC-labeled phalloidin in the front to that in the back was  $1.04 \pm 0.15$  (Table 2). This result reveals that there is no technical or instrumental artifact that will generate a ratio significantly different from 1.0 when this analysis is performed.

By contrast, comparison of the ratio of F-actin labeled with phalloidin to the 34 kDa protein stained with monoclonal antibody B2C in the trailing region (Fig. 7C,D) divided by that in the leading edge is  $1.38 \pm 0.38$  ( $n=19$ ). This result shows that

**Table 2. Ratio of proteins in the trailing/leading edges of *Dictyostelium* obtained by fluorescence microscopy**

Trailing/leading edges	Mean $\pm$ s.d	Cells ( $n$ )	Student's $t$ -test $^\ddagger$
F-actin/F-actin*	$1.04 \pm 0.15$	13	
F-actin/34 kDa*	$1.38 \pm 0.38$	19	$P < 0.01$
F-actin/34 kDa-GFP $^\dagger$	$1.19 \pm 0.15$	22	$P < 0.01$
F-actin/34 kDa $\Delta$ EF2 $^\dagger$	$1.09 \pm 0.29$	22	$P = 0.286$

\*Ratios obtained using wild-type AX2.

$^\dagger$ Ratios obtained using 34-kDa-null amoebae with indicated 34 kDa protein expressed.

$^\ddagger$ The  $P$  values obtained from the student's  $t$ -test show that the ratio of F-actin/34 kDa protein and F-actin/34-kDa-GFP in the trailing versus the leading edges are significantly different than the distribution of F-actin/F-actin in these regions. By contrast, the ratio of F-actin/34 kDa  $\Delta$ EF2 protein in the trailing versus the leading edges are not significantly different from the distribution of F-actin in these regions.

the trailing edge of the cell has a lower amount of 34 kDa protein compared with F-actin than the leading edge. Furthermore, the ratio of F-actin to 34 kDa protein is significantly different from the F-actin to F-actin ratio described above ( $P < 0.01$ ). To verify that this result is a true reflection of the distribution of the 34 kDa protein and F-actin rather than an artifact resulting from limited accessibility of the monoclonal antibody used to detect the 34 kDa protein, the analysis was repeated to determine the relative distribution of F-actin to a 34 kDa GFP construct (Fig. 7E,F). This 34 kDa GFP fusion protein is functional, since it can restore normal cell size to 34 kDa/ $\alpha$ -actinin double mutants (Table 1). The ratio of F-actin to 34-kDa-GFP in the trailing compared to the leading edge of the cell is  $1.19 \pm 0.15$  ( $n=22$ ) (Table 2). The enrichment for F-actin relative to the 34 kDa protein in the rear of the cell detected using the GFP is significantly different from the F-actin to F-actin control ( $P = 0.005$ ). Thus, the monoclonal antibody B2C and the 34-kDa-GFP probe both reveal a relative decrease in the 34 kDa protein compared with F-actin in the rear of the cell.

To determine whether calcium regulation of the 34 kDa protein was important for this relative decrease in the presence of the 34 kDa protein in the trailing portion of motile amoebae, the same analysis was performed in 34-kDa-null cells expressing the 34 kDa  $\Delta$ EF2 protein (Fig. 7G,H). The results reveal that the ratio of F-actin stained with phalloidin to the 34 kDa  $\Delta$ EF2 protein stained with monoclonal antibody B2C in the trailing versus the leading regions of cells was  $1.09 \pm 0.29$  ( $n=22$ ) (Table 2). This value was not significantly different from the F-actin to F-actin control that showed no difference in the front versus the trailing edge of the cell. Moreover, these results with the 34 kDa  $\Delta$ EF2 protein were significantly different from those with the wild-type 34 kDa protein ( $P < 0.05$ ). Thus, calcium regulation of the 34 kDa protein is required for regulation of the distribution of the localization of the 34 kDa protein with respect to F-actin leading to a decrease in the relative amount of the 34 kDa protein in the trailing edge of the cell.

## Discussion

We have tested the hypothesis that calcium regulation of actin crosslinking is important for the function of the actin cytoskeleton *in vivo*. Site-directed mutagenesis and

equilibrium dialysis were employed to show that the wild-type 34 kDa protein binds 1 mole of calcium with a  $K_d$  of 2.4  $\mu\text{M}$ , but an altered form of the protein with a mutation in the second putative EF hand (34 kDa  $\Delta\text{EF2}$ ) lacks high affinity calcium binding (Fig. 2). The actin-binding activity of the 34 kDa  $\Delta\text{EF2}$  protein was similar to that of the wild-type protein at low ( $<10^{-8}$  M) calcium but failed to show inhibition of F-actin binding and bundling in the presence of  $10^{-6}$  M calcium that is observed with the wild-type protein (Figs 3 and 4). Thus, this calcium-insensitive 34 kDa  $\Delta\text{EF2}$  protein was employed for studies of the physiological significance of calcium regulation of F-actin crosslinking by expression in 34 kDa null and 34 kDa/ $\alpha$ -actinin double null cells.

We assayed a range of cellular functions in the presence of expressed wild-type and  $\Delta\text{EF2}$  34 kDa proteins. When the 34 kDa  $\Delta\text{EF2}$  protein is expressed in 34-kDa-null cells, it has a negative effect reducing the growth rate at 20°C (Fig. 5A), and it fails to rescue the slow growth of these cells at 15°C (Fig. 5B). In addition, the 34 kDa  $\Delta\text{EF2}$  protein fails to restore normal growth rates to 34kDa/ $\alpha$ -actinin double null cells at normal (20°C) and reduced (15°C) temperatures (Fig. 6A,B), and it fails to rescue the small cell phenotype of these cells that lack two actin crosslinking proteins (Table 1). These results show that calcium regulation of actin crosslinking is essential for normal 34 kDa function and for the function of the actin cytoskeleton in vivo.

It is intriguing to ask why the 34 kDa  $\Delta\text{EF2}$  protein exhibits a dominant-negative effect in 34-kDa-null cells but not in the 34 kDa/ $\alpha$ -actinin double null cells. A possible explanation emerges from consideration of the growth rates of the strains. Wild-type cells, 34-kDa-null and  $\alpha$ -actinin-null all have at least one calcium-sensitive actin crosslinking protein and grow at a normal rate at 20°C. The 34 kDa/ $\alpha$ -actinin double null cells have no calcium-regulated actin-crosslinking proteins and grow more slowly. Addition of the 34 kDa  $\Delta\text{EF2}$  protein to the 34 kDa/ $\alpha$ -actinin double null cells does not restore calcium-sensitive actin-crosslinking but neither does it slow growth in this strain since the cells were already deficient in calcium-sensitive actin crosslinking. By contrast, the 34-kDa-null cells contained one calcium-sensitive actin crosslinking protein and grew normally. Addition of the calcium-insensitive 34 kDa  $\Delta\text{EF2}$  protein to these cells may have added additional calcium-insensitive crosslinks so that filament rearrangements are impeded even though the cells contain a normal  $\alpha$ -actinin molecule. Consistent with this interpretation is that the growth rate of the 34 kDa/ $\alpha$ -actinin double null cells is as slow as the 34-kDa-null cells expressing the 34 kDa  $\Delta\text{EF2}$  protein. Thus, calcium sensitivity of actin crosslinks in the cytoskeleton is required for dynamic rearrangements of the cytoskeleton needed for normal growth.

The ability of an actin crosslinking protein with compromised calcium regulation to supply function in vivo was examined previously in studies of modified  $\alpha$ -actinin (Rivero et al., 1999; Witke et al., 1993). When the second EF hand of  $\alpha$ -actinin is modified ( $\alpha$ -A M2), the resultant protein requires 500 times more calcium than wildtype for inhibition of actin binding and is not regulated by physiological levels of calcium. Surprisingly, this  $\alpha$ -actinin M2 restored the ability to proceed through normal development to a cell line lacking both  $\alpha$ -actinin and gelation factor (Witke et al., 1993). An  $\alpha$ -actinin protein mutant in the first EF hand ( $\alpha$ -A M1) exhibits

reduced actin binding and failed to restore normal development to these cells (Witke et al., 1993). Expression of these same two modified  $\alpha$ -actinin proteins in the 34 kDa/ $\alpha$ -actinin double null cells rescued the slow growth phenotype (Rivero et al., 1999). Expression of the modified  $\alpha$ -actinin M1 and M2 proteins resulted in a partial and complete rescue, respectively, of the small cell size phenotype (Rivero et al., 1999). The  $\alpha$ -actinin M2 protein is not sensitive to physiological calcium levels and supplies  $\alpha$ -actinin function to both the 34-kDa/ $\alpha$ -actinin null and 120/ $\alpha$ -actinin double null, in apparent contrast to the results with the calcium-insensitive 34 kDa  $\Delta\text{EF2}$  protein. This difference in the results might be explained if the 34 kDa protein has a higher affinity for F-actin than  $\alpha$ -actinin. This is because the rheological behavior of crosslinked actin structures is a function of the shear rate with higher resistance to mechanical force observed at high shear rates. At low shear rates, the crosslinked actin structures reorganize rapidly, and the network exhibits less stiffness (Sato et al., 1987; Wachsstock et al., 1993). The dynamic behavior of the 34 kDa protein may be slower than  $\alpha$ -actinin, so that in the absence of calcium-induced release from actin, an impediment to actin filament rearrangement, is detected. If  $\alpha$ -actinin is sufficiently dynamic, a calcium-insensitive form may be able to supply crosslinking function. This explanation is supported by studies of the localization of  $\alpha$ -actinin and the 34 kDa protein in *Dictyostelium*. Both the 34 kDa protein and  $\alpha$ -actinin are associated with cortical actin filaments. However, the 34 kDa protein is tightly associated with cortical actin, whereas  $\alpha$ -actinin reveals both cortical staining and a significant amount of diffuse cytoplasmic localization (Brier et al., 1983; Fechheimer, 1987). Further, biochemical studies in vitro show directly that the 34 kDa protein has a higher affinity for actin filaments than does amoeba  $\alpha$ -actinin (Fechheimer, 1987; Lim et al., 1999; Wachsstock et al., 1993). Thus, the results can be reconciled and do demonstrate a requirement for calcium-regulation of the actin-crosslinking in vivo.

Calcium fluxes, either from extracellular or intracellular stores, are required for *Dictyostelium* spreading, locomotion and chemotaxis as shown by chelation of cytoplasmic calcium (Unterweger and Schlatterer, 1995). Elevation of calcium is most prominent at the rear of a locomoting amoeba (Taylor et al., 1980; Yumura et al., 1996). These results agree with a model of locomotion in which calcium elevation at the rear of the cell promotes simultaneous contraction of actin and myosin II and conversion of gel to sol at the rear of the cell (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Mast, 1926; Taylor and Fechheimer, 1982). Our results both support and extend this concept, which continues to provide a working model of molecular events at the trailing region of a crawling cell. First, the 34 kDa protein and actin filaments are highly colocalized in *Dictyostelium*, and the only difference that can be reproducibly detected is a relative lack of the 34 kDa protein at the rear of motile cells (Table 2). The relative absence of the 34 kDa protein at the rear of motile amoebae was noted in an independent report (Okazaki and Yumura, 1995). We found this difference using both monoclonal antibody B2C and a 34-kDa-GFP probe (Table 2). This decrease in the 34 kDa protein at the rear of the cell could be a result of calcium-induced release from actin. This explanation is supported by the observation that the difference in localization of the 34 kDa



protein and F-actin at the trailing edge of moving cells is not observed in cells expressing the calcium-insensitive 34 kDa  $\Delta$ EF2 protein (Table 2). Further, the trailing edge of cells expressing 34 kDa  $\Delta$ EF2 was sometimes extended and contained large aggregates of actin filaments that appeared to have been released from the posterior cortex but not yet disassembled (Fig. 7G,H). These results suggest that the calcium-insensitive 34 kDa  $\Delta$ EF2 protein is not readily disassociated from actin at the rear of the cell, remains bound to F-actin in the tail and inhibits depolymerization of actin at the rear. This scenario is supported by two independent observations. First, binding of the 34 kDa protein to actin filaments slows the depolymerization of actin filaments in vitro (Zigmond et al., 1992). Thus, filaments in the trailing edge with bound 34 kDa protein would be impeded from disassembly during tail retraction. Second, measurements of calcium ion fluxes in *Dictyostelium* cells reveal that calcium elevation following application of chemoattractant correlates not with the initial period of actin assembly but rather with the later phase of actin depolymerization (Nebl and Fisher, 1997). These data provide independent evidence for a role of calcium in stimulation of actin depolymerization. Our results suggest that calcium-induced release of 34 kDa protein from F-actin in the trailing region of the cell may be required for depolymerization of F-actin and dynamic rearrangement of actin structures.

Calcium-induced events in tail retraction during movement of mammalian cells include calpain-mediated proteolysis to promote cell detachment from the substrate (Huttenlocher et al., 1997) and activation of myosin II (Eddy et al., 2000). Although myosin II plays a prominent role in the locomotion *Dictyostelium* amoebae (Fukui et al., 1990; Shelden and Knecht, 1996; Zhang et al., 2002), the role of calpain is worthy of additional study. *Dictyostelium* and mammalian cells may differ in the manner of calcium-induced tail retraction either because of the speed of motility, the strength of adhesion to the substrate or other factors. Alternatively, calpain-mediated proteolysis and calcium-sensitive actin crosslinking may be general features of locomotion shared by diverse types of cells. This possibility is worthy of additional investigation.

The finding that calcium-sensitive actin filament crosslinking is significant in vivo provides additional support for a model of partial redundancy for the function of the multiple actin crosslinking proteins present in eukaryotic cells that has been proposed previously (Rivero et al., 1996; Witke et al., 1992). The unique functions of actin-crosslinking proteins are observed in single mutants, whereas redundant functions are revealed by synthetic phenotypes in double mutants (Rivero et al., 1999). Additional roles of actin-crosslinking proteins can be discerned by observation of cells under physiologically relevant conditions not normally encountered in the laboratory (Ferrary et al., 1999; Ponte et al., 2000; Rivero et al., 1999) and by expression of altered forms of the proteins that lack sites for regulation by binding of secondary messengers or covalent modification [e.g. this work and Yamashiro et al. (Yamashiro et al., 2001)]. Additional insight is gained from the ability of the altered forms to provide function to some mutants, but not others, as discussed above. A thorough understanding of the structure and function of actin-crosslinking proteins is beginning to emerge but will require much additional investigation owing to the overlapping

and partially redundant functions of these ubiquitous and essential structural proteins.

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