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Effects of single amino acid substitutions in the actin-binding site on the biological activity of bovine profilin I

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SUMMARY

For a detailed analysis of the profilin-actin interaction, we designed several point mutations in bovine profilin I by computer modeling. The recombinant proteins were analyzed in vitro for their actin-binding properties. Mutant proteins with a putatively higher affinity for actin were produced by attempting to introduce an additional bond to actin. However, these mutants displayed a lower affinity for actin than wild-type profilin, suggesting that additional putative bonds created this way cannot increase profilin's affinity for actin. In contrast, mutants designed to have a reduced affinity for actin by eliminating profilin-actin bonds displayed the desired properties in viscosity assays, while their binding sites for poly(L)proline were still intact. The profilin mutant F59A, with an affinity for actin reduced by one order of magnitude as compared to wild-type profilin, was analyzed further in cells. When microinjected into fibroblasts, F59A colocalized with the endogenous profilin and actin in ruffling areas, suggesting that profilins are targeted to and tethered at these sites by ligands other than actin. Profilin null cells of *Dictyostelium* were transfected with bovine wild-type profilin I and F59A. Bovine profilin I, although expressed to only approximately 10% of the endogenous profilin level determined for wild-type *Dictyostelium*, caused a substantial rescue of the defects observed in profilin null amoebae, as seen by measuring the growth of colony surface areas and the percentage of polynucleated cells. The mutant protein was much less effective. These results emphasize the highly conserved biological function of profilins with low sequence homology, and correlate specifically their actin-binding capacity with cell motility and proliferation.

Key words: Profilin, Actin, Point mutation, *Dictyostelium discoideum*

INTRODUCTION

The structure of profilin and of the profilin-actin complex has been revealed by the pioneering X-ray studies of Schutt et al. (1993). Profilins were originally described as ubiquitous G-actin-binding proteins (Carlsson et al., 1977) and were therefore thought mainly responsible for G-actin sequestering in cells. Many eukaryotes synthesize at least two isoforms as discrete gene products, which can be expressed in a tissue-specific (cf. Witke et al., 1998) or in a developmental stage-specific manner (cf. Haugwitz et al., 1991). There is a large body of evidence showing that profilins are essential for correct microfilament organisation, which is consistent with their actin-binding ability. Mutants of *Dictyostelium discoideum* lacking both profilin isoforms expressed in this organism show an increase in F-actin content, which is correlated with abnormal size and defects in motility, cytokinesis and development (Haugwitz et al., 1994). Similar abnormalities were found in profilin null cells of *Saccharomyces cerevisiae* (Haarer et al., 1990). *Drosophila* mutants deficient in the ovary-specific profilin are not viable, probably because the lack of correctly organized actin filament bundles in nurse cells prevents nutrient transport to the oocyte (Cooley et al., 1992; Verheyen and Cooley, 1994). Disruption of the gene for profilin I, one of the two isoforms identified in the mouse, results in very early death of the mouse embryo (Witke et al., 1993). Overexpressing profilin in mammalian tissue culture cells leads to an abnormal stabilization of F-actin (Finkel et al., 1994; Rothkegel et al., 1996), while a local increase of profilin by microinjection disrupts the cortical microfilament web (Cao et al., 1992; Hajkova et al., 1997). The precise role of profilins in these events, however, is unknown. Profilin-actin interaction and its consequences on microfilament organization seem subject to a variety of physiological conditions which are poorly understood. Depending on the ionic conditions and the presence of other actin-binding proteins, profilins may block actin polymerization by sequestering G-actin (Pollard and Cooper, 1984; Nishida et al., 1984; Goldschmidt-Clermont et al., 1991b), cap the fast growing end of actin filaments (Tilney et al., 1983; Pring et al., 1992) or promote actin polymerization (Pantaloni and Carlier, 1993; Perelroizen et al., 1996; Ballweber et al., 1998).

With the discovery of profilin ligands other than actin, some of which are members of signal transduction pathways, it became evident that the activity of profilins on the actin cytoskeleton is itself subject to regulatory mechanisms. In this

respect, the interaction of profilins with phosphatidylinositol-4,5 bisphosphate (PtdIns 4,5-P_2; Lassing and Lindberg, 1985, 1988; Goldschmidt-Clermont et al., 1990), proteins of the Ena/VASP family, which are targets of Ser/Thr-kinases (Reinhard et al., 1995; Gertler et al., 1996), and the formin-related proteins (for example p140mDia, which in addition to profilin binds to the GTP-bound form of Rho; Watanabe et al., 1997) is especially noteworthy. Being thus positioned at the crossroads between the actin cytoskeleton and several signalling pathways, profilins are considered to mediate between signal transduction and the actin cytoskeleton (Aderem, 1992; Machesky and Pollard, 1993; Sohn and Goldschmidt-Clermont, 1994; Schlüter et al., 1997), and the notion that they localize in the ruffling, highly dynamic regions of locomoting and spreading cells (Buss et al., 1992; Balasubramanian et al., 1994; Machesky et al., 1994; Rothkegel et al., 1996; Mayboroda et al., 1997; Neely and Macaluso, 1997) enforces this concept.

Transmission of external signals to the actin cytoskeleton via profilins is currently thought to be by two discrete routes. One is based on the finding that the binding to PtdIns 4,5-P_2 and actin is mutually exclusive and hence postulates that profilins may be complexed to PtdIns 4,5-P_2 in the plasma membrane, until it is released by signal-triggered cleavage of PtdIns 4,5-P_2. This event would then liberate profilins to stimulate actin polymerization at the site of signal perception (Lassing and Lindberg, 1985, 1988; Goldschmidt-Clermont et al., 1990, 1991a). A second model postulates that profilin is engaged in several other signalling pathways by its binding to VASP/Ena or formin proteins, which should be independent of its actin-binding (Gertler et al., 1996; Reinhard et al., 1995). Currently, it is unknown to what extent these links modulate the microfilament organizing activity of profilins in motility and cytokinesis, and whether the various signalling routes may act in concert or counteract each other in the regulation of the actin cytoskeleton.

With the aim of identifying the individual role of the different ligand binding sites for cellular functions of profilins, we designed several point mutations of bovine profilin I to obtain mutant proteins with altered actin-binding capacity, without affecting other ligand binding sites. In contrast to our expectations, we did not obtain mutants with an increased affinity for actin. Instead, we created mutants with drastically reduced actin-binding ability. The properties of one of these mutants and of wild-type profilin I, were further characterized in cells, by microinjection into fibroblasts and by transfection into a profilin null strain of *Dictyostelium*. Our results suggest that the actin-binding capacity of profilin is not solely determining its intracellular targeting and show that a mammalian profilin with an intact actin-binding site can substitute quite well for amoeba profilin.

MATERIALS AND METHODS

Computer modeling of point mutations affecting the actin-binding activity of profilin I

Modeling of mutant profilins was based on the X-ray structure of the bovine profilin-actin complex (Schutt et al., 1993), using the program BRAGI, which includes the program AMBER for force field calculations (Schomburg and Reichelt, 1988). The interface between actin and profilin I was screened for interacting amino acids. Profilin I mutants with a putatively weakened affinity for actin were planned by exchanging phenylalanine 59 and lysine 125 on the profilin molecule for alanine. To obtain profilin I mutants with a putatively higher affinity for actin, we first searched for aromatic or charged amino acids in the actin molecule that have no profilin-interacting partner, and thus probably do not participate in intramolecular interactions. Regarding the profilin partner, residues were selected that are involved neither in intramolecular nor in intermolecular interactions, but are located in a position suitable for an additional bond to actin. According to these criteria, valine 60 and glycine 120 were exchanged for glutamic acid and phenylalanine, respectively. Modeling was completed by energy minimizing in the environment of the exchanged amino acids.

Vector construction and expression of bovine profilin I and the profilin mutants

The cDNA sequence for bovine profilin I was cloned into the expression vector pTZ19SD as described (Schlüter et al., 1998). By DNA sequencing the 423 bp cDNA sequence in the resulting expression plasmid, pTZRP4, was found to be identical to the coding sequence determined for calf spleen profilin I (Nystrom et al., 1979). To introduce point mutations into the cDNA of profilin I, site-directed mutagenesis was performed by fusion PCR: first, the 5′- and 3′-fragments of the mutants were amplified with overlapping internal primers carrying the mutation and then these fragments were fused by a second PCR with the outer primers. The fusion PCR products, with the expected length of 437 bp, were cloned into the plasmid pGEM-T (Promega, Heidelberg, Germany) and subsequently, the cDNAs were ligated as *Hpa*I/*Kpn*I fragments into the multiple cloning site of pTZ19SD. The cDNAs coding for the profilin I mutants were verified by sequencing.

To construct the *Dictyostelium discoideum* expression vectors, the profilin I cDNAs were modified by PCR. Primers were used to exchange the first eight codons of the bovine cDNA for the most suitable codons, according to the codon usage of *Dictyostelium* (Sharp and Devine, 1989). The PCR products were cloned into the plasmid pGEM-T, verified by DNA sequencing and subsequently cloned as *Hin*dIII fragments into the expression vector pT84bsr/H (Neujahr et al., 1998). This vector allows the expression of the insert under control of an actin-15 promoter and an actin-8 terminator, and carries a blasticidin resistence cassette (Sutoh, 1993).

Purification of recombinant and genuine proteins

Recombinant wild-type bovine profilin I and its mutants were expressed in *E. coli* JM101 transformed with the respective plasmids. 4 hours after induction with IPTG and M13mGP1-2 phage containing the T7 RNA polymerase gene (Dunn and Studier, 1983), bacteria were sedimented and disrupted by ultrasonication in a buffer containing 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 10 mM $MgCl_2$, 10 mM glucose, 1.5% Triton X-100, pH 7.2, protease inhibitors, and 10 mg lysozyme and 15 μg DNase I per litre of bacterial culture.

After centrifugation, the clear supernatant was applied to a poly(L)proline (PLP) affinity column and eluted with a urea gradient (Kaiser et al., 1989; Lindberg et al., 1988; Tanaka and Shibata, 1985). Profilin I-containing fractions were identified by SDS-PAGE on tricine-containing gels (Schagger and von Jagow, 1987) and immunoblots (for antibodies, see below), pooled and dialyzed against 0.01 M Tris-HCl, pH 7.6, 0.2 mM $CaCl_2$ and 0.5 mM DTE. The purity of the protein preparations was better than 95%, as judged by Coomassie Blue-stained SDS-gel profiles. The preparations were sterilized by filtration and concentrated in Amicon cells (Amicon, Witte, Germany) under nitrogen pressure. Protein yield was determined by using the extinction coefficient for profilin I (Larsson and Lindberg, 1988). Approximately 15 mg protein were obtained from a 3 l bacterial culture and stored at 4°C for up to 3 weeks.

Rabbit skeletal muscle actin was prepared from acetone powder as

described (Spudich and Watt, 1971), with an additional gel filtration step, as described in (Giehl et al., 1994). Protein yield was determined by the extinction coefficient (Houk and Ue, 1974).

Actin polymerization assays

The influence of profilin I wild type and mutants on the kinetics of skeletal muscle actin was measured by high shear viscometry, using Ostwald viscometers, essentially as described (Giehl et al., 1994). Briefly, actin (8 μM) in 0.01 M imidazole, pH 7.0, 0.15 mM $CaCl_2$, 1.5 mM DTE, 1 mM ATP (G-buffer), with or without additional proteins (12 μM final concentration), was polymerized by the addition of KCl (final concentration 0.05 M). Viscosity was monitored for 240 minutes. The solutions were then adjusted to 2 mM $MgCl_2$ and viscosity was followed for an additional hour.

The influence of profilin I wild type and mutants on actin polymerization was determined either by low shear viscometry, using the falling ball assay, or by fluorimetry with pyrene-labeled actin (actin reacted on Cys 374 with N-(1-pyrenyl)iodoacetamide; Kouyama and Mihashi, 1981). Increasing concentrations of G-actin (in G-buffer) were incubated with constant concentrations of profilin I wild-type or mutant proteins. Polymerization of actin was started by adjusting the solution to 0.05 M KCl and allowed to continue at room temperature for at least 12 hours. Viscosity was monitored in calibrated glass capillaries set up at a 30° angle, following the time required for a stainless steel ball to pass a standard distance. Fluorimetry was performed with 10% pyrene-labeled and 90% unlabeled actin at 366 nm excitation and 407 nm emission in an LS50B fluorimeter (Perkin-Elmer, Langen, Germany). Dissociation constants (K_d values) were calculated according to Giehl et al. (1994) and Tseng and Pollard (1982), assuming that under these conditions profilins act as sequestering factors for Ca^{2+}-actin (Perelroizen et al., 1996).

Antibodies and immunoblotting

The following primary antibodies were used in this study: (1) a monoclonal anti-profilin I (2H11), classified as IgGγ1, which reacts with a wide variety of mammalian profilin I, except for rodent profilins (Mayboroda et al., 1997); (2) a polyclonal rabbit antiserum against bovine profilin I (Buss and Jockusch, 1989); (3) a monoclonal anti-actin raised against *Dictyostelium discoideum* actin (Act I; Simpson et al., 1984); (4) a monoclonal antibody against *Dictyostelium* profilin I (153-246-10; Haugwitz et al., 1991).

For immunofluorescence, goat anti-mouse IgG or goat anti-rabbit IgG coupled to TRITC (Dianova, Hamburg, Germany) were used as secondary antibodies. For double labeling of profilin and F-actin, FITC-phalloidin was used in conjunction with the different anti-profilins. Nuclear staining was performed with DAPI (4′,6-diamino-2-phenylindol; Mobitec, Göttingen, Germany).

Proteins were separated by SDS-PAGE and blotted onto Immobilon P (Millipore, Eschborn, Germany) membranes. The membranes were incubated with second antibodies against rabbit and mouse IgG, coupled to horse radish peroxidase (HRP; Sigma, Deisenhofen, Germany), and antibody binding was monitored by enhanced chemical luminescence (ECL; Amersham, Braunschweig, Germany).

Cell culture, microinjection and transformation

The fibroblast line SW3T3 (mouse) was used for localization studies. Cells were grown on collagenated coverslips in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Microinjection experiments were performed by using the micromanipulator 5170 and the microinjector 5242 (Eppendorf, Berlin, Germany) at a needle concentration of 2 mg profilin/ml in phosphate-buffered saline. Subsequently, the injected cells were returned to the incubator for at least 30 minutes before being fixed and processed for fluorescence microscopy.

Dictyostelium discoideum wild-type strain AX2 and profilin double minus mutant strain PI/II-minus (Haugwitz et al., 1994) were cultivated at 21°C, either on SM agar plates with *Klebsiella aerogenes* (Williams and Newell, 1976) or axenically in HL-5 medium (Sussman, 1987) with 40 μg/ml streptomycin, either in shaking suspension (120 rpm) or submerged in plastic culture dishes. For transformation, PI/II-minus cells were treated as described by Karakesisoglou et al. (1996). After incubation for 24 hours at 21°C without selection, 4 μg/ml blasticidin (ICN Biochemicals, Eschwege, Germany) was added for selection of transformed cells. Transformants were cloned on *Klebsiella aerogenes* by spreader dilutions. To determine the effect of bovine profilin I on solid phase growth, the area of individual colonies was measured.

Immunohistochemistry

Mammalian cells were fixed in 3.7% formaldehyde for 20 minutes and extracted with 0.2% Triton X-100 for 10 minutes. *Dictyostelium* cells were allowed to spread on coverslips for 45 minutes before being fixed and extracted with ice-cold methanol for 10 minutes at −20°C. Then the coverslips were dried at room temperature for 30 minutes.

All samples were then processed for fluorescence microscopy and examined either in a conventional light microscope equipped with epifluorescence (Axiophot, Zeiss, Oberkochen, Germany), or in a confocal laser scanning microscope (MRC 600 Imaging System, Bio-Rad, Munich, Germany). Optical sections were obtained at 0.25 μm intervals.

To determine the number of nuclei, *Dictyostelium* cells from shaking cultures were fixed as described and stained with DAPI.

RESULTS

Design of mutants

We designed and characterized four profilin I mutants which, according to computer-based models, should differ in their affinity for actin as compared to the wild type. The modeling was based on the structure of the bovine profilin I actin complex as revealed by X-ray analysis (Schutt et al., 1993).

In modeling profilin I mutants with a possibly higher affinity for actin, two amino acid exchanges appeared attractive. In the actin molecule, arginine 290, located within the interface with profilin, neither participates in the interaction with profilin nor has an intramolecular binding partner. Valine 60 on the profilin I molecule shows similar properties: it has neither intra- nor intermolecular interacting partners. By exchanging valine 60 of profilin I for glutamic acid, an additional ionic bond should be formed, involving actin arginine 290 (V60E, Fig. 1, left bottom). Indeed, after energy minimizing, the distance between the functional groups of arginine 290 (actin) and glutamic acid 60 (profilin) was found in the optimal range for such an ionic bond. Furthermore, an additional interaction between the glutamic acid at position 60 (profilin) and lysine 291 (actin) might be conceivable. A second mutant with a presumptive increase in affinity for actin was designed by adding a hydrophobic interaction. According to the criteria given above, i.e. lack of intramolecular and intermolecular interactions, tyrosine 169 (actin) and glycine 120 (profilin) seemed suitable candidates. Glycine 120 was exchanged for phenylalanine (G120F, Fig. 1, right top).

To obtain profilin I mutants with a putatively weaker binding to actin, two point mutations were introduced. In the first mutant, the amino acid phenylalanine 59 was exchanged for alanine (F59A, Fig. 1, left top), with the aim of destroying the interaction between the aromatic rings of this residue and histidine 173 on the actin molecule. In a second mutant, the

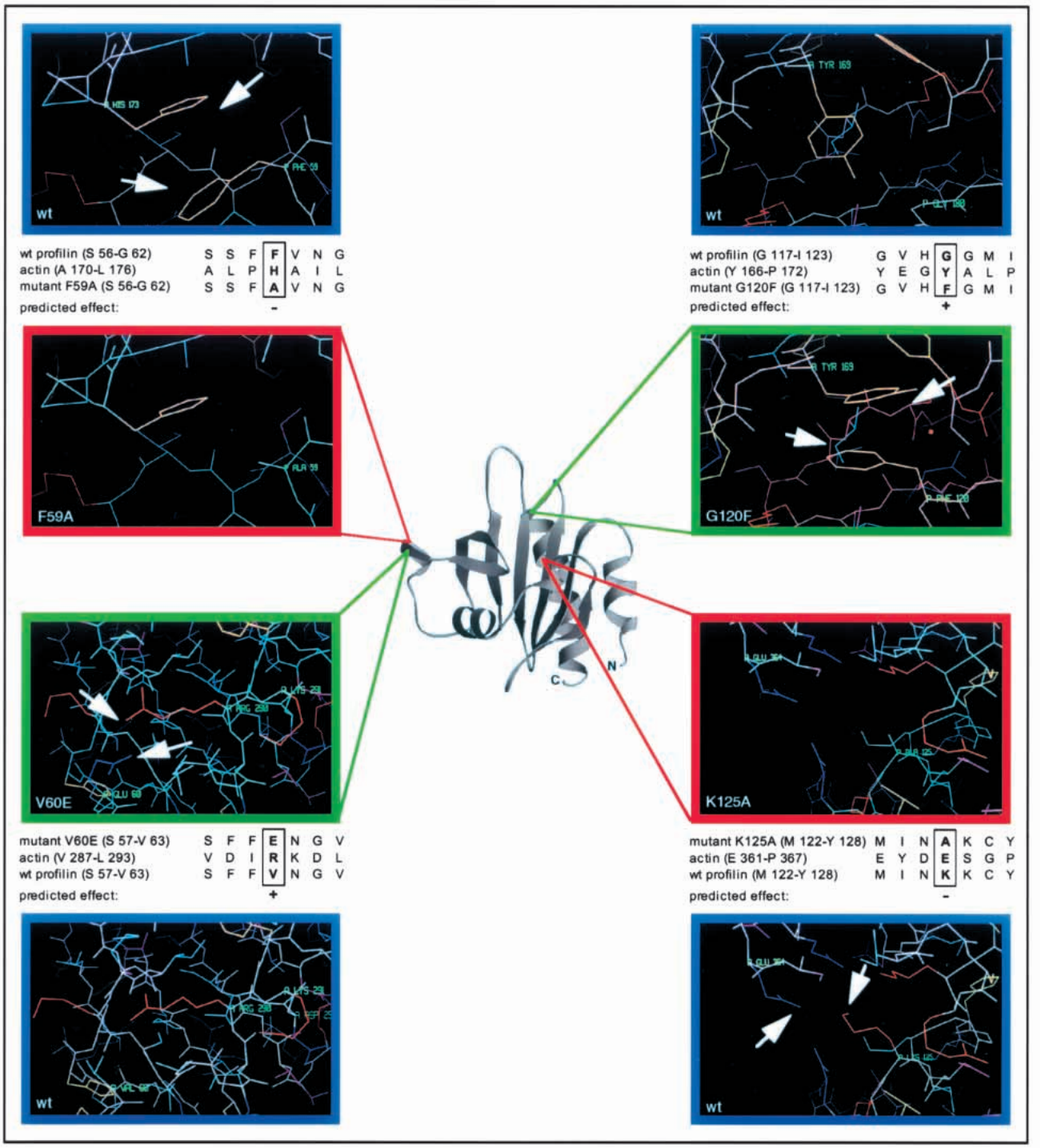

Fig. 1. Computer-modelled topography of residues in the profilin-actin interface and predicted effects of single amino acid substitutions. The structure of bovine profilin I, as determined by Schutt and coworkers (Schutt et al., 1993) is shown in the center. Details of the profilin-actin interface are shown on both sides (wild-type complex (wt), blue; modeled complex with the respective profilin mutant, green or red). The actin part is depicted on the top, the profilin parts are on the bottom in each panel. The green and red lines point to the position of the mutated amino acid in the profilin I molecule. The corresponding regions in the amino acid sequences, the mutated residues and the predicted effects are indicated between each pair.

ionic bond between lysine 125 (profilin) and glutamic acid 364 (actin) should be disconnected by exchanging lysine for alanine (K125A, Fig. 1, right bottom).

All these point mutations should not affect the overall structure of profilin I and were expected not to be defective in binding to other ligands. To test these predictions, the mutated profilins were expressed as recombinant proteins in *E. coli* and analysed in biochemical and biophysical assays.

In vitro analysis of the profilin I mutants V60E, G120F, F59A and K125A

All four mutants were successfully expressed in *E. coli* and purified on poly(L)proline (PLP) affinity columns (Fig. 2). The yields of recombinant wild-type and mutant profilins were similar (5 mg protein per litre *E. coli* culture). As shown in Fig. 2 (left), the profilin I mutants eluted from the PLP column at the same urea concentration as wild-type profilin, which indicates that the binding to PLP is not reduced in the mutant proteins. As judged by SDS-PAGE on tricine-containing gels (Schagger and von Jagow, 1987), purity of the preparations was >95% and all mutants displayed an apparent molecular mass of approximately 14.5 kDa (Fig. 2, right). The identity of the purified proteins was verified by immunoblotting. All mutants reacted specifically with a monoclonal antibody against wild-type bovine profilin I, 2H11 (Fig. 2, right).

The actin-binding activity of the profilin I mutants was analysed in polymerization assays. First, we studied the influence of profilin I on the polymerization kinetics of actin by high shear (Ostwald) viscometry. Skeletal muscle actin and profilins were mixed in molar ratios of 1:1.5 (8 μM:12 μM) and actin polymerization was started by increasing the ionic strength to 0.05 M KCl. As seen in Fig. 3, under the conditions used here, the control actin solution showed the expected sigmoidal polymerization curve, with an initial lag phase of about 20 minutes, followed by a rapid linear phase, and a steady state level at 70 minutes. The addition of wild-type profilin I totally suppressed actin filament formation for 150 minutes and the final viscosity value was only 15% of the control. In contrast, all mutant proteins inhibited the actin polymerization much less than wild-type profilin (Fig. 3). Only the mutant K125A showed some profilin-type activity, inducing a prolongation of the lag phase to 40 minutes and a steady state level of only 70% of the control. The other mutants, including those designed for a higher affinity for actin, had no significant effect on actin polymerization, suggesting that their actin-binding ability was severely affected. The addition of Mg^{2+} (2 mM final concentration) to the solution at a time point where the steady state level had been reached in the control induced a further increase in viscosity, which is consistently seen in such assays and probably due to the addition of both Mg^{2+}-ATP-actin and profilin-actin complexes to the fast growing ends of filaments (Perelroizen et al., 1996).

To determine the affinity of the various profilin proteins for actin, their influence on the critical concentration of Ca^{2+}-actin polymerization was monitored, by falling ball viscometry and by fluorescence spectroscopy with pyrene-labeled actin. Fig. 4A-C shows the effects of profilin I wild-type or mutant proteins on the steady state viscosity of different concentrations of skeletal muscle actin, as measured in single experiments. The linear regression curves show the difference in critical concentration obtained in the presence of either profilin I wild type or mutants, as compared to the actin control. All profilin I mutants increased the critical concentration of actin much less than profilin I wild type. This finding is consistent with the effect of the profilin I mutants on actin polymerization kinetics (Fig. 3). The mean values of several experiments were combined with analogous results obtained by following the polymerization of pyrene-labeled actin, to calculate the K_d values for different profilin/actin complexes

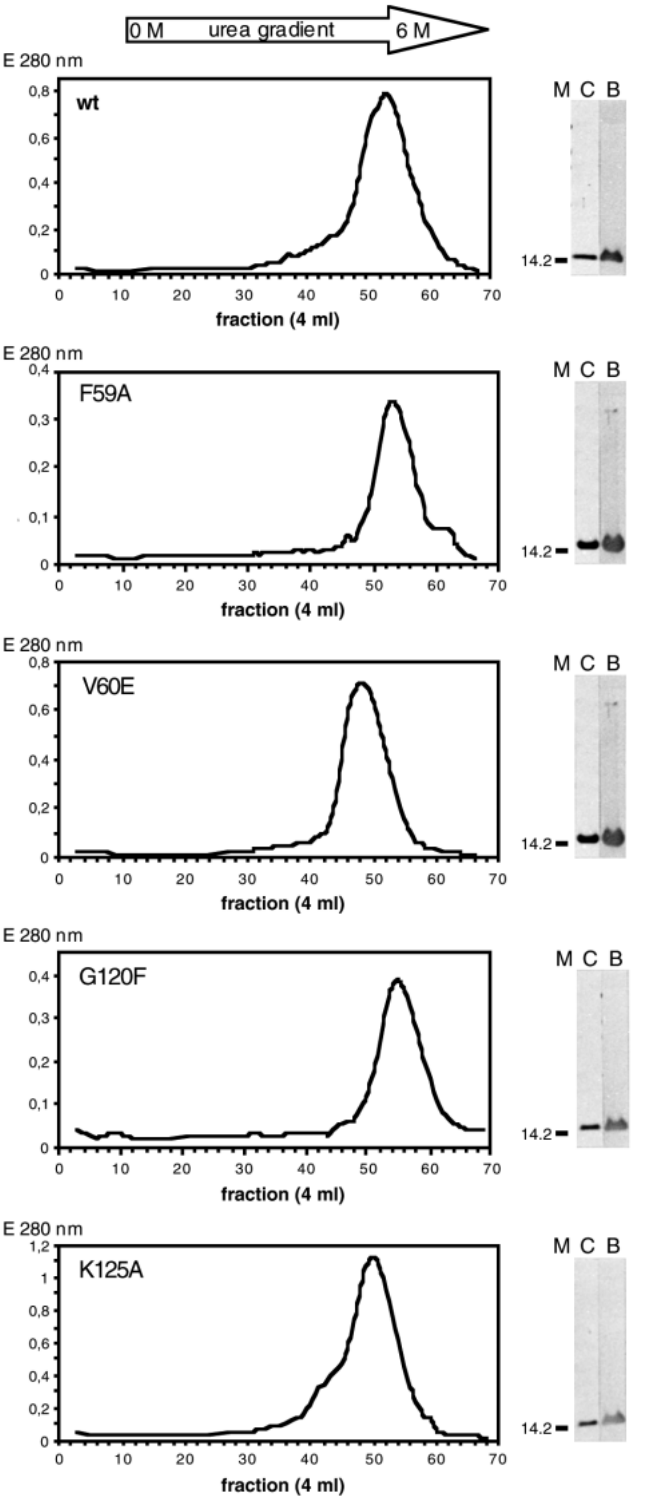

Fig. 2. Purification of recombinant bovine profilin I and mutant proteins. Left, elution profiles of wild-type and mutant profilins from poly(L)proline by a urea gradient. Right, Coomassie Blue-stained profiles of the peak fractions after SDS-PAGE (C) and corresponding immunoblots with anti-profilin (B). The purified protein comigrates with the marker protein α-lactalbumin of 14.2 kDa (M).

(Fig. 4D). While the standard deviations in individual experiments (n=4) were fairly large, the difference of the data

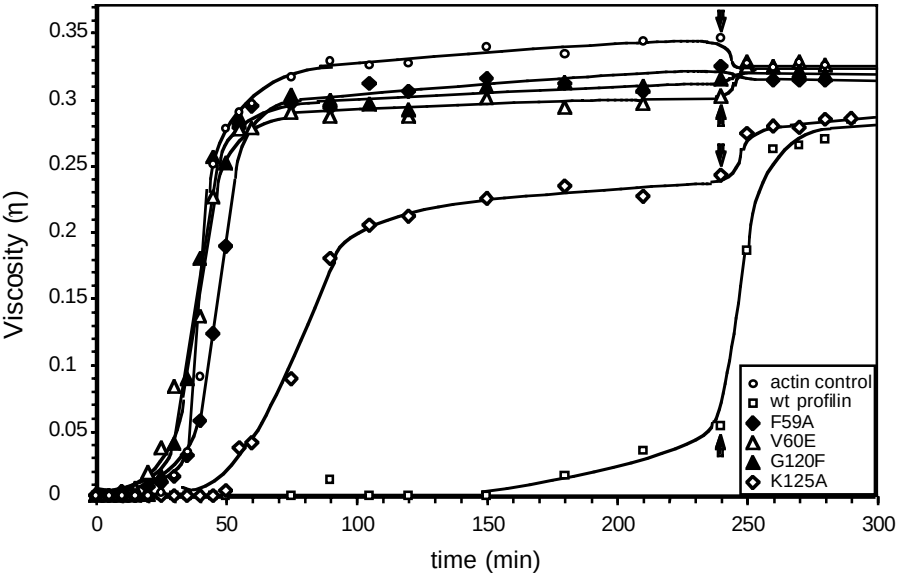

Fig. 3. The influence of profilin I mutants on actin polymerization, as seen by high shear (Ostwald) viscometry. Skeletal muscle actin (control) and mixtures of actin and different profilins (molar ratio of 1:1.5) were induced to polymerize in 0.05 M KCl. After 4 hours, the solutions were adjusted to 2 mM $MgCl_2$ (arrow). The slight drop in actin viscosity (η) after addition of Mg^{2+} is due to the dilution caused by adding the Mg^{2+} stock solution.

obtained for mutant and wild-type profilins was significant and almost constant in each experiment. Consistent with the high shear viscosity data (Fig. 3), all four mutants displayed a decreased affinity for actin. Hence, and disappointingly, the mutants which had been designed to contain additional bonds to actin, V60E and G120F, were apparently defective in their actin-binding site. In contrast, and in accordance with the expectation, both mutants with a reduced number of bonds to actin showed a decreased affinity for actin. K125A showed an affinity for actin reduced to 50% of wild-type profilin, while F59A, lacking an interaction between two aromatic rings, was reduced in its actin binding activity to less than 10% of the parental protein.

The profilin I mutant F59A targets to the same cellular sites as wild-type profilin

Cellular localization was investigated by microinjecting fibroblasts with F59A, which shows an actin affinity one order of magnitude lower than wild-type profilin.

Microinjection was performed with SW3T3 mouse fibroblasts. Cells were microinjected with mutant and wild-type profilin I (needle concentration 2 mg/ml in each case). After 30-60 minutes incubation past microinjection, the cells were fixed with formaldehyde, permeabilized and double stained with FITC-phalloidin to visualize the actin filament organization, and with the monoclonal antiprofilin I 2H11, which does not react with rodent profilins (Mayboroda et al., 1997). Microinjected cells were easily identified with this antibody (Fig. 5). The cellular distribution of wild-type profilin I and the mutant F59A was identical, and was similar to the images obtained previously for the endogenous profilin I (Buss et al., 1992; Rothkegel et al., 1996; Mayboroda et al., 1997). Microinjected as well as endogenous profilins were found dispersed in the cytoplasm and enriched in highly motile peripheral areas of the cells, such as ruffles and lamellipods, where they colocalized with cortical actin filaments (Fig. 5). As revealed by analysing optical sections by confocal laser scanning microscopy, the prominent fluorescence signal in these areas was not due to differences in cellular thickness or trapping effects. Additionally, a nuclear localization of both microinjected wild-type and mutant profilin I was observed (for example Fig. 5B′,D′), which had also been described for endogeneous profilin (Mayboroda et al., 1997).

Bovine profilin I and its mutant F59A partially rescue profilin-deficient *Dictyostelium discoideum* amoebae

To further study the biological activity of profilin I wild type and the mutant, we used the *Dictyostelium discoideum* mutant PI/II-minus, which lacks both profilin isoforms expressed in this amoeba (Haugwitz et al., 1994). The most important differences between PI/II-minus and wild-type *Dictyostelium discoideum* are reduced growth on bacterial lawns, impaired cytokinesis and incomplete development of the mutant (Haugwitz et al., 1994). Two questions were addressed in this study. (1) To what extent can mammalian profilin I, i.e. wild-type bovine profilin I, compensate for the defects observed in this mutant? (2) What is the difference in biological activity between wild-type bovine profilin I and the mutant F59A, which displays a drastically reduced affinity for actin?

To ascertain that bovine profilin I can interact with *Dictyostelium discoideum* actin in a *Dictyostelium* background, affinity precipitation with extracts of *Dictyostelium* PI/II-minus cells and bovine wild-type profilin I coupled to Sepharose was performed. Using SDS-PAGE and immunoblotting with an antibody against *Dictyostelium* actin, we showed that *Dictyostelium* actin was specifically precipitated by bovine profilin-Sepharose (data not shown).

Dictyostelium PI/II-minus cells were then transformed with expression vectors carrying the cDNA for wild-type bovine profilin I and for F59A. The expression of bovine profilin I in *Dictyostelium* cells was monitored by immunoblots using the monoclonal antibody 2H11. Three different clones were isolated by selection in blasticidin-containing medium: the wild-type profilin I-expressing clone Ddwt1 and the F59A-expressing clones Dd59A1 and Dd59A11 (Fig. 6A, upper panel). All three clones showed a 2H11-reactive band at the

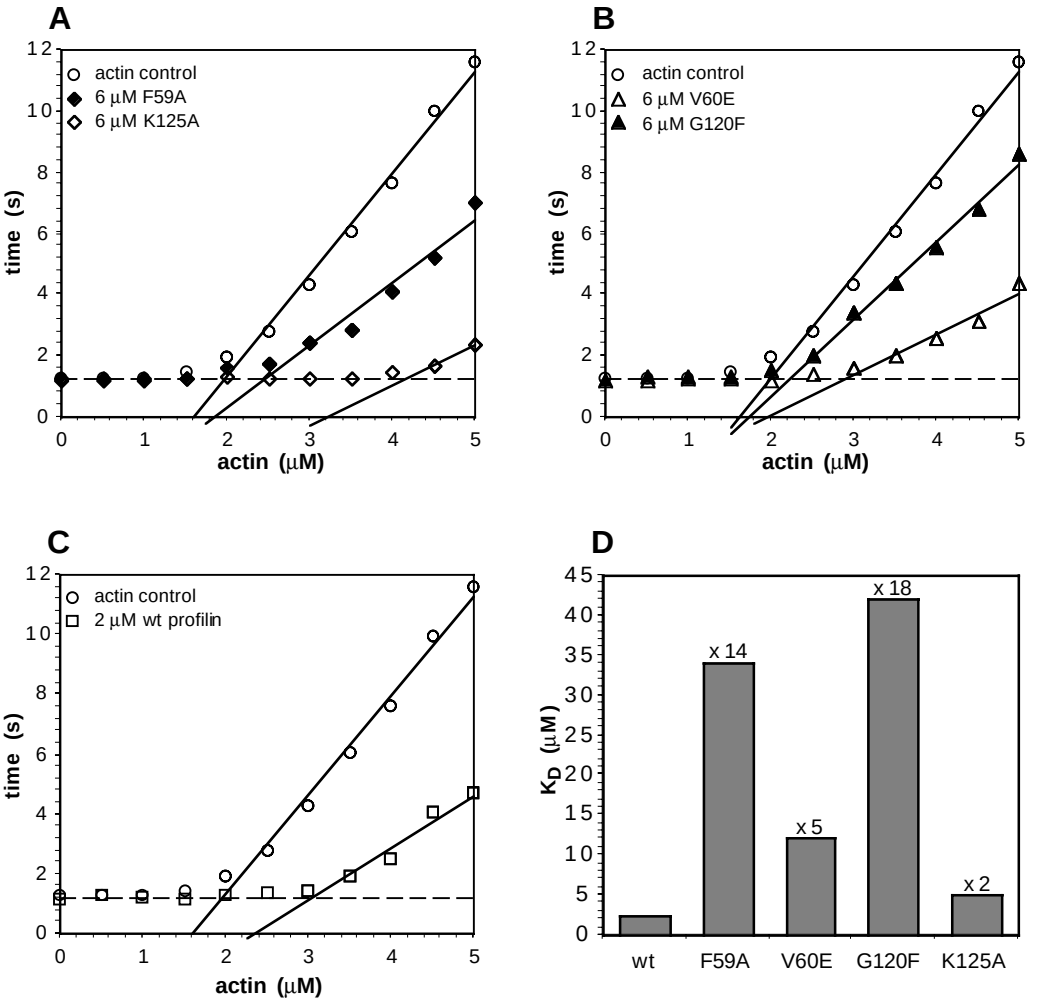

Fig 4. K_d values of profilin-actin complexes, as calculated from values obtained for polymerized actin at steady state in the absence or presence of profilin proteins. (A-C) Single experiments monitoring the effects of various profilin proteins on the low shear viscosity of polymerized Ca^{2+}-actin. The steady state viscosity of each probe is expressed as the time required for a stainless steel ball to pass a standard distance through the sample in a glass capillary (ordinate). Dotted line, the value obtained for buffer only. The critical concentration required for poylmerization can be deduced from the regression plots. (D) Mean K_d values of wild-type and mutant profilins, obtained from the steady state data of several falling ball and fluorimetric experiments. The columns illustrate the K_d values (wild type, 2.3±0.9 μM; F59A, 34±15 μM; V60E, 12±3.7 μM; G120F, 42±13 μM; K125A, 5±2.0 μM) and the numbers given at the top demonstrate the factor of deviation from the K_d value of wild-type profilin.

position expected for bovine profilin I, but in addition, minor bands were seen at higher positions. These might be due to some modification of the exogeneous profilin by *Dictyostelium* cells. The expression levels were quantitated on immunoblots with 2H11, using purified recombinant bovine profilin I as calibration standard. The concentration of the heterologous protein was calculated according to Podolski and Steck (1990) and Haugwitz et al. (1994). The expression levels for bovine profilin I in Ddwt1 and Dd59A1 were similar (3.2 μM and 5.2 μM, respectively), whereas the expression level in Dd59A11 was only about one tenth of that obtained for the other clones (0.5 μM, Fig. 6A, lower panel). As the concentration of profilin I and II in AX2 *Dictyostelium* wild-type strain has been determined to 19 μM and 28 μM, respectively (Haugwitz et al., 1994), the expression level obtained for bovine profilin I and its mutant is only approximately 10% of that determined for the endogenous profilins in the *Dictyostelium* wild-type strain AX2.

The expression of the mammalian profilins in *Dictyostelium* cells was also demonstrated by immunofluorescence (Fig. 6B). Bovine profilin I was found distributed throughout the cytoplasm of Ddwt1 cells (right panel), a pattern also seen for the endogenous *Dictyostelium* profilin I in the wild-type strain AX2 (left panel). In general, fluorescence intensity was low, probably reflecting the fact that the expression level was low. No difference in cell morphology between wild-type and mutant bovine profilin-expressing cells was seen (not shown).

A potential rescue of the defects seen in PI/II minus *Dictyostelium* cells was analysed by monitoring development, growth and cell division fidelity of the bovine profilin wild type expressing clone Ddwt and the mutant expressing clones Dd59A1 and Dd59A11.

Expression of bovine profilin I was not capable of overcoming the developmental arrest at the stage of culmination, and the finger-like structures formed were indistinguishable from those of PI/II minus cells (data not shown).

Partial rescue of growth and cytokinesis defects, however, was seen in PI/II-minus cells transfected with bovine profilin I. Growth was monitored on a lawn of *Klebsiella aerogenes*. The surface area of colonies emerging after seeding was measured. As previously described (Haugwitz et al., 1994) and depicted in Fig. 7, colony growth of *Dictyostelium* PI/II-minus cells, as determined by surface increase, was retarded as compared to the wild-type strain AX2. Expression of bovine profilin I in such cells partially rescued the colony growth defect, as seen for the clones Ddwt1 (Fig. 7). In Dd59A1, expressing the profilin mutant defective in actin-binding, this rescue was much less pronounced, indicating that colony growth depends on an efficient profilin-actin interaction.

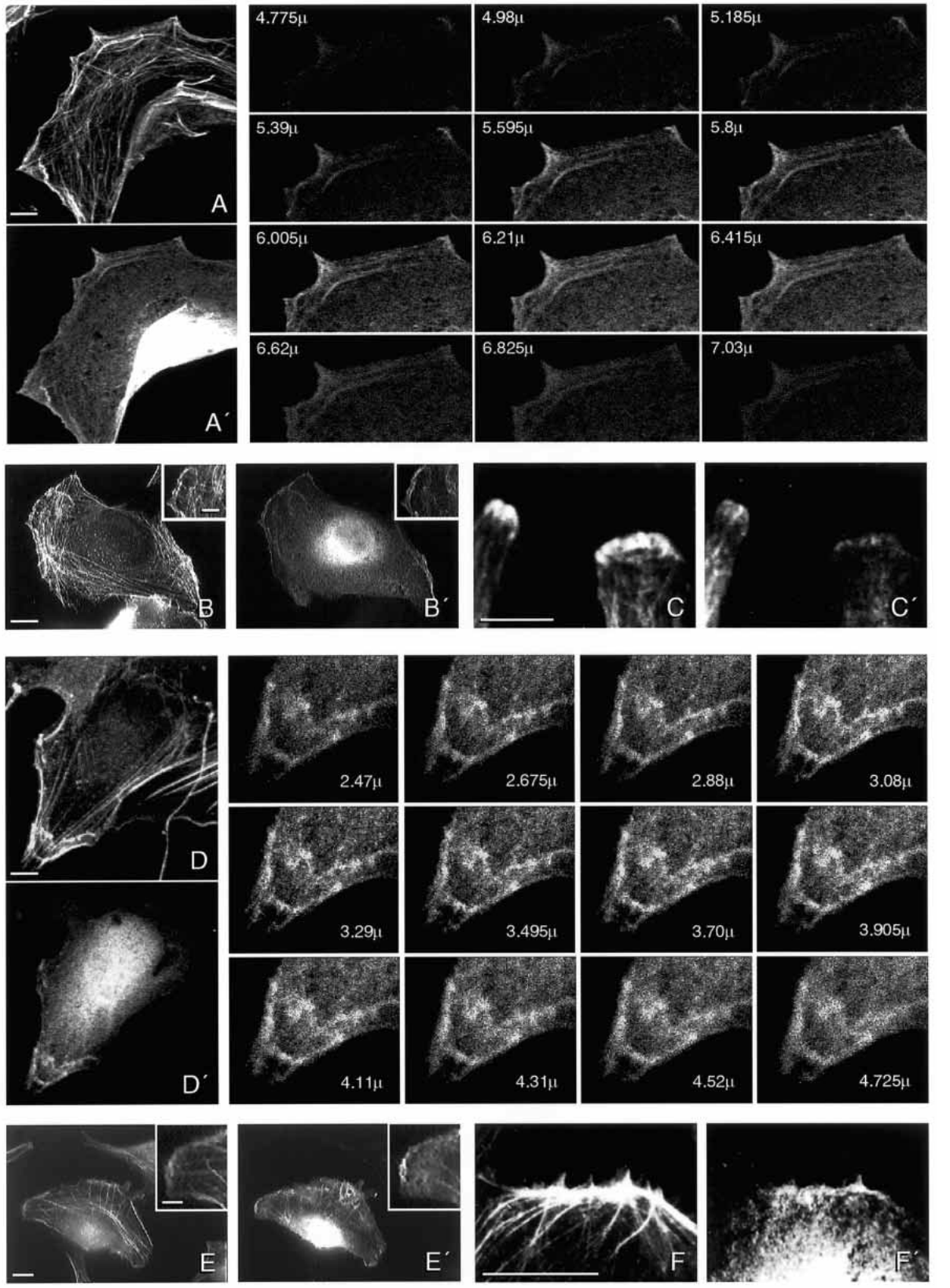

Fig. 5. Immunofluorescence images of mouse fibroblasts stained with the anti-profilin 2H11 after microinjection of bovine profilin I wild-type (A′-C′) and mutant F59A (D′-F′), and counterstained for actin filaments with FITC-phalloidin (A-F). 2H11 does not recognize endogeneous rodent profilin. The images B,B′-C,C′ and E,E′-F,F′ were taken by conventional microscopy, while the cells depicted in A,A′ and D,D′ represent the sum of confocal sections. The individual sections are shown enlarged next to A,A′ and D,D′, and are taken from A′ and D′. The level of scanning (in µm) in each individual section is shown. Bars, 5 µm (in insets, 2 µm).

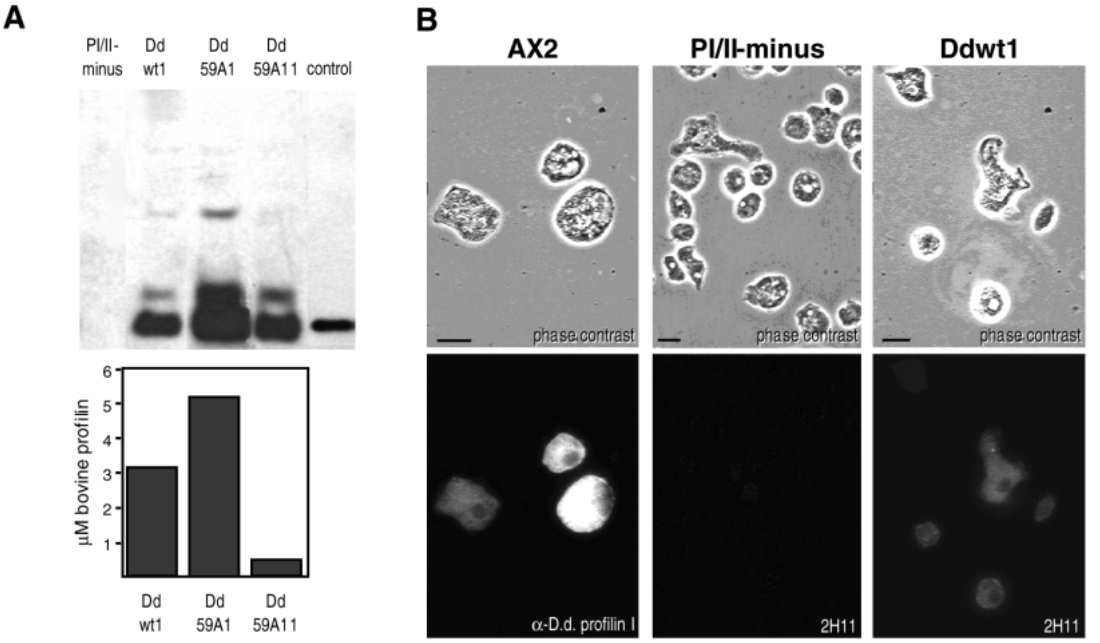

Fig. 6. Expression of bovine profilin I in *Dictyostelium* cells. (A) Cell extracts of 3×10^5 cells per lane were separated on tricine-containing SDS gels, blotted onto Immobilon P membrane and incubated with anti-profilin 2H11. Antibody binding was monitored with ECL (upper panel). In addition to a polypeptide comigrating with purified profilin (control), the different clones show 2H11-reacting bands at higher positions, apparently modified profilins. The overall content in profilin I (lower panel) was determined using bovine profilin standards, assuming an average volume of 1 pl per cell (Podolski and Steck, 1990). AX2, *Dictyostelium* wild-type cells; PI/II-minus, profilin null cells; Ddwt1, bovine Profilin I wild-type expressing cells; Dd59A1 and Dd59A11, bovine Profilin I mutant F59A expressing cells. (B) Immunofluorescence images of *Dictyostelium* cells stained for *Dictyostelium* profilin I (AX2) or bovine profilin I (PI/II-minus and Ddwt1). Bars, 5 µm.

Dd59A11, containing only one-tenth of the profilin I level of the other clones, was almost indistinguishable from PI/II-minus colonies. The reduced growth rates of mammalian profilin-expressing cells after several days is probably due to transient expression under blasticidin-free growth conditions, and was also observed for expression of plant profilin in *Dictyostelium* cells (Karakesisoglou et al., 1996).

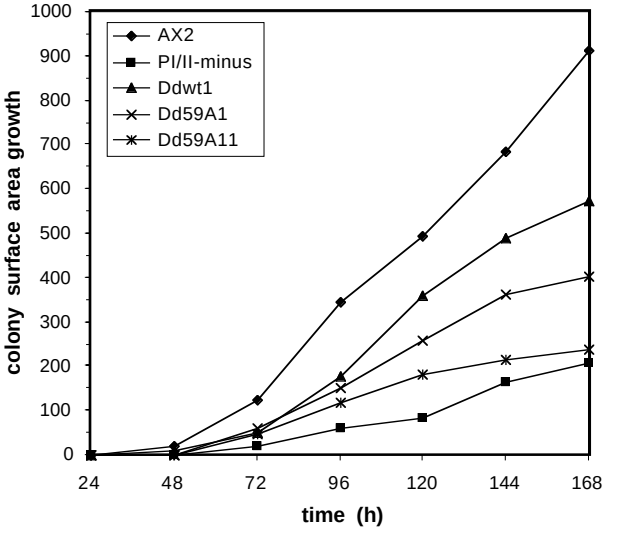

Fig. 7. Rescue effect of bovine profilin I in PI/II-minus cells: growth on bacteria. Agar plates were inoculated on a lawn of *K. aerogenes* with a 5 µl drop containing 5×10^5 cells of the different clones (see Fig. 6) and incubated at 21°C. The surface area of each colony was measured after different times as indicated. Plotted are the means of individual measurements (n=6).

Impairment of cytokinesis was examined by determining the number of nuclei per cell. Cells grown in shaking culture were allowed to adhere to coverslips, fixed and stained with DAPI. As demonstrated in Fig. 8, PI/II-minus cells show an increase in cells with four nuclei as compared to wild-type strain AX2. This again was attenuated by the expression of bovine profilin I. In Ddwt1, the number of tetranucleate cells was significantly decreased, while in Dd59A1 and Dd59A11 this effect was less pronounced.

DISCUSSION

In this study, we designed several profilin I point mutants with the aim of altering their binding to actin. Bovine profilin I forms two major contacts with actin in the crystal. The more extended one comprises a region of profilin I defined by helix 3, the amino-terminal portion of helix 4, and strands 4, 5 and 6 (Schutt et al., 1993). Binding to actin of this contact site involves actin subdomains 1 and 3, and this leads to the formation of a closely packed, complementary interface of ionic, polar and hydrophobic interactions, spanning a surface of 2.25 $Å^2$ (Schutt et al., 1993).

Our attempts to strengthen this interaction by introducing into this interface additional bonds based on charged or aromatic residues led to a weakening of the profilin actin complex. In V60E, computer modeling did not suggest sterical

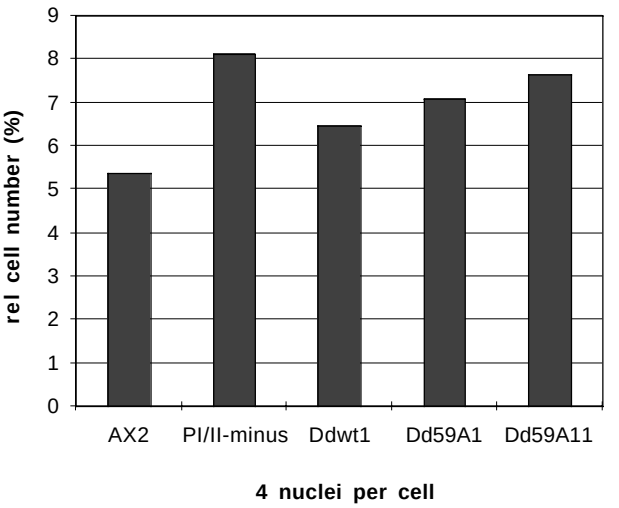

Fig. 8. Rescue effect of bovine profilin I in PI/II-minus cells: relative number of cells with 4 nuclei. Cells were grown in shaking culture for 5 days, allowed to adhere to a coverslip, fixed and stained with DAPI. (AX2, *n*=2338; PI/II-minus, *n*=1790; Ddwt1, *n*=1622; Dd59A1, *n*=1607; Dd59A11, *n*=1478).

hindrance for an additional bond to actin arginine 90, yet, this mutant profilin diplayed a lower affinity to actin than wild-type profilin I. Possibly, the charged amino acid introduced in position 60 disturbes the interaction between the aromatic rings of phenylalanine 59 (profilin I) and histidine 173 (actin). In G120F, a distortion of tyrosine 169 (actin), whose aromatic ring was expected to interact with phenylalanine 120 of the mutant profilin, was observed after energy minimizing. This might suggest sterical hindrance, although energy minimizing of the wild-type complex resulted in a similar distortion. Furthermore, energy minimizing led to a repositioning of phenylalanine 375 (actin). In the wild-type complex, this residue plays a central role in a large hydrophobic contact between actin and profilin I and additionally, its terminal carboxyl group forms an ionic bond with arginine 74 (profilin) (Schutt et al., 1993). It is conceivable that these interactions are disturbed in the complex with the mutant G120F. Although many more mutants would have to be designed to arrive at a final conclusion, our data suggest that the introduction of additional putative bonds by the method used here cannot improve profilin-actin affinity.

In contrast, designing profilin I mutants with a decreased affinity for actin by computer modeling yielded the desired proteins. By exchanging interacting residues against alanine, discrete bonds were apparently disconnected. In the mutant F59A, the removal of the striking interaction between two aromatic ring systems, phenylalanine 59 (profilin) and histidine 173 (actin) resulted in a drastic reduction of affinity for actin, the K_d value being increased by a factor of 14. In accordance with the fact that in a hydrophilic environment, hydrophobic interactions are much stronger than ionic bonds, disconnecting an ionic bond resulted in a much less drastic change: in the mutant K125A, the K_d value was increased only by a factor of 2 over wild-type profilin I. Lysine 125 is thought to be homologous to lysine 115 of *Acanthamoeba* profilin I (Rozycki et al., 1994; Schutt et al., 1993; Haugwitz et al., 1991), which can be crosslinked with glutamic acid 364 on the actin molecule (Vandekerckhove et al., 1989).

As all mutation sites used in this study are located opposite to the PLP binding region of profilin (Table 1; Haarer et al., 1993; Archer et al., 1994; Bjorkegren et al., 1993; Metzler et al., 1994), this activity of profilin should not be disturbed. Indeed, all mutants bound to PLP columns and eluted at the same urea concentration as wild-type profilin I, confirming that none of the mutated residues is involved in PLP binding. Furthermore, as both termini of the profilin polypeptide are involved in PLP binding (Sohn et al., 1995; Archer et al., 1994; Bjorkegren et al., 1993; Haarer et al., 1993; Metzler et al., 1994), effective binding to PLP implicates an intact overall conformation of the mutant proteins. The integrity of the PLP-binding site can indeed be regarded as a sensitive indicator for faithful profilin folding, since mutating residues in this site has severe consequences on profilin's binding to other ligands, like actin (Bjorkegren-Sjogren et al., 1997). Hence, we suggest that the effects of the point mutations seen on actin binding capacity are not due to incorrect folding of the mutant proteins but indeed are caused by the planned addition and respective removal of aromatic and charged amino acids at the interface to actin. In addition, we assume that our mutants are not altered in their affinity for PtdIns 4,5-P_2. The positively charged region comprising the C-terminal part of β-strand 5 and the large loop to β-strand 6 shown to be involved in PtdIns 4,5-P_2-binding (Sohn et al., 1995; Haarer et al., 1993) is not touched by the point mutations designed in this study. This assumption is corroborated by the fact that the point mutations of human profilin I K53I, R74L, H119D, G121D, and K125Q, which are quite close to the mutation sites used here, were also not affected in their affinity to PtdIns 4,5-P_2 (Sohn et al., 1995; compare also Table 1).

A complete catalogue of profilin I mutants with an altered affinity for actin and the effect on binding to other ligands is given in Table 1. This list also includes four recently described point mutations in the interface of human profilin I with actin (Korenbaum et al., 1998). Remarkably, one of these, K125N, displayed a moderately increased affinity for actin, which the authors explain by the possibility that the elimination of an ionic bond in the profilin-actin interface facilitates the establishment of hydrophobic interactions (Korenbaum et al., 1998). For the same protein, R88L was found to display a weakened affinity for both PtdIns 4,5-P_2 and actin, indicating the involvement of this residue in binding to both ligands, and W3N and H133S were found defective in binding to actin as well as to PLP, suggesting that their overall conformation was altered (Table 1; Bjorkegren-Sjogren et al., 1997). In addition to point mutations which allowed for a detailed mapping of residues involved in profilin-ligand interactions, larger operations were performed. For example, by deleting proline 96 and threonine 97 in profilin I, Hájková and coworkers repositioned the entire loop connecting β-strands 5 and 6 in profilin I, which is located closely to actin (Hajkova et al., 1997; Schutt et al., 1993). As expected, this deletion mutant showed a decreased affinity for actin (see Table 1; Hajkova et al., 1997).

We used the mutant F59A to study the effect of a weakened affinity for actin on the biological behaviour of profilin I in a physiological environment. Microinjection into fibroblasts revealed that this protein still associates with highly dynamic

Table 1. Effects of single amino acid substitutions on ligand binding in profilins

Species	Mutation (rel. position)	Effects on affinity for PLP	PtdIns4,5-P_2	Actin	Reference
Human	W3N (^H1)*	↓	↑	↓	Björkegren et al., 1997‡
Human	Y6F (H1)	↓	n.d.	n.d.	Sohn et al., 1995‡
Human	D8A (H1)	=	↑	n.d.	Sohn et al., 1995‡
Human	G14A (H1^β1)	=	=	=	Björkegren et al., 1993‡
Human	K25Q (β1^β2)	↓	n.d.	n.d.	Sohn et al., 1995‡
S. pombe	E43K (H2)*	↓	↓	↓	Balasubramanian et al., 1994
Human	K53I (H2^H3)	=	=	n.d.	Sohn et al., 1995‡
Human	R55D (H2^H3)*	n.d.	n.d.	↓	Korenbaum et al., 1998‡
Bovine	F59A (H3)	=	n.d.	↓	This study
Bovine	V60E (H3)	=	n.d.	↓	This study
Human	K69N (β4)	n.d.	n.d.	↓	Korenbaum et al., 1998‡
S. cerevisiae	R72E (β4^β5)	↓	↓	=, ↓[+]	Haarer et al., 1993
Human	R74L (β4^β5)	=	=	n.d.	Sohn et al., 1995‡
Human	R74E (β4^β5)	n.d.	n.d.	↓	Korenbaum et al., 1998‡
S. cerevisiae	R76E (β5)	=	↓	=, ↓[+]	Haarer et al., 1993
S. cerevisiae	R76G/R81K (β5)	↓	=	=, ↓[+]	Haarer et al., 1993
S. cerevisiae	R81G (β5)	↓	=	=, ↓[+]	Haarer et al., 1993
Human	R88L (β5)	=	↓	↓	Sohn et al., 1995‡
Human	K90E (β5)	=	=	n.d.	Sohn et al., 1995‡
Human	K90E (β5)	n.d.	n.d.	↓	Korenbaum et al., 1998‡
Human	ΔP96ΔT97 (β5^β6)	=	=	↓	Hájková et al., 1997
Human	H119D (β7^H4)	=	=	n.d.	Sohn et al., 1995‡
Bovine	G120F (H4)	=	n.d.	↓	This study
Human	G121D (β7^H4)	=	=	n.d.	Sohn et al., 1995‡
Bovine	K125A (H4)	=	n.d.	↓	This study
Human	K125Q (H4)	=	=	n.d.	Sohn et al., 1995‡
Human	K125N (H4)	n.d.	n.d.	↑	Korenbaum et al., 1998‡
Human	H133S (H4)	↓	=	↓	Björkegren et al., 1997‡
S. cerevisiae	Δ3-C-terminal (H4)	↓	n.d.	=	Haarer et al., 1993
S. cerevisiae	Δ8-C-terminal (H4)*	↓	n.d.	=	Haarer et al., 1993

The relative positions of mutations within the molecule are indicated in parentheses (H, helix; β, β-strand; ^, loop between two secondary structural elements), assuming a similar structure of yeast profilin and *Acanthamoeba* profilin.

↓, decreased affinity; ↑, increased affinity; =, similar affinity; n.d., not determined.

[+]Results depending on the assay.

*Effect on structure possible.

‡No in vivo data available.

regions of the cell, such as ruffling areas, a pattern indistinguishable from that obtained with microinjected wild-type bovine profilin I (this study) or plant profilin (Mayboroda et al., 1997), and identical with the distribution of endogeneous profilin in ruffling fibroblasts and epithelial cells (Buss et al., 1992; Rothkegel et al., 1996; Mayboroda et al., 1997). This result strongly suggests that profilin is not primarily tethered to actin in motile cortical regions of the cell, but, additionally or preferentially, to other ligands. These may involve membrane-bound PtdIns 4,5-P_2 and PtdIns 3-kinase (Hartwig et al., 1989; Ostrander et al., 1995; Lange et al., 1998; Lu et al., 1996), but also protein ligands for the PLP-binding site on profilin, such as VASP and p140mDia, which were both found to colocalize with profilin in these sites (Reinhard et al., 1995; Watanabe et al., 1997).

To study the biological activity of profilin I wild type and F59A in vivo, the corresponding cDNAs were introduced into *Dictyostelium discoideum* profilin null mutant (PI/II-minus), which shows reduced growth on bacterial lawns, impaired cytokinesis and incomplete development (Haugwitz et al., 1994).

Three bovine profilin I expressing clones were isolated, the wild-type profilin I expressing clone Ddwt1 and the F59A expressing clones Dd59A1 and Dd59A11. The expression levels of the exogenous profilins obtained with this expression system were low, i.e. not more than 10% of the concentration of both endogenous profilin isoforms in *Dictyostelium* wild-type strain AX2. The effective profilin concentration in these clones may be even lower, as we observed the appearance of profilin polypeptides with aberrant apparent molecular masses, suggesting that the amoebae partially modified the foreign proteins. The exogenous profilins were found diffusely distributed throughout the cells, a pattern also observed for the endogenous *Dictyostelium* profilin I in AX2 cells. Despite the relatively low expression levels of the exogenous profilins in PI/II-minus cells and despite the possibility that heterologous profilin-actin complexes of *Dictyostelium* and mammalian partners may not be of the same affinity as the homologous

counterparts (cf. Haugwitz et al., 1991), a partial rescue of the aberrant phenotype was observed with respect to colony growth and precision of cytokinesis, and this rescue was less effective in cells expressing the mutant protein F59A. In Dd59A11, containing only one-tenth of the profilin level as compared to both other clones, no significant effects on colony growth and cytokinesis were observed, which indicates that the changes seen were specific for bovine profilin. A more efficient rescue than seen here had been previously described by the introduction of maize profilin into PI/II-minus (Karakesisoglou et al., 1996), but as in this case the expression levels were not determined, it is difficult to compare these two systems. However, it is conceivable that plant profilins are a better surrogate for *Dictyostelium* profilins than mammalian profilins, since the sequence homology of *Dictyostelium* profilins is higher to plant profilins than to mammalian profilins (maize profilin I and bovine profilin I to *Dictyostelium* profilin I, 45% and 24%, respectively; Schlüter et al., 1997). To ascertain whether this is indeed important, and to screen for more prominent differences in biological activity between wild-type bovine profilin I and F59A, higher expression levels in PI/II-minus might have been necessary. However, our results show that 10% of the normal profilin levels in AX2 are already sufficient for specific rescue effects and emphasize the importance of profilins for actin-based processes in colony growth, such as proliferation and locomotion.

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