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Plant and animal profilins are functionally equivalent and stabilize microfilaments in living animal cells

Martin Rothkegel[1], Oleg Mayboroda[1,2], Manfred Rohde[3], Carmen Wucherpfennig[1], Rudolf Valenta[4] and Brigitte M. Jockusch[1,*]

[1]Cell Biology, Zoological Institute, Technical University of Braunschweig, D-38092 Braunschweig, Germany
[2]Institute of Cytology, Russian Academy of Science, St Petersburg, Russia
[3]Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany
[4]Institute of General and Experimental Pathology, University of Vienna, A-1080 Vienna, Austria

*Author for correspondence

SUMMARY

We have analyzed the degree of functional similarity between birch and mammalian profilins, two members of the profilin family which show only a moderate sequence homology (22%) in living animal cells. The plant profilin, derived from birch pollen, was stably expressed in BHK-21 cells. Plant and endogenous profilin synthesis and cellular distribution were monitored by specific monoclonal antibodies. Quantitation of profilin and actin on calibrated immunoblots showed that two stable clones contained in total 1.4 and 2.0 times as much profilin as the parental cells. Using double fluorescence and confocal laser scanning microscopy, it was seen that the endogenous and the plant profilin colocalized with dynamic microfilaments, in particular with F-actin-rich foci and cortical microfilament webs of spreading cells, with dynamic microfilament bundles induced by serum deprival, and with cytochalasin D- and latrunculin-induced transient F-actin aggregates. The increase in the overall profilin concentration correlated with a significantly higher resistance of actin filaments to these drugs. Our data indicate that even profilins of highly distant evolutionary origin can functionally substitute for each other and support the hypothesis that in animal cells, profilins are engaged in regulating either the stability or the kinetic properties of actin filaments.

Key words: Plant profilin, Microfilament dynamics, Profilin overexpression

INTRODUCTION

Profilins (Carlsson et al., 1977) are considered as universal regulators of actin filament assembly. Consistent with this view is their expression, concomitant with actin, in all eukaryotic cells from protozoa to vertebrates (see Haarer and Brown, 1990; Jockusch et al., 1995; Sohn and Goldschmidt-Clermont, 1994, for references), their concentration in cellular regions with dynamic microfilaments (Buss et al., 1992; Theriot et al., 1994; Verheyen and Cooley, 1994) and their direct interaction with actin in vitro. Experiments on the latter point revealed that profilins efficiently complex G-actin (Carlsson et al., 1977; Tseng et al., 1984), cap the fast growing ends of actin filaments (Kaiser et al., 1986; Pollard and Cooper, 1984), and catalyze the adenine nucleotide exchange on actin (Goldschmidt-Clermont et al., 1991b; Mockrin and Korn, 1980; Perelroizen et al., 1995). These results suggest that the intracellular profilin concentration should be an important factor in regulating cytoplasmic actin filament assembly, although the precise role of profilin is obviously complex and still poorly understood (Pantaloni and Carlier, 1993; Theriot and Mitchison, 1993). The extent of profilin-actin interaction in the cell may in turn depend on the concentration of polyphosphoinositides like phosphatidylinositol-4,5-bisphosphate (PIP_2), which interferes with the formation of profilin-actin complexes (Goldschmidt-Clermont et al., 1991a; Lassing and Lindberg, 1985, 1988). Therefore, it has been speculated that profilins can act as molecular links between signal transduction and microfilaments (Aderem, 1992; Goldschmidt-Clermont et al., 1990, 1991a; Machesky et al., 1990), triggering the stimulus-dependent, rapid and localized assembly of microfilaments in animal cells. There is also evidence for a linkage of profilin to signalling cascades in yeast and in plants (Machesky and Pollard, 1993; Vojtek et al., 1991).

From these observations, one might expect dramatic consequences for cellular motility and transport in cells with experimentally modulated profilin concentrations. In agreement with such predictions, severe defects in budding and cell division have been observed in yeast and *Dictyostelium* mutants with disrupted profilin genes, respectively, concomitant with an abnormal organization of the actin filament system (Balasubramanian et al., 1994; Haarer et al., 1990; Haugwitz et al., 1994). In *Drosophila*, profilin mutants that lack both mRNAs generated from a single profilin gene do not survive after late embryogenesis, while selective deletion of only the germ line-specific transcript results in viable mutants that are defective

in germ cell differentiation and bristle formation. In all cases, abnormal actin organization in different cell types was seen (Cooley et al., 1992; Verheyen and Cooley, 1994). In the mouse, disruption of the profilin I gene is apparently already incompatible with life at a very early embryonic stage (W. Witke, personal communication).

Conversely, a rise in intracellular profilin concentration also has severe consequences for the afflicted cells. Microinjection of profilin disrupts the highly dynamic actin filaments in fibroblasts (Cao et al., 1992) and plant cells (Staiger et al., 1994), respectively, and CHO cells transfected with human profilin display an altered stability of actin filaments (Finkel et al., 1994). Overexpression of profilin in *Schizosaccharomyces pombe* is incompatible with the formation of a cleavage ring, but the cells can be rescued by actin overexpression, indicating that cell division in the fission yeast depends critically on the profilin:actin balance (Balasubramanian et al., 1994). Similar results were obtained for *Saccharomyces cerevesiae* (Magdolen et al., 1993). Thus, although the details of profilin-actin interactions remain elusive, all observations made are consistent with the hypothesis that profilins have a stringent control on actin filament organization in the cytoplasm.

We have asked whether this function can also be exerted when the partner molecules, actin and profilin, are from very remote members of the evolutionary pedigree. We have introduced a plant profilin from birch pollen into a mammalian cell line, BHK-21 cells, and examined the profilin localization, microfilament organization and stability in the resulting stable clones. Our results suggest that the plant profilin, although only moderately related in sequence to the endogenous profilin, can functionally substitute for the endogenous profilin. Furthermore, we observed a positive correlation of F-actin stability with the overall profilin content.

MATERIALS AND METHODS

Vectors and plasmids

A full length cDNA coding for birch profilin (Valenta et al., 1991) was inserted into the *Eco*RI-site of the eukaryotic expression vector pBEHpac18 (Artelt et al., 1988), which also encodes a puromycin resistance sequence (Vara et al., 1986). Identity and orientation of the insert in the resulting plasmid, pEXBP12, were confirmed by restriction enzyme analysis and DNA sequencing.

Cells

BHK-21 cells were obtained from the American Type Culture Collection (ATCC no. CCL10) and grown in DMEM, supplemented with 10% fetal calf serum and 10 μg/ml gentamycin (Sigma, Deisenhofen, FRG). Stable transfectants were obtained from exponentially growing cultures at 5×10^5 cells/10 cm dish by transforming the parental cells with pEXBP12 (containing the birch profilin insert) or pBEHpac18 (no insert, control), using calcium phosphate precipitation (Gorman et al., 1983), and selecting for viable clones on puromycin (2.5-5.0 μg/ml)-containing medium. Expression of the birch profilin in puromycin-resistant subclones that were obtained by limiting dilution was monitored with a birch profilin-specific antibody (see below). For treatment, fixation and immunofluorescence processing, cells were grown on glass coverslips.

To induce the formation of transient stress fibers, all cells were kept in DMEM without serum for at least two days. To analyse locomotory activity, cells were grown to confluency in 24-well dishes. Wounding assays were performed essentially as decribed (Rodríguez Fernández et al., 1992).

Subcellular fractionation and protein preparation

To obtain a crude fractionation between filamentous and unpolymerized actin, a confluent culture corresponding to 20-100 μg total protein was lysed in lysis buffer (0.01 M Hepes, 0.15 M KCl, 2 mM $MgCl_2$, 2 mM KPO_4, 5 mM EGTA, 5 mM ATP, 0.01 M β-glycerophosphate, 1% NP-40, protease inhibitor cocktail containing benzamidine, trasylol, leupeptin, pefabloc, iodoacetamide and pepstatin; Cano et al., 1992; Haugwitz et al., 1994). After centrifugation at 100,000 ***g*** for 1 hour in an airfuge (Beckman Instruments, Munich, Germany), aliquots of the soluble fraction, containing G-actin, and the sediment, containing the cytoskeletal elements, were both subjected to SDS-PAGE (see below) for quantification of G- and F-actin fractions, respectively. Skeletal muscle actin was prepared from acetone powder according to standard procedures (Spudich and Watt, 1971), with an additional gel filtration step (Buss and Jockusch, 1989). Calf thymus profilin and recombinant birch profilin were prepared as described (Lindberg et al., 1988; Giehl et al., 1994).

Gel analysis, immunoblotting and protein quantitation

To monitor the expression of the transfected birch profilin in BHK 21 derived clones and compare it with the level of endogenous proteins, cell extracts, derived from 10^7 cells obtained in lysis buffer (Finkel et al., 1994) by ultrasonication, and purified proteins (actin, calf thymus and recombinant birch profilin, respectively) were subjected to SDS-PAGE (10-15% polyacrylamide). In some cases, tricine was used as a buffer instead of glycine (Schägger and von Jagow, 1987), and either stained with Coomassie Brilliant Blue or blotted onto nitrocellulose. For the determination of G- and F-actin, cell lysates were fractionated into supernatants and a cytoskeletal fraction sedimented by centrifugation. Protein content was determined by bicinchoninic acid, using bovine serum albumin as a standard (Smith et al., 1985), and equal amounts of protein were loaded onto the gels. Protein concentration in BHK cells was assayed by determining cell volume in addition to the protein content. Antibody (see below) binding to the blotted polypeptide profiles was visualized by enhanced chemiluminescence (ECL, Amersham, Braunschweig, FRG). Quantitation of profilin and actin in total cell extracts and in subfractions was performed on ECL blots with profilin- and actin-specific antibodies (see below), using calibration curves obtained with purified proteins and scanning the blots with the QuantiScan program (Biosoft, Cambridge, UK).

The content of F-actin was monitored by phalloidin-binding, according to the method of Howard and Oresajo (1985). Cells grown to confluency in a 10 cm dish were formaldehyde-fixed, extracted with Triton X-100 and incubated with 0.2 μM TRITC-phalloidin (provided by Dr Faulstich, Heidelberg, FRG) in phosphate-buffered saline. After extensive washing, the bound phalloidin was extracted with 3 ml methanol and the rhodamine fluorescence was monitored in a spectrofluorometer (RF 6003, Shimadzu, Düsseldorf, FRG), using excitation and emission at 544 and 590 nm, respectively.

Antibodies

The following specific antibodies were used: (1) a monoclonal antibody against bovine thymus actin. This antibody, of the IgM type, recognizes all vertebrate actin isoforms on blots derived from 2-D gels (H. Hinssen and B. M. Jockusch, unpublished). (2) A monoclonal antibody against recombinant birch profilin, of the IgG type. This antibody specifically reacts with birch profilin in total birch pollen extracts (Giehl et al., 1994). (3) A poly- and a monoclonal antibody against calf thymus profilin which selectively recognize mammalian profilin on blots of one- and two-dimensional gels (Buss et al., 1992; Giehl et al., 1994). The secondary antibodies were as follows: (1) For ECL blots, horseradish peroxidase-coupled goat anti-mouse and goat anti-rabbit immunoglobulins (Sigma, Deisenhofen, FRG). (2) For

immunofluorescence, goat anti-mouse antibodies, coupled to either FITC, TRITC (Sigma) or Cy3 (Dianova, Hamburg, FRG).

Treatment with cytochalasin D and latrunculin A

BHK-21 parental cells and the various clones were incubated with two actin-specific compounds, cytochalasin D and latrunculin A. Cells grown to confluency in a culture dish or on coverslips were incubated with 0.5-2 μM cytochalasin D (Sigma) in DMEM and 0.1% dimethylsulfoxide (DMSO), for 0.5-6 hours. Controls were incubated in DMEM containing 0.1% DMSO only. The effect of latrunculin was observed in cells grown on coverslips and incubated in 0.1 μM latrunculin A (obtained from Dr I. Spector, SUNY) in DMEM, for 3 hours.

Fluorescence microscopy and photography

Cells on coverslips were fixed in 3.7% formaldehyde and extracted with 0.2% Triton X-100. As profilin is a small, soluble protein which might be easily lost when not fixed properly, we determined the amount of profilin released after fixation in the supernatant of detergent-lysed cells by immunoblotting. Less than 10% of the total protein was detected in the soluble phase, emphasizing the validity of the fixation procedure used and suggesting that the majority of profilin is indeed bound to other proteins in the cytoplasm. For double labeling profilin and F-actin, they were incubated with FITC-phalloidin (obtained from Dr Faulstich, Heidelberg), anti-profilin and the corresponding second antibodies. The specimens were examined either in a conventional microscope equipped with epifluorescence (Axiophot, Zeiss, Oberkochen, FRG), and photographed on Kodak Tri-X-Pan Film, or by confocal laser microscopy (MRC 600 imaging system, Bio-Rad, Munich, FRG). Optical sections were obtained at 0.1 μm intervals and printed on a color video printer (Mitsubishi, FRG).

RESULTS

BHK-21 clones transfected with birch profilin display an altered profilin:actin ratio

The expression of birch profilin in transfected mammalian cells was monitored with monoclonal antibodies against bovine profilin to probe for the endogenous mammalian protein, and against birch profilin, respectively. The latter antibody is restricted in its reactivity to profilins of birch and related trees (beech family) (Giehl et al., 1994; Wiedemann et al., unpublished data). A monoclonal anti-actin was used to analyse actin expression. Purified proteins were used for calibration. Fig. 1 shows the expression of profilin and actin in BHK-21 parental cells, in cells transfected with the vector pBEH-pac3 only and in the stable clones BHK-BP3 and BHK-BP4. As expected, the plant profilin is seen only in the homogenates of transfected cells (Fig. 1A), while endogenous profilin, as well as actin is recognized in all extracts (Fig. 1B,C). Table 1 shows the quantitative data obtained from immunoblots, expressed as a percentage of the total protein. The overall protein concentration of BHK cells was determined to be 0.06 mg/ml. Thus, all cell lines tested contain approximately 100 μM actin and 20 μM endogenous profilin. The transfected clones differ in the amount of plant profilin expression: BHK-BP4 contains less than half of the amount determined for BHK-BP3. In terms of the overall profilin content, BHK-BP3 contains twice and BHK-BP4 1.4 times as much profilin as the parental cells. Since neither the expression of the endogenous profilin nor actin was altered by introducing the plant gene into the mammalian cell line, the resulting transfectants show a correspondingly altered molar ratio of profilin:actin. These values remained stable for more than two years.

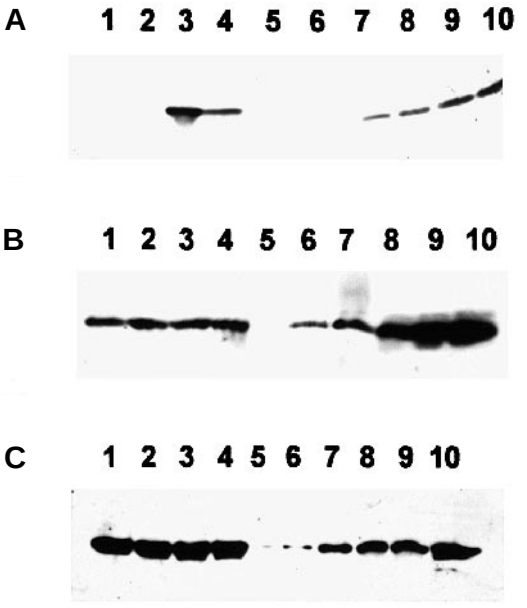

Fig. 1. ECL immunoblots with total cell lysates, a monoclonal antibody specific for birch profilin (A), a polyclonal antibody against thymus profilin which does not recognize the plant profilin (B) and a monoclonal anti-actin (C). The numbers specify the lanes of the SDS gels which received equal amounts of protein in total extracts of BHK-21 parental cells (1), BHK-21 cells transfected with the vector pBEH-pac3 (2), and two stable clones expressing the birch profilin, BHK-BP3 (3) and BHK-BP4 (4). Lanes 5-10, increasing amounts of purified standard proteins: (A) 0.01, 0.02, 0.04, 0.06, 0.08 and 0.10 μg birch profilin; (B) 0.05, 0.15, 0.25, 0.35, 0.45 and 0.55 μg thymus profilin; (C) 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 μg brain actin. Such immunoblots and calibration curves were used to quantify profilins and actin, as shown in Table 1.

Table 1. Profilin and actin content (in % of total protein) of the various BHK cell lines, as determined by ECL blots

Protein	BHK-21*	BHK-pac3	BHK-BP3	BHK-BP4
Actin	7.1	6.8	6.8	6.9
BHK profilin	0.54	0.52	0.49	0.54
Birch profilin	–	–	0.52	0.21
Molar Ratio Profilin:Actin	0.23	0.22	0.43	0.31

*BHK-21, parental cells; BHK-pac3, BHK-21 stably transfected with the vector pBEH-pac3; BHK-BP3 and BHK-BP4, two clones expressing birch profilin.

The G:F-actin ratio was determined by quantitating immunoblots of the soluble and cytoskeletal fractions of cell lysates. In some experiments, phalloidin was added to prevent disassembly of actin filaments (Haugwitz et al., 1994). The values obtained for the various cell lines were not significantly different and a clearcut correlation with the expression of the plant protein in BHK-21 cells could not be seen (Table 2). Thus, profilin overexpression did neither result in a change in total actin content nor in a shift to higher G-actin values. Interestingly, values obtained for the G:F-actin ratio were always lower than 1, demonstrating that BHK-21 cells contain a higher

Table 2. The G:F actin ratio in the different BHK cell lines

Cells	G:F-actin ratio (– phalloidin)*	G:F-actin ratio (+ phalloidin)*
BHK-21†	n.d.	0.74±0.09
BHK-pac3	0.75±0.02	0.68±0.05
BHK-BP3	0.74±0.17	0.59±0.06
BHK-BP4	0.83±0.18	0.59±0.06

*Refers to the addition of phalloidin to the lysis buffer.
†The cell lines listed are specified in Table 1.
n.d., not determined.

percentage of filamentous actin than is found for many other cell types.

Plant and endogenous profilins colocalize with dynamic microfilaments

The distribution of endogenous and plant profilin was examined by indirect fluorescence in spreading BHK-21, BHK-BP3 and BHK-BP4, respectively, and compared to the F-actin distribution (Fig. 2). Within two hours after seeding, the cells had settled down and started spreading rapidly. At that time point, the actin organization was rather variable, as seen by antibody- or phalloidin-staining. In some cells, only a dot-like pattern at the cell periphery (Fig. 2A′) or a cortical staining was visible (Fig. 2B′), whereas in others, nascent focal contacts and the beginning of stress fiber organization could be seen (Fig. 2C′). The endogenous profilin, as monitored with the antibody against bovine profilin, colocalized with the cortical microfilament seam and with actin-rich foci in spreading control and transfected cells, respectively (Fig. 2A,A′,B,B′). Thus, in BHK-21, profilin colocalizes with dynamic microfilaments, as previously described for other fibroblasts (Buss et al., 1992; Reinhard et al., 1995). In the transfectants (Fig. 2C,C′), the plant protein, as revealed by the birch profilin-specific antibody, displayed an analogous pattern.

Similar to mouse 3T3 fibroblasts, but in contrast to many other cultivated vertebrate cells, BHK-21 cells react to serum deprivation by flattening and concomitant expression of prominent microfilament bundles. Morphologically, and with respect to their complement of actin-associated proteins, these bundles resemble stress fibers in quiescent fibroblasts. However, in contrast to these, they are highly dynamic, being continuously formed at the periphery of the cell and moving towards the cell center, where they are dissolved (Giuliano and Taylor, 1990, 1994). These transient stress fibers were found associated with a high concentration of profilin, as seen in optical sections obtained by confocal microscopy, for the endogenous protein in BHK-21 control cells and for the birch protein in the BHK-BP3-transfected clone (Fig. 3).

The colocalization of both profilins with transient actin-containing structures was also seen in another situation. Treatment of BHK cells, grown for 6 hours in normal serum-containing medium supplemented with 0.5 μM cytochalasin D or with 0.1 μM of the marine macrolide latrunculin A (Spector et al., 1983), resulted in the disruption of stress fibers and the appearance of large, phalloidin-stainable aggregates. These, again, were associated with endogenous and with birch profilin in the respective cells (Fig. 4). Profilin accumulated at these F-actin bodies in peculiar, dot-like structures. The aggregates proved rather unstable and disappeared gradually when the cells were confronted with these toxins for longer periods.

Increased profilin levels correlate with an increase in actin filament stability

When the microfilament distribution of BHK-21 parental cells, BHK-21 cells containing the pBEHpac vector only and profilin-overexpressing cells (BHK-BP4, BHK-BP3) was examined in phalloidin-stained cells, no significant difference was seen. In general, the microfilament organization in the control and transfected cells was inhomogeneous, and even in cultures of well attached, fully spread cells only a minority

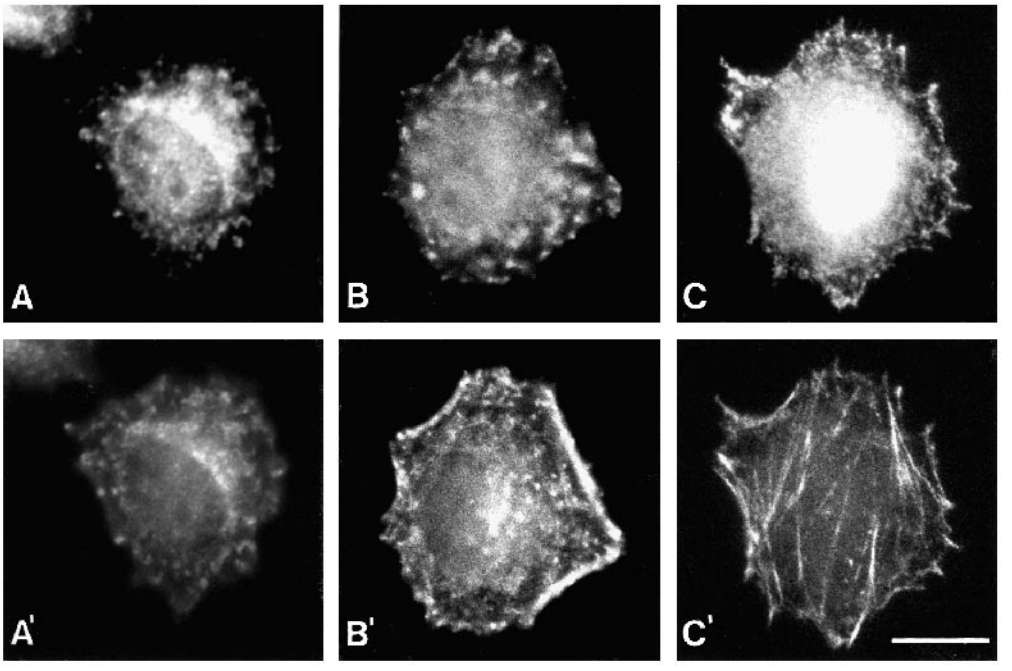

Fig. 2. Colocalization of endogenous (A,B) and birch (C) profilin with F-actin in spreading BHK cells. (A,A′) A BHK-21 parental cell, (B,B′ and C,C′) BHK-BP3 birch profilin expressing cells. (A-C) Staining with the monoclonal antibodies against thymus profilin (A,B) and against birch profilin (C). (A′-C′) corresponding F-actin distribution, as revealed by FITC phalloidin. During their rapid spreading after seeding, BHK cells show a rather inhomogeneous distribution of actin (A′-C′). Both profilins are associated with dot-like actin spots and peripheral extensions and ruffles, rather than with stress fiber-like bundles, and a large proportion is concentrated in the perinuclear area. Bar, 10 μm.

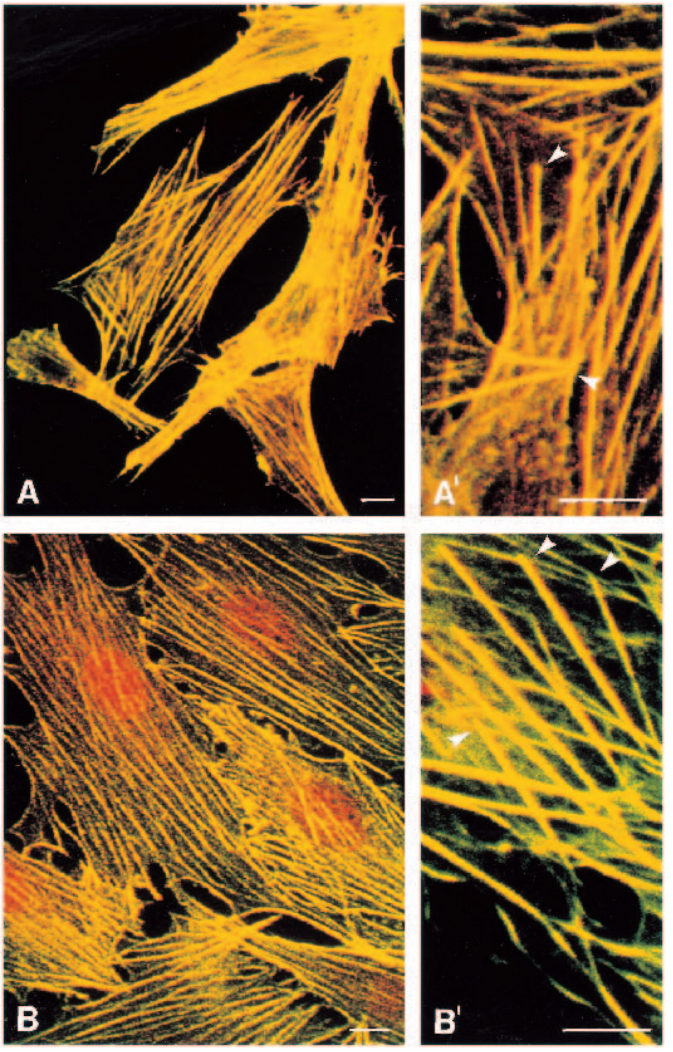

Fig. 3. Confocal laser scanning micrographs of serum-deprived BHK-BP3 cells, double-stained with antibodies for (A) endogenous profilin or (B) birch profilin (red) and actin with FITC-phalloidin (green). The yellow color demonstrates the extensive overlap of both profilins and actin in the microfilament bundles induced by serum deprival in these cells. Profilin is also concentrated in their focal contact-like terminal portions (arrowheads in the enlargements A′ and B′). Bars, 10 μm.

contained well developed stress fibers. On the average, the birch profilin-expressing cells were slightly larger and flatter, but this was not very conspicuous (the size difference was less than 10%). Cell proliferation and locomotion, as examined in a wounding assay, were indistinguishable between the parental and the transfected clones. All cell lines migrated at approximately 4 μm/hour, which is within the range normally found for fibroblasts.

However, when the stability of actin filaments against cytochalasin D and latrunculin A was tested, a significant difference was observed. By applying these drugs at lower concentrations or for shorter time periods than described above, conditions were found which in control cells did not yet result in the formation of actin aggregates, but already displayed an overall decrease in microfilaments. This was, for example, seen with 0.5 μM cytochalasin D or 0.1 μM latrunculin A for 2 hours only, or with 2 μM cytochalasin D for 30 minutes. The microfilaments of the birch profilin-expressing cells were much less affected by these conditions. This was directly seen by surveying the phalloidin fluorescence of confluent cultures at low magnification: The cultures of the transfected clones appeared much more brightly stained than the controls. This result was quantified by determining spectroscopically the amount of methanol-extractable rhodamine-phalloidin previously bound to the cytoskeleton of the fixed, permeabilized cells of the different clones. Fig. 5 shows the values obtained by treating the various lines with 0.5 μM cytochalasin D (hatched bars) or 0.1 μM latrunculin (solid bars) for 2 hours each, expressed as a percentage of untreated controls. While this treatment leads to a reduction of F-actin to 70-75% of the value determined for the untreated cells, the actin filaments in BHK-BP3 cells, having twice as much profilin as the parental strain, proved more resistant: these cells contain more than 90% of the normal F-actin value. The degree of resistance seemed to correlate with the amount of profilin expressed, as the BHK-BP4 clone, expressing an intermediate amount of profilin, displayed a drug-sensitivity in between that of the control and the BHK-BP3 cells (Fig. 5).

DISCUSSION

In this report, we have shown that a plant profilin can be stably expressed in BHK-21 cells. Both clones described here have been expressing the alien protein for more than 24 months now, without any visible adverse effect. Several lines of evidence suggest that the plant profilin may contribute to or functionally replace the endogenous profilin in the transfectants, supporting the concept of conserved ligand binding sites in these proteins whose overall sequence similarity is less than 25% (Staiger et al., 1993). First, in vitro, the birch profilin shows the same affinity to mammalian cytoplasmic actin as compared to the homologous profilin (Giehl et al., 1994). Second, CHO cells in which human profilin has been overexpressed, show similar effects on microfilament stability (Finkel et al., 1994) as described here. Thus, our studies imply that at least some microfilament proteins of phylogenetically very distant relationship may functionally substitute for each other.

Birch profilin expression did neither change the actin nor the endogenous profilin content in the transfected clones. Based on the assumption that both profilins functionally add up in the transfectants, this indicates that actin and profilin synthesis are not coupled, although a precise balance between these proteins must be important, as has been demonstrated for microfilament organization and function in yeast (Magdolen et al., 1993) and *Drosophila* (Cooley et al., 1992). Moreover, in the BHK derivatives used here, we found that the F:G actin ratio was not substantially altered by the expression of additional profilin. In contrast, in CHO cells, the expression of additional profilin at up to 8 times the parental level caused an increase in F-actin by a factor of two (Finkel et al., 1994). For BHK-21 cells, we did not obtain stable clones overexpressing profilin by more than a factor of 2, which may not be sufficient to cause an increase in F-actin. It is, however, difficult to draw more general conclusions on the importance of profilin:actin and of F:G actin ratios for cellular viability and function from the two

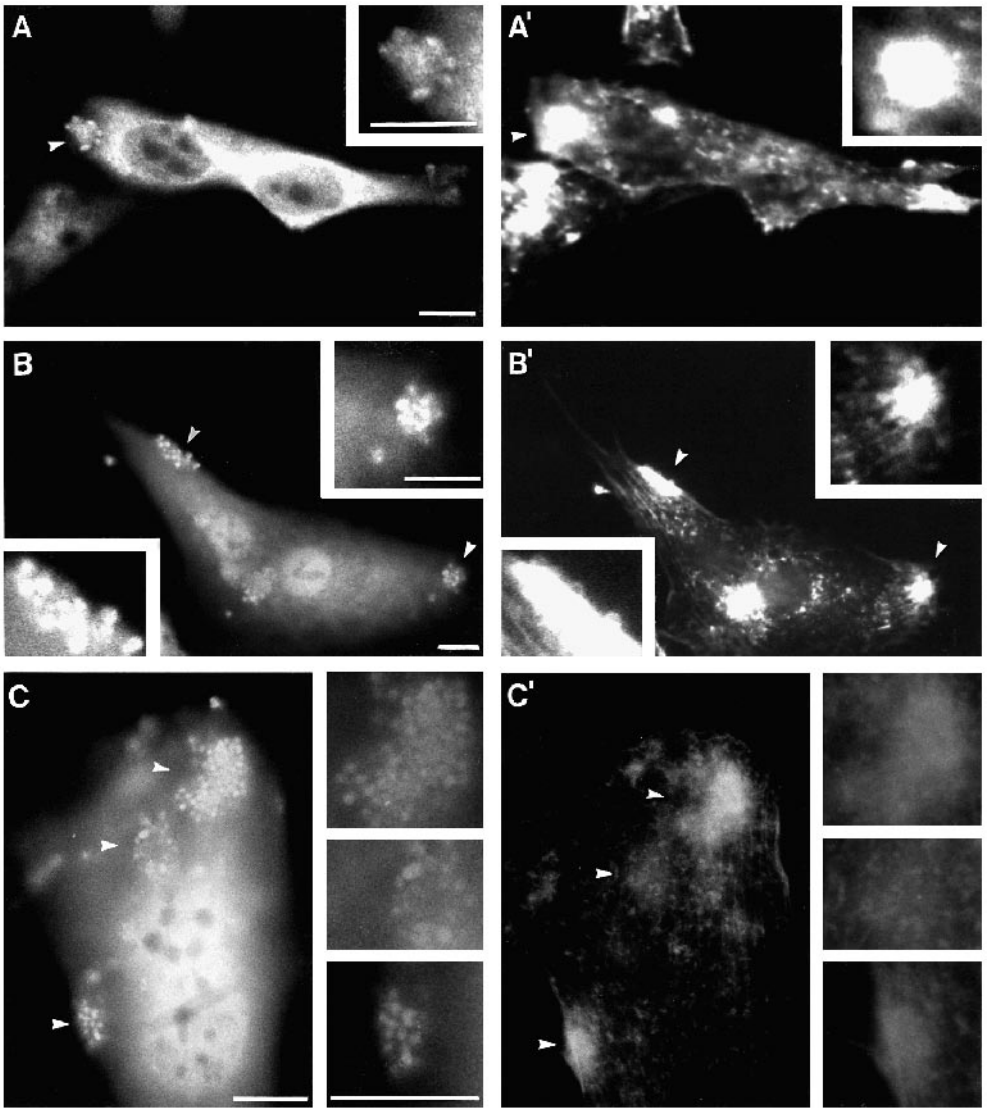

Fig. 4. Localisation of profilin and F-actin in BHK-21 (A,A′) and BHK-BP3 (B,B′ and C,C′) cells after treatment with 0.5 μM cytochalasin D for 6 hours. Profilins were revealed by the corresponding monoclonal antibodies (A-C), actin was stained by FITC-phalloidin (A′-C′). The endogenous (A,B) and the plant (C) profilin both associate with cytochalasin D-induced F-actin aggregates in BHK-21 (A) and transfected BHK-BP3 (B,C) cells, respectively, in a peculiar dot-like pattern (arrowheads). Bars, 10 μm.

examples, CHO and BHK-21, since for both cell lines no data exist on other potentially actin-recruiting proteins. In particular, the concentration of thymosin β4 (Safer et al., 1991) and other thymosins might be important in this context.

However, for both, CHO and BHK-21 cells, it was found that profilin overexpression stabilizes actin filaments. Thus, the major role of profilin in these cells does not seem to be a mere G-actin sequestering function, but rather a contribution to net actin polymerization or increase in filament half life. Our data on the close association of profilin with dynamic microfilament bundles and toxin-induced transient F-actin aggregates suggest that profilin may regulate the kinetics of actin polymers. Similar conclusions can be drawn from results obtained with insect and mammalian cells. In *Drosophila* oogenesis, as well as during the generation of microfilament tails at the bacterial walls in *Listeria*-infected vertebrate cells, profilin apparently assists in actin filament assembly (Cooley et al., 1992; Sanger et al., 1995; Theriot et al., 1994). In mammalian fibroblasts, an increase in the affinity of actin for profilin, obtained by altering the actin partner, leads to an increased incorporation of actin into the distal ends of stress fibers (Giuliano and Taylor, 1994).

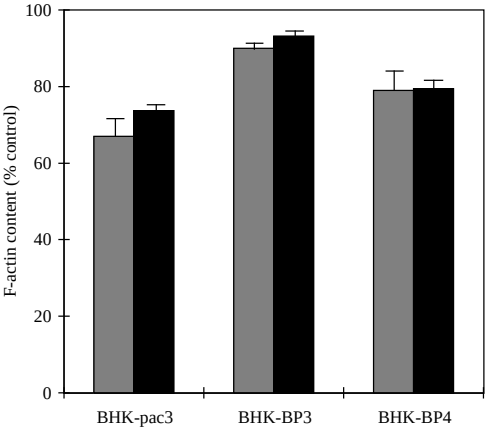

Fig. 5. F-actin content of control (BHK-pac3) and birch profilin-expressing (BHK-BP3 and BHK-BP4) cells after treatment with 0.5 μM cytochalasin D (hatched) or 0.1 μM latrunculin A (solid) for 2 hours. For the method used to quantitate F-actin, see Material and Methods. The columns represent the results of five independent experiments, with standard deviation.

Although these experiments cannot directly be compared with ours, the results indicate that profilin in vertebrate cells is primarily regulating or modulating actin filament dynamics, rather than sequestering G-actin. This is in contrast to studies on simpler eukaryote cells like yeast and *Dictyostelium*, where lack of profilins causes phenotypes which can be explained best by a G-actin-sequestering role for profilins (Haugwitz et al., 1994; Magdolen et al., 1993). More studies with different cell types, and a detailed quantitative analysis on the molar ratios of actin and profilins, on the one hand, and on true sequestering molecules like thymosin β4, on the other, will be necessary to yield a complete picture on the roles of profilins in the various organisms.

As seen in immunofluorescence, the birch profilin concentrates in areas of dynamic actin-rich foci and cortical microfilaments in spreading and ruffling transfected cells, quite similar to the endogenous BHK profilin or to rat profilin in rat fibroblasts (Buss et al., 1992; Reinhard et al., 1995). From these studies, one can of course not conclude whether profilin in these locations is directly bound to actin filaments or to other components. It is conceivable that profilin associates with other actin-binding proteins in these areas. A likely candidate for such an interaction is VASP, the vasodilator-stimulated phosphoprotein. This protein, expressed in almost all mammalian cell types, is an actin-binding protein highly concentrated in focal contacts, ruffling membranes and along stress fibers (Reinhard et al., 1992, 1995). Recently, it has been identified as the first natural ligand exploiting the poly-proline-binding domain of profilin, and mammalian VASP interacts with both mammalian and birch profilins (Reinhard et al., 1995). VASP is a substrate for cAMP- and cGMP-dependent kinases and is thus part of an elaborate signal transduction pathway (Butt et al., 1994). At present, it is not clear in which way VASP might regulate profilin's function. However, in contrast to the PIP_2-binding of profilin, VASP-binding is compatible with profilin's actin-binding, since engagement of the poly-proline-binding domain of profilin does not interfere with actin-binding (Kaiser et al., 1989; Lassing and Lindberg, 1988). Thus, profilin's role in actin filament formation and/or stability may be directly modulated by VASP-dependent signalling pathways. This is in contrast to the shuttle mechanism assumed for profilin-PIP_2 and profilin-actin binding, suggesting that two different signaling routes may be involved in the regulation of microfilament assembly and organization.

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