

Interaction of plant profilin with mammalian actin

Klaudia GIEHL^{1,2,3}, Rudolf VALENTA³, Martin ROTHKEGEL^{1,2}, Melanie RONSIEK¹, Hans-Georg MANNHERZ⁴ and Brigitte M. JOCKUSCH^{1,2}

¹ Cell Biology, University of Bielefeld, Germany

² Cell Biology, Zoological Institute, Technical University of Braunschweig, Germany

³ Institute of General and Experimental Pathology, University of Vienna, Austria

⁴ Institute of Cytobiology and Cytopathology, University of Marburg, Germany

(Received June 7/September 2, 1994) – EJB 94 0819/3

The mode of interaction of birch and bovine profilins with actin was compared using a number of techniques. Birch profilin was purified from pollen or as a recombinant protein from *Escherichia coli*, using poly(L-proline) affinity chromatography and a monoclonal antibody for the identification of the isolated product. On two-dimensional gels, the genuine and recombinant proteins were identical in molecular mass and isoelectric point and revealed that birch profilin, in contrast to the basic profilins found in mammals, is an acidic protein, analogous to maize profilins. Bovine profilin was obtained from calf thymus.

In viscometric assays, the birch protein was seen to modulate actin filament formation analogous to animal profilin. Birch profilin increased the critical concentration required for muscle and brain actin polymerization in a concentration-dependent manner, supporting the notion of the formation of a heterologous complex between the plant protein and animal actin. The effect was Mg^{2+} -sensitive, as had been described for homologous complexes. The dissociation constants obtained for the plant/vertebrate and the vertebrate/vertebrate system were both in the micromolar range. The affinity of birch profilin for muscle actin was slightly lower than that for nonmuscle (brain) actin. A binary complex of birch profilin and skeletal muscle actin could be isolated by gel chromatography.

Cross-linking experiments with actin, birch profilin, the G-actin binding peptide thymosin $\beta 4$ and gelsolin segment 1, the N-terminal fragment of an actin capping protein, showed that profilin competed with thymosin $\beta 4$, but had no effect on segment 1 binding to actin.

These data indicate that the actin-binding domains in plant and animal profilins are functionally highly conserved, although the overall sequence similarity is less than 25%.

Profilins are small (14–17 kDa), ubiquitous proteins probably involved in the regulation of actin dynamics (cf. Haarer and Brown, 1990; Machesky and Pollard, 1993; Theriot and Mitchison, 1993; Pantaloni and Carlier, 1993). Originally, profilin was discovered in calf spleen extracts, as a 1:1 complex with G-actin (Carlsson et al., 1977). More recently, profilin homologues have been found in all eukaryotic cells examined, ranging from man, animals and plants to protozoa, and even as a constituent of the *Vaccinia* virus (Blasco et al., 1991). In multicellular organisms, profilin concentration and isoform expression can vary considerably between different tissues (Buß and Jockusch, 1989; Staiger et al., 1993). In addition to capturing the attention of cell biologists as potential regulators of actin organisation (see below), they have recently also become of interest to biomedical science, since plant profilins are major allergens in man (Valenta et al., 1991, 1993). Sequence alignments revealed only a moderate overall similarity (22–40%) between different

profilins, with very few invariant amino acids (Pollard and Rimm, 1991; Rozycki et al., 1994). However, all profilins seem to share a high degree of structural and functional similarity. NMR (Vinson et al. 1993; Metzler et al., 1993, 1994; Archer, 1994) and X-ray (Schutt et al., 1993) analyses of protozoan, human and bovine profilins, respectively, have revealed a highly conserved structure. The sequences are folded into a compact molecule, containing a core of five anti-parallel β -pleated sheets which are flanked by amino- and carboxy-terminal α -helices on one side, and two β -pleated sheets with one (*Acanthamoeba*) or two (bovine and human) α -helices on the other side.

Regarding their function, all profilins known interact with actin, all eukaryotic profilins bind to poly(L-proline) (Tanaka and Shibata, 1985) and most to polyphosphoinositol lipids, in particular phosphatidylinositol 4,5-bisphosphate (PtdIns P_2) (Lassing and Lindberg, 1988). From comparison with other actin-binding proteins (Ampe and Vandekerckhove, 1987), chemical cross-linking experiments (Vandekerckhove et al., 1989) and X-ray crystallographic studies on the profilactin complex (Schutt et al., 1993), it became evident that the N- and C-terminal α -helices participate in the actin-binding domain. The poly(L-proline) binding is confined to a hydrophobic surface-exposed patch distinct from this domain, involving six highly conserved aromatic resi-

Correspondence to B. M. Jockusch, Cell Biology, Zoological Institute, Biocenter, TU Braunschweig, D-38092 Braunschweig, Germany

Fax: +49 531 391 8203.

Abbreviations. EGF, epidermal growth factor; PtdIns P_2 , phosphatidylinositol 4,5-bisphosphate; PhMeSO $_2$ F, phenylmethyl-sulfonyl fluoride.

dues (Schutt et al., 1993; Björkegren et al., 1993; Archer et al., 1994; Metzler et al., 1994). The binding domain for PtdInsP₂ is less well characterized. In *Acanthamoeba* profilin I, it has been ascribed to a cluster of basic residues, adjacent to the actin binding site (Vinson et al., 1993). The resulting model of the topographic relation of three ligand binding domains at the surface of the molecule is consistent with the observation that poly(L-proline) binding is compatible with actin binding (Tanaka and Shibata, 1985), while the interactions with PtdInsP₂ and actin are mutually exclusive (Lassing and Lindberg, 1988; Goldschmidt-Clermont et al., 1991b).

The biological significance of poly(L-proline) binding is as yet unknown. Actin and PtdInsP₂ binding, on the other hand, probably enable profilins to mediate between microfilament organisation and signal transduction. Their interaction with actin is obviously complex: profilins have been described to sequester G-actin, thus shifting the balance between monomeric and filamentous actin (Carlsson et al., 1977). Additionally, they have been reported to catalyze the ADP/ATP exchange on G-actin (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991b), to bind to the rapidly growing end of actin filaments (Pollard and Cooper, 1984) and even to 'desequester' actin, i.e. to release polymerization-competent ATP-G-actin from a cellular pool (Pantaloni and Carlier, 1993; Theriot and Mitchison, 1993). These reports emphasize that the intracellular profilin concentration must be an important factor in microfilament dynamics. Conversely, signal transduction-mediated fluctuations in the PtdInsP₂ concentration may regulate the concentration of free profilin in cells. Support for this view comes from data showing that EGF-dependent activation of phospholipase C_γ1 leads to dissociation of a profilin/PtdInsP₂ complex (Goldschmidt-Clermont et al., 1991a). Obviously, a molecule acting at the crossroads between signal transduction and actin filament polymerization should be localized at the plasma membrane, and cortical enrichment of profilin has indeed been described for highly dynamic cells, such as locomoting and spreading fibroblasts (Buß et al., 1992), leukocytes and platelets (Hartwig et al., 1989).

Many details, however, on the precise role of profilins in cells are still elusive (cf. Theriot and Mitchison, 1993) and it seems imperative to learn more about profilin/ligand interactions, *in vitro* as well as in cells. For plant profilins, which have been identified more than a decade later than protozoan and animal profilins, this is even more evident.

In this study, we have analyzed the functional homology of the actin-binding properties between birch and bovine profilins. We found that the plant profilin, as purified from birch pollen or as a recombinant protein, interacts with mammalian actin. It interferes with actin polymerization in a Mg²⁺-sensitive manner and competes with several mammalian actin-binding proteins for the same binding site. Thus, it behaves analogously to the mammalian profilin. These data emphasize the close functional relationship between profilins of only moderate sequence similarity.

MATERIALS AND METHODS

Tissues

Bovine and porcine tissues were obtained from freshly killed animals at the slaughterhouse. Calf thymus was frozen at -20°C, porcine brain was only used as fresh material, cooled on ice. Rabbit skeletal muscles were obtained from

freshly killed rabbits. Acetone powder was prepared from leg and back muscles according to standard procedures.

Protein preparation

Recombinant birch profilin

The birch protein was expressed in *Escherichia coli* BL21 (DE3) (Studier et al., 1990) using a T7 RNA polymerase system. The bacteria were transformed with the plasmid pMW175 Prof (gift from M. Susani, Salzburg), containing the cDNA of birch profilin (Valenta et al., 1991) and grown in Luria broth with 0.2% glucose and 150 µg/ml ampicillin. Expression of birch profilin was induced with 0.5 mM isopropyl β-thiogalactopyranoside at A₆₀₀ = 0.5 (≈2 × 10⁸ cells/ml) and allowed to continue for at least 6 h at 37°C. The bacteria were harvested from a 3-l culture by centrifugation, homogenized using an ultraturrax and lysed in 100 ml PHEM-TX lysis buffer (Schliwa and Blerkon, 1981) with 20 mg lysozyme, 50 µg DNase I (grade II) at room temperature for 40 min. The supernatant of a high-speed centrifugation step (100000 × g, 1 h) was applied to a poly(L-proline)-Sephacrose column (2 × 10 cm) (Tanaka and Shibata, 1985) equilibrated in washing buffer (20 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.5 mM ATP). The column was washed with 5 vol. washing buffer and washing buffer including 2 M urea. Bound profilin was eluted with 5 vol. 6 M urea in washing buffer. Fractions of 4 ml were collected and analysed on Tricine/SDS/polyacrylamide gels (see below). Profilin-containing fractions were pooled and dialysed extensively against 10 mM imidazol pH 7.2, 0.1 mM CaCl₂, 0.1 mM ATP, 1 mM dithioerythritol. The protein was stored at 4°C.

Birch pollen profilin

Birch (*Betula verrucosa*) pollen (8 g), obtained from Allergon AB (Engelholm, Sweden), was homogenized in the same lysis buffer and under the same conditions as described for the recombinant protein. Likewise, purification and identification were as described above.

Calf thymus profilin

Calf thymus (300 g) was homogenized in 300 ml extraction buffer containing 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 20 mM MgCl₂, 5 mM EGTA, 1 mM dithioerythritol, 1 mM phenylmethylsulfonyl fluoride (PhMeSO₂F), 1 ml/l aprotinin (Trasylol, Bayer). The crude extract was cleared by two centrifugation steps (60 min at 13000 × g and 90 min at 100000 × g) and the supernatant was applied to a poly(L-proline) affinity column (2 × 10 cm) equilibrated in washing buffer. All steps were performed on ice or at 4°C. Washing and elution steps were performed as described for birch profilin. The 6 M urea fractions containing profilin were dialysed against 25 mM Tris/HCl pH 7.6, 0.5 mM dithioerythritol, 0.1 mM ATP, 2.1 M urea (buffer A) and subsequently applied to a Mono-Q anion-exchange column (FPLC system, Pharmacia) equilibrated with the same buffer. The purified bovine profilin was recovered from the flow-through fractions and dialysed against the same buffer as birch profilin. The column was washed with buffer A and regenerated with a gradient of 0–1 M KCl in buffer A.

Actin

Actin was prepared from rabbit skeletal muscle as described (Spudich and Watt, 1971), with an additional gel

filtration step on a column (2.6×100 cm) of Sephadex G-150 (Pharmacia). Cytoplasmic actin was obtained from fresh, chilled porcine brain as described (Gordon et al., 1976; Weir and Frederiksen, 1982), with some modifications: 500 g porcine brain was homogenized in 500 ml extraction buffer with 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.2 mM MgCl₂, 0.2 mM ATP, 0.2 mM dithioerythritol, 0.1 mM PhMeSO₂F, 0.1% protease-inhibitor mix, and extracted for 30 min at 4°C. The crude extract was cleared by sedimentation at 13000×g for 1 h and at 120000×g, 4°C, for 3 h. The supernatant was dialysed overnight against buffer B (10 mM imidazole, pH 7.5, 0.1 M KCl, 0.1 mM CaCl₂, 0.5 mM ATP, 0.2 mM dithioerythritol, 0.02% NaN₃) and applied to a Servacel DE52-column (2.6×20 cm, Serva) equilibrated with buffer B. The column was washed with buffer B and actin was eluted with a linear gradient of 0.1–0.5 M KCl in buffer B. Actin-containing fractions were analysed by SDS/PAGE and pooled. Actin was polymerized by the addition of MgCl₂ to 5 mM final concentration for either 1 h at room temperature, or overnight at 4°C. Filamentous actin was sedimented by centrifugation at 120000×g for 3 h and homogenized in 10–20 ml 5×buffer G (5 mM Tris/HCl pH 7.2, 0.2 mM CaCl₂, 1 mM ATP, 2 mM dithioerythritol, 2 mM NaN₃). After extensive dialysis, actin aggregates were removed by sedimentation and the G-actin-containing supernatant was applied to a Sephadex G-150 gel filtration column, equilibrated in 2×buffer G. The column was washed with 2×buffer G and fractions were analysed by SDS/PAGE.

Additional proteins

Equine thymosin β 4 (purified as described in Hannappel et al., 1989) and the recombinant segment S1 of human cytoplasmic gelsolin (Way et al., 1990) were obtained from Drs E. Hannappel (Erlangen) and A. Weeds (Cambridge), respectively.

Protein quantification

Protein concentrations were either determined using the bicinchoninic acid assay (Sigma) with bovine serum albumin as a standard (Smith et al., 1985), or spectrophotometrically. Profilin concentration was measured at 280 nm using an absorption coefficient of $1.2 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Larsson and Lindberg, 1988); actin concentration was determined at 290 nm with an absorption coefficient of $0.63 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Houk and Ue, 1974).

Antibodies

A polyclonal antibody against calf thymus profilin was raised in rabbits. The serum, diluted 1:250, reacted specifically with profilin on two-dimensional gels prepared with total soluble calf thymus proteins (Buß et al., 1992). A monoclonal antibody against birch profilin was prepared according to standard procedures, using Balb/c mice and the Ag8 myeloma line. The antibody obtained was classified as IgG subclass 2a and used as hybridoma tissue culture supernatant, diluted 1:2–1:5. Its specificity and reactivity was tested by using various crude extracts from animals and plants, as well as purified profilins, in immunoblots (see below). The epitope was mapped by using synthetic oligopeptides (unpublished). The polyclonal antibody against skeletal muscle actin has been described (Polzar et al., 1989).

Gel electrophoresis and immunoblotting

Tricine/SDS/PAGE was performed as described (Schägger and Jagow, 1987) with an acrylamide concentration of 10% for actin and 13% for profilin preparations. Two-dimensional gel electrophoresis of birch profilin was performed according to O'Farrell (1975) with slight modification, using carbamoylated anhydride (Pharmacia) as a standard. Gels were stained with Coomassie brilliant blue. For immunoblotting, crude tissue extracts and purified proteins were separated on either conventional 10% SDS/polyacrylamide gels or on Tricine/SDS gels, blotted onto nitrocellulose (Schleicher & Schüll) and incubated with the first antibody. Second antibodies (either goat anti-rabbit or rabbit anti-mouse immunoglobulin, diluted 1:4000, Sigma) labeled with horseradish peroxidase were used for labeling, and monitored either by 4-chloro-1-naphthol/H₂O₂ or diaminobenzidine/H₂O₂.

Polymerization assay

Actin polymerization was measured by high shear viscometry (Cooper and Pollard, 1982), using Ostwald viscometers size 150 (Cannon instruments, State College, Pennsylvania), with a buffer flow time of about 30 s. For the time course of actin polymerization and the influence of profilin, actin was incubated in 10 mM imidazole pH 7.0, 1 mM ATP, in the absence or presence of profilin in different molar ratios. After 30 min, polymerization was started by addition of KCl (50 mM final concentration) and the change of viscosity was monitored for 4 h. After addition of 2 mM MgCl₂, viscosity was recorded for an additional hour. The dissociation constants (K_d) of the different complexes were determined as described (Tobacman and Korn, 1982; Tseng and Pollard, 1982; Larsson and Lindberg, 1988; Haugwitz et al., 1991). Skeletal muscle actin (0.5–12 μM) was incubated without or with 5–10 μM calf or birch profilin in polymerization buffer.

Isolation of the profilactin complex

Complex formation of actin with birch profilin was also analyzed by gel chromatography. Equimolar concentrations (0.15 mM) of skeletal muscle actin and recombinant birch profilin were incubated in 5 mM Hepes pH 7.5 containing 0.1 mM CaCl₂, 0.5 mM NaN₃ and 0.1 mM ATP for at least 1 h on ice. Thereafter, the mixture was applied to a column (45×1.5 cm) of sephacryl S 300 (Pharmacia) and eluted with the same buffer. The peak fractions were pooled, concentrated by pressure filtration and analysed by SDS/PAGE.

Competition experiments

G-actin from skeletal muscle was preincubated for 30 min with either thymosin 4 or gelsolin segment 1 at room temperature in 5 mM Hepes pH 7.5, 0.1 mM CaCl₂, 0.5 mM NaN₃ and 0.1 mM ATP. Thereafter birch profilin, that had been dialysed overnight against the Hepes buffer, was added at various concentrations and further incubated for 30 min at room temperature. The crosslinking reaction was initiated by the addition of 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide (stock: 0.1 M in 0.5 M Mes pH 8.0) to a final concentration of 1 mM. The reaction was stopped after various time intervals (ranging over 0.5–24 h) by addition of boiling

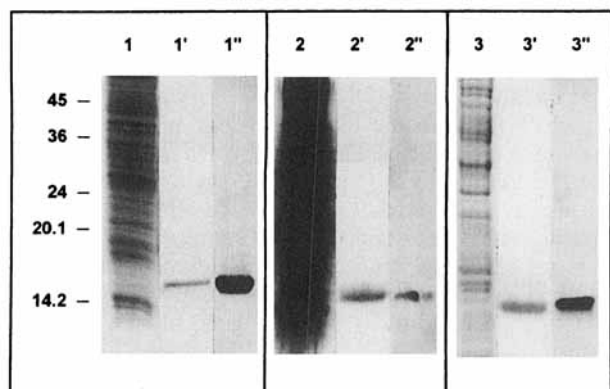


Fig. 1. Purification and identification of profilins. Lanes 1, 2 and 3, Coomassie-stained gel profiles of total soluble proteins from bovine thymus (1), birch pollen (2) and *E. coli* (3) transformed with the birch profilin cDNA; lanes 1', 2' and 3', the corresponding profilins after affinity purification on poly(L-proline)-Sepharose; lanes 1'', 2'', 3'', immunoblots obtained with the polyclonal antibody against thymus profilin (1'') and the monoclonal antibody against recombinant birch profilin (2'', 3''). Apparent molecular mass values for marker proteins (in kDa) are given on the left.

SDS sample buffer. Subsequently, the samples were analysed by SDS/PAGE and immunoblotting.

RESULTS

Biochemical and immunochemical characterization of purified profilins

As shown in Fig. 1, isolation of profilins by affinity chromatography on poly(L-proline) yield homogeneous protein preparations from extracts of calf thymus (Fig. 1, 1 and 1'), birch pollen (Fig. 1, 2 and 2') and transformed bacteria (Fig. 1, 3 and 3'). The identity of the purified proteins could be verified in immunoblots, using the corresponding antibodies (Fig. 1, 1'', 2'', 3''). In this context, the availability of a monoclonal antibody against birch profilin proved very useful. This antibody specifically recognized birch profilin but not timothy grass (Valenta et al., 1994), tobacco or mammalian profilins. Its epitope was localized between amino acid residues 40–49, within a region highly variable in different profilins (Pollard and Rimm, 1991; Wiedemann et al., unpublished).

The birch protein was further characterized on two-dimensional SDS gels. Fig. 2 shows that the major fraction in the protein preparation isolated from pollen extracts was identical with the recombinant protein in both molecular mass and isoelectric point. In addition, the genuine product showed a minor, smaller and more acidic component. This might either correspond to a second isoform of birch profilin, or to a degradation product of the major component. As the proportion between the two polypeptides varied with the preparation and storage time, the second possibility seems more likely.

These results show that the genuine and recombinant forms do not differ in charge and that the plant protein is rather acidic. In this respect, it is similar to some profilins of protozoa and slime moulds, and also to the three isoforms isolated from maize, but is in contrast to the highly basic calf thymus protein (Table 1).

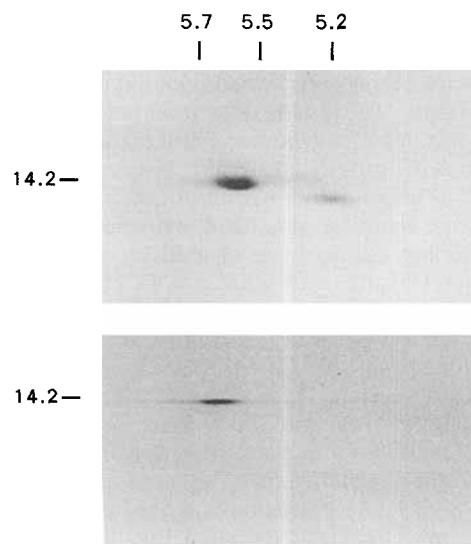


Fig. 2. Two-dimensional gel electrophoresis of native (top) and recombinant birch profilin (bottom). pH marker positions for isoelectric focussing are given on the top, apparent molecular mass values (in kDa) are given on the left.

Table 1. Isoelectric points of various profilins.

Profilin	pI	Reference
<i>A. castellanii</i> profilin IA/IB	5.5	Ampe et al., 1985
<i>A. castellanii</i> profilin II	9.2	Ampe et al., 1985
<i>D. discoideum</i> profilin I	6.6	Haugwitz et al., 1991
<i>D. discoideum</i> profilin II	7.3	Haugwitz et al., 1991
<i>P. polycephalum</i>	5.4	Ozaki et al., 1983
Calf spleen	9.1	Malm et al., 1983
Human platelet	7.1	Lind et al., 1987
Maize pollen (3 isoforms)	≈5.8	Staiger et al., 1993
Birch pollen	5.6	this study

Interaction of birch profilin with mammalian actins

Binding of the birch profilin to muscle and nonmuscle actins was monitored in viscometric assays, measuring the high shear viscosity of polymerizing actin solutions, and using calf thymus profilin as a reference (Fig. 3). As expected (cf. Buß and Jockusch, 1989), the bovine protein decreased the high shear viscosity of polymerizing brain actin in a concentration-dependent manner, resulting in a complete inhibition of viscosity increase at a molar ratio of 2:1 (profilin/actin). This effect of profilin has originally been ascribed to its G-actin sequestering activity (Carlsson et al., 1977). The profilin-actin (profilactin) complex is sensitive to Mg^{2+} , as can also be demonstrated by viscometry: after the addition of $MgCl_2$ to a final concentration of 2 mM, the viscosity reaches the level of the actin control without profilin, indicating the dissociation of profilactin complexes (Fig. 3A).

These data were compared with the influence of recombinant birch protein on the polymerization of brain actin. As seen in Fig. 3B, the birch protein also modulated the viscosity increase of polymerizing actin, indicating that a heterologous complex of the plant protein with the mammalian actin was formed. The effect was somewhat less drastic: the same molar ratio of both partners that led to a total inhibition of

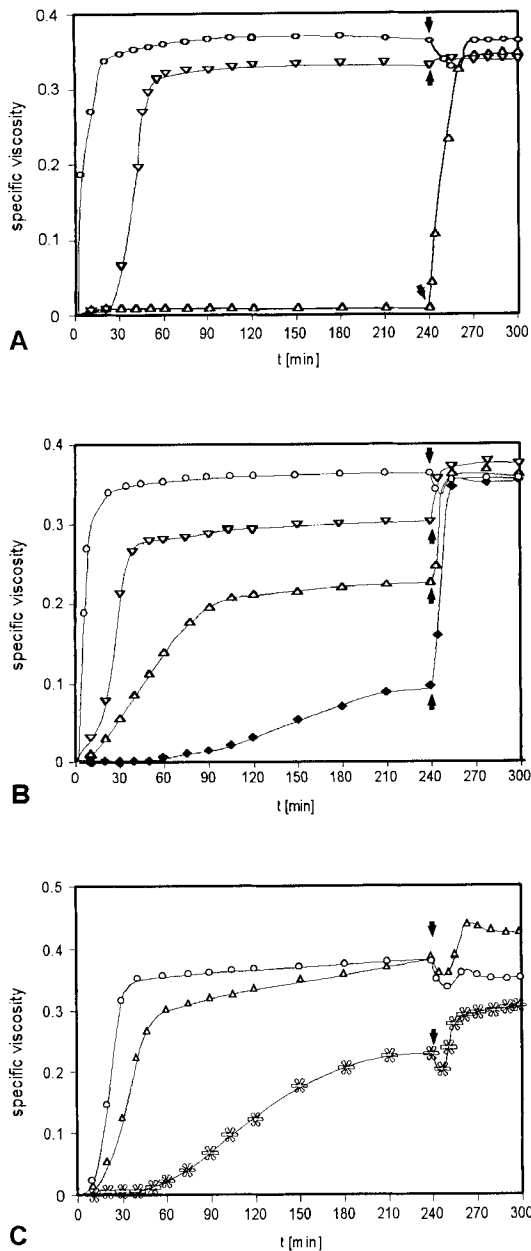


Fig. 3. The effect of profilin on the polymerization of mammalian actin, as measured by Ostwald-type viscometry. Brain (A, B) or skeletal muscle (C) actin, 11 μM each, was either incubated alone ($\circ$, control), or with various concentrations of thymus (A) or recombinant birch (B, C) profilin, in the following molar ratios (profilin to actin): 1:1 (∇), 2:1 ($\triangle$), 3:1 ($\diamond$) and 4:1 ($\circ$). Polymerization was started by the addition of KCl (final concentration 50 mM). ($\downarrow$) Addition of MgCl_2 , final concentration 2 mM.

viscosity increase in case of the bovine protein (profilin/actin = 2:1) delayed the polymerization process, but did not block it completely (cf. Fig. 3A with 3B). At a molar ratio of 3:1 (profilin/actin), the effect was more pronounced, but a complete inhibition was again not seen. Similar results were obtained when brain actin was replaced by skeletal muscle actin. In this case, the interaction was weaker as compared with the brain protein (cf. Fig. 3B with 3C, where profilin/actin ratios of up to 4:1 were used). This indicates that the plant profilin, quite similar to thymus profilin (Buß and Jockusch, 1989, and data not shown), forms complexes with

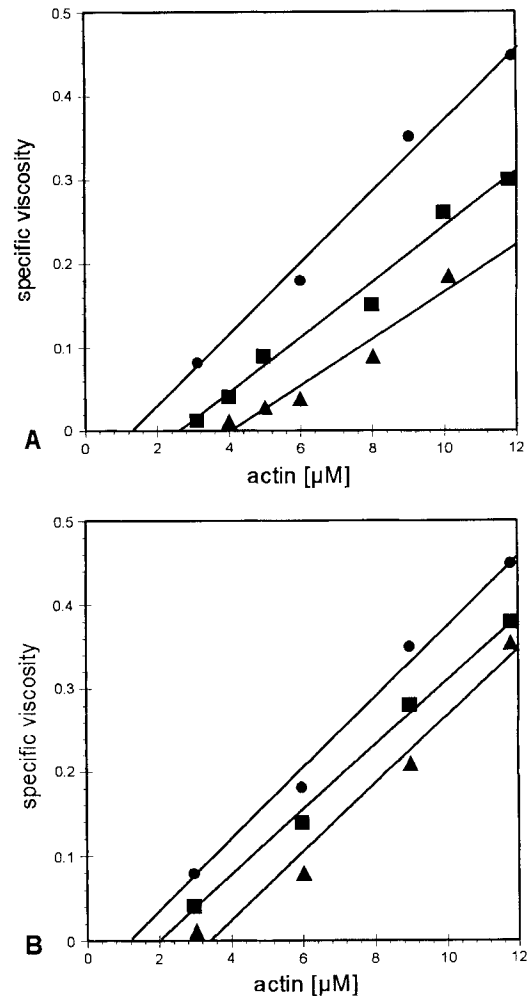


Fig. 4. The effect of profilin on the steady-state viscosity of skeletal muscle F-actin. Skeletal muscle actin (0.5–12 μM) was incubated either alone ($\bullet$, control), or with 5 μM ($\blacksquare$) or 10 μM ($\blacktriangle$) thymus (A) and recombinant (B) birch profilin, respectively. Steady-state viscosity was measured 4 h after KCl-induced polymerization. The critical actin concentration required for polymerization can be deduced from the regression curves.

the β/γ nonmuscle (and possibly α smooth muscle actin isoforms) of brain preparations that are more stable than those obtained with α skeletal muscle actin.

The affinity of the birch protein for mammalian actin was determined by monitoring the profilin influence on the steady state of F-actin viscosity. Fig. 4 shows the values obtained for skeletal muscle actin and bovine profilin (Fig. 4A) and the recombinant birch protein (Fig. 4B), respectively. The plot fitted for linear regression shows the critical concentration of actin required for polymerization as a function of various profilin:actin ratios. From these data, the K_d value for the birch profilin/muscle actin complex was determined to 5 μM , and for the calf thymus profilin/muscle actin complex to 3 μM . As seen in Table 2, these values are within the range obtained for most protozoan and animal profilins with the homologous actin, or in heterologous complexes with muscle actin. Thus, despite the only moderate degree of sequence similarity between plant and animal profilins, their interaction with α actin, as measured in this assay, is of comparable affinity.

Table 2. Dissociation constants of various profilin/actin complexes.

Profilin	Actin	K_d	Reference
		μM	
<i>A. castellanii</i>	<i>A. castellanii</i>	1–10	Tseng & Pollard, 1982
<i>A. castellanii</i>	rabbit skeletal muscle	50–80	Mockrin & Korn, 1980
<i>D. discoideum</i> profilin I	<i>D. discoideum</i>	5.1	Haugwitz et al., 1991
<i>D. discoideum</i> profilin II	<i>D. discoideum</i>	1.8	Haugwitz et al., 1991
Rat spleen (recomb.)	rabbit skeletal muscle	1–3	Babcock & Rubenstein, 1989
Rabbit macrophage	rabbit skeletal muscle	3	DiNubile & Southwick, 1985
Porcine brain	porcine brain	0.5	Nishida, 1985
Porcine brain	rabbit skeletal muscle	1.1–1.5	Nishida, 1985
Calf spleen	calf spleen	0.4–0.01	Larsson & Lindberg, 1988
Calf spleen	rabbit skeletal muscle	1	Larsson & Lindberg, 1988
Sheep spleen	rabbit skeletal muscle	1–5	Carlier et al., 1993
Human platelet	human platelet	3.7–4.5	Lind et al., 1987
Human platelet	human platelet	1–5	Goldschmidt-Clermont et al., 1991 a
Human platelet	rabbit skeletal muscle	1–5	Goldschmidt-Clermont et al., 1991 a
Birch (recomb.)		5	this study

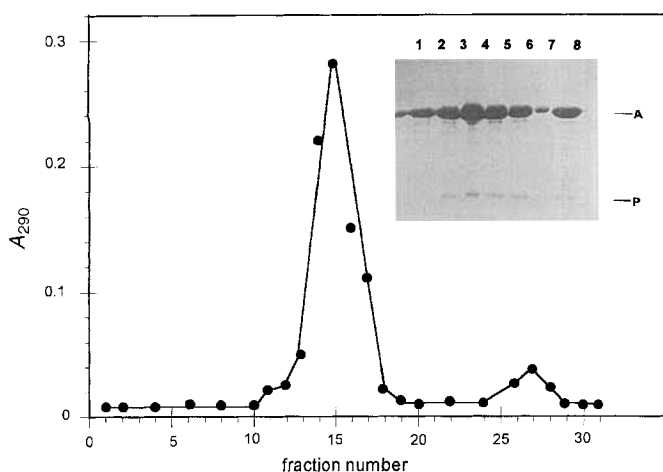


Fig. 5. Isolation of the binary complex between birch profilin and skeletal muscle actin. 7.9 mg actin in 5 mM Hepes pH 7.5, 0.1 mM CaCl_2 , 0.5 mM NaN_3 and 0.1 mM ATP was mixed with 2.46 mg profilin, incubated for 2 h on ice and subsequently loaded onto a Sephacryl S 300 column (dimensions 45×1.5 cm) equilibrated with the Hepes buffer. The column was eluted with the same buffer and fractions of 3.4 ml were collected. The graph shows the elution profile as obtained from gel chromatography, and the corresponding polypeptide patterns on SDS/PAGE. Lanes 1–7, aliquots from fractions 12–18; lane 8, aliquot after concentration by pressure filtration.

The interaction of birch profilin with mammalian actin could be further substantiated by coelution after gel filtration. Using this method, a binary heterologous profilactin complex could be isolated. Fig. 5 shows that the affinity between these two proteins is sufficient to withstand gel filtration and pressure concentration.

Competition experiments with other actin-binding proteins

The interaction of recombinant birch profilin with actin was also analysed in the presence of several mammalian actin binding proteins. To obtain information on a possible competition for the same binding site on actin, skeletal muscle actin and the various ligand proteins were incubated with

increasing concentrations of profilin. In all cases, actin was in molar excess over the ligand proteins. After subjecting the mixtures to the bivalent crosslinker 1-ethyl-3[3-(dimethyl-amino)propyl]carbodiimide, the products were analyzed by SDS/PAGE. Fig. 6A shows the stained gel pattern obtained for actin, the small G-actin-binding polypeptide thymosin $\beta 4$ and profilin (Fig. 6A, a) and the corresponding immunoblots with anti-profilin (Fig. 6A, b) and anti-actin (Fig. 6A, c). With increasing profilin concentrations, bands of crosslinked profilactin appeared. Although they were faint on the stained gels, they could be clearly identified by the corresponding immunoblots (cf. Fig. 6A, a, PA with b). A concomitant reduction in the thymosin $\beta 4$ /actin complex is seen, but is more obvious in Fig. 6B, lanes 8–11. No difference in the relative ratios of the crosslinked to non-crosslinked material was observed up to 24 h during the crosslinking reaction. On the Coomassie-stained gels (Fig. 6A, a and 6B) very little crosslinked actin-profilin is visible. However, after Western blotting, an increase of crosslinked actin-profilin was detectable that parallels the decrease in crosslinked actin-thymosin $\beta 4$ (Fig. 6A, b). These data suggest that birch profilin competes with thymosin $\beta 4$ for the same binding site on actin. In contrast, in analogous experiments, profilin was unable to reduce the amount of a complex formed between actin and the N-terminal domain of gelsolin, segment 1 (Fig. 6B, lanes 2–5).

DISCUSSION

In this study, we have shown that birch profilin is capable of interacting with mammalian actin. A comparison of the recombinant profilin with the native one on two-dimensional gels showed that the protein coded for by our cDNA probe was the major, if not the only, isoform expressed in birch pollen and that there is no major post-translational modification of the plant protein. Thus, the results obtained in this study with the recombinant protein can be considered as valid also for pollen profilin.

To characterize the affinity of the birch profilin for mammalian actin, we monitored its influence on the actin polymerization and the steady-state viscosity of F-actin, using bovine thymus profilin for a reference. The determination of K_d values by this method can actually be criticized, since a

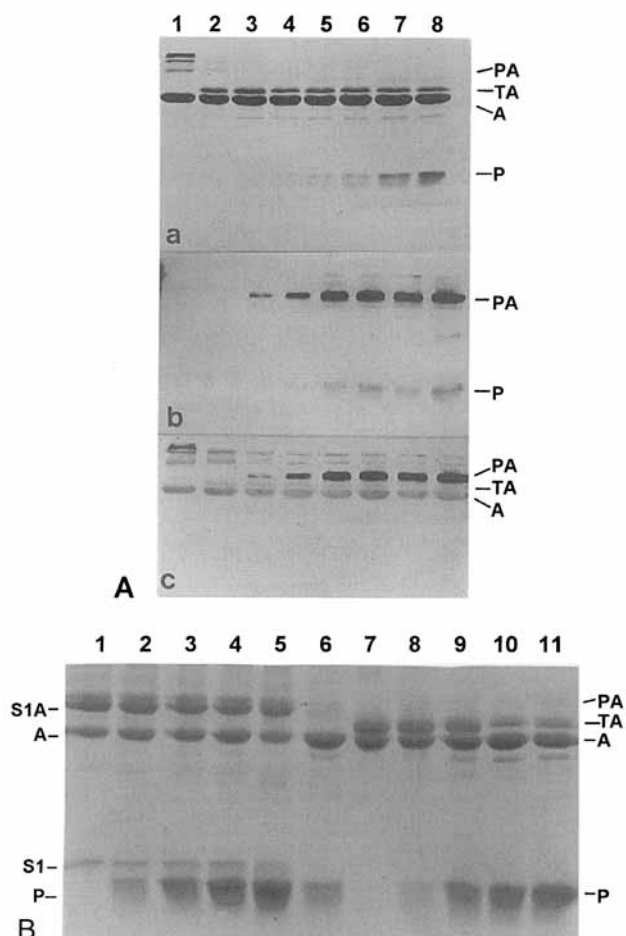


Fig. 6. Competition between birch profilin and other actin ligand proteins. (A) Competition between recombinant birch profilin and thymosin $\beta 4$ for binding to skeletal muscle G-actin. (a) Coomassie-stained gel profile of 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide-crosslinked products, obtained after 6 h of crosslinking from the following mixtures: lane 1, actin alone (15 μM); lane 2, actin mixed with 15 μM thymosin $\beta 4$; lanes 3–8, like 2, but with 4.8, 9.6, 14.4, 19.2, 28.8 and 38.4 μM recombinant birch profilin, respectively. (b) Immunoblot of a parallel gel, reacted with the monoclonal anti-(birch profilin), a second horseradish-peroxidase-coupled antibody and chloronaphthol/ H_2O_2 . (c) The same blot as shown in (b), but subsequently reacted with a polyclonal anti-actin, a second horseradish-peroxidase-coupled antibody and diaminobenzidine/ H_2O_2 . The differently coloured reaction products allowed for the assignment of the respective antigens. With these blots, the polypeptides and crosslinking products could be identified as follows: P = profilin; A = actin; TA = thymosin $\beta 4$ -actin; PA = profilactin. The TA band decreases with increasing profilin concentration. (B) Competition between birch profilin, gelsolin segment 1 and thymosin $\beta 4$, respectively. Coomassie-stained gel profile of reaction products obtained after 30 min 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide crosslinking. Lane 1, 15 μM actin and 14 μM gelsolin segment 1; lanes 2–5, like 1 but with 30, 60, 90, 120 and 120 μM profilin, respectively; lane 6, 15 μM actin with 60 μM profilin crosslinked in the absence of segment 1 to demonstrate the inability of 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide to form enough crosslinked actin-profilin to become detectable on a Coomassie-stained gel so that the crosslinked material shown in lanes 1–5 is predominantly actin-segment-1 complex; lane 7, 15 μM actin crosslinked to 6 μM thymosin $\beta 4$ in the absence of profilin; lanes 8–11, like lane 7, but with 30, 60, 90 and 120 μM profilin. Crosslinking was performed as described in Materials and Methods. While the gelsolin-segment-1-actin complex (S1A) does not change with increasing amounts of profilin, the decrease of the thymosin-actin complex (TA) is obvious.

prerequisite for the calculation of free and profilin-bound actin by this method would be that profilin exclusively interacts with G-actin. The weak F-actin capping activity of profilin (see introduction for references to this point) may therefore influence the calculation somewhat. However, we deliberately chose this method since the main purpose of this study was to compare the affinities of birch profilin for actin with data available in the literature for other profilin/actin complexes, as obtained by the same or comparable methods.

We found that birch profilin interacts with mammalian skeletal muscle actin with an affinity quite comparable to other profilins. In general, the affinities for heterologous profilactin complexes reported are in the micromolar range (Table 2). In contrast, affinities higher by 10–100-fold have been found for the homologous calf spleen complex, with a K_d of 10 nM in the absence of Mg^{2+} ions (Larsson and Lindberg, 1988). Recently, the interaction of birch profilin and also of mammalian (pig brain) profilin with plant actin filaments has been reported (Staiger et al., 1994). However, these studies were confined to microinjecting these proteins into living cells and no data on affinities are available. It should be interesting to determine the affinity of birch profilin to birch actin in homologous complexes. Unfortunately, the isolation of pure isoforms of plant actins in sufficient quantities is still not trivial and recombinant plant actin, although it can be efficiently expressed in bacteria (cf. Heilmann et al., 1994), cannot be reconstituted easily into a fully polymerization-competent form. Thus, these experiments have to await further technical improvements in plant actin isolation.

The interaction of birch profilin with mammalian actin could be further substantiated by competition experiments and crosslinking of the actin/ligand complexes. In previous studies, crosslinking of *Acanthamoeba* profilin Lys115 with actin Glu364 by 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide and subsequent proteolytic digestion had been used to identify the contact site in the profilactin complex in solution (Vandekerckhove et al., 1989). For bovine profilin, Lys125 seems to occupy a similar position (Rozycki et al., 1994). Our results show that the birch protein can also be crosslinked to actin. However, the crosslinked adduct was only detectable by using immunoblotting rather than Coomassie staining. A reason for the low yield of crosslinked actin-profilin may reside in the primary structure of plant profilins, since a possible Lys residue cannot easily be identified in the sequence.

Birch profilin competed with thymosin $\beta 4$, another major G-actin sequestering protein in mammalian cells (Safer et al., 1991), for actin binding, suggesting that the respective binding sites are at least closely spaced. Although so far no direct evidence for competition of mammalian profilin with thymosin $\beta 4$ on actin has been reported, competitive binding can be deduced from recently published experiments (Pantaloni and Carlier, 1993). Regarding gelsolin segment 1, we found no competition between this ligand and birch profilin. The gelsolin contact site on G-actin has been defined to locate mainly to actin subdomain 1 and two Tyr residues in subdomain 3 (McLaughlin et al., 1993). Bovine profilin forms crystal contacts with the same actin domains (Schutt et al., 1993). Although the orientation of these two ligands is different (cf. Rozycki et al., 1994), an at least partial overlap of both binding sites can be deduced from structural data. However, as the affinities differ grossly (the gelsolin segment 1/actin complex has a K_d value of 10 pM, i.e. 10^4 -fold lower than the profilactin complex), the lack of competition be-

tween these ligands, as observed in this study, cannot be interpreted as proof for discrete and independent binding sites.

In summary, our results show that birch profilin interacts specifically and with a reasonably high affinity with mammalian actin. They suggest that the contacts formed involve the same areas on the actin molecule as found for bovine profilin. Further insight into the interaction of plant profilins with actin will have to await detailed structural information on these proteins.

We thank Drs E. Hannappel (Erlangen) and for thymosin β_4 and Dr A. G. Weeds (Cambridge) for gelsolin segment 1, and Mrs B. Niggemeyer (Marburg) for expert technical assistance. H. G. M. and B. M. J. acknowledge the financial support of the *Deutsche Forschungsgemeinschaft* 'Schwerpunkt 'Funktionelle Domänen von Strukturproteinen' and SFB 223). R. V. was supported by the *Fonds zur Förderung der wissenschaftlichen Forschung*, Austria (P 10432).

REFERENCES

- Ampe, C. & Vandekerckhove, J. (1987) The F-actin capping proteins of *Physarum polycephalum*: Cap42(a) is very similar, if not identical, to fragmin and is structurally and functionally very homologous to gelsolin; cap42(b) is *Physarum* actin, *EMBO J.* 6, 4149–4157.
- Ampe, C., Vandekerckhove, J., Brenner, S. L., Tobacman, L. & Korn, E. D. (1985) The amino acid sequence of *Acanthamoeba* profilin, *J. Biol. Chem.* 260, 834–840.
- Archer, S. J., Vinson, V. K., Pollard, T. D. & Torchia, D. A. (1994) Elucidation of the poly-L-proline binding site in *Acanthamoeba* profilin I by NMR spectroscopy, *FEBS Lett.* 337, 145–151.
- Babcock, G. & Rubenstein, P. A. (1989) Synthesis of mammalian profilin in *Escherichia coli* and its characterization, *Cell Motil. Cytoskeleton* 14, 230–236.
- Björkegren, C., Rozycki, M., Schutt, C. E., Lindberg, U. & Karlsson, R. (1993) Mutagenesis of human profilin locates its poly(L-proline) binding site to a hydrophobic patch of aromatic residues, *FEBS Lett.* 133, 123–126.
- Blasco, R., Cole, N. B. & Moss, B. (1991) Sequence analysis, expression and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein, *J. Virol.* 65, 4598–4608.
- Buß, F. & Jockusch, B. M. (1989) Tissue specific expression of profilin, *FEBS Lett.* 249, 31–34.
- Buß, F., Temm-Grove, C., Henning, S. & Jockusch, B. M. (1992) Distribution of profilin in fibroblasts correlates with the presence of highly dynamic actin filaments, *Cell Motil. Cytoskel.* 22, 51–61.
- Carlier, M. F., Jean, C., Rieger, K. J., Lenfant, M. & Pantaloni, D. (1993) Modulation of interaction between G-actin and thymosin β_4 by the ATP/ADP ratio: possible implication in the regulation of actin dynamics, *Proc. Natl Acad. Sci. USA* 90, 5034–5038.
- Carlsson, L., Nystrom, L. E., Sundkvist, I., Markey, F. & Lindberg, U. (1977) Actin polymerizability is influenced by profilin, a low molecular mass protein in nonmuscle cells, *J. Mol. Biol.* 115, 465–483.
- Cooper, J. A. & Pollard, T. D. (1982) Methods to measure actin polymerization, *Methods Enzymol.* 85, 182–210.
- DiNubile, U. J. & Southwick, F. S. (1985) Effects of macrophage profilin on actin in the presence and absence of acumentin and gelsolin, *J. Biol. Chem.* 260, 7402–7409.
- Goldschmidt-Clermont, P., Kim, J., Machesky, L., Rhee, S. & Pollard, T. D. (1991a) Regulation of phospholipase C $_1$ by profilin and tyrosine phosphorylation, *Science* 251, 1231–1233.
- Goldschmidt-Clermont, P., Machesky, L., Doberstein, S. & Pollard, T. D. (1991b) Mechanism of the interaction of platelet profilin with actin, *J. Cell Biol.* 113, 1081–1089.
- Gordon, D. J., Eisenberg, E. & Korn, E. D. (1976) Characterization of cytoplasmic actin isolated from *Acanthamoeba castellanii* by a new method, *J. Biol. Chem.* 251, 4778–4786.
- Haarer, B. K. & Brown, S. S. (1990) Structure and function of profilin, *Cell Motil. Cytoskel.* 17, 71–74.
- Hannappel, E., Wartenberg, F. & Bustelo, X. R. (1989) Isolation and characterization of thymosin β_9^{net} from pork spleen, *Arch. Biochem. Biophys.* 273, 396–402.
- Hartwig, J. H., Chambers, K. A., Hopcia, K. L. & Kwiatkowski, D. J. (1989) Association of profilin with filament-free regions of human leukocyte and platelet membranes and reversible membrane binding during platelet activation, *J. Cell Biol.* 109, 1571–1579.
- Haugwitz, M., Noegel, A. A., Rieger, D., Lottspeich, F. & Schleicher, M. (1991) *Dictyostelium discoideum* contains two profilin isoforms that differ in structure and function, *J. Cell Sci.* 100, 481–489.
- Heimann, P., Herkströter, E., Rothkegel, B. & Giehl, K. (1994) Expression and localisation of profilin and plant actin in *E. coli*: an immunoelectron microscopic study, *Eur. J. Cell Biol.* 63, Suppl. 40, 97.
- Houk, T. W. J. & Ue, K. (1974) The measurement of actin concentration in solution: a comparison of methods, *Anal. Biochem.* 62, 66–74.
- Larsson, H. & Lindberg, U. (1988) The effect of divalent cations on the interaction between calf spleen profilin and different actins, *Biochim. Biophys. Acta* 953, 95–105.
- Lassing, I. & Lindberg, U. (1988) Specificity of the interaction between PIP $_2$ and the profilin:actin complex, *J. Cell. Biochem.* 37, 255–267.
- Lind, S. E., Janmey, P. A., Chaponnier, C., Herbert, T. J. & Stossel, T. P. (1987) Reversible binding of actin to gelsolin and profilin in human platelet extracts, *J. Cell Biol.* 105, 833–842.
- Machesky, L. M. & Pollard, T. D. (1993) Profilin as a potential mediator of membrane-cytoskeleton communication, *Trends Cell Biol.* 3, 381–385.
- Malm, B., Larsson, H. & Lindberg, U. (1983) The profilin-actin complex: further characterization of profilin and studies on the stability of the complex, *J. Muscle Res. Cell Motil.* 4, 569–588.
- McLaughlin, P. C., Gooch, J. T., Mannherz, H. G. & Weeds, A. G. (1993) Structure of gelsolin segment 1-actin complex and the mechanism of filament severing, *Nature* 364, 685–692.
- Metzler, W. J., Constantine, K. L., Friedrichs, M. S., Bell, A. J., Ernst, E. G., Lavoie, T. B. & Mueller, L. (1993) Characterization of the three-dimensional solution structure of human profilin: ^1H , ^{13}C , and ^{15}N NMR assignments and global folding pattern, *Biochemistry* 32, 13818–13829.
- Metzler, W. J., Bell, A. J., Ernst, E., Lavoie, T. B. & Mueller, L. (1994) Identification of the poly-L-proline-binding site on human profilin, *J. Biol. Chem.* 269, 4620–4625.
- Mockrin, S. & Korn, E. (1980) *Acanthamoeba* profilin interacts with G-actin to increase the exchange of actin bound adenosine 5'-triphosphate, *Biochemistry* 19, 5359–5362.
- Nishida, E. (1985) Opposite effects of cofilin and profilin from porcine brain on rate of exchange of actin-bound adenosine 5'-triphosphate, *Biochemistry* 24, 1160–1164.
- O'Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins, *J. Biol. Chem.* 250, 4007–4021.
- Ozaki, K., Sugino, H., Hasegawa, T., Takahashi, S. & Hatano, S. (1983) Isolation and characterization of *Physarum* profilin, *J. Biochem. (Tokyo)* 93, 295–298.
- Pantaloni, D. & Carlier, M. F. (1993) How profilin promotes actin filament assembly in the presence of thymosin β_4 , *Cell* 75, 1007–1014.
- Pollard, T. D. & Cooper, J. A. (1984) Quantitative analysis of the effect of *Acanthamoeba* profilin in actin filament nucleation and elongation, *Biochemistry* 23, 6631–6641.
- Pollard, T. D. & Rimm, D. L. (1991) Analysis of cDNA clones for *Acanthamoeba* profilin I and profilin II shows end to end homology with vertebrate profilins and a small family of profilin genes, *Cell Motil. Cytoskel.* 20, 169–177.
- Polzar, B., Rösch, A. & Mannherz, H. G. (1989) A simple procedure to produce monospecific polyclonal antibodies of high affinity against actin from muscular sources, *Eur. J. Cell Biol.* 50, 220–229.

- Rozycki, M. D., Myslik, J. C., Schutt, C. E. & Lindberg, U. (1994) Structural aspects of actin-binding proteins. *Curr. Opin. Cell Biol.* 6, 87–95.
- Safer, D., Elzinga, M. & Nachmias, V. T. (1991) Thymosin β 4 and F $_{\alpha}$, an actin sequestering peptide, are indistinguishable. *J. Biol. Chem.* 266, 4029–4032.
- Schägger, H. & von Jagow, G. (1987) Tricine-sodium dodecyl sulfate polyacrylamide gelelectrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368–379.
- Schliwa, M. & von Blerkon, J. (1981) Structural interaction of cytoskeletal components. *J. Cell. Biol.* 90, 222–235.
- Schutt, C. E., Myslik, J. C., Rozycki, M. D., Goonesekere, N. C. W. & Lindberg, U. (1993) The structure of crystalline profilin- β -actin. *Nature* 365, 810–816.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85.
- Spudich, J. A. & Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* 246, 4866–4871.
- Staiger, C. J., Goodbody, K. C., Hussey, P. J., Valenta, R., Drobak, B. K. & Lloyd, C. W. (1993) The profilin multigene family of maize: differential expression of three isoforms. *Plant J.* 4, 631–641.
- Staiger, C. J., Yuan, M., Valenta, R., Shaw, P. J., Warn, R. M. & Lloyd, C. W. (1994) Microinjected profilin affects cytoplasmic streaming in plant cells by rapidly depolymerizing actin microfilaments. *Curr. Biol.* 4, 215–219.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185, 60–89.
- Tanaka, M. & Shibata, H. (1985) Poly(L-proline)-binding proteins from chick embryos are profilin and profilactin. *Eur. J. Biochem.* 151, 291–297.
- Theriot, J. A. & Mitchison, T. J. (1993) The three faces of profilin. *Cell* 75, 835–838.
- Tobacman, L. S. & Korn, E. D. (1982) The regulation of actin polymerization and the inhibition of monomeric actin ATPase activity by *Acanthamoeba castellanii* profilin. *J. Biol. Chem.* 257, 4166–4170.
- Tseng, P. C. H. & Pollard, T. D. (1982) Mechanism of action of *Acanthamoeba* profilin: demonstration of actin species specificity and regulation by micromolar concentrations of MgCl₂. *J. Cell Biol.* 94, 213–218.
- Valenta, R., Ball, T., Vrtale, S., Duchêne, M., Kraft, D. & Scheiner, O. (1994) cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. *Biochem. Biophys. Res. Commun.* 199, 106–118.
- Valenta, R., Duchêne, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D. & Scheiner, O. (1991) Identification of profilin as a novel pollen allergen; IgE-autoreactivity in sensitized individuals. *Science* 253, 557–560.
- Valenta, R., Ferreira, F., Grote, M., Swoboda, I., Vrtala, S., Duchêne, M., Deviller, P., Meagher, R. B., McKinney, E. & Heberle-Bors, E. (1993) Identification of profilin as an actin-binding protein in higher plants. *J. Biol. Chem.* 268, 22777–22781.
- Vandekerckhove, J. S., Kaiser, D. A. & Pollard, T. D. (1989) *Acanthamoeba* actin and profilin can be cross-linked between glutamic acid 364 of actin and lysine 115 of profilin. *J. Cell Biol.* 109, 619–626.
- Vinson, V. K., Archer, S. J., Lattman, E. E., Pollard, T. D. & Torchia, D. A. (1993) Three-dimensional solution structure of *Acanthamoeba* profilin-I. *J. Cell Biol.* 122, 1277–1283.
- Way, M., Pope, B., Gooch, J. & Weeds, A. G. (1990) Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J.* 9, 4103–4109.
- Weir, G. P. & Frederiksen, D. W. (1982) Preparation of cytoplasmic actin. *Methods Enzymol.* 85, 371–375.

Note added in proof. After this work had been submitted for publication Ruhlandt et al. [Ruhlandt, G., Lange, U. & Grolig, F. (1994) *Plant Cell Physiol.* 35, 849–854] described the interaction of plant profilin with cardiac muscle actin.