The mammalian profilin isoforms display complementary affinities for PIP₂ and proline-rich sequences

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We present a study on the binding properties of the bovine profilin isoforms to both phosphatidylinositol 4,5-bisphosphate (PIP₂) and proline-rich peptides derived from vasodilator-stimulated phosphoprotein (VASP) and cyclase-associated protein (CAP). Using microfiltration, we show that compared with profilin II, profilin I has a higher affinity for PIP₂. On the other hand, fluorescence spectroscopy reveals that proline-rich peptides bind better to profilin II. At micromolar concentrations, profilin II dimerizes upon binding to proline-rich peptides. Circular dichroism measurements of profilin II reveal a significant conformational change in this protein upon binding of the peptide. We show further that PIP₂ effectively competes for binding of profilin I to poly-L-proline, since this isoform, but not profilin II, can be eluted from a poly-L-proline column with PIP₂. Using affinity chromatography on either profilin isoform, we identified profilin II as the preferred ligand for VASP in bovine brain extracts. The complementary affinities of the profilin isoforms for PIP₂ and the proline-rich peptides offer the cell an opportunity to direct actin assembly at different subcellular localizations through the same or different signal transduction pathways.

Keywords: actin cytoskeleton/phosphatidylinositol 4,5-bisphosphate/profilin isoforms/signal transduction/vasodilator-stimulated phosphoprotein

Introduction

Profilin is a ubiquitous, small (12–15 kDa) actin-binding protein that plays an important role in the regulation of actin polymerization in a number of motility functions (Carlsson *et al.*, 1977; Stossel, 1993). *In vitro* experiments suggest that profilin can promote actin assembly from the G-actin-thymosin-4 pool when barbed filament ends are free. In the presence of gelsolin, a barbed end capping protein, profilin can act as an actin-sequestering protein resulting in actin depolymerization (Pantaloni and Carlier, 1993). This dual function of profilin is thought to be essential for rapid filament turnover during cell motility processes, e.g. in the growing end of fast moving lamellipodia of fibroblasts (Buss *et al.*, 1992; Small, 1995) and at

the tail of *Listeria monocytogenes*, a bacterial pathogen that uses actin assembly to move forward (Theriot *et al.*, 1994).

Profilin also binds phosphatidylinositol 4,5-bisphosphate (PIP₂) (Lassing and Lindberg, 1985, 1988). Phospholipase C1 cannot hydrolyse profilin-bound PIP₂ *in vitro*, unless the phospholipase is phosphorylated (Goldschmidt-Clermont *et al.*, 1990, 1991; Machesky *et al.*, 1990). These experiments led to a model in which profilin plays an important role as mediator in cell signalling to the cytoskeleton. PIP₂-associated profilin is not able to bind actin monomers but, after hydrolysis of PIP₂, profilin is free and can diffuse into the cytoplasm to interact with actin (Lassing and Lindberg, 1985, 1988).

Profilins, with the exception of vaccinia virus profilin (Machesky *et al.*, 1994), bind poly-L-proline, but the significance of this interaction remained an enigma for a long time. However, several proteins associated with the cytoskeleton or its dynamics contain such a sequence: cyclase-associated protein (CAP), a surface protein from the cytotoxic pathogen *L.monocytogenes* (ActA) and the vasodilator-stimulated phosphoprotein (VASP). Already in 1991, Vojtek *et al.* proposed a functional link between profilin and CAP, which is the 70 kDa subunit of the *Saccharomyces cerevisiae* adenylyl cyclase complex (Field *et al.*, 1990). CAP is a bifunctional protein of which the N-terminal domain is necessary and sufficient for a Ras-responsive adenylyl cyclase complex, and of which the C-terminal domain binds actin (Freeman *et al.*, 1995) and is important for normal cell morphology and responsiveness to nutrient extremes (Gerst *et al.*, 1991). CAP mutants that lack the C-terminal domain can be rescued by overexpression of profilin (Vojtek *et al.*, 1991). Since CAP has a proline-rich stretch in its middle domain, it may be a ligand for profilin (Goldschmidt-Clermont and Janmey, 1991).

Similarly, ActA contains three single stretches of four proline residues. A *Listeria* mutant which does not express ActA on the surface is unable to form actin tails, to spread intracellularly and to infect adjacent cells (Domann *et al.*, 1992), although host factors also seem to be required for actin tail formation (Theriot *et al.*, 1994; Chakraborty *et al.*, 1995).

VASP was identified originally as a protein present in human platelets which becomes phosphorylated by cAMP- and cGMP-dependent protein kinases in response to both cAMP- and cGMP-elevating vasodilators and platelet inhibitors (Waldmann *et al.*, 1986, 1987; Halbrügge *et al.*, 1990; Nolte *et al.*, 1991). The sequence reveals a central domain with a proline-rich motif, with five consecutive proline residues, that occurs as a single copy and a 3-fold repeat (Haffner *et al.*, 1995). Only very recently, Reinhard and co-workers (1995) showed that VASP interacts directly with profilin.

It was shown that human cells have two profilin isoforms

and, given the different expression levels of each isoform in different tissues (Honoré *et al.*, 1993), researchers have used, unknowingly, preparations containing different ratios of profilin isoforms in the past, e.g. in microinjection studies (Cao *et al.*, 1992) and bio- and immunochemical studies (Buss and Jockusch, 1989).

Human and bovine profilin I (Ampe *et al.*, 1988; Kwiatkowski and Bruns, 1988) and II (Honoré *et al.*, 1993; Lambrechts *et al.*, 1995) show only 65.5% identity. Most of the mutated residues map at the exterior of the known profilin I structure, suggesting that both isoforms have a similar overall fold but display different biochemical properties. Their affinity for actin is quite similar (Lambrechts *et al.*, 1995), consistent with the observation that the residues of profilin, forming the interface with actin, are conserved. However, one of our previous experiments suggested that the isoforms display a different affinity for poly-L-proline at acidic pH (Lambrechts *et al.*, 1995). To probe this difference further, we investigated the interaction of each isoform with proline-rich model peptides derived from VASP, CAP and ActA using fluorescence spectroscopy at more physiological pH. We also searched for differences in interaction with the other known profilin ligand, PIP₂, using a microfiltration assay. We show that profilin I has higher affinity for PIP₂, while profilin II has higher affinity for proline-rich sequences, and can form dimers upon binding these sequences. In addition we show that PIP₂ is an effective competitor for poly-L-proline binding of profilin I, but not of profilin II. Our data suggest that in cells profilin I may be preferentially associated with PIP₂, and profilin II with proteins such as VASP, which we show is recruited preferentially from bovine brain extracts by profilin II.

Results

Bovine profilin I has a higher affinity for PIP₂ than the profilin II isoform

An important characteristic of profilins is their ability to bind PIP₂ (Lassing and Lindberg, 1985, 1988). Our previous results showed that bovine profilin II binds PIP₂, but nevertheless they hinted at a reduced affinity compared with profilin I (Lambrechts *et al.*, 1995). To investigate this potential difference between the profilin isoforms further, we used an assay described by Haarer *et al.* (1993). In a microfiltration experiment, we incubated a constant amount of profilin (I or II) with increasing amounts of PIP₂ (0- to 100-fold molar excess over profilin), and centrifuged them on a filter with a molecular weight cut-off of 30 000. The flow-through contains profilin not bound to PIP₂ and is shown in Figure 1A. The result of scanning the gels is given in Figure 1B. With a 25-fold molar excess of PIP₂ over profilin, nearly all profilin I is bound to PIP₂. On the other hand, even with a 100-fold molar excess of PIP₂, 60% of profilin II (relative to the sample without PIP₂) is still present in the flow-through. This microfiltration assay clearly demonstrates the differences in affinity for PIP₂ between the profilin isoforms.

PIP₂ binding induces a greater change in the structure of profilin I than in profilin II

Local structure changes induced by phospholipids can be observed in near UV (250–320 nm) circular dichroism

Fig. 1. Microfiltration of profilin-PIP₂ complexes. (A) Samples containing a constant amount of profilin (4 μ M) and increasing concentrations of PIP₂ were made. The flow-through was analysed by SDS-PAGE, of which only the 14–20 kDa region is shown. The molar excess of PIP₂ is indicated. (B) Result of scanning the Coomassie-stained spots shown in (A). Boxes represent the percentage of non-bound profilin. White boxes are profilin I and black boxes profilin II. The amount of profilin found in the flow-through when no PIP₂ is present was set to 100%.

(CD) spectra, as has been shown for profilin I (Raghunathan *et al.*, 1992) and the pleckstrin homology domain of spectrin (Hyvönen *et al.*, 1995). The CD measurements were carried out for samples containing 15 μ M profilin with different concentrations of PIP₂ ranging from 0 to 300 μ M (Figure 2). Profilin I and II alone have different spectra. This is probably because of the difference in their tyrosine, tryptophan and phenylalanine content and the environment of these residues. The ellipticity of profilin I in the presence of PIP₂ shows a large decrease with only a 10-fold molar excess of PIP₂ and only a slight further decrease when the PIP₂ concentration is doubled. In contrast, the decrease in ellipticity of profilin II is smaller and nearly doubles when the PIP₂ concentration is doubled.

Profilin I and II display different affinities for proline-rich peptides

Whereas profilin I has a higher affinity for PIP₂, profilin II binds more strongly to poly-L-proline (Lambrechts *et al.*, 1995). Poly-L-proline as such is not present in living cells, though several proteins have proline-rich sequences. At the start of our investigations, no natural profilin ligands with stretches of proline residues were identified. However, a number of proteins were likely candidates for a profilin ligand. CAP contains six and five prolines in a row separated by a single glycine residue (Matviw *et al.*,

Fig. 2. Circular dichroism spectra in the near UV of 15 μM profilin (thick line) I or II with 150 (....) and 300 μM (---) PIP_2 . The molar ellipticity per residue weight is shown as a function of the wavelength.

1992). A repeat of four prolines preceded by a phenylalanine occurs three times in ActA, each time separated by 30 amino acids (Domann *et al.*, 1992), and, more recently, a three times repeated sequence of five consecutive proline residues separated by a glycine was identified

in VASP (Haffner *et al.*, 1995). A shorter sequence containing only five proline residues is also found in the VASP sequence. As these three proteins have been implicated in actin dynamics or are associated with the actin cytoskeleton, we chemically synthesized peptides derived from human CAP and VASP and from *Listeria* ActA (Table I) and studied their interaction with both profilin isoforms. We incubated a constant amount of profilin (1 μM) with an increasing amount of peptide (0–55 μM) under physiological conditions and monitored binding by spectrofluorimetry (Figure 3).

The ActA peptide, peptACTwt, which contains only four proline residues, induces no significant increase in fluorescence even at high peptide concentrations, indicating that it has a very low affinity for both of the profilin isoforms (Figure 3A and B). This is consistent with the finding of Zeile *et al.* (1996) that the ActA peptide does not bind to profilin at a concentration of 100 μM . The same is observed for peptVASPs, a short proline-rich sequence derived from VASP. The other peptides studied, peptCAPwt and peptVASPwt, show a change in the fluorescence intensity which means they do bind to profilin.

We tried to fit a hyperbolic curve to the measured values but could not find a sufficiently good fit. Also, attempts to linearize the data using the Surewicz and Epand equation (1984) were unsuccessful. This suggests that the binding is heterogenous, as will be demonstrated further below. Nevertheless, the difference in the steepness of the curves in the low concentration range in Figure 3A and B suggests a difference in affinity of these peptides for each of the profilin isoforms. They appear to bind better to profilin II than to profilin I.

As peptCAPwt and peptVASPwt (with 11 and 15 proline residues respectively) seem to have a comparable affinity and peptACTwt (with only four prolines) showed no binding, we synthesized increasingly shorter mutant CAP peptides (see Table I) to assay the minimal length required for binding (Figure 3C). PeptCAP Δ 1 lacking one proline residue has almost the same affinity as peptCAPwt, while peptCAP Δ 2 and peptCAP Δ 4, lacking two and four prolines respectively, have a strongly reduced affinity compared with wild-type CAP peptide.

Profilin II dimerizes upon binding of proline-rich peptides

Modelling experiments suggest that one repeat of five proline residues is sufficient to fill the hypothetical poly-L-proline binding pocket (data not shown). We demonstrated above that two repeats are necessary for profilin binding. This prompted us to study whether dimers of profilin can be formed on the proline-rich peptides. Since profilin II appears to bind better to these peptides, we chose to study this isoform. Profilin II elutes at 47 min on a Superdex 200 gel filtration column (Figure 4a). With a 1.2-fold molar excess of peptVASPwt, profilin II shifts partly to an earlier position (Figure 4b). When more peptide is added in 12-fold molar excess over profilin II, a second shift can be observed (Figure 4c) relative to the profilin II peak. In the simplest scenario, the first shift is a 1:1 complex of profilin and peptVASPwt and the second, larger shift would then be a 2:1 complex consisting of a dimer of profilin bound to one peptide. We observed the

Table I. Sequence of proline-rich peptides

Peptide	Sequence	Protein	Reference
peptCAPwt	Ac.SGPPPPPPGPPPPVVS.OH	human CAP	Matviw <i>et al.</i> (1992)
peptVASPwt	Ac.GPPPPPGPPPPGPPPPGL.OH	human VASP	Haffner <i>et al.</i> (1995)
peptVASPs	Ac.GGPPPPGL.OH	human VASP	Haffner <i>et al.</i> (1995)
peptACTwt	Ac.FPPPPTD.OH	<i>Listeria</i> ActA	Domann <i>et al.</i> (1992)
peptCAPΔ1	Ac.SGPPPPPGPPPPVVS.OH	CAP mutant	
peptCAPΔ2	Ac.SGPPPPPGPPPPVVS.OH	CAP mutant	
peptCAPΔ4	Ac.SGPPPGPPPPVVS.OH	CAP mutant	

same shift with profilin II and peptCAPwt (data not shown), but observed no shift when an 18-fold molar excess of the short peptVASPs is added to profilin II, indicating that no binding occurs (Figure 4d).

A proline-rich peptide induces a conformational change in profilin II

Local structural changes in proteins induced by peptide binding can also be observed in CD spectra in the region from 250 to 290 nm. We measured spectra for profilin I and II with three different concentrations of peptCAPwt (Figure 5), and the results suggest that aromatic amino acids are involved in peptide binding, consistent with results from mutational analysis of profilin I (Björkegren *et al.*, 1993).

The first decrease in profilin II ellipticity is very large, while subsequent decreases at larger peptide concentrations are small. This suggests that a conformational change accompanies the binding of profilin II to peptCAPwt. On the other hand, for profilin I, the ellipticity decreases more gradually as the peptide concentration increases, consistent with a lower affinity for the peptide.

The polyproline binding site of profilins is not accessible when PIP₂ is present

We also investigated whether PIP₂ and poly-L-proline compete for profilin binding. In the control experiment, profilin I or II were bound to poly-L-proline and, after washing, were eluted with 8 M urea. A small fraction of profilin I did not bind or bound very weakly to the poly-L-proline column. The majority of profilin I is in the early eluting 8 M urea fractions (Figure 6A, lanes 5 and 6) while the majority of profilin II elutes later (lanes 7–11). This result is consistent with our previously published data where a similar experiment was carried out at a different pH (Lambrechts *et al.*, 1995) and again points to a difference in affinity for poly-L-proline of these two isoforms.

We next loaded the poly-L-proline affinity column with each profilin isoform and, after washing, eluted them with increasing concentrations of PIP₂ (Figure 6B). We observed that PIP₂ was capable of eluting profilin I from the column although a significant amount still remained bound to poly-L-proline. By contrast, PIP₂ was not able to elute profilin II.

In the reverse experiment, the profilin isoforms were incubated with a 25-fold molar excess of PIP₂ micelles prior to loading them onto the column. A large fraction of profilin I is found in the flow-through and the wash (combined, ~53%), though still ~47% is eluted with 8 M urea (Figure 6C). In the profilin II sample, only a small

amount of profilin is found in the flow-through (17%). No profilin II is found in the wash, and the remaining 83% can only be eluted with 8 M urea. These results show that profilin I has a higher affinity for PIP₂ than for poly-L-proline.

Identification of VASP as a ligand for profilin II but not for profilin I

Reinhard and colleagues (1995) identified VASP as a ligand for profilins and, since our experiments with the VASP-derived peptide suggest that the profilin isoforms have a different affinity for this ligand, we assayed the binding of VASP to each isoform using profilin I or II affinity columns. We first show that the monoclonal antibodies against human VASP also recognize bovine VASP (Figure 7A).

We loaded an equal amount of bovine brain extract on the columns and, after washing, eluted the remaining proteins with a step gradient of the proline-rich peptide derived from VASP (peptVASPwt) as indicated in Figure 7B. We tested the eluted fractions for the presence of VASP on Western blot using a monoclonal antibody (Figure 7B). Interestingly the capacity of the profilin isoforms to retain VASP appears different. While only a barely noticeable amount of VASP is eluted from the profilin I column, a much larger amount of VASP bound to the profilin II column. The elution starts with 50 μM peptVASPwt but the majority of the protein is eluted with 500 μM peptVASPwt. This result confirms the other *in vitro* experiments with purified proteins and peptides and indicates that profilin II rather than profilin I is a possible ligand for VASP.

Discussion

In this study, we demonstrate that the profilin isoforms have complementary affinities for two ligands. Profilin I interacts strongly with PIP₂ and more weakly with poly-L-proline sequences. On the other hand, profilin II has a higher affinity for proline-containing peptides and binds less tightly to PIP₂.

Our data on binding of profilin I to proline-rich peptides are in agreement with previous results of Perelroizen and co-workers (1994). These authors postulated the optimal length of the poly-L-proline sequence to be 15–20 residues. However, we find that there is little difference in affinity between peptCAPwt and peptVASPwt containing 11 and 15 proline residues, and a slight decrease in affinity of a mutant CAP peptide lacking one proline (peptCAPΔ1). Shorter peptides, such as those that occur in the *Listeria* surface protein ActA and the shorter repeats in VASP,

Fig. 3. Binding of profilin I and II to different proline-rich peptides. The relative fluorescence change (rfc) is plotted against peptide concentration. The best fitting logarithmic curves are drawn as full lines. The profilin concentration is 1 μM in all samples, and the peptide concentration ranges from 0 to 55 μM . (A) Profilin I and peptides peptCAPwt ($\blacklozenge$), peptVASPwt ($\square$), peptVASPs ($\blacktriangledown$) and peptACTwt ($\triangle$). (B) Profilin II and peptides peptCAPwt ($\blacklozenge$), peptVASPwt ($\square$), peptVASPs ($\blacktriangledown$) and peptACTwt ($\triangle$). (C) Profilin II and CAP wild-type and mutant peptides peptCAPwt ($\blacklozenge$), peptCAP Δ 1 ($\diamond$), peptCAP Δ 2 ($\blacksquare$), and peptCAP Δ 4 (∇).

have very low affinity for both of the profilin isoforms. This result suggests that the role of profilin in *Listeria* movement is not by direct interaction with ActA, consistent with recent results showing that VASP may be the host factor that links profilin to ActA (Chakraborty *et al.*,

Fig. 4. Gel filtration of profilin peptide complexes. Only the relevant part of the chromatograms between 40 and 50 min is shown. (a) Profilin II alone at 33 μM , (b) profilin II (33 μM) and a 1.2-fold molar excess of peptVASPwt, (c) profilin II (30 μM) and a 12-fold molar excess of peptVASPwt, (d) profilin II (20 μM) and an 18-fold molar excess of peptVASPs. Detection was at 280 nm, a wavelength where the peptides do not absorb, but their presence was investigated using reversed phase HPLC (data not shown). The original absorption units to full scale are set at 0.1 for (a) and (b) and 0.2 for (c) and (d).

1995). The precise cellular function of VASP, a focal adhesion protein, is not known but its interaction with profilin, which is known from *in vitro* experiments, suggests that it may be an anchor for profilin-mediated actin polymerization (Pantaloni and Carlier, 1993; Reinhard *et al.*, 1995). This is exemplified by the fact that after *Listeria* infection, VASP is recruited to the bacterial surface and binds ActA, prior to actin polymerization (Chakraborty *et al.*, 1995), and that depletion of profilin from extracts used for *in vitro* motility assays slows down *Listeria* bacteria (Theriot *et al.*, 1994).

So far, a direct binding of CAP to profilin has not been demonstrated, although a functional link between the two proteins exists in yeast (Vojtek *et al.*, 1991). Over-expression of profilin is able to rescue morphological defects associated with deletion of the CAP C-terminal domain. Interestingly the part of CAP still present in this mutant contains the proline-rich sequence, used in the present study. This suggests that the poly-L-proline site may be cryptic most of the time and only made accessible after an, as yet unknown, type of regulation. Cryptic sites have been identified in other cytoskeletal proteins (Menkel *et al.*, 1994; Turunen *et al.*, 1994; Gilmore and Burridge, 1995).

We also found that profilin II forms dimers upon binding to proline-rich peptides. If this also occurs *in vivo* it gives the cell a more efficient machinery to direct localized actin assembly. For each tetrameric VASP molecule (Haffner *et al.*, 1995) eight profilin molecules would be recruited at focal adhesions (or other sites) where VASP is active. In view of the polymerization-promoting activity of profilin (Pantaloni and Carlier, 1993), this concentrating effect could have a dramatic effect on the actin dynamics

Fig. 5. Circular dichroism spectra in the near UV. Profilin I or profilin II at a concentration of 15 μM was measured in the absence (thick line) or presence of peptide peptCAPwt at a concentration of 20 (....), 50 (---) or 100 μM (thin line). The molar ellipticity per residue weight is shown as a function of the wavelength. The profilin I and II spectra are somewhat different from those in Figure 2, due to a different buffer composition and dilution effects.

Fig. 6. Competitive interaction between PIP₂ and poly-L-proline for the profilin isoforms. (A) Profilin I (white boxes) and II (black boxes) are loaded onto a poly-L-proline column and eluted with 8 M urea. Fractions 1 and 2, flow-through; 3 and 4, wash; 5–12, 8 M urea elution. (B) Profilin I (white boxes) and II (black boxes) are bound to a poly-L-proline column and eluted with PIP₂. The PIP₂ gradient is indicated by full lines. Fraction 1, flow-through; 2, wash; 3, 50 μM PIP₂; 4, 100 μM PIP₂; 5, 200 μM PIP₂; 6, 300 μM PIP₂; 7, 400 μM PIP₂; 8, wash; 9–12, 8 M urea. (C) Samples containing profilin I (white boxes) or II (black boxes), pre-incubated with a 25-fold molar excess of PIP₂, are loaded on a poly-L-proline column and eluted with 8 M urea. Fraction 1 and 2, flow-through; 3–10, buffer wash; 11–16, 8 M urea elution.

Fig. 7. Binding of VASP to profilin. (A) Immunoprecipitation of bovine VASP from brain extracts with monoclonal antibodies against human VASP. (B) Profilin affinity chromatography and Western blotting using anti-VASP monoclonal antibody. Profilin I or II Sepharose columns are loaded with bovine brain extracts and, after washing, eluted with increasing concentrations of peptVASPwt. The eluted fractions are precipitated in 10% trichloroacetic acid and, after gel electrophoresis and Western blotting, tested for the presence of VASP using monoclonal antibodies.

at these subcellular localizations. This dimerization may also explain the slightly sigmoidal behaviour of the fluorescence data, from which it appears that profilin II has a higher affinity for proline-rich peptides than has profilin I. This is evidenced further by other experiments in the present study. An excess of peptCAPwt only induces a small change in the near UV CD spectrum of profilin I, while similar concentrations result in a large change in the CD spectrum of profilin II. In addition, elution of profilin I from poly-L-proline affinity columns requires less urea than does profilin II (control experiment in Figure 5 and Lambrechts *et al.*, 1995). Finally, in brain extracts, we could recover VASP only from profilin II-Sepharose columns (see also below). Although the profilins from eukaryotic organisms display this difference in affinity, in *Acanthamoeba* both profilin isoforms appear to have similar dissociation constants for poly-L-proline (Kaiser and Pollard, 1996).

We also show that, in contrast to poly-L-proline binding, profilin I has a higher affinity for PIP₂ than does profilin II. Using a 50-fold molar excess of PIP₂ over profilin, all of isoform I and only ~24% of isoform II was bound to PIP₂ vesicles. The CD spectra after PIP₂ binding show a large decrease for profilin I and only a small decrease for profilin II. This may indicate that profilin II binding to PIP₂ is not accompanied by structural changes or, more probably, that only a small amount of profilin II is bound to PIP₂. The competition experiment shows that, under the conditions used, PIP₂ is a competitor for poly-L-proline in the case of profilin I, but not of profilin II. In addition, PIP₂ binding makes the poly-L-proline binding site inaccessible on both profilin isoforms. Evidence from other investigators (for references, see below) indicates that poly-L-proline and PIP₂ occupy different binding sites. These are illustrated in Figure 8 where we show the crystal structure of profilin I (Cedergren-Zeppezauer *et al.*, 1994) and a calculated structure of profilin II. Our CD data and those from Raghunathan and co-workers (1992) indicate that PIP₂ and proline-rich peptides alter the conformation of profilin. The competition between these two ligands which we observed would, therefore, result rather from a conformational switch. Interestingly, in the calculated profilin II structure (after molecular dynamics

calculation in vacuum), the N- and C-terminal α -helices move apart, thus widening the hypothetical poly-L-proline binding groove formed by aromatic and hydrophobic amino acids from these helices and from the underlying β -strands (Björkegren *et al.*, 1993; Archer *et al.*, 1994; Metzler *et al.*, 1994). Comparison of the crystal and NMR structure of human profilin I by Metzler and co-workers (1995) shows that the N- and C-terminal helices are more closely packed in the NMR ensemble than in the crystal structure, suggesting that this movement of the terminal α -helices may occur under certain conditions even in profilin I. Moving these terminal helices would make the hydrophobic patch more accessible, and this motion appears energetically more favourable in profilin II.

The location of the PIP₂ binding site is less clearly defined. It is generally accepted that the polyanionic headgroups of this phospholipid contact basic amino acids. An interesting observation is that bovine profilin I is more basic than profilin II (Honoré *et al.*, 1993; Lambrechts *et al.*, 1995) and binds better to PIP₂ (this work). A similar correlation is found for the *Acanthamoeba* profilin isoforms where profilin II is more basic and binds more strongly to PIP₂ (Machesky *et al.*, 1990). Some basic amino acids implicated in PIP₂ binding are indicated in Figure 8. Mutagenesis of Arg72 to Glu in yeast profilin decreased the affinity for PIP₂ (Haarer *et al.*, 1993). This arginine corresponds to Arg74 in bovine profilin. Sohn and co-workers (1995) recently showed that Arg88 in human profilin I also is important in PIP₂ binding, because mutation to Leu resulted in decreased inhibition of phospholipase C γ 1 (PLC γ 1) activity. However, these residues are conserved between the profilin isoforms and, therefore, they cannot be responsible for the difference in affinity. On the other hand, substitution of Ser56 in profilin I by Glu in profilin II may exert a negative effect on binding of PIP₂ to profilin II, since it is only 11.06 and 16.36 Å away from the basic residues Arg74 and Arg88 respectively. Comparison of electrostatic potential calculations of profilin I and II show that residue 56 is located between two ridges of positive potential (data not shown). An extra negative charge in between these could interfere with PIP₂ binding.

Our binding studies of the isolated isoforms indicate

Fig. 8. Ribbon structure of bovine profilin I and II after molecular dynamic calculations in vacuum. The N- and C-terminal helices are shown in white. The hydrophobic side chains of the residues in the postulated poly-L-proline binding pocket are shown in green (bottom of the figure). N and C indicate the N- and C-terminal ends. The amino acids postulated to be in the PIP₂ binding site are also indicated. The basic residues Arg74 and Arg88 are shown in blue and the acidic residue Glu56 in profilin II is shown in red, while the corresponding Ser56 in profilin I is shown in green on top of the molecule.

that they both are capable of binding to proline-containing peptides. One is also able to purify profilin I by poly-L-proline affinity chromatography (Tanaka and Shibata, 1985; Kaiser *et al.*, 1989; Lambrechts *et al.*, 1995). Nevertheless, VASP in brain extracts does not bind to profilin I–Sepharose, while it is retained by a profilin II column. This is not because the profilin I coupled to Sepharose is no longer able to interact with VASP; VASP from platelet extracts does bind to these profilin I columns and can be eluted with peptVASPwt (Reinhard *et al.*, 1995, and data not shown). The failure of VASP in brain extracts to interact with profilin I columns may have several causes. VASP in brains may be modified differentially (compared with VASP in platelets) and this modification could interfere only with binding to profilin I. As pointed out by Reinhard *et al.* (1995), the phosphorylated form of VASP is capable of binding profilin. It is more probable that the binding reaction is regulated by the balance of the concentrations of profilin isoforms and their cellular ligands. Indeed, profilin II is the predominant isoform in brain (Honoré *et al.*, 1993; Lambrecht *et al.*, 1995) while platelets are enriched for profilin I (A.Lambrechts, J.-L.Vershelde, V.Jonckheere, M.Goethals, J.Vandekerckhove and C.Ampe, unpublished results). Thus, the fact that we do not observe profilin I binding to VASP in brain extracts may be due to the endogenous profilin II which is abundant in this tissue and competes efficiently with the Sepharose-bound profilin I.

The fact that profilin I and II have complementary affinities for the phospholipid PIP₂ and for poly-L-proline-containing sequences, such as they occur in VASP and CAP, may have important implications for signal transduc-

tion. It opens the way to differential regulation of these isoforms *in vivo*. This is especially relevant in view of the fact that VASP seems to be downstream of a cAMP or cGMP signal transduction pathway (Nolte *et al.*, 1991) while PIP₂ is a substrate of PLC γ 1 and downstream of receptor tyrosine kinases (Rhee, 1991), and that in some cells (e.g. platelets) these pathways work in an antagonistic manner (see Halbrügge and Walter, 1993, and references therein). Alternatively, it may enable cells to direct actin assembly at different subcellular localizations through the different profilin isoforms.

Materials and methods

Protein preparation and peptide synthesis

We purified profilin I and II from bovine spleen and brain respectively, as described previously (Kaiser *et al.*, 1989; Cao *et al.*, 1992; Lambrechts *et al.*, 1995).

Peptides were synthesized on a model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. Peptides were purified using reversed phase HPLC, and the mass and purity were assessed by electrospray mass spectrometry (VG-platform, Fisons, UK).

Microfiltration

PIP₂ (Sigma) at a concentration of 1 mg/ml in H₂O was sonicated for 5 min to obtain micelles. Increasing concentrations of these micelles were incubated with either profilin I or II in 10 mM Tris–HCl, 75 mM KCl, 0.5 mM dithiothreitol (DTT) pH 7.5 for 30 min on ice. Afterwards, the samples were loaded on a Millipore filter with a molecular weight cut-off of 30 000 and centrifuged for 1 min at 2000 g. The flow-through was analysed on a 20% SDS–polyacrylamide gel, stained with Coomassie Brilliant Blue.

Circular dichroism

CD in the near UV region (250–320 nm) was done in a JASCO J-170 spectropolarimeter using a 1 cm pathway cell. The step resolution was 0.5 nm and the scan speed used was 20 nm/min. For each sample, the average of nine scans was obtained. The sample composition for the peptCAPwt binding assay is 15 μ M profilin (I or II) in 20 mM Tris–HCl, 0.7 mM EDTA pH 8.2 and 0, 20, 50 or 100 μ M peptCAPwt. In the PIP₂ binding assay we used samples containing 15 μ M profilin in 20 mM Tris–HCl pH 8.2, 0.7 mM EDTA with 150 or 300 μ M PIP₂. PIP₂ was sonicated for 10 min prior to addition of profilin and the mixture was incubated for 30 min at room temperature before measurement.

Fluorescence spectroscopy

Perelroizen *et al.* (1994) established that binding of poly-L-proline to profilin results in a change in fluorescence. Because the increase in fluorescence is a saturation function of the peptide concentration, we made a series of samples with an increasing amount of peptide (0–60 μ M) and a constant amount of profilin (1 μ M) in a total sample volume of 400 μ l. The buffer used was 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM DTT. The samples were incubated for 2 h at room temperature. Fluorescence was measured (excitation was at 275 nm and emission at 320 nm) with a SFM 25 fluorescence spectrophotometer (Kontron Instruments). Because the profilin isoforms have a different number of tyrosines (profilin I has four and profilin II seven), they have a different intrinsic fluorescence (F_0), and therefore we calculated the relative increase in fluorescence, defined as $(F-F_0)/F_0$.

Gel filtration

The Superdex 200 column (Pharmacia) was equilibrated with 20 mM Tris–HCl, 150 mM NaCl, pH 7.5. Profilin II samples in the same buffer were reduced with 40 mM DTT prior to addition of peptVASPwt, peptCAPwt or peptVASPs. Profilin and peptide were mixed at the concentrations and ratios indicated in the legend to Figure 4 and were incubated for 30 min at room temperature and then loaded on the column. The flow rate was 0.4 ml/min and detection was at 280 nm.

Poly-L-proline affinity chromatography

Samples (1 ml) containing 16 μ M profilin were loaded on a poly-L-proline column (0.5 ml) equilibrated with buffer A (20 mM Tris–HCl, 1 mM DTT, 1 mM EDTA, pH 7.5). After washing the column with 5 ml of buffer A, the remaining profilins were eluted with 5 ml of 8 M urea in buffer A (Figure 5A). In the parallel experiment, profilin was eluted with increasing concentrations of PIP₂ in buffer A, as indicated in Figure 5B.

Samples (1 ml) containing 10 μ M profilin and 250 μ M PIP₂ micelles in 10 mM Tris–HCl, 0.3 mM EDTA, 1 mM DTT, pH 7.5 were incubated for 30 min on ice and loaded on the poly-L-proline column. The elution protocol was the same as for the samples containing only profilin.

Profilin affinity chromatography

One milligram of bovine profilin I or II was coupled to 0.39 g of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's manual. In parallel, Sepharose columns were made without coupling any protein to the resin. The column was equilibrated with buffer B + 50 mM NaCl (buffer B = 20 mM NaH₂PO₄, 2 mM EDTA, 0.5 mM DTT pH 6.8).

Bovine brain extracts (20 ml) in 20 mM Tris–HCl pH 8.1, 50 mM NaCl, 5 mM DTT, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mg/l leupeptin [see Lambrechts *et al.* (1995) for detailed description] were first loaded on a Sepharose column to clear the extracts of non-specific binding proteins. The flow-through was then loaded onto a profilin I or II column. After washing the column with buffer B + 50 mM NaCl (15 ml), proteins were eluted with increasing concentrations of peptVASPwt as indicated in Figure 6. Then buffer B + 100 mM NaCl was added to elute most of the remaining proteins. Fractions were precipitated in 10% trichloroacetic acid, redissolved in sample buffer, loaded on SDS–polyacrylamide gels and Coomassie stained or used for Western blotting.

Western blotting

The VASP monoclonal antibodies (IE226, IE310 and IE273) were kindly provided by J. Wehland (GBF, Braunschweig) and U. Walter (University of Würzburg). The antibodies were raised against purified human platelet VASP (Halbrügge and Walter, 1989) according to the methods described (Niebuhr *et al.*, 1993; Abel *et al.*, 1996).

The proteins were electrophoretically blotted onto nitrocellulose mem-

branes applying 30 V for 4 h in 50 mM Tris, 50 mM borate buffer pH 8. The blots were blocked in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4) + 2% non-fat milk powder at room temperature for 1.5 h. The primary and secondary antibodies were diluted in PBS + 2% non-fat milk powder to the appropriate concentration. In between each step, the blots were washed five times in PBS. The primary antibody used was IE310. The secondary antibody was an alkaline phosphatase conjugate and the staining of the blots was done in 0.1 M NaHCO₃, 0.1 M MgCl₂ pH 9.8, nitroblue tetrazolium (Sigma) in 70% dimethylformamide and 5-bromo-4-chloro,3-indolylphosphate (Sigma) in dimethylformamide.

Immunoprecipitation

Bovine brain extract was incubated with an anti-VASP monoclonal antibody (IE273) for 2 h at room temperature. The antibodies were pelleted with protein G–Sepharose (Pharmacia). After three washes with buffer B, the Sepharose pellet was dissolved in sample buffer and loaded on an SDS–polyacrylamide gel. Detection of VASP was done with a second monoclonal antibody (IE310) after electroblotting and was processed as described above.

Model building of profilin II

We built a model of bovine profilin II starting from the known profilin I three-dimensional structure (Schutt *et al.*, 1993) using the software Homology (BIOSYM, 1994). The sequence identity (65%) of the two bovine isoforms is sufficiently high to use this homology building strategy. First, we aligned the primary sequence of profilin II with the profilin I sequence; no gaps or insertions needed to be introduced. The coordinates of all backbone atoms and atoms of the conserved side chain atoms of profilin I were then assigned to profilin II. Non-conserved side chains were positioned in their most probable conformation by using a rotamer library (Ponder and Richards, 1987). We successively minimized the energy of side chains in mutated loops, the mutated side chains in structurally conserved regions and finally all side chains. We assigned the AMBER force field to the profilin II model. Acidic and basic residues were charged –1 and +1 respectively. A charge of 0.5 was assigned to histidines. We used this model for molecular dynamics simulations ('DISCOVER', Biosym 1994) in vacuum to investigate the possible dynamic behaviour of the conformation of each isoform. We applied an energy minimization using a conjugated gradient optimizer whereby a non-bond cut-off algorithm smooths the interaction over a range from 15 to 16 Å. Throughout, we used an internal distance-dependent dielectric constant of 2. The structures of both profilins were equilibrated by running dynamics at 300 K during 10 ps. We explored the local conformation space during 300 ps at 300 K.

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