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Isolation and characterization of two mutants of human profilin I that do not bind poly(L-proline)

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Abstract A simple procedure for the isolation of profilin mutants having a reduced capacity to bind poly(L-proline) is used to isolate two mutants of human profilin I, W3N and H133S. Binding of the mutants to poly(L-proline), actin, and phosphatidylinositol (4,5)-bisphosphate (PIP2) was studied. Both mutations abolished the poly(L-proline)-binding activity of profilin. This suggests that the arrangement of the N- and C-terminal helices forming the poly(L-proline)-binding site depends on the stabilizing interaction between W3 and W31 in the underlying β-strand, and that the H133S mutation in the C-terminal helix also must have distorted the arrangement of the terminal helices.

Both mutations caused a reduced affinity for actin, with the W3N replacement having the most pronounced effect. This shows that structural changes in the poly(L-proline)-binding region of profilin can affect the distantly located actin-binding site. Thus, ligands influencing the structure of the poly(L-proline)-binding site may regulate actin polymerization through profilin. This is consonant with the finding that PIP2, which changes the tryptophan fluorescence in wild-type profilin and dissociates the profilin:actin complex in vitro, binds more strongly to the W3N mutant profilin. Thus, the poly(L-proline)-binding surface represents a crucial regulatory site of profilin function.

Key words: Profilin; Actin polymerization; Cytoskeleton control; Phosphoinositide-binding; Proline-binding

1. Introduction

The profilins are ubiquitous actin monomer-binding proteins thought to be of central importance in the regulation of the dynamics of the microfilament system [1]. The fact that the profilins specifically bind poly(L-proline) [2,3] and polyphosphoinositides [4] is consonant with this idea. Several proteins containing proline-rich sequence motifs, like the GP_5 motif [5] of the vasodilator-stimulated phosphoprotein (VASP), have recently been reported to bind to mammalian profilin I [6].

The poly(L-proline)-binding site on profilins is a well conserved surface consisting of aromatic and hydrophobic amino acid residues [7,8], which makes this binding site analogous to proline-recognizing surfaces of SH3 and WW domains. However, the poly(L-proline)-binding site on profilin is specific, since SH3 or WW domains bind to proline-rich sequences, but not to poly(L-proline) [9]. In addition to VASP and its homologues, Ena and Mena, in Drosophila [10,11], the poly(L-proline)-binding site on profilin appears to bind formins, which is a group of proteins involved in the control of several developmentally related processes such as polarized cell growth and cell patterning [6]. The significance of these interactions is unknown, but it has been proposed that they are important in directing profilin to regions where its influence on actin dynamics is required.

Thus, many observations implicate profilin in signal transduction as well as in the control of the microfilament system [12]. It is known that binding of polyphosphoinositides to profilin dissociates the profilin:actin complex [4,13], but in most respects the mechanisms by which the different binding activities influence the functioning of profilin remain to be explored. Recently, characterization of mammalian profilin mutants with altered binding affinities for actin and PIP2 were reported [14,15].

In the presence of gelsolin to block the (+)-end (barbed end) of filaments, profilin sequesters actin monomers efficiently, since the profilin:actin complex does not contribute to actin polymerization at the (−)-end (pointed end) of filaments [16]. When the (+)-end of the actin filament is free, the profilin:actin complex can be added to that end [16–18]. This results in a lowering of the concentration of free monomeric actin (A_{free}) at steady state as compared with the situation with actin alone where $A_{free} = A_{cc}$ (the critical concentration of polymerization) [18]. In the presence of thymosin β_4, low concentrations of profilin increase the level of F-actin at steady state, as if profilin catalyzed the transfer of actin monomers from the thymosin β_4:actin complex to actin filaments under these conditions [18,19]. Profilin also accelerates nucleotide exchange from actin [20,21], except in the case of plant profilins [22].

Studies of the biochemical properties of several *Acanthamoeba* profilin mutants and monoclonal antibodies to *Acanthamoeba* profilin were reported recently [23] confirming the location of the binding sites for actin and poly(L-proline) and the importance of the C-terminus for the stability of the profilins. In addition, structural requirements and thermodynamic parameters for the interaction of profilin with poly(L-proline) were described [24].

This paper presents a convenient, two-step procedure for the isolation of human profilin I from yeast cell extracts that is independent on the affinity of profilin for poly(L-proline). It allowed the isolation of two mutants, W3N and H133S, which lack poly(L-proline)-binding capacity [7]. The results of investigating the effect of wild-type and the two mutant profilins with respect to kinetics of actin polymerization, participation of the profilin:actin complex in the polymerization at the (+)-end, binding of PIP2, and nucleotide exchange, are described.

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2. Materials and methods

Oligodeoxyribonucleotide-directed mutagenesis of the human profilin gene was performed according to [25]. Mutant genes were sequenced by the dideoxy method [26] and subcloned into the *S. cerevisiae* expression vector [27] following standard methodology.

Fermenter cultures of recombinant yeast [28], expressing wild-type or mutant human profilin, were lysed in a bead mill in the presence of 5 mM KPO_4, pH 7.1, containing 5 mM KCl and protease inhibitors as reported previously [29]. The yeast extract, having a total volume of approximately 1500 ml, was clarified by centrifugation, filtered through glass wool, and then added to a column (70 ml) of phosphocellulose (P11, Whatman) equilibrated with 5 mM KPO_4, pH 7.1, containing 5 mM KCl and 0.5 mM DTT. After the whole sample had entered (approximately 5 h), the column was washed with the KPO_4-buffer until the absorbance at 280 nm of the flow-through was below 0.1. Bound proteins were then eluted with a 300 ml salt gradient of 5 to 400 mM KCl in 5 mM KPO_4-buffer, pH 7.1, containing 0.5 mM DTT. The flow rate was 60 ml per h and fractions of 3.5 ml were collected. After analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate SDS-PAGE [30], fractions containing profilin were combined into two separate pools (see Section 3), which were dialyzed against saturated ammonium sulfate to a final concentration of 80% saturation. The precipitated material was collected by centrifugation, dissolved in the low salt KPO_4-buffer containing 0.5 mM DTT, and further purified by gel filtration (Sephacryl S-200 (Pharmacia) on a column (1.5×80 cm) using a flow rate of 60 ml/h. Fractions containing profilin were combined, and stored at −80°C. The concentration of profilin was determined by UV-absorption at 280 nm using an $E^{1cm}_{mg/ml}$-value of 1.2 [31]. To correct for the change of extinction coefficient due to the amino acid replacement in W3N profilin, the absorption at 205 nm was used as described earlier [32].

Binding of profilin to poly(L-proline) was measured using a poly(L-proline)-Sepharose matrix. Samples of the different profilins at different concentrations were incubated separately with a fixed amount of poly(L-proline)-Sepharose. After thorough washing, the beads with associated profilin were boiled in SDS-PAGE sample buffer, and the resulting solution was analyzed by SDS-PAGE and densitometry of the stained profilin bands.

Phosphatidylinositol (4,5)-bisphosphate was isolated from calf brain by homogenization of 80 g of frozen brain tissue in the presence of 100 ml chloroform:methanol (1:2), followed by addition of 0.6 volumes of each of 1 M KCl and chloroform, and centrifugation at 5000 rpm for 3 min (Sorvall RC5, AH650 rotor). After centrifugation the lower phase of the sample was saved, while the inter- and upper phases were reextracted with one volume of chloroform and centrifuged once more. The lower phases were then combined, filtered through filterpaper (Munktell, grade 3), and extracted with one volume of a mixture of methanol:1 M HCl (1:1). The lower phase was collected, supplied with one volume of 200 mM ammoniumformate in methanol and then subjected to neomycin affinity chromatography for the isolation of PIP2 as described by Palmer [33]. The purity of the lipid was assessed by thin layer chromatography according to Palmer and its concentration was determined by phosphate analysis as described by Hess and Derr [34]. It was then dried in aliquots under nitrogen and stored at −80°C. Assay of profilin-binding to PIP2 micelles was performed as described in detail earlier [35,14]. In brief, profilin was incubated with the micelles at different molar ratios, and then the mixture was transferred to a Millipore PLTK filter with a molecular weight cut off of 30 kDa. The filters were washed with 400 µl of 10 mM KPO_4-buffer, pH 7.6, containing 80 mM NaCl, 0.1 mM $CaCl_2$, 0.12 mM EGTA and 1 mM DTT before use. The PIP2:micelle mixtures were centrifuged in an Eppendorf centrifuge until a fraction of the mixture had passed through the filter. The flow-through, containing profilin not bound to PIP2, was then analyzed for profilin by SDS-PAGE. For densitometry of the profilin bands after SDS-PAGE, the NIH Image software, version 1.60, was used.

The β/γ-actin was isolated from bovine profilin: β/γ-actin as described [36]. It was kept in 5 mM Tris-HCl-buffer, pH 7.6, containing 0.5 mM ATP, 0.1 mM $CaCl_2$ and 0.5 mM DTT (G-buffer), and the concentration was determined by absorbance measurements at 290 nm using $E^{mg/ml}_{1cm}$ of 0.63 [37]. Pyrenyl-labelling was performed as described in [38] except that the *N*-(1-pyrenyl)iodoacetamide was dissolved in dimethylsulfoxide (DMSO). The profilin-actin interaction studies were performed in the presence of 2% pyrenyl-labelled actin as described in detail elsewhere [39]. Briefly, for the polymerization studies 2 mM $MgCl_2$ or 100 mM KCl were used to initiate the polymerization of 12 µM actin in G-buffer in the absence or presence of equimolar concentrations of profilin. Filament formation was followed by monitoring the increase in pyrenyl fluorescence at 410 nm using a Fluoroskan II microplate reader (Labsystems) and an excitation wavelength of 365 nm. The measurements of steady-state filament concentration were performed in the presence of 2 mM $MgCl_2$ and 100 mM KCl. The samples containing different concentrations of actin were incubated in the presence and absence of 2 µM profilin until steady state was reached. To block (+)-end filament assembly, gelsolin (Sigma) was added to actin at a molar ratio of 1:330. Calculations of the dissociation constants (K_{app} and K_d), and the concentrations of unpolymerized free and profilin-bound actin (A_{free} and PA, respectively) were performed as described [18].

The increase in fluorescence when actin binds 1,N^6-ethenoadenosine 5′-triphosphate (εATP) was used to determine nucleotide exchange on the actin essentially following the method of Nishida [40] as described recently [39].

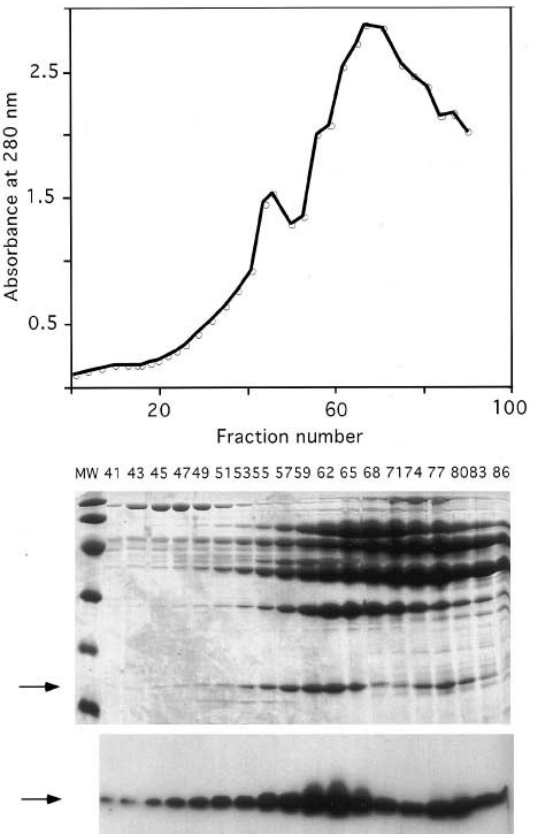

Fig. 1. Chromatography of a lysate of recombinant yeast cells containing W3N mutant human profilin I on phosphocellulose P11. The elution profile (upper panel) was generated as described in Section 2. Subsequent SDS-PAGE and Western blot analyses (middle and lower panels, respectively) of the fractions revealed two peaks of profilin (arrows) in fractions 54–65 and 75–83, corresponding to amino-terminally blocked and unblocked forms of the protein, respectively. Wild-type profilin gave rise to a similar elution profile, while H133S mutant profilin only gave rise to the second pool of material. Molecular weight markers are denoted MW and represent from top to bottom; 94, 67, 43, 30, 20.1 and 14.4 kDa. The W3N mutant profilin migrates somewhat slower than wild-type profilin on SDS-PAGE [7].

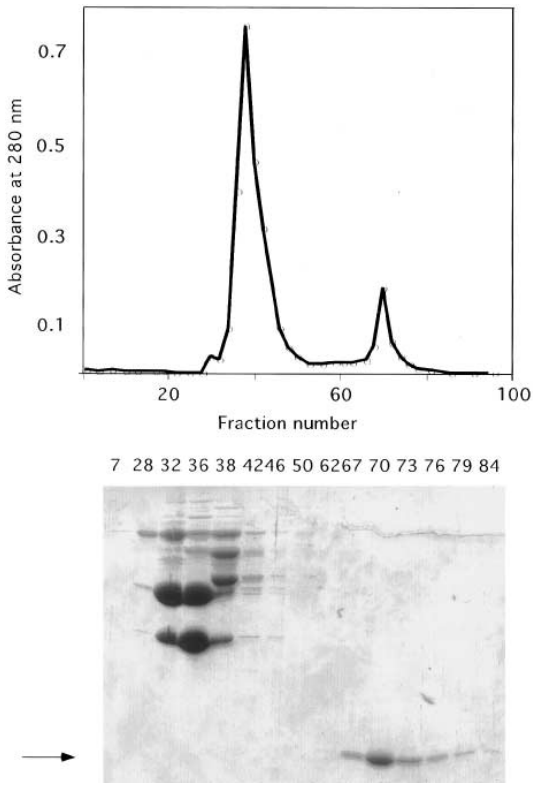

Fig. 2. Isolation of W3N profilin by gel filtration on Sephacryl S-200 of profilin-containing material obtained by P11 ion exchange chromatography (Fig. 1, fractions 75–83). The upper panel shows the elution profile, and the lower panel the SDS-PAGE analysis of collected fractions. The position of profilin is indicated by the arrow. Similar profiles were obtained with the other profilin preparations.

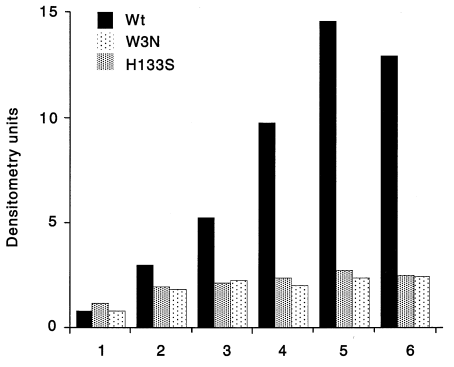

Fig. 3. Histogram illustrating the poly(L-proline)-binding expressed by wild-type and mutant profilins. As described in Section 2, the different profilins were incubated with poly(L-proline)-Sepharose in 3 series of 6 samples containing increasing concentrations of protein. After washing, the amount of profilin bound to the Sepharose beads was determined by SDS-PAGE and densitometry of the resulting profilin band. Filled bars represent the densitometry values obtained for wild-type profilin, while those observed for W3N and H133S mutant profilin are illustrated with open and grey bars, respectively. In the first group of bars (group 1) the corresponding samples contained 1 μmol profilin; in group 2, the amount of profilin was 5 μmol; in 3, 10 μmol; in 4, 48 μmol; and in 5, and 6, it was 72 and 97 μmol, respectively.

3. Results

3.1. Isolation of profilin

Extracts of yeast cells expressing wild-type human profilin I and the W3N profilin mutant, respectively, both gave rise to two broad peaks containing profilin, when fractionated on phosphocellulose as revealed by SDS-PAGE and Western blot analysis of the fractions (Fig. 1). The H133S profilin mutant, on the other hand, gave rise to only one peak, which was eluted from the phosphocellulose in a position corresponding to the second peak of wild-type profilin. To separate the profilin from components of higher molecular weight, the protein from the profilin-containing fractions was chromatographed on Sephacryl S-200. As shown in Fig. 2, this step led to the isolation of recombinant profilin.

Separations of yeast-expressed profilin into two forms, one with a blocked and one with an unblocked N-terminal, was described earlier [14,41]. It was confirmed here by protein sequencing that the profilin isolated from the first and second peak obtained in the phosphocellulose step, contained N-terminally blocked and unblocked profilin, respectively. Acetylation is the most likely posttranslational modification of profilin [42]. The protein sequencing also confirmed that the W3N amino acid replacement had taken place. The single peak of H133S profilin contained unblocked profilin.

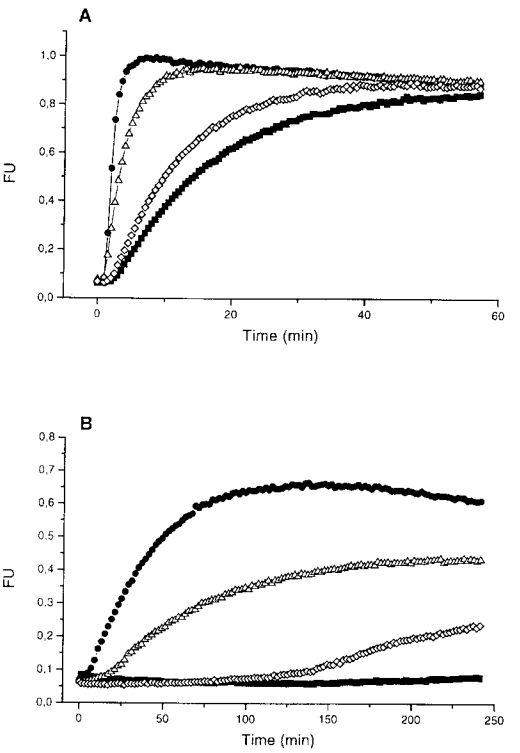

Fig. 4. Polymerization of β/γ-actin in the absence and presence of wild-type and mutant profilins. Equimolar concentrations actin and profilin (12 μM) in G-buffer were polymerized by the addition of $MgCl_2$ (panel A) or KCl (panel B) to final concentrations of 2 and 100 mM, respectively. The subsequent formation of filamentous actin was followed by monitoring the increase in pyrenyl fluorescence due to the presence of 2% pyrenyl-labeled β/γ-actin in the samples. Filled circles, actin alone; open triangles with W3N mutant profilin present; open diamonds, H133S profilin; and closed diamonds, wild-type profilin.

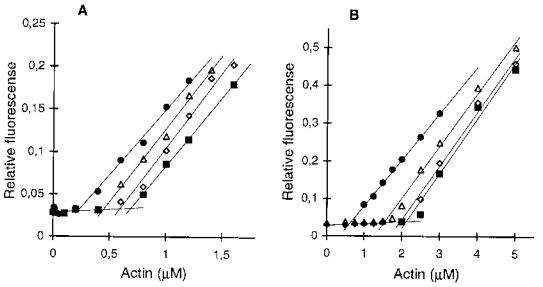

Fig. 5. The effect of wild-type and mutant profilins on the steady-state concentration of filamentous actin, in the absence and presence of gelsolin (panels A and B, respectively). Serial dilutions of polymerized actin in G-buffer, containing 2 mM $MgCl_2$ and 100 mM KCl, and the different profilins at 2 μM, were assayed for pyrenyl fluorescence after overnight incubation at room temperature. Gelsolin (panel B) was added to actin at a molar ratio of 1:330. Regression curves were fitted to the values obtained, and the concentrations of total unpolymerized actin (free and profilin-bound) were determined for the different cases as described in the text. Closed circles, actin alone; closed squares, in the presence of wild-type profilin; open triangles, W3N mutant profilin; and open diamonds, H133S profilin.

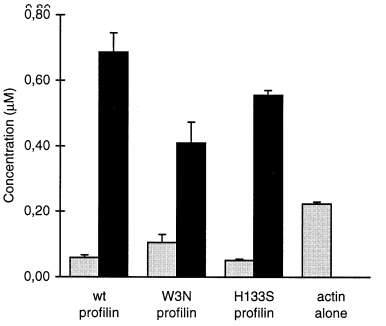

Fig. 6. Steady-state concentrations of unpolymerized free (open bar) and profilin-bound actin (closed bar) in the presence of the different profilins. The values were calculated using the results presented in Table 1. The concentration of free actin (A_{free}) in the absence of profilin (= A_{cc}) was 0.22 μM, and the ability of profilin to sequester monomeric actin and to participate in (+)-end monomer assembly changed due to the amino acid replacements as shown by the histogram.

3.2. Profilin:poly(L-proline) interaction

To compare the capacity of wild-type and mutant profilins to bind poly(L-proline), samples of the different profilins were incubated separately with a fixed amount of poly(L-proline)-Sepharose. The amount of profilin that bound to the matrix was analyzed as described in Section 2. With wild-type profilin, the staining intensity of the profilin band increased with the amount of profilin added to the poly(L-proline)-Sepharose (Fig. 3). When the two mutants were analyzed, the amount of matrix-bound profilin remained at a low level regardless of the amount of protein added. This showed that the isolated W3N and H133S mutant profilins did not bind to poly(L-proline)-Sepharose in agreement with previous observations [7].

3.3. Profilin:actin interaction

3.3.1. Polymerization kinetics. The effects of profilin on polymerization kinetics are shown in Fig. 4A. Actin alone (12 μM) polymerized with a short lag phase (< 1 min), and reached steady-state level of polymerization 3–4 min after addition of 2 mM $MgCl_2$. The rate of elongation was 0.45 fluorescence units per min (FU/min). In the presence of equimolar concentration of wild-type profilin, the onset of filament formation was delayed by a lag phase of 2–3 min and the elongation rate was reduced to 0.04 FU/min, illustrating the interference with the polymerization process. With the W3N mutant profilin, there was no effect on the lag phase, but the elongation rate was slower (0.19 FU/min) than with actin alone. The H133S profilin delayed the onset of polymerization like wild-type profilin, and the elongation rate was slowed down (0.06 FU/min), but not as much as with the non-mutated profilin. Thus the H133S mutation interfered less with the activity of profilin than the W3N replacement did. Similar results were obtained when the polymerization was induced with 100 mM KCl (0.1 mM Ca^{2+}), although the effects of the profilins appeared more pronounced under these conditions (Fig. 4B).

3.3.2. Steady-state experiments. Fig. 5A and B show the effects of 2 μM of the different profilins on the level of F-actin at various concentrations of actin at steady state of polymerization, without and with gelsolin added, respectively. From the intercepts between the lines representing unpolymerized and filamentous actin obtained in the presence of gelsolin, the equilibrium dissociation constants, K_d, of the profilin:actin complexes can be calculated [18]. Similarly, the apparent dissociation constants, K_{app}, are obtained from the results recorded in the absence of gelsolin. These latter values represent the situation where profilin:actin and free actin both participate in polymer growth. The K_d and K_{app} for the different profilin:actin complexes are given in Table 1 together with the respective steady-state concentrations of free actin monomers (A_{free}) and profilin:actin (PA). It is clear also from these analyses (Table 1) that the W3N mutation in profilin affected the profilin:actin interaction more than the H133S mutation. The K_d obtained with profilin containing the latter mutation (0.32 μM) was close to that of wild-type profilin (0.28 μM), whereas that of the W3N mutant profilin was significantly higher (0.93 μM) indicating a strong interference with complex formation. With all three profilins, the concentration of A_{free} at steady state in the absence of gelsolin was significantly

Table 1
The steady-state concentrations of free actin monomers and profilin:actin complexes and the K_d and K_{app} for the profilin:actin interaction determined in the presence and absence of gelsolin, respectively

Profilin	Capped (+)-ends			Uncapped (+)-ends		
	K_d	A_{free}	PA	K_{app}	A_{free}	PA
–	–	0.58	–	–	0.22	–
	–	0.42	–	–	0.28	–
Wild-type	0.26	0.58	1.38	1.66	0.05	0.64
	0.30	0.42	1.16	1.37	0.07	0.73
W3N	0.76	0.58	0.87	3.68	0.08	0.37
	1.09	0.42	0.43	2.35	0.14	0.45
H133S	0.30	0.58	1.32	2.14	0.05	0.56
	0.34	0.42	1.11	2.10	0.06	0.57

Experimental data shown in Fig. 5 were used to derive the values given here. The K_d and K_{app} values were calculated from the formula $K = P_{free}A_u/PA$, where P_{free} ('free' profilin) = $P_{added} - PA$, and $PA = A_u - A_{cc}$, where A_u is total unpolymerized actin, and A_{cc} is critical concentration for polymerization. A_{free} and PA in the absence of gelsolin were calculated using the following formula [18]: $A_{free} = \{A_u - P_{added} - K_d + \sqrt{((P_{added} + K_d - A_u)^2 + 4K_dA_u)}\}/2$. This takes into account that A_{free} in the absence of gelsolin may vary from A_{cc} due to participation of profilin-bound actin in monomer assembly at (+)-ends.

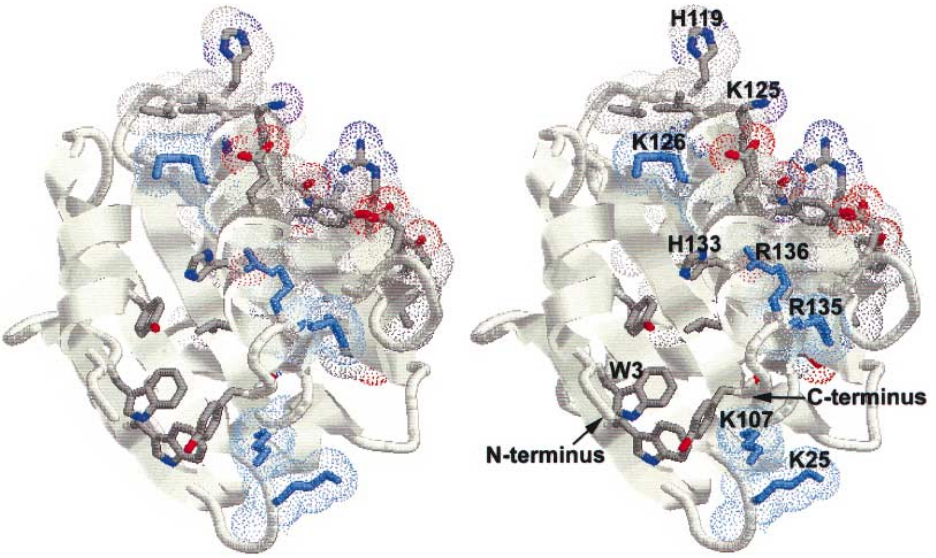

Fig. 7. Illustration of the actin-, poly(L-proline)-, and putative PIP2-binding sites of human profilin I. The structure of human profilin I [51] is shown with the actin-binding residues visualized as CPK-coloured sticks with clouds of dots around, the putative PIP2-binding residues as cyan-coloured sticks with clouds around, and finally poly(L-proline)-binding residues as CPK-coloured sticks between the N- and C-terminal helices.

lower than that with actin alone (where $A_{free} = A_{cc}$). It is noted that the H133S mutant profilin, which only had a slightly changed K_d, had an effect on A_{free} that anything was even more pronounced than that of wild-type profilin. This illustrates that the participation of profilin:actin in (+)-end filament growth was reduced by the W3N replacement, while it remained unaffected by the H133S mutation (Fig. 6).

None of the mutations introduced into profilin here altered the capacity of the profilin to accelerate the exchange of nucleotide on actin monitored as a change in fluorescence when ATP on actin is exchanged with εATP (data not shown).

3.4. Profilin:PIP2 interaction

The binding of PIP2 to profilin was studied using a filtration assay, where free profilin is separated from profilin bound to PIP2 micelles. Analysis of filtrates containing free profilin by SDS-PAGE showed that both profilin mutants could bind the micelles. Densitometry of the resulting profilin bands indicated that W3N profilin had a higher affinity for the lipid compared to wild-type profilin (Table 2), while the H133S mutant profilin was unchanged in this respect.

Table 2
Binding of profilin mutants to PIP2 as compared to binding of wild-type profilin

Profilin/PIP2 (mole/mole)	W3N wild-type/mutant	H133S wild-type/mutant
1:0	1.13	1.06
1:10	1.97	1.04
1:20	2.93	0.97
1:35	–	0.98

The assay measures the amount of non-PIP2-bound profilin as described in Section 2. To illustrate the binding of PIP2 by the mutants relative to wild-type profilin, the values obtained with the wild-type protein were divided by the corresponding values obtained with mutant proteins (wild-type/mutant). In samples containing 35 mole of PIP2 per mole of the W3N mutant protein, there was no detectable profilin in the flow-through, i.e. all had bound to the PIP2 micelles. The values given are averages of two experiments.

4. Discussion

It was shown earlier that the W3N and H133S mutant profilins are unable to bind to poly(L-proline) [7]. The present investigation provides an easy method that might be useful for the isolation not only of profilin variants that cannot bind poly(L-proline), but also of other mutant and wild-type profilins. It also separates profilin with a blocked N-terminus from that having a free N-terminus. Even though preliminary results did not reveal any differences between profilin molecules with a blocked and unblocked N-terminus with respect to the activities of the profilins in the actin polymerization assays (not shown), it may turn out to be important in the interaction of profilin with other proteins. Interestingly, this post-translational modification of profilin seemed to be more effective on W3N than on both wild-type and H133S mutant profilin (not shown).

Profilin is involved in the regulation of actin polymerization as a sequesterer of actin monomers, and it participates in (+)-end filament growth as profilin:actin [18,22]. In addition, the binding of proteins containing proline-rich sequences and of polyphosphoinositides may modulate the function of profilin as an actin-regulating protein.

Solving the profilin:β-actin crystal structure revealed two sites of interaction between profilin and actin. The most extensive interaction buries 2260 Å^2 solvent-accessible surface area (b.s.a.), and forms the profilin:actin complex in solution [43–45,23]. It involves the first half of the C-terminal helix (α4), with a crucial residue being K125, the exposed β-sheet consisting of strands β5, β6, and β7, and helix α3. The second, less extensive interaction site (1187 Å^2 b.s.a.) is located on the opposite side of the profilin molecule and involves residues in the beginning of the N-terminal helix. There is no direct evidence, as yet, that this second actin-binding site plays a role in the functioning of profilin vis-à-vis actin [45]. However, the binding site for poly(L-proline) is located to this side of the profilin molecule.

Poly(L-proline) binds to a conserved feature of the profilin molecule consisting of a cluster of aromatic and hydrophobic residues at the interface between the ends of the N- and C-terminal helices (Fig. 7). It consists of W3, Y6 on the N-terminal helix (α1) and W31 (adjacent to W3) on strand 2 on one side, and L134, H133, and Y139 on the C-terminal helix on the other [44]. Presumably, this is the site where the profilin-binding protein VASP [5] and its homologues ENA and Mena interact with profilin via their GP_5 sequence motifs [10,11]. Binding of poly(L-proline) does not influence the affinity nor the rate of association of actin monomers to profilin, and it does not affect the promotion of actin assembly by profilin in the presence of thymosin β_4 [45]. What effect binding of VASP or being homologues have on the structure and function of profilin or the profilin:actin complex remains to be elucidated.

The W3 is centrally located in this aromatic/hydrophobic cluster of residues, and the introduction of an asparagine residue (W3N mutation) disrupts the poly(L-proline)-binding site. Why this structural change affected the binding of the profilin to actin is unclear. It is possible, however, that the W3N substitution destabilizes the entire N-terminal helix, whose interaction with the underlying β-sheet and the adjacent C-terminal helix is hydrophobic in nature, except for a hydrogen bond between K126 and T15. Disruption of the relationship between the N- and C-terminal helices could influence the spatial location of actin-binding amino acid residues in the beginning of the C-terminal helix and thereby cause the change in actin-binding observed here. Furthermore, the binding of antibodies directed against the tryptophanes in the poly(L-proline)-binding site have been reported to enhance the binding of *Acanthamoeba* profilin to actin [23], also pointing to a structural relationship between these sites on the profilin molecule. Ligands interacting with profilin at this site may therefore affect its actin-binding properties.

The W3N amino acid replacement caused a reduced affinity for actin and an increased PIP2-binding, pointing to a close relationship also between the actin- and PIP2-binding surfaces. The PIP2-binding site of mammalian profilin I (Fig. 7) has been suggested to involve a number of basic residues (K25, K107, K126, H133, R135, and R136) partially encircling the poly(L-proline)-binding site [44]. Binding of PIP2 to profilin changes the environment of the two tryptophanes of profilin [48] and there is a PIP2-induced exposure of S137 to phosphorylation by protein kinase C [49]. Dissociation of the profilin:actin complex [4] by PIP2 could be due to interference with the basic residues K125 and K126 in the primary profilin:actin interaction [43,39], but perturbations of the N- and C-terminal helices could also be involved. The introduction of a polar residue, W3N, in the nearness of the implicated site might explain the increase in PIP2-binding observed. The involvement of R88 and R74 in the binding of PIP2 suggested in other studies [15,35] is more difficult to explain since these amino acid residues are buried in the interface between profilin and actin, and direct binding studies have shown that a tertiary complex between profilin:actin and PIP2 micelles can form [4].

The W3N and H133S mutations did not abolish the participation of the respective profilin:actin complexes in actin polymerization, Fig. 6 and Table 1. The A_{free} values obtained in the presence of wild-type, as well as the mutant profilins, were significantly decreased as compared with A_{free} ($=A_{cc}$) for actin alone. However, the A_{free} for the W3N mutant profilin did not decrease as much as with the other two profilins. This is reasonable since this mutant bound actin with a higher K_d. It may also reflect a lower affinity for the (+)-end of the filament of this mutant profilin:actin. The latter appears to agree with the fact that, in the kinetic experiments, this mutant had no effect on the nucleation of actin while it slowed down the rate of elongation. This suggests that interaction with W3N profilin alters the (+)-end binding surface on the actin monomer, since actin associated to wild-type profilin becomes incorporated at the (+)-end with the same rate constant as free actin [22].

Evidence that the C-terminal helix is important for the stability of profilin has been given earlier [23,46]. However, although the H133 is important for binding of poly(L-proline) [7] and GP_5 motifs [47], it does not seem to be crucial to the interaction between the N- and C-terminal helices (Fig. 7), which may be the reason why the H133S mutation only slightly affected the affinity of profilin for actin. In fact, removal of eight C-terminal amino acid residues does not preclude the binding of the modified profilin to actin [23]. However, it was shown recently that profilin becomes phosphorylated on two residues in the poly(L-proline)-binding region, S137 and Y139, by kinases present in an EGF-receptor preparation obtained by immunoabsorbtion from lysates of EGF-stimulated cells [50]. This C-terminal phosphorylation interferes with the binding of poly(L-proline) to profilin. Thus, the surface formed by the N- and C-terminal helices is of great interest, since it appears to be the site where regulation of the activities exhibited by profilin takes place.

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References

[1] Machesky, L.M. and Pollard, T.D. (1993) Trends Cell Biol. 3, 381–385.
[2] Tanaka, M. and Shibata, H. (1985) Eur. J. Biochem. 151, 291–297.
[3] Lambrechts, A., Van Damme, J., Goethals, M., Vandekerckhove, J. and Ampe, C. (1995) Eur. J. Biochem. 230, 281–286.
[4] Lassing, I. and Lindberg, U. (1985) Nature 314, 472–474.
[5] Reinhard, M., Giehl, K., Abel, K., Haffer, C., Jarchau, T., Hoppe, V., Jockusch, B.M. and Walter, U. (1995) EMBO J. 14, 1583–1589.
[6] Frazier, J.A. and Field, C.M. (1997) Curr. Biol. 7, R414–R417.
[7] Björkegren, C., Rozycki, M., Schutt, C.E., Lindberg, U. and Karlsson, R. (1993) FEBS Lett. 333, 123–126.
[8] Thorn, K.S., Christensen, H.E.M., Shigeta Jr., R., Huddler Jr., D., Shalaby, L., Lindberg, U., Chua, N.-H. and Schutt, C.E. (1996) Structure 5, 19–32.
[9] Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D. (1993) Nature 259, 1157–1161.
[10] Gertler, F.B., Comer, A.R., Juang, J.-L., Ahern, S.M., Clark, M.J., Liebl, E.C. and Hoffmann, F.M. (1995) Genes Dev. 9, 521–533.
[11] Gertler, F.B., Niebuhr, K., Reinhard, M., Wehland, J. and Soriano, P. (1996) Cell 87, 227–239.
[12] Sohn, R.H. and Goldschmidt-Clermont, P.J. (1994) BioEssays 16, 465–472.
[13] Lu, P.-J., Shieh, W.-R., Rhee, S.G., Yin, H.L. and Chen, C.-S. (1996) Biochemistry 35, 14027–14034.

[14] Hájková, L., Björkegren Sjögren, C., Korenbaum, E., Nordberg, P. and Karlsson, R. (1997) Exp. Cell Res. 234, 66–77.
[15] Sohn, R.H., Chen, J., Koblan, K.S., Bray, P.F. and Goldschmidt-Clermont, P.J. (1995) J. Biol. Chem. 270, 21114–21120.
[16] Pollard, T.D. and Cooper, J.A. (1984) Biochemistry 23, 6631–6641.
[17] Pring, M., Weber, A. and Bubb, M.R. (1992) Biochemistry 31, 1827–1836.
[18] Pantaloni, D. and Carlier, M.-F. (1993) Cell 75, 1007–1014.
[19] Carlier, M.-F. and Pantaloni, D. (1994) Sem. Cell Biol. 5, 183–191.
[20] Mockrin, S.C. and Korn, E.D. (1980) Biochemistry 19, 5359–5362.
[21] Goldschmidt-Clermont, P.J., Furman, M.I., Wachsstock, D., Safer, D., Nachmias, V.T. and Pollard, T.D. (1992) Mol. Biol. Cell 3, 1015–1024.
[22] Perelroizen, I., Didry, D., Christensen, H., Chua, N.H. and Carlier, M.-F. (1996) J. Biol. Chem. 271, 12302–12309.
[23] Kaiser, D.A. and Pollard, T.D. (1996) J. Mol. Biol. 256, 89–107.
[24] Petrella, E.C., Machesky, L.M., Kaiser, D.A. and Pollard, T.D. (1996) Biochemistry 35, 16535–16543.
[25] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[26] Sanger, F., Niclen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[27] Karlsson, R. (1988) Gene 68, 249–257.
[28] Aspenström, P. and Karlsson, R. (1991) Eur. J. Biochem. 200, 35–41.
[29] Aspenström, P., Lassing, I. and Karlsson, R. (1991) J. Muscle Res. Cell Motil. 12, 201–207.
[30] Matsudaira, P.T. and Burgess, D.R. (1978) Anal. Biochem. 87, 386–396.
[31] Larsson, H. and Lindberg, I. (1988) Biochim. Biophys. Acta 953, 95–105.
[32] Scopes, R.K. (1994) Protein Purification. Principles and Practice, 3rd Edn., Springer-Verlag, New York.
[33] Palmer, F.B.St.C. (1981) J. Lipid Res. 22, 1296–1300.
[34] Hess, H.H. and Derr, J.E. (1975) Anal. Biochem. 63, 607–613.
[35] Haarer, B.K., Petzold, A.S. and Brown, S.S. (1993) Mol. Cell. Biol. 13, 7864–7873.
[36] Lindberg, U., Schutt, C.E., Hellsten, E., Tjäder, A.-C. and Hult, T. (1988) Biochim. Biophys. Acta 967, 391–400.
[37] Houk Jr., T.W. and Ue, K. (1974) Anal. Biochem. 62, 66–74.
[38] Kouyama, T. and Mihashi, K. (1981) Eur. J. Biochem. 114, 33–38.
[39] Korenbaum, E., Nordberg, P., Björkegren-Sjögren, C., Lindberg, U. and Karlsson, R. (1997), submitted.
[40] Nishida, E. (1985) Biochemistry 24, 1160–1164.
[41] Aspenström, P., Schutt, C.E., Lindberg, U. and Karlsson, R. (1993) FEBS Lett. 329, 163–170.
[42] Nyström, L.-E., Lindberg, U., Kendrick-Jones, J. and Jakes, R. (1979) FEBS Lett. 101, 161–165.
[43] Schutt, C.E., Myslik, J.C., Rozycki, M., Goonesekere, N.C.W. and Lindberg, U. (1993) Nature 365, 810–816.
[44] Cedergren-Zeppezauer, E.S., Goonesekere, N.C.W., Rozycki, M.D., Myslik, J.C., Dauter, Z., Lindberg, U. and Schutt, C.E. (1994) J. Mol. Biol. 240, 459–475.
[45] Perelroizen, I., Marchand, J.-B., Blanchoin, L., Didry, D. and Carlier, M.-F. (1994) Biochemistry 33, 8472–8478.
[46] Malm, B., Larsson, H. and Lindberg, U. (1983) J. Muscle Res. Cell Motil. 4, 569–588.
[47] Kang, F., Laine, R.O., Bubb, M.R., Southwick, F.S. and Purich, D.L. (1997) Biochemistry 36, 8384–8392.
[48] Raghunanthan, V., Mowery, P., Rozycki, M., Lindberg, U. and Schutt, C.E. (1992) FEBS Lett. 297, 46–50.
[49] Singh, S.S., Chauhan, A., Murakami, N., Styles, J., Elzinga, M. and Chauhan, V.P.S. (1996) Recept. Signal Transduct. 6, 77–86.
[50] Björkegren-Sjögren, C., Rönnstrand, L., Wernstedt, C., Kullander, K. and Karlsson, R. (1997), submitted.
[51] Fedorov, A.A., Pollard, T.D. and Almo, S.C. (1994) J. Mol. Biol. 241, 480–482.