Localization of a Binding Site for Phosphatidylinositol 4,5-Bisphosphate on Human Profilin*

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Profim is a small 12–15-kDa actin-binding protein, which in eukaryotic organisms is ubiquitous and necessary for normal cell growth and function. Although profilin's interactions with its three known ligands (actin monomers, phosphatidylinositol 4,5-bisphosphate (PIP2), and poly-L-proline (PLP)) have been well characterized in vitro, its precise role in cells remains largely unknown. By binding to clusters of PIP2, profilin is able to inhibit the hydrolysis of PIP2 by phospholipase Cγ1 (PLCγ1). This ability is the result of profilin's affinity for PIP2, but the specific residues of profilin's amino acid sequence involved in the binding of PIP2 are not known. Using site-directed mutagenesis, we sought to localize regions of profilin important for this interaction by generating the following mutants of human profilin (named according to the wild-type amino acid altered, its position, and the amino acid substituted in its place): Y6F, D8A, L10R, K25Q, K53I, R74L, R88L, R88L/K90E, H119D, G121D, and K125Q. With the exception of L10R, all of the mutants were successfully expressed in Escherichia coli and purified by affinity chromatography on PLP-Sepharose. Only Y6F and K25Q demonstrated moderately less stringent binding to PLP, indicating that most of the mutations did not induce marked alterations of profilin's structure. When tested for their relative abilities to inhibit the hydrolysis of PIP2 by PLCγ1, most of the mutants were indistinguishable from wild-type profilin. Exceptions included D8A, which demonstrated increased inhibition of PLCγ1, and R88L, which demonstrated decreased inhibition of PLCγ1. To assess the importance of the region surrounding residue 88 of human profilin, three synthetic decapeptides selected to correspond to non-overlapping stretches of the human profilin sequence were tested for their abilities to inhibit PLCγ1. We found that only the decapeptide that matched the peptide stretch centered around residue 88 was able to inhibit PLCγ1 activity substantially and was able to do so at nearly wild-type profilin levels. Taken together with the finding that mutating residue 88 resulted in decreased inhibition of PLCγ1 activity, these data provide strong evidence that this region of human profilin represents an important binding site for PIP2.

Since the molecule's discovery 20 years ago (1), profilin's interactions with its three ligands (actin monomers, phosphatidylinositol 4,5-bisphosphate (PIP2), and PLP) have been well established through in vitro studies (for review, see Ref. 2). For example, by binding to actin monomers in a 1:1 complex, profilin decreases the critical concentration of monomeric actin in the presence of thymosin β4 (3), inhibits the spontaneous nucleation of actin filaments (4), and catalyzes the exchange of adenosine nucleotides bound to actin monomers (5). By binding to PIP2 and to a lesser degree its precursor PIP (6), profilin prevents PLCγ1 from hydrolyzing PIP2 (7). However, when PLCγ1 is phosphorylated on specific tyrosine residues, such as the effects of profilin and hydrolyze PIP2 (8). Since PIP2 binding to profilin precludes the formation of profilin–actin complexes, one can conjecture that in resting cells, PIP2 sequesters profilin from actin and that upon growth factor-induced cell activation, profilin is released from PIP2 by the hydrolytic actions of phosphorylated PLCγ1 and diffuses freely to the actin cytoskeleton, where it then exerts effects as a regulator of actin polymerization.

How PIP2 is able to displace actin so effectively remains unclear, and indeed, efforts to localize a binding site for PIP2 on profilin have been supplanted until only recently by the more extensive efforts to identify the binding site for actin. The quest for the latter began as early as 1982 with biochemical studies involving peptidases applied to actin (9), whereas the first mention of a putative binding site for PIP2 on profilin did not occur until 1991 when Pollard and Rimm (10) noted that the charge differences between Acanthamoeba profilin-I and -II occur between residues 24 and 66 (corresponding to residues 25–69 of human profilin) and that a polylysine region exists between residues 80 and 115 (corresponding to residues 88–126 of human profilin). These regions were supposed to be involved in PIP2 binding because first, positively charged residues are assumed to be involved in the binding of acidic head groups of PIP2 and second, the more positively charged isoform, profilin-II, has ~100 times greater affinity for PIP2 (11).

A year later, Yu et al. (12) implicated the region spanning residues 126–136 of human profilin as a binding site for PIP2.

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† The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; PLP, poly-L-proline; PIP, phosphatidylinositol 4-monophosphate; PLC, phospholipase C; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; G, globular; F, filamentous; eATP, 1N9-ethenoadenosine 5'-triphosphate.
by proposing the sequence KXXXXXXHXXR to be a modification of the KXXXXXXK and KXXXXXXR motifs of gelsolin, which by themselves bind to PIP2 (12). These motifs are also found in CapG, villin, coflin, and the PLC family (12), all of which bind PIP2. Another region was implicated by Raghunathan et al. (13), who used circular dichroism spectroscopy to show that binding of profilin to PIP2 resulted in marked fluorescence quenching of Tyr-3 and Tyr-31. However, they also showed through circular dichroism spectroscopy that upon binding to PIP2, profilin undergoes a significant conformational change involving an increase in α-helical content from a base line of 5% to one as high as 35%. This true, being it is difficult to know whether the changes in the fluorescence of Tyr-3 and Tyr-31 are due simply to the proximity of a binding site or due to local conformational changes transmitted from a distant binding site.

Finally, Vinson et al. (14), in elucidating the three-dimensional structure for Acanthamoeba profilin-I, proposed a PIP2-binding site consisting of the loop between β-strands 1 and 2, the loop between β-strands 6 and 7, and the region immediately after α-helix 2 (see Fig. 1B). However, Fedorov et al. (15) recently showed through calculations of electrostatic surface potentials that a second distinct region of positive potential present on Acanthamoeba profilin-II but markedly less so on profilin-I is located on the opposite side of the protein. The positively charged residues here include Arg-66, Arg-71, Lys-69, Arg-74, Arg-88, Lys-90, and Lys-125 of human profilin (16).

To further define the importance of the residues to be involved in PIP2 binding, we mutated single base pairs distributed across the human profilin cDNA sequence, corresponding to the substitution of several conserved residues on both terminal α-helices (Tyr-6, Asp-8, Leu-10, His-119, Gly-121, Lys-125), basic residues implicated by Vinson et al. (14) (Lys-25, Lys-53), and basic residues located on the second region of positive potential identified by Fedorov et al. (15) (Arg-74, Arg-88, Lys-90). Here, we report the results of our testing these mutations for the effects they have on profilin’s ability to inhibit the hydrolysis of PIP2 by PLC1, an effect that has been shown previously to correlate precisely with profilin’s affinity for PIP2 (11). We found that most of the mutations did not significantly alter profilin’s ability to inhibit PLC1 activity. However, the mutation D8A caused a marked increase of PLC1 inhibition, and R88L resulted in a marked decrease of PLC1 inhibition. Based on our work, we propose that a crucial binding site for PIP2 on human profilin is contained within five amino acids of residue 88 since this stretch, by itself, inhibits PLC1 activity as well as the entire mole of profilin.

**EXPERIMENTAL PROCEDURES**

DNA Techniques—The DNA for human profilin was obtained from David J. Kwiatkowski (17) and subcloned into pTrc99A (Pharmacia Biotech Inc.), a prokaryotic expression vector. To generate mutant profilins, single amino acid substitutions were introduced through single base pair changes using a previously described method for site-directed mutagenesis using mutagenic primers, T4 DNA polymerase, and T4 DNA ligase (18). Identities of the resultant mutants were confirmed by DNA sequencing kit. Before expressing the profilins in bacteria, the DNA was first subcloned into pMW172 (constructed by Michael Way) to allow higher levels of expression.

Protein Purification—Escherichia coli BL21(DE3) cells transformed with plasmids containing the appropriate cDNAs were grown in 1-liter cultures to an optical density (at λ = 600 nm) of 0.6–0.7, at which point expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside. The cells were incubated for an additional 4 h before harvesting by centrifugation. Subsequent steps were performed at 0–4°C. To each cell pellet, 100 ml of lysing buffer (6 M urea, 145 mM NaCl, 0.1 mM MgCl2, 15 mM HEPES, 10 mM EGTA, 1 mM sodium vanadate, 0.5% Triton X-100, 30 μg/ml leupeptin and 1 mM 4-(2-aminophenyl)benzenesulfonyl fluoride) was added, and the cells were resuspended by sonication (Sonifier 450, Branson). The lysates were clarified by centrifugation at 6,000 × g for 15 min and dialyzed (Spectra/Por membrane, molecular weight cutoff, 6–8,000) for 72 h against three changes of Dulbecco’s phosphate-buffered saline, pH 7.1, supplemented with 0.5 mM DTT. The dialyzed vesicles were then filtered through a 0.2-μm filter and applied separately to a 20-ml column of PLP-linked Sepharose, to which profilins are known to bind with high specificity (19). For each profilin mutant, the column was washed with 200 ml of PLP buffer (10 mM Tris, pH 7.8, 0.1 mM NaCl, 0.1 mM glycine, 0.01 mM DTT), followed by 100 ml of PLP buffer containing 3.5 mM urea. Each profilin was then eluted with 100 ml of PLP buffer containing 7.5 mM urea (20). Fractions of 0.5 ml each were tested for protein content using the Bio-Rad protein assay (Bio-Kinetics Reader EL312e, Bio-Tek Instruments), and the purity of profilins was checked by SDS-PAGE (21). The appropriate fractions were pooled and dialyzed for 72 h against three changes of 2 mM Tris, pH 8.5, supplemented with 0.5 mM DTT. Final profilin concentrations were measured by the Bio-Rad protein assay after dialysis to wild-type human profilin standards determined by UV absorbance at 280 nm using an extinction coefficient of A0.015 μM⁻¹ cm⁻¹ (22).

Actin was purified from rabbit skeletal muscle (23). G-actin was separated from residual F-actin by size-exclusion chromatography on a Bio-Gel P60 gel column after dialysis against G buffer (2 mM Tris, pH 7.5, 0.1 mM ATP, 0.5 mM DTT, 0.1 mM CaCl2) and used within 7 days. Some of the actin was labeled with pyrenylodocadamide (24) and stored in G buffer.

Recombinant phosphoinositide-specific rat brain PLCγ1 was purified from bacterial cell extracts using a three-amino acid C-terminal tag (Glu-Glu-Phe) engineered into the PLCγ1 cDNA (25). The recombinant PLCγ1 displayed calcium dependence, pH sensitivity, and substrate specificity indistinguishable from that of wild-type bovine PLCγ1. Synthetic decapeptides cross-linked to an octabranched matrix core were obtained from Research Genetics (26). The anti-human profilin antibody (H44) was a generous gift from Donald A. Kaiser and Thomas D. Pollard (26).

Lipid Preparation—Unilamellar vesicles containing 7 μM PIP2 trace [3H]inositol phospholipid and 50 μM phosphorylcholine-ammonium were incubation in deionized water as described (7). The unilamellar character of vesicles was confirmed by incubating the vesicles with PLCγ1 for extended periods of time (24–48 h) to show that ~50% of the total cpm translocated from the lipid phase to the aqueous phase (see below).

PLCγ1 Activity—To measure hydrolysis of PIP2 by PLCγ1, IP1 production was measured after incubating PIP2 vesicles with 25 nM recombinant PLCγ1 at various concentrations of profilin for 15 min at 22°C in 100 μl of 50 mM HEPES, pH 7.1, 1.4 mM Tris, 0.2 mM CaCl2, 70 mM KCl, 0.4 mM EGTA, and 0.35 mM DTT. IP1 produced in the absence of profilin was measured in triplicate and defined as 100% activity. The relative PLCγ1 activity obtained in the presence of various profilin concentrations was equal to IP1 production in the presence of profilin, expressed as a percentage of the mean IP1 production in the absence of profilin, for each profilin concentration. (7) Since the Michaelis constant, Kₘ, for PLCγ1 is much greater (by at least 50-fold) than the substrate concentration, [S], of PIP2, (7,27), the Michaelis-Menten equation V = V₀max[S]/(Kₘ + [S]) simplifies to V = k[S], where k is a proportionality constant. Thus, the relative PLCγ1 activity in the presence of profilin is simply S/Kₘ, where S is the concentration of PIP2 pentamers not bound to profilin and Kₘ is the total PIP2 pentamer concentration. By substituting in the equation S = (1/2)(P₁ – S₁ + Kᵣ) + 4P₂, S₃)²⁻¹ – (P₁ – S₁ + Kᵣ), where P₁ is the total profilin concentration, and Kᵣ is the dissociation constant for the profilin-PIP₂ complex, a value for Kᵣ can be calculated for each data point by solving the simplified equation Kᵣ = A/P₁ – A – S₁, where A is the relative PLCγ1 activity expressed as a fraction of unity.

Actin Polymerization—The concentration of F-actin was determined from the fluorescence of 10% pyrenylodocadamide-labeled actin using an excitation wavelength of 365 nm and emission wavelength of 407 nm (28). To measure the steady-state concentrations of F-actin, labeled actin was polymerized, dialyzed, and applied to destack pellets at 22°C in the absence or presence of profilins (29). Before measuring fluorescence, the samples were depleted for 1 h. Steady-state experiments were performed in G buffer containing 50 mM KCl and 1 mM MgCl₂. To initiate polymerization for the time course experiments, 2 mM MgCl₂ was added to G buffer containing 10 μM labeled G-actin.

Actin Monomer Nucleotide Exchange—The concentration of G-actin complexed with bound free nucleotide was measured using an excitation wavelength of 360 nm and emission wavelength of 410 nm (30,31). The experiments were performed in G buffer contain-
ing 1.5 μM G-actin, 3 μM CaCl$_2$, and 3 μM ATP. Time 0 corresponds to the addition of 75 μM eATP. Profilin is known not to alter the fluorescence of eATP (21).

Measuring PLP Binding—Serial dilutions of wild-type profilin and R88L were made in 2 mM Tris, pH 8.5, supplemented with 0.5 mM DTT, and added to 80 μl of PLP-linked Sepharose for a total volume of 160 μl, with and without 7.5 M urea. After incubation with mixing for 30 min at 4°C, the concentration of unbound profilin was determined with the Bio-Rad protein assay as the protein concentration in the supernatant after centrifugation.

Protease Sensitivity—To assess the sensitivity of proteins to trypsin, 50 μl of either 12 μM wild-type profilin or R88L was added to 5.5 μl of washed insoluble trypsin attached to beaded agarose (Sigma) and was incubated in a shaker at room temperature. Samples of 10 μl each were removed at 0, 5, 10, 20, and 40 min and centrifuged. The supernatants (5 μl of each) were tested by SDS-PAGE using both Coomassie Blue staining and Western blot analysis.

Results

Mutagenesis of Human Profilin—By designing mutagenic primers to induce single base pair changes in the cDNA sequence for human profilin, we generated 11 mutant clones of profilin to which we assigned the names Y6F, D8A, L10R, K25Q, K53I, R74L, R88L, R88L/K90E, H119D, G121D, and K125Q, according to the amino acid altered (17), its position in the wild-type sequence, and the amino acid substituted in its place. The locations of these mutations in the primary amino acid sequence as well as on the three-dimensional map for human profilin are shown in Fig. 1, along with excerpts of sequencing gels verifying their identities.

Expression and Purification of Profilin Mutants—Milligram amounts of highly pure wild-type and mutant profilins were eluted from the PLP-Sepharose column with 7.5 M urea, as demonstrated by SDS-PAGE (Fig. 2). Only in the case of L10R did the column fail to bind a 14.5-kDa protein. Western blot analysis in this case revealed the presence of a high molecular mass protein (120 kDa) in the bacterial extract that was not retained by the column but which cross-hybridized with a polyclonal antibody specific for human profilin (data not shown). These findings suggested that the L10R mutation may have induced the aggregation of profilin into large, high affinity complexes that were unable to bind PLP. The fact that the remaining mutants were able to bind normally to PLP indicates that the mutations did not induce marked alterations of profilin’s structure. Interestingly, the mutants Y6F and K25Q eluted from the column under less stringent conditions (3.5 M urea) (data not shown) than with either the wild-type profilin or any of the other mutant profilins (all of which required 7.5 M urea for elution). These same residues are known to localize to the PLP-binding site on human profilin (32, 33).

Inhibition of PLC$_\gamma$1 Activity—The ability of profilins to inhibit PLC$_\gamma$1 activity has been previously shown to be directly proportional to the affinity of profilins for PIP$_2$ (11). Therefore, to determine which of the residues in question are important for the profilin-PIP$_2$ interaction, we tested the mutants Y6F, D8A, K25Q, K53I, R74L, R88L, H119D, and G121D for their relative abilities to inhibit PLC$_\gamma$1 activity. All except two mu-
tants exhibited concentration-dependent inhibition that was indistinguishable from that of wild-type human profilin (Fig. 3A), corresponding to a best-fit dissociation constant, $K_d$, of $\sim 0.21 \mu M$ between profilin and PIP$_2$, assuming a stoichiometric ratio of 1:5 (7). The mutants that showed altered interactions with PLC$_{\gamma 1}$ were D8A and R88L (Table I). The mutant D8A demonstrated increased inhibition of PLC$_{\gamma 1}$ activity, corresponding to a best-fit $K_d$ of $\sim 25 \mu M$, and the mutant R88L showed lessened inhibition of PLC$_{\gamma 1}$ activity, corresponding to a best-fit $K_d$ of $\sim 0.60 \mu M$ (Fig. 3A). The reason for the increased affinity demonstrated by D8A is unclear but is presumably charge-related and may suggest involvement of $\alpha$-helix 1 in profilin’s binding to PIP$_2$.

To rule out the possibility that a separate direct interaction between PLC$_{\gamma 1}$ and the profilins accounts for these alterations in activity, we have shown that PLC$_{\gamma 1}$ and profilin do not coprecipitate under a variety of conditions (data not shown).

In light of the work by Fedorov et al. (15), we were particularly interested in the reduction of profilin’s affinity for PIP$_2$ caused by mutating residue 88 since this result suggests that a region near the loop between $\beta$-strands 5 and 6 may be involved in the binding of PIP$_2$. To assess the importance of this region, we tested three different synthetic decapeptides containing sequences that matched that of three non-overlapping 10-amino acid stretches in the wild-type human profilin sequence for their abilities to inhibit PLC$_{\gamma 1}$ activity. Besides selecting the peptide segment surrounding residue 88, we also selected segments implicated by Vinson et al. (14) and Yu et al. (12) as being involved in PIP$_2$ binding. The only decapeptide that had

![Fig. 2. Purification of mutant profilins.](image)

**TABLE 1**

| Protein-PIP$_2$ binding | $K_d$ (mean ± S.E.) | P-value |
|-------------------------|---------------------|---------|
| Wild type ($n = 15$)    | $0.21 \pm 0.04$     | n/a     |
| D8A ($n = 5$)           | $0.025 \pm 0.007$   | 0.0301  |
| R88L ($n = 10$)         | $0.60 \pm 0.06$     | <0.0001 |

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a significant effect on PLCγ1 activity was that which corresponded to the segment centered around residue 88, spanning residues 83–92 (Fig. 3B). Furthermore, the degree of inhibition observed was comparable to that of wild-type profilin (Kd = 0.63 ± 0.13 μM, calculated from peptide concentrations greater than 1 μM). Thus, it appears that this 10-amino acid segment, by itself, could account for much of profilin’s ability to inhibit PLCγ1 activity. These data implicate this region of human profilin (residues 83–92) as a key binding site for PIP2.

Effects of R88L on Actin Polymerization and Monomer Nucleotide Exchange—Since residue 88 also lies in the binding site for actin (9, 16, 34, 35), we sought to determine whether mutating residue 88 also induced changes in profilin’s interactions with actin. We measured the effects of R88L on the critical concentration of actin, the rate of actin polymerization, and the rate of actin monomer nucleotide exchange. While wild-type profilin decreased the steady-state concentration of F-actin, no difference between steady-state concentrations of F-actin in the absence and presence of R88L was detectable by our assay at the concentrations tested (Fig. 4A). Correlated with this observation was the fact that R88L inhibited the time course of actin polymerization by much less than wild-type profilin (Fig. 4B).

When we tested the effect of R88L on the rate of actin monomer adenosine nucleotide exchange, we found its effect to be greatly diminished compared to that of wild-type profilin, such that at least 25 times higher concentrations of R88L were required to achieve comparable levels of catalysis (Fig. 5).

Ruling Out Unstable Folding and Global Denaturation in R88L—Although the decreased actin and PIP2 interactions exhibited by R88L can be explained by the overlap in binding sites for actin and PIP2 on human profilin, an alternative explanation for this effect is that unstable folding results in large-scale denaturation. To rule out this possibility, we tested the abilities of wild-type profilin and R88L to bind PLP and performed protease sensitivity assays. As shown by Scatchard plot analyses, the affinity of R88L for PLP was not decreased in comparison to that of wild-type profilin, and both proteins lost their affinities for PLP when denatured by 7.5 M urea (Fig. 6A). This demonstrates that global denaturation does not occur in R88L since profilin’s binding to PLP requires the proper alignment of both terminal α-helices (32, 33, 35, 36). Protease sensitivity assays further ruled out large-scale conformational changes by demonstrating no significant difference between wild-type profilin and R88L in their time courses for digestion by trypsin (Fig. 6B).

**DISCUSSION**

The many effects of profilin on its ligands have been well worked out through numerous in vitro studies, but how these effects are coordinated inside of cells to produce vital functions has been more difficult to ascertain. For example, the precise manner in which profilin’s three effects on actin are balanced in vivo remains unclear. The relative contributions of these effects likely depend on the ratio of profilin-to-actin concentrations, the relative availabilities of ADP and ATP, and the concentration of thymosin β4 and other sequestering proteins. Since these parameters probably vary greatly between different subcompartments of the same cell, profilin may actually inhibit actin polymerization in some regions of a cell while promoting actin polymerization in others (37). Recent evidence suggests that even across species, profilin’s role may vary depending on its total intracellular concentration and the relative availability of other actin monomer sequestering proteins (38).

Indeed, a variety of elegant in vivo studies including the microinjection, deletion, and overexpression of profilin in cells (26, 38–44) has demonstrated the dramatic phenotypic changes caused by simply altering the level of total profilin in cells. Unfortunately, the specific mechanisms responsible for such changes fail to be revealed with any certainty by these studies.

Mutagenesis offers an alternative and more targeted approach for dissecting out profilin’s functions and functional domains (35), made all the more feasible by the recent elucidation of the three-dimensional structures for Acanthamoeba and bovine profilin (14, 16). Using this approach, we substituted a variety of residues in the primary structure of human profilin to clarify further the importance of various regions of the molecule in binding to PIP2. In particular, we tested the effects of point mutations on the ability of human profilin to inhibit PLCγ1 activity, a property of profilin that is directly related to its ability to bind PIP2 (11). We mutated residues in the proposed binding sites for PIP2 (10, 12–15), as well as a number of other residues including several in both the N- and C-terminal α-helices, which constitute the most highly conserved regions of profilin.

If the binding site proposed by Vinson et al. (14) were correct,
we would expect the mutations K25Q and K53I, if any, to decrease profilin's ability to inhibit PLC\(_\gamma\) activity since both of these are in the region analogous to the propeled site, and they both involve substitution of a positively charged residue analogous to one present in \(A\)canthamoeba profilin-II but not in profilin-I. Contrary to this, neither mutation had any observable effect on profilin's ability to inhibit PLC\(_\gamma\) activity, a result not entirely surprising since we now know that the loop between \(\beta\)-strands 1 and 2 makes up part of profilin's binding site for PLP (32, 33) and that profilin can bind both PLP and PIP\(_2\) simultaneously (33). This correlates with our observation that the mutants Y6F and K25Q, both with a substitution in the region of the PLP-binding site, exhibited diminished binding to PLP.

We found that mutating residue Arg-88 on the opposite side of profilin caused a decrease in profilin's inhibition of PLC\(_\gamma\) activity, suggesting that the binding site actually exists on the opposite side of the protein. This result is consistent with the presence of a positive electrostatic potential over the analogous region of \(A\)canthamoeba profilin-II (15). Mutagenesis applied to yeast profilin has shown that substituting Arg-72 decreased PIP\(_2\) binding (35), providing further evidence that the binding site for PIP\(_2\) is localized to this area. We also showed that a decapeptide comprised of the sequence around residue 88 was able to inhibit PLC\(_\gamma\) activity just as well as the entire molecule of wild-type profilin, thereby providing strong evidence that a binding site for PIP\(_2\) on human profilin is located near the loop between \(\beta\)-strands 5 and 6.

If the loop between \(\beta\)-strands 5 and 6 represents a binding site for PIP\(_2\), then the binding sites for PIP\(_2\) and actin would overlap since biochemical studies, x-ray crystallography, and mutational analysis of yeast profilin have implicated the region spanning \(\alpha\)-helix 3, \(\beta\)-strands 4–6, and the first portion of \(\alpha\)-helix 4 as the binding site for actin (9, 16, 34, 35). Such overlap has already been demonstrated for cofilin (45) and yeast profilin (35), and this could account for the ability of PIP\(_2\) to dissociate profilin-actin complexes, although PIP\(_2\)-induced changes in conformation may also be important in precluding actin binding (13). Overlap of the two binding sites is consistent with our finding that R88L exhibited markedly diminished interactions with actin, an effect not simply explained by large-scale denaturation since R88L demonstrated unaltered protease sensitivity and exhibited normal binding to PLP. Smaller scale effects on conformation, however, are more difficult to exclude. In fact, just as PIP\(_2\) binding induces a conformation that may disfavor actin binding, the mutation R88L may be stabilizing an intermediate conformation that favors neither PIP\(_2\) or actin binding. Crystallographic analysis is underway and should provide us with specific structural information concerning this mutant.

**FIG. 5.** Effect of R88L on actin monomer nucleotide exchange.
Time courses for the exchange of eATP for ATP bound to 1.5 \(\mu\)M G-actin in the presence of varying concentrations (indicated on graphs) of wild-type profilin (A) and R88L (B).

**FIG. 6.** Confirmation that R88L is not globally denatured. A, Scatchard plot analyses of wild-type profilin (closed circles) and R88L (open circles) binding to PLP in the absence (top row) and presence (bottom row) of 7.5 \(M\) urea. The values of \(K_d\) for native wild-type profilin and R88L were calculated from the slopes of the best-fit lines to be 4.6 and 2.7 \(M\), respectively. B, time courses for the digestion of wild-type profilin and R88L by trypsin (see “Experimental Procedures”).
Why the charge differences between Acanthamoeba profilin-I and -II, two isoforms displaying markedly different affinities for PIP$_2$, cluster to the side of the molecule that does not bind PIP$_2$ remains uncertain. It may be that the overall positive charge of profilin is important in facilitating an interaction with PIP$_2$, as would be consistent with the increased affinity of DBA for PIP$_2$, but that a separate non-electrostatic interaction is crucial for the cooperative binding of multiple PIP$_2$ molecules. This is supported by the fact that the relative abilities of our three synthetic decapeptides to inhibit PLC$_{Y1}$ activity did not correlate with the net charge of each peptide, a result also seen with PIP$_2$-binding peptides derived from gelsolin (46).

Examination of the loop at the proposed site shows that it protrudes from the molecule and could potentially serve as a core for the clustering of PIP$_2$ molecules, an arrangement perhaps stabilized by a hydrophobic interaction between the proximal aspects of the acyl chains of PIP$_2$ and the aliphatic residues at the tip of the loop (Gly-93, Gly-94, and Ala-95). This may explain why profilin does not bind to PIP$_2$ (11), the acidic head group of PIP$_2$ cleaved by PLC$_{Y1}$ from the remainder of the molecule. Recently, the PIP$_2$-binding region of the N-terminal homology domain of pleckstrin was found to include a loop contained in the sequence KKGSVNFTWK (47). This bears a striking resemblance to the loop between $\beta$-strands 5 and 6 of Acanthamoeba profilin-II contained in the sequence KKG-SAGVITVK. Admittedly, the corresponding sequence for human profilin is not so similar, but it is interesting to note that human profilin has a 10-fold greater affinity than Acanthamoeba profilin-II for PIP$_2$ (11) and that the single major difference between the two profilin tertiary structures is in this same loop, which is much larger and more protrusive in human profilin. Furthermore, the affinity of pleckstrin for PIP$_2$ ($K_D \sim 30 \mu M$) (47) is much closer to that of Acanthamoeba profilin-II than to that of human profilin for PIP$_2$.

The details of how profilin binds PIP$_2$ is certainly complicated and will require additional research to establish more clearly the structures and mechanisms involved. Mutational studies directed to other residues in the vicinity of residue 88 are currently underway, and three-dimensional studies such as nuclear magnetic resonance spectroscopy or x-ray crystallography of the profilin-PIP$_2$ complex will be needed to confirm our proposed region of human profilin as the true PIP$_2$-binding site. Meanwhile, the generation of profilin mutants, which are deficient for the profilin-PIP$_2$ interaction but not for the actin interactions, should help us to determine through their over-expression in mammalian cells the physiologic importance of the profilin-PIP$_2$ interaction in vivo. As for R88L, its intact PLP-bind-activity with diminished PIP$_2$ and actin interactions may be useful in determining the importance of the recently reported interaction between profilin and vasodilator-stimulated phosphoprotein, an interaction mediated by proline-rich domains on vasodilator-stimulated phosphoprotein (48), by acting as a competitive inhibitor of wild-type profilin. As such, site-directed mutagenesis provides us with a powerful tool for deciphering the molecular interactions between this multifunctional protein and its many ligands.

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2 S. C. Almo, personal communication.