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The affinities of human platelet and *Acanthamoeba* profilin isoforms for polyphosphoinositides account for their relative abilities to inhibit phospholipase C

Laura M. Machesky,* Pascal J. Goldschmidt-Clermont,† and Thomas D. Pollard*
Johns Hopkins University School of Medicine
*Department of Cell Biology and Anatomy
and †Department of Medicine
(Division of Cardiology)
Baltimore, Maryland 21205

In light of recent work implicating profilin from human platelets as a possible regulator of both cytoskeletal dynamics and inositol phospholipid–mediated signaling, we have further characterized the interaction of platelet profilin and the two isoforms of *Acanthamoeba* profilin with inositol phospholipids. Profilin from human platelets binds to phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) with relatively high affinity ($K_d \sim 1$ μM for PIP_2 by equilibrium gel filtration), but interacts only weakly (if at all) with phosphatidylinositol (PI) or inositol trisphosphate (IP_3) in small-zone gel-filtration assays. The two isoforms of *Acanthamoeba* profilin both have a lower affinity for PIP_2 than does human platelet profilin, but the more basic profilin isoform from *Acanthamoeba* (profilin-II) has a much higher (~10-μM K_d) affinity than the acidic isoform (profilin-I, 100 to 500-μM K_d). None of the profilins bind to phosphatidylserine (PS) or phosphatidylcholine (PC) in small-zone gel-filtration experiments. The differences in affinity for PIP_2 parallel the ability of these three profilins to inhibit PIP_2 hydrolysis by soluble phospholipase C (PLC). The results show that the interaction of profilins with PIP_2 is specific with respect to both the lipid and the proteins. In *Acanthamoeba,* the two isoforms of profilin may have specialized functions on the basis of their identical (~10 μM) affinities for actin monomers and different affinities for PIP_2.

Introduction

Profilin was originally identified as a small actin monomer-binding protein (Carlsson *et al.,* 1977), but has been recently been shown to associate with plasma membranes (Hartwig, *et al.,* 1989) and to inhibit soluble phospholipase C (PLC) by binding to phosphatidylinositol 4,5 bisphosphate (PIP_2) (Goldschmidt-Clermont *et al.,* 1990). Profilin may provide a link between the processes that regulate transmembrane signaling and the cytoskeleton, but sorting out the complex relationships at these regulatory crossroads will require careful characterization of a variety of molecular interactions, a task still in its infancy.

Members of the profilin family vary considerably in their primary structures. Only 18 of 125 residues are conserved among the profilins from vertebrates (Nystrom *et al.,* 1979; Kwiatkowski and Bruns, 1988), *Acanthamoeba* (Ampe *et al.,* 1985, 1988), and yeast (Oechsner *et al.,* 1987). But comparison of any two profilins (such as human platelet and yeast) yields considerably more sequence identity. There are single isoforms in yeast and man, but at least three isoforms in *Acanthamoeba* (2 neutral and 1 basic).

In spite of these differences in primary structure, all well-characterized profilins bind actin monomers with micromolar affinity (Pollard and Cooper, 1986), increase the rate of exchange of ATP bound to actin (Mockrin and Korn, 1980; Nishida, 1985), inhibit the actin monomer ATPase (Tobacman and Korn, 1982), and inhibit actin polymerization (Reichstein and Korn, 1979; Dinubile and Southwick, 1985; Kaiser *et al.,* 1986). Formation of a nonpolymerizable 1:1 complex with actin monomers was originally thought to explain all of these effects on actin, but more recent data (Pollard and Cooper, 1984; Kaiser *et al.,* 1986; Goldschmidt-Clermont, unpublished observations) show that the mechanism of action is more complex. For example, in *Acanthamoeba,* binding to actin monomers can account for the inhibition of nucleation and elongation at the pointed end of actin filaments, but not for the weak inhibition of elongation at the barbed end (Tseng and Pollard, 1982; Tseng *et al.,* 1984; Tilney *et al.,* 1983; Kaiser *et al.,* 1986). These observations can be explained by low-affinity binding of profilin to the barbed end

of filaments (Pollard and Cooper, 1984). In the cell, these properties of profilin should suppress spontaneous nucleation but allow actin filaments to grow at one end. However, these effects of profilin require stoichiometric concentrations of profilin, whereas the actin concentration in both human platelets and *Acanthamoeba* exceeds the profilin concentration by at least two- to sixfold (Tseng *et al.*, 1984; Lind *et al.*, 1987; Goldschmidt-Clermont, unpublished observations). On the other hand, the catalytic effect of profilin on exchange of both nucleotide and divalent cation on actin monomers could contribute to the regulation of actin polymerization, even at the substoichiometric concentrations found in cells.

The potential regulatory roles of profilin in cells were expanded by the discoveries that PIP_2 dissociates the actin-profilin complex (Lassing and Lindberg, 1985, 1988) and that profilin binds to PIP_2 in a manner that protects the lipid from hydrolysis by a soluble, phosphoinositide-specific PLC-II from human platelets (Goldschmidt-Clermont *et al.*, 1990). Because platelet profilin has a higher affinity for PIP_2 than actin, much of the profilin might be bound to lipids and regulate both phosphatidylinositol (PI) turnover and actin dynamics. These findings have raised many questions about the specificity of the interaction between profilin and lipids, the mechanism of dissociation of the profilin-PIP_2 complex, and the significance of both the profilin-actin and profilin-PIP_2 associations.

In this paper, we provide evidence that the interaction of profilin with polyphosphoinositides is specific for both the lipid and the protein. On the lipid side, profilins bind to phosphoinositides with a phosphate in the 4 position, but not to PI or to other acidic phospholipids, such as phosphatidylserine (PS). On the basis of the low affinity of profilin for inositol trisphosphate (IP_3), we think that the affinity for individual phosphoinositide head groups is very low. Binding is achieved by the association of profilin with small clusters of PIP_2. On the protein side, two basic profilins (human platelet profilin and *Acanthamoeba* profilin-II) bind to PIP_2 much more strongly than profilin-I, the acidic isoform from *Acanthamoeba.* This is the first evidence for a functional difference between profilin-I and profilin-II. Given that these profilins bind actin monomers with equal affinity, we hypothesize that profilin-II is mainly membrane associated and involved with phosphoinositide metabolism, while profilin-I acts primarily as a regulator of the actin cytoskeleton. The single isoform of profilin in platelets has a high affinity for both actin and phosphoinositides and may have dual regulatory functions.

Results

Three profilins interact differently with polyphosphoinositides during small-zone gel filtration

In small-zone gel-filtration experiments, human platelet profilin (Goldschmidt-Clermont *et al.*, 1990), *Acanthamoeba* profilin-I, and *Acanthamoeba* profilin-II all bound to PIP_2 micelles, but each bound in a different manner (Figure 1). A given concentration of PIP_2 micelles bound human platelet profilin > profilin-II > profilin-I. The concentration of each profilin carried from the starting sample by the micelles was directly proportional to the concentration of PIP_2, but the slopes of the plots differed for each profilin (Figure 1A). One human platelet profilin bound for an average of 4.3 PIP_2 molecules. This is a slightly larger ratio than the 1:7 ratio in our previous report (Goldschmidt-Clermont *et al.*, 1990). The lower ratios for profilin-II (~1:15) and profilin-I (~1:50) are unlikely to result from differences in the stoichiometries, as the data in the following sections show that this can be explained by lower affinities. Because of the lower affinities of the *Acanthamoeba* profilins for PIP_2, dissociation of the PIP_2-profilin complex occurs during chromatography, and higher concentrations of PIP_2 are required to bind these profilins.

Platelet profilin and *Acanthamoeba* profilin-II bound to micelles of PIP_2 under a variety of conditions. By small-zone gel filtration, binding was similar in 0 and 75 mM KCl at pH 7.5 and over a range of pH 5.5–8.5 in 75 mM KCl (data not shown).

When incorporated into large unilamellar vesicles made by an extrusion technique (LUVETS) of mixed composition, PIP_2 bound human platelet profilin > profilin-II > profilin-I (Goldschmidt-Clermont *et al.*, 1990; and Figure 1B). The PIP_2 concentration dependence of binding was similar for LUVETS and micelles. The slopes of the plots gave ratios of 1 human platelet profilin per 5 PIP_2 (Goldschmidt-Clermont *et al.*, 1990) and 1 *Acanthamoeba* profilin-II per 12 PIP_2 (Figure 1B). Binding of profilin-I required much more PIP_2 than we could use in this assay. Because steric hindrance should not affect the binding of profilin to large unilammellar vesicles composed mostly of PC, we attribute these differences in binding ratios to the lower affinities of the *Acanthamoeba* profilins for PIP_2 and the resulting dissociation of the lipid-profilin complex during chromatography.

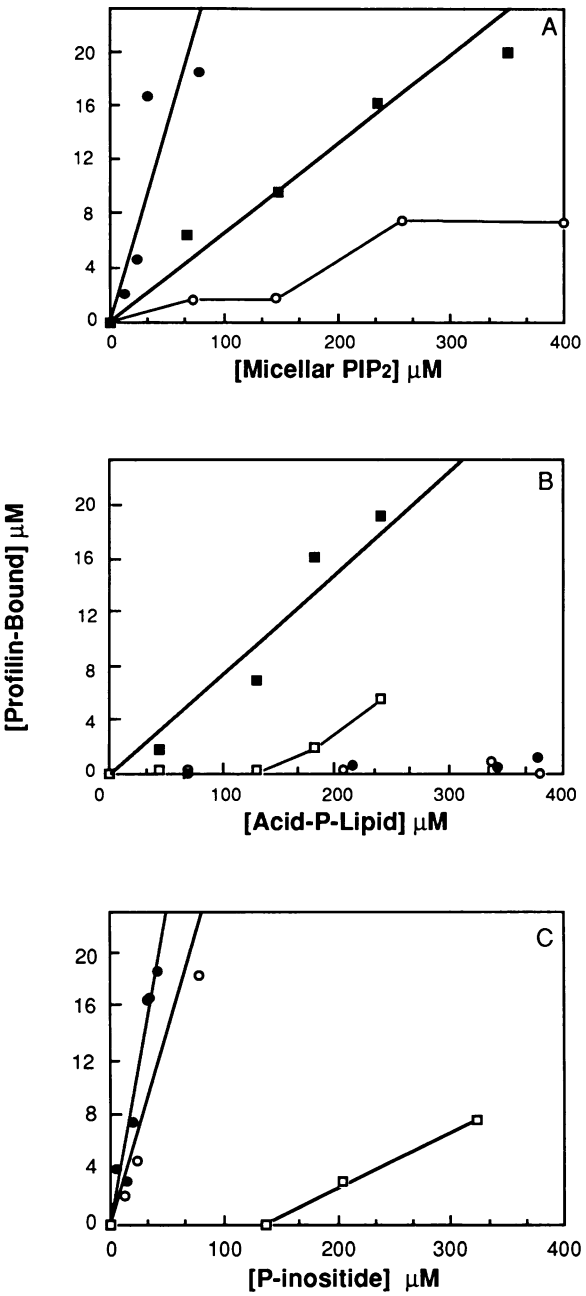

Figure 1. **Binding of various profilins to lipids measured by small-zone gel filtration.** Small-zone gel filtration was performed with human platelet profilin, *Acanthamoeba* profilin-I, and *Acanthamoeba* profilin-II with micelles of pure PIP_2 or PIP, SUVs of PI, or LUVETS of mixed lipid composition. Conditions: samples of 100 μl containing 23 μM profilin and various concentrations of lipids in VB were preincubated for 15–30 min and chromatographed on a 0.7 × 7-cm column of Sephadex G-100 equilibrated with VB at 22°C. The concentration of free profilin was measured by the Bradford assay (Bradford, 1976) and the fraction bound to lipid calculated by difference. (A) Profilin from human platelets (●), amoeba profilin-I (○), or amoeba profilin-II (■) with micelles of pure PIP_2. (B) Amoeba profilin-I with LUVETS of PC:PIP_2 5:1 (□) and PC:PS 5:1 (○) and amoeba profilin-II with LUVETS of PC:PIP_2 5:1 (■) and PC:PS 5:1 (●). [Acidic-P-Lipid] corresponds to the concentration of the acidic phospholipid species in the outer leaflet of the vesicles (assumed to be 50% of the total concentration), e.g., PIP_2, PI, or PS. (C) Human platelet profilin with micelles of PIP (●), PIP_2 (○), and SUVs of PI (□).

Binding of profilins is specific for phosphatidylinositol-4-monophosphate (PIP) and PIP_2. In small-zone gel-filtration experiments, human profilin bound equally well to micellar PIP and PIP_2, but only weakly to small unilamellar vesicles (SUVs) of PI (Figure 1C). The stoichiometry one profilin per three or four molecules of PIP, and the fact that binding was directly proportional to the concentration of lipid added, are only compatible with a high-affinity interaction. There was no detectable interaction between human platelet profilin and IP_3 by gel filtration (Figure 2), so the affinity of profilin for individual PIP_2 headgroups is low relative to the affinity for PIP_2 pentamers in a membrane.

No detectable *Acanthamoeba* profilin-I or profilin-II bound to LUVETS of phosphatidylcholine (PC):PS (5:1) during small-zone gel filtration. This agrees with our studies using human platelet profilin, where we also showed no detectable binding to LUVETS of PC:PS (5:1) or PC:PI (5:1) (Goldschmidt-Clermont *et al.*, 1990). These results support the specificity of the interaction between profilin and polyphosphoinositides.

Affinities of profilins for PIP_2 determined by equilibrium gel filtration

We used equilibrium gel filtration to measure the affinities of the three profilins for PIP_2 and other lipids (Figure 3 and Table 1). These measurements confirmed that human platelet profilin has the highest affinity for PIP_2, profilin-II has an intermediate affinity, and profilin-I from *Acanthamoeba* has the lowest affinity for PIP_2, in agreement with the small-zone gel-filtration assays. As in the small-zone gel-filtration assays, no detectable interaction occurred between LUVETS of PC:PS and either *Acanthamoeba* profilin-II (Figure 3, C and D) or *Acanthamoeba* profilin-I (not shown).

Because this is a novel application of the method of Hummel and Dreyer (1962), a few comments are in order regarding the equilibrium gel-filtration method. This method is traditionally used to measure the binding of small molecules to macromolecules. We adapted it to measure the binding of small proteins to large lipid assemblies (LUVETS). First, a sample con-

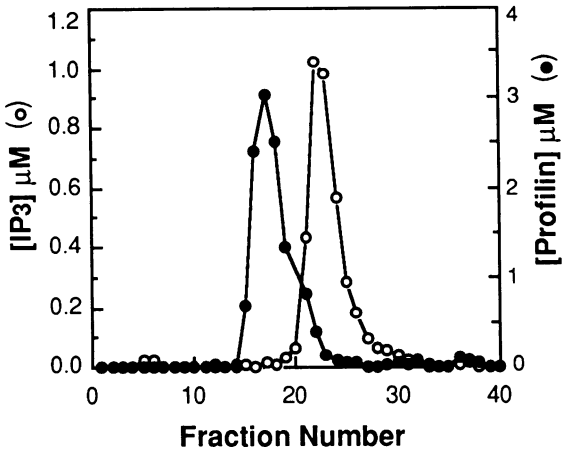

***Figure 2.* Chromatograph of human platelet profilin with IP_3.** Small-zone gel filtration of 4 μM IP_3 with 12 μM human platelet profilin was performed using a 0.7 × 10 cm column of Biorad P30 gel equilibrated with buffer (see Methods). Concentration of human platelet profilin in each fraction was measured by the method of Bradford (1976), and the concentration of IP_3 was measured in each fraction by liquid scintillation counting of an aliquot. The first peak is due to free profilin, and the later eluting peak is free IP_3.

taining lipid and the same concentration of profilin as in the column buffer are added to the column. As the column runs, the lipid in the sample migrates faster than the protein that is included in the buffer used to equilibrate the column. The lipid, which is quantitated by counting ^3H PIP_2 or ^{14}C PC, elutes early, in fractions corresponding to the void volume in small-zone experiments (not shown). The lipid carries with it a peak of protein and leaves behind a trough in the profilin concentration at the position where free profilin elutes on this column in small-zone experiments. This is called equilibrium gel filtration because the lipid sample is at equilibrium with profilin from the time of loading throughout the entire running of the column. In Figure 3, A and B, we show the protein concentration as measured by the Bradford protein determination (Bradford, 1976). The two chromatograms shown differ because the lipid interferes with the Bradford protein determination (Figure 3A), producing either an increase or a decrease in the determination, depending on the lipid species. Protein measurements made after lipid extraction (Figure 3B) confirm that the quantity of profilin removed from the trough is equal to the quantity of profilin in the peak eluting with the lipid, but the extra manipulations introduce more scatter in the data. Consequently, data obtained with the Bradford assay to determine the concentration of profilin in the later eluting trough were used to calculate the fraction of the profilin bound to lipid and the equilibrium constants for binding (Table 1).

Evidence that profilin does not substantially disrupt micellar structure from TNS fluorescence

Profilin had a minimal effect on the fluorescence of mixtures of PIP_2 micelles and 2-p-toluidinylnapthylene-6-sulfonate (TNS), a fluorescent dye sensitive to the disorder of the aliphatic core of the micelles (Figure 4). TNS has been used in the past to detect changes in protein conformation and can also detect changes in the structure of lipid assemblies (Langner *et al.*, 1990). This is evidence that profilin binds to PIP_2 head groups on the surface of the micelle, rather than penetrating the core of the micelle in such a way as to grossly affect the environment of the TNS. In contrast, $MgCl_2$ aggregated PIP_2 micelles into large multilammellar structures (not shown) and increased the fluorescence of TNS by eightfold. Profilin did not cause such a change, and although we cannot rule out a slight penetration into hydrophobic regions of the bilayer, this is evidence that there is no gross disruption of the hydrophobic regions of micelles when profilin interacts with PIP_2.

Profilins inhibit the hydrolysis of PIP_2 by PLC

All three profilins tested can inhibit the hydrolysis of PIP_2 by phosphoinositide-specific PLC, although the rate of reaction is a complex function of several factors, including the concentrations of PLC, PIP_2, and profilin; the types of profilin and PLC; the physical state and composition of the lipid substrate; and the time of incubation. When platelet PLC hydrolyzes micellar PIP_2, the initially high rate of reaction declines with time (Figure 5, ●). Platelet profilin can inhibit the hydrolysis completely (Figure 5, □; and Goldschmidt-Clermont *et al.*, 1990). This effect of profilin is not directly on the enzyme, because PLC activity is partially restored by additional substrate (Figure 5, ○). Rather, the profilin seems to compete with the enzyme for the substrate, because activity is also partially restored by high concentrations of enzyme (Figure 5, ■)—although we observed a lag of several minutes after mixing the PLC with the PIP_2 and profilin, which is not observed in the absence of profilin. Given these variables, we restricted the subsequent analysis of the effects of profilin on PLC activity to low concentrations of enzyme and initial rates of reaction (generally the first 3–5 min).

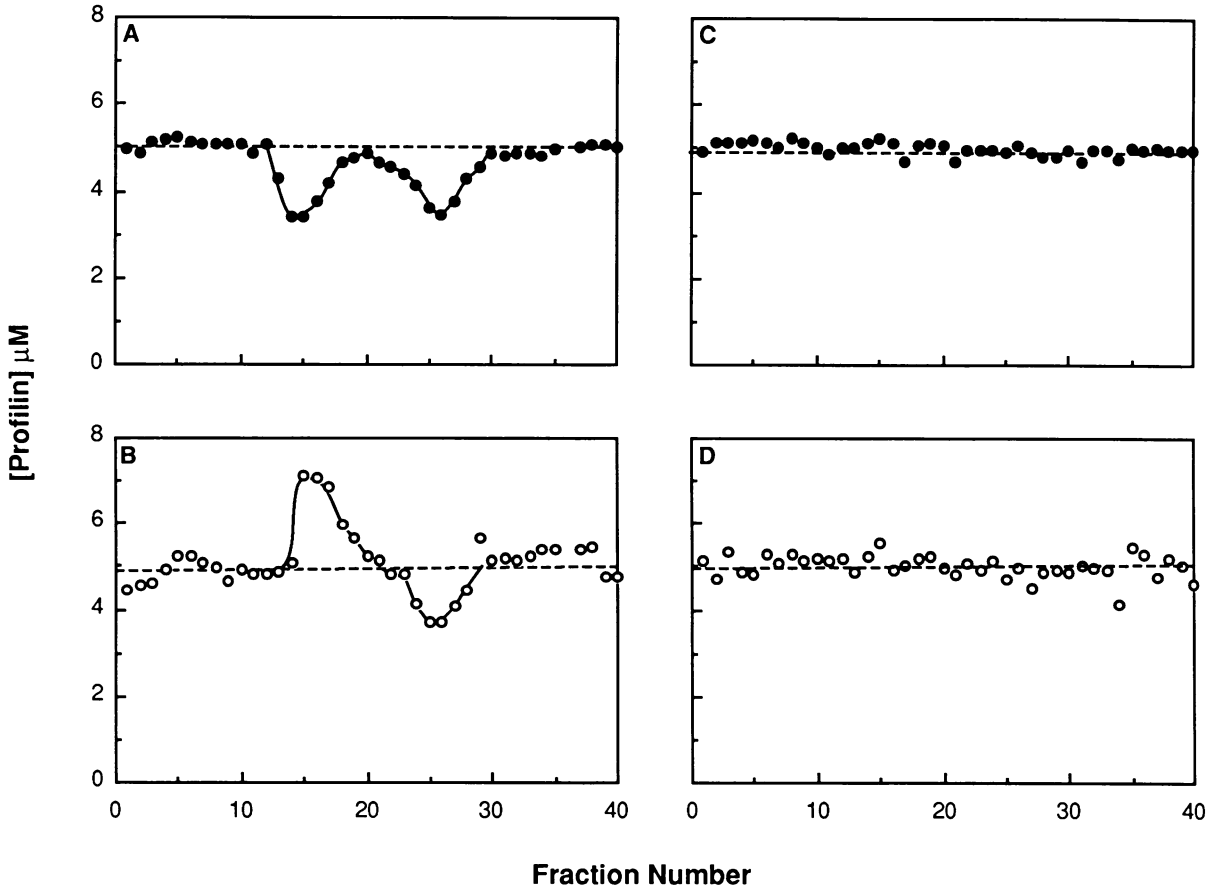

Figure 3. **Binding of *Acanthamoeba* profilin-II to lipids measured by equilibrium gel filtration.** In these experiments, the 0.7 × 50-cm column of Sephadex G-100 was equilibrated with VB containing 5 μM profilin-II; and 600-μl samples, containing both *Acanthamoeba* profilin-II (5 μM) and lipids, were chromatographed through the column. (A) Chromatogram of *Acanthamoeba* profilin-II with micellar PIP_2. The profilin concentration in 0.45-ml fractions was assayed by the method of Bradford (1976). The first trough is due to negative interference of the lipid with the assay, whereas the second trough represents the depletion of profilin because of interaction with lipid. (B) The same chromatogram as in A, assayed for protein by the BCA method after extraction of the lipids. This demonstrates that the profilin concentration is higher in the fractions where the lipids elute (here fractions 15–20, corresponding to the void volume in small-zone experiments). (C) The sample contained 300 μM PS in PC:PS (2:1) LUVETS. No interference was observed of the PC:PS LUVETS with the Bradford assay. (D) The same chromatogram as in C, assayed by the BCA method after extraction of the lipids.

The ability of a given profilin to inhibit PIP_2 hydrolysis by PLC (Figure 6 and Table 2) correlates well with its affinity for PIP_2 as measured by equilibrium gel filtration (Table 1), with human platelet profilin being the strongest inhibitor and *Acanthamoeba* profilin-I being the weakest. The type and concentration of profilin are major determinants of the PLC activity (Figure 6, A–C). We obtained our cleanest data for all three profilins with PLC-γ from bovine brain (Figure 6A), where the inhibition of PIP_2 hydrolysis parallels the affinities of the three profilins for PIP_2. The apparent dissociation constants for a complex of profilin with a pentamer of PIP_2 can be estimated from the theoretical curves in Figure 6A to be ~3 μM for platelet profilin, 70 μM for *Acanthamoeba* profilin-II, and 500 μM for *Acanthamoeba* profilin-I.

The composition and concentration of lipids in the vesicles are major determinants of the PLC activity in the presence of profilins, so the apparent affinity of profilin for PIP_2 varies depending on the lipid substrate. Although PLC hydrolyzed PIP_2 at the same rate with a high (Figure 6C) or low (Figure 6B) mole fraction of PIP_2 in the outer leaflet of the LUVETS (data not shown), the *Acanthamoeba* profilins inhibited the enzyme more strongly when assayed with a high mole fraction of PIP_2 in the outer leaflet. These *Acanthamoeba* profilins with low affinity for PIP_2 may not compete well with PLC for PIP_2 when the PIP_2 is widely dispersed in the bilayer.

Table 1. Determination of binding constants by equilibrium gel filtration

	K_d μM	
	PIP_2 micelles	PC:PIP_2 LUVETS
Acanthamoeba profilin-I	143 (D = 17, n = 2)	101 (D = 15, n = 2)
Acanthamoeba profilin-II	8 (SD = 3, n = 5)	3 (SD = 2, n = 3)
Human platelet profilin	0.6 (D = 0.3, n = 2)	3 (n = 1)

Dissociation constants (K_d) for profilin and PIP_2 (7:1 PIP_2:profilin for micelles, and 5:1 PIP_2:profilin for LUVETS) as determined by equilibrium gel filtration. SD corresponds to the standard deviation where more than two replicate experiments were performed (n > 2); D, the deviation from the mean where two experiments were performed (n = 2).

Profilin inhibited equally well the two soluble PLCs that we tested, phosphoinositide-specific PLC-γ from bovine brain (Figure 6A) and soluble phosphoinositide-specific PLC-II from human platelets (Figure 6, B and C). In contrast, profilin did not inhibit PLC beta from bovine brain, an enzyme thought to be associated with membranes (Goldschmidt-Clermont, unpublished observations).

Discussion

Mechanism of interaction of profilin with polyphosphoinositides

The available evidence suggests that members of the profilin family interact specifically with PIP and PIP_2 but that the mechanism is complicated because the protein binds to a small cluster of lipid molecules. In this section we will define the overall mechanism as we now understand it.

The lipid requirements for binding profilin include an inositol head group with at least one phosphate in addition to the phosphodiester linkage to the glycerol. A phosphate in the 4 position is apparently sufficient. Single phosphates at other positions have not been tested. Neither PI nor PS is a good ligand, so the interaction is not simply an electrostatic association with a negatively charged head group. The lack of detectable binding of IP_3, at concentrations where profilin binds to PIP_2, provides an important clue discussed further below. The physical state of the phosphoinositide does not appear to be important, because PIP_2 binds profilins equally well, whether it is concentrated in small micelles or dispersed among neutral lipids in a bilayer. The concentration of lipid is important, as in any association reaction. The mole fraction of polyphosphoinositide in a bilayer is probably an important variable with respect to the ability of profilin to interact with lipid clusters, as indicated by the PLC experiments with amoeba profilins (Figure 6C), but we have not yet studied this systematically.

The stoichiometry of the molecules in the complex is one of the most important parameters. From the small-zone gel-filtration binding assays and the PLC inhibition experiments with human platelet profilin, we estimate the stoichiometry to be one profilin bound to four or five PIP_2s. This number needs further verification, because neither assay is accurate enough to determine the stoichiometry to within more than $\sim \pm 2$ lipid molecules. The experiments with the amoeba profilins provide no evidence regarding the stoichiometry, because their affinity is too low to obtain an accurate measurement. Therefore, we assumed that the stoichiometry of the complex of amoeba profilins with PIP_2 is also 1:5.

Our current interpretation of this data is that profilins have multiple weak binding sites for individual phosphoinositide head groups and that a stable complex is formed by possibly sequential association with up to five lipid head groups. We have quantitated this interaction by assuming that each profilin molecule binds simultaneously to a pentamer unit of lipid molecules. Such clusters with the appropriate geometry are probably readily available on the surface of pure PIP_2 micelles, but when the PIP_2 is dispersed in a bilayer with other lipids, the cluster of PIP_2 probably has to form by multiple associations of individual lipid molecules with the protein. We speculate that this would start with a weakly bound 1:1 complex and grow as PIP_2 molecules diffuse into association with the protein. Consequently, the dissociation constants that we measure are not equilibrium dissociation constants in the sense of a typical bimolecular reaction. Rather, these apparent equilibrium con-

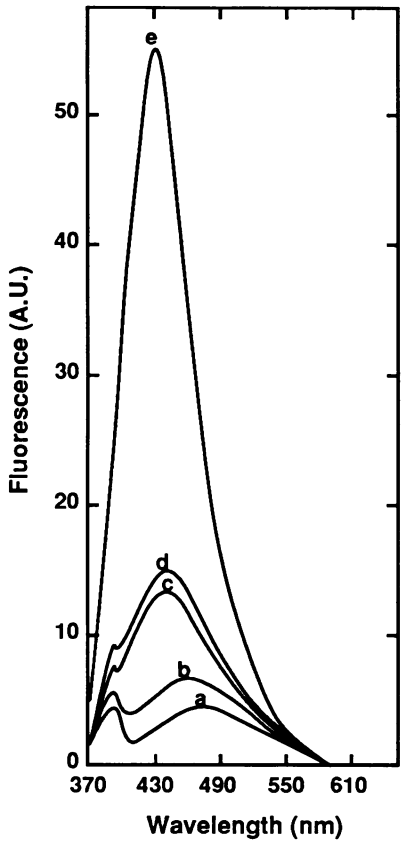

Figure 4. **The effect of human platelet profilin on the structure of PIP_2 micelles measured by TNS fluorescence.** The fluorescence emission spectrum of 30 μM TNS excited at 360 nm, in the presence of PIP_2 micelles and various molecules, was measured to analyze their effect on the structure of the micelles. Conditions: (a) 100 μM TNS alone; (b) TNS with 10 μM PIP_2; (c) TNS and 7 μM profilin; (d) TNS with 7 μM profilin and 10 μM PIP_2; (e) 10 mM $MgCl_2$ with TNS and 10 μM PIP_2. Profilin does not significantly change the fluorescence of TNS in the presence of PIP_2 micelles, whereas $MgCl_2$ has a marked effect on TNS fluorescence in the presence of PIP_2.

stants are a composite value reflecting multiple, possibly cooperative, interactions. Given the low affinities, we suspect that these complexes of profilin with PIP_2 have a relatively short lifetime, but the weak binding may also be the consequence of slow association rates rather than rapid dissociation rates. We hope that the formation and dynamics of these complexes can be studied further by both kinetic and structural methods.

The evidence suggests that electrostatic interactions between the negatively charged phosphosphate on the inositol and the protein are important but not sufficient to explain the observed binding. Because other acidic head groups do not bind profilin (Figures 1, B and C; and 3, C and D), the geometry of the phosphoinositide must play an essential role. The stable binding of platelet profilin and profilin-II in 0–75 mM KCl over the pH range 5.5–8.5 does not rule out electrostatic interactions, because there is evidence that PIP_2 is expected to have a net charge of ~ −3 (Toner *et al.*, 1988) over this pH range in 75 mM KCl. However, there is also evidence suggesting that in 100 mM NaCl, the pK of PIP_2 is variable over this pH range (van Paridon *et al.*, 1986).

Clues about polyphosphoinositide binding from comparison of profilin sequences

The sequences of all profilins are weakly homologous from end to end, but the vertebrate profilins differ so much from the amoeba profilins that a detailed sequence comparison to explain human platelet profilin's higher affinity for PIP_2 was not useful in the absence of knowledge about their three-dimensional structures. Nonetheless, the two profilins that bind well to PIP_2 have one common feature that may be relevant. Both human platelet profilin and *Acanthamoeba* profilin-II have basic isoelectric points (pI ~ 9), whereas *Acanthamoeba* profilin-I is acidic (pI ~ 5.5).

Because *Acanthamoeba* profilin-I and profilin-II differ at only 22 out of 125 residues (Table 3),

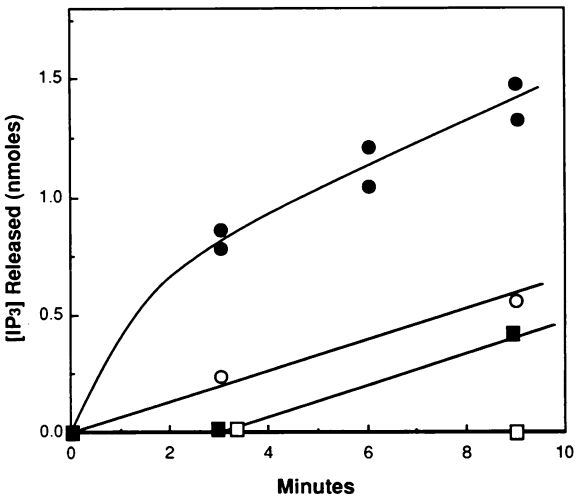

Figure 5. **Influence of platelet profilin, substrate, and enzyme concentrations on the time course of PIP_2 hydrolysis by platelet PLC.** Release of IP_3 from micellar PIP_2 was assayed according to Methods. Conditions: (●) Control with 47 μg/ml PLC and 7.9 μM micellar PIP_2; (□) 47 μg/ml PLC, 7.9 μM PIP_2, and 1.25 μM profilin, which completely inhibits activity; (○) 47 μg/ml PLC, 1.25 μM profilin, and excess (19.1 μM) PIP_2, which restores activity in the presence of the profilin; (■) 7.9 μM PIP_2, 1.25 μM profilin in the presence of a high (110 μg/ml) concentration of PLC, which gives a low level of activity.

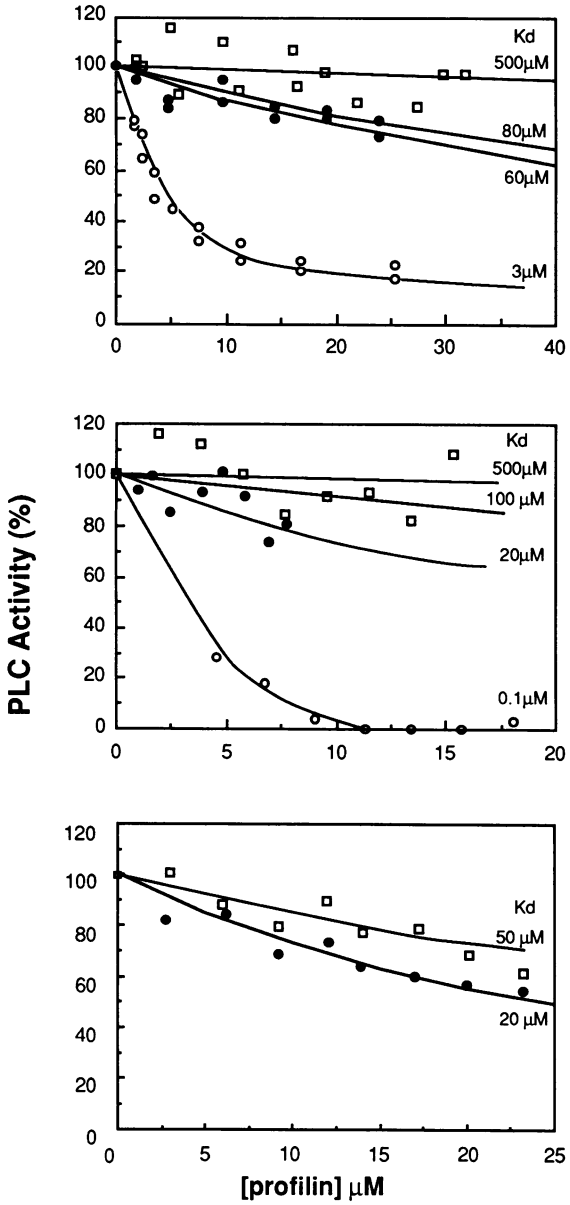

Figure 6. **Inhibition by profilins of the hydrolysis of PIP_2 by soluble PLC.** The samples contained profilin, a PLC isozyme, and various lipids (micelles or LUVETS). Theoretical curves for PLC activity were calculated by a simple model, where dissociation constants (K_d) between 0.1 and 1000 μM are indicated. (A) Activity of bovine brain PLC-γ using LUVETS of PC:PIP_2 (5:1) in the presence of (□) *Acanthamoeba* profilin-I, (●) *Acanthamoeba* profilin-II, or (○) human platelet profilin. Theoretical curves for a complex of profilin with PIP_2 pentamers are drawn for K_ds of 500 μM, 80 μM, 60 μM, and 3 μM. (B) Activity of human platelet PLC using LUVETS of PC:PE:PIP_2 (5:5:1) in the presence of (□) *Acanthamoeba* profilin-I, (●) *Acanthamoeba* profilin-II, or (○) human platelet profilin. Theoretical curves are drawn for K_ds of 500 μM, 100 μM, 20 μM, and 0.1 μM. (C) Activity of human platelet PLC using LUVETS of PC:PE:PIP_2 (1:1:1) in the presence of (□) *Acanthamoeba* profilin-I, or (●) *Acanthamoeba* profilin-II. Theoretical curves are drawn for 50 μM and 20 μM.

these differences provide some clues about the 10-fold difference in their affinities for PIP_2. Most of these substitutions involve uncharged residues or the exchange of two residues with the same charge. Substitutions at residues 24, 50, and 66 result in differences in charge, account for the higher isoelectric point of profilin-II, and give it a net charge of +2 higher than profilin-I at pH 7. Because profilin-II binds well to PIP_2 at pH 5.5 and 8.5, lysine-50 is more likely to account for its higher affinity than histidine-24 (leucine in profilin-I) or arginine-66 (histidine in profilin-I).

Although the substitution of a lysine for an asparagine may seem like a minor difference between profilin-I and profilin-II, single charged residues can have a profound effect on the affinity of peptides for acidic phospholipids. In model studies of the binding of small positively charged peptides derived from protein kinase C (PKC) to vesicles composed of PS and PS:PC, each lysine or arginine residue contributed about one order of magnitude (a free energy of 1.4 kcal/mol) to the affinity (Kim *et al.*, unpublished observations). PKC peptides are an imperfect model for profilin-lipid interactions, because they bind to vesicles containing PS or phosphatidylglycerol (PG), but the conclusion regarding the energic contribution of a single electrostatic bond may be sufficient to explain the higher affinity of profilin-II for PIP_2.

Mechanism of PLC inhibition by profilin

The best explanation for the inhibition of PLC by profilin is sequestration of substrate PIP_2 (Figure 7). Under a wide variety of conditions, the ability of the three profilins to inhibit the enzyme parallels their affinity for PIP_2 measured in binding reactions (Tables 1 and 2). The agreement is not precisely quantitative, likely because of the complexity of the interactions among PIP_2, profilin, and PLC in the enzyme assay. For example, even at high concentrations, *Acanthamoeba* profilin-II never inhibited PLC as well as platelet profilin (Figure 6). Perhaps the subtle difference in the affinity of human platelet profilin and *Acanthamoeba* profilin-II for PIP_2 is magnified when competing with PLC. This seems particularly likely, given the possibly cooperative interaction of one profilin with five PIP_2 molecules. Further systematic studies of both the binding reactions and the enzyme activity will be required to substantiate the mechanism of inhibition.

Functions of profilins in cells

The actin and lipid binding capabilities of profilins suggest that they are unique regulatory

Table 2. Affinity of profilins for PIP_2 estimated from their effects on PLC activity

	Substrates			
	K_d μM (profilin-LUVETS)			K_d μM (profilin-micelles)
	1:1:1, PC:PE:PIP_2	5:5:1, PC:PE:PIP_2	5:1, PC:PIP_2	PIP_2
Platelet PLC-II				
Amoeba profilin-I	40–50	100–500		
Amoeba profilin-II	15–20	20–100		
Platelet profilin	0.1–10	0.1–1.0	1–5	0.01–0.1
Brain PLC-γ				
Amoeba profilin-I			1000	
Amoeba profilin-II			60–80	
Platelet profilin			1–10	

We measured the activity of PLC-II from human platelets and PLC-γ from bovine brain over a range of profilin concentrations and vesicle compositions. The K_ds of the profilin:PIP_2 complex (7:1 PIP_2:profilin for micelles and 5:1 PIP_2:profilin for LUVETS) were estimated from theoretical curves, calculated assuming that all bound PIP_2 is inaccessible to the PLC (Figure 6 and Materials and methods).

proteins functioning at the interface of a major signaling pathway and the actin cytoskeleton, and our results show that these functions can be partitioned in at least three different ways. Platelet profilin appears to be bifunctional with relatively high affinities for both polyphosphoinositides and actin monomers; *Acanthamoeba* profilin-II has moderate affinities for both ligands; and *Acanthamoeba* profilin-I seems to be specialized for binding to actin. This is the first known functional difference between the two isoforms of profilin from *Acanthamoeba.* Not enough is known about profilin isoform expression in other cells to predict whether the vertebrate or amoeba solution is more widespread.

To estimate the potential physiological significance of these interactions, we calculated the concentrations of the various species present at equilibrium in a mixture with cellular concentrations of profilin, actin, and PIP_2 (Table 4). Accepting for now that cells are much more complicated than three-component systems, these calculations show that platelet profilin would be partitioned between unpolymerized actin monomers and membrane PIP_2, whereas amoeba profilin-II would mostly be associated with PIP_2, and amoeba profilin-I would be largely bound to actin monomers. In all cases, most of the profilin is bound to one of its ligands, but in no case is all of the unpolymerized actin or membrane PIP_2 complexed with profilin.

These predictions, based on binding constants measured with purified proteins, agree in a general way with observations on cells and crude cellular fractions. For example, about one-third of total platelet profilin is associated with actin when isolated on poly-L-proline Sepharose (Lind *et al.,* 1987; Goldschmidt-Clermont, unpublished observations). Second, by electron microscopy, part of the profilin in blood cells is associated with the plasma membrane (Hartwig *et al.,* 1989). Third, part of cellular PIP_2 is not accessible for binding neomycin, presumably because it is already bound to a protein (King *et al.,* 1987; Toner *et al.,* 1988), perhaps profilin. It is an open question how many other proteins may also sequester PIP and PIP_2. Three candidates are the plasma membrane protein glycophorin (Anderson and Marchesi, 1985) and the cytoplasmic proteins gelsolin (Janmey and Stossel, 1987) and cofilin (Yonezawa *et al.,* 1990).

These new insights regarding interactions of profilin, actin, and phosphoinositides provide an opportunity to reevaluate the potential physiological functions of profilin. With the partial information currently available, it is not yet clear which molecules are the regulators and which are regulated. For example, does profilin regulate phosphoinositide metabolism, or do the phosphoinositides regulate profilin, or is the regulation reciprocal?

Originally profilin was viewed as an actin monomer sequestering protein responsible in full or in part for the pool of unpolymerized actin in nonmuscle cells. However, given the affinity of the profilins for lipids and the expected large excess of unpolymerized actin over the concentration of profilin-actin complexes (Table 4),

Table 3. Comparison of the variable residues of the three isoforms of *Acanthamoeba* profilin

	22	24	38	41	43	44	47	50	51	53	54	57	58	61	65	66	70	77	82	84	94	97
Profilin-IA	L	L	T	Q	Q	T	S	N	N	D	P	A	S	D	V	H	L	I	G	A	S	V
Profilin-IB	L	L	T	Q	T	T	S	N	N	D	P	A	G	D	V	H	L	I	G	S	A	V
Profilin-II	I	H	S	N	A	A	N	K	D	T	A	S	N	E	T	R	I	V	S	A	A	I

The primary structures of profilin-IA and profilin-IB were determined by amino acid sequencing of a mixture of the two proteins (Ampe et al., 1985) and from the nucleotide sequence of profilin-IA (Pollard and Rimm, unpublished observations). The sequence of profilin-II was determined by amino acid sequencing (Ampe et al., 1988) and confirmed and corrected by cDNA sequencing (T.D. Pollard and D.L. Rimm, unpublished observations). Because there are only three amino acid differences between profilin-IA and profilin-IB, and because these isoforms cannot be separated by our current purification procedure, we have referred to them collectively as profilin-I throughout this paper. Differences at residues 24, 50, and 66, involving charged residues, may be particularly important in determining the differences between the affinities of profilin-I and profilin-II for the polyphosphoinositides.

it is unlikely that the profilins alone account for the pool of unpolymerized actin monomer in cells. The predicted concentration of unpolymerized actin not bound to profilin is well above the critical concentration required for polymerization, so it seems likely that part of the unpolymerized actin is bound to other sequestering proteins, such as members of the actophorin/depactin/actin depolymerizing factor (ADF) group (Giuliano *et al.*, 1988; Mabuchi, 1983; Cooper *et al.*, 1986), or to a recently discovered 5-kDa protein (Safer *et al.*, 1990). If profilin is not adequate to sequester the unpolymerized actin in the cell, we should consider other roles that are compatible with the low concentration of free profilin in the cytoplasm. One possibility is fine-tuning actin turnover in the cell by enhancing nucleotide exchange. Profilin accelerates the exchange of nucleotide on actin monomers (Mockrin and Korn, 1980; Nishida, 1985). The profilin can accomplish this at low concentrations because it acts catalytically (Goldschmidt-Clermont, unpublished observations). At the time of cell activation, profilin appears to associate with actin (Lind *et al.*, 1987), presumably as a result of the activation of PLC, which allows it to overcome the profilin block (Goldschmidt-Clermont, unpublished observations) and thus begin rapid PI-turnover and subsequent release of profilin from the membrane. There is no information about the pools of ADP-actin in cells, but if present, this released profilin could promote the exchange of its ADP for ATP and stimulate polymerization of actin. Profilin could also inhibit spontaneous nucleation of the ATP-actin (Pollard and Cooper, 1984), so that polymerization takes place in an organized fashion from preformed nucleating sites in the cell. In these mechanisms we envision the phosphoinositides as the regulators of profilin, which in turn regulates the assembly of actin.

The other side of this scheme is that profilin participates in the regulation of phosphoinositide metabolism. In both platelets and *Acanthamoeba,* profilin binds to PIP and PIP_2 with a high enough affinity that a substantial portion of these lipids could be complexed with profilin in a cell (Table 4). Together with other phosphoinositide-binding proteins, profilin may protect PIP_2 from hydrolysis by PLC until a cell becomes activated (Forscher, 1989; Goldschmidt-Clermont *et al.*, 1990). We have investigated only the effects of profilin on the hydrolysis of PIP_2 by PLC, but because profilin also binds to PIP, it may regulate phosphoinositide metabolism in other ways, including the syn-

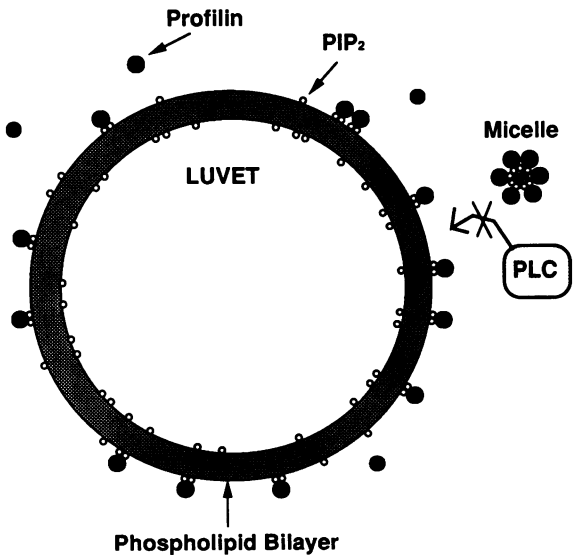

Figure 7. **Model for the mechanism of PLC inhibition by profilin.** When profilin is added in a high concentration, it binds to clusters of PIP_2 on the micelles or outer leaflet of vesicles, forming complexes that are poor substrates for hydrolysis by soluble PLC.

Table 4. Estimates of the concentrations of the species present in mixtures of physiological concentrations of profilin, actin, and PIP_2

		Bound to				
	Total μM	Profilin-I μM	Profilin-II μM	Actin μM	PIP_2 μM	Free μM
Acanthamoeba						
Profilin-I	67			50.9	3.2	12.9
Profilin-II	33			9.9	20.5	2.6
PIP_2	240	15.8	102.7			121.5
Actin	200	50.9	9.9	100*		39.2
		Bound to				
	Total μM	Profilin μM	5 kDa μM	Actin μM	PIP_2 μM	Free μM
Platelets						
Profilin	40			6.0	32.0	2.0
PIP_2	240	160				80
Actin	200	6.0	86.4	100*		7.6
5 kDa	200			86.4		113.6

Concentrations and equilibrium dissociation constants were obtained from the following sources:

Concentrations: *Acanthamoeba* profilins—Tseng and Pollard, 1982; Kaiser *et al.*, 1986; *Acanthamoeba* actin—Gordon *et al.*, 1976; Tseng and Pollard, 1982; *Acanthamoeba* PIP_2—P.A. Allen, personal communication; platelet profilin and actin—Lind *et al.*, 1987; Goldschmidt-Clermont, unpublished observations; platelet PIP_2—Cohen *et al.*, 1971; and platelet 5 kDa—Safer *et al.*, 1990.

Dissociation constants: Profilins and PIP_2—this paper and Goldschmidt-Clermont *et al.*, 1990; *Acanthamoeba* profilin and actin—Pollard and Cooper, 1984; platelet profilin and actin—Lind *et al.*, 1987; Goldschmidt-Clermont, unpublished observations; and platelet profilin and 5 kDa—Safer *et al.*, 1990; Goldschmidt-Clermont, unpublished observation.

We used the KINSIM program of Barshop *et al.* (1983) to calculate equilibrium distributions of each species. We understand that cells are much more complex than simple four-component systems, and, actually, the free species are probably interacting with other components of the cell. Until numbers are available for affinities and concentrations of other actin-binding proteins as well as profilin's affinity for PIP and PIP's concentration in cells, however, a better model is not possible. * Refers to actin filament concentration found in a resting cell.

thesis of PIP_2 from PIP. Profilin or other PIP_2-binding proteins may also be responsible for maintenance of a hormone-insensitive pool of PIP_2 (Koreh and Monaco, 1986). Furthermore, it will be interesting to examine the interaction of the profilins with phosphatidylinositol-3,4-bisphosphate and other minor phosphoinositides, because so little is known about these novel phospholipids, and profilin may actually have a higher affinity for one or more of these minor species than for the common isomers that we have studied.

Insight about the physiological relevance of the profilin-phosphoinositide interactions in *Acanthamoeba* will have to await further studies. Accurate quantitation of PIP and PIP_2 concentrations in the membrane has not been done, although estimates have been made that put the concentration of PIP_2 close to that found in other cells (>0.5% of total lipid, P.G. Allen, personal communication), which would be ~140–240 μM (Cohen *et al.*, 1971). *Acanthamoeba* chemotax toward bacteria on an agar substrate (McIntyre and Jenkins, 1969), and PI turnover increases with phagocytosis of either yeast or high-mannose glycoproteins (P.G. Allen, personal communication), but neither the PLC nor other parts of the signaling machinery have been identified. This information will be necessary to appreciate fully the meaning of the differences between the two profilin isoforms from *Acanthamoeba.* The available evidence points to two different roles, with profilin-II being more like human platelet profilin and profilin-I being the major actin monomer-sequestering profilin.

Perhaps the most interesting model is that the interactions of phosphoinositides and profilins are part of a reciprocal regulatory network. The interaction of profilin with PIP_2 might help to explain the heterogeneity in cellular phosphoinositide pools available for hydrolysis by PLC, whereas the interaction of profilin with actin (regulated by the phosphoinositide concentrations) might be key in reorganization of the cytoskeleton.

Materials and methods

Preparation of proteins

Profilins were purified from *Acanthamoeba* and outdated human platelets by affinity chromatography on poly-L-proline agarose (Kaiser *et al.*, 1989). The profilin-I and profilin-II isoforms from *Acanthamoeba* were separated by carboxymethylSepharose affinity chromatography (Kaiser *et al.*, 1986). Profilins were dialyzed into vesicle buffer (VB; 10 mM tris(hydroxymethyl)aminomethane [Tris], pH 7.5, 75 mM KCl, 0.5 mM dithiothreitol, and 1.8 mM NaN_3). Protein concentrations were measured by ultraviolet absorbance with an extinction coefficient of 0.015 $OD \cdot \mu M^{-1} \cdot cm^{-1}$ at 280 nm. Phosphoinositide-specific PLC-II was purified from human platelet cytosol (Baldassare *et al.*, 1989) (a generous gift of Dr. J.J. Baldassare) and PLC-γ from bovine brain (Sung *et al.*, 1987) (a generous gift of Dr. S.G. Rhee).

Sources of lipids

Lipids were obtained from the following sources: PIP_2 from Calbiochem (La Jolla, CA); PIP from Sigma Chemical (St. Louis, MO); PC, PS, phosphatidylethanolamine (PE), and PI in chloroform from Avanti Polar Lipids (Pelham, AL); phosphatidyl [2-^{3}H] inositol-4,5-bisphosphate ([^{3}H] PIP_2) in dichloromethane:ethanol:water 20:10:1 (v/v/v), L-3 phosphatidylcholine 1,2-di(1 ^{14}C) oleoyl ([^{14}C] PC) in toluene:ethanol 1:1 (v/v) IP_3, and [^{3}H]IP_3 from Amersham (Arlington Heights, IL). The purity of the phosphoinositides PI, PIP, and PIP_2 was confirmed by thin-layer chromatography using 10 μg of each lipid loaded onto silica gel 60 plates in a 90:90:7:22 chloroform:methanol:ammonium hydroxide:water solvent system.

Preparation of lipid substrates

Homogenous PIP_2 and PIP micelles were prepared by suspending 1 mg of PIP_2 or PIP in 0.5 or 1 ml of deionized water and sonicating in a Bransonic 32 (bath type) sonicator for 5 min at room temperature. SUVs of PI were prepared by drying the lipid under a stream of nitrogen, resuspending in the appropriate amount of deionized water by vortexing, and sonicating in a Bransonic 32 (bath type) sonicator as above. Large unilamellar vesicles of various compositions were made by an extrusion technique (LUVETS, Hope *et al.*, 1985; Mayer *et al.*, 1986). Lipids obtained in organic solvents, including the radiolabeled lipids (see below) were dried in a glass tube under a stream of nitrogen. PIP_2, which had been resuspended in deionized water, was added to the dried lipids, along with enough additional deionized water to produce the desired final concentration; all lipids were then resuspended by vortexing. After five cycles of freezing in liquid nitrogen and thawing in a 37°C water bath, samples were passed 10 times through a filter (polycarbonate, 0.1-μM pore size, Nucleopore, Pleasanton, CA) in an Extruder (Lipex Biomembranes, Vancouver, Canada) under a pressure of 400 psi. The concentration of lipid in each mixture was measured by liquid scintillation counting of an aliquot of the sample after extrusion.

Gel-filtration assay for profilin binding to lipids

For small-zone experiments, samples were prepared in VB at room temperature and incubated for 15–30 min before chromatography. Micelles and LUVETS with profilin were run on a 0.7 × 7-cm column of Sephadex G-100, preequilibrated with VB, at room temperature at 20 ml/h, collecting fractions of 0.45 ml. Protein concentrations of each fraction were measured with the Bradford Coomassie Blue dye-binding assay (Bradford, 1976) with purified profilin as the standard. Because some lipids interfere with the Bradford assay, we used only the free profilin peak in our calculations. Lipid concentrations of each fraction were measured by liquid scintillation counting of an aliquot. The fraction of profilin bound to lipids was calculated as the difference between the total applied to the column and the amount recovered in the entire peak of free protein.

Dissociation constants for the profilin-PIP_2 complex were measured by equilibrium gel filtration (Hummel and Dreyer, 1962) with a 0.7 × 50-cm column of Sephadex G-100 equilibrated with 3–20 μM profilin in VB. Samples of 600 μl contained various concentrations of lipid preequilibrated for 15–30 min with the same concentration of profilin as that used in the column buffer. The following lipid concentrations and compositions were used: micelles of pure PIP_2 at 150–420 μM, LUVETS of PC:PIP_2 2:1 with 100–300 μM PIP_2 in the outer leaflet, and LUVETS of PC:PS 2:1 with 300 μM PS in the outer leaflet. The column was run at 20 ml/h, and 0.45-ml fractions were collected. Profilin bound to lipid was measured as the amount of profilin depleted from the fractions where free profilin elutes (approximately fractions 21–29). Profilin bound to lipid was measured directly for two representative experiments, one with micelles and one with LUVETS. In these control experiments, 100-μl aliquots of each fraction were acidified to 12 mM HCl and the lipids were extracted with 10 vol of acetone for 1 h at 20°C. After spinning for 10 min in an Eppendorf centrifuge at 4°C, we removed acetone with a pasteur pipette, and the protein pellet was dried in a Speed Vac centrifuge (Savant, Farmingdale, NY). Pelleted protein was assayed by the BCA method (Pierce Chemical Co., Rockford, IL) using 96-well plates and a microplate reader at 540 nm. Dissociation constants for the profilins and PIP_2 were calculated assuming a stoichiometry of one profilin molecule per seven PIP_2 molecules in micelles and one profilin molecule per five PIP_2 molecules with LUVETS.

Gel filtration of IP_3 and profilin

The interaction of profilin with IP_3 was tested by small-zone gel filtration using a 0.7 × 10-cm column of Biorad P30 gel preequilibrated with 2 mM Tris pH 7.3, 50 mM KCl, 100 μM $CaCl_2$, 100 μM NaN_3, and 0.5 mM dithiothreitol. A 400-μl sample of 4 μM IP_3 and 12 μM human platelet profilin, in column buffer, was run at 4°C; and 0.45-ml fractions were collected. Trace amounts of [^{3}H] IP_3 (specific activity 0.1 Ci/mole) were added to detect the position of elution of IP_3, whereas protein concentration in each fraction was measured by the Bradford assay.

TNS fluorescence

Fluorescence of TNS (30 μM in buffer: 2 mM Tris pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol) was measured at 22°C with a Perkin-Elmer 650-10S Fluorescence Spectrophotometer. Fluorescence excitation was at 360 nm and emission was scanned from 360–560 nm (McClure and Edelman, 1966; Goldschmidt-Clermont *et al.*, 1987). The signal obtained with TNS alone was compared with that obtained with TNS in the presence of PIP_2 (10 μM) and/or human platelet profilin (7 μM) and in separate assays, $MgCl_2$ (10 mM) and/or PIP_2 (10 μM). $MgCl_2$ was chosen because it aggregates PIP_2 into large multilammellar structures, thus disrupting the micellar structure.

PLC assays

These assays were run at 37°C in VB with 80 μM $CaCl_2$ added for human platelet PLC and 20 μM $CaCl_2$ for bovine

brain PLC-γ. PLC from human platelets was used at various concentrations (47–172 μg/ml) in experiments with micelles and at ~78 μg/ml in experiments with LUVETS. PLC-γ from bovine brain was used at ~4 μg/ml. Micelles of pure PIP_2 or LUVETS of mixed lipid composition were made using [^{3}H]-PIP_2 as the labeled substrate for PLC. The following compositions and concentrations of LUVETS were used: PC:PE:PIP_2 1:1:1 at 0.3 mg/ml total lipid, with 45 μM PIP_2 in outer leaflet; PC:PE:PIP_2 5:5:1 at 0.2 mg/ml total lipid, with 2.3 μM PIP_2 in outer leaflet; and PC:PIP_2 (5:1) at 0.24 mg/ml total lipid, with 18 μM PIP_2 in outer leaflet. We assumed that the PIP_2 would distribute equally between inner and outer leaflets, except with the PC:PE:PIP_2 (5:5:1) LUVETS, where we added PIP_2 to the outer leaflet by incubating the LUVETS of PC:PE with micelles of PIP_2 (Goldschmidt-Clermont *et al.,* 1990). Micelles of pure PIP_2 were used at various concentrations, as indicated in Figure 5. Hydrolysis was stopped by addition of ice cold methanol/chloroform/HCl to separate the IP_3 in the aqueous phase from lipids in the organic phase (Dawson, 1965; Baldassare and Fisher, 1986; Raben, 1987). The [^{3}H]-IP_3 in the aqueous phase was measured by liquid scintillation counting. Because the concentration of PIP_2 was much lower than the K_m of the enzyme (Goldschmidt-Clermont *et al.,* 1990), the rate of hydrolysis was directly proportional to the PIP_2 concentration according to the Michaelis-Menton equation $V = (V_{max}[S])/([S] + K_m)$. The effect of PIP_2 sequestration by profilin on the hydrolysis rate can be calculated directly from the Michaelis-Menton equation where [S] is replaced by $1/2\{([K_d + P_T - S_T]^2 + 4K_d \times S_T)^{1/2} - [K_d + P_T - S_T]\}$, where P_T is the total concentration of profilin, S_T is the total concentration of PIP_2, and K_d is the dissociation constant for the profilin:PIP_2 complex. Each point corresponds to one individual time course.

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