The Actin-Binding Protein Profilin Binds to PIP$_2$ and Inhibits Its Hydrolysis by Phospholipase C

Author(s): Pascal J. Goldschmidt-Clermont, Laura M. Machesky, Joseph J. Baldassare, Thomas D. Pollard


Stable URL: http://www.jstor.org/stable/2874183

Accessed: 08/04/2009 11:17
The Actin-Binding Protein Profilin Binds to PIP$_2$ and Inhibits Its Hydrolysis by Phospholipase C

PASCAL J. GOLDSCHMIDT-CLERMONT, LAURA M. MACHESKY, JOSEPH J. BALDASSARE,* THOMAS D. POLLARD

Profilin is generally thought to regulate actin polymerization, but the observation that acidic phospholipids dissociate the complex of profilin and actin raised the possibility that profilin might also regulate lipid metabolism. Profilin isolated from platelets binds with high affinity to small clusters of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) molecules in micelles and also in bilayers with other phospholipids. The molar ratio of the complex of profilin with PIP$_2$ is 1:7 in micelles of pure PIP$_2$ and 1:5 in bilayers composed largely of other phospholipids. Profilin competes efficiently with platelet cytosolic phosphoinositide-specific phospholipase C for interaction with the PIP$_2$ substrate and thereby inhibits PIP$_2$ hydrolysis by this enzyme. The cellular concentrations and binding characteristics of these molecules are consistent with profilin being a negative regulator of the phosphoinositide signaling pathway in addition to its established function as an inhibitor of actin polymerization.

Table 1. Characterization of the PIP$_2$-profilin complex. Stoichiometry and $K_d$ were estimated by fitting theoretical curves (obtained by varying these two parameters independently) to the data from the binding filtration assay (22) and the PLC inhibition assay (25) (Fig. 1, E and F; Fig. 2, B and C). Because the bound and free profilin are separated during the gel filtration assay, the equilibrium is perturbed, causing the complex to dissociate and profilin to trail behind the micelles and vesicles. In the PLC inhibition assay, the equilibrium is also perturbed, as PIP$_2$ is hydrolyzed when profilin comes off the PIP$_2$ clusters. Consequently, the $K_d$’s estimated by these assays are maximum values. The 95% confidence intervals (in parentheses) were calculated from 8 to 12 separate filtrations for the binding filtration assay, and from two separate experiments (each representing eight individual time courses) for the PLC inhibition assay.

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>Stoichiometry (number of PIP$_2$ molecules per profilin molecule)</th>
<th>$K_d$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelles of PIP$_2$</td>
<td>7.4 (6.7 to 8.2)</td>
<td>&lt;0.1 (&lt;0.1)</td>
</tr>
<tr>
<td>LUVETs of PIP$_2$:PC (1:5)</td>
<td>5.4 (3.4 to 7.3)</td>
<td>&lt;1.0 (&lt;1.0)</td>
</tr>
<tr>
<td>LUVETs of PIP$_2$:PC:PE (1:1:1)</td>
<td>4.8 (3.9 to 5.8)</td>
<td>&lt;1.0 (&lt;1.0)</td>
</tr>
<tr>
<td>LUVETs of PIP$_2$:PC (1:12)</td>
<td>5.0</td>
<td>&lt;5.0 (&lt;5.0)</td>
</tr>
<tr>
<td>LUVETs of PIP$_2$:PC:PE (1:5:5)</td>
<td>5.0</td>
<td>&lt;1.0 (&lt;1.0)</td>
</tr>
<tr>
<td>LUVETs of PI:PC (1:5)</td>
<td>&lt;1000</td>
<td>&lt;1000</td>
</tr>
</tbody>
</table>
cules per PIP₂ molecule (Fig. 1F and Table 1). The molar ratio was 1 profilin per 5 PIP₂ molecules in the outer leaflet. This result suggests that the affinity of profilin for PIP₂ is relatively high even in the presence of excess PC. The data are also consistent with the induction by profilin of aggregation of PIP₂ into small patches of ~5 molecules in the PC lipid bilayer.

We tested LUVETs with a variety of different lipid compositions, but only those with PIP₂ bound profilin with high affinity (Fig. 1, F and G). LUVETs composed of pure PC or PC in a 5:1 ratio with PI or phosphatidylserine (PS) did not bind profilin; LUVETs composed of mixtures of PS and phosphatidylethanolamine (PE) evinced high lipid concentrations, also did not bind profilin.

We then tested the possibility that the PIP₂-profilin complex might be a poor substrate for phospholipase C (PLC). Profilin inhibited the hydrolysis of micellar PIP₂ by a platelet soluble PLC (9) in a concentration-dependent manner (Fig. 2, A and B, and Table 1). At molar ratios of >1 profilin per 10 PIP₂ molecules, the rate of hydrolysis by a low concentration of enzyme was near zero. The inhibition of hydrolysis is mediated by the binding of profilin to the substrate PIP₂ micelles rather than by interaction with the enzyme, as profilin concentrations that completely inhibited hydrolysis of a low concentration of substrate did not inhibit hydrolysis with excess PIP₂ (10). Profilin inhibited the hydrolysis of micellar PIP₂ by a greater extent than expected from the 1:7 stoichiometry of the profilin-PIP₂ complex measured by the gel filtration assay. A possible explanation, consistent with the experimental data, is that each profilin obstructs the access of PLC (which is larger than profilin (9)) to 10 molecules of PIP₂. High concentrations of enzyme gave low but measurable rates of hydrolysis even in the presence of an excess of profilin over PIP₂ (10).

At an optimal concentration of Ca²⁺ (80 μM in our assay) and at pH 6.5, PLC activity varied according to the type and concentration of the lipids mixed with PIP₂. Compared to PLC activity measured with PIP₂ micelles as the substrate, the activity was higher with LUVETs composed of PE and PIP₂ and lower with LUVETs of PC and PIP₂. This agrees with reports that indicated an enhancing effect of PE and an inhibitory effect of PC on PLC-catalyzed hydrolysis of PIP₂ in mixed SUVs (11).

Profilin inhibited hydrolysis of PIP₂ by PLC in all LUVET compositions tested (Fig. 2C and Table 1). In experiments with high concentrations of LUVETs, each profilin molecule protected ~5 PIP₂ molecules from hydrolysis by PLC at all profilin concentrations. This suggests that profilin aggregates PIP₂ into patches. These patches are likely to be small, because formation of large patches of PIP₂ would induce steric hindrance among profilin molecules (as observed with micelles). Estimation of the K₅₄ was difficult with high concentrations of LUVETs, because the binding of profilin was relatively tight. The best estimates were obtained with small amounts (0.1 to 10 μM) of PIP₂ added to the outer leaflet of PC:PE vesicles. Under these conditions, the PLC inhibition data fit with models for the binding of profilin to a PIP₂ pentamer in LUVETs with a submicromolar K₅₄ (Fig. 2C and Table 1). In control experiments, neither ovalbumin nor rabbit skeletal muscle actin inhibited hydrolysis of PIP₂ in LUVETs.

Lassing and Lindberg (1) drew attention to the interaction of profilin with acidic phospholipids, particularly polyphosphoinositides, as a possible mechanism to regulate the sequestration of actin monomers by profilin. Their evidence was based on the ability of the polyphosphoinositides to reverse the inhibition of actin polymerization by profilin. With a variety of different assays, we have confirmed their results (10). Human platelet profilin binds to actin monomers with a K₅₄ of ~3 μM and increases the rate of exchange of divalent cations and adenosine triphosphate (ATP) bound to actin (10, 12). Profilin also inhibits nucleation, and to a lesser extent elongation, of actin filaments (10, 12). PIP₂ and PIP₃ micelles overcome these effects of profilin on actin, whereas IP₃ and SUVs of PI and PS have no effect (10).

The properties of the complex of profilin with PIP₂ described in this report explain why PIP₂ micelles can reverse the effects of profilin on actin. About 12 molecules of profilin bind to each micelle of PIP₂ with submicromolar affinity. At saturation, each profilin is associated with 7 PIP₂ molecules. The protein molecules are likely to be tightly packed on the surface of the micelle, presumably bound by electrostatic forces be-
tween the negatively charged polar head groups of the micelle and the basic side chains on profilin (13). The geometry of such a complex makes it unlikely that the profilin penetrates the hydrophobic core of the micelle, which is composed of packed aliphatic chains (4). This conclusion is supported by our observation that addition of PI(3,4,5)P_3 did not change the fluorescence of mixtures of 2-p-toluidinylnaphthalene-6-sulfonate and profilin, as might be expected if profilin disrupted the micellar structure (10). The structure of platelet profilin has not been determined, but a preliminary model for Acanthamoeba profilin based on x-ray diffraction data (6) has a large cluster of basic residues in one place on the surface. If the structure of platelet profilin is similar [as would be expected from the sequences of the proteins (14)], such basic patch is a likely candidate for the PI(3,4,5)P_3-binding site. Chemical cross-linking has identified an actin contact site in the middle of the basic patch near the COOH-terminus of Acanthamoeba profilin (15). Such an overlap of binding sites and the higher affinity of profilin for PI(3,4,5)P_3 micelles than for actin suggest that simple competition between actin and the acidic lipids for binding to profilin may explain the actin polymerization data (1, 10).

A substantial fraction of the PI(3,4,5)P_3 in the inner leaflet of the platelet plasma membrane may be bound to profilin. This conclusion is based on the high concentration of profilin [30 to 40 μM (10, 16)], the concentration of PI(3,4,5)P_3 [140 to 240 μM (17)], the 1:5 stoichiometry and submicromolar affinity of the complex, and the apparent ability of profilin to aggregate PI(3,4,5)P_3 into small patches. Other phospholipids form patches in lipid bilayers (18), and an association of profilin with the cytoplasmic face of the plasma membrane of human platelets (and leukocytes) has been observed independently by electron microscopy (19). The high concentration of actin in platelets will compete with PI(3,4,5)P_3 for profilin, but the affinity of profilin for PI(3,4,5)P_3 pentamers is at least one order of magnitude higher than that for actin under physiological conditions, and the concentration of PI(3,4,5)P_3 is about the same as that of unpolymerized actin in platelets (10).

Profilin bound to PI(3,4,5)P_3 on the membrane could be the negative regulator of PLC activity that has been postulated to account for the low rate of PI(3,4,5)P_3 hydrolysis in resting cells (20). Profilin is an effective inhibitor of PI(3,4,5)P_3 hydrolysis by PLC, even when PI(3,4,5)P_3 is incorporated into lipid bilayers composed mainly of other lipids and when the concentration of Ca^{2+} is optimal for PLC activity.

Fig. 2. Profilin inhibits the hydrolysis of PI(3,4,5)P_3 by platelet soluble PLC. (A) Time course of micellar PI(3,4,5)P_3 hydrolysis by PLC (25) in the absence (circles) or presence (squares) of 9.5 μM profilin. Incubations were performed at 36°C, with PLC (78 μg/ml) and 45 μM PI(3,4,5)P_3 in 100 mM tris (pH 6.5), 1 mM CaCl_2, and 0.1% deoxycholate. Open and solid symbols represent two separate experiments. (B) Profilin concentration dependence of the hydrolysis of micellar PI(3,4,5)P_3 by PLC. Conditions were as in (A) except that PI(3,4,5)P_3 concentration was 9 μM. Hydrolysis rates expressed as percentage of maximal activity (in the absence of profilin) were obtained from time courses. Both curves are theoretical: the dashed curve was calculated by assuming that each profilin molecule binds to 7 PI(3,4,5)P_3 molecules with an infinitely small K_eq and protects only these heptamers from PLC. The solid curve corresponds to each profilin molecule binding to 10 PI(3,4,5)P_3 molecules with a K_d of 0.1 μM. (C) The effect of profilin or ovalbumin on the hydrolysis of PI(3,4,5)P_3 incorporated into LUVs. Incubations were performed at 36°C in buffer B with 80 μM CaCl_2 and PLC (30 μg/ml). PI(3,4,5)P_3 was added to LUVs containing PE and PC (1:1) and purified away from micelles (26). The concentration of PI(3,4,5)P_3 was 2.3 μM. If we assume that the PI(3,4,5)P_3 was confined to the outer leaflet (26), the ratio of PI(3,4,5)P_3/PC:PE was 1:5 in the outer leaflet. The concentrations of human platelet profilin (●) or ovalbumin (○) were varied as indicated. Both curves are theoretical. The solid curve was calculated for a K_d of 1 μM for the complex of profilin with PI(3,4,5)P_3 pentamers. The dashed curve corresponds to a K_d of 1 mM.

In the cytoplasm, the low Ca^{2+} concentration together with profilin should reduce the activity of PLC even further.

Because all platelet PLC isoforms, including membrane-associated forms, have similar kinetic constants for PI(3,4,5)P_3 hydrolysis (9), the interaction of profilin with the substrate would be expected to inhibit all PLC isoforms. The human platelet soluble PLC-II used in these studies can be distinguished from membrane-bound isoforms with specific antibodies and by a difference in their Michaelis constants (K_m) for the hydrolysis of PI, but not for PI(3,4,5)P_3 (9). Studies suggest that membrane-associated PLC isoforms are responsible for the hydrolysis of PI(3,4,5)P_3 in response to stimulation of membrane receptors (20). However, the amino acid sequences available for the cytosolic and the membrane-associated PLCs are similar (20). Posttranslational mechanisms (20) may be responsible for the association of PLC isoforms with membranes.

If profilin inhibits PI(3,4,5)P_3 hydrolysis in cells, there must be some way for activated PLC to compete effectively with profilin. One possibility is that the activated PLC has a higher affinity for PI(3,4,5)P_3 than that of the purified enzyme used in our experiments (20). A central regulatory role for profilin in both the cytoskeleton and the polyphosphoinositide pathway may explain why deletion of the profilin gene can be lethal in yeast (21).

REFERENCES AND NOTES

9. J. J. Baldassare, P. A. Henderson, G. J. Fisher, Biochemistry 28, 6010 (1989). We used phosphoinositide-specific PLC-II that was isolated from the cytosol of outdated human platelets through four chromatographic steps, to a specific activity of 0.5 to 1.0 μmol min^{-1} mg^{-1}. The enzyme was stable at −70°C for at least 8 weeks after purification.


22. After incubation, micelles were run on a 0.7 cm by 50 cm column of Sepharcl S-300 at 4°C. SUVs (PI) and LUVs (~500±200 nM) were run on a 0.7 cm by 10 cm column in a 25 cm column of Sephadex G-100 at room temperature. Both columns were equilibrated with sample buffer, and the flow rate was 20 ml/hour. Fractions of 0.4 to 0.6 ml were assayed for protein (Bradford, Anal. Biochem. 72, 248 (1976)). Because lipids quench the Bradford dye-binding assay, the data in Fig. 1, A to D, are given in arbitrary units. The fraction of bound profilin was calculated as the difference between the total amount applied to the column and the amount in the entire included peak of free protein (centered on fraction 45 in Fig. 1, A to D).

23. Profilin was purified from outdated human platelets by affinity chromatography on poly-L-proline--Sepharcl S-300 columns by the following method: A. Spudich and S. Watt, J. Biol. Chem. 246, 4866 (1971); T. D. Pollard, J. Cell Biol. 103, 2747 (1986) and dialyzed in buffer B [5 mM tris (pH 7.5) 150 mM NaCl, 1.5 mM diethrotetanol, and 0.1 mM NaNO3 before the PC assay. Ovalum (Sigma) was dissolved in buffer B.

24. The purity of the profilinoides PI, PIP, and PIP, was determined by thin-layer chromatography with 10 μg of each lipid loaded onto silica gel 60 plates in a 95:8:1:2 chloroform, methanol, ammonium acetate, and water solvent system. Homogeneous PIP micelles were prepared by suspending 1 mg of PIP (Calbiochem) in 1 ml of deionized water and sonicated in a Branson 32 (batch-type) sonicator for 5 min at room temperature. PI (Sigma) SUVs were prepared in the same manner as PIP micelles. Large unilamellar vesicles of various dimensions were obtained by the extrusion technique (7). PIPs were dried in a glass vial under a stream of nitrogen and then resuspended in deionized water (0.5 to 1.2 ml) by vortexing. The PC, PS, and PE were obtained in chloroform (Avanti Polar Lipids, Pelham, AL) [2-32P]phosphatidyl inositol 4,5-bisphosphate ([32P]PIP2) was obtained in dichloromethane, ethan-

The product of the ras proto-oncongenc Ras, is a guanine nucleotide binding protein (1). By analogy with Saccharomyces cerevisiae Ras gene products and the mammalian G proteins that couple membrane receptors to effector molecules such as adenylate cyclase, it is thought that the guanosine diphosphate (GDP) form of Ras is inactive, that the exchange of bound GDP for guanosine triphosphate (GTP) stimulates interaction between Ras and an effector molecule, and that GTP hydrolysis returns Ras to an inactive state (7, 8).

Ras is implicated in the control of cell growth. Oncogenic mutations in ras cause unregulated cell proliferation. In Xenopus oocytes, microinjection of oncogenic forms of Ras stimulate maturation (1). Microinjection of a monoclonal antibody to Ras into resting fibroblasts blocks mitogenic responsiveness to serum and to purified PDGF and epidermal growth factor (EGF) (4).

A guanosine triphosphatase (GTPase) activator protein known as GAP has properties of a mediator of signals generated by Ras (5–7). GAP was isolated on the basis of its ability to enhance the weak GTPase activity of normal Ras. Oncogenic forms of Ras are not sensitive to GAP, and persist as GTP complexes. GAP action on normal Ras converts it to a GDP complex. In this way, GAP may attenuate signaling by normal Ras-GTP. Several results suggest that GAP may itself be the effector through which Ras-GTP transmits a mitogenic signal to the cell. Mutations of the GAP interaction domain on oncogenic forms of Ras blocks signaling (6, 8). In the Xenopus oocyte system, injection of a truncated form of Ras that has increased affinity for GAP is able to block some effects of oncogenically activated Ras, and excess GAP protein overcomes this