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The Actin-Binding Protein Profilin Binds to PIP_2 and Inhibits Its Hydrolysis by Phospholipase C

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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.

THE INHIBITION OF THE INTERACTION of actin with profilin, the most abundant actin-binding protein, by the membrane phospholipid PIP_2 (*1*) is one of a growing number of examples where phospholipids can influence cytoplasmic proteins (*2*). In the case of profilin and actin, it has been assumed that PIP_2 binds to the profilin. PIP_2 is also the precursor of two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, which regulate a variety of cellular processes (*3*). We have characterized the interaction of human platelet profilin with PIP_2 in vitro and present evidence that profilin can regulate phospholipid metabolism.

In a gel filtration assay (Fig. 1, A to E, and Table 1), 12 molecules of platelet profilin bound to micelles composed of 83 molecules of PIP_2 (*4*). This corresponds to 1 profilin molecule bound to 7 lipid molecules. The profilin that was bound to micelles eluted in the void volume and was well-separated from the free profilin. As the molar ratio of profilin bound to PIP_2 was the same (1:7) at all micelle concentrations tested, the affinity of the interaction must be relatively high. The amount of profilin trailing behind the micelle peak was small, which is further evidence that the complex has a dissociation constant (K_d) in the submicromolar range. The amount of profilin bound was the same in buffer with and without 75 m*M* KCl. Micelles of phosphatidylinositol 4-phosphate (PIP) gave similar results as micelles of PIP_2 (*5*), whereas much higher concentrations of small unilamellar vesicles (SUVs) of pure phosphatidylinositol (PI) were required to bind profilin.

At a 1:7 binding ratio, the profilin molecules should be tightly packed on the surface of a PIP_2 micelle. This conclusion is based on the dimensions of the profilin molecule [~3.0 nm by 3.0 nm by 3.5 nm (*6*)] and the 5.5- to 6.0-nm diameter of PIP_2 micelles (*4*). Thus, steric hindrance is likely to limit the binding of more profilin molecules to the micelles and to result in an overestimate of the number of PIP_2 molecules actually associated with each profilin molecule.

To obtain a better estimate of the molecular stoichiometry of the complex, we carried out parallel experiments with small amounts of PIP_2 incorporated into large unilamellar vesicles composed of other lipids. We used large unilamellar vesicles produced by the extrusion technique (LUVETs) (*7*) that were composed of a 5:1 molar ratio of phosphatidylcholine (PC) to PIP_2. Electron microscopy of negatively stained vesicles showed that they were unilamellar and had a mean diameter of 0.12 μm (SD = 0.03 μm, n = 31). Freeze-fracturing (*7*) showed that these vesicles were unilamellar, and nuclear magnetic resonance (*8*) showed that PIP_2 partitions between the two leaflets of such vesicles. Thus, we have assumed that 50% of the PIP_2 in the LUVETs is exposed to the medium and available for binding to profilin.

In the gel filtration assay, all concentrations of PC-PIP_2 LUVETs that we tested bound the same number of profilin mole-

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.

Lipid composition	Stoichiometry (number of PIP_2 molecules per profilin molecule)		K_d (μ*M*)	
	Filtration	PLC assay	Filtration	PLC assay
Micelles of PIP_2	7.4 (6.7 to 8.2)	10.0	<0.1	<0.1
LUVETs of PIP_2:PC (1:5)	5.4 (3.4 to 7.3)		<1.0	
LUVETs of PIP_2:PC:PE (1:1:1)		4.8 (3.9 to 5.8)		<1.0
LUVETs of PIP_2:PC (1:12)		5.0		<5.0
LUVETs of PIP_2:PC:PE (1:5:5)		5.0		<1.0
LUVETs of PI:PC (1:5)			<1000	
SUVs of PI			<1000	

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cules per PIP_2 molecule (Fig. 1F and Table 1). The molar ratio was 1 profilin per 5 PIP_2 molecules in the outer leaflet. This result suggests that the affinity of profilin for PIP_2 is relatively high even in the presence of excess PC. The data are also consistent with the induction by profilin of aggregation of PIP_2 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 PIP_2 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), even at high lipid concentrations, also did not bind profilin.

We then tested the possibility that the PIP_2-profilin complex might be a poor substrate for phospholipase C (PLC). Profilin inhibited the hydrolysis of micellar PIP_2 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 PIP_2 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 PIP_2 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 PIP_2 (*10*). Profilin inhibited the hydrolysis of micellar PIP_2 to a greater extent than expected from the 1:7 stoichiometry of the profilin-PIP_2 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 PIP_2. High concentrations of enzyme gave low but measurable rates of hydrolysis even in the presence of an excess of profilin over PIP_2 (*10*). These observations are consistent with competition between profilin and PLC for interaction with PIP_2.

At an optimal concentration of Ca^{2+} (80 μM in our assay) and at *p*H 6.5, PLC activity varied according to the type and concentration of the lipids mixed with PIP_2. Compared to PLC activity measured with PIP_2 micelles as the substrate, the activity was higher with LUVETs composed of PE and PIP_2 and lower with LUVETs of PC and PIP_2. This agrees with reports that indicated an enhancing effect of PE and an inhibitory effect of PC on PLC-catalyzed hydrolysis of PIP_2 in mixed SUVs (*11*).

Profilin inhibited hydrolysis of PIP_2 by PLC in all LUVET compositions tested (Fig. 2C and Table 1). In experiments with high concentrations of LUVETs, each profilin molecule protected ~5 PIP_2 molecules from hydrolysis by PLC at all profilin concentrations. This suggests that profilin aggregates PIP_2 into patches. These patches are likely to be small, because formation of large patches of PIP_2 would induce steric hindrance among profilin molecules (as observed with micelles). Estimation of the K_d 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 PIP_2 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 PIP_2 pentamer in LUVETs with a submicromolar K_d (Fig. 2C and Table 1). In control experiments, neither ovalbumin nor rabbit skeletal muscle actin inhibited hydrolysis of PIP_2 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 K_d 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*). PIP_2 and PIP micelles overcome these effects of profilin on actin, whereas IP_3 and SUVs of PI and PS have no effect (*10*).

The properties of the complex of profilin with PIP_2 described in this report explain why PIP_2 micelles can reverse the effects of profilin on actin. About 12 molecules of profilin bind to each micelle of PIP_2 with submicromolar affinity. At saturation, each profilin is associated with 7 PIP_2 molecules. The protein molecules are likely to be tightly packed on the surface of the micelle, presumably bound by electrostatic forces be-

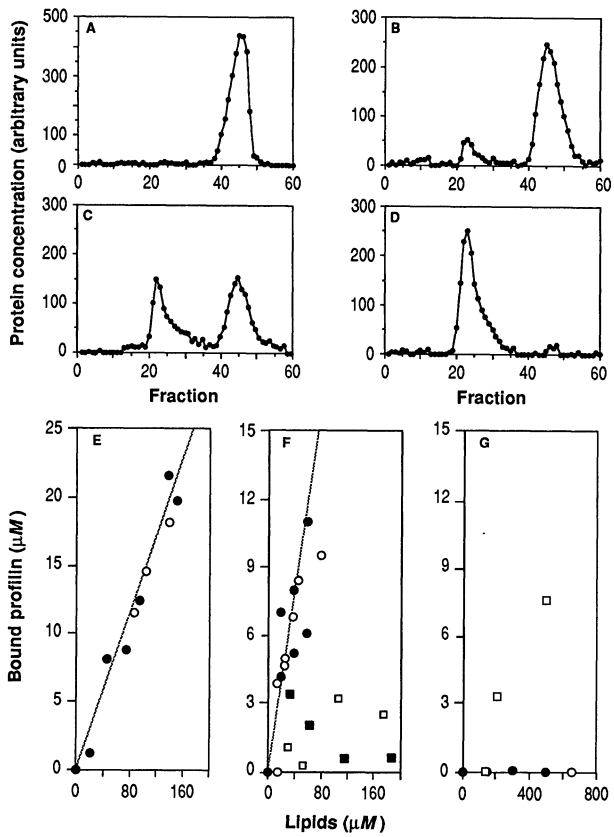

Fig. 1. Gel filtration assay for the binding of profilin to lipid micelles and vesicles. (**A**) Chromatogram (*22*) of profilin (*23*) alone at 4°C in buffer A [2 m*M* tris (*p*H 7.2), 0.2 m*M* ATP, 0.5 m*M* dithiothreitol, and 0.1 m*M* NaN_3]. (**B** to **D**) Chromatograms of mixtures of 22 μM profilin and various concentrations of micellar PIP_2 (*24*) (PIP_2-profilin molar ratios 2.1:1, 4.3:1, and 6.3:1, respectively) that had been incubated for 1 to 6 hours and chromatographed in buffer A at 4°C. (**E**) Binding of profilin to PIP_2 micelles. The results of gel filtration are expressed as the concentration of profilin bound to micelles in the original sample volume as a function of the PIP_2 concentration. Symbols are as follows: (●) profilin (22 μM) in buffer A; and (○) profilin (18.5 μM) in buffer B [5 m*M* tris (*p*H 7.5), 75 m*M* KCl, 0.5 m*M* dithiothreitol, and 0.1 m*M* NaN_3]. The theoretical line corresponds to a stoichiometry of 1 profilin per 7 PIP_2 molecules with all sites occupied. (**F**) Binding of profilin to LUVETs containing 5:1 molar ratios of PC and acidic phospholipids. The results are expressed as the concentration of profilin bound to LUVETs in the original sample volume as a function of the concentration of PIP_2, PI, or PS exposed on the surface (assumed to be 50% of the total). All experiments were performed in buffer B at room temperature. Symbols are as follows: (●) 23 μM profilin with LUVETs composed of PIP_2 and PC or (○) 13 μM profilin with LUVETs composed of PIP_2 and PC; (□) 23 μM profilin with LUVETs composed of PI and PC; and (■) 23 μM profilin with LUVETs composed of PS and PC. The theoretical line corresponds to a stoichiometry of 1 profilin per 5 PIP_2 molecules with all sites occupied. (**G**) Binding of profilin to lipids without PIP_2. All experiments were performed at room temperature with 23 μM profilin in buffer B. Symbols are as follows: (●) LUVETs of PC; (○) LUVETs of a 1:1 molar ratio of PE and PS; and (□) SUVs of PI.

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 PIP_2 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 a basic patch is a likely candidate for the PIP_2-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 PIP_2 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 PIP_2 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 μ*M* (*10, 16*)], the concentration of PIP_2 [140 to 240 μ*M* (*17*)], the 1:5 stoichiometry and submicromolar affinity of the complex, and the apparent ability of profilin to aggregate PIP_2 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 PIP_2 for profilin, but the affinity of profilin for PIP_2 pentamers is at least one order of magnitude higher than that for actin under physiological conditions, and the concentration of PIP_2 is about the same as that of unpolymerized actin in platelets (*10*).

Profilin bound to PIP_2 on the membrane could be the negative regulator of PLC activity that has been postulated to account for the low rate of PIP_2 hydrolysis in resting cells (*20*). Profilin is an effective inhibitor of PIP_2 hydrolysis by PLC, even when PIP_2 is incorporated into lipid bilayers composed mainly of other lipids and when the concentration of Ca^{2+} is optimal for PLC activity.

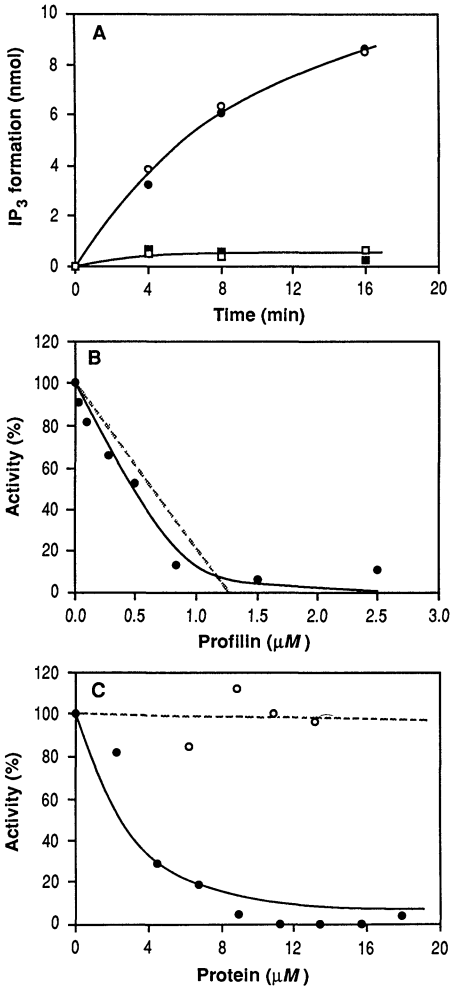

Fig. 2. Profilin inhibits the hydrolysis of PIP_2 by platelet soluble PLC. (**A**) Time course of micellar PIP_2 hydrolysis by PLC (*25*) in the absence (circles) or presence (squares) of 9.5 μ*M* profilin. Incubations were performed at 36°C, with PLC (78 μg/ml) and 45 μ*M* PIP_2, in 100 m*M* tris (*p*H 6.5), 1 m*M* $CaCl_2$, and 0.1% deoxycholate. Open and solid symbols represent two separate experiments. (**B**) Profilin concentration dependence of the hydrolysis of micellar PIP_2 by PLC. Conditions were as in (A) except that PIP_2 concentration was 9 μ*M*. 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 PIP_2 molecules with an infinitely small K_d and protects only these heptamers from PLC. The solid curve corresponds to each profilin molecule binding to 10 PIP_2 molecules with a K_d of 0.1 μ*M*. (**C**) The effect of profilin or ovalbumin on the hydrolysis of PIP_2 incorporated into LUVETs. Incubations were performed at 36°C in buffer B with 80 μ*M* $CaCl_2$ and PLC (30 μg/ml). PIP_2 was added to LUVETs containing PE and PC (1:1) and purified away from micelles (*26*). The concentration of PIP_2 was 2.3 μ*M*. If we assume that the PIP_2 was confined to the outer leaflet (*26*), the ratio of PIP_2:PC:PE was 1:5:5 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 μ*M* for the complex of profilin with PIP_2 pentamers. The dashed curve corresponds to a K_d of 1 m*M*.

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

Because all platelet PLC isozymes, including membrane-associated forms, have similar kinetic constants for PIP_2 hydrolysis (*9*), the interaction of profilin with the substrate would be expected to inhibit all PLC isozymes. The human platelet soluble PLC-II used in these studies can be distinguished from membrane-bound isozymes with specific antibodies and by a difference in their Michaelis constants (K_m) for the hydrolysis of PI, but not for PIP_2 (*9*). Studies suggest that membrane-associated PLC isozymes are responsible for the hydrolysis of PIP_2 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 isozymes with membranes.

If profilin inhibits PIP_2 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 PIP_2 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*).

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22. After incubation, micelles were run on a 0.7 cm by 50 cm column of Sephacryl S-300 at 4°C. SUVs (PI) and LUVETs were run on a 0.7 cm by 10 cm or 0.7 cm by 26 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 [M. 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–Sepharose (*16*). Actin was purified from rabbit skeletal muscle [J. 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 m*M* tris (*p*H 7.5), 75 m*M* KCl, 0.5 m*M* dithiothreitol, and 0.1 m*M* NaN_3] before the PLC assay. Ovalbumin (Sigma) was dissolved in buffer B.
24. The purity of the phosphoinositides PI, PIP, and PIP_2 was confirmed by thin-layer chromatography with 10 μg of each lipid loaded onto silica gel 60 plates in a 90:90:7:22 chloroform, methanol, ammonium hydroxide, and water solvent system. Homogeneous PIP_2 micelles were prepared by suspending 1 mg of PIP_2 (Calbiochem) in 1 ml of deionized water and sonicating in a Bransonic 32 (bath-type) sonicator for 5 min at room temperature. PI (Sigma) SUVs were prepared in the same manner as PIP_2 micelles. Large unilamellar vesicles of various composition were obtained by the extrusion technique (*7*). Mixtures of lipids were dried in a glass tube under a stream of nitrogen and were 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-^{3}H]Phosphatidyl inositol 4,5-bisphosphate ([^{3}H]PIP_2) was obtained in dichloromethane, ethanol, and water (20:10:1) (Amersham). After five cycles of freezing in liquid nitrogen and thawing in a water bath (at 36°C), samples were passed ten times through a filter (polycarbonate, 0.1 μm pore size, Nuclepore) in the extruder under a pressure of 400 psi. The concentration of lipid in each mixture was measured by liquid scintillation counting of a portion of the sample after extrusion.
25. For the PLC assay, [^{3}H]PIP_2 (final specific activity of 0.05 to 0.10 Ci/mol) was mixed with unlabeled PIP_2 to form micelles or with unlabeled PIP_2 and other lipids to form LUVETs of known composition (*24*). Hydrolysis was stopped by addition to the samples (100 μl) of ice-cold methanol, chloroform, and HCl [(3:1:1), 625 μl], which results in the separation of IP_3 from lipids. The [^{3}H]IP_3 in the aqueous phase was measured by liquid scintillation counting [M. G. Low and W. B. Weglicki, *Biochem. J.* **215**, 325 (1983); J. J. Baldassare and G. J. Fisher, *J. Biol. Chem.* **261**, 11942 (1986); D. M. Raben, K. Y. Yasuda, D. D. Cunningham, *Biochemistry* **26**, 2759 (1987)]. Because the PIP_2 concentration (S) was much smaller than the K_m of the enzyme (*9*), the rate of hydrolysis (v) was directly proportional to the PIP_2 concentration according to the Michaelis-Menten equation $v = V_{max}\,S/K_m + S$, where V_{max} is the maximal rate of hydrolysis. The effect of PIP_2 sequestration by profilin on the hydrolysis rate can be directly calculated from the Michaelis-Menten equation where S is replaced by: $1/2\{[(K_d + P_T - S_T)^2 + 4K_d S_T]^{1/2} - (K_d + P_T - S_T)\}$, where P_T and S_T are the total profilin and total PIP_2 concentrations, respectively, and K_d is the dissociation constant for the profilin-PIP_2 complex.
26. To add PIP_2 to the outer leaflet of LUVETs [P. A. Janmey and T. P. Stossel, *J. Biol. Chem.* **264**, 4825 (1989)], we incubated LUVETs (0.20 μm in diameter; SD = 0.05, n = 29) of known composition [PC or PC:PE (1:1)] with PIP_2 micelles in deionized water at 36°C for 5 hours. Separation of LUVETs from micellar PIP_2 was performed by filtration of the mixture on a 0.7 cm by 10 cm Sepharose 2B column at room temperature, with a flow rate of buffer B of 30 ml/hour, and a fraction size of 0.4 ml. The fraction of PIP_2 incorporated in the LUVETs, about 50% of the total, was quantitated by liquid scintillation counting of the void volume. After filtration, the LUVETs remained 0.20 μm in diameter (SD = 0.02 μm, n = 27) and unilamellar. To be sure that the PIP_2 remained incorporated in these LUVETs, a sample was refiltered on Sepharose 2B 6 hours after preparation, by which time the PLC assays had been completed. As transverse diffusion of phospholipids in a bilayer (flip-flop) is slow, we considered in our calculations that all the PIP_2 was in the outer leaflet of the LUVETs. We could not rule out that some of the PIP_2 was incorporated into the inner leaflet of the vesicles or trapped as micelles inside the vesicles. However, the stoichiometry and affinity of profilin binding to these LUVETs as measured in the PLC assay was consistent with our other determinations if we assumed that all of the PIP_2 in these fused LUVETs was in the outer leaflet.
27. We are grateful to R. E. Pagano, A. Ting, D. M. Raben, A. S. Mildvan, and J. M. Baraban for helpful discussions and comments on the manuscript. Supported by NIH research grant GM-26338 to T.D.P. L.M.M. is supported by a graduate fellowship from NSF.

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Binding of GAP to Activated PDGF Receptors

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The *ras* proto-oncogene products appear to relay intracellular signals via the Ras guanosine triphosphatase (GTPase) activator protein, GAP. In dog epithelial cells expressing human platelet-derived growth factor (PDGF) receptors, binding of PDGF caused approximately one-tenth of the total GAP molecules to complex with the receptor. Studies with mutant PDGF receptors showed that maximum association required both receptor kinase activity and phosphorylatable tyrosine residues at both the identified sites of receptor autophosphorylation.

THE PRODUCT OF THE *ras* PROTO-oncogene, 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 (*2, 3*).

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. Mutagenesis 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

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