Lipid Products of Phosphoinositide 3-Kinase Bind Human Profilin with High Affinity†

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ABSTRACT: To gain insight into the physiological function of phosphoinositide 3-kinase (PI 3-kinase) lipid products, this study examines the interactions of the D-3 phosphoinositides with profilin and the consequent effects on actin dynamics and phosphoinositide turnover. Profilin, a ubiquitous actin-regulating protein, plays a putative role in regulating actin assembly and PLC-γ1 signaling in light of its unique interactions with actin and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. Here we raise evidence that the affinity of profilin with the D-3 phosphoinositides is substantially higher than that of PtdIns(4,5)P_2. The dissociation constants (K_d) are estimated to be 1.1 μM, 5.7 μM, and 11 μM for phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2], phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], and PtdIns(4,5)P_2, respectively. Spectroscopic data show that while all these phosphoinositides alter the tryptophan fluorescence of profilin in a similar fashion, the respective conformational effect on profilin is vastly different. Based on CD data, the α-helical contents of profilin in the presence of 8 molar equiv of PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, and PtdIns(3,4)P_2 are 17.4%, 11.5%, and 1.4%, respectively, vis-à-vis 9.4% for profilin alone. In contrast, no appreciable change in the fluorescence and CD spectra is observed when related inositol phosphates such as Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, or Ins(1,3,4)P_3 at comparable concentrations are tested. Evidence suggests that this differential recognition bears functional significance concerning the intricate roles of profilin and inositol lipids in modulating actin polymerization and PtdIns(4,5)P_2 turnover. The relative potency of individual phosphoinositides in offsetting the inhibitory effect of profilin on actin assembly is PtdIns(3,4)P_2 > PtdIns(3,4,5)P_3 > PtdIns(4,5)P_2, consistent with their relative binding affinity with profilin. Moreover, the inhibitory effect of profilin on PLC-γ1-mediated PtdIns(4,5)P_2 hydrolysis is overcome by PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 through a combined effect of PLC-γ1 activation and preferential profilin binding. This D-3 phosphoinositide-mediated regulation may represent a new mechanism for controlling PtdIns(4,5)P_2 turnover by PLC-γ1.

Profilin is a ubiquitous cytoskeletal protein found in many eukaryotic systems. Because of its unique ability to bind actin and PtdIns(4,5)P_2[1] *in vitro*, profilin has been suggested to function as a cellular regulator that links transmembrane signal transduction and microfilament-based motility [for reviews, see Theirot and Mitchison (1993) and Sohn and Goldschmidt-Clermont (1994)]. By forming a 1:1 complex with actin monomers, profilin modulates the rate of actin polymerization by affecting actin dynamics, in which several distinct mechanisms may be involved (Mockrin & Korn, 1980; Pollard & Cooper, 1984; Pantaloni & Carlier, 1993). However, it is thought that profilin preferentially binds to PtdIns(4,5)P_2 in the plasma membrane in resting cells (Lassing & Lindberg, 1985). The putative utility of this PtdIns(4,5)P_2−profilin association is 2-fold. First, profilin is sequestered from actin monomers, thus preventing actin−profilin complex formation. Second, this binding protects PtdIns(4,5)P_2 from being hydrolyzed by phospholipase C-γ1 (PLC-γ1), which implicates profilin in the regulation of Ins(1,4,5)P_3 production (Goldschmidt-Clermont et al., 1990). Evidence indicates that this inhibition is overcome when PLC-γ1 is phosphorylated by a receptor tyrosine kinase, leading to the hydrolysis of PtdIns(4,5)P_2 accompanied by the dissociation of profilin from the plasma membrane (Goldschmidt-Clermont et al., 1991).

The intricate relationship among profilin, PtdIns(4,5)P_2, and actin in cells has been the focus of many *in vivo* investigations. Several lines of data dispute the premise that PtdIns(4,5)P_2 plays a regulatory role in the actin cytoskeleton.

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[1] Abbreviations: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; PtdEA, phosphatidylethanolamine; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4)P_3, D-*myo*-inositol 1,3,4-trisphosphate; PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; PDGF, platelet-derived growth factor; DTT, dithiothreitol; CD, circular dichroism, K_d, dissociation constant.

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For example, Traynor-Kaplan and co-workers showed that *N*-formyl peptide-induced actin polymerization in neutrophils correlated with the intracellular levels of PtdIns(3,4,5)P_3 rather than with PtdIns(4,5)P_2 concentrations (Eberle et al., 1990). Cantley et al. (1991) reported that the cellular event that most closely correlated with PI 3-kinase activation was actin filament rearrangement. In addition, cells with mutations that lacked PI 3-kinase binding sites in the platelet-derived growth factor (PDGF) β-receptor failed to undergo actin rearrangement or other motility responses such as membrane ruffling and chemotaxis in response to PDGF (Severinsson et al., 1990; Wennstrom et al., 1994; Kundra et al., 1994). Exposure of fibroblasts to wortmannin, a PI 3-kinase inhibitor, blocked PDGF-mediated actin rearrangement (Wymann & Arcaro, 1994). Moreover, the small GTP-binding protein Rho that regulated cytoskeletal reorganization in growth factor-treated cells was found to activate PI 3-kinase in platelet extracts (Zhang et al., 1993). Rac, another small GTP-binding protein that was implicated in membrane ruffling, was suggested to be a major effector protein for the PI 3-kinase signaling pathway (Hawkins et al., 1995). All these findings have prompted a notion that activation of PI 3-kinase is crucial to actin reorganization.

It has been shown that the output signal of PI 3-kinase activation is transient accumulations of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in cells (Traynor-Kaplan et al., 1989; Stephens et al., 1991). These D-3 phosphoinositides are not susceptible to PLC hydrolysis, indicating that they do not serve as precursors to inositol phosphate second messengers (Serunian et al., 1989). Although the crucial role of PI 3-kinase activation in many cellular functions has been demonstrated [see reviews by Stephens et al. (1993) and Kapeller and Cantley (1994)], the molecular targets of its lipid products remain unclear. In an effort to understand the mode of mechanism for the D-3 phosphoinositides in actin reorganization, we examined the molecular interactions of profilin with various phosphatidylinositol phosphates including PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, and PtdIns(3,4)P_2. Here, we report that profilin displays differential recognition among these phospholipids, which bears functional significance concerning the cellular roles of PI 3-kinase and profilin in actin rearrangement and second messenger production.

EXPERIMENTAL PROCEDURES

Materials. 1-*O*-(1,2-Di-*O*-palmitoyl-*sn*-glycerol-3-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], 1-*O*-(1,2-di-*O*-octanoyl-*sn*-glycerol-3-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate [di-C_8-PtdIns(3,4,5)P_3], and 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycerol-3-phosphoryl)-D-*myo*-inositol 3,4-bisphosphate [PtdIns(3,4)P_2] were synthesized as previously reported (Gou & Chen, 1994; Wang & Chen, 1996). The purity of these synthetic phosphoinositides was confirmed by ^{1}H and ^{31}P NMR and FAB mass spectrometry, in which no trace amounts of isomeric impurity could be detected. PtdIns(4,5)P_2, phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEA), and 1,2-dipalmitoyl-*sn*-glycerol were purchased from Sigma. Inositol phosphates used in this study were synthesized according to the reported procedures (Lu et al., 1994).

Homogeneous micelles containing various phospholipids were prepared according to the procedure of Goldschmidt-Clermont et al. (1990) by sonicating in distilled water in a Model 1210 Bransonic Ultrasonic Cleaner for 5 min at room temperature.

Poly(L-proline) (M_r 12 000−15 000; Sigma) was coupled to CNBr-activated Sepharose (Sigma) according to a modification of the method developed by Lindberg et al. (1988). Poly(L-proline) (2 g) was stirred in 100 mL of distilled water for 2 days, and added to 40 mL of 0.1 M $KHCO_3$, pH 8.3, containing 5 g of CNBr-activated Sepharose. The mixture was incubated at 4 °C for 20 h with gentle shaking. The matrix beads were separated by filtration, washed with 0.1 M $KHCO_3$, pH 8.3, and added to the same buffer containing 0.2 M ethanolamine to remove the remaining active groups on the gel. The affinity matrix was stored in a high-salt buffer (buffer A) containing 10 mM Tris, pH 7.8, 0.1 M NaCl, 0.1 M glycine, and 1 mM dithiothreitol (DTT). After each use, the gel was washed with 8 M urea for 6 h to remove bound material.

Profilin was purified from outdated human platelets (Central Kentucky Blood Center) by affinity chromatography on poly(L-proline)−Sepharose based on the method of Kaiser et al. (1989). In brief, the cytosolic fraction of human platelets was diluted with buffer A at a 1:1 ratio, and applied onto a poly(L-proline)-conjugated Sepharose column (1 × 15 cm) pre-equilibrated with the same buffer. After the column was washed with 10 bed volumes of buffer A, actin and profilin were eluted by 4 and 8 M urea, respectively. The homogeneity of the affinity-purified profilin was indicated by a single protein band on 12.5% SDS−PAGE with a molecular mass of 15.5 kDa. The profilin-containing fractions were collected and dialyzed against 2 mM Tris/HCl, pH 7.2, containing 0.1 mM ATP, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.5 mM DTT for 48 h. The profilin was concentrated by ultrafiltration and used for assays. The profilin concentration was determined by measuring the UV absorbance at 280 nm using an extinction coefficient of 0.015 $\mu M^{-1}cm^{-1}$ (Tseng et al., 1984). PLC-γ1 was purified from bovine brain as previously described (Ryu et al., 1987).

Gel Filtration Assay for the Binding of Profilin to Lipid Micelles. This assay was similar to that described for studying the binding of profilin (Goldschmidt-Clermont et al., 1990) and gelsolin (Yu et al., 1992) to PtdIns(4,5)P_2. For comparison, phosphoinositide-containing micelles were prepared in distilled water as originally described. Profilin (32.1 μM) was incubated with micelles containing varying amounts of phospholipids for 30 min at 25 °C. One hundred microliters of the mixture was chromatographed at room temperature on a Sephacryl S-200 column (1 × 15 cm) equilibrated with 5 mM Tris/HCl, pH 7.5, containing 75 mM KCl and 0.5 mM DTT. The column was eluted with the same buffer at 0.5 mL/min, and fractions of 0.45 mL were collected. Protein assays were performed by the Coomassie blue dye-binding method using a commercial protein assay reagent (Bio-Rad) with bovine serum albumin as a standard. Because phospholipids interfere with the dye-binding assay, protein concentrations 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 (Goldschmidt-Clermont et al., 1990). Free phospholipid concentrations were calculated as the difference between the total

lipid concentration and the amount of profilin-associated lipid. Accordingly, the apparent dissociation constant was determined by using eq 1:

$$K_d = [\text{profilin}]_{\text{free}}[\text{lipid}]_{\text{free}}/[\text{profilin}-\text{lipid}] \quad (1)$$

Fluorescence Spectroscopy. Fluorescence spectra were recorded at 30 °C with a Hitachi F-2000 spectrophotometer. Human profilin contains two N-terminal tryptophan residues at positions 3 and 31. To assess the interactions between phospholipids and these tryptophans, the fluorescence excitation wavelength was set to 292 nm as described by Raghunathan et al. (1992). The buffer used for the fluorescence experiments consisted of 10 mM Tris, 150 mM KCl, 1 mM EDTA, and 5 mM β-mercaptoethanol, pH 7.5. Individual phosphoinositides, in the form of micelles, were gradually introduced into 700 μL of the buffer containing 14 μM profilin. The total volume of the micelles added was less than 10 μL. Within the concentration range of phosphoinositides used in this study, the bulk solution remained clear. Moreover, no appreciable spectra were noted when individual phospholipids at the indicated concentrations were incubated alone.

Changes in the fluorescence intensity were used as a measure of the binding affinity of the protein−lipid complex according to eq 2 (Ward, 1985):

$$1/\{[1 - (\Delta F/\Delta F_{\max})]K_a\} = \{[\text{phosphoinositide}]_{\text{total}}/(\Delta F/\Delta F_{\max})\} - p[\text{profilin}]_{\text{total}} \quad (2)$$

where K_a denotes the association constant and p represents the number of binding sites.

Circular Dichroism Spectroscopy. CD spectra were recorded with a JASCO J720 spectropolarimeter at 30 °C in a 20-mm path length cell. The solution contained 17 μM profilin and varying amounts of phospholipids in 10 mM Tris, 150 mM KCl, and 5 mM β-mercaptoethanol, pH 7.5. The following settings were used: wavelength range, 200−300 nm; bandwidth, 1 nm; step resolution, 0.5 nm; scan speed, 10 mdeg/min. Each spectrum represented an average of 10 scans with base line subtraction. Phospholipids alone at the indicated concentrations did not give measurable spectra within this region. The recorded spectra were presented in terms of mean residue ellipticity on the basis of the protein concentration. The helical content of profilin (f_H) was calculated based on the molecular ellipticity at 222 nm ($[\theta]_{222}$) according to the equation developed by Chen et al. (1972) (eq 3):

$$[\theta]_{222} = -30\,300 f_H - 2340 \quad (3)$$

where −30 300 is the ellipticity of pure helix.

Actin Polymerization. Actin polymerization was analyzed by using a fluorescence assay described by Lee et al. (1988). The amount of F-actin was measured from the fluorescence of pyrene-labeled actin (rabbit muscle) with an excitation wavelength of 365 nm and an emission wavelength of 407 nm at 30 °C. Experiments were performed in G buffer that contained 0.5 mM ATP, 0.5 mM DTT, 0.1 mM $CaCl_2$, and 10 mM Tris/HCl, pH 7.5, and the polymerization was initiated by adding 100 mM KCl and 2 mM $MgCl_2$ to the assay mixture.

PLC Assay. The assay was carried out by measuring the formation of Ins(1,4,5)P_3 from PtdIns(4,5)P_2 hydrolysis by PLC-γ1 according to the procedure of Goldschmidt-Clermont et al. (1991). The reaction mixture consisted of 50 mM Hepes, pH 7.0, PtdIns(4,5)P_2 vesicles containing 0.015 μCi of [^{3}H]PtdIns(4,5)P_2 (specific activity, 2−10 Ci/mmol), 10 μM cold PtdIns(4,5)P_2, 30 μM PtdEA, and 30 μM PtdSer with or without 10 μM PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, varying amounts of profilin, 2 mM $MgCl_2$, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, and 1 μM free form $CaCl_2$, in a total volume of 45 μL. The reaction was initiated by adding 5 ng of PLC-γ1 in 5 μL of 50 mM Hepes, pH 7.0, containing 10 mg/mL bovine serum albumin. After incubating at 30 °C for 10 min, the reaction was stopped by the addition to the mixture of ice-cold 300 μL of 10% trichloroacetic acid and 100 μL of 10 mg/mL BSA. The sample stood on ice for 3 min and was centrifuged at 10000*g* for 5 min. The [^{3}H]Ins(1,4,5)P_3 in the supernatant was measured by liquid scintillation spectrometry.

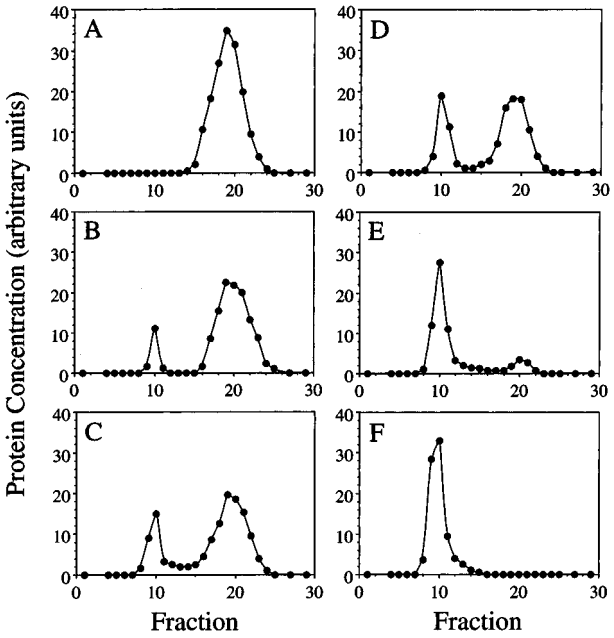

FIGURE 1: Gel filtration assay for profilin binding to PtdIns(3,4,5)-P_3-containing micelles. (A) Elution profile of profilin (32.1 μM) alone at 25 °C on a Sephacryl S-200 column (1 × 15 cm). (B−F) Elution profiles of mixtures of profilin and varying amounts of micellar PtdIns(3,4,5)P_3 under the same conditions. The molar ratios of PtdIns(3,4,5)P_3 to profilin were 1.2:1, 2.5:1, 3.7:1, 4.9:1, and 6:1, respectively, for panels B−F. Due to the interference of phospholipids in the protein assay, the concentrations of eluted profilin were expressed in arbitrary units.

RESULTS

Gel Filtration Analysis of Profilin Binding to Phosphatidylinositol Polyphosphates. The interfacial interactions of profilin with PtdIns(4,5)P_2, PtdIns(3,4)P_2, and PtdIns(3,4,5)-P_3 were first analyzed by a gel filtration method described by Goldschmidt-Clermont et al. (1990). Profilin (32.1 μM) was incubated with micelles composed of varying amounts of the pure phosphoinositide, and was chromatographed on a short-path Sephacryl S-200 column. Figure 1 illustrates the representative elution profiles of profilin alone (A) and with increasing amounts of PtdIns(3,4,5)P_3 (B−F). As shown, the micelle-bound profilin was eluted in the void volume and was well separated from the free profilin, and PtdIns(3,4,5)P_3 shifted the elution of profilin in a dose-dependent manner. At a 6:1 molar ratio, the free profilin

peak completely disappeared (Figure 1F), indicating the high binding affinity with PtdIns(3,4,5)P_3. The elution profiles in the presence of PtdIns(3,4)P_2 or PtdIns(4,5)P_2 were analogous to those of PtdIns(3,4,5)P_3 (not shown). From the data of individual experiments, the apparent dissociation constants were estimated, using eq 1, to be 1.1 ± 0.5 μM (n = 5), 5.7 ± 1.2 μM (n = 5), and 11.0 ± 1.7 μM (n = 6) for PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, and PtdIns(4,5)P_2, respectively. In addition, the apparent binding stoichiometry of the lipid−profilin complex was determined by plotting the amount of profilin bound to the micelle against the theoretical concentration of individual inositol lipids (not shown). For all three phosphoinositides, a linear relationship was observed up to the completion of complex formation. Accordingly, the molar ratios of PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, and PtdIns(4,5)P_2 to profilin were estimated to be 5.5:1, 6:1, and 10:1, respectively. It is worth noting that the stoichiometry of the PtdIns(4,5)P_2−profilin complex (10:1) was in line with the reported range of 5:1 to 10:1 (Goldschmidt-Clermont et al., 1990).

Fluorescence Spectroscopy. Further evidence that profilin exhibited differential binding among these three phosphoinositides was provided by fluorescence spectroscopy. As human profilin contains two N-terminal tryptophan residues (Trp-3 and Trp-31), its interactions with ligands could be examined by monitoring the tryptophan fluorescence with the excitation wavelength set to 292 nm (Raghunathan et al., 1992). The emission maximum for profilin was observed at 334 nm, which is consistent with the reported data and indicates a partially buried environment for these tryptophan residues (Metzler et al., 1994). As shown in Figure 2 A−C, all three phosphoinositides quenched the fluorescence of profilin in a dose-dependent and saturable manner, accompanied by a gradual shift of the fluorescence maximum from 334 to 331 nm. Two control experiments were carried out here to ensure that such spectral modifications were not caused by a micellar effect. First, no appreciable fluorescence spectra were noted when individual phosphoinositides alone, even up to 100 μM, were examined. Second, fluorescence titration of profilin with di-C_8-PtdIns(3,4,5)P_3, a water-soluble counterpart of PtdIns(3,4,5)P_3 (a di-C_{16} derivative), gave a concentration-dependent spectral change similar to that of PtdIns(3,4,5)P_3 (not shown).

Moreover, addition of PtdCho, PtdSer, PtdIns, PtdEA, or related inositol phosphates such as Ins(1,3,4)P_3, Ins(1,4,5)P_3, or Ins(1,3,4,5)P_4 at comparable concentrations did not cause an appreciable change in profilin fluorescence. These data clearly showed the specificity of profilin binding to phosphatidylinositol phosphates.

The results of the fluorescence titration showed that the phosphoinositide-binding site on human profilin was saturated at lipid:profilin ratios of 1.8:1, 2.7:1, and 5.6:1 for PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, and PtdIns(4,5)P_2, respectively. A linear relationship was observed between $1/[1 - (\Delta F/\Delta F_{max})]$ and $[\text{phosphoinositide}]_{total}/(\Delta F/\Delta F_{max})$. Accordingly, dissociation constants were calculated from the slopes of the lines, as depicted in eq 2, which were 2 μM, 7 μM, and 35 μM for PtdIns(3,4)P_2, PtdIns(3,4,5)P_3, and PtdIns(4,5)P_2, respectively. It is worth mentioning that the K_d values determined by fluorescence titration were consistent with those estimated by gel filtration despite differences in their basic principles.

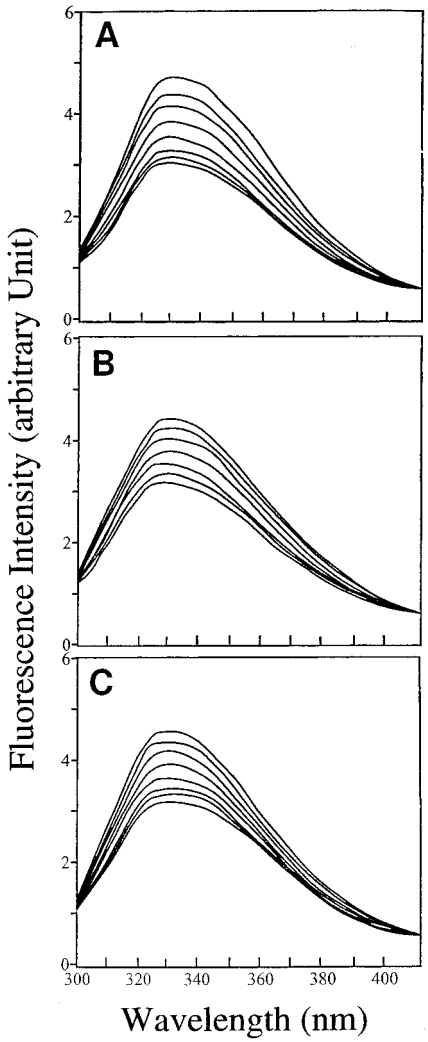

FIGURE 2: Tryptophan fluorescence emission spectra of profilin in the presence of varying amounts of (A) PtdIns(3,4)P_2, (B) PtdIns(3,4,5)P_3, and (C) PtdIns(4,5)P_2. Spectra were recorded with 17 μM profilin according to the method described under Experimental Procedures. Molar ratios of the phosphoinositide to profilin were (A) for PtdIns(3,4)P_2 (top to bottom): 0:1, 0.03:1, 0.06:1, 0.09:1, 0.26:1, 0.66:1. 1.2:1, and 1.8:1; (B) for PtdIns(3,4,5)P_3 (top to bottom): 0:1, 0.14:1, 0.29:1, 0.43:1, 1:1, 1.6:1, and 2.7:1; (C) for PtdIns(4,5)P_2 (top to bottom): 0:1, 0.43:1, 0.85:1, 1.29:1, 2.1:1, 3.0:1, 3.9:1, and 5.6:1.

Circular Dichroism Spectroscopy. Information of the effect of phosphoinositide binding on profilin conformations was obtained by CD spectroscopy. Figure 3 shows the CD spectra of profilin (14 μM) alone (curve a) and in the presence of 8 molar equiv of PtdIns(3,4)P_2 (curve b), PtdIns(3,4,5)P_3 (curve c), and PtdIns(4,5)P_2 (curve d). None of these three phosphoinositides alone (112 μM) gave a measurable spectrum within this region, indicating that the aforementioned changes in the CD spectra were not caused by micellar scattering or self-absorption. Especially noteworthy is that PtdIns(3,4)P_2 and PtdIns(4,5)P_2 affected the CD profiles of profilin, between 205 and 240 nm, in an opposite manner, revealing that these two inositol lipids exerted vastly different effects on profilin conformations. In contrast, the spectral change by PtdIns(3,4,5)P_3 was less pronounced as compared to that by the other two phosphoinositides.

It is well understood that the molecular ellipticity at 222 nm ($[\theta]_{222}$) provides a reliable measure of the helicity (Chen

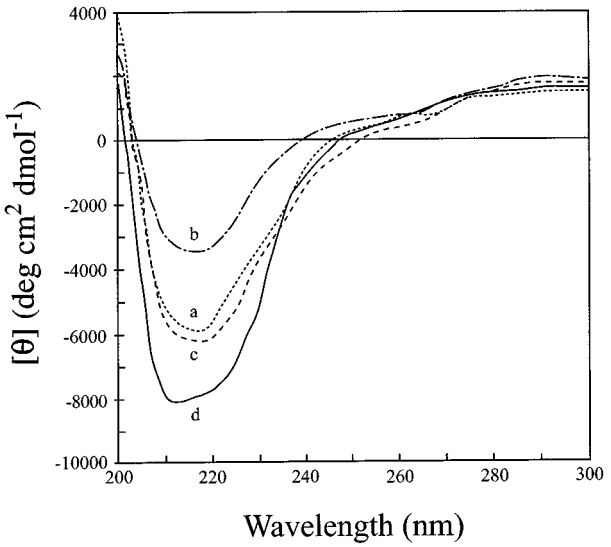

FIGURE 3: Circular dichroism spectra of profilin (17 μM) alone (a, ···) and in the presence of 8 molar equiv of PtdIns(3,4)P_2 (b, − · −), PtdIns(3,4,5)P_3 (c, − −), and PtdIns(4,5)P_2 (d, —). Individual phosphoinositides alone at 138 μM did not give any appreciable spectra, as represented by the straight line at $y = 0$.

et al., 1972), and eq 3 has previously been used to determined the α-helicity of profilin (Raghunathan et al., 1992). Considering the $[\theta]_{222}$ value of −5200 deg cm^2 dmol^{-1}, the α-helical content of profilin in the absence of any phospholipid was estimated to be 9.4%, a value that lied within the reported range of 8−15% helical content for profilin (Malm et al., 1983). In the presence of an 8:1 molar ratio of phospholipid to profilin, the corresponding values were the following: PtdIns(4,5)P_2, −7600 deg cm^2 dmol^{-1} and 17.4%; PtdIns(3,4,5)P_3, −5842 deg cm^2 dmol^{-1} and 11.5%; PtdIns(3,4)P_2, −2756 deg cm^2 dmol^{-1} and 1.4%.

Reversal of Profilin-Exerted Inhibition of Actin Polymerization by Phosphoinositides. The effect of phosphoinositides on profilin-exerted inhibition of actin polymerization was assessed by two types of experiments (Figure 4). In the competitive binding experiment (Figure 4A), individual phosphoinositides (10 μM) competed with actin monomers (8 μM) for profilin (8 μM) binding. Curves a and b display the time course of actin polymerization for actin alone and in the presence of an equivalent amount of profilin. As expected, profilin inhibited actin polymerization as a result of actin−profilin complex formation. However, this inhibitory effect was overcome by all three phosphoinositides examined, in particular PtdIns(3,4)P_2, as a result of competitive profilin binding. The order of this compensatory effect was PtdIns(3,4)P_2 (curve c) > PtdIns(3,4,5)P_3 (curve d) > PtdIns(4,5)P_2 (curve e), which was in accord with their relative binding affinity with profilin.

Correlated with this finding was the observation that PtdIns(3,4)P_2 enhanced actin polymerization by facilitating the dissociation of actin−profilin complexes (Figure 4B). As shown, the rate of actin polymerization was suppressed in the presence of profilin (curve b), but significantly arose upon addition of PtdIns(3,4)P_2 to the complex (curve c). It is worthy to note that PtdIns(3,4)P_2 was able to completely offset profilin inhibition after a few hours as the extent of actin polymerization (curve c) reached the same level as that of the control (curve a) (data not shown). This reversibility test confirmed the regulatory effect of PtdIns(3,4)P_2 on the dynamics of actin−profilin interactions. In comparison,

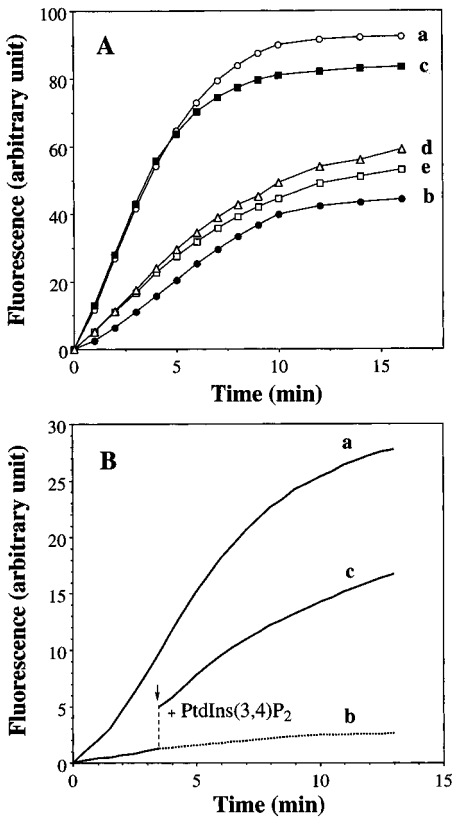

FIGURE 4: Effect of phosphoinositides on profilin-exerted inhibition of actin polymerization. In the competitive experiment (A), 10 μM pyrene-labeled actin was incubated in G buffer that contained 10 mM Tris/HCl, pH 7.5, 0.5 mM ATP, 0.5 mM DTT, and 0.1 mM $CaCl_2$ under various conditions: (a) control (none was added); (b) +8 μM profilin; (c) +8 μM profilin and 10 μM PtdIns(3,4)P_2; (d) +8 μM profilin and 10 μM PtdIns(3,4,5)P_3; (e) +8 μM profilin and 10 μM PtdIns(4,5)P_2. After 10 min incubation, actin polymerization was initiated by adding 100 mM KCl and 2 mM$MgCl_2$ to the assay mixture. All concentrations indicated above were based on a final volume of 1 mL. The extent of actin polymerization was indicated by the increase in fluorescence intensity. In the reversibility experiment (B), 2.4 μM pyrene-labeled actin and 5 μM unlabeled actin were incubated alone (curve a) or in the presence of 8 μM profilin (curve b) under polymerization conditions. The arrow indicates the addition of 32 μM PtdIns(3,4)P_2 to a mixture of actin and profilin at 3.5 min, and curve c represents the resulting time course.

PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 also exhibited activity in dissociating actin−profilin complexes, however, at lower potencies than PtdIns(3,4)P_2.

Effect of D-3 Phosphoinositides on Profilin-Mediated Inhibition of PLC-γ1. Profilin imposed a dose-dependent inhibition of PtdIns(4,5)P_2 hydrolysis by PLC-γ1 due to the effect of substrate depletion (Figure 5, curve a). Even at a molar ratio of PtdIns(4,5)P_2 to profilin of 2.5:1, 70% of the enzyme activity was inhibited, which might, in part, be due to the high binding stoichiometry of the PtdIns(4,5)P_2−profilin complex (10:1). Conceivably, competition for PtdIns(4,5)P_2 binding between profilin and PLC-γ1 inhibited PLC-γ1-mediated PtdIns(4,5)P_2 hydrolysis (Goldschmidt-Clermont et al., 1990). This inhibition, however, could be overcome by adding PtdIns(3,4,5)P_3 (curve b) or PtdIns(3,4)P_2 (curve c). The reversal of PLC-γ1 inhibition might be accounted for by two distinct mechanisms. First, both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were able to stimulate PLC-γ1 (Rhee et al., unpublished data), which is evidenced by the relative PLC-γ1 activities in the absence of profilin in

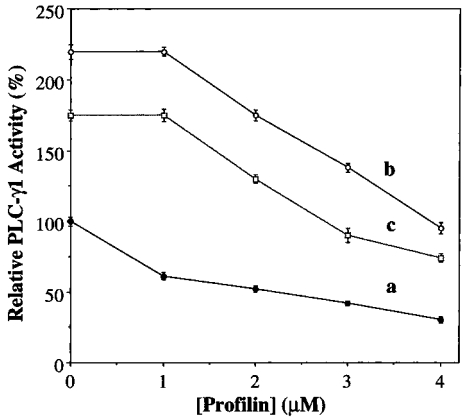

FIGURE 5: Effect of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 on profilin-elicited PLC-γ1 inhibition. PLC-γ1-mediated PtdIns(4,5)P_2 hydrolysis was analyzed according to the method described under Experimental Procedures. The PLC activity was assayed in the presence of varying amounts of profilin under different conditions: (a) control (none was added); (b) +10 μM PtdIns(3,4,5)P_3; (c) +10 μM PtdIns(3,4)P_2. Each point represents the mean of three determinations.

Figure 5. Second, this protective effect could also be attributed to preferential binding of profilin to the D-3 phosphoinositides. As shown, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at 10 μM were able to protect PLC-γ1 from losing activity with profilin concentrations up to 1 μM.

DISCUSSION

Our study shows that micellar PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 bind profilin with high affinity, with K_d values 10- and 2-fold, respectively, lower than that of PtdIns(4,5)P_2. This differential recognition bears functional significance concerning the intricate roles of profilin and inositol lipids in the regulation of actin assembly and Ins(1,4,5)P_3 production. In unstimulated cells, profilin partitions between the plasma membrane and the cytosol in response to membrane PtdIns(4,5)P_2 levels (Ostrander et al., 1995), of which the ratio appears to be crucial to the regulation of actin dynamics. The present data raise a possibility that PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 may also play a role in controlling the locality of profilin owing to their high affinity with this cytoskeletal protein. This premise is corroborated by *in vivo* findings that transient accumulations of the D-3 phosphoinositides and a rapid decrease in PtdIns(4,5)P_2 levels were noted in *N*-formyl peptide-induced actin assembly in neutrophils (Eberle et al., 1990), and that activation of platelets led to a significant increase in the membrane association of profilin (Hartwig et al., 1989). Conceivably, the D-3 phosphoinositides produced in response to agonist stimulation spontaneously take over profilin from PtdIns(4,5)P_2 or actin (Figure 6). Though the exact mechanism remains unclear, profilin at the periphery of activated cells triggers cytoskeletal reorganization (Buss et al., 1992). However, to establish a link between PI 3-kinase activation and actin assembly, an important factor that warrants consideration is the relatively small total mass of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 produced versus that of the classical phosphoinositides. For example, it was reported that after 10 s of *N*-formyl peptide stimulation of human neutrophils, the concentration of PtdIns(4,5)P_2 was higher than that of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 by 1 order of magnitude (Stephens et al., 1991).

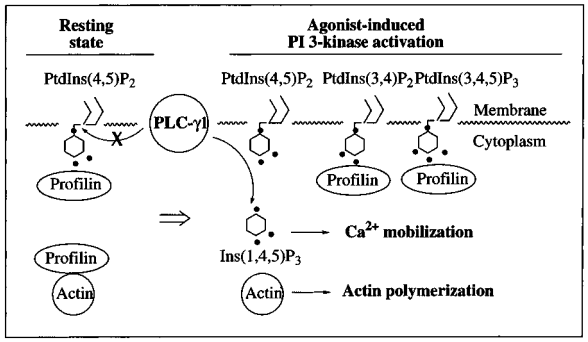

FIGURE 6: Diagrammatic representation of the effect of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 on profilin locality, Ins(1,4,5)P_3 production, and actin assembly.

Thus, a plausible scenario to account for the functional role of the D-3 phosphoinositides is that upon activation, the displacement of actin and PtdIns(4,5)P_2 from profilin is localized to a specific area of the plasma membrane, thereby promoting local actin assembly.

This study also provides evidence that the D-3 phosphoinositides modulate the turnover of PtdIns(4,5)P_2 by counteracting the inhibitory effect of profilin on PLC-γ1. Conceivably, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 facilitate the dissociation of PtdIns(4,5)P_2−profilin complexes, which leads to increased exposure of PLC-γ1 to its substrate PtdIns(4,5)P_2. In addition, both inositol lipids were found to activate PLC-γ1, of which the underlying mechanism is currently under investigation (Rhee et al., unpublished). This D-3 phosphoinositide-mediated regulation represents a new mechanism, auxiliary to the PTK-mediated phosphorylation, for modulating PLC-γ1 activity. It is noteworthy that activation of PLC-γ1 by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 may provide a cross-talk mechanism between PI-3 kinase- and PLC-γ1-mediated signaling pathways.

As evidenced by the CD spectra (Figure 3), the modes of interactions of various phosphoinositides with profilin and the consequent conformational effects appear to be different. Among the phosphoinositides examined, PtdIns(3,4)P_2 displays the most stringent binding to profilin, which leads to a significant reduction in the α-helical content, from 9.5% to 1.4%. An opposite effect on the α-helicity is noted with PtdIns(4,5)P_2 that is the weakest ligand among these three inositol lipids. In contrast, addition of PtdIns(3,4,5)P_3 does not cause a significant change in the CD spectrum. The structural basis for such effects on CD spectroscopy may be rationalized as follows.

Although the three-dimensional structures of profilin have been elucidated, the exact location of the phosphoinositide-binding site remains in dispute. Yu et al. (1992) proposed a consensus sequence for PtdIns(4,5)P_2-specific binding, KXXXXXXHXRR, among a variety of PtdIns(4,5)P_2-binding proteins, which corresponded to the region spanning residues 126−136 of human profilin. According to the crystal structure of *Acanthamoeba* profilin, Almo and coworkers suggested that the two distinct areas of high positive potential on the surface of profilin II were candidate binding sites for PtdIns(4,5)P_2 (Fedorov et al., 1994). The positively charged residues on one area included Arg-66, Arg-71, Lys-80, Lys-81, and Lys-115, which were analogous to residues Lys-69, Arg-74, Arg-88, Lys-90, and Lys-125 of human profilin. In addition, Schutt et al. (1993) proposed that the four positively charged residues in the COOH-terminal helix

(Lys-125, Lys-126, Arg-135, and Arg-136) might be responsible for interacting with the phosphate function. More recently, Sohn et al. (1995) demonstrated through site-directed mutagenesis that the region surrounding residue Arg-88 of human profilin represented an important binding site for PtdIns(4,5)P_2. As shown, all these putative binding regions contain multiple basic residues for interacting with the charged head groups of PtdIns(4,5)P_2. In view of the largely shared structural motifs, it is plausible that PtdIns-(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 bind to the same area of profilin. Different basic residues, however, may be involved in the electrostatic recognition of the vicinal 3,4- and 4,5-bisphosphates. Consequently, the respective binding strength as well as the induced conformational change varies between PtdIns(3,4)P_2 and PtdIns(4,5)P_2. Presumably, both sets of charged residues take part in the binding with PtdIns(3,4,5)P_3, resulting in a conformational effect intermediary to that of PtdIns(3,4)P_2 and PtdIns(4,5)P_2. Thus, no apparent change in the CD spectrum is noted.

On the other hand, all these phosphoinositides modify the tryptophan fluorescence of profilin in a similar fashion, i.e., a dose-dependent and saturable decrease in the fluorescence intensity accompanied by a slight blue shift in the optimal emission wavelength. In comparison, interaction of profilin with other types of ligands gave rise to different patterns of fluorescence spectra. For example, Metzler et al. (1994) reported that titration of profilin with poly(L-proline) resulted in a substantial increase in the fluorescence intensity and a 10-nm blue shift in the emission maximum. As tryptophan fluorescence is highly sensitive to local conformational changes, our data indicate that Trp-3 and Trp-31 are not involved, directly or indirectly, in the interaction with the charged head groups of phosphoinositides. According to the three-dimensional structure of bovine profilin, Trp-3 and Trp-31 are among the highly conserved hydrophobic residues that form a solvent-exposed hydrophobic surface located between NH_2- and COOH-terminal helices (Schutt et al., 1993). It is plausible that this hydrophobic surface facilitates the interfacial interactions with the fatty acyl chains of phosphoinositides. Thus, the microenvironment surrounding these tryptophan residues remains virtually the same after binding to individual phosphoinositides despite the variation in their head groups.

To date, considerable evidence indicates that PtdIns(3,4,5)-P_3 and PtdIns(3,4)P_2 represent crucial cellular signals in diverse physiological responses. Previously, these D-3 phosphoinositides have been shown to activate specific PKC isozymes (Nakanishi et al., 1993; Toker et al., 1994) and to modulate PI 3-kinase association with tyrosine-phosphorylated proteins through the interaction with SH2 domains (Rameh et al., 1995). The data presented here further implicate these signaling molecules in actin assembly and second messenger production. It is interesting that PtdIns-(3,4)P_2 has a higher affinity with profilin than PtdIns(3,4,5)-P_3, which coincides with the finding that PtdIns(3,4)P_2 is more potent than PtdIns(3,4,5)P_3 in activating Ca^{2+}-independent PKC isoforms (Toker et al., 1994). Taken together with the fact that PtdIns(3,4)P_2 has a longer life span than PtdIns(3,4,5)P_3 (Traynor-Kaplan et al., 1989; Stephens et al., 1991), it is plausible that PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 assume different physiological roles by interacting with different cellular targets. Based on this reasoning, understanding the mode of recognition of individual D-3 phosphoinositides is a viable approach to elucidate the physiological functions of PI 3-kinase, which represents the current focus of this investigation.

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REFERENCES

Buss, F., Temm-Grove, C., Henning, S., & Jockusch, B. M. (1992) *Cell Motil. Cytoskeleton 22*, 51−61.
Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281−302.
Chen, Y.-H., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120−4131.
Eberle, M., Traynor-Kaplan, A. E., Sklar, L. A., & Norgauer, J. (1990) *J. Biol. Chem. 265*, 16725−16728.
Fedorov, A. A., Magnus, K. A., Graupe, M. H., Lattman, E. E., Pollard, T. D., & Almo, S. C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8636−8640.
Goldschmidt-Clermont, P. J., Machesky, L. A., Baldassare, J. J., & Pollard, T. D. (1990) *Science 246*, 1575−1578.
Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G., & Pollard, T. D. (1991) *Science 251*, 1231−1233.
Gou, D.-M., & Chen, C.-S. (1994) *J. Chem. Soc., Chem. Commun.*, 2125−2126.
Hartwig, J. H., Chambers, K. A., Hopcia, K. L., & Kwiatkowski, D. J. (1989) *J. Cell. Biol. 109*, 1571−1579.
Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennstrom, S., Claesson-Welsh, L., Evans, T., Symons, M., et al. (1995) *Curr. Biol. 5*, 393−403.
Kaiser, D. A., Goldschmidt-Clermont, P. J., Levine, B. A., & Pollard, T. D. (1989) *Cell Motil. Cytoskeleton 14*, 251−262.
Kapeller, R., & Cantley, L. C. (1994) *BioEssays 16*, 565−576.
Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T., & Zetter, B. R. (1994) *Nature 367*, 474−476.
Lassing, I., & Lindberg, U. (1985) *Nature 314*, 472−474.
Lee, S., Li, M., & Pollard, T. D. (1988) *Anal. Biochem. 168*, 148−155.
Lindberg, U., Schutt, C. E., Hellston, E., Tjader, A. C., & Hult, T. (1988) *Biochim. Biophys. Acta 967*, 391−400.
Lu, P.-J., Gou, D.-M., Shieh, W.-R., & Chen, C.-S. (1994) *Biochemistry 33*, 11586−11597.
Malm, B., Larsson, H., & Lindberg, U. (1983) *J. Muscle Res. Cell Motil. 4*, 569−588.
Metzler, W. J., Bell, A. J., Ernst, E., Lavoie, T. B., & Mueller, L. (1994) *J. Biol. Chem. 269*, 4620−4625.
Mockrin, S. C., & Korn, E. D. (1980) *Biochemistry 19*, 5359−5362.
Nakanishi, H., Brewer, K. A., & Exton, J. H. (1993) *J. Biol. Chem. 268*, 13−16.
Ostrander, D. B., Gorman, J. A., & Carman, G. M. (1995) *J. Biol. Chem. 270*, 27045−27050.
Pantaloni, D., & Carlier, M.-F. (1993) *Cell 75*, 1007−1014.
Pollard, T. D., & Cooper, J. A. (1984) *Biochemistry 23*, 6631−6641.
Raghunathan, V., Mowery, P., Rozycki, M., Lindberg, U., & Schutt, C. (1992) *FEBS Lett. 297*, 46−50.
Rameh, L. E., Chen, C. S., & Cantley, L. C. (1995) *Cell 83*, 821−830.
Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G., & Rhee, S. G. (1987) *J. Biol. Chem. 261*, 12511−12518.
Schutt, C. E., Myslik, J. C., Rozycki, M. D., Goonesekere, N. C. W., & Lindberg, U. (1993) *Nature 365*, 810−816.
Serunian, L. A., Haber, M. T., Fukui, T., Kim, J. W., Rhee, S. G., Lowenstein, J., M., & Cantley, L. C. (1989) *J. Biol. Chem. 264*, 17809−17815.
Severinsson, L., Ek, B., Mellstrom, K., Claesson-Welsh, L., & Heldin, C. H. (1990) *Mol. Cell. Biol. 10*, 801−809.

Sohn, R. H., & Goldschmidt-Clermont, P. J. (1994) *BioEssays 16*, 465−472.
Sohn, R. H., Chen, J., Koblan, K. S., Bray, P. F., & Goldschmidt-Clermont, P. J. (1995) *J. Biol. Chem. 270*, 21114−21120.
Stephens, L. R., Hughes, K. T., & Irvine, R. F. (1991) *Nature 351*, 33−39.
Stephens, L. R., Jackson, T. R., & Hawkins, P. T. (1993) *Biochim. Biophys. Acta 1179*, 27−75.
Theirot, J. A., & Mitchison, T. J. (1993) *Cell 75*, 835−838.
Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., & Cantley, L. C. (1994) *J. Biol. Chem. 269*, 32358−32367.
Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Omann, G. M., & Sklar, L. A. (1989) *J. Biol. Chem. 264*, 15668−15673.
Tseng, P. C.-H., Runge, M. S., Cooper, J. A., & Pollard, T. D. (1984) *J. Cell Biol. 98*, 214−221.
Wang, D.-S., & Chen, C.-S. (1996) *J. Org. Chem. 61*, 5905−5910.
Ward, L. D. (1985) *Methods Enzymol. 117*, 400−414.
Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A. K., Heldin, C. H., Mori, S., & Claesson-Welsh, L (1994) *Oncogene 9*, 651−660.
Wymann, M., & Arcaro, A. (1994) *Biochem. J. 298*, 517−520.
Yu, F.-X., Sun, H.-Q., Janmey, P. A., & Yin, H. L. (1992) *J. Biol. Chem. 267*, 14616−14621.
Zhang, J., King, W. G., Dillon, S., Hall, A., Feig, L., & Rittenhouse, S. E. (1993) *J. Biol. Chem. 268*, 22251−22254.

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