Actin Directly Interacts with Phospholipase D, Inhibiting Its Activity*

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Mammalian phospholipase D (PLD) plays a key role in several signal transduction pathways and is involved in many diverse functions. To elucidate the complex molecular regulation of PLD, we investigated PLD-binding proteins obtained from rat brain extract. Here we report that a 43-kDa protein in the rat brain, \( \beta \)-actin, acts as a major PLD2 direct-binding protein as revealed by peptide mass fingerprinting in combination with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. We also determined that the region between amino acids 613 and 723 of PLD2 is required for the direct binding of \( \beta \)-actin, using bacterially expressed glutathione S-transferase fusion proteins of PLD2 fragments. Intriguingly, purified \( \beta \)-actin potently inhibited both phosphatidylinositol-4,5-bisphosphate and oleate-dependent PLD2 activities in a concentration-dependent manner (IC_{50} \approx 5 \text{ nM}). In a previous paper, we reported that \( \alpha \)-actinin inhibited PLD2 activity in an interaction-dependent and an ADP-ribosylation factor 1 (ARF1)-reversible manner (Park, J. B., Kim, J. H., Kim, Y., Ha, S. H., Kim, J. H., Yoo, J.-S., Du, G., Frohman, M. A., Suh, P.-G., and Ryu, S. H. (2000) J. Biol. Chem. 275, 21295–21301). In vitro binding analyses showed that \( \beta \)-actin could displace \( \alpha \)-actinin binding to PLD2, demonstrating independent interaction between cytoskeletal proteins and PLD2. Furthermore, ARF1 could steer the PLD2 activity in a positive direction regardless of the inhibitory effect of \( \beta \)-actin on PLD2. We also observed that \( \beta \)-actin regulates PLD1 and PLD2 with similar binding and inhibitory potencies. Immunocytochemical and co-immunoprecipitation studies demonstrated the in vivo interaction between the two PLD isozymes and actin in cells. Taken together, these results suggest that the regulation of PLD by cytoskeletal proteins, \( \beta \)-actin and \( \alpha \)-actinin, and ARF1 may play an important role in cytoskeleton-related PLD functions.

Mammalian phospholipase D (PLD)\(^1\) hydrolyzes phosphatidylcholine (PC) to generate phosphatidic acid and choline in response to a variety of signals, which can include hormones, neurotransmitters, and growth factors (1). Phosphatidic acid itself has been shown to be an intracellular lipid second messenger and to be involved in multiple physiological events such as the promotion of mitogenesis, stimulation of respiratory bursts, secretory processes, actin cytoskeletal reorganization, and the activation of Raf-1 kinase and phosphatidylinositol 4-phosphate (PtdIns4P) 5-kinase isoforms in a large number of cells. These relationships suggest that agonist-induced PLD activation may play roles in multiple signaling events (2–7).

The mammalian PLD isoforms identified thus far, PLD1 and PLD2, share a sequence homology of ~50%, but they have very different regulatory properties. PLD1 has low basal activity in the presence of phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) and can be activated by several cytosolic factors including protein kinase C \( \alpha \) and small GTP-binding proteins such as Rho A, Rac-1, ARF1, RaIA, and CDC42 (8–15). PLD2 also depends on PIP\(_2\) but has a higher basal activity than PLD1 (16), and it has been proposed that PLD2 may be closely associated with different cellular inhibitors. Although many studies continue to focus on the functional relationships and the isozyme specificities of the PLD isoforms, the molecular mechanism of the regulation of the PLDs has not been fully elucidated. In this regard, the identification of PLD-binding partners may provide clues toward the understanding of the complex regulatory mechanism of PLD in different cells.

It has been observed in many studies that PLD is crucially implicated in the actin-based cytoskeleton of cells. More recently, PLD activity has been found in the detergent-insoluble fraction of various cell types that contain a wide range of cytoskeletal proteins (17–18). Several cytoskeletal proteins such as fodrin and gelsolin have been found to act as PLD-specific inhibitors in vitro (19–21), and agonist-induced PLD stimulation can provoke changes in cell morphology through cytoskeletal rearrangement (5, 22–24). Furthermore, we reported previously that \( \alpha \)-actinin, an F-actin cross-linking protein, also binds to PLD2 to inhibit its activity (25). Thus, there is a strong body of evidence supporting a possibly close regulatory association between PLD and the actin cytoskeleton.

In our present study, we found for the first time that \( \beta \)-actin, a major cytoskeletal protein, negatively regulates PLD by direct binding. We also looked at the relationships and modes of action of ARF1 and other cytoskeletal proteins on PLD using PLD2 as a model system for signaling through PLD.
model enzyme, and the results obtained suggest possible mechanisms for the regulation of PLD by these cellular components.

**EXPERIMENTAL PROCEDURES**

**Materials**—The enhanced chemiluminescence kit (ECL system), diapalmitoylphosphatidyl-[methyl-3H]choline, chelating-Sepharose, DEAE-Sepharose, and Sephadex-15 resin were purchased from Amersham Pharmacia Biotech. Dipalmitoyl-phosphatidylcholine, PIP<sub>2</sub>, dioleoyl-phosphatidylethanolamine, paraformaldehyde, and sodium oleate were purchased from Sigma. Anti-actin antibody was purchased from ICN Pharmaceuticals. GTPγS was obtained from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Dulbecco’s modified Eagle’s medium was purchased from Life Technologies, Inc. Immobilized protein A and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody were purchased from Pierce. β-octylglucopyranoside was obtained from Calbiochem. Rhodamine-phalloidin was obtained from Molecular Probes. A polyclonal antibody that recognizes both PLD1 and PLD2 was generated as described previously (25). Anti-ARF monoclonal antibody was provided kindly by Dr. Richard A. Kahn (Emory University, Atlanta, GA). Full-length cDNAs of murine PLD2 and its N-terminal deletion mutants were provided generously by Dr. Michael A. Frohman (State University of New York, NY).

**Purification of Recombinant PLD from Baculovirus-transfected sf9 Cells**—Hexa-histidine (His<sub>6</sub>)-tagged PLD1 and PLD2 were purified from detergent extracts of baculovirus-infected sf9 cells by chelating-Sepharose affinity column chromatography as described previously (26).

**Preparation of Rat Brain Extract**—Rat brains (3 g) were homogenized in homogenization buffer (20 mm Tris/HCl, pH 7.5, 1 mm MgCl<sub>2</sub>, 1 mm EDTA, 1 mm EGTA, and 150 mm NaCl) using a polytron homogenizer. After centrifugation at 100,000 × g for 1 h at 4 °C, the resulting supernatant was used to investigate potential PLD2-binding partners. Protein concentrations in the brain extract were determined using the methods developed by Bradford (27).

**Co-precipitation of PLD2-binding Proteins**—Affinity-purified anti-PLD antibodies immobilized on protein A resin (PLD antibody complex) were first incubated with purified recombinant PLD2 (3 μg) for 2 h. After a brief centrifugation, the immune complexes were washed three times with radioimmuneprecipitation buffer (50 mm Tris/HCl, pH 8.5, 0.1% SDS, 150 mm NaCl, 1% TX-100, and 1% deoxycholate). The prepared brain extract (3 mg of protein) was then incubated with the complexes for 2 h at 4 °C. Finally, the co-precipitated proteins were washed again three times with radioimmuneprecipitation buffer, loaded onto a gel, and visualized by Coomassie Brilliant Blue staining.

**Identification of a 43-kDa Protein Using Peptide Mass Fingerprinting**—By Matrix-assisted Laser Desorption Ionization/Time-of-Flight Mass Spectrometry—The technique used was performed as described previously (25). A brief, the fraction containing the 43-kDa protein (p43) after co-immunoprecipitation from rat brain extract was separated by 8% polyacrylamide gel. The resultant co-precipitates were washed with the same buffer containing 0.25% CHAPS as described previously (25). The masses of the tryptic peptides obtained were determined with a Voyager DE time-of-flight mass spectrometer (Perceptive Biosystems, Inc., Framingham, MA) in the Korea Basic Science Institute. Delayed ion extraction resulted in peptide masses with better than 50 ppm mass accuracy on average. Using the amino acid sequences and the mass modifications. In brief, the reaction was carried out at 37 °C for 15 min in a 125-μl assay mixture containing the PLD assay buffer, the PLD preparation, and 25 μl of phospholipid vesicles composed of dioleoylphosphatidylethanolamine, PIP<sub>2</sub>, dipalmitoyl-phosphatidylcholine, and dipalmitoyl-[methyl-3H]choline (a total of 150,000 cpm/assay) in a molar ratio of 16:1.4:1. Oleate-dependent PLD activity was assayed as described earlier (26). In brief, PC vesicles (25 μl) containing 5 nmol of dipalmitoylphosphatidylcholine and 200,000 dpm of dipalmitoylphosphatidyl-[methyl-3H]choline were added to a reaction mixture (175 μl) containing 50 mm HEPES/NaOH, pH 7.0, 2 mm EGTA, 1.7 mm CaCl<sub>2</sub>, 2 mm MgCl<sub>2</sub>, and 80 mm KC1 containing 0.5% β- octylglucopyranoside. After brief centrifugation, the co-precipitated complexes were washed three times in the same buffer before being loaded onto a polyacrylamide gel. The in vitro binding of the GST-PLD2 fragments with β-actin was also performed in the same buffer containing 1% TX-100. All procedures using α-actinin binding were similar to those used for β-actin binding. In brief, PLD2 immune complexes were incubated with α-actinin and β-actin at 37 °C for 15 min in the PLD assay buffer, and the resulting co-precipitates were washed with the same buffer containing 0.25% CHAPS as described previously (25).

**PLD Activity Assay**—PIP<sub>2</sub>-dependent PLD activity was assayed by measuring choline release from phosphatidylcholine (12) with minor modifications. In brief, the reaction was carried out at 37 °C for 15 min in a 125-μl assay mixture containing the PLD assay buffer, the PLD preparation, and 25 μl of phospholipid vesicles composed of dioleoylphosphatidylethanolamine, PIP<sub>2</sub>, dipalmitoyl-phosphatidylcholine, and dipalmitoyl-[methyl-3H]choline in 0.5 ml of 1N HCl/5 mM EGTA and 1 ml of chloroform/methanol/HCl (50:50:0.3). After a brief centrifugation, the amount of [methyl-3H]choline in 0.5 ml of the aqueous phase was quantified by liquid scintillation counting.

**Immunoblot Analysis**—Proteins were denatured by boiling for 5 min at 95 °C in a Laemmli sample buffer (32), separated by SDS-PAGE, and transferred to nitrocellulose membranes by electrophoretic transfer using the Bio-Rad wet transfer system. After blocking in TTBS buffer (10 mm Tris/HCl, pH 7.5, 150 mm NaCl, and 0.05% Tween 20) containing 5% skim milk powder, the membranes were incubated with individual monoclonal or polyclonal antibodies, which was subsequently followed by another incubation with anti-mouse or anti-rabbit IgG, as required, coupled with horseradish peroxidase. Detection was performed using an enhanced chemiluminescence kit according to manufacturer instructions.

**Cell Culture**—The tetracycline-regulated (Tet-off) expression system (Life Technologies, Inc.) was used to induce the expression of PLD2 in...
PC12 cells (33). Clonal cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.5 mg/ml tetracycline, 10% (v/v) equine serum, and 5% fetal calf serum. To induce PLD2, the cells were grown in the same medium without tetracycline. COS-7 cells were maintained in a growth medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified CO2-controlled (5%) incubator. For transfection and the transient expression of PLD isoforms, COS-7 cells were plated at a density of 1 × 10^6 cells/well in 100-mm dishes and transfected using LipofectAMINE (Life Technologies, Inc.) as described previously (34).

Co-immunoprecipitation—PLD2-inducible PC12 cells cultured in the presence or absence of tetracycline or COS-7 cells overexpressing PLD1 were lysed with PLD assay buffer containing 1% cholate, 1% TX-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 5 μg/ml aprotinin. After brief sonication, the cell lysates were incubated for 2 h with constant agitation and centrifuged at 100,000 × g for 1 h. The cell extracts (1 mg of protein) recovered were incubated with anti-PLD antibody-immobilized on protein A resin for 2 h. After brief centrifugation, the co-immunoprecipitated complexes were washed three times with ice-cold radioimmune precipitation buffer before being loaded onto a polyacrylamide gel for immunoblot analysis.

Preparation of COS-7 Cell Membranes—Cells transfected with the wild type and the N-terminal deletion mutant of murine PLD2 (D1–185) were disrupted by sonication in ice-cold PLD assay buffer. The lysates were then centrifuged at 100,000 × g for 1 h at 4 °C, and the pellet was resuspended in the same buffer and referred to as membranes.

Immunocytochemistry—Immunocytochemistry was performed as described previously (35). In brief, PC12 cells grown on coverslips in the presence or absence of tetracycline were rinsed with PBS four times and fixed with 3.7% (w/v) paraformaldehyde for 10 min at 37 °C. After rinsing with PBS and blocking with PBS containing 1% goat serum and 0.1% TX-100 for 4 h at 4 °C, the cells were incubated with 2 mg/ml primary polyclonal antibody specific to PLD overnight at 4 °C. The cells were washed six times with PBS containing 0.05% TX-100 and then incubated in this washing medium with fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody and rhodamine-phalloidin for 1 h to visualize PLD2 and filamentous actin (F-actin), respectively. To visualize F-actin in COS-7 cells overexpressing GFP, GFP-PLD1, or GFP-PLD2 (36), they were incubated with rhodamine-phalloidin as described above. Slides were then examined under a fluorescence microscope (Nikon, Melville, NY).

RESULTS

The 43-kDa Protein Precipitated with PLD2 from a Rat Brain Extract Was Identified as β-Actin—Because the regulation of PLD could possibly occur through direct interaction between PLD and other binding partners, we started our investigation by looking for cellular PLD2-binding proteins from rat brain extracts using purified PLD2 complexed with anti-PLD antibody. After the precipitation (PLD2 precipitate) and protein analysis by SDS-PAGE, we found that the co-precipitate contained major PLD2-binding proteins with relative molecular masses of 48 (p48), 43 (p43), and 35 kDa (p35) and some minor differences. To identify the 43-kDa protein, the co-immunoprecipitated complexes were analyzed by mass spectrometry. The results are shown in Table I.

**Table I**

| Peptide Sequence | M + H<sup>a</sup> | M + H<sup>b</sup> |
|------------------|------------------|------------------|
| **Peptide**      | Observed         | Calculated       |
| P1 LDLAGR (178–183) | 644.36           | 644.37           |
| P2 ILAPPER (329–335) | 795.49           | 795.47           |
| P3 GYSFTTAER (197–206) | 1132.54          | 1132.52          |
| P4 HQGVMVGMQK (40–50) | 1187.57          | 1187.56          |
| P5 QYDESQPSIVHR (360–372) | 1516.72          | 1516.70          |
| P6 SYELPGQVTIGNER (239–254) | 1790.90          | 1790.89          |
| P7 VAEEHPVLLTEALNPK (96–113) | 1954.03          | 1954.06          |

<sup>a</sup> The matched peptides cover 21% (81 of 375 amino acids) of the proteins.

<sup>b</sup> Monoisotopic mass.

PC12 cells (33). Clonal cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.5 μg/ml tetracycline, 10% (v/v) equine serum, and 5% fetal calf serum. To induce PLD2, the cells were grown in the same medium without tetracycline. COS-7 cells were maintained in a growth medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified CO2-controlled (5%) incubator. For transfection and the transient expression of PLD isoforms, COS-7 cells were plated at a density of 1 × 10^6 cells/well in 100-mm dishes and transfected using LipofectAMINE (Life Technologies, Inc.) as described previously (34).
proteins. As shown in Fig. 1, these bands appeared in distinctive patterns only in PLD2 immunoprecipitates. A major band corresponding to p43 in the PLD2 precipitates was excised from the gel for identification by peptide mass fingerprinting. A trypsinized peptide mixture of p43 was then subjected to matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. Fig. 2A shows the matrix-assisted laser desorption ionization mass spectrum of the digested peptides of p43. The masses obtained, marked as P1–P7, were compared with proteins in the Swiss-Prot data base using the MS-Fit peptide mass search program. As shown in Table I, the peptides exhibited molecular masses that were almost identical to the calculated masses of the corresponding theoretically predicted tryptic peptides of β-actin. The accuracy of this peptide search result was obtained with 50 ppm, and the analyzed peptides covered 21% of the β-actin sequence (Table 1). To substantiate the identity of this protein further, the presence of actin in the PLD2 precipitate was confirmed using a monoclonal antibody to actin. As shown in Fig. 2B, actin was strongly detected in the PLD2 precipitate but not in a control immune complex. On the basis of these results, we concluded that the 43-kDa protein in the PLD2 precipitate from the rat brain extract was β-actin.

β-Actin Directly Associates with a Region (Amino Acids 613–723) of PLD2—To determine whether β-actin associates directly with PLD2, β-actin from rat brain was purified to over 90% (data not shown) using the methods of Bray and Thomas (28) and incubated with the PLD2-bound immune complexes. As shown in Fig. 3A, the resulting co-precipitation demonstrated that β-actin interacts directly with PLD2. To identify the PLD2 sequence involved in the β-actin binding, we constructed the GST fusion proteins shown in Fig. 3B and tested them for their ability to bind to purified β-actin. GST-PLD2 (amino acids 613–723) was found to be the region that most potently bound to β-actin (Fig. 3C). It seems therefore that the region of the protein encoded between amino acids 613 and 723 may be important for the direct interaction with β-actin.

β-Actin Specifically Inhibits PLD2 Activity in Vitro—We monitored PLD2 activity to determine the effect of β-actin on

![Fig. 3. Direct interaction of β-actin with PLD2. A, Anti-PLD antibody complexes were first incubated in the absence (−) or presence (+) of purified PLD2. After three washings with ice-cold radioimmune precipitation buffer, the complexes were incubated with purified β-actin (30 nM) as described under “Experimental Procedures.” After precipitation, the samples were subjected to immunoblot analysis. B, PLD2 cDNA was fragmented into individual domains consisting of F1 (1–314), F2 (315–475), F3 (476–612), F4 (613–723), F5 (724–825), and F6 (826–933). The fragments were cloned as GST fusion proteins, expressed in E. coli, and purified using glutathione-Sepharose beads. Boxes are the regions of highly conserved sequences in PLD. PX, phox; PH, pleckstrin homology; I–IV, conserved regions I–IV; CT, C-terminal region. C, equal amounts (1 µg) of GST or GST fusion proteins (GST-PLD2 fragments, F1–F6) were incubated with purified β-actin (150 nM) as described under “Experimental Procedures.” The precipitated proteins were subjected to immunoblot analysis using antibodies directed against actin (upper panel). GST was used as the control. The amounts of the GST fusion proteins were visualized on the nitrocellulose membrane by Ponceau staining (lower panel). The results shown are representative of two separate experiments.](image-url)
presence of various concentrations of $b_28256$.

The data represent the means ± S.D. obtained from two separate experiments.

B

FIG. 5. Effect of $\beta$-actin on the interaction and regulation of $\alpha$-actinin with PLD2. A, PLD2 (0.1 $\mu$g)-bound PLD immune complexes were incubated with $\alpha$-actinin (267 nM) and the indicated amounts of $\beta$-actin under PLD assay conditions at 37 °C for 15 min. After a brief centrifugation, the precipitates were washed as described under “Experimental Procedures” and subjected to immunoblot analysis. PLD2, $\alpha$-actinin, and $\beta$-actin were detected using antibodies to PLD, $\alpha$-actinin, or actin, respectively. The data shown here represent one of two separate experiments. B, COS-7 cells were transfected with the empty vector (MOCK), N-terminal 185-amino acid acid-truncated PLD2 (PLD2Δ1–185) or wild-type PLD2 (PLD2). After the preparation of PLD immune complexes, these complexes were incubated with 30 nM of $\beta$-actin as described under “Experimental Procedures.” The final co-precipitates were subjected to immunoblot analysis. The results shown are representative of two separate experiments. C, COS-7 cell membranes overexpressing N-terminal 185-amino acid acid-truncated PLD2 (PLD2Δ1–185), or wild-type PLD2 (PLD2), or were prepared as described under “Experimental Procedures” and used as a source of PLD2. PLD activity was determined with 0.3 $\mu$g of membrane in the presence of various concentrations of $\beta$-actin purified from rat brain. The data represent the means ± S.D. obtained from two separate experiments performed in duplicate.

PLD2. As shown in Fig. 4A, the PIP$_2$-dependent PLD2 activity was inhibited specifically in a $\beta$-actin concentration-dependent manner. Using $\beta$-actin purified from rat brain, the concentration required for half-maximal inhibition was about 5 nM. To further confirm the inhibitory effect of $\beta$-actin on PLD2 activity, we constructed and purified a GST-$\beta$-actin for reconstitution assays of PLD activity. As expected, we observed that this GST-$\beta$-actin had an inhibitory effect that was similar to that of the $\beta$-actin purified from rat brain. To exclude the possibility that the $\beta$-actin-inhibited PLD2 activity might be caused by PIP$_2$ sequestration or masking in the substrate phospholipid vesicles, we also performed a PLD2 activity assay in the absence of PIP$_2$. We previously reported that PLD2 could be activated specifically by oleate (18:1) in the absence of PIP$_2$ (26). As shown in Fig. 4B, PLD2 activated by oleate was inhibited progressively by increasing either the concentration of $\beta$-actin purified from rat brain or GST-$\alpha$-actinin with similar inhibitory potency. The inhibitory efficacy of $\beta$-actin, under the condition of the oleate assay, was close to that observed under the condition that included PIP$_2$ (Fig. 4A). Taken together, these results suggest that the inhibitory effect of $\beta$-actin on PLD2 might be mediated by direct interaction.

$\beta$-Actin and $\alpha$-Actinin Act Independently on PLD2 in a Reversible Manner—In a previous study, we found that $\alpha$-actinin binds directly to the N-terminal region (amino acids 1–185) of PLD2 and inhibits activity of the enzyme (25). To clarify the relationship between the two cytoskeletal proteins, $\beta$-actin and $\alpha$-actinin, in terms of PLD2 binding, in vitro binding assays were performed. Fig. 5A demonstrates that an increase in $\beta$-actin reduced $\alpha$-actinin binding to PLD2 in a competitive and concentration-dependent manner. In other words, the binding of $\beta$-actin to PLD2 induced the release of $\alpha$-actinin already bound to PLD2. To exclude the possibility that this mode of competition occurs through the same binding site on the two proteins, we used an N-terminal $\alpha$-actinin binding region deletion mutant of PLD2 (PLD2Δ1–185). In vitro binding analysis showed that $\beta$-actin binds to PLD2Δ1–185 with an affinity comparable with that of wild-type PLD2 (Fig. 5B), proving that $\beta$-actin does not bind to the $\alpha$-actinin binding region of PLD2.
To further substantiate this mode of competition, we also performed a PLD activity assay and compared the inhibitory effects of β-actin on wild type and PLD2Δ (1–185). As shown in Fig. 5C, β-actin potently inhibited the activities of both PLD2Δ (1–185) and wild type to similar extents. These results indicate that β-actin may inhibit PLD2 activity by interacting directly with a site other than the α-actinin binding region and suggest further that β-actin and α-actinin act independently on PLD2 in a reversible manner.

β-Actin-inhibited PLD2 Activity Can Be Activated by ARF1—This study was undertaken to determine whether the repression of PLD2 activity by β-actin could be modulated by cellular factors. Previously, we had reported that the α-actinin inhibited PIP2-dependent PLD2 activity could be completely restored by ARF1 and that this recovery was caused by the release of α-actinin from PLD2 by ARF1 binding (25). Therefore, we studied the effect of ARF1 in this system by increasing the concentration of ARF1 in the PLD activity assay in the presence and absence of 30 nm purified β-actin. As shown in Fig. 6A, β-actin strongly inhibited PIP2-dependent PLD2 activity, this inhibition could be activated by varying the concentration of ARF1, and this was independent of the effect of β-actin to PLD2. It was suspected that this simple activation may not be caused by competition but by different forms of binding between PLD2 and ARF1 or β-actin. To further elucidate the mode of action of ARF1 on the actin-PLD interaction, we added ARF1 in the presence of PLD2 and β-actin. Fig. 6B shows that even though the amount of ARF1 was increased to 3.2 μg in the presence of GTPγS, ARF1 expectedly did not compete out any PLD2-bound β-actin. Taken together, these results suggest that β-actin might bind to PLD2 independently of ARF1 and that β-actin-induced PLD2 inhibition might be regulated by a mode of action of ARF1 that differs from that of α-actinin.

PLD2 Specifically Interacts and Co-localizes with the Actin Cytoskeleton in PC12 Cells—The possibility that actin may interact with PLD2 inside the cell was tested by using a co-immunoprecipitation method on PLD2-overexpressing PC12 cells, which are able to induce the expression of PLD2 upon tetracycline withdrawal (33). As shown in Fig. 7A, a very low level of PLD2 was expressed in PC12 cells in the presence of tetracycline, which we considered representative of the level of endogenous PLD2. Actin did co-immunoprecipitate with PLD2 from extracts of the PLD2-overexpressing PC12 cells cultured in the absence of tetracycline, demonstrating a specific interaction between actin and PLD2 in vivo. In these cells, the overexpression of PLD2 did not affect the endogenous expression of actin (data not shown). To further check whether PLD2 co-localized with the actin cytoskeleton in PC12 cells, the PLD2-overexpressing PC12 cells were stained immunofluorescently with the anti-PLD antibody. As shown in Fig. 7B, the overexpressed PLD2 was found to be localized near the plasma membrane of the PC12 cells, and co-staining of filamentous actin with rhodamine-phalloidin showed that the PLD2 co-localized with F-actin in the PC12 cells. Taken together, these results suggest that actin regulation of PLD2 might occur through direct interaction within the actin cytoskeleton regions.

β-Actin Inhibits PLD1 as well as PLD2 in an Interaction-dependent Manner—Mammalian PLD has two isozymes, PLD1 and PLD2, that have a sequence homology of ~50%. To check the effect of purified β-actin on these two PLD isozymes, in vitro binding analysis was performed. Fig. 8A shows that β-actin interacted directly with both PLD isozymes with almost the same affinity. To further check the inhibitory effect of β-actin on PLD isozymes, we reconstituted purified β-actin and PLD isozymes in a PLD activity assay in the presence of ARF1. In this case, we measured the activity of both PLD isozymes activated by ARF1 to compare the effect of β-actin under the same conditions. Fig. 8B shows that β-actin similarly inhibited PLD1 and PLD2 activity in a concentration-dependent manner. To determine whether actin also forms a complex with PLD1 in vivo, we transfected a vector containing PLD1 in COS-7 cells. The cell extracts were subjected to co-immunoprecipitation and immunoblot analysis. As shown in Fig. 8C, actin immunoreactivity was detected not in immunoprecipitates of the vector-transfected cells but in those of the PLD1-transfected cells, showing that actin associates tightly with PLD1 in cells. We also confirmed, in these cells, that actin was co-immunoprecipitated with PLD2 as was shown in PC12 cells (data not shown). To further examine the interaction between PLD isozymes and the actin cytoskeleton in cells, we transfected GFP, GFP-PLD1, or GFP-PLD2 into COS-7 cells. As shown in Fig. 8D, F-actin co-localized with the two PLD isozymes, which suggests an association between PLD and the actin cytoskeleton. Taken together, these results suggest that β-actin may inhibit the activities of both PLD isozymes through direct interaction in these cells.

**DISCUSSION**

Although some evidence exists that suggests that PLD activity is confined to the cytoskeletal fraction and may be involved closely in actin cytoskeletal rearrangement in vivo, the regulatory mechanism of PLD involvement in the actin cy-
Actin-induced PLD Inhibition through Direct Interaction

**Fig. 8.** The effect of β-actin on PLD isozymes. A, the same amounts (0.1 μg) of PLD1 and PLD2-bound immune complexes were incubated with 50 nM of β-actin as described under “Experimental Procedures.” After the precipitation and washing steps, the final co-precipitates were subjected to immunoblot analysis. PLD1, PLD2, and β-actin were detected using anti-PLD or actin antibodies. The results shown represent one of two experiments performed with independent preparations. B, purified PLD1 (■) and PLD2 (□) activated by ARF1 were assayed to determine the concentration-dependent effect of β-actin on PLD isozymes as described under “Experimental Procedures.” The concentration of purified β-actin was varied as indicated. The assays also contained 10 μM GTPγS. The results shown represent the means ± S.D. from three different experiments performed in duplicate. C, COS-7 cells were transfected with empty vector (Vec) or pCDNA 3.1 vectors encoding PLD1 as described under “Experimental Procedures.” The total amount of vector DNA used per transfection was 5 μg. The transfected cells were lysed, extracted, and co-immunoprecipitated with polyclonal PLD antibody as described under “Experimental Procedures.” Final PLD immune complexes were subjected to immunoblot analysis and the bands were detected with antibodies to PLD or actin. The results shown are representative of two separate experiments. D, COS-7 cells overexpressing GFP, GFP-PLD1, or GFP-PLD2 were grown on coverslips and stained as described under “Experimental Procedures” to visualize F-actin. The arrowheads point to candidate regions in which the two PLD isozymes and F-actin overlap. The results shown are representative of two separate experiments.
Actin-induced PLD Inhibition through Direct Interaction

Proteins (46–47). In recent papers, PLD activity has been reported in the detergent-insoluble fraction of HL-60 and U937 cells (17–18), demonstrating the possibility of in vivo interaction between PLD and the actin-based cytoskeleton. It has been further suggested that several F-actin-binding proteins such as fodrin and α-actinin act as PLD-specific inhibitors in vitro (19–20, 25). However, there had been no report that has proved an in vivo interaction between PLD2 and the actin cytoskeleton. Therefore, our immunocytochemical study is the first study to demonstrate that PLD2 is localized near the plasma membrane of PLD2-overexpressing PC12 cells (as has been reported previously in other cells (24)) and that it is co-localized with filamentous actin (Fig. 7B). Moreover, our co-immunoprecipitation results also show a specific interaction between PLD2 and the actin cytoskeleton in PC12 cells (Fig. 7A). Therefore, our results suggest that the actin cytoskeleton may be associated closely with and possibly involved in the regulation of PLD2.

In this study, we tried to develop a possible model for the complex regulatory mechanisms operating between the cytoskeletal proteins, ARF1, and PLD2. Our findings show that β-actin uses a binding site (amino acids 613–723) on PLD2, which differs from that used by α-actinin and ARF1. It seems that ARF1 does not share either an identical or an overlapping binding site with β-actin on PLD2. As reported previously, α-actinin interacts with the N-terminal region (amino acids 1–185) of PLD2, and it seems that ARF1 can induce the release of α-actinin from PLD2 by causing a conformational change in the enzyme (25). However, the regulatory mode of the β-actin-induced PLD2 inhibition by ARF1 must differ from its regulation of α-actinin-induced inhibition. Therefore, we suggest that the regulation of PLD2 by β-actin or α-actinin occurs via different binding sites on PLD2, that they may occur independently, and that they may be activated by different modes of action of ARF1. Our observation that the binding/inhibitory capacity of β-actin to PLD2 is almost similar in both wild type and N-terminal deletion mutant, α-actinin binding region (Δ1–185), further supports the notion that β-actin and α-actinin use different binding sites and perhaps act by inducing conformational changes.

We also suggest that the independent interaction between the two cytoskeletal proteins and PLD2 is probably not caused by an interaction between β-actin and α-actinin, because the interaction between α-actin, another actin muscle isoform, and α-actinin have been reported to be very weak (Kd = 2 μM) (48) and because we found that GST-β-actin did not bind to α-actinin under the same conditions (data not shown). Therefore, our findings lead to the possible speculations that PLD2 may exist in various populations, its regulation by either β-actin or α-actinin may change according to the spatial and temporal conditions in the cells, and that this would be reflected in a different responsiveness to ARF1. We suggest that the complex mechanisms involved in the regulation of PLD2 by cytoskeletal proteins may be related to the actin cytoskeletal changes required when cells are stimulated by diverse signals.

ARF plays a major role in the regulation of PLD (49–51) in various cell types, and it also may be involved in actin cytoskeletal rearrangements in these cells. It has been reported recently that ARF1 stimulates wild-type human PLD2 a little less than 2-fold, whereas an N-terminal deletion (1–308) mutant of PLD2 was stimulated about 13-fold in vitro (16, 52). In HIRb cells, insulin activated PLD2 by a mechanism sensitive to brefeldin A (6). PLD, ARF, GRP1, and ARNO (guanine-nucleotide exchange factors with specificity for ARF) exist in the detergent-insoluble fraction of several cell types (17–18, 54–55), and ARF1 also potentiated Rho-stimulated stress fiber formation in Swiss 3T3 fibroblasts (56). Our finding that the inhibition of PLD2 by β-actin can be activated by ARF1 (Fig. 6A) further supports the hypothesis that ARF1 may be an important factor in the regulation of PLD2. Although we concentrated on the effect of ARF1 on the regulation of PLD2, we cannot exclude the possibility that ARF6 may be implicated in both the regulation of PLD2 and the actin cytoskeletal changes in cells. Increasing numbers of reports have shown that ARF6 can activate rat brain PLD (57) or plasma membrane-associated PLD in chromaffin cells (58). Furthermore, Honda et al. (7) reported that ARF6 co-localizes with PLD2 in membrane ruffles upon treatment with epidermal growth factor. All these observations suggest that various ARF proteins might be deeply involved in the complex mechanism of the regulation of PLD2 in the actin cytoskeleton.

The mammalian PLD isozymes, PLD1 and PLD2, have a sequence homology of over 50% and are found in highly conserved regions in the PLD superfamily (53, 59). Although many previous reports have suggested that the two PLD isozymes may exhibit different regulatory properties, one interesting possibility arises from the observation that β-actin interacts with and inhibits both PLD isozymes with similar potency in vitro (Fig. 8, A and B). This suggests that actin may have a broad interaction and regulation spectrum for PLD in cells. This is supported further by our result that actin was co-immunoprecipitated and co-localized with two PLD isozymes in COS-7 cells. On the basis of our results, we also suggest that conserved region III, the β-actin binding region of PLD2, may contribute to the interaction and regulation of the two PLD isozymes. Although this needs further detailed work, many reports support this suggestion because this region is highly conserved in PLD family members that exhibit bona fide PLD activity. Furthermore, we suggest that the regulation of PLD by the actin cytoskeleton may depend on β-actin binding to this conserved region III of PLD in cells.

In summary, we have identified one of the PLD-binding proteins as β-actin and found that direct interaction between β-actin and PLD specifically mediates PLD activity. On the basis of our results, we suggest that the close association between the actin cytoskeleton and PLD may inhibit PLD activity in nascent cells. Upon stimulation by appropriate agonists, the translocation of ARF to the membrane may activate the repressed activity of PLD by the actin cytoskeleton. In the future, we hope to study the details and fine-tuning of this regulatory mechanism in the actin cytoskeleton.

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