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Regulation of Phospholipase D Activity by Actin

ACTIN EXERTS BIDIRECTIONAL MODULATION OF MAMMALIAN PHOSPOLIPASE D ACTIVITY IN A POLYMERIZATION-DEPENDENT, ISOFORM-SPECIFIC MANNER*

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David J. Kusner‡§¶‖, James A. Barton‡§, Kuo-Kuang Wen, Xuemin Wang‡‡, Peter A. Rubenstein**, and Shankar S. Iyer‡§**

*From the ‡Department of Internal Medicine, Division of Infectious Diseases, §Inflammation Program, ¶Graduate Programs in Immunology and Molecular Biology, and **Department of Biochemistry, University of Iowa and Veterans Affairs Medical Center, Iowa City, Iowa 52242 and ‡‡Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506*

Many critical cellular processes, including proliferation, vesicle trafficking, and secretion, are regulated by both phospholipase D (PLD) and the actin microfilament system. Stimulation of human PLD1 results in its association with the detergent-insoluble actin cytoskeleton, but the molecular mechanisms and functional consequences of PLD-actin interactions remain incompletely defined. Biochemical and pharmacologic modulation of actin polymerization resulted in complex bidirectional effects on PLD activity, both *in vitro* and *in vivo*. Highly purified G-actin inhibited basal and stimulated PLD activity, whereas F-actin produced the opposite effects. Actin-induced modulation of PLD activity was independent of the activating stimulus. The efficacy and potency of the effects of actin were isoform-specific but broadly conserved among actin family members. Human $\beta\gamma$-actin was only 45% as potent and 40% as efficacious as rabbit skeletal muscle α-actin, whereas its inhibitory profile was similar to the single actin species from the yeast, *Saccharomyces cerevisiae*. Use of actin polymerization-specific reagents indicated that PLD1 binds both monomeric G-actin, as well as actin filaments. These data are consistent with a model in which the physical state of the actin cytoskeleton is a critical determinant of its regulation of PLD activity.

Phospholipase D (PLD)[1] enzymes have been identified throughout the animal and plant kingdoms and are located in all cells and tissues of metazoans (1–3). PLD functions in several essential cellular processes, including cytoskeletal remodeling, proliferation, motility, and membrane trafficking as well as in several highly specialized activities characteristic of terminally differentiated cells (4). The ubiquitous distribution and diverse physiologic functions of PLD enzymes underscore the critical importance of defining the molecular mechanisms of regulation and characterizing the biochemical pathways that link its catalytic activity to such a broad range of cellular responses. Two mammalian PLD isoforms, PLD1 and PLD2, have been molecularly characterized (2, 5–7). PLD1 is activated by low molecular weight (LMW) GTPases of the Rho and ARF families, as well as by protein kinase C (PKC). PLD2 exhibits high basal activity and appears to be primarily subject to negative regulation *in vivo*, although evidence for specific activators has recently been presented (8, 9). Both PLD1 and PLD2 require phosphatidylinositol 4,5-bisphosphate (PI $(4,5)P_2$) as a cofactor.

A distinctive feature of the mammalian PLDs is their functional association with the actin-based microfilament cytoskeleton (2, 4, 5, 10–15). In phagocytic leukocytes (monocytes, macrophages, and neutrophils), the major antimicrobial and tissue-damaging responses, *i.e.* phagocytosis, oxidant generation, and secretion, require both activation of PLD and dynamic rearrangements of actin filaments (16–24, 26). The agonist-stimulated generation of actin stress fibers in fibroblasts and endothelial cells is tightly coupled to activation of PLD (10–13). Furthermore, stress fiber formation is induced by addition of purified PLD or its product, phosphatidic acid, and is blocked by inhibitors of PLD. We recently demonstrated (27) that physiologic stimulation of PLD activity in human monocytic U937 cells via plasma membrane receptors as well as pharmacologic activation of GTP-binding proteins result in stable association of PLD1 with a detergent-insoluble fraction that contains F-actin and the cytoskeletal proteins α-actinin, vinculin, paxillin, and talin. A similar association of PLD activity with the Triton X-100-insoluble fraction of HL-60 cell membranes has also been reported (28). However, because the detergent-insoluble fraction is molecularly heterogeneous (27), the regulatory interactions responsible for this reported localization of PLD1 require further definition. Lee *et al.* (29) have recently reported that both PLD1 and PLD2 bind actin, resulting in inhibition of PLD activity. Because cellular actin exists in a dynamic equilibrium between monomeric, G-actin, and filamentous F-actin, we sought to characterize further the physical and functional interactions between actin and PLD to answer the following questions. 1) Do PLD enzymes bind both G-actin and F-actin? 2) Do these interactions occur both *in vitro* and *in vivo*? 3) Do monomeric and filamentous actin exhibit similar effects on PLD activity?

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‖ To whom correspondence and reprint requests should be addressed: Dept. of Internal Medicine, University of Iowa, 200 Hawkins Dr., SW 54-I, GH, Iowa City, IA 52242. Tel.: 319-353-6525; Fax: 319-356-4600; E-mail: david-kusner@uiowa.edu.

[1] The abbreviations used are: PLD, phospholipase D; DPPC, dipalmitoylphosphatidylcholine; PEt, phosphatidylethanol; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; LMW, low molecular weight; GTPγS, guanosine 5′-(γ-thio)triphosphate; ECL, enhanced chemiluminescence; AMB, actin monomer buffer; APB, actin polymerization buffer; Ab, antibody; mAb, monoclonal Ab; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PVDF, polyvinylidene difluoride; Fx, fraction.

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EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, materials were from previously published sources (27, 30–32). PLD1 and PLD2 protein standards were generously provided by Dr. Andrew J. Morris (State University of New York, Stony Brook). Polyclonal Abs to PLD1 or PLD2 were from Quality Controlled Biochemicals Corp. (Hopkington, MA). Rabbit polyclonal anti-PLD1 Ab was a generous gift from Nicholas Ktistakis (Babraham Institute, Cambridge, MA). Polyclonal anti-RhoA Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and polyclonal anti-ARF Ab was a kind gift of Richard Kahn (Emory University, Atlanta, GA). Actin from rabbit skeletal muscle (α-actin) or human platelets (5:1 ratio of β/γ-actin), both >99% pure, were purchased from Cytoskeleton Inc. (Denver, CO) and stored as G-actin in AMB. *Saccharomyces cerevisiae* actin was purified, as previously described (33). Phalloidin was obtained from Roche Molecular Biochemicals, and jasplakinolide was from Molecular Probes (Eugene, OR). Purified protein kinase C (rat brain), latrunculin B, and DNase I were purchased from Calbiochem. The DNase I preparation was >99% pure and lacked detectable RNase or protease activity. Sepharose 4B and DNase I-Sepharose were from Amersham Biosciences.

Cell Fractionation—U937 human promonocytic leukocytes were obtained from ATCC and maintained in Iscove's medium, 10% fetal bovine serum, 1% penicillin/streptomycin at 37 °C, 7.5% CO_2. Approximately 10^9 cells were washed in H/S buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 0.7 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, 1 mg/ml bovine serum albumin) (27, 31), incubated with 4 mM diisopropyl fluorophosphate for 25 min at 4 °C, and resuspended in H/K buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) prior to disruption by N_2 cavitation (450 pounds/square inch, 25 min, 4 °C). Following removal of undisrupted cells and nuclei by centrifugation at 900 × g, the cavitate was layered over 50% sucrose and centrifuged at 150,000 × g for 60 min at 4 °C. The resulting supernatant (cytosol) was re-centrifuged at 225,000 × g and filtered through a 0.2-μm filter. The membrane fraction at the sucrose interface was pelleted at 225,000 × g for 60 min, washed in H/K buffer, then resuspended in the same and homogenized with a tissue grinder. This membrane fraction has been shown previously to be highly enriched in plasma membrane protein markers, *e.g.* it contains virtually all of the HLA class I antigen of the total cell lysate, and >90% of the total membrane-associated PLD1 in U937 cells (27). This fraction also contains Golgi membranes, defined by immunoreactivity for β-COP. This HLA class I-enriched membrane fraction was used for all experiments. The more dense membrane fraction, which sedimented through the 50% sucrose, was enriched in the primary granule marker CD63 and contained <10% of total membrane protein mass and <10% of cellular immunoreactivity for PLD1 (not shown). Protein concentrations in membrane and cytosolic fractions were determined by the method of Bradford (34).

Assay of Phospholipase D Activity in the Cell-free Reconstitution System—Substrate vesicles containing phosphatidylethanolamine/PI(4,5)P_2/PC (molar ratio of 16:1.4:1), with 10 μCi/sample of [^{3}H]DPPC, were prepared by sonication for 5 min at 25 °C (35). 75 μg of the membrane fraction, 100 μg of cytosol, and 10 μl of substrate vesicles were incubated with 1.5% ethanol to permit detection of the PLD-specific transphosphatidylation product, phosphatidylethanol (PEt), in a total volume of 100 μl. Actin or buffer control was added for 2 min at 37 °C, prior to initiation of the reaction with GTPγS (30 μM). In select experiments, GTPγS was omitted, and reactions were initiated by adding 10 nM purified PKC and 100 nM PMA. Reactions were conducted for 30 min at 37 °C and terminated by addition of 500 μl of chloroform/methanol (2:1, v/v). Lipids were extracted, dried under N_2, and analyzed by TLC in an ethyl acetate/isooctane/acetic acid (9:5:2) solvent system (27, 31, 32), [^{3}H]PEt was identified by co-migration with pure standard. [^{3}H]PEt counts/min were quantitated by liquid scintillation spectrometry, and counts were normalized for the total amount of ^{3}H-labeled phospholipid in each experiment. [^{3}H] counts/min co-migrating with PEt were determined for each set of samples in the absence of ethanol, and these background counts were subtracted from each data point.

Assay of Phospholipase D Activity in Intact Cells—U937 promonocytes were radiolabeled with [^{3}H]oleate (5 μCi/ml) for 18 h in Iscove's medium, 10% fetal bovine serum, 1% penicillin/streptomycin at 37 °C, 7.5% CO_2. Cells were washed 3 times in H/S buffer and resuspended in the same (27). 10^6 cells/sample were incubated with 1.0% ethanol for 2 min, followed by PMA (1–100 nM), or buffer control, in a total volume of 500 μl. In select experiments, jasplakinolide, latrunculin B, or the appropriate 0.1% ethanol or Me_2SO controls, respectively, were added to the cells 15 min prior to stimulation with PMA. Reactions were terminated at 30 min and PLD activity quantitated as noted above.

Co-immunoprecipitation of PLD1 and Actin—Immunoprecipitations experiments were performed essentially as described by Ktistakis and co-workers (36), with the following modifications. Purified membranes were solubilized in Lysis buffer (H/K buffer containing 1% Triton X-100, 1% octyl glucoside, and 1% deoxycholate) by incubation for 1 h on ice. Following centrifugation at 14,000 × g for 15 min at 4 °C, to pellet the insoluble fraction, supernatants were pre-cleared by incubation with pre-immune serum for 120 min at 4 °C, followed by a 30-min incubation with 50 μl of a 10% protein A-Sepharose slurry prepared in the same buffer. Lysates were centrifuged at 1,000 × g for 5 min at 4 °C, and supernatants were incubated with rabbit polyclonal anti-PLD1 Ab for 5 h at 4 °C, followed by an additional 1-h incubation with 50 μl of 10% protein A-Sepharose. The immunoprecipitates were washed five times with Lysis buffer and subjected to SDS-PAGE on 8% gels. Following transfer to PVDF, Western blotting was performed with anti-actin IgM mAb, with detection by enhanced chemiluminescence (ECL). The three polyclonal anti-PLD1 Abs were generated to the following sequences of PLD1: 1) peptide 525–541 (Quality Controlled Biochemicals, Inc.), 2) peptide 1–15, and 3) peptide 1057–1074. The latter two Abs were generously provided by Dr. Nicholas Ktistakis.

Assessment of PLD1 Binding to Membrane-localized G-actin—Membranes (500 μg/sample) were solubilized in Lysis buffer, as noted above. In select samples, purified actin (rabbit skeletal muscle α-actin, human platelet $\beta\gamma$-actin, or *S. cerevisiae* actin, 0.01–0.2 mg/ml) was added to the membrane extracts. Lysates were pre-cleared with washed Sepharose beads and then incubated with DNase I-Sepharose beads for 16 h at 4 °C on a rotator. Beads were sedimented by centrifugation (2,000 × g, 5 min, 4 °C) and washed five times with Lysis buffer. The final wash was removed; 100 μl SDS-sample buffer was added, and SDS-PAGE was performed on 8% gels. Following transfer of DNase I-binding proteins to PVDF, Western blotting was preformed with anti-PLD1 polyclonal Ab, with detection by ECL. Blots were stripped and re-probed with mAb to actin. In control samples, uncomplexed Sepharose 4B beads were substituted for DNase I-Sepharose.

Velocity Sedimentation of Membrane Lysates on Sucrose Density Gradients—Freshly isolated membranes were prepared as noted above, and 300-μl aliquots were layered onto 4-ml linear sucrose gradients (20–55%) in H/K buffer containing 0.5% octyl glucoside (37). A 0.5-ml sucrose cushion (88%) was placed at the bottom of each tube to prevent loss of material by pelleting. Samples were centrifuged at 100,000 × g for 16 h at 8 °C. In select assays, 10 μM phalloidin was incubated with each of the membrane fractions for 30 min prior to initiation of velocity sedimentation. Fractions (350 μl) were collected from the top of the gradient, and the density of each was calculated from its refractive index. Fractions were analyzed by SDS-PAGE on 8% gels, and proteins were transferred to PVDF membrane. Following blocking with 5% non-fat dry milk, Western blotting was performed with polyclonal anti-PLD1 Ab or anti-actin IgM mAb, with detection via horseradish peroxidase-coupled 2° Ab and ECL, as described (37).

Preparation of Phalloidin-stabilized F-actin—G-actin from rabbit skeletal muscle was polymerized, and the resultant actin filaments were cut with plasma gelsolin and stabilized by addition of phalloidin, as described previously (38). Briefly, highly purified rabbit skeletal muscle G-actin (1 mg/ml, 23.2 μM) was incubated in polymerization buffer (5 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM ATP) in the presence of gelsolin (46.4 nM, 3.39 μg/ml) for 10 min at 0 °C (38). The suspension was warmed to 25 °C, and phalloidin was added to a final concentration of 50 μM, followed by incubation at 25 °C for 2 h and 4 °C for 18 h.

Analysis of Data—Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of normally distributed data were analyzed for statistical significance using Student's t test. Non-parametric evaluation of other data sets was performed with the Mann-Whitney Rank Sum test (39).

RESULTS

Addition of Purified Actin Inhibits PLD Activity—To characterize the effects of actin on PLD activity, we utilized a cell-free reconstitution system of purified cytosol and membranes from U937 human promonocytic leukemia cells. U937 cells express PLD1, but they contain no detectable PLD2 protein (25, 27, 40). PLD activity was determined via formation of the specific product [^{3}H]PEt from [^{3}H]phosphatidylcholine presented in mixed

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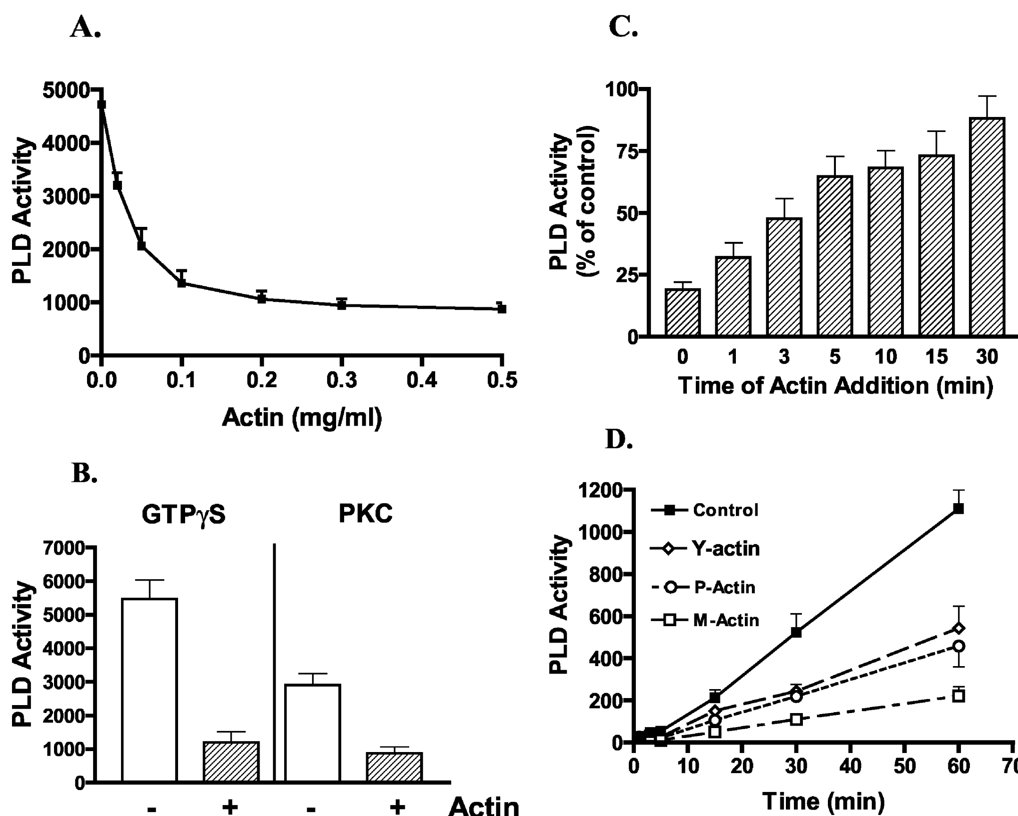

FIG. 1. **Addition of purified actin inhibits PLD activity.** *A*, membrane (75 μg) and cytosol (100 μg) were incubated with buffer control or the indicated concentrations of rabbit skeletal muscle α-actin, prior to addition of 30 μM GTPγS, 1.5% ethanol, and [^{3}H]PC substrate vesicles. PLD activity was determined at 30 min by quantitation of [^{3}H]PEt and normalized per 10^5 total [^{3}H]cpm in phospholipid. *B*, PLD activity was determined in the cell-free assay following stimulation by 30 μM GTPγS or 10 nM PKC + 100 nM PMA. Samples were preincubated with either 0.5 mg/ml α-actin (+) or buffer control (−) for 2 min prior to stimulation. *C*, skeletal muscle actin (0.5 mg/ml) was added either concurrent with 30 μM GTPγS (0 min) or at the indicated times after GTPγS. The PLD activity is expressed as the percentage of control samples (± range) to which no actin was added. *D*, kinetics of PLD activity in the presence of buffer control or 0.5 mg/ml α-actin from skeletal muscle (*M-Actin*), platelet βγ-actin (*P-Actin*) or actin from the yeast, *Saccharomyces cerevisiae* (*Y-actin*). Data represent mean ± S.E. of five identical experiments, each performed in triplicate.

lipid vesicles, in the presence of 1.5% ethanol. Addition of highly purified (>99% pure) rabbit skeletal muscle G-actin (α-actin, 0.02–0.5 mg/ml) resulted in significant dose-dependent inhibition of GTPγS-stimulated PLD activity (Fig. 1*A*). The IC_{50} for actin was 0.98 μM (0.042 mg/ml), and the highest dose of actin tested, 0.5 mg/ml (11.6 μM), produced an 84% inhibition of PLD activity (range 79–88%, $p < 0.001$, $n = 8$).

These inhibitory concentrations of actin are well within the normal range of cytosolic actin in mammalian cells (41, 42), suggesting that PLD activity may be physiologically regulated by actin. To evaluate further the potential physiologic relevance of actin-mediated inhibition of PLD activity, we substituted a natural substrate for the exogenous mixed lipid vesicles. The natural substrate consisted of purified membranes from U937 cells that had been biosynthetically radiolabeled in endogenous lipids with [^{3}H]oleate. Actin produced significant inhibition of GTPγS-stimulated PLD activity *versus* endogenous [^{3}H]oleate-labeled membranes (mean 76% reduction at 0.5 mg actin/ml, range 73–88%, $n = 4$, $p < 0.001$), to a level that was quantitatively similar to that observed with the exogenous substrate assays. Thus, inhibition of PLD activity occurs *in vitro* at physiologically relevant concentrations of actin, utilizing either native membranes or purified lipid vesicles as substrate.

PLD1 activity is stimulated by PKC, as well as by LMW GTPases (2, 5). To determine whether the inhibitory effect of exogenous actin on PLD activity was restricted according to the pathway of stimulation, we evaluated the effect of actin on activation of PLD by purified PKC. As demonstrated in Fig. 1*B*, addition of actin to PKC-stimulated samples resulted in a level of PLD inhibition (reduction of 71%, range 62–81%, $n = 4$, $p < 0.003$) that was very similar to that observed in samples stimulated by GTPγS. Thus, actin-induced inhibition of PLD activity was not restricted according to the class of activating stimulus.

To begin to characterize the mechanism of actin-induced inhibition of PLD activity, we determined the kinetic parameters of its maximal effect. The magnitude of the inhibitory effect of actin on PLD activity was critically dependent on the interval between the additions of actin and GTPγS (Fig. 1*C*). Maximal inhibition occurred when actin was added prior to guanine nucleotide. When this sequence was reversed, the extent of inhibition was inversely proportional to the interval between the additions of GTPγS and actin. For example, incubation of purified membrane and cytosol fraction with 0.5 mg/ml actin, prior to stimulation by GTPγS, resulted in an 84% reduction of PLD activity (range 79–88%, assayed at 60 min). In contrast, the same concentration of actin produced only a 31% inhibition (range 27–36%) of PLD activity when added 15 min after GTPγS, and an 8% inhibition (range 6–11%) when added 30 min after stimulation. These data are consistent with the hypothesis that the inhibitory effect of actin is exerted primarily during the initial activation of PLD.

Utilizing the conditions of maximal actin-induced inhibition, *i.e.* addition of actin 2 min prior to stimulation with GTPγS, the level of PLD activity was determined at 2, 5, 15, 30, or 60 min following stimulation. Compared with control samples treated with GTPγS alone (Fig. 1*D, solid squares*), the PLD activity of samples treated with α-actin was decreased by ∼75–85% at each

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TABLE I

Effect of actin monomer and polymerization buffers on PLD activity

Membranes from U937 cells were buffer-exchanged into AMB or APB by washing twice, followed by resuspension in that buffer. Cytosol was exchanged into the respective buffers by ultrafiltration, utilizing a 10-kDa exclusion limit. PLD activity of samples containing 75 μg of membrane protein and 100 μg of cytosol was determined in response to 30 μM GTPγS or buffer control, via accumulation of [^{3}H]PEt, in the presence of 1.5% ethanol.

Buffer	PLD activity[a]
AMB	110 ± 35
AMB + 30 μM GTPγS	192 ± 25
APB	223 ± 31
APB + 30 μM GTPγS	3874 ± 203
AMB + 30 μM GTPγS + 2 mM $MgCl_2$	1276 ± 185
AMB + 30 μM GTPγS + 50 mM KCl	177 ± 31
AMB + 30 μM GTPγS + 2 mM $MgCl_2$ + 25 mM KCl	2893 ± 262
AMB + 30 μM GTPγS + 2 mM $MgCl_2$ + 50 mM KCl = APB	4106 ± 374
AMB + 30 μM GTPγS + 2 mM $MgCl_2$ + 100 mM KCl	5259 ± 319

[a] PLD activity is expressed as [^{3}H]PEt cpm per 10^5 total [^{3}H]cpm in phospholipid.

of these time points (Fig. 1*D*, *open squares*). The fact that the relative magnitude of actin-induced inhibition remained constant throughout the course of the 60-min assay supports the hypothesis that inhibitory function of actin occurred primarily during the formation of a catalytically active PLD complex.

Several distinct isoforms of actin are differentially expressed in a cell- and tissue-restricted manner (41). Although structurally quite homologous (>90%), distinct differences exist in several characteristics, *e.g.* interaction with actin-binding proteins and cellular localization (41–44). To determine whether actin-mediated inhibition of PLD activity demonstrated isoform-specific characteristics, the effects of α-actin from rabbit skeletal muscle were compared with human platelet actin, which is a complex of βγ-actin in a 5:1 ratio. The purity of both actin preparations was >99%. Platelet βγ-actin was only 45% as efficacious (range 42–49%) and 41% as potent (IC_{50} = 2.4 μM) as muscle α-actin at inhibiting PLD activity (Fig. 1*D, open circles*). However, similar to α-actin, the relative magnitude of βγ-actin-mediated inhibition of PLD activity was constant throughout the duration of the 60-min assay. The level of native actin in the two actin samples was very similar, as evidenced by the extent of polymerization (data not shown).

Actin is also highly conserved among all eukaryotic species. The single actin species of the yeast *S. cerevisiae* is 87% identical to rabbit skeletal muscle α-actin and interacts with the majority of mammalian actin-binding proteins (69, 70). Because studies of yeast actin have provided valuable insights regarding structure-function relationships in this protein family (43, 69, 71), we tested the hypothesis that interactions with PLD would be conserved among actin species. Addition of highly purified yeast actin (43, 69, 71) resulted in time- and concentration-dependent inhibition of GTPγS-stimulated PLD activity (Fig. 1*D* and data not shown). The inhibitory efficacy of yeast actin (49% reduction in PLD activity at 0.5 mg/ml actin, range 44–53%, $p < 0.01$, $n = 4$) was slightly less than that of mammalian platelet actin (61% reduction, range 55–64%, $p < 0.01$, $n = 6$).

Actin-induced Modulation of PLD Activity Is Dependent on Its State of Polymerization—The physiologically most important attribute of actin is its ability to exist in a dynamically regulated equilibrium between the monomeric, globular G-actin form, and polymeric, filamentous F-actin. Therefore, we sought to determine whether the PLD-inhibitory effect of exogenously added actin was modulated by its state of polymerization. Several experimental considerations complicated this analysis. First, certain components of the buffer system that regulate the state of actin polymerization (*e.g.* concentrations of Ca^{2+} and Mg^{2+}, ionic strength, pH) also modulate PLD activity (31, 45, 46). Second, several actin-binding proteins that regulate both the critical concentration of free actin monomer and the dynamics of filament assembly/disassembly (including fodrin, gelsolin, and α-actinin) have recently been shown to potently modulate PLD activity (47–50). Third, the cell-free assay, composed of membrane and cytosol fractions from undifferentiated U937 promonocytes, contains a significant amount of both G- and F-actin. Fourth, in addition to activating PLD, stimulation of LMW GTPases with GTPγS promotes actin polymerization (51, 52). Thus, multiple modulators of actin polymerization also affect PLD activity and vice versa.

To begin to address the complexities of this analysis, we compared the ability of GTPγS to stimulate PLD activity in Actin Monomer Buffer (AMB: 5 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$) *versus* Actin Polymerization Buffer (APB: 5 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM $CaCl_2$). AMB, as the name signifies, does not support actin polymerization (46). Addition of $MgCl_2$ or KCl to AMB results in actin polymerization, and the combination of $MgCl_2$ and KCl is synergistic. For this analysis, purified membranes were washed twice in the respective buffers and resuspended in the same, and cytosol (containing G-actin) was exchanged into each buffer by ultrafiltration. PLD activity was determined in response to 30 μM GTPγS, or buffer control, via accumulation of [^{3}H]PEt.

Reconstitution of membrane and cytosol in AMB resulted in minimal GTPγS-stimulated PLD activity, whereas a significant level of PLD activity was detected in APB (Table I). We reasoned that one or both of the components that are present in APB, but not AMB ($MgCl_2$ and KCl), were critical for stimulation of PLD activity by GTPγS. Addition of 2 mM $MgCl_2$ (the concentration present in APB) to AMB resulted in a significant increase in PLD activity (Table I). In contrast, the addition of 50 mM KCl to AMB resulted in the same low level of PLD activity seen with AMB alone. Addition of both $MgCl_2$ and KCl to AMB resulted in a significant potentiation of GTPγS-stimulated PLD activity, compared with samples to which only $MgCl_2$ was added. Furthermore, in the presence of $MgCl_2$, the level of PLD activity was directly proportional to the concentration of KCl (Table I).

The comparison between the requirements for PLD activation and actin polymerization may be summarized as follows: addition of $MgCl_2$, but not KCl, to AMB was necessary and sufficient for GTPγS-induced PLD activity. In the presence of $MgCl_2$, KCl potentiated the level of PLD activation. In contrast, the addition of either $MgCl_2$ or KCl alone to AMB is both necessary and sufficient for actin polymerization, and the combination is synergistic (46). In the absence of Mg^{2+}, KCl promotes actin polymerization but not GTPγS-dependent stimulation of PLD activity, probably because Mg^{2+} is required for

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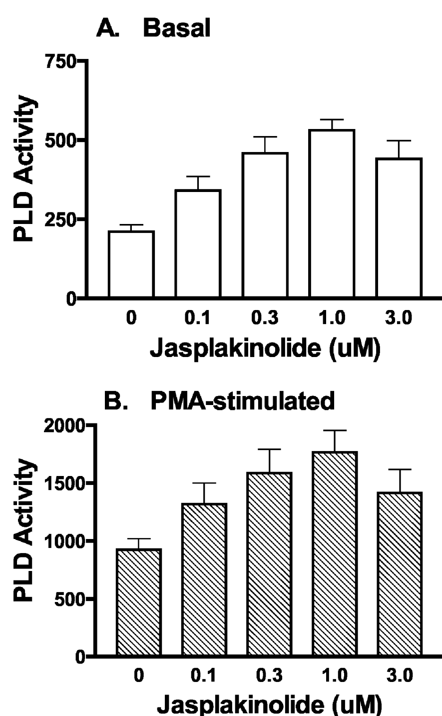

FIG. 2. **Induction of actin polymerization in intact cells by jasplakinolide increases basal and PMA-stimulated PLD activity.** Intact U937 promonocytes were radiolabeled for 18 h with [^{3}H]oleate, washed three times, and resuspended in H/S buffer. The indicated concentrations of jasplakinolide, or 0.1% methanol solvent control were added for 15 min at 37 °C. Samples in *A* received no other additions, and in *B*, cells were stimulated with 1 nM PMA. PLD activity was determined at 30 min, by accumulation of [^{3}H]PEt. Data were normalized per 10^5 [^{3}H] counts/min in total phospholipid to correct for any differences in cell labeling between experiments. Results are mean ± S.E. of four identical experiments, each performed in triplicate.

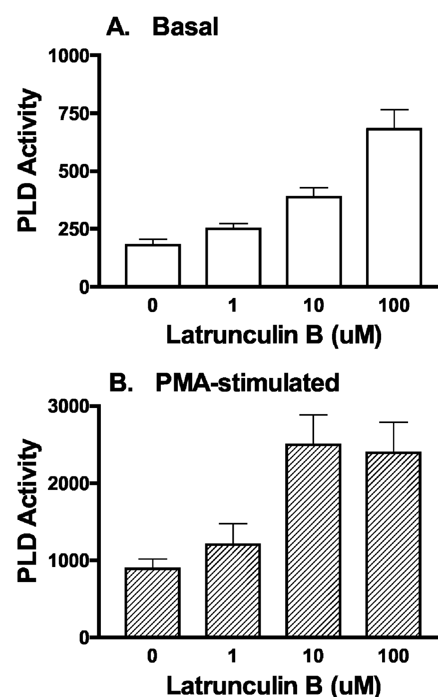

FIG. 3. **The actin monomer-sequestering agent, latrunculin B, increases basal and stimulated PLD activity in intact U937 promonocytes.** U937 cells, labeled with [^{3}H]oleate, were incubated with the indicated concentrations of latrunculin B or 1.0% ethanol solvent control for 15 min at 37 °C. PLD activity was determined under either basal conditions in resting cells (*A*) or following addition of 1 nM PMA (*B*). Data are corrected for ^{3}H labeling of phospholipids, as described in the legend to Fig. 2. Results are mean ± S.E. of three identical experiments, each performed in triplicate.

GTPγS exchange on Rho and ARF GTPases (which is necessary for activation of PLD1).

Taken together, these data support two hypotheses: 1) actin polymerization promotes PLD activity, and 2) monomeric actin inhibits PLD activity. The data presented in Fig. 1 are compatible with these hypotheses, because the exogenous actin was added as G-actin (in AMB) to membrane and cytosol that had been reconstituted in a polymerization competent buffer (H/K). The major inhibitory effect of exogenous actin occurred at the onset of PLD stimulation, when it would primarily be in the G-actin form. Of note, the aliquots of purified G-actin (in AMB) that were added to the cell-free PLD assay (in H/K buffer, Fig. 1) constituted ≤3% of the total assay volume. In control experiments, the addition of this concentration (v/v) of AMB (lacking actin) to H/K did not alter the kinetics or magnitude of PLD activity or actin polymerization (not shown). Considering the relation between the two hypotheses, above, the preceding data cannot distinguish whether they are (*a*) mechanistically linked, *i.e.* actin polymerization increased PLD activity by decreasing the level of its inhibitory complex with G-actin, or (*b*) mechanistically distinct, actin filaments and monomeric actin exhibit independent, and opposite, effects on PLD activity.

To evaluate further whether the physical state of actin, *i.e.* its degree of polymerization, modulates its effects on PLD activity, we utilized two well characterized pharmacologic agents, jasplakinolide and latrunculin B. Because these agents permeate cell membranes, they provided the opportunity to evaluate whether the physical state of actin modulates PLD activity *in vivo*. Jasplakinolide is a marine toxin that induces actin polymerization by increasing actin nucleation and stabilizing actin filaments (53, 54). Addition of jasplakinolide (0.1–3.0 μM) to intact U937 promonocytes (labeled with [^{3}H]oleate) resulted in a dose-dependent increase in basal PLD activity (Fig. 2*A*). The maximal increase, produced by 1 μM jasplakinolide, was 210% of the PLD activity of resting U937 cells. Coincident with this increase in PLD activity, jasplakinolide also resulted in increased levels of actin filaments, as determined by staining with rhodamine phalloidin (data not shown).

To determine whether jasplakinolide would also enhance PLD activity in response to agonist stimulation, we utilized sub-maximal doses of PMA, which is a potent activator of PLD in intact cells (2, 5). Pretreatment of U937 cells with jasplakinolide resulted in significant potentiation of PMA-stimulated PLD activity (Fig. 2*B*). The maximal augmentation occurred with 1 μM jasplakinolide, and was 191% of the level of PLD activity stimulated by 1 nM PMA alone. Thus, induction of actin polymerization by jasplakinolide is associated with increases in both basal and PMA-stimulated PLD activity in intact U937 cells.

Latrunculin B, another cell-permeant marine toxin, binds G-actin in a 1:1 stoichiometric complex (55, 56). The resulting sequestration of actin monomers causes depolymerization of actin filaments. Incubation of [^{3}H]oleate-labeled U937 cells with latrunculin B (1–100 μM) resulted in concentration-dependent increases in basal PLD activity (Fig. 3*A*), with a maximal value that was 378% that of control, untreated cells. Similar to the effects of jasplakinolide, latrunculin B also potentiated the level of PMA-stimulated PLD activity, with a maximal level 280% that of cells stimulated by PMA alone (Fig. 3*B*). Thus, sequestration of actin monomers by latrunculin B was accompanied by stimulation of both basal and stimulated PLD activity in intact U937 cells. Neither latrunculin B nor jasplakinolide, at the concentrations utilized, affected the viability of U937 cells, as determined by trypan blue exclusion (not

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shown). These reagents also produced no detectable PLD activity when incubated with the standard mixed lipid vesicle substrate (*i.e.* in the absence of cells, data not shown).

The fact that jasplakinolide, which increases actin polymerization, and latrunculin B, which promotes disassembly of actin filaments, both stimulated PLD activity suggested that the key determinant of the effects of actin on PLD activity is not simply the absolute levels of G- or F-actin. Consideration of the physiologic states of G-actin, and the specific effects of the toxins on these states, suggested an alternative hypothesis. First, the concentration of actin in non-muscle cells has been estimated at ~400 μM (17.2 mg/ml), with relatively half of this existing in the monomeric G-actin state and half as filamentous F-actin (41, 42). However, of the total G-actin pool (200 μM, 8.6 mg/ml), only ~0.1% (0.2 μM, 8.6 μg/ml) exists as free actin monomer. The remaining 99.9% of cellular G-actin is complexed to monomer-binding proteins, predominantly thymosin β4 and profilin (42). Second, although latrunculins and jasplakinolide exert opposite effects on levels of F-actin, they both decrease the levels of free G-actin but via different mechanisms. Latrunculins directly sequester free G-actin monomers (55, 56), whereas jasplakinolide indirectly depletes the pool of free G-actin by increasing the number of actin nucleation sites and stabilizing the resultant actin filaments (53, 54).

These considerations, and the preceding data, support the hypothesis that G-actin is primarily responsible for the observed inhibition of PLD. According to this model, latrunculin B and jasplakinolide stimulate PLD activity by decreasing the level of free G-actin. To evaluate further this hypothesis, we tested the effect of a well characterized G-actin-binding protein, DNase I, on PLD activity in the cell-free reconstitution assay. Because DNase I forms a stable 1:1 complex with G-actin (57, 58), we hypothesized that DNase I would increase PLD activity, via a mechanism analogous to that of latrunculin B in intact cells, *i.e.* sequestration of actin monomers. Membrane and cytosolic fraction were incubated with DNase I for 2 min, prior to addition of GTPγS or buffer control. Samples treated with DNase I demonstrated a significant increase in both basal and GTPγS-stimulated PLD activity (Fig. 4). The level of basal PLD activity increased 2.1-fold (range 2.0–2.3-fold, $p < 0.003$, $n = 4$) in DNase I-treated samples. An even larger relative increase, 3.4-fold (range 3.2–3.6-fold, $p < 0.001$, $n = 4$), was noted in samples stimulated by GTPγS + DNase I, compared with GTPγS alone. Of note, this highly purified preparation of DNase I lacked any detectable protease activity, eliminating the possibility that the enhancement of PLD activity was due to degradation of endogenous actin (data not shown). Incubation of DNase I with the lipid vesicle PLD substrate resulted in no detectable PLD activity, excluding a direct artifactual effect of DNase I (data not shown). These data are consistent with the hypothesis that G-actin inhibits PLD activity and that reductions in the level of free G-actin are associated with increases in basal and stimulated activity of PLD.

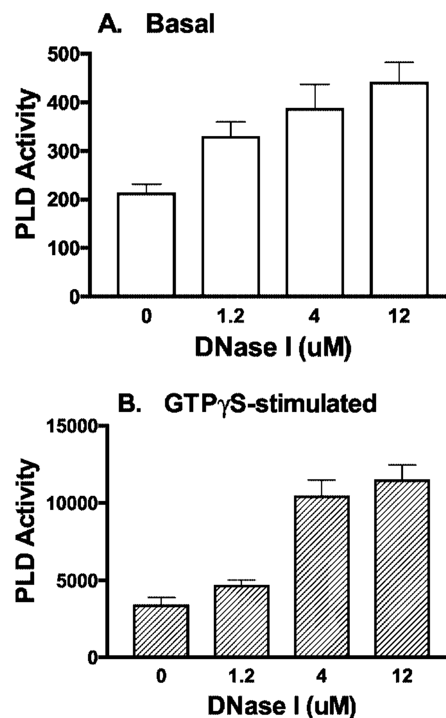

FIG. 4. **The G-actin-binding protein, DNase I, increases basal and GTPγS-stimulated PLD activity.** *A*, membrane and cytosol fractions from U937 promonocytes were incubated with buffer control or the indicated concentrations of DNase I and mixed lipid vesicles containing [^{3}H]PC substrate. PLD activity was determined at 30 min by quantitation of [^{3}H]PEt, in the presence of 1.5% ethanol. *B*, experimental conditions were identical to those described in *A* except that 30 μM GTPγS was added to each sample.

PLD1 and Actin Are Associated in Membranes from U937 Cells—The *in vitro* and *in vivo* inhibitory effects of G-actin on PLD activity may be mediated by a direct or indirect mechanism. Therefore, we sought to determine whether PLD1 and actin could be co-immunoprecipitated from U937 cell membranes. Purified membrane fractions were solubilized in lysis buffer containing 1% Triton X-100, 1% octyl glucoside, and 1% deoxycholate. Lysates were pre-cleared by incubation with preimmune serum and protein A-Sepharose, prior to immunoprecipitation with rabbit polyclonal anti-PLD1 Ab, or control, irrelevant Ab, bound to protein A-Sepharose. Immunoprecipitates were subjected to SDS-PAGE and Western blotting with anti-actin mAb. Three different polyclonal anti-PLD1 Abs were utilized for immunoprecipitation. These anti-peptide Abs were generated to the following amino acid sequences of PLD1: 1–15 (N terminus, PLD1-N); 525–541 (internal, PLD1-I); and 1057–1074 (C terminus, PLD1-C) (6, 36). Control experiments demonstrated that each of the three anti-PLD1 Abs immunoprecipitated a protein that co-migrated with baculovirus-expressed recombinant PLD1 and was recognized by the alternative anti-PLD1 Abs in Western blots (see Ref. 27 and not shown).

Fig. 5*A* presents the results of immunoprecipitation of membrane lysates with the anti-PLD1 Abs, followed by Western blotting of the immunoprecipitates with anti-actin mAb. Actin was co-immunoprecipitated by all three of the anti-PLD Abs but not by the control, irrelevant polyclonal Ab. The increased levels of actin co-immunoprecipitated by PLD1-N and PLD1-C, compared with PLD1-I, are consistent with the comparative efficacies of these Abs as reported previously (36). The results of the co-immunoprecipitation assay are consistent with the hypothesis that PLD1 constitutively associates with actin in membranes from resting cells. However, the data do not distinguish between a direct binary interaction *versus* an indirect mechanism involving one or more intermediary molecules.

PLD1 Binds to Membrane-associated G-actin—Both monomeric G-actin and actin filaments are associated with eukaryotic membranes (59, 60). Therefore, we sought to determine whether the membrane-localized interaction between actin and PLD1, as evidenced by the co-immunoprecipitation results, was due to G-actin, F-actin, or both. For each species of actin, we evaluated the possibility of both physical and functional interactions with PLD1. To determine whether membrane-associated G-actin binds to PLD1, we took advantage of the fact that DNase I binds specifically to G-actin but not F-actin (57, 58). Membranes were solubilized in Lysis buffer containing 1%

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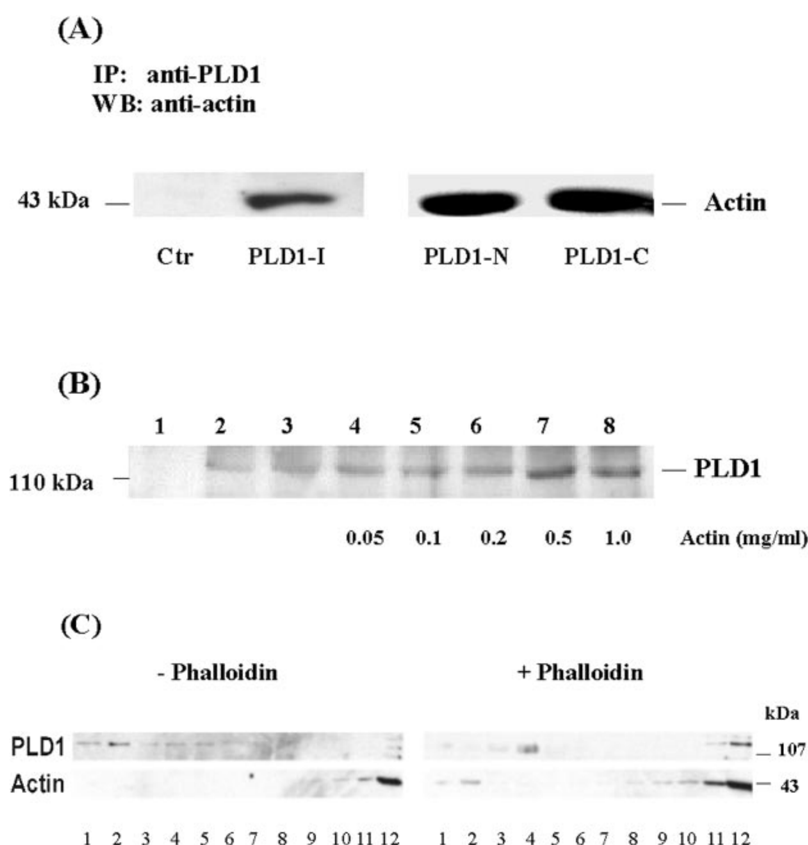

FIG. 5. **PLD1 is associated with both G- and F-actin in membranes from U937 cells.** *A*, co-immunoprecipitation of PLD1 and actin. Membranes were incubated in Lysis buffer for 1 h on ice and subjected to immunoprecipitation (*IP*) with control irrelevant Ab (*Ctr*) or polyclonal Abs generated to the following sequences of PLD1: 525–541 (PLD1-I), 1–15 (PLD1-N), or 1057–1074 (PLD1-C). Following washing of the immunoprecipitates, samples were analyzed by SDS-PAGE/Western blotting (*WB*) with anti-actin IgM mAb, with detection by horseradish peroxidase-conjugated secondary Ab and ECL. *B*, co-sedimentation of PLD1 and membrane-associated G-actin. Purified membranes were solubilized in Lysis buffer and incubated with uncomplexed Sepharose beads (*lane 1*) or DNase I-Sepharose (*lanes 2–8*). The designated amounts of purified α-actin were added to *lanes 4–8*, prior to sedimentation by centrifugation. Sedimented beads were washed in Lysis buffer, and associated proteins were analyzed by SDS-PAGE/Western blotting with anti-PLD1 Ab. *C*, purified membranes were incubated in H/K buffer, in the absence (*left panels*) or presence (*right panels*) of 10 μM phalloidin for 30 min at 25 °C, followed by incubation in 0.5% octyl glucoside for 1 h at 4 °C. Samples were loaded on 20–55% sucrose gradients and subjected to centrifugation for 16 h at 8 °C. Western blotting was performed with polyclonal Ab to PLD1 or anti-actin IgM mAb, with detection by ECL.

Triton X-100, 1% octyl glucoside, and 1% deoxycholate. Lysates were incubated for 16 h at 4 °C with DNase I that was covalently linked to Sepharose 4B beads. In control samples, uncomplexed Sepharose 4B was substituted for DNase I-Sepharose. Beads were pelleted by centrifugation and washed five times in lysis buffer. Proteins bound to the beads were analyzed by SDS-PAGE and Western blotting with anti-PLD1 Ab. PLD1 was specifically co-precipitated by DNase I-Sepharose beads (Fig. 5*B*, *lanes 2* and *3*) but not by the uncomplexed-Sepharose control (Fig. 5*B*, *lane 1*), consistent with the hypothesis that PLD1 associates with G-actin in membranes. In select samples, various concentrations of purified α-actin (0.05–1.0 mg/ml) were added to the membranes lysates prior to incubation with DNase I-Sepharose. Addition of actin was associated with dose-dependent increases in the levels of co-sedimented PLD1 (Fig. 5*B*, *lanes 4–8*), with a saturation at 0.5 mg/ml of added actin. This latter finding suggests that membranes contain 2 pools of PLD1 with respect to its association with G-actin: 1) PLD1 that is bound to membrane-associated G-actin, and 2) PLD1 that is not associated with G-actin (and thus sedimented by DNase I-Sepharose only when exogenous actin is added).

The Membrane-localized Interaction with G-actin Inhibits PLD1 Activity—Our hypothesis is that the physical interaction with G-actin results in inhibition of PLD activity. Based on the previous demonstration that G-actin-sequestering agents increase PLD activity in intact cells (latrunculin B) and the cell-free reconstitution system of membrane and cytosol (DNase I), we similarly evaluated the potential functional consequences of the association of PLD1 with G-actin in purified membranes, *i.e.* in the absence of cytosol. Membranes from resting U937 cells were incubated with DNase I (50 μM), or buffer control, for 15 min, followed by addition of [^{3}H]DPPC-containing mixed lipid substrate vesicles. DNase I-treated membranes exhibited a significant increase in PLD activity,

TABLE II
Effects of DNase I and F-actin on PLD activity of purified membranes

100 μg of membrane from U937 promonocytes was incubated in H/K buffer and the indicated components for 2 min at 37°C, prior to addition of phosphatidylethanolamine/PI(4,5)P_2/[^{3}H]DPPC substrate vesicles and 1.5% ethanol. PLD activity was determined via quantitation of [^{3}H]PEt at 30 min.

Conditions[a]	PLD activity[b]
Membrane	249 ± 23
Membrane + DNase I	440 ± 38
Membrane + F-actin	336 ± 42
Membrane + G-actin	47 ± 11
Membrane + GTPγS[c]	8370 ± 603
Membrane + GTPγS + DNase I	30356 ± 2531
Membrane + GTPγS + F-actin	13894 ± 986
Membrane + GTPγS + G-actin	1603 ± 141

[a] DNase I = 50 μM, phalloidin-stabilized F-actin and G-actin = 0.05 mg/ml.

[b] Data represent mean ± S.E. of three separate experiments, each performed in triplicate.

[c] Membrane (100 μg) and cytosol (75 μg) were incubated 100 μM GTPγS for 30 min. Membranes were re-isolated by ultracentrifugation, washed, and incubated with the indicated compounds or buffer control, and PLD activity was determined at 30 min.

compared with control membranes (Table II). Furthermore, membranes "pre-activated" by incubation with GTPγS (27, 31), also exhibited significantly greater PLD activity following addition of DNase I (Table II). Similar enhancement of basal and GTPγS-stimulated PLD activity in purified membranes was obtained by treatment with latrunculin B (not shown). Thus, G-actin-binding compounds enhance basal and stimulated PLD activity in intact cells (Fig. 3), a complete cell-free system (Fig. 4), and isolated membranes (Table II). Taken together, these data support the hypothesis that the physical interaction between PLD1 and G-actin inhibits PLD activity.

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PLD1 Binds to Actin Filaments during Equilibrium Velocity Sedimentation on Sucrose Density Gradients—F-actin is normally complexed with multiple cytoskeletal proteins, which together comprise the polymeric state of the actin microfilament cytoskeleton. To test the hypothesis that PLD1 binds to actin filaments, we utilized a previously established method based on equilibrium velocity sedimentation, in the absence and presence of phalloidin (37, 38). Because phalloidin cross-links F-actin and stabilizes the resultant microfilaments, it changes the density profile of F-actin in velocity sedimentation to fractions of greater density. Proteins that are bound to F-actin demonstrate a similar "shift" to higher density fractions in the presence of phalloidin (37). Purified membranes were subjected to extraction with 0.5% octyl glucoside, followed by equilibrium velocity sedimentation of the entire lysate (detergent-soluble and -insoluble components) on a 20–55% continuous sucrose gradient for 16 h at 4 °C (37). Aliquots of each fraction were analyzed by SDS-PAGE and Western blotting with polyclonal anti-PLD1 Ab or mAb to actin. PLD1 exhibited a bimodal distribution in the density gradient. One subset of PLD1 was located in the low density fractions 1–6 with a peak in fraction (Fx) 2 (Fig. 5*C*, *left*). The second subset of PLD1 was located in the densest fraction, Fx 12. Comparison with the position of protein standards (not shown), cytochrome *c* (13.4 kDa), Fx 2; hemoglobin (64.4 kDa), Fx 3; hexokinase (100 kDa), Fx 6, indicated that PLD1 was localized to fractions of both greater (Fx 12) and lesser density (Fxs 1–5) than predicted by its calculated molecular mass of 120 kDa. In agreement with previous work (27), PLD2 was not detected in any fraction (not shown). Western blotting for actin demonstrated its localization to the densest fractions of the gradient, 9–12, with the majority in Fx 12 (Fig. 5*C*, *left*).

In parallel samples, 10 μM phalloidin was incubated with membranes for 30 min at 25 °C, prior to detergent extraction and equilibrium velocity sedimentation. Inclusion of phalloidin resulted in a shift of the density profile of PLD1 (Fig. 5*C*, *right*), compared with membranes processed in the absence of phalloidin. Specifically, phalloidin-treated membranes exhibited the major peak of PLD1 immunoreactivity in Fx 4 (density 1.11 mg/dl), compared with control samples not treated with phalloidin, in which peak PLD was located in Fx 2 (density 1.08 mg/dl). Phalloidin treatment also resulted in increased amounts of PLD1 and actin in the "heavy" fractions of the gradient (Fig. 5*C*) and decreased extraction of both proteins during the washing steps (not shown). These effects of phalloidin on the density distribution of PLD1 are consistent with the proposed hypothesis that PLD1 binds actin filaments.

F-actin Augments PLD Activity—Direct evaluation of whether the interaction between PLD1 and F-actin modulates PLD activity required a form of F-actin that is stable under the conditions of the PLD assay. To this end, highly purified G-actin was polymerized, and the resultant actin filaments were severed by gelsolin and stabilized by addition of phalloidin, as described previously (38). In this preparation, essentially all the actin is present as F-actin, due to stabilization of filaments by phalloidin and capping of the barbed ends of filaments by gelsolin. This molar ratio of actin/gelsolin has been reported to yield F-actin filaments with an average length of 500 monomers (38). Addition of (phalloidin-stabilized) F-actin to the cell-free PLD reconstitution assay resulted in significant dose-dependent enhancement of GTPγS-stimulated PLD activity (Table II). In the absence of prior polymerization and phalloidin cross-linking, addition of the same amounts of actin (in the G-actin form) resulted in significant inhibition of PLD activity, similar to the results presented in Fig. 1. Taken together, these data are consistent with a model in which actin exhibits polymerization-dependent modulation of PLD activity; G-actin inhibits PLD, whereas F-actin augments the activity of PLD.

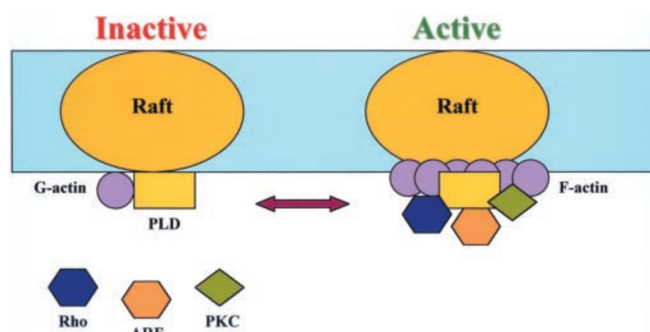

FIG. 6. **Model for regulation of mammalian PLD by G- and F-actin.** In resting cells, PLD is kept in an inactive state (*left side*) in part via complexation with G-actin. Cell stimulation is accompanied by activation of PLD (*right side*) by low molecular weight GTPases (*e.g.* Rho, ARF) and PKC. Concurrent with its direct binding to these activators, PLD is released from its inhibitory complex with G-actin and becomes associated with F-actin-containing filaments. F-actin promotes the binding of PLD to the membrane substrate in part via interaction with membrane lipids. Both PLD and nascent F-actin formation are preferentially localized to glycosphingolipid-enriched domains, termed membrane "rafts."

DISCUSSION

The critical importance of cellular functions that are coordinately regulated by PLD and actin, including proliferation, migration, vesicle trafficking, and secretion, underscores the need to define the physical and functional interactions between these molecular families. We (27) and others (28) have demonstrated that PLD1 physically associates with the detergent-insoluble actin cytoskeleton, in a constitutive and stimulation-enhanced manner. On the functional level, evidence has been presented for the involvement of PLD in formation of actin stress fibers in intact cells (10–13). However, the converse possibility that actin may regulate PLD activity has remained relatively unexplored. Recently, Lee *et al.* (29) reported that β-actin inhibited mammalian PLD2 and PLD1 *in vitro*. Our results confirm and significantly extend the work of Lee *et al.* (29) in several important ways. First, actin exerted *bimodal* modulation of PLD1 activity; monomeric G-actin inhibited PLD, whereas F-actin enhanced stimulation of PLD. Second, this biphasic modulation of PLD1 activity was demonstrated both *in vivo* and *in vitro*. Third, actin-mediated modulation of PLD1 activity was stimulus-independent, affecting its activation by both GTP-binding proteins and PKC. Fourth, actin monomer-sequestering agents, including the physiologic G-actin-binding protein, DNase I, activated PLD1 in resting cells, cell extracts, and purified membranes, suggesting a novel mechanism of PLD stimulation. Fifth, PLD1 was physically associated with both G-actin and actin filaments, supporting the hypothesis that actin is a physiologic regulator of PLD activity. Sixth, the functional effects of actin on PLD were isoform-specific; α-actin was more than twice as potent and efficacious as $\beta\gamma$-actin, suggesting the possibility of tissue selectivity/specificity in actin-mediated regulation of PLD. Seventh, the modulatory effects of actin on PLD activity were broadly conserved throughout eukaryotic species, from single-celled yeast to humans.

The most important advance provided in this report is that actin bidirectionally modulates PLD activity in a polymerization-dependent manner. The critical aspect of this modulation is the potent inhibition of PLD by G-actin. Because reductions of free G-actin by either monomer sequestration or induction of polymerization are both associated with increases in PLD activity (despite differing effects on levels of F-actin), we hypothesize

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that the amount of G-actin complexed to PLD is the primary determinant of the effects of actin. Notably, these effects of G-actin depletion are evident in both resting cells and the unstimulated cell-free reconstitution assay. To our knowledge, this is the first demonstration of activation of mammalian PLD1 by a mechanism distinct from LMW GTPases or PKC. The increases in PLD activity induced by phalloidin-stabilized F-actin support an independent positive effect of actin filaments on PLD activity. Along with previous work establishing a role for PLD in actin cytoskeletal rearrangements (10–13), the current findings and the work of Lee *et al.* (29) suggest that PLD enzymes and G- and F-actin function in a coordinately regulated system. These complex functional interactions are likely to account for the co-involvement of PLD and the actin cytoskeleton in many essential cellular processes (2, 5).

The data in this article concur with those of Lee *et al.* (29) to the extent that addition of purified β-actin inhibits activation of PLD by LMW GTPases *in vitro*. An important extension of our work is the demonstration that PKC-induced PLD activity is inhibited in a quantitatively and kinetically similar manner. This stimulus independence of actin-mediated inhibition, as well as its kinetic characteristics (Fig. 1), strongly suggests that G-actin blocks the formation of a catalytically active PLD complex. Because all eukaryotic membranes (plasma membrane, Golgi, nuclear membrane, etc.) have a tightly associated actin skeleton (61–63), PLD enzymes will necessarily encounter actin in the context of substrate binding and catalysis. Thus, we propose a model in which actin, both monomeric G-actin and F-actin filaments, are fundamental participants in the catalytic cycle of mammalian PLD (Fig. 6). This model is complemented by recent evidence that membrane rafts, the glycosphingolipid-enriched membrane domains in which PLD1 and PLD2 are preferentially localized (27, 64, 65), are loci of nascent F-actin formation (66). Thus, actin-mediated regulation of PLD is likely to be spatially coupled to dynamic rearrangements of the actin cytoskeleton.

The physical interactions responsible for these functional effects are beginning to be defined. Lee *et al.* (29) demonstrated the presence of actin in anti-PLD immunoprecipitates from cells overexpressing PLD. We have extended their important findings by demonstrating the following: (*a*) endogenous PLD1 associated with both G-actin and F-actin, (*b*) these physical interactions differentially modified PLD activity, and (*c*) these interactions occurred in intact cells, a cell-free reconstitution system, and in purified membranes. Mammalian PLD enzymes have been extremely difficult to purify. This challenge has limited our ability to resolve the binding interactions between PLD1 and G- or F-actin at the molecular level. Anti-PLD immunoprecipitates, obtained with the three different anti-peptide Abs described above, and utilizing a broad spectrum of detergents, all contained many proteins in addition to actin (not shown). This is not surprising because the affinity of PLD for detergent-insoluble membrane domains has been characterized previously (27, 65, 67), and many protein and lipid components of these membrane rafts co-precipitate with PLD isoforms. It is important to note that Lee *et al.* (29) did not report the purity of the PLD preparations used in their study. Because purified preparations of mammalian PLDs are required to test the hypothesis of a direct interaction with actin, we conclude that the mechanism by which mammalian PLDs bind actin is not yet defined. In contrast, we have utilized bacterial and plant PLDs that have been purified to homogeneity to address definitively whether PLD enzymes bind directly to G- and/or F-actin.[2]

Another important difference between the work of Lee *et al.* (29) and this study is the degree of purity of the actin preparation utilized for *in vitro* studies. Because several actin-binding proteins (ABPs), including fodrin and α-actinin, potently inhibit PLD activity (IC_{50} 1–10 nM) (47, 68) and are common contaminants of actin preparations, the issue of purity is critical. Both the α-actin and βγ-actin used in this work were greater than >99% pure (by protein staining with Coomassie Blue and Sypro Ruby Red). Furthermore, Western blotting with Abs to prevalent ABPs, including fodrin, α-actinin, paxillin, talin and vinculin, were negative (not shown). Lee *et al.* (29) reported a purity of >90% for their β-actin preparation, and no information regarding potential contamination by ABPs was provided. Gelsolin, another ABP, has been alternatively reported to activate (48) or inhibit PLD (49). Perhaps differential effects and/or amounts of actin in these experimental systems contributed to the reported divergent impacts of exogenous gelsolin on PLD activity.

In summary, mammalian PLD1 associates with both monomeric G-actin and filamentous F-actin, with significant functional consequences for the regulation of PLD activity. These physical and functional interactions with actin are isoform-specific and independent of the PLD-activating stimulus. Further study will be required to establish the molecular details and physiologic roles of PLD-actin interactions in the multiple critical cellular responses associated with these protein families.

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Regulation of Phospholipase D Activity by Actin: ACTIN EXERTS BIDIRECTIONAL MODULATION OF MAMMALIAN PHOSPOLIPASE D ACTIVITY IN A POLYMERIZATION-DEPENDENT, ISOFORM-SPECIFIC MANNER

David J. Kusner, James A. Barton, Kuo-Kuang Wen, Xuemin Wang, Peter A. Rubenstein and Shankar S. Iyer

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