Association of Phospholipase D Activity with the Detergent-insoluble Cytoskeleton of U937 Promonocytic Leukocytes*

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Phospholipase D (PLD) regulates cytoskeletal-dependent antimicrobial responses of myeloid leukocytes, including phagocytosis and oxidant generation. However, the mechanisms responsible for this association between PLD activity and the actin cytoskeleton are unknown. We utilized a cell-free system from U937 promonocytes to test the hypothesis that stimulation of PLD results in stable association of the activated lipase with the detergent-insoluble membrane skeleton. Plasma membrane and cytosol were incubated ± guanosine 5′-3′-O-(thio)triphosphate (GTPγS), followed by re-isolation and extraction of the washed membranes with octyl glucoside. The detergent-insoluble fraction derived from membranes incubated with GTPγS (DIFGTPγS) exhibited 22-fold greater PLD activity than that derived from control membranes (DIFg), when both were assayed in the presence of GTPγS. The DIF contained PLD1, RhoA, and ARF, and the level of each was increased by GTPγS in a dose-dependent manner. The DIF also contained F-actin, vinculin, talin, paxillin, and α-actinin, consistent with its identification as the membrane skeleton. The physiologic relevance of these findings was demonstrated by a similar increase in DIF-associated PLD activity after stimulation of intact U937 cells with opsonized zymosan. These results indicate that stimulation of PLD1 is accompanied by stable association of the activated lipase, RhoA, and ADP-ribosylation factor with the actin-based membrane skeleton.

Activation of phospholipase D (PLD)† results in the generation of the lipid second messengers, phosphatidic acid (PA) and diglycerides, and has been linked to multiple physiologic processes, including secretion, vesicle trafficking, mitosis, and mitogenesis (1–3). In phagocytic leukocytes (monocytes, macrophages, and neutrophils), activation of PLD is coupled to the major antimicrobial responses of phagocytosis, generation of reactive oxidants, and granule secretion (1, 4). The mechanisms that regulate PLD and the means by which its lipid products function in such diverse physiologic processes are beginning to be elucidated. Two mammalian PLD genes designated PLD1 and PLD2 have recently been cloned (5, 6). PLD1 is regulated by low molecular weight GTP-binding proteins (GTPases) of the ARF and Rho families and by protein kinase C (1, 2). The mechanisms that regulate PLD2 are undefined, but this isoform is unaffected by the activators of PLD1. The relations between these PLD isoforms and the various PLD activities demonstrated in diverse cells and tissues requires further study.

Several lines of evidence suggest that PLD may be functionally associated with the actin-based microfilament cytoskeleton. First, many of the physiologic processes in which activation of PLD is believed to play an important role also require rearrangements of the cytoskeleton, including motility, secretion, and cell division (1, 7–9). This association of PLD with cytoskeletal-dependent responses is particularly notable for the primary antimicrobial functions of phagocytic leukocytes (4, 10–14). Second, stimulation of PLD in fibroblasts and endothelial cells is coupled to formation of actin stress fibers, and this effect is mimicked by the addition of purified PLD or PA and blocked by inhibitors of PLD-dependent generation of PA (15, 16). Third, overexpression of PLD2 promotes cytoskeletal reorganization in serum-stimulated fibroblasts (6). Fourth, several cytoskeletal-associated proteins, including fodrin, synaptopodin, and clathrin-associated protein 3, are potent inhibitors of PLD activity (17–19), suggesting that mechanisms exist for feedback regulation of PLD-dependent cytoskeletal rearrangements. Fifth, the downstream effectors of PLD modulate actin microfilament dynamics. PA stimulates the synthesis of phosphatidylinositol-(4,5)-bisphosphate via activation of phosphoinositide 4- and 5-kinases (20, 21), and phosphatidylinositol-(4,5)-bisphosphate, in turn, regulates actin polymerization (22–24). Diglycerides promote actin nucleation at the plasma membrane, enhancing the formation of F-actin (25). These observations suggest a functional association between PLD and the actin cytoskeleton. The objective of this study was to determine whether stimulation of GTP-binding proteins results in stable association of PLD activity with the F-actin-containing membrane skeleton of U937 promonocytes.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, materials were from previously published sources (26, 27). [1H]Dipalmitoylphosphatidylcholine (DPPC) was obtained from Amersham Pharmacia Biotech. GTPγS, Gpp(NH)p, and GDPβS were from Boehringer Mannheim. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol-(4,5)-bisphosphate, phosphatidyl serine, zymosan, octyl glucoside, sodium cholate, and molybdenum blue reagent were obtained from Sigma. Silica gel plates (200 μm) for high performance thin layer chromatography were from Fisher. Triton X-100 and the reagents for Western blot detection by enhanced chemiluminescence were obtained from Pierce. PLD1 and PLD2 protein standards were generously provided by Dr. Andrew J. Morris (State University of New York, Stony Brook, NY).

Antibodies—Polyclonal antibodies specific for PLD1 or PLD2 were

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‡ The abbreviations used are: PLD, phospholipase D; PA, phosphatidic acid; PEt, phosphatidylethanol; PE, phosphatidylethanolamine; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; GDPβS, guanosine 5′-O-2-thio(diphosphate); Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; DIF, detergent-insoluble fraction; DPPC, dipalmitoylphosphatidylcholine; Ab, antibody; COZ, complement-opsonized zymosan; PAGE, polyacrylamide gel electrophoresis; DIFP, detergent-insoluble fraction from permeabilized cells; ARF, ADP-ribosylation factor.
from Quality Controlled Biochemicals Corp. (Hopkinton, MA). Monoclonal Abs to vinculin, τ-actin, and β-COP were from Sigma, and monoclonal Ab to paxillin was from ICN ImmunoBiologicals (Costa Mesa, CA). Monoclonal Ab to human major histocompatibility class I was obtained from Dakopatts, Inc. (Denmark). Polyclonal α-RhoA Ab was a gift from Santa Cruz Biotechnology (Santa Cruz, CA). The murine α-ARF was a kind gift of Dr. Richard A. Kuhn (National Cancer Institute, MD).

Cell Fractionation—U937 promonocytic leukocytes maintained at 37 °C, 7.5% CO₂ in Iscoves medium, 10% fetal bovine serum, were washed in H/S buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 0.7 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, 1 mg/ml bovine serum albumin) (27–29), incubated with 4 mM diisopropylfluorophosphate, and resuspended in H/K buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 2 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol) before disruption by N₂ cavitation (450 p.s.i., 25 min). After 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, resuspended in H/K buffer, and homogenized with a Tenbroeck tissue grinder. This membrane fraction was enriched in plasma membrane (defined by the presence of virtually all of the human major histocompatibility class I antigen, as determined by a specific antibody [data not shown] and was utilized for all subsequent studies. Because this fraction also contained the Golgi marker β-COP, it will be referred to as the “plasma membrane-enriched” or “membrane” fraction, for simplicity. The more dense fraction, which sedimented through the 50% sucrose, was enriched in the primary granule marker CD63 and was not utilized in this study. Protein concentrations in membrane and cytosolic fractions were determined by the method of Bradford (30).

Assay of Membrane Phospholipase D Activity—75 μg of the membrane fraction was incubated with 100 μg of cytosol + GTP.S (1–100 μM) for 30 min at 37 °C in H/K buffer in a volume of 100 μL. Membranes were re-isolated by centrifugation at 150,000 × g for 60 min, washed twice with H/K, and resuspended in same (29). Washed membranes prepared from incubation with cytosol in the absence of GTP.S are designated Mₑ, whereas those prepared in the presence of GTP.S are referred to as MₑGTP.S. Substrate vesicles containing phosphatidylethanolamine:phosphatidylinositol-(4,5)-bisphosphate:phosphatidylcholine (molar ratio of 16:1:4:1) with 10 μCi/sample [3H]DPPC were prepared by sonication for 10 min at 25 °C (31). 100 μM GTP.S was included in each sample, and 1.5% ethanol was added to permit detection of PLD activity. The transphosphatidylcholine reaction products were detected as diphosphatidylethanol (DPE). Reactions were terminated at 60 min by the addition of 500 μL of chloroform:methanol (2:1, v/v). Lipids were extracted, dried under N₂, and analyzed by thin layer chromatography (TLC) in an ethyl acetate:isoctane:acetic acid (9:5:2, v/v) solvent system (26, 27, 29). PEt and PA were identified by comigration with purified standards, [3H]PEt and [3H]PA cpm were quantitated by liquid scintillation spectrophotometry. Normalized data were analyzed by an analysis of variance (32). Briefly, samples were assayed in Mg(NO₃)₂ in ethanol, solubilized in 0.5 M HCl, and heated at 100 °C for 15 min. After cooling to 25 °C, Ames colorimetric reagent (34) was added, samples were incubated at 37 °C for 1 h then cooled to 25 °C, and Аₕ₂₅ was determined. Quantitation was performed by reference to a standard curve derived from KH₂PO₄ solution containing known concentrations of [3H]PEt.

Analysis of Phospholipid Content of Membrane and Detergent-insoluble Fractions—Lipid phosphorus content of membrane and detergent-insoluble fractions were determined by the ashing procedure of Ames (34). Briefly, samples were ashed in Mg(NO₃)₂, in ethanol, solubilized in 0.5 M HCl, and heated at 100 °C for 15 min. After cooling to 25 °C, Ames colorimetric reagent (34) was added, samples were incubated at 37 °C for 1 h then cooled to 25 °C, and Аₕ₂₅ was determined. Quantitation was performed by reference to a standard curve derived from KH₂PO₄ solution containing known concentrations of [3H]PEt.

SDS-PAGE and Western Blot Analysis—Membrane, cytosolic, and detergent-insoluble fractions from U937 cells were prepared as above. 5 × 10⁶ cell equivalents of the membrane or DIF or 100 μg of cytosol were subjected to SDS-PAGE as described previously (27). 12.5% gels were utilized for analysis of RhoA and ARF, whereas 9% gels were used for detection of PLD, vinculin, τ-actin, paxillin, and actin. Proteins were transferred to polyvinylidene difluoride membrane and blocked with 5% nonfat dry milk. Western blotting, with detection via horseradish peroxidase-coupled 2° Ab and enhanced chemiluminescence (ECL) was performed as described (27).

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 by a Student’s t test. Nonparametric evaluation of other data sets was performed with the Mann-Whitney Rank Sum test (12).

RESULTS

GTP.γS Induces a Stable Association of PLD Activity with the Plasma Membrane and Its Detergent-insoluble Fraction—We utilized a cell-free system from U937 promonocytic leukocytes to study GTP-binding protein-dependent stimulation of PLD activity. Undifferentiated U937 cells were disrupted by nitrogen cavitation and subjected to sucrose density centrifugation as described under “Experimental Procedures.” Plasma membrane-enriched and cytosolic fractions were incubated in the presence or absence of GTP.S, followed by re-isolation and washing of the membrane fraction. The PLD activity of these membranes was then determined in the presence of GTP.S by quantitation of [3H]PEt formation from DPPC-labeled mixed lipid vesicles in the presence of 1.5% ethanol (31). Membranes that had been incubated with cytosol in the absence of GTP.S (Mₑ) exhibited little basal or GTP.S-dependent PLD activity upon subsequent isolation (Fig. 1A). In contrast, incubation of membrane and cytosolic fractions in the presence of GTP.S resulted in stable association of PLD activity with the isolated, washed membrane fraction (MₑGTP.S), which was 21-

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Stimulation of Permeabilized or Intact U937 Cells Also Results in Stable Association of PLD Activity with the Detergent-insoluble Fraction—To determine whether GTP\(\gamma\)S induces the association of PLD with the DIF in a more complex experimental system, we utilized U937 cells permeabilized with \(\beta\)-escin (26, 29). U937 cells in H/K buffer were incubated with 75 \(\mu\)M GTP\(\gamma\)S for 15 min at 37 °C. Permeabilized cell preparations were pelleted by centrifugation then washed in buffer followed by \(N_2\) cavitation and isolation of membranes. Preparation of the octyl glucoside-insoluble fractions was performed exactly as for the cell-free system, described above. The octyl glucoside-insoluble fraction from permeabilized cells treated with GTP\(\gamma\)S (DIF-GTP\(\gamma\)S) contained 2.2-fold greater PLD activity than the corresponding detergent-insoluble fraction from control cells incubated with \(\beta\)-escin alone (DIF\(\beta\)escin) (range 2.0–2.3, \(p < 0.01\), Fig. 2A). The PLD activity of DIF-GTP\(\gamma\)S accounted for approximately 45% of the total PLD activity of the plasma membrane fraction from permeabilized cells, MP-GTP\(\gamma\)S. These results indicate that activation of GTP-binding proteins in permeabilized cells results in elevated PLD activity in the DIF, similar to that demonstrated in the cell-free system. The decreased PLD activity of DIF-GTP\(\gamma\)S (632 ± 47 H cpm) compared with the DIF derived from the cell-free system, DIF-GTP\(\gamma\)S (3263 ± 320 H cpm) may be because of several factors, including decreased activation of GTP-binding proteins, decreased access of PLD to the exogenous substrate vesicles, and/or inclusion of a second detergent (\(\beta\)-escin), which may directly decrease PLD activity.

To further evaluate the potential physiologic relevance of DIF-associated PLD activity, we determined the effects of stimulation of intact U937 cells with a receptor-dependent agonist, COZ. U937 cells were incubated with COZ (at a particle/cell ratio of 10:1) or H/S buffer control for 15 min at 37 °C. Incubations were terminated by sedimenting the cells at 3000 \(\times\) g for 1 min at 4 °C, followed by resuspension in H/K buffer and disruption by \(N_2\) cavitation. Membrane fractions from control (M\(\beta\)) or COZ-treated cells (M\(\beta\)-COZ) were extracted with 0.5% octyl glucoside, and the detergent-insoluble fractions were isolated, washed, and resuspended in H/K buffer. The PLD activity of DIF\(\beta\)-COZ was 2.0-fold greater than the octyl glucoside-insoluble fraction from control cells, DIF\(\beta\), (range 1.9–2.2, \(p < 0.01\), Fig. 2B). Therefore, a physiologic stimulus, COZ, that binds to complement receptors on the plasma membrane resulted in stable association of PLD activity with the DIF, which was qualitatively similar to that produced by GTP\(\gamma\)S in permeabilized cells or in the cell-free system. The level of PLD activity of DIF\(\beta\)-COZ (437 ± 32 H cpm) approximated that of DIF-GTP\(\gamma\)S (derived from permeabilized cells, 632 ± 47 H cpm). Because the cell-free assay provided the greatest level of DIF-associated PLD activity and was most accessible to experimental manipulations, further characterization of the association of PLD activity with the detergent-insoluble fraction was performed with this system.
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M<sub>GT</sub>S were very similar (Table I). After extraction with 0.5% octyl glucoside, approximately 35–40% of the protein content of M<sub>0</sub> and M<sub>GT</sub>S remained in the detergent-insoluble fractions, DIF<sub>0</sub> and DIF<sub>GT</sub>S. In contrast to their similar protein levels, the membrane and octyl glucoside-insoluble fractions contained significantly different levels of phospholipid, measured as nmol of lipid phosphorus via the ashing procedure of Ames (34). The inclusion of GTP<sub>S</sub> in the initial incubation of membrane and cytosol resulted in an 18% increase in lipid phosphorus in M<sub>GT</sub>S compared with M<sub>0</sub> (p < 0.01, n = 3) (Table I). We hypothesize that this GTP<sub>S</sub>-dependent increase in membrane phospholipid content was because of stimulation of de novo synthesis, because the lipid phosphorus content of M<sub>GT</sub>S exceeded that of the freshly isolated membrane fraction, M, although this has not been tested directly. A similar 26% increase in lipid phosphorus occurred in DIF<sub>GT</sub>S compared with DIF<sub>0</sub> (p < 0.01).

It is notable that extraction with 0.5% octyl glucoside resulted in relatively small decreases in phospholipid content in the DIFs compared with the membrane fractions from which they were derived, suggesting that this detergent concentration resulted in only modest disruption of membrane structure. Utilizing both biochemical and electron microscopic methods, Hartwig et al., (35) demonstrated similar effects of low concentrations of octyl glucoside on the structure of the platelet plasma membrane and submembranous actin cytoskeleton. Concentrations of octyl glucoside >1.0% resulted in extraction of a significantly greater fraction of phospholipid from U937 cells (data not shown).

The specific activity of M<sub>GT</sub>S, 331 ± 20.2 [3H]P<sub>E</sub> cpm/μg of protein/h, was 20-fold greater than that of M<sub>0</sub>, 16.1 ± 1.21 (p < 0.001, n = 3). Because octyl glucoside has a direct inhibitory effect on PLD activity (detailed below), it is not possible to compare the specific activities of the membrane and detergent-insoluble fractions. If one assumes similar concentrations of octyl glucoside remain in DIF<sub>GT</sub>S and DIF<sub>0</sub> after resuspension in detergent-free buffer, the specific activity of DIF<sub>GT</sub>S, 515 ± 47.3 [3H]P<sub>E</sub> cpm/μg of protein/h was 22-fold greater than that of DIF<sub>0</sub>, 23.8 ± 3.40 (p < 0.001, n = 3).

Detergent-dependence of the PLD Activity of DIF<sub>GT</sub>S—We evaluated the effect of incubating M<sub>GT</sub>S in various concentrations of octyl glucoside on the PLD activity of the resultant detergent-insoluble fractions (after resuspension in detergent-free buffer). The level of PLD activity associated with the DIF was inversely proportional to the octyl glucoside concentration used for extraction of the membrane over the range 0.1–1.0% (3.4–34 mM). The 0.1% octyl glucoside-insoluble fraction exhibited a level of PLD activity that was 91% (range 87–95%) that of the total PLD activity of the activated membrane fraction, M<sub>GT</sub>S, whereas extraction with 1% octyl glucoside resulted in retention of 42% (range 36–46%) of the PLD activity in the DIF. It is notable that significant levels of PLD activity were associated with the detergent-insoluble fraction when the concentration of octyl glucoside was either below or above the critical micellar concentration of 20–25 mM (0.59–0.74% octyl glucoside).

To evaluate whether the significant level of PLD activity associated with the octyl glucoside-insoluble fraction was due, in part, to a direct enhancement of PLD activity by the detergent itself, we determined the effect of adding various concentrations of octyl glucoside to the PLD assay. Octyl glucoside produced dose-dependent inhibition of the PLD activity of M<sub>GT</sub>S, ranging from a 13 ± 3% reduction at 0.02% octyl glucoside to a 92 ± 2% decrease in PLD activity at a detergent concentration of 2.0%. These results are in agreement with previous reports of detergent-induced inhibition of GTP<sub>S</sub>-dependent PLD activity (1–3). Although we cannot determine the exact concentration of residual octyl glucoside, which remains associated with DIF<sub>GT</sub>S, its direct inhibitory effect on PLD activity strongly suggests that the significant levels of DIF-associated PLD activity presented in Figs. 1 and 2 do not represent artifactual detergent-mediated enhancement of lipase activity.

To determine whether the GTP<sub>S</sub>-dependent association of PLD activity with the DIF was specific to the use of octyl glucoside, similar analyses were conducted with sodium cholate (critical micellar concentration 9–15 mM) and Triton X-100 (critical micellar concentration 0.2–0.3 mM). Extraction
of M<sub>GTP<sub>S</sub></sub> with 1% sodium cholate (23 mM) under conditions identical to those employed with octyl glucoside resulted in a 15.2-fold increase in PLD activity in the cholate-insoluble fraction compared with that obtained from extraction of control membranes (M<sub>o</sub>) when both were assayed in the presence of 100 μM GTP<sub>S</sub> (range 14.3–16.1-fold, p < 0.001, n = 3). The PLD activity of the cholate-insoluble fraction represented 31% (range 28–34%) of the total PLD activity of M<sub>GTP<sub>S</sub></sub>. Use of 1% Triton X-100 (15 mM) also resulted in elevated PLD activity in the DIF derived from M<sub>GTP<sub>S</sub></sub> (3.2-fold increase over control, range 1.4–6.4, p < 0.01, n = 4). However, only 2.2% of the original PLD activity of M<sub>GTP<sub>S</sub></sub> was retained in the Triton X-100-insoluble fraction. This low level of Triton X-100-insoluble PLD activity was likely because of the significant inhibitory effect of this detergent on PLD activity. In fact, of the detergents tested, Triton X-100 exerted the greatest inhibitory effect on M<sub>GTP<sub>S</sub></sub> when added directly to the PLD assay (data not shown). Because the actual amount of each detergent remaining in the DIF is unknown, it is not possible to directly compare the PLD activities of the various DIFs resulting from extraction with octyl glucoside, cholate, or Triton X-100. However, these results clearly demonstrate that the GTP<sub>S</sub>-dependent association of PLD activity with the detergent-insoluble fraction is a general property exhibited by detergents with significantly different chemical structures (both nonionic and ionic) and critical micellar concentrations.

**Guanine Nucleotide-dependence of Detergent-insoluble PLD Activity**—Membrane and cytosolic fractions were incubated in the presence of various concentrations of GTP<sub>S</sub> (1.0–100 μM), followed by re-isolation of membranes, extraction with 0.5% octyl glucoside, and determination of PLD activity in the presence of 100 μM GTP<sub>S</sub>. The PLD activities of both the re-isolated, washed membrane (M<sub>GTP<sub>S</sub></sub>) and octyl glucoside-insoluble (DIF<sub>GTP<sub>S</sub></sub>) fractions were directly proportional to the concentration of GTP<sub>S</sub>, which was present in the initial incubation with cytosol (Fig. 3A). The PLD activity associated with the DIF represented 28%, 37%, and 54% of the total PLD activity of the membrane fraction, resulting from incubations with cytosol in the presence of 1, 10, and 100 μM GTP<sub>S</sub>, respectively.

Further evidence for the GTP-binding protein dependence of the association of PLD activity with the DIF was obtained with the use of another nonhydrolyzable analogue of GTP, Gpp(NH)p. Substitution of Gpp(NH)p for GTP<sub>S</sub> in the initial incubation of membrane and cytosol also resulted in increased PLD activity in the membrane (M<sub>Gpp(NH)p</sub>) and detergent-insoluble (DIF<sub>Gpp(NH)p</sub>) fractions, compared with M<sub>GTP<sub>S</sub></sub> and DIF<sub>GTP<sub>S</sub></sub> (Fig. 3B).

Gpp(NH)p was less efficacious than GTP<sub>S</sub> in promoting stable association of PLD activity with the membrane and DIF, which parallels the decreased efficacy of Gpp(NH)p, relative to GTP<sub>S</sub>, in direct stimulation of PLD activity in permeabilized or cell-free preparations from phagocytic leukocytes (27, 28, 36).

The GDP analogue GDPβS, which cannot be phosphorylated to the triphosphate form, inhibits GTP-binding protein-mediated responses. Inclusion of a 10-fold molar excess of GDPβS in the initial incubation of membrane and cytosol resulted in significant inhibition of GTP<sub>S</sub>-dependent, DIF-associated PLD activity (56% reduction, range 51–62%, p < 0.001, Fig. 3B). The incomplete inhibition of GTP<sub>S</sub>-stimulated PLD activity by GDPβS is consistent with the known biochemical properties of these guanine nucleotide analogues. GDPβS inhibits the stimulation of GTP-binding proteins via competition with guanine nucleoside triphosphates for binding to the nucleotide-free form of the GTPase. Although GDPβS cannot be phosphorylated to its corresponding triphosphate, it can be displaced from the GTP-binding protein by GTP or GTP<sub>S</sub>. In contrast, because release of guanine nucleoside triphosphates requires their hydrolysis, nonhydrolyzable analogues such as GTP<sub>S</sub> are more stably bound to the GTPase than GDPβS. Therefore, GDPβS-induced responses are only partially inhibited by GDPβS (27, 28, 36). In summary, the studies with guanine nucleotide analogues strongly support the hypothesis that activation of a GTP-binding protein(s) mediates stable association of PLD activity with the detergent-insoluble fraction derived from the plasma membrane.

**Effects of GTP<sub>S</sub> on the Association of RhoA and ARF with the DIF**—To test the hypothesis that GTP<sub>S</sub>-dependent association of PLD activity with the DIF is accompanied by the stable co-localization of RhoA and/or ARF, the levels of these GTPases in membranes and DIFs were determined by Western blotting. In subcellular fractions from resting U937 cells (control), RhoA and ARF are present in both cytosol and membranes, with the majority of each located in cytosol (Fig. 4A). A significant fraction of RhoA and, to a lesser extent, ARF, were extracted from control membranes by 0.5% octyl glucoside (compare M and DIF<sub>GTP<sub>S</sub></sub> in Fig. 4A). GTP<sub>S</sub>-induced concentration-dependent increases in the levels of RhoA and ARF in membrane (M<sub>GTP<sub>S</sub></sub>) and octyl glucoside-insoluble (DIF<sub>GTP<sub>S</sub></sub>) fractions. Previous work has demonstrated that guanine nucleotides induce translocation of Rho and ARF GTPases from the cytosol to membranes (37, 38). Our results confirm these findings and extend them by demonstrating that GTP<sub>S</sub>-dependent membrane translocation is accompanied by increased association of RhoA and ARF with the DIF derived from these membranes. Inclusion of GDPβS in the initial incubation of membrane and cytosol resulted in inhibition of the GTP<sub>S</sub>-induced association of RhoA and ARF with the membrane and DIF (Fig. 4B). Of note, SDS-PAGE of the GDPβS-containing samples demonstrated two species of RhoA, one of which migrated at the standard position for this GTPase, and a second, which exhibited slightly decreased mobility. At present, we have no information on the identity of, or the mechanism responsible for, this slower-migrating form of RhoA.

To directly evaluate the hypothesis that RhoA functions in the GTP<sub>S</sub>-dependent association of PLD activity with the DIF, we utilized C. botulinum C3 exotoxin, which catalyzes the ADP-ribosylation and inactivation of RhoA, RhoB, and RhoC, but not Rac or Cdc42 (32, 39). Membrane and cytosolic fractions from resting U937 cells were incubated with 1 μg/ml C3 exotoxin prior to the addition of GTP<sub>S</sub> and the subsequent prep-
aration of MGTP and DIFGTP. Treatment with C3 exotoxin decreased the levels of RhoA associated with MGTP and DIFGTP without significantly affecting the amount of ARF in these fractions (Fig. 5A), confirming its specific inactivation of Rho GTPases. C3 exotoxin also inhibited the level of PLD activity associated with MGTP (57% inhibition, range 52–64%, \( p < 0.001 \)) and DIFGTP (62% inhibition, range 55–66%, \( p < 0.001 \), Fig. 5B). These results are consistent with a role for Rho GTPases in regulating the association of PLD with the membrane fraction and the DIF derived from it.

**Effects of GTPγS on the Membrane and Cytoskeletal Localization of PLD**—To determine whether GTPγS affected the level of PLD protein associated with the plasma membrane and DIF, we utilized polyclonal antibodies to human PLD1 and PLD2. Extracts from Sf9 cells infected with baculovirus constructs expressing human PLD1 or PLD2 served as positive controls for the Western blot experiments. The anti-PLD2 Ab detected a single species at approximately 125 kDa in the appropriate positive control (Fig. 6A, 9th lane); however no PLD2 was detected in any U937 cell fraction (data not shown). The anti-PLD1 Western blot of the Sf9 cell positive control (Fig. 6A, 3rd lane) demonstrated a major immunoreactive species at 93 kDa. Two proteins, of approximately 95 and 93 kDa, were detected in both membrane (M, Fig. 6A, 8th lane) and cytosol (C, 1st lane) from U937 cells by the anti-PLD1 Ab. Because of its co-migration with the PLD1 positive control, the 93-kDa protein in U937 cell membrane and cytosol is likely to represent PLD1. The identity of the 95-kDa protein is unknown, but it may represent an isoform of PLD1 and/or stable (covalent) modifications. For simplicity of discussion, both the 93- and 95-kDa immunoreactive proteins will be referred to as PLD1 and further specified by their approximate molecular weight.

Incubation of membrane and cytosolic fractions from U937 cells (in the absence of GTPγS) for 30 min at 37 °C resulted in...
Fig. 4. Effect of GTP S and GDP S on the localization of Rho A and ARF to membrane and DIF. A, plasma membrane (M) and cytosol (C) isolated from U937 cells by nitrogen cavitation and density gradient centrifugation were incubated in the presence of the indicated concentrations of GTP S or buffer to prepare M GTP S (1–100 μM) and M C. Membrane fractions were extracted with 0.5% octyl glucoside and the respective detergent-insoluble fractions (1–100 μM DIF GTP S, DIF C) were isolated by centrifugation. 5 × 10 6 cell equivalents of each sample were subjected to SDS-PAGE on 12.5% gels, followed by transfer to polyvinylidene difluoride membranes and Western blotting with polyclonal α-Rho A or monoclonal α-ARF Abs. The approximate molecular masses for Rho A and ARF were 22 and 20 kDa, respectively, based on the migration of protein standards. B, membrane and cytosol were incubated with the indicated guanine nucleotide analogues (100 μM GTP S, 1 mM GDP S) or buffer for 30 min at 37°C followed by re-isolation and washing of the membrane fractions and preparation of the octyl glucoside-insoluble fractions. Western blotting for Rho A and ARF was performed as indicated in panel A, above. Results are representative of three replicates for each experiment.

a significant decrease in membrane-associated PLD1 (M GTP S, Fig. 6A, 7th lane) and its accumulation in the supernatant and membrane-wash fractions (data not shown). In marked contrast, inclusion of GTP S in the membrane + cytosol incubation resulted in a significant increase in PLD1 in the re-isolated, washed membrane, M GTP S (Fig. 6A, 6th lane). Specifically, GTP S induced a concentration-dependent increase in the level of the 95-kDa PLD1 (but not the 93-kDa species) in M GTP S compared with M C (Fig. 6B).

The GTP S-dependent association of PLD1 with the plasma membrane fraction, M GTP S, was accompanied by similar association of this lipase with the octyl glucoside-insoluble fraction derived from it, DIF GTP S (Figs. 6A, 4th lane; 6B, 6th-8th lanes). The 95-kDa species was the major anti-PLD1-immunoreactive protein present in DIF GTP S, and its accumulation was dependent on the concentration of GTP S over the 1.0–100 μM range. The level of the 93-kDa PLD1 in DIF GTP S did not exhibit a consistent relation to the concentration of GTP S. DIF C contained very little 95-kDa PLD1 and a variable amount of the 93-kDa species.

PLD1 Is Stably Associated with the F-actin-containing Fraction Derived from M GTP S—The results of the previous studies suggest that stimulation by GTP S induces the stable association of PLD1, Rho A, and/or ARF into an active lipase complex that is localized to the membrane surface via interactions with the F-actin-based membrane skeleton. To test this hypothesis, M GTP S was subjected to a broad range of extraction conditions that varied with respect to detergent concentration, ionic strength, and pH, followed by separation of soluble and pellet fractions by centrifugation at 200,000 × g and Western blotting for PLD1, Rho A, ARF, and actin. The association of PLD1, Rho A, ARF, and actin with the pelleted containing sedimentable actin (F-actin) was stable to incubation with NaCl over the concentration range of 125–400 mM (Fig. 7A). At 1.0 M NaCl, the majority of PLD1 was extracted from M GTP S and localized to the 10% sucrose layer (data not shown), whereas Rho A and ARF remained predominantly in the pellet. 0.1 M Na 2 CO 3, which extracts many peripheral membrane proteins (40), completely solubilized PLD1, as did 1.0 M NaOH. Extraction of M GTP S with octyl glucoside (0.5–1.0%) or Triton X-100 (0.2–1.0%) resulted in retention of the majority of PLD1 in the F-actin-containing pellet (Fig. 7B). At an octyl glucoside concentration of 0.5%, most of the Rho A and ARF were similarly localized to the pellet fraction, whereas 1.0% octyl glucoside and 0.2–1.0% Triton X-100 resulted in solubilization of the majority of these GTPases. These results demonstrate that GTP S induces the association of PLD1 with the F-actin-con-
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Fig. 6. Antibody to PLD1 detects two major species in membrane, cytosol, and detergent-insoluble fractions from U937 cells. 5 × 10⁶ cell equivalents of membrane, cytosol, and cytoskeletal fractions were analyzed by 9% SDS-PAGE, followed by Western blotting and detection by horseradish peroxidase-conjugated ₪ Ab/ECL. A, the 1st-8th lanes were probed with polyclonal Ab to PLD1. The 3rd lane is the positive PLD1 control from baculovirus-infected Sf9 cells. The 9th lane contains the PLD2 protein standard and is from a separate blot probed with polyclonal Ab to PLD2. The designation of the remaining lanes follows the nomenclature in the text. B, the concentration-dependence of GTPγS-induced association of PLD1 with membrane and DIFs was determined by Western blotting 5 × 10⁶ cell equivalents of each fraction with anti-PLD1 Ab. Data in panels A and B are from two representative experiments utilizing separate preparations of membrane and cytosol from a total of three replicates for each set of conditions.

 Activation of PLD, like that of other phospholipases, must involve the stable association of the enzyme as well as required regulatory proteins and co-factors with the membrane surface (1, 42). The molecular interactions responsible for stabilization of an active PLD complex at the lipid bilayer are not fully defined. The objective of this study was to investigate the hypothesis that activation of PLD by GTP-binding proteins in a plasma membrane-enriched fraction from U937 promonocytes involves an association of the lipase with the detergent-insoluble membrane skeleton. Extraction of membranes with octyl glucoside resulted in significant retention of PLD activity in the DIF, which also contained F-actin. This DIF-associated PLD activity accounted for approximately 45–90% of the total PLD activity of the membrane fraction, dependent on the concentration of octyl glucoside used for extraction. Similar DIF-associated PLD activities resulted from detergent extraction of membranes with cholate or Triton X-100. The physiologic relevance of this observation was demonstrated by similar enhancement of PLD activity in the membrane-derived DIF after stimulation of intact U937 cells with COZ. To our knowledge, this is the first demonstration of an association between PLD activity and the detergent-insoluble membrane skeleton.

The presence, in the DIF, of several cytoskeletal proteins, including vinculin, talin, paxillin, and α-actinin, and the co-sedimentation of PLD1 with F-actin under widely varying extraction conditions indicates that the DIF has many characteristics of the actin microfilament cytoskeleton. The plasma membrane-enriched fraction prepared by sucrose density gradient centrifugation also contains Golgi-derived membranes (43), as evidenced by the presence of the Golgi marker, β-COP (data not shown). Because the Golgi complex is associated with its own distinct actin cytoskeletal network (44, 45), we cannot, at present, distinguish the relative contributions of the plasma membrane and Golgi complex to the PLD activity studied in this report. The term “membrane skeleton” has been used to indicate the membrane origin (plasma membrane or intracellular membrane-bound organelles) of the detergent-insoluble fraction and to distinguish it from the cytosolic actin-based cytoskeleton (46–48). In the case of stimulation of intact cells by the phagocytic particle, COZ (12), it seems reasonable to hypothesize that a significant fraction of the membrane- and DIF-associated PLD activity is derived from the plasma membrane, although this has not been formally demonstrated.

Our attempts to further characterize the association of PLD1 with F-actin by immunofluorescence or immunoprecipitation have been limited by the specificity and sensitivity of the currently available Abs to PLD1 (data not shown). To our knowledge, the only data on the subcellular localization of PLD derived from immunofluorescence was based on expression of epitope-tagged PLD1 or PLD2 in fibroblasts (6). The location of endogenous PLD1 and PLD2 in fibroblasts and other cell types requires further study. Exton and co-workers (49) and Cockcroft and co-workers (43, 50) characterize the subcellular distribution of multiple ARF and RhoA-regulated PLD activities (which are likely to represent PLD1) and present evidence consistent with agonist-dependent translocation of PLD between these compartments (43).

A regulated association of PLD activity with the actin-based membrane skeleton would be relevant to our understanding of the many physiologic processes in which stimulation of PLD and cytoskeletal rearrangements have been linked, including mitosis, meiosis, secretion, and motility (1, 7–9). In the case of myeloid leukocytes, the primary antimicrobial responses of phagocytosis, generation of reactive oxidants, and granule secretion involve the actin cytoskeleton, and each has been strongly associated with stimulation of PLD (4, 10–14). Cytoskeletal-localization of PLD activity may result in spatially and temporally restricted generation of the lipid second messengers PA and diglycerides, which may be important in regulation of these highly focal leukocyte responses. The membrane skeleton may serve as a focal point or scaffold for the coordinate assembly of a multiprotein complex containing regulatory (RhoA, ARF, protein kinase C) and catalytic (PLD) components (51, 52). Furthermore, because PA and diglycer-
The mechanisms that regulate stable association of PLD activity with the DIF are incompletely defined. Incubation of membrane and cytosol with GTPγS induced significant increases in the amounts of RhoA and ARF present in the detergent-insoluble fraction. Although regulation of the actin cytoskeleton by Rho GTPases has previously been demonstrated (58, 59), we believe that this is the first description of guanine nucleotide-dependent association of ARF with the detergent-insoluble, F-actin-containing fraction. Inhibition of DIF-associated PLD activity by C. botulinum C3 exotoxin suggests that Rho family GTPases function in this process. Because C3-treated samples retain approximately 50% of the PLD activity of untreated controls, both Rho-dependent and Rho-independent pathways may mediate stable association of PLD activity with the membrane skeleton. Rac, CDC42, ARF, or incompletely inactivated Rho GTPases may be responsible for stimulation of the residual membrane- and DIF-associated PLD activity in C3-treated samples. Experiments to distinguish between these possibilities are currently in progress.

The GTPγS dependence of the DIF-associated PLD activity and the results of Western blotting with PLD-specific Abs suggest that PLD1 is responsible, at least in part, for the PLD activity of the DIF. Furthermore, co-migration with the recombinant standard identifies the 93-kDa immunoreactive protein and signal transduction from cell surface receptors (53, 55, 56). We (data not shown) and others (53, 57) have been unable to detect caveolin in mononuclear phagocytes by Western blot. However, the demonstration that 0.5% octyl glucoside results in solubilization of only a minority of plasma membrane phospholipid (Table I) would be consistent with the presence of detergent-insoluble, glycolipid-enriched (DIG (detergent-insoluble glycolipid-enriched)) domains in U937 cell plasma membranes. The potential relations between the DIFs studied in this report and other detergent-insoluble preparations, including caveolae, will require further study.
as PLD1. Although overexpressed, PLD2 localized to the plasma membrane in transfected fibroblasts (6), the GTPS dependence and lack of immunoreactivity with the α-PLD2 Ab indicates that PLD2 is unlikely to account for the membrane and DIF-associated PLD activities in this report. The approximate molecular weight of GTPS-stimulated PLD activities from mammalian cells, including PLD1a and PLD1b, have ranged from 95 to 120 kDa (51, 52, 60). Morris and co-workers (52) note the propensity of both PLD1 isoforms to aggregate during SDS-PAGE (52), which may contribute to this variability in migration. The identity of the α-PLD1-immunoreactive 95-kDa protein in the membrane and DIF of U937 cells is currently unknown. PLD1a and PLD1b could account for the 95- and 93-kDa proteins (because the polyclonal Ab utilized in this study recognizes a shared peptide sequence). However, this probably would not account for the GTPS-dependent increase in the level of the 95-kDa immunoreactive protein in MCTPS and DIFGTPS (compared with M0 and DIF0), because the alternative splicing responsible for the generation of PLD1a and PLD1b would not be expected to occur in the cell-free system. An alternative explanation for the results of the anti-PLD1 Western blot is the presence of a single PLD protein, which exhibits altered mobility because of covalent modification (60). We cannot exclude the formal possibility that the 95-kDa protein is a previously uncharacterized PLD, distinct from PLD1, that is co-reactive with the α-PLD1 Ab. Approximately 15% of the membrane PLD activity was recovered in the 0.5% octyl glucoside-soluble supernatant. This likely represents an underestimate because of the inhibition of PLD activity by this concentration of octyl glucoside and because PLD activity was assayed in the absence of added cofactors such as low molecular weight GTPases or protein kinase C. Sternweis and co-workers extensively characterize a GTPase- and protein kinase C stimulated PLD activity in detergent extracts from membranes of HL-60 cells (31) or porcine brain (51, 61), which was assayed in the presence of 0.05% octyl glucoside. The relation between the PLD activity extracted from plasma membranes of U937 cells and that reported by Sternweis and co-workers will require further analysis.

In summary, this report demonstrates that stimulation of GTP-binding proteins or plasma membrane receptors of U937 promonocytes results in stable association of PLD activity with the detergent-insoluble fraction, which has many characteristics of the actin-based membrane skeleton. We propose that cytoskeletal localization of PLD activity may promote focal regulation of the actin microfilament system and function in the control of physiologic processes, which require both stimulation of PLD and cytoskeletal rearrangements.

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