Characterization of Two Alternately Spliced Forms of Phospholipase D1

ACTIVATION OF THE PURIFIED ENZYMES BY PHOSPHATIDYLSERINE 4,5-BISPHOSPHATE, ADP-RIBOSYLATION FACTOR, AND RHO FAMILY MONOMERIC GTP-BINDING PROTEINS AND PROTEIN KINASE C-α

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We previously reported the cloning of a cDNA encoding human phosphatidylcholine-specific phospholipase D1 (PLD1), an ADP-ribosylation factor (ARF)-activated phosphatidylcholine-specific phospholipase D (Hammond, S. M., Tsung, S., Autscheller, Y., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29645). We have now identified an evolutionarily conserved shorter splice variant of PLD1 lacking 38 amino acids (residues 585–624) that arises from regulated splicing of an alternate exon. Both forms of PLD1 (PLD1a and 1b) have been expressed in SF9 cells using baculovirus vectors and purified to homogeneity by detergent extraction and immunoaffinity chromatography. PLD1a and 1b have very similar properties. PLD1a and 1b activity is Mg2+-dependent but insensitive to changes in free Ca2+ concentration. Phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate activate PLD1a and 1b but a range of other acidic phospholipids are ineffective. PLD1a and 1b are highly responsive to activation by GTP-γ-S-ligated ADP-ribosylation factor-1 (ARF-1) and can also be activated to a lesser extent by three purified RHO family monomeric GTP-binding proteins, RHO A, RAC-1, and CDC42. Activation of PLD1a and 1b by the RHO family monomeric GTP-binding proteins is GTP-dependent and synergistic with ARF-1. Purified protein kinase C-α activates PLD1a and 1b in a manner that is stimulated by phorbol esters and does not require ATP. Activation of PLD1a and 1b by protein kinase C-α is synergistic with ARF and with the RHO family monomeric GTP-binding proteins, suggesting that these three classes of regulators interact with different sites on the enzyme.

Phosphatidylcholine (PC)1-specific phospholipase D enzymes (PLD) are emerging as key components of pathways of cell regulation leading to transduction of extracellular signals, regulation of intracellular protein trafficking and secretion, and control of meiosis in budding yeast (see Refs. 1, 2 for review). The common reaction catalyzed by these enzymes is hydrolysis of PC to form phosphatic acid (PA) and choline. PA has a number of biological activities including direct regulation of a range of cell-specific target proteins in vitro (3, 4). Dephosphorylation of PA by phosphatidate phosphohydrolase produces diacylglycerol that activates members of the Ca2+- and phospholipid-dependent protein kinase C family (5). Other PA-derived molecules with regulatory properties include lyso-PA and arachidonic acid metabolites, both of which act on specific cell-surface receptors (6, 7).

PLD activities are present in prokaryotic and eukaryotic organisms including many mammalian tissues and cell lines. cDNA sequences of several bacterial and plant PLDs have been determined (8, 9). Mutation or deletion of the Saccharomyces cerevisiae spo14 gene produces defects in meiosis, and the spa14 gene product has recently been identified as a phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-dependent PC-specific PLD (10–12). We used sequence shared between this protein and the plant enzymes to clone a human cDNA encoding a PC-specific PLD which we termed PLD1. Heterologous expression of this cDNA generated a PI(4,5)P2-dependent PLD activity that was stimulated by GTP-y-S-activated ARF-1 (13). The plant, yeast, and human PLDs share four regions of homology. Two of these are present in bacterial PLDs from Streptomyces species and presumably contain regions essential for catalysis. Apart from these regions, the sequences of the proteins diverge widely. Although the PLD enzymes catalyze the same reactions, these differences in primary structure suggest that they are regulated in different ways and have different functions (2, 9).

Regulation of PLD activities in mammalian systems is com-

The abbreviations used are: PC, phosphatidylcholine; PLD, phosphatidylcholine-specific phospholipase D; PA, phosphatic acid; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; ARF, ADP-ribosylation factor; PKC, protein kinase C; P(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PI, phosphatidylinositol; β-Dog, β-d-octylglucoside; FMA, phorbol myristate acetate; PBS, phosphate-buffered saline; GTP-γ-S, guanosine 5′-3′-O-(thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; PE, phosphatidylethanolamine; PA, phosphatic acid; CL, cardiolipin; PS, phosphatidylserine; PG, phosphatidylglycerol.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U38545.

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plex. In intact cells, a range of agonists acting through G-protein-coupled receptors and receptor tyrosine kinases stimulate PLD-catalyzed hydrolysis of PC. In many systems, receptor-mediated activation of PLD appears to be dependent on a prior activation of inositol lipid-specific phospholipase C (PLC) and mediated by a protein kinase C (PKC)-dependent process (14–16). In vitro studies suggest that PKC regulates PLD by two different mechanisms. Addition of a purified PKC preparation to membranes from rat liver produced a modest PLD activation that was independent of ATP; in addition, a partially purified PLD preparation from porcine brain was strongly activated by PKC-α (and its isolated lipid-binding regulatory domain) in an ATP-independent manner (17, 18). PKC-α and, to a lesser extent, PKC-β also activate an HL-60 membrane PLD activity in an ATP-independent manner (19).

In contrast, similar studies using human neutrophil membranes and a number of purified PKC isoforms indicate that PKC-catalyzed phosphorylation of a membrane component is required for PLD activation (20).

Other work has identified roles for monomeric GTP-binding proteins in PLD regulation. Brain extracts appear to contain two PLD activities that can be separated by column chromatography and further distinguished by their differential dependences on PI(4,5)P₂ and fatty acids for activity (21, 22). PI(4,5)P₂-dependent PLD activities in brain and HL-60 monocytic cells are stimulated by members of the ADP-riboseylation factor (ARF) family of monomeric GTP-binding proteins and several RHO family monomeric GTP-binding proteins including RHO A, RAC-1, and CDC42 (23–28). The ARF proteins are key regulators of protein trafficking that control the assembly of the coatomer complex on the surface of Golgi membranes (29). ARF-activated PLD is enriched in Golgi membranes, and PLD may play a role in ARF-dependent coatomer assembly (30). The RHO proteins control both changes in organization of the actin cytoskeleton and activation of protein kinase cascades leading to gene transcription (31). The role of PLD in these processes is untested. Studies using bacterial toxins implicated the RHO proteins in receptor regulation of PLD in some systems (32, 33). Increases in PLD activity have also been reported in cells transformed by members of the ras oncogene family and by the src nonreceptor tyrosine kinase (34, 35).

The complex regulation of PLD activities reported in intact cells and the variety of lipid and protein factors capable of PLD activation in vitro has led to the proposal that distinct mammalian PLDs exist (21–28). Purification of a 180-kDa oleate-dependent PLD from porcine lung has been described (21), but other work has identified roles for monomeric GTP-binding proteins as key regulators of protein trafficking that control the assembly of the coatomer complex on the surface of Golgi membranes (29). ARF-activated PLD is enriched in Golgi membranes, and PLD may play a role in ARF-dependent coatomer assembly (30). The RHO proteins control both changes in organization of the actin cytoskeleton and activation of protein kinase cascades leading to gene transcription (31). The role of PLD in these processes is untested. Studies using bacterial toxins implicated the RHO proteins in receptor regulation of PLD in some systems (32, 33). Increases in PLD activity have also been reported in cells transformed by members of the ras oncogene family and by the src nonreceptor tyrosine kinase (34, 35).

The complex regulation of PLD activities reported in intact cells and the variety of lipid and protein factors capable of PLD activation in vitro has led to the proposal that distinct mammalian PLDs exist (21–28). Purification of a 180-kDa oleate-activated PLD from porcine lung has been described (21), but no mammalian monomeric GTP-binding protein-regulated PLD has been purified to homogeneity. During studies of the regulation of PLD1 mRNA levels in HL-60 cells, we identified a second form of PLD1 that arises by alternate splicing of a 38-amino acid exon. We designate the "long" and "short" forms of PLD1 as PLD1α and 1b, respectively. The present study was undertaken to investigate the catalytic and regulatory properties of PLD1α and 1b. Both proteins have been expressed in insect cells using baculovirus vectors and purified to homogeneity. Our results demonstrate that PLD1α and 1b can be activated by PI(3,4,5)P₃, PI(4,5)P₂, ARF-1, RHO A, RAC-1, CDC42, and protein kinase C-α. The simplest explanation of our findings is that this diverse range of regulators exert their effects by direct interaction with the PLD1α catalyst.

MATERIALS AND METHODS

General Reagents—Unless otherwise stated, reagents were from previously noted sources (13). Unlabeled phospholipids were obtained from Avanti Polar Lipids. PI(4,5)P₂ was purified from a lipid extract of bovine brain as described (13). Di-C₁₆ PtdIns(3,4,5)P₃ was synthesized as described (36).

RNA Extraction and Reverse Transcriptase-PCR Analysis of PLD1

Human, Rat, and Mouse PLD1 Sequences—Total RNA was isolated from HL-60 cells by the acid guandine thiocyanate method and reverse transcribed using random hexamer mixed primers. Primers used for amplification of hPLD1 were synthesized as follows: primer A (forward), nucleotides 1475–1491, 5′-TGGGCTCACCATGAGAA; primer B (reverse), nucleotides 2133–2113, 5′-GTCATGGCCAGGGTCATCGGG. Amplification conditions used were 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min at 25–27 cycles. The rat PLD1 sequences were obtained by PCR amplification from a reverse-transcribed rat brain RNA template. The mouse PLD1 sequences were obtained from cDNA clones isolated from a mouse embryo cDNA library. The genomic sequence of PLD1 was obtained from cDNA sequences corresponding to a partially processed mRNA (accession number NR77576).

Preparation of Monomeric GTP-binding Proteins—Some of our experiments also employed human ARF-1 that was bacterially expressed and purified as described (37) with a final step of hydroxylapatite chromatography (Bio-Rad, Bio-Gel HTP). Human RHO A, RAC-1, and CDC42 were modified to contain the sequence MEEEEYMPME at the carboxyl terminus, and these proteins were expressed in Sf9 cells using baculovirus vectors and purified by affinity chromatography using an immobilized monoclonal antibody (38). All preparations of monomeric GTP-binding proteins were estimated to be greater than 90% pure by SDS-PAGE. The purified monomeric GTP-binding proteins were solubilized in buffer containing 25 mM Tris, pH 7.5, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine (Buffer A). PKC-α was purified from the supernatant obtained after ultracentrifugation of this extract by sequential chromatography on Source 15 Q (Pharmacia Biotech Inc.), threonine-Sepharose 4BCL (prepared as described in Ref. 38), and phenyl-Superose (Pharmacia). PKC-α was followed during this procedure by measurements of calciuim and phospholipid-dependent protein kinase activity using casein as substrate. The final PKC-α preparation was homogeneous as determined by SDS-PAGE and silver staining. The purified PKC-α was concentrated to approximately 0.1 mg/ml using an Amicon pressure concentrator with a PM-10 membrane and stored in aliquots at −80 °C.

Preparation of Affinity Purified PLD1 Anti-peptide Antibodies—Peptides corresponding to residues 1–15 and 525–541 of the sequence of human PLD1 were prepared, conjugated to keyhole limpet hemocyanin, suspended in saline, and emulsified with Freund’s adjuvant. Two rabbits were immunized with the peptide corresponding to residues 1–15, and two different rabbits were immunized with the peptide corresponding to residues 525–541 by subcutaneous injection. After a second immunization, serum was obtained from the animals, and antibody titers were determined by enzyme-linked immunosorbent assay using the individual peptide antigens as the solid phase. Antibodies were affinity purified from serum obtained from the animals with the highest antibody titers by affinity chromatography using immobilized peptide antigens as the solid phase as described (40). The purified antibodies were adjusted to approximately 1 mg/ml and stored as aliquots in buffer containing 10 mM NaPO₄, 20 mM NaCl, and 0.1 mM NaN₃ at −80 °C. These antibodies recognize PLD1α and 1b by Western blotting and can immunoprecipitate their antigens under denaturing and non-denaturing conditions. The peptide antigens were chosen to generate antibodies that can distinguish PLD1α from a structurally related mammalian PLD enzyme, PLD2.

Preparation of Immunoaffinity Resin—1 mg of a mixture of the two affinity purified antibodies was adsorbed to 0.6 mg of protein A coupled to Sepharose CL4B (Sigma) in phosphate-buffered saline (PBS) for 1 h

2 W. C. Colley and M. A. Frohman, unpublished observations.

3 W. C. Colley, T.-C. Sung, R. Roll, J. Jenco, S. M. Hammond, Y. Altschuller, D. Bar-Sagi, A. J. Morris, and M. A. Frohman, submitted for publication.
at room temperature. The resin was washed with 0.2 M NaOH borate, pH 9.0, and the antibodies were covalently linked to the immobilized protein A by reaction with 20 mM dimethylpimelimidate in 0.2 M NaOH borate, pH 9.0, for 30 min at room temperature with constant agitation. The reaction was quenched by washing the resin in 0.2 M ethanolamine, pH 8.0, and the resin was washed with 100 mM glycine, pH 3.0, to remove antibodies that had not been covalently attached. The resin was then washed extensively and stored in PBS containing 0.1% NaN3.

**Baculovirus Expression and Purification of PLD1—**Recombinant baculoviruses for expression of PLD1a and 1b were generated, selected, purified, and propagated using methods described in Ref. 41. In brief these baculoviruses (PLD1a and 1b) were inserted into the multiple cloning sites of the pAcHLT and PVL392 transfer vectors, respectively (Pharmingen and Invitrogen Inc.). Monolayers of SF9 cells were transfected with mixtures of the PLD1a and 1b transfer vectors and linearized wild-type baculovirus DNA. Recombinant baculoviruses were plaque-purified from media removed from these transfected cells. The pure viruses were amplified by infection of suspension cultures of SF9 cells, and the high titer virus stocks were stored at 4°C. All of our studies used SF9 cells grown at 27 °C in complete Grace’s medium supplemented with lactalbumin, yeastolate, and 10% fetal bovine serum containing antibiotic-antimycotic agents.

For expression of PLD1a and 1b, monolayers of exponentially growing SF9 cells (3 × 10^6 cells/225-cm^2 flask, generally two flasks of cells were used for each purification) were infected with recombinant baculoviruses at a multiplicity of 10 for 1 h with gentle rocking. The virus-containing medium was replaced and replaced with fresh supplemented Grace’s medium. The infected cells were grown for 48 h; the medium was removed, and the cells were washed once with ice-cold phosphate-buffered saline. The cells were lysed on ice by addition of 5 ml/225-cm^2 flask of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 1 mM EGTA, 0.1 mM benzamidone, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin. After 30 min on ice, the cells were scraped up, and the suspension was centrifuged at 50,000 × g for 30 min at 4 °C. The supernatant obtained (10 ml) was mixed with 0.5 ml of the immunofafinity resin and kept at 4 °C with constant agitation for 1 h. The resin was then washed by gentle centrifugation, and the supernatant containing unbound protein was removed. The resin was washed three times with 25 volumes of lysis buffer. After the final wash, the resin was resuspended in 5 ml of lysis buffer and placed in a 10-ml Bio-Rad disposable chromatography column. The resin was washed with 10 ml of 10 mM phosphate buffer, pH 6.8, containing 1% β-octyl-glucoside (β-DOG). Bound protein was eluted with 100 mM glycine buffer, pH 3.0, containing 1% β-DOG as 3 × 0.5-ml fractions. The eluant was collected on ice into tubes containing 0.075 ml of 1× phosphate buffer, pH 8.0. ARF-activated PLD activity in these fractions was determined as described below, and the fractions were also analyzed by SDS-PAGE on 7.5% gels and Western blotting with detection by alkaline phosphatase (and other enzymes from tissue sources by other investigators. We therefore developed an immunoaffinity procedure for isolation of the proteins that use immobilized affinity purified anti-peptide antibodies. This procedure is extremely rapid and produces essentially homogeneous preparations of the proteins in reasonable yield. For the experiment shown in Fig. 2, two 225-cm^2 flasks each containing 3 × 10^7 cells were infected with baculoviruses for expression of PLD1a, 1b, or an irrelevant control protein (PLC-β3). Proteins were extracted from PBS-washed monolayers of cells with 1% Nonidet P-40 as described above. Supernatants obtained after ultracentrifugation of these extracts (approximately 45 mg of protein) were adsorbed to 0.5 ml of antibody-coupled Protein A-Sepharose 4B/CL, and the resin was washed as described above. Bound proteins were released with elution buffer at pH 3. Rapid neutralization of the eluant was essential for preservation of enzymatic activity. Fig. 2 shows a silver-stained 7.5% SDS gel of purified PLD1a, PLD1b, and material obtained from a purification using an extract from cells infected with an irrelevant control baculovirus and a Western blot of the same fractions using a mixture of the two anti-peptide antibodies. We obtained approximately 10 μg each of PLD1a and 1b from two 225-cm^2 flasks which corresponded independently (data not shown).

These results could arise from regulated splicing of an optional 38-amino acid exon or from partial processing of an intron which would imply that our original report described a partially processed mRNA (13). Examination of the PLD1 genomic sequence at codon corresponding to amino acid 623 (the 3' junction of the missing sequence) revealed that an acceptable splice donor site (and 11 nucleotide T/C-rich sequence followed by any nucleotide and AG) is present and that the adjacent genomic sequence does not encode the nucleotide sequence found in either of the PCR products detected (Fig. 1B). We therefore conclude that the originally reported sequence of PLD1 includes an alternately spliced exon. Subsequent analysis of cDNAs obtained by screening a HeLa cell cDNA library with a PLD1 nucleotide probe (13) revealed that two clones obtained encoded the “long” form of PLD1 and three the short form (hereafter designated PLD1a and PLD1b, respectively). PCR analysis of reverse-transcribed rat brain mRNA using these primer sets also led to the amplification of fragments corresponding to PLD1a and 1b. Partial length cDNA clones corresponding to PLD1a and PLD1b have also been isolated from a mouse embryo cDNA library using human PLD1-derived probes (Fig. 1C). The position of this alternately spliced region of PLD1 and its relationship to sequences conserved among other PLD enzymes is shown in Fig. 1D.

**Expression and Purification of PLD1a and 1b—**We have expressed and purified PLD1a and PLD1b using baculovirus vectors and investigated their regulation by divalent cations, phospholipids, monomeric GTP-binding proteins, and PKC-α. Baculovirus-mediated expression of both forms of PLD1 is considerably better when monolayers (as opposed to suspension cultures) of insect cells are used. In both cases, large quantities of insoluble proteins accumulate. The active fraction of these recombantly expressed enzymes is predominantly membrane-associated. Despite considerable efforts, we were unable to purify PLD1a and 1b to homogeneity from SF9 cell extracts using conventional chromatographic techniques. The major problems were instability of the enzymes during purification and a pronounced tendency to behave heterogeneously or “smear” during column chromatography presumably due to interactions with other proteins in the extracts and/or nonspecific interactions with the chromatography resins used. We surmise that similar problems coupled with low starting levels of the proteins have hampered attempts to purify these enzymes from tissue sources by other investigators. We therefore developed an immunoaffinity procedure for isolation of the proteins that use immobilized affinity purified anti-peptide antibodies. This procedure is extremely rapid and produces essentially homogeneous preparations of the proteins in reasonable yield. For the experiment shown in Fig. 2, two 225-cm^2 flasks each containing 3 × 10^7 cells were infected with baculoviruses for expression of PLD1a, 1b, or an irrelevant control protein (PLC-β3). Proteins were extracted from PBS-washed monolayers of cells with 1% Nonidet P-40 as described above. Supernatants obtained after ultracentrifugation of these extracts (approximately 45 mg of protein) were adsorbed to 0.5 ml of antibody-coupled Protein A-Sepharose 4B/CL, and the resin was washed as described above. Bound proteins were released with elution buffer at pH 3. Rapid neutralization of the eluant was essential for preservation of enzymatic activity. Fig. 2 shows a silver-stained 7.5% SDS gel of purified PLD1a, PLD1b, and material obtained from a purification using an extract from cells infected with an irrelevant control baculovirus and a Western blot of the same fractions using a mixture of the two anti-peptide antibodies. We obtained approximately 10 μg each of PLD1a and 1b from two 225-cm^2 flasks which corresponded independently (data not shown).
to a 20–30% yield of the ARF-stimulated PLD activity present in the starting S9 cell detergent extract (PLD activity in the detergent extracts was determined after detergent removal by gel filtration chromatography). Identical purifications from S9 cells infected with an irrelevant baculovirus control produced no detectable protein by SDS-PAGE, Western blotting, or activity measurement so presumably the protein(s) responsible for endogenous S9 cell PLD activity are present at extremely low levels compared with the recombinantly expressed human proteins and/or are not bound by the immunoaffinity resin. We have purified PLD1a and 1b over 20 times using two separate preparations of immunoaffinity resin with similar results. As described below, purified PLD1a and 1b are both PC-specific PLD enzymes that catalyze hydrolysis and (in the presence of a primary alcohol) transphosphatidylation reactions (not shown).
Regulation of Phospholipase D1a and 1b

FIG. 3. Dependence of PLD1a and 1b activity on polyphosphoinositides and other acidic phospholipids. A, activity of purified PLD1a (open symbols) and 1b (closed symbols) was determined under standard assay conditions in the presence of 4.7 μM GTPγS-activated ARF-1. The concentration of the indicated phospholipids in the substrate-containing phospholipid vesicles was varied as shown, PI(3,4,5)P3 ( ), PI(4,5)P2 (●, ○), B, purified PLD1a (gray bars) and 1b (black bars) activity was determined in the presence of 4.7 μM GTPγS-activated ARF5-1 under standard assay conditions except that the PI(4,5)P2 component of the substrate-containing phospholipid vesicles was replaced with the indicated phospholipids each at a final concentration of 7.5 μM in the assay. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin. The data shown are means of triplicate determinations. Standard errors were within 15% of the mean. These experiments have been repeated once with independently isolated preparations of PLD1a and 1b with similar results.

investigated the dependence of PLD1a and 1b activity on divalent cations, phospholipids, monomeric GTP-binding proteins, and PKC-α.

Regulation by Polyphosphoinositides—We previously reported that PLD1 activity was dependent on PI(4,5)P2 (13). We have now studied the effects of a range of acidic phospholipids on the activity of purified PLD1a and 1b using our standard assay conditions. Vesicles contained 7% acidic lipid in a background of phosphatidylethanolamine (PE)/phosphatidylserine (PS). Of the lipids tested, only PI(4,5)P2 and PI(3,4,5)P3 stimulated the activity of PLD1a and 1b significantly, and the two forms of PLD1 showed a similar dependence on these lipids (Fig. 3B). Activity of PLD1a and 1b depends on the molar fraction of polyphosphoinositide in the vesicles. Maximal activation was observed with approximately 7% PI(4,5)P2 or PI(3,4,5)P3, and PI(4,5)P2 was a more effective activator (Fig. 3A). At concentrations of up to 100 μM, soluble inositol 1,4,5-trisphosphate or glycerophosphoinositol 4,5-bisphosphate neither activated PLD1a or 1b nor blocked activation by PI(4,5)P2 or PI(3,4,5)P3 (data not shown).

Dependence on Ca2+ and Mg2+—ARF-stimulated PLD1a and 1b activity was determined as the free concentrations of these ions were varied in the assay medium. PLD1a and 1b activity was insensitive to changes in [Ca2+] over a wide range (<10^-8–10^-2 M). By contrast, ARF-stimulated activity of both PLD1a and 1b was dependent on Mg2+ with half-maximal activity observed at approximately 10^-4 M (Fig. 4).

Activation by ARF and RHO Family Monomeric GTP-binding Proteins—Purified PLD1b was incubated with increasing concentrations of purified GTPγS-activated ARF-1, RHO A, RAC-1, and CDC42. Half-maximal activation was observed with approximately 0.2 μM ARF-1. The three RHO family monomeric GTP-binding proteins were somewhat less potent activators of PLD1 with half-maximal effects observed at approximately 1 μM. ARF-1 was the most effective activator producing an approximately 50-fold stimulation of the enzyme. RHO A and RAC-1 stimulated the enzyme approximately 10- and 13-fold, respectively, and CDC42 produced an approximately 5-fold activation. Effects of ARF and RAC-1 were clearly saturable and, although the concentrations of purified RHO A and CDC42 we obtained limited the final concentrations achieved in the assay, at the highest concentrations used, effects of these activators also appeared to be approaching saturation (Fig. 5A). We compared the effects of maximal concentrations of these monomeric GTP-binding proteins on activity of purified PLD1a and 1b. ARF-1, RHO A, RAC-1, and CDC42 activated PLD1a and 1b to similar extents, and in all cases the activation observed was strictly dependent on GTPγS (Fig. 5B).

Activation by Protein Kinase C-α—Regulation of PLD activities by PKC is complex, and phosphorylation-dependent and -independent mechanisms for PKC-dependent PLD activation have been reported. We investigated the effects of purified
PKC-α on purified PLD1b activity and found that this protein could stimulate the enzyme in a concentration-dependent and saturable manner. Half-maximal effects of PKC-α were observed with approximately 10 nM protein, and the maximal effect of this activator (25-fold stimulation) was approximately 50% that observed with a maximally effective concentration of ARF-1. Inclusion of 100 nM phorbol myristate acetate (PMA) in the assay medium increased the potency with which PKC-α-stimulated PLD1b by approximately 10-fold and the maximal effect by approximately 1.5-fold (Fig. 6A).

A maximally effective concentration of PKC-α activated purified PLD1a and 1b to a similar extent. Activation of PLD1a and 1b by PKC-α was ATP-independent irrespective of the inclusion of PMA in the assay medium (Fig. 6B). Inclusion of apryrase in the assays to degrade any ATP that might have contaminated our purified protein preparations had no effect on activation of PLD1a and 1b by PKC-α (not shown). In fact, when 0.1 mM ATP was included in the assays, PKC-stimulated PLD activity was somewhat lower than observed in the absence of ATP, and the effects of PKC on PLD1a and 1b activity appeared to be more strongly dependent on PMA. This inhibitory effect of ATP was concentration-dependent and half-maximal at approximately 10 μM (not shown). Activation of PLD1 by ARF was unaffected by the inclusion of ATP in the assay (data not shown).

Synergistic Effects of Monomeric GTP-binding Proteins and PKC-α on PLD1a and 1b Activity—As described above, the ARF and RHO family monomeric GTP-binding proteins and PKC-α activate PLD1a and 1b independently. We examined interactions between these regulators as activators of purified PLD1. The results obtained are shown in Fig. 7. Consistent with the data shown in Figs. 3 and 4 above, in this experiment maximally effective concentrations of ARF-1 (4.7 μM), RHO A (3.8 μM), RAC-1 (5.3 μM), CDC42 (5.5 μM), and PMA-activated PKC-α (0.043 μM) produced approximately 50-, 13-, 13-, 10- and 30-fold activations of PLD1a and 1b, respectively (Fig. 7A). When this experiment was repeated including 4.7 μM GTP-S-activated ARF-1 in each set of assays, the response to additional ARF-1 was unchanged as expected, but substantial increases in response to the RHO proteins were observed. In the presence of ARF-1, activation was increased to an approximately 140-fold stimulation over basal activity. Similarly, responses to RAC-1 and CDC42 were both increased to approximately 100-fold of basal. Combination of PKC-α with the RHO family monomeric GTP-binding proteins also produced a substantial activation of PLD1. When combined with RHO A, RAC-1, or CDC42, PKC-α-stimulated PLD1 activity was increased approximately 60-, 70-, and 70-fold of basal, respectively, whereas ARF-1 increased the response to PKC-α to an approximately 140-fold stimulation. By contrast, combinations of the three RHO proteins did not result in greater PLD1a or 1b activity than observed with each of the proteins alone. For example, RHO A alone produced a 13-fold activation of PLD1, and PLD activity was not further increased by addition of concentrations of RAC-1 or CDC42 that were sufficient to cause a maximal activation of PLD1a or 1b when added alone. Similar observations were made for combinations of RAC-1 and CDC42. Finally, combination of maximally effective concentrations of ARF-1 and PKC-α with each of the RHO family monomeric GTP-binding proteins produced a dramatic increase in PLD1a and 1b activity. As discussed above combination of ARF-1 and PKC-α stimulated PLD1 activity to a level 145-fold over basal. In the presence of RHO A, RAC-1, and CDC42 this was increased to approximately 280-, 270-, and 250-fold of basal activity (Fig. 7B).

DISCUSSION

The complex regulation of PLD activities in intact cells and cell extracts coupled with a failure to isolate these enzymes from tissue sources has led to considerable speculation about...
FIG. 7. Synergistic activation of PLD1 by ARF and RHO family monomeric GTP-binding proteins and PKC-α. A, PLD1a (gray bars) and 1b (black bars) activity was determined under standard assay conditions. Assays contained 50 μM GTP-S and PLD activators at the following concentrations: ARF-1 (4.7 μM), RHO A (3.8 μM), RAC-1 (5.5 μM), CDC42 (5.5 μM), and PKC-α (0.045 μM). Incubations with PKC-α contained 100 nM PMA. B, the various PLD activators were combined as indicated and their effects on PLD1a (gray bars) and 1b (black bars) activity determined. The data shown are means of triplicate determinations. Standard errors were within 15% of the mean. This experiment has been repeated once with similar results.

the number and nature of PLD enzymes present in mammalian cells. Recent advances in identification of plant and yeast PLD genes provided an important insight into the structure of a new multigene family, and we reported the cloning and expression of the first mammalian PLD enzyme, PLD1. We describe an alternately spliced form of PLD1 in this paper. Our initial description of the properties of PLD1 used Sf9 cell membranes from baculovirus-infected cells as a source of enzyme activity (13). Even though increases in PLD activity in PLD1 virus-infected cells are substantial, the membranes themselves contain endogenous PLD activities, phospholipids, G-proteins, protein kinases, and possibly other factors that may influence activity of the recombinant PLD enzymes. Definitive characterization of PLD1a and 1b therefore required isolation of the protein to homogeneity, and the experiments reported in this manuscript were designed to address this issue.

Purification of PLD1—Recombinant PLD1 was purified from detergent extracts of baculovirus-infected Sf9 cells. Our results demonstrate that, as reported for crude preparations of the enzyme, the isolated PLD1 protein functions as a PC-specific PLD enzyme that catalyzes both the hallmark hydrolysis and trans-phosphatidylation reactions that characterize this class of phospholipases.

Although our purification of PLD1 used detergent extraction and the purified protein appeared more stable when maintained in detergent-containing solution, we believe the enzyme to be a tightly associated extrinsic membrane protein rather than an integral membrane protein. Approximately half of the membrane-associated recombinant PLD1a and 1b activity of Sf9 cells can be extracted with 0.5 M NaCl and PLD1 can be purified under detergent-free conditions using the immunoaffinity procedure. Consistent with these findings, the PLD1 sequence does not contain large stretches of hydrophobic residues indicative of regions involved in membrane insertion. PLD1a and 1b do not contain pleckstrin homology domains or C2 domains, protein motifs known to be involved in protein phospholipid interactions.

Dependence of PLD1a and 1b Activity on PI(4,5)P2 and PI(3,4,5)P3—In assays employing exogenously provided substrates, PLD1a and 1b activity is stimulated by PI(4,5)P2 and PI(3,4,5)P3. This effect is highly selective for these two lipids as a variety of other acidic phospholipids and phosphoinositides with different positional phosphate group substitutions were ineffective. The mechanism by which PI(4,5)P2 activates PLD1a and 1b is unclear. It is possible that the presence of a low molar fraction of PI(4,5)P2 alters the substrate-containing phospholipid surface in a manner that renders the PC substrate more readily hydroyzed by the enzyme. Not all PLD activities are stimulated by PI(4,5)P2 (21, 22), and the high degree of phospholipid headgroup selectivity coupled with our observation that PI(3,4,5)P3 activates the purified PLD1 isoenzymes suggest that activation involves a direct interaction between PLD1a and 1b and PI(3,4,5)P3. Phosphatidylinositol-specific phospholipase C-δ1 (PLC-δ1) is activated by PI(4,5)P2, which binds to an NH2-terminal noncatalytic site (a pleckstrin homology domain) anchoring the enzymes to the membrane and allowing them to function in a scooting mode of catalysis (42). Although inspection of the primary sequence of PLD1a and 1b, or of the S. cerevisiae SPO14 protein which is also a PI(4,5)P2-dependent PLD, does not reveal homologies to other proteins known to interact with inositol lipids and phosphates, it is possible that an analogous mechanism underlies the PI(3,4,5)P3-stimulated increase in catalytic activity. At present, the physiological role of PI(3,4,5)P3 in PLD1a and 1b regulation is not known. Since PI(4,5)P2 and PI(3,4,5)P3 are approximately equipotent activators of PLD1 in vitro (PI(4,5)P2 is approximately 1.5-fold more effective), given the relative abundance of these two lipids in mammalian cells it is reasonable to speculate that PI(4,5)P2 is the most likely candidate for a physiologic PLD activator. Studies using permeabilized U937 cells suggest a role for stimulated PI(4,5)P2 synthesis in controlling monomeric GTP-binding protein-regulated PLD activity (43). On the other hand, in general, agonist-promoted changes in PI(4,5)P2 levels in stimulated cells are modest, whereas PI(3,4,5)P3 levels can increase dramatically (44).

Activation of PLD1 by ARF and RHO Family Monomeric GTP-binding Proteins and PKC-α—As we reported for crude preparations of the enzyme expressed in Sf9 or COS-7 cells, purified PLD1a and 1b are strongly activated by ARF-1. These results firmly establish PLD1a and 1b as direct effectors for ARF and further emphasize the importance of defining the role of PLD in ARF function. The ARF proteins are central regulators of protein trafficking (29). Several independent lines of evidence suggest a role for PLD in ARF-dependent protein trafficking and secretion. The most specific proposal is that ARF-dependent PLD activation plays a role in coated vesicle formation in the endoplasmic reticulum and Golgi apparatus, and experimental evidence in support of this idea has been presented (30, 45).

Protein kinase C-α is also an effective activator of PLD1a and 1b and does so in an ATP-independent manner. Phorbol esters increase both the potency and efficacy with which PKC-α activates PLD1a and 1b. This unexpected mode of PLD regulation has been reported by others using crude or partially purified PLD preparations (17, 18), and our results make it likely that this effect results from a direct interaction between PLD1a and 1b and PKC-α. Phorbol esters are effective stimuli of PLD
activities in many cells, but since in vitro activation of PLD1 by PKC-α is not absolutely dependent on phorbol esters, at present it is unclear if the phosphorylation-independent regulatory mechanism that operates in vitro underlies PKC-dependent PLD activation in vivo. The observation that, in several systems, inhibitors that block the catalytic activity of PKC inhibit PLD activation by cell-surface receptors is also suggestive of a phosphorylation-dependent mechanism for PKC-mediated PLD activation (20).

Although the effects are modest by comparison with the responses to ARF-1 and PKC-α, PLD1a and 1b are also activated by three RHO family monomeric GTP-binding proteins, RHO A, RAC-1, and CDC42. One component of the signal generated by the RHO-type monomeric GTP-binding proteins is mediated by protein kinase cascades ultimately leading to transcriptional activation. Microinjection studies indicate that RHO, RAC, and CDC42 can also promote changes in cell morphology, motility, and organization of the actin cytoskeleton on a time scale that is clearly too rapid to involve alterations in gene expression so they must control other regulatory pathways (31). Our finding that PLD1a and 1b are directly activated by these monomeric GTP-binding proteins focuses attention on this enzyme as a mediator of these processes. Although appropriate accessory proteins (guanine nucleotide-dissociation inhibitors and guanine nucleotide exchange factors) have been identified, the mechanisms controlling activation of the RHO proteins are not fully defined (31). One possibility suggested by two recent studies is that the RHO proteins play a role in coupling cell-surface receptors to PLD activation (32, 33).

Synergistic Effects of ARF and RHO Family Monomeric GTP-Binding Proteins and PKC-α on PLD1a and 1b Activity—As described above, three classes of protein factors, the ARF and RHO family monomeric GTP-binding proteins, and PKC-α activate PLD1a and 1b independently. We also investigated the effects of combinations of these factors on PLD1a and 1b activity. Whereas combinations of maximally effective concentrations of RHO A, RAC-1, and CDC42 did not increase PLD1 activity further than observed when each of these monomeric GTP-binding proteins were included in the assays alone, we found that combinations of the ARF and RHO family monomeric GTP-binding proteins and PKC-α increased PLD1a and 1b activity to levels that were considerably greater than would be observed if their effects on PLD1a and 1b activity were additive. It therefore appears that these three classes of activators interact synergistically to increase PLD1a and 1b activity.

The simplest explanation of our findings is that the PLD1a and 1b proteins contain separate sites for interaction with P[4,5]P₂/P[3,4,5]P₃, ARF, the RHO family monomeric GTP-binding proteins, and protein kinase C-α and that occupancy of these sites by their respective ligands results in a cooperative increase in catalytic efficiency of the enzyme. Comparison of the primary sequences of plant, yeast, and human PLD enzymes identifies four regions of homology including two regions containing sequences conserved among a family of related proteins that catalyze phospholipid synthesis reactions. We and others (9, 46) have therefore suggested that these sequences are important in catalysis, so it seems reasonable to postulate that other regions of the protein are involved in regulatory interactions with the lipid and protein factors. A more detailed kinetic analysis of PLD1a and 1b activity will be required to understand how these factors increase catalytic efficiency of the enzyme and why cooperative interactions between combinations of the ARF and RHO monomeric GTP-binding proteins and PKC-α result in synergistic increases in PLD1 activity.

Photoaffinity labeling studies with PtdIns(4,5)P₂ and PtdIns-(3,4,5)P₃ analogs (36) and mutagenesis studies of PLD1a and 1b promise to define the sites of interaction between PLD1 and these lipid and protein regulators and may lead to identification of mutant forms of the enzymes with altered selectivities for activation by ARF and RHO family monomeric GTP-binding proteins and PKC. These will clearly be of great potential value for elucidating the roles played by the three classes of regulators in PLD regulation in cells.

Significance of PLD1a and 1b—The studies reported in this paper indicate that PLD1a and 1b have identical catalytic and regulatory properties. The alternate splicing of PLD1 transcripts would appear to be of biological importance since it is conserved across three different mammalian species. The 38-amino acid sequence that distinguishes PLD1a from PLD1b is located in a 150-amino acid region that is unique to PLD1 (Fig. 1C). This 150-amino acid region is located between conserved sequence domains I and II identified in plant, yeast, and bacterial PLD enzymes that do not encompass conserved sequences shared among a growing family of PLD-related proteins (9, 46). Our results indicate that the 38-amino acid alternately spliced region is not involved in catalysis or regulation by ARF, RHO family monomeric GTP-binding proteins, P[4,5]P₂/P[3,4,5]P₃, or PKC-α. Further work will be required to determine the role of this region in PLD1 function.

Possible Functions for PLD1a and 1b—All of the experiments reported in this study employed in vitro assays with purified proteins so it will clearly be important to establish the roles played by the monomeric GTP-binding proteins and protein kinase C in regulation of PLD1 in cells. With this in mind, our findings suggest that PLD1a and 1b may be uniquely positioned to receive and integrate different kinds of extracellular signals, transducing them to generate lipid-derived molecules that, in turn, mediate cell-specific responses. Given the growing evidence for involvement of ARF-activated PLD in intracellular protein trafficking, clearly receptor-regulated secretion would be a good candidate for a PLD1-mediated response. Another possibility (not necessarily incompatible with the first idea) is that PLD1 is present in different membrane compartments of the cell where different modes of regulation predominate and different downstream effectors are present. For example, ARF-dependent activation of PLD1 in the Golgi apparatus might generate PA for coated vesicle formation, whereas PKC and/or RHO-dependent PLD activation of PLD1 in the plasma membrane could result in changes in cell morphology mediated by the actin cytoskeleton or lead to formation of diglyceride for PKC activation.

Relationship of PLD1 to Other Mammalian Monomeric GTP-Binding Protein-regulated PLD Activities—Sternweis and colleagues (18, 28, 47) have reported several studies on an extensively purified PLD preparation from porcine brain. This activity is P[4,5]P₂-dependent and activated by ARF and RHO family monomeric GTP-binding proteins and PKC-α in a synergistic manner (18, 28, 47). It therefore seems likely that these workers were studying a porcine homolog of PLD1a, 1b, or a closely related PLD enzyme. Work from other laboratories has suggested that distinct ARF and RHO-activated PLD enzymes exist (25–27). Given the pronounced synergy between the three classes of protein regulators for activation of PLD1a and 1b, it is possible that crude or partially resolved PLD preparations containing PLD1a or 1b could exhibit different sensitivities to activation by exogenously added ARF and RHO proteins depending on the presence of endogenous monomeric GTP-binding proteins or PKC isoenzymes. Cell and tissue extracts also contain a variety of factors that exert inhibitory effects on monomeric GTP-binding protein-regulated PLD activities measured using exogenously provided substrates (48, 49).
These include several as yet unidentified protein inhibitors of PLD1a and 1b.\(^4\) Taken together, these two considerations clearly complicate attempts to classify crude PLD preparations on the basis of their responses to ARF and RHO family monomeric GTP-binding proteins. The availability of PLD1-selective antibodies should aid resolution of this issue.

Concluding Comments—In summary, we report the purification and characterization of two closely related forms of PLD1. Our results establish PLD1a and 1b as targets for direct regulation by ARF and RHO family monomeric GTP-binding proteins and PKC-\(\alpha\). Although further work will be required to establish the roles played by these different factors in regulation of PLD1 in cells, our results focus attention on PLD1a and 1b as mediators of processes of protein trafficking and alterations in cell motility and morphology controlled by the ARF and RHO family monomeric GTP-binding proteins. The purified proteins, PLD isoenzyme-selective antisera, and PLD cDNAs now available should prove effective tools for defining the role of PLD1a and 1b in cell regulation.

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\(^4\) J. M. Jenco, S. M. Hammond, M. A. Frohman, and A. J. Morris, unpublished observations.