Structural Analysis of Human Phospholipase D1*

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Activation of phosphatidylcholine-specific phospholipase D (PLD) has been proposed to play roles in numerous cellular pathways including signal transduction and membrane vesicular trafficking. We previously reported the cloning of two mammalian genes, PLD1 and PLD2, that encode PLD activities. We additionally reported that PLD1 is activated in a synergistic manner by protein kinase c-α (PKC-α), ADP-ribosylation factor 1 (ARF1), and Rho family members. We describe here molecular analysis of PLD1 using a combination of domain deletion and mutagenesis. We show that the amino-terminal 325 amino acids are required for PKC-α activation of PLD1 but not for activation by ARF1 and RhoA. This region does not contain the sole PKC-α interaction site and additionally functions to inhibit basal PLD activity in vivo. Second, a region of sequence unique to PLD1 (as compared with other PLDs) known as the “loop” region had been proposed to serve as an effector regulatory region but is shown here only to mediate inhibition of PLD1. Finally, we show that modification of the amino terminus, but not of the carboxyl terminus, is compatible with PLD enzymatic function and propose a simple model for PLD activation.

During the past 4 years, phosphatidylcholine-specific phospholipase D (PLD) cDNAs have been cloned from a wide variety of species ranging from bacteria to humans (reviewed in Refs. 1 and 2). Isolation of the first PLD cDNA sequence from animals (human PLD1) revealed that an evolutionarily related PLD family was widespread and encoded several regions of conserved sequence (3). Use of these conserved regions for motif searches of sequence data bases then revealed the existence of additional related genes of known and unknown function from bacteria, viruses, and mammals (4–8). A representative of this PLD superfamily (human PLD1) and the regions conserved in it are shown in Fig. 1. Two separate mammalian PLD genes approximately 50% identical have been reported (PLD1 and PLD2; Ref. 4), but only a single gene appears to exist in worms, flies, and yeast (as suggested by searches of GenBank™). Sequence comparisons suggest that the mammalian genes arose through duplication of an ancestral gene after divergence occurred between the lower eukaryotes and animals (4, 9, 10).

PLD proteins from most species exhibit constitutive activity when assayed in vitro, and their regulation in vivo is effected through mechanisms such as phosphorylation and translocation (11, 12). Mammalian PLD2 is similarly constitutively active in vitro (4, 13). How endogenous PLD2 is regulated in vivo is not fully understood (4, 14), although it is clear that the activity of both transfected PLD1 and PLD2 increase after agonist stimulation (15). In contrast, however, to PLD2 and PLDs from nonmammalian species, it has been reported that PLD1 exhibits a low basal activity when expressed in tissue culture cell lines or as a recombinant, purified protein in vitro and that it is stimulated in vivo in the presence of recombinant, purified protein kinase C-α (PKC-α) or ARF or Rho small GTP-binding protein family members (3, 16). These findings also demonstrated that the effectors interact directly with PLD1, because no other proteins were present in the assays. Each class of effectors can act alone to stimulate PLD1, and in combination they elicit a synergistic activation (16, 17). However, because PLD1 does not contain motifs frequently found in other kinds of signaling enzymes, such as C2, SH2, and SH3 domains, Rho binding sequences, or membrane localization signals, it was not readily apparent how the small GTP-binding protein and PKC-α interactions would be mediated. Moreover, PKC-α stimulation of PLD1 is conducted through a novel mechanism, because the stimulation is ATP-independent and appears to be mediated by the regulatory domain of PKC-α instead of its catalytic one (16, 18).

In our initial studies on this topic, we undertook site-directed mutagenesis of regions held in common among PLDs from different species (CRII, CRIII, and CRIV; Ref. 8). We found that these regions were critical for catalysis in vitro and for PLD function in vivo and developed a hypothetical model for the catalytic cycle involving a covalent phosphatidyl-enzyme intermediate (8). However, although many of the mutants displayed diminished or no enzymatic activity, none of them exhibited selective responsiveness to ARF, Rho, or PKC-α (8).

In this report, we targeted for analysis the regions of PLD1 that are the least conserved relative to mammalian PLD2 or PLD genes from other species. We define several regions in terms of their potential regulatory roles and suggest a model for PLD1 activation.

EXPERIMENTAL PROCEDURES

General Reagents—All phospholipids were purchased from Avanti Polar Lipids. PIP₂ was isolated as described (3). t-α-Dipalmitoylphosphatidylincholine (choline-methyl-³H) (³H)phosphatidylincholine) was obtained from American Radiolabeled Chemicals. All other reagents were obtained from previously noted standard sources and were of analytical grade unless otherwise specified (3).

Site-directed and Deletion Mutagenesis—Site-directed mutagenesis of expression plasmids was carried out using the Quik-Change kit (Stratagene). Plasmids were sequenced to confirm the intended mutation and the integrity of the surrounding sequences for at least 100 base pairs using Sequenase (U. S. Biochemical Corp.). Deletion mutants

* This work was supported by a Onyx Pharmaceutical Inc. grant and by National Institutes of Health Grants GM54813 (to M. A. F.) and GM50388 (to A. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PLD, phospholipase D; ARF, ADP-ribosylation factor; CR, conserved region; PMMA, phosphoribosylamine acetate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC-α, protein kinase C-α; PX, phox; HA, hemagglutinin; GTP·γS, guanosine 5’-3-O- (thio)triphosphate; GFP, green fluorescent protein.
were constructed using convenient restriction sites or polymerase chain reaction-based strategies and were sequenced at all junctions to ensure that the reading frame was maintained. Insertional mutants were constructed by linearizing pCGN-PLD1 at unique sites in the open reading frame and religating in the presence of a linker that inserted 3 or 4 amino acids while maintaining the open reading frame.

Cell Culture—COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For transfections, the cells were grown in 35-mm dishes and then switched into Opti-MEM I (Life Technologies, Inc.). Western blot analysis was performed as described previously (4, 8, 16). In brief, 5 μl of cell lysate was added to 5 μl of 2X sample loading dye containing 8 M urea but not boiled (if boiled in standard dye, PLD1 were mixed on ice, and the reactions were initiated by transfer to a present in the assay tubes to which PKC was added. The components lipids containing [3H]phosphatidylcholine, water, and in some cases, were grown in 35-mm dishes and then switched into Opti-MEM I (Life Technologies, Inc.) was observed to be approximately 5–20%.

Mammalian PLD activity was measured in vitro using the head-group-labeled phosphatidylincholine release assay as described previously (4, 8, 16, 19–21). In brief, after allowing 24 h for expression of the recombinant plasmids, the transfected cells were harvested by scraping in 60 μl of phosphate-buffered saline containing protease inhibitors and then sonicated. 10 μl of lysate was added to assay buffer, sonicated lipids containing [3H]phosphatidylcholine, water, and in some cases, PLD effectors. Recombinant ARF, RhoA, Rac-1, and PKCα were purified as described previously (16). The small GTP-binding proteins were used at 1 μM after being EDTA-stripped and preloaded with GTPyS as described previously (16). PKCα was activated in the assay tube by the addition of phorbol ester to a final concentration of 100 nM. ATP was not present in the assay tubes to which PKC was added. The components were mixed on ice, and the reactions were initiated by transfer to a 37 °C water bath and then stopped after 30 min by addition of bovine serum albumin and trichloroacetic acid. The samples were centrifuged to precipitate unreacted lipid, and the supernatants containing the released [3H]choline were quantitated in a scintillation counter.

Mammalian PLD activity was measured in vivo using the transphosphatidylation assay exactly as described previously (4, 8, 28, 21). In brief, COS-7 cells were transfected with PLD1 plasmids, labeled with [3H]oleate for 24 h, washed, and cultured with 0.3% butanol with or without 100 nM PMA for 30 min. After removal of the medium, cold methanol was added to stop the reaction, and the lipids were extracted, dried, resuspended, and separated on a TLC plate. The phosphatidyl-

PKCα-PLD1 Co-immunoprecipitation—Wild-type or truncated PLD1 was transiently overexpressed in COS-7 cells in 35-mm dishes as described above. After 24 h of culture in medium containing serum, the cells were washed in Opti-MEM I lacking serum and cultured in fresh Opti-MEM I for an additional 20 min. The medium was then replaced by Opti-MEM I containing PMA at 100 nM. After an additional 5 min of culture, the cells were washed using ice-cold phosphate-buffered saline, harvested from the dishes by scraping using ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM diethiothreitol, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride), and sonicated until lysis occurred. All subsequent steps were carried out at 4 °C unless noted otherwise. The resulting lysate was precleared using a 3,000 × g spin for 5 min. The supernatant was then centrifuged at 30,000 × g for 30 min to pellet the membranes. Pilot experiments had shown that virtually all of the PLD1 segregates to this pellet under these conditions. The membrane pellet was then resuspended in lysis buffer containing 2% cholate and immunoprecipitated through incubation with 10 μl of 12CA5 coupled to protein A-Sepharose on a rotator (12CA5 is a monoclonal antibody that recognizes the Flu epitope tag fused to the amino terminus of the PLD1 constructs as expressed in pCGN). The beads were spun for 5 s at 16,000 × g and washed three times with 1 ml of 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 150 mM NaCl, 0.1% Triton X-100. The beads were resuspended in 30 μl of wash buffer following which an equal volume of 2X SDS sample buffer containing 8 M urea was added (16). The samples were incubated at room temperature (but not boiled) before protein electrophoresis (16). Western blot analyses were performed as described previously, using 12CA5 or a rabbit PKD1-specific antiserum (16) to detect PLD1 (4) and an antipKCα monoclonal antibody (Santa Cruz) to detect PKCα.

RESULTS

Rationale for Deletion Analysis—PLD1 contains several regions of sequence not found in mammalian PLD2 or PLDs from other species, and we and others have hypothesized that these regions confer the unusual regulatory properties of PLD1 (1, 2, 17).

The most unique region is the "loop" sequence in the center of the protein (amino acids 505–620 in human PLD1α, Fig. 1). Mammalian PLD2 lacks these 116 amino acids; yeast PLD1 (Spo14p) lacks an additional 30 amino acids, and the loop region is similarly absent from all other known PLD proteins except for one from Caenorhabditis elegans, in which the loop peptide is increased in size to 300 amino acids. The regulatory properties of this C. elegans PLD have not been reported (GenBankTM accession number U55854). Part of the PLD1 loop region undergoes alternative splicing, removing 33 amino acids to generate the PLD1b isoform (9, 16). This regulated splicing is conserved in mouse, rat, and human and confers no obvious changes in regulation to human PLD1 (16), although it has been suggested that there are subtle changes in Rho responsiveness for rat PLD1 (17). The loop region is the least well-conserved region when mouse, rat, and human PLD1 sequences are compared.

The amino termini are well conserved between different mammalian PLD1 proteins but exhibit little similarity to the amino termini of mammalian PLD2 or PLDs from nonmammalian species. This is particularly true for the first 100 amino acids. Weak homology is observed from amino acids 100–330.
**A**

[Diagram showing the structure of PLD1 with deletion mutants.

**B**

Western blot analysis of wild-type and mutant proteins.

**C**

In vivo analysis of Δ1–325 PLD1.

**D**

PLD activity in vivo was assessed after a 4-h preincubation with PMA.

**In vitro activity (%)**

|        | Basal | ARF1 | PKCα | Rac1 | RhoA |
|--------|-------|------|------|------|------|
|        | 100   | 100  | 100  | 100  | 100  |
| Δ(1-100)| 0 ± 30| 7 ± 3 | 2 ± 7 | 4 ± 0 | 3 ± 1 |
| Δ(1-157)| 43 ± 78| 8 ± 1 | 0 ± 1 | 4 ± 1 | 1 ± 3 |
| Δ(1-228)| 6 ± 2 | 6 ± 0 | 2 ± 1 | 4 ± 3 | 4 ± 2 |
| Δ(1-325)| 68 ± 43| 133 ± 42 | 2 ± 2 | 160 ± 59 | 36 ± 16 |

Fig. 2. An amino-terminal PLD1 deletion mutant exhibits selective loss of responsiveness to PKC-α. A, structure and in vitro activity of PLD mutants in lysates after expression in COS-7 cells (not shown at the left is an amino-terminal 20-amino acid Flu epitope tag appended by the pCGN expression plasmid). Values in parentheses on the right beneath the top line indicate the fold activation of wild-type PLD1 when stimulated by a series of effectors as compared with basal activity. The values represent the average of four experiments and were calculated after subtraction of the background activity observed subsequent to transfection of a non-PLD cDNA (Gbx2) expressed from pCGN and stimulated under the same conditions (actual cpm: basal activity in cell lysates from Gbx2-transfected cells (basal background), 541 cpm; basal activity in cell lysates from wild-type PLD1-transfected cells, 871 cpm; ARF1-stimulated activity in Gbx2-transfected cells (ARF-stimulated background), 937 cpm; ARF1-stimulated activity in PLD1-transfected cells, 20250 cpm; see also Figs. 2C and 6A as examples). The background-corrected wild-type PLD1 activities were then normalized to 100% for each category and used as a base to calculate relative activities of the mutants shown below (after similar background corrections). Mutant activities shown represent the average of four to nine separate experiments. The intra-assay variance was 7%, and the inter-assay variance in some cases was higher due to day-to-day differences in assay parameters. B, Western blot analysis of wild-type and mutant proteins. Lysates were electrophoresed in SDS-polyacrylamide gel electrophoresis gels, and recombinant proteins were visualized using a monoclonal antibody to detect the Flu epitope tag as described previously (8) and under “Experimental Procedures.” Black arrowhead, PLD1 is detected at a range of sizes from 122 to 130 kDa due to post-translational modifications. Infrequently, a breakdown product is observed (white arrowhead). Asterisk, the monoclonal antibody detects an 80-kDa cytoplasmic nonspecific protein in COS-7 cell lysates, which coincides with the truncated but correctly sized protein observed for Δ1–325. Black dots indicate the expected bands for wild-type and truncated PLD1 proteins. C, in vivo analysis of Δ1–325 PLD1. In vivo assays were carried out as described previously and under “Experimental Procedures.” Black arrowhead, PLD1 is detected at a range of sizes from 122 to 130 kDa due to post-translational modifications. Infrequently, a breakdown product is observed (white arrowhead). Asterisk, the monoclonal antibody detects an 80-kDa cytoplasmic nonspecific protein in COS-7 cell lysates, which coincides with the truncated but correctly sized protein observed for Δ1–325. Black dots indicate the expected bands for wild-type and truncated PLD1 proteins. C, in vivo analysis of Δ1–325 PLD1. In vivo assays were carried out as described previously and under “Experimental Procedures.” The results shown are representative of four separate experiments conducted. PLD1-K989R is a catalytically inactive point mutant as described previously (8). In all experiments, the Δ1–325 basal activity was higher than the wild-type PLD1 basal activity. The left panel depicts the absolute levels of PLD activity observed in separate experimental dishes. In the right panel, the averaged basal (endogenous) PLD activities (K989R) were subtracted from the averaged experimental values to show the activity specifically generated by the overexpressed wild-type and mutant PLD proteins. The numbers indicate the fold induction in the presence of PMA. D, PLD activity in vivo was assessed after a 4-h preincubation with PMA. The basal and +PMA results shown were averaged from three separate experiments. The values for PMA-stimulated PLD activity in cells transfected with pCGN and stimulated under the same conditions (i.e., raw values in a single, representative experiment: basal: pCGN (7781 cpm), wild-type PLD1 (11383 cpm); Δ1–325 (15474 cpm). PMA-stimulated: pCGN (39251 cpm), wild-type PLD1 (73851); Δ1–325 (44799 cpm)).
of protein-protein interactions (23), and PH domains frequently mediate inositol lipid and phosphate binding (PIP2 is a requisite co-factor for PLD1 and PLD2). Many membrane-associated proteins require free amino termini to successfully interact with membrane surfaces, particularly if they encode prenylation sequences. PLD1 does not encode such sequences, and it was known from earlier studies that a free amino terminus was not required because the protein appears to behave normally when fused to an amino-terminal Flu epitope peptide (3, 4).

In contrast to the amino terminus, the final 41 amino acids at the carboxyl terminus are relatively well conserved between PLD1, PLD2, and some of the PLDs from nonmammalian species (2). To assess the potential role of PLD1-specific sequences in PLD1 activation by effectors, a series of deletion mutants were constructed, transfected into COS-7 cells, and assayed for basal and stimulated PLD activities.

The Amino Terminus Is Required for PKC-α Activation of PLD1—As shown in the top line of Fig. 2A (and see figure legend), PLD activity in COS-7 cell lysates overexpressing wild-type PLD1 increases dramatically (30–60-fold) in the presence of small GTP-binding proteins or PKC-α. However, three proximal deletion mutant proteins (∆1–100, 1–157, and 1–228) were found to be inactive (Fig. 2A) and to differing degrees, unstable (Fig. 2B), suggesting that critical regions had been deleted or that protein misfolding occurred as a result of the missing sequences. However, ∆1–325 was both stable and active, demonstrating that the minimal sequence required for catalysis does not include the amino-terminal third of the protein. The atypical stability of this truncated protein may result from the fact that the site surrounding amino acid 325 appears to constitute a protein domain boundary, as suggested by the fact that amino acid similarity between yeast and mammalian PLD dramatically increases carboxyl-terminal to this point (1, 3).

Strikingly, however, ∆1–325 was capable of being activated only by ARF and Rho small GTP-binding protein family members; no response to PKC-α was observed (the in vitro assay was also conducted on ∆1–325 generated and purified from baculovirus with similar results; data not shown). The result demonstrates that a sequence uniquely required for activation of PLD1 by PKC-α is present in the amino terminus of the protein. To extend this observation, ∆1–325 was expressed in COS-7 cells and assayed using the transphosphatidylation assay (Fig. 2C). A low endogenous PLD basal activity and a modest response to PMA was observed for wild-type PLD1 (11-fold above basal, after subtraction of the endogenous component of the response). However, no PMA-stimulated increase in activity was observed for PLD1-∆1–325, confirming that the amino terminus appears to be required for PLD1 to respond to PKC-α.

FIG. 3. PX domain mutants are largely inactive in vitro but exhibit a higher basal activity in vivo and respond to PMA. A, PLD1 residues matching amino acids conserved in PX subdomains (23) and mutant activities. The mutations generated were made to the most highly conserved residues of the PX motif and are listed above the subdomain boxes. Relative activities were calculated as described in the legend to Fig. 2. Mutant activities shown represent the average of three separate experiments. Intra-assay variance was 7%. B, mutant PLD1 activities in vivo. PLD activity in intact cells was assayed as described in the legend to Fig. 2. The experiment is representative of three separate experiments performed in duplicate, and the values shown were corrected for endogenous PLD activity as described in the legend to Fig. 2C. Higher basal activity and a response to PMA was observed for the PX mutants in each experiment. Numbers above each bar signify the fold increase of nonstimulated mutant PLD1 activities over the nonstimulated wild-type PLD1 activity.
Similar results were observed after down-regulation of PKC-α through a 4-h pretreatment with PMA (Fig. 2D).

Unexpectedly, Δ1–325 assayed in vivo exhibited a significantly and reproducibly increased (2-fold) basal activity in comparison with wild-type PLD1 (Fig. 2, C and D), suggesting that a component of the normal physiological regulation of PLD1 in vivo requires the amino terminus to be intact. The mechanism underlying the increased basal activity in vivo is not apparent. Possibilities include increased sensitivity in vivo to small GTP-binding proteins or changes in subcellular location to sites more conducive to PLD1 activation. Δ1–325 remains membrane-localized similar to PLD1 as determined by subcellular fractionation (data not shown), but the specific sites to which it is targeted may be altered.

These results also suggested that the PH-like region encoded by amino acids 220–329 is unlikely to underlie the absolute requirement for PIP2 exhibited by PLD1 (3), because the truncated protein retains wild-type catalytic activity despite the elimination of most of this sequence. To test this issue, wild-type PLD1 and Δ1–325 were assayed in vitro in the presence of ARF and in the presence or absence of PIP2. In the absence of PIP2, wild-type PLD1 retained only 7% of the activity that it exhibited in the presence of PIP2, and Δ1–325 was completely inactive (data not shown). These results clearly demonstrate that the activity manifested by Δ1–325 is PIP2-dependent and thus that the PIP2-interacting site lies outside the amino terminus. As one possibility, a region present between the loop region and CRIII (Fig. 1) also contains a minimal PH domain basic amino acid motif.

The simplest explanation for selective loss of PKC-α responsiveness would be that the sole PKC-α interaction site lies in the amino terminus and that the amino terminus functions as a regulatory domain for PKC-α. However, two sets of experiments argue against this model. The first line of argument arises from our attempts to generate PLD1 point mutants that are selectively nonresponsive to PKC-α. To accomplish this, we targeted highly conserved residues in the PX domain (Figs. 1 and 3). The PX domain has been proposed to be a protein-protein interaction motif that can act as a SH3- or Pak kinase-targeted highly conserved residues in the PX domain (Figs. 1 and 3). The PX domain was excised. This different mutant proteins (two point mutants and one insertional mutant) were generated and assayed in vitro (Fig. 3A). The proteins were stably expressed (data not shown), but surprisingly, not only was PKC-α-responsiveness lost, but all three proteins were largely unresponsive to ARF and Rho. This result suggests that although the amino terminus is not required for catalysis or for activation by small GTP-binding proteins, it is nonetheless capable of locking the protein into an “off” state if nonfunctional. This implies that one step in the small GTP-binding protein activation of PLD1 is to mediate a conformational change in the amino terminus that permits catalysis. Nonetheless, when the PX point mutants were expressed in vivo, they were active, exhibited a high level of basal activity, and responded to PKC-α (PMA; Fig. 3B). These results confirm that the role of the PX domain is not to mediate interaction with PKC-α. Moreover, the results suggest that loss of PX domain functionality accounts for the increased basal activity observed for Δ1–325.

In the second set of experiments, we examined the physical interaction between PKC-α and PLD1. It was previously reported that PKC-α co-immunoprecipitates with PLD1 in a PMA-dependent manner (24). Using a modification of the published protocol (24), we examined whether Δ1–325 fails to co-immunoprecipitate PKC-α (Fig. 4; a representative experiment is shown). A modest increase in the co-immunoprecipitation of PKC-α with PLD1 in a PMA-dependent manner was observed, consistent with the previously reported finding (24). However, PKC-α also co-immunoprecipitated very effectively with Δ1–325, revealing that at least one PKC-α interaction site lies outside of the amino terminus. Taken together, the PX mutagenesis and PKC-α-PLD1 co-immunoprecipitation experiments demonstrate that the amino terminus has a complex function that involves more than simply mediating PKC-α activation of PLD1 and that PKC-α most likely interacts with multiple sites on PLD1.

The PLD1-specific Loop Region Does Not Mediate Effector Activation but Does Contain a Negative Regulatory Element—Two deletion constructs were generated in the loop region (Fig. 5A). The first (Δ497–645) replaced 150 amino acids unique to PLD1 when compared with yeast PLD (Spo14p) with the 11 amino acids present in the corresponding region of Spo14p. This mutant protein was stably expressed (data not shown) but was inactive under all conditions tested (Fig. 5A). In the second construct (Δ505–621), the 116 amino acids unique to PLD1 when compared with PLD2 were excised. Δ505–621 exhibited basal activity that was increased 3-fold both in vitro and in vivo as compared with wild-type PLD1 (Fig. 5, A and B). Δ505–621 was stimulated effectively in vitro by all three classes of activators and consistently exhibited approximately a 2-fold higher level of activation in vitro than wild-type PLD1. These findings demonstrate that the loop region does not act as an effector regulatory region and suggests that it accounts for part of the low basal activity characteristic of wild-type PLD1.

A Mini-PLD1 Protein Lacking the Amino Terminus and Loop Region Is Active and Responsive to Rho and ARF Effectors—The amino terminus and loop deletion findings were extended by constructing a PLD1 cDNA lacking both regions (Fig. 5A). The resulting cDNA (Δ1–325,505–621) encodes a 633-amino acid protein that is only slightly larger than bacterial (Streptomyces) PLD (556 amino acids). Δ1–325,505–621 was expressed in COS-7 cells and assayed. The in vitro basal activity was not elevated, and the extent of activation by ARF and Rho and the lack of activation by PKC-α was comparable with that observed for Δ1–325 (Fig. 5A). In vivo, again similar to Δ1–325, the basal activity was elevated (2.4-fold), and no response was observed to PMA (Fig. 5B). Because the small GTP-binding protein activation was decreased relative to Δ505–621, and high basal activity was not observed in vitro, it is possible that...
the mini-PLD1 protein is not fully functional. However, the fact that it is activated well by ARF relative to wild-type PLD1 suggests that it could be employed for structural determination or in settings in which a PLD1 primarily responsive to ARF would be useful for dissecting PLD1 activation or function.

**The Carboxyl Terminus of PLD1 Is Critical for PLD1 Function**—Unlike the amino terminus, which is quite variable, the carboxyl-terminal 40 amino acids are well conserved in PLD proteins ranging from *C. elegans* to mammals. A construct that truncated the carboxyl-terminal 80 amino acids (D976–1074) was generated and assayed (Fig. 5A). D976–1074 was stably expressed (data not shown) but was inactive under all conditions tested. This result suggested that unlike the amino terminus, an intact carboxyl terminus is required for PLD1 function. Although it is theoretically possible that a construct truncated at a different or more distal site might be active, other studies involving transposon-mediated scanning of PLD1 at the extreme carboxyl terminus or in which Flu epitope or Ras membrane localization tags were fused to the carboxyl terminus of yeast PLD or PLD2 resulted in similar losses of activity, supporting this conclusion (data not shown). In contrast, an amino acid insertion into PLD1 just outside of the conserved carboxyl-terminal region (1028E-DRRV-D1029) did not inactivate the enzyme (Fig. 5A), although the absolute levels of activity observed were 2-fold lower. This suggests that the critical sequence lies within the final 45 amino acids.

In contrast to the carboxyl terminus, the amino terminus can be truncated (D1–325) or fused to heterologous peptide tags without loss of activity. In addition to the Flu epitope tag described previously (3, 4, 8, 16) and used in this report, GFP-tagged PLD1 is also active and exhibits wild-type basal and stimulated activities and subcellular localization (Fig. 6).

**DISCUSSION**

Partially purified mammalian PLD activities began to be examined several years ago and were found to exhibit low basal activities and the capacity for synergistic activation by PKC-α and small GTP-binding proteins (18, 20, 25, 26). With the cloning and characterization of two mammalian PLDs as well as PLDs from a variety of other species, two realizations became apparent. First, the previously characterized partially purified activity was probably mediated by the enzyme now known as PLD1. Second, PLD1 is atypical when compared with nonmammalian PLDs, which exhibit regulatory properties...
more similar to PLD2 (i.e. constitutive activity \textit{in vitro}).

In a previous report, we showed that the PLD catalytic site is composed of two (CRII and CRIV) and possibly three (CRIII) noncontiguous blocks of sequence that presumably are folded into apposition in active protein (8). In this report, we define two regions, the amino terminus and the loop region, that contribute to the low basal activity of purified PLD1. The amino terminus is also required for PKC-\(\alpha\) activation of PLD1 but does not contain (all of) the PKC-\(\alpha\) interaction site(s). However, a partially truncated (presumably nonfunctional) amino terminus also blocks PLD1 activation by ARF and Rho, demonstrating that the function of this region is more complicated than as a simple PKC-\(\alpha\) regulatory domain. Moreover, PLD1 proteins mutated in the PX domain are inactive \textit{in vitro} but exhibit a high basal activity and are responsive to PKC-\(\alpha\) in \textit{vivo}, confirming that the role of the PX domain is something other than to mediate interaction with PKC-\(\alpha\). Potential roles include inter- or intramolecular interactions or subcellular targeting.

These findings extend our working model for PLD1 activation \textit{in vitro}, which no doubt will continue to undergo modification until structural data become available. We propose that the amino-terminal region blocks catalytic activity and that for wild-type protein, ARF, Rho, and PKC-\(\alpha\) induce conformational changes that remove this inhibition. When made nonfunctional, as for example when mutations are made to the conserved PX domain, ARF, Rho, and PKC-\(\alpha\) are unable to overcome the inert amino terminus \textit{in vitro} and activate PLD1. \textit{In vivo}, however, these proteins exhibit elevated basal activity and are responsive to PKC-\(\alpha\), suggesting that PLD1 regulation \textit{in vivo} is more complicated.

Nonetheless, because basal activity increases only modestly when the amino terminus is removed, there are clearly other mechanisms that act to silence PLD1 activity which ARF and Rho also modulate. One such component appears to involve the loop region, which confers a significant inhibitory effect on PLD1 and appears to limit the degree of activation by all three classes of activators. Because the loop region is relatively poorly conserved even between different mammalian PLD1 proteins, the precise sequence in it may be unimportant, so long as it acts as a “spacer” to misalign CRII and CRIV until effector interaction occurs. Studies in progress on a large set of randomly mutagenized PLD1 alleles have failed to identify specific sequences in the loop region important for PLD1 regulation,\footnote{Y. Zhang and M. A. Frohman, unpublished observations.} supporting this proposal. Deletion of the loop region has the strongest effect on some of the Rho family members, consistent with the modest alteration in Rho responsiveness reported for rat PLD1a and PLD1b (17), which differ in the inclusion or exclusion of the carboxyl-terminal 33 amino acids of the loop region (16).
Finally, because PKC-\(\alpha\) binds amino-terminally deleted PLD1 (\(\Delta T–325\), a PKC-\(\alpha\) interaction site must be present in the carboxyl-terminal two-thirds of the protein. It has previously been reported that PKC-\(\alpha\) activates PLD1 in a PMA-dependent but ATP-independent manner (16). In addition, it has been shown that this activation is mediated by the PKC-\(\alpha\) regulatory subunit, which can function to activate PLD1 even in the absence of the PKC-\(\alpha\) catalytic subunit, although at greatly reduced potency (18).\(^3\) Nonetheless, PLD1 does become phosphorylated by PKC-\(\alpha\) (16),\(^3\) demonstrating that there is interaction between PLD1 and the PKC-\(\alpha\) catalytic domain, presumably at a site separate from where the PKC-\(\alpha\) regulatory domain interacts. Finally, as shown in this report, PKC-\(\alpha\) interacts with the carboxyl-terminal two-thirds of PLD1, but this does not lead to activation. Taken together, we think that the most likely explanation for these findings is that there are at least two PKC-\(\alpha\) interaction sites on PLD1: an amino-terminal one that interacts with the PKC-\(\alpha\) regulatory domain and a central or carboxyl-terminal one that interacts with the PKC-\(\alpha\) catalytic domain. Because the three-dimensional PLD1 structure most likely places CRII and CRIV in opposition, it is unreasonably to propose that the amino-terminal and carboxyl-terminal PKC-\(\alpha\) interacting sites may be occupied simultaneously by a single molecule of PKC-\(\alpha\). It has been reported that the interaction of PLD1 with PKC-\(\alpha\) after PMA stimulation is a transient one that peaks at 5 min and that lasts less than 20 min (24), even though PLD1 remains fully activated for more than 1 h (Fig. 2D and Ref. 4). One possibility arising from these findings is that interaction of PKC-\(\alpha\) with PLD1 may lead to a conformation change in PLD1 that persists subsequent to the interaction with PKC-\(\alpha\).

Our findings also raise the issue of what role the PLD1 PX region plays, because its function does not appear to involve interaction with PKC-\(\alpha\). One possibility is that it may interact with vesicular trafficking machinery because this is a documented role for this motif in other proteins (reviewed in Ref. 23), and PLD1 has been shown to affect the rate of budding of vesicles from the trans-Golgi (27) and is negatively regulated by vesicular trafficking machinery components (28, 29). Consistent with this hypothesis, the amino-terminal region in yeast PLD is also dispensable for enzymatic activity and instead regulates changes in subcellular localization from the cytoskeleton to the spindle apparatus during meiosis (11).

Although the amino-terminally truncated PLD1 appears useful on the surface as a tool for dissecting PLD1 regulation and function in vivo, its high basal activity in vivo and other altered regulatory properties suggest that interpretation of its effects might be complicated. It is probable that more appropriate selectively responsive PLDs will be generated through the use of mutagenic scanning or other approaches.

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\(^3\) S. M. Hammond and A. J. Morris, submitted for publication.