Molecular Analysis of Mammalian Phospholipase D2*

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The mammalian phosphatidylcholine-specific phospholipase D (PLD) enzymes PLD1 and PLD2 have been proposed to play roles in signal transduction and membrane vesicular trafficking in distinct subcellular compartments. PLD1 is activated in a synergistic manner in vitro by protein kinase C-α, ADP-ribosylation factor 1 (ARF1), and Rho family members. In contrast, PLD2 is constitutively active in vitro. We describe here molecular analysis of PLD2. We show that the NH2-terminal 308 amino acids are required for PLD2's characteristic high basal activity. Unexpectedly, PLD2 lacking this region becomes highly responsive to ARF proteins and displays a modest preference for activation by ARF5. Chimeric analysis of PLD1 and PLD2 suggests that the ARF-responsive region is in the PLD carboxyl terminus. We also inserted into PLD2 a region of sequence unique to PLD1 known as the “loop” region, which had been proposed initially to mediate effector stimulation but that subsequently was shown instead to be required in part for the very low basal activity characteristic of PLD1. The insertion decreased PLD2 activity, consistent with the latter finding. Finally, we show that the critical role undertaken by the conserved carboxyl terminus is unlikely to involve promoting PLD association with membrane surfaces.

Phosphatidylcholine-specific phospholipase D (PLD)1 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 animal PLD cDNA sequence (human PLD1) and subsequent studies revealed that an evolutionarily related PLD superfamily was widespread and encoded several regions of conserved sequence (1, 3–7). Two distinct mammalian PLD genes approximately 50% identical have been isolated from humans, rats, and mice (3, 4, 8–12). PLD proteins examined thus far from nonmammalian species exhibit constitutive activity when assayed in vitro, and their regulation in vivo is effected through mechanisms such as phosphorylation and trypsin digestion (13, 14). Mammalian PLD2 is similarly constitutively active in vitro (4, 9, 11, 15) and is regulated in vivo through unknown mechanisms (15, 16). In contrast, PLD1 exhibits a low basal activity when expressed in tissue culture cell lines or as a recombinant, purified protein in vitro and is directly stimulated by the presence of recombinant, purified protein kinase C-α (PKC-α) or ARF or Rho small GTP-binding protein family members (3, 9, 17). Each class of effectors can act alone to stimulate PLD1 and in combination to elicit a synergistic activation, suggesting that for each there is a separate site of interaction on PLD1 (10, 17).

In our initial studies on this topic, we undertook site-directed mutagenesis of regions held in common among PLDs from different species (conserved regions II, III, and IV; Ref. 7). 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 (7). However, although many of the mutants displayed diminished or no enzymatic activity, none of them exhibited selective responsiveness to ARF, Rho, or PKC-α (7). We subsequently undertook molecular analysis of PLD1 and found that the amino terminus conferred PKC-α responsiveness. In addition, we showed that both the amino terminus and the central loop region mediated a negative regulatory effect that maintained PLD1’s low basal activity and finally that a conserved carboxyl-terminal region is critical for PKC-α-mediated catalysis.

In this report, we targeted for analysis the NH2-terminal region of PLD2 and unexpectedly generated a strongly ARF-responsive isoform, which we characterize and discuss in the context of PLD2 regulation in vivo.

EXPERIMENTAL PROCEDURES

General Reagents—All phospholipids were purchased from Avanti Polar Lipids. Phosphatidinositol 4,5-bisphosphate was isolated as described (3). [3H]Dipalmitoylphosphatidylcholine was obtained from American Radiolabeled Chemicals, and [32P]Phosphoric acid was from ICN Pharmaceuticals. 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 and chimeric PLD1/PLD2 cDNAs 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. For two constructs, a Ras membrane localization sequence was appended to the 3'-end of the open reading frame using a linker encoding the sequence PCCSCKCVLS.

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.). For in vivo assays, the cells were washed into serum and phosphoric acid-free Dulbecco’s modified Eagle’s medium after transfection and labeled with 5 μCi of [32P]Phosphoric acid (P) per dish for 18 h (19).

PLD Assays—Recombinant mammalian ARF, RhoA, Rac-1, and PKC-α were purified and activated using GTPγS or phorbol 12-myristate 13-acetate and are therefore marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PLD, phospholipase D; ARF, ADP-ribosylation factor; mARF, mammalian ARF; yARF, yeast ARF; HA, hemagglutinin; PKC-α, protein kinase C-α; GTPγS, guanosine 5’-3-O-thio(triphosphate).

2 Sung, T.-C., Zhang, Y., Morris, A. J., and Frohman, M. A. (1999) J. Biol. Chem. 274, in press.
tate 13-acetate as described previously (17). Yeast ARF, mutant mammalian ARFs, and chimeric mammalian/yeast ARFs were generated, prepared, and activated as described previously (20). Mammalian PLD activity was measured using the \textit{in vitro} head group release assay and the \textit{in vivo} transphosphatidylation assay as described previously (4, 17, 21, 22). PLD cDNAs were transiently overexpressed in COS-7 cells as described previously using the mammalian expression vector, pCGL (3, 4), or immunoaffinity-purified from baculovirus-infected SF9 cells as described previously (4, 15, 17). The transfection efficiency was observed to be approximately 5–20%. Western blots were performed as described previously, using affinity-purified rabbit anti-PLD peptide antisera to detect PLD1 and PLD2 (4, 15, 17) or the monoclonal antibody 12C5 to detect HA-tagged PLD1 and PLD2 (7).

**RESULTS**

The Amino Terminus of PLD2 Is Required for Its High in Vitro Basal Activity—Conservation of protein sequence between yeast PLD and mammalian PLD1 begins at amino acid 325 of PLD1 (amino acid 308 of PLD2). We previously found that the nonconserved region (denoted the amino terminus) of PLD1 mediates responsiveness to PKC but is not required for PLD1’s intrinsic enzymatic activity or for small GTP-binding protein stimulation of PLD1 (Fig. 1). We also observed that deletion of either the amino terminus or the unique “loop” sequence from PLD1 increased its basal activity, suggesting that these regions in combination were responsible for at least part of the difference in the PLD1 and PLD2 states of activation \textit{in vitro}. These findings prompted us to investigate the function of the PLD2 NH$_2$ terminus, since PLD2 exhibits high basal activity and is not PKC-responsive.

The amino termini are well conserved between the different mammalian PLD2 proteins but exhibit little similarity to the PLD1 amino termini, particularly over the first 80 amino acids. Weak homology is observed from amino acid 83 to 196, including a phox domain (Fig. 1, Ref. 2) that has been proposed to mediate a wide variety of protein-protein interactions (23). Many membrane-associated proteins require free amino termini to successfully interact with membrane surfaces, particularly if they encode prenylation sequences. PLD2 does not encode such sequences, and it was known from earlier studies that a free amino terminus is not required, since the protein appr...
An NH$_2$-terminal PLD2 deletion mutant becomes ARF-responsive. A, structure and in vitro activity of PLD mutants in lysates after expression in COS-7 cells (not shown at the left is an NH$_2$-terminal 20-amino acid HA epitope tag appended by the pCGN expression plasmid). B, activities exhibited by the PLDs. The values represent the average of three experiments. The intraassay variance was 7%, and the interassay variance in some cases was higher due to small differences in transfection efficiency for individual plasmids in the separate experiments. The basal values were relatively variable due to the smaller numbers involved. The ARF and Rho effectors were preloaded with GTP$_\gamma$S, and PKC-α was activated using phorbol ester as described previously (17). The stimulation triggered by the addition of GTP$_\gamma$S alone is not shown, but the lack of response of PLD2Δ(1–308) to GTP$_\gamma$S-loaded Rho family members demonstrates that the response to ARF1 is specific and not due to an unknown GTP$_\gamma$S effect on the assay. C, in vivo analysis of PLD2Δ(1–308). In vivo assays were carried out as described previously and under “Experimental Procedures.” The results shown are representative of three separate experiments conducted. PLD2-K758R is a catalytically inactive point mutant as described previously (7). CTL, control (pCGN). D, Western analysis of wild-type and mutant proteins. Lysates were electrophoresed in SDS-polyacrylamide gel electrophoresis gels, and recombinant proteins were visualized as described under “Experimental Procedures” using a monoclonal antibody to detect the HA epitope tag as described previously (7) or an affinity-purified polyclonal rabbit antisera directed against PLD1-specific or PLD2-specific peptides (17, 18). Predicted sizes were as follows: PLD1, 120–124 kDa; PLD2, 106 kDa; PLD1Δ(1–325), 84 kDa; PLD2Δ(1–308), 66 kDa. Breakdown or truncated products are observed for PLD2. The monoclonal antibody nonspecifically detects unknown 50- and 80-kDa cytoplasmic proteins in COS-7 cell lysates. The latter coincides with the truncated but correctly sized protein observed for PLD1Δ(1–325). The goat anti-rabbit secondary antiserum nonspecifically detects an unknown 45-kDa protein. The anti-PLD peptide antisera, which were generated using peptides chosen from the amino terminus and the middle of PLD1 and PLD2, detect the amino termini much more effectively than the sites in the middle of the proteins. Thus, the NH$_2$-terminally truncated PLDs are recognized relatively poorly by the anti-peptide antisera as compared with detection by the anti-HA epitope tag monoclonal antibody. SK, pBluescript.
gests that the truncated protein is subcellularly localized to sites containing activated ARF (in contrast to PLD1) or that negative regulatory regions active only \textit{in vivo} are present in the NH$_{2}$ terminus. Since PLD1 and PLD2 subcellularly localize to different regions (4) and since PLD1 encodes an \textit{in vivo} specific negative regulatory region in its amino terminus, both hypotheses are tenable.

Finally, we examined PLD2$\Delta$(1–308) protein expression (Fig. 2D). Immunoreactive protein is observed near the predicted size of 66 kDa, in addition to proteolyzed fragments, which are also observed for wild-type PLD2, but not PLD1 or PLD1$\Delta$(1–325). The proteolyzed fragments observed for PLD2$\Delta$(1–308) are unlikely to be catalytically active, since both larger NH$_{2}$-terminal deletions or deletion of any sequence from the COOH terminus yields inactive protein (data not shown). In the full-length PLD2 COS-7 cell lysate, however, catalytically active NH$_{2}$-terminally truncated proteins could be present that could contribute to the partial ARF responsiveness observed.

**ARF1 Acts Directly on PLD2$\Delta$(1–308)—** To rule out indirect effects of ARF1, we expressed PLD2$\Delta$(1–308) in baculovirus and immunoaffinity-purified it using an anti-peptide antiserum (4). Both full-length PLD2 and PLD2$\Delta$(1–308) prepared in this manner are largely intact (unproteolyzed) (data not shown and Ref. 4). As shown in Table I, whereas full-length PLD2 is minimally stimulated by ARF1 (less than 2-fold), PLD2$\Delta$(1–308) exhibits a 13-fold increase. The stimulation is observed at relatively low levels of ARF1 (0.1 mM), is GTP$\gamma$S-dependent, and is not elicited by irrelevant carrier protein.

**Fig. 3.** PLD2 preferentially responds to yARF2 and mARF5 instead of mARF1. A, COS-7 cells transfected with PLD constructs were lysed and assayed for PLD activity in the presence of 1 mM GTP-loaded ARF proteins. The values represent the average of three experiments. Basal activity (after subtraction of pCGN background) was defined as 1.0 for each individual PLD. Fold stimulation is shown above each bar relative to the appropriate basal activity. The intrassay variance was 7%, and the interassay variance in some cases was higher due to small differences in transfection efficiency for individual plasmids in the separate experiments. The basal values were relatively variable due to the smaller numbers involved. B, PLD1 and PLD2$\Delta$(1–308) stimulation by a panel of GTP-loaded ARFs (1 mM each) was measured. Values are presented relative to the stimulation observed for mARF1 after subtraction of background. C, dose-response curves for GTP-loaded mARF1 and mARF5 stimulation of PLD1, PLD1$\Delta$(1–325), and PLD2$\Delta$(1–308). A representative experiment (of three) is shown.
(bovine serum albumin). Since both the ARF1 and PLD proteins were recombinantly expressed and were purified, these data demonstrate that ARF interacts directly with PLD2 (1–308).

PLD1 and PLD2Δ(1–308) Respond Best to Different ARFs—PLD1 and PLD2 localize to different subcellular regions (4). At least six mammalian ARFs exist, and these also localize to different subcellular regions (reviewed in Refs. 24 and 25). There are three major groups of the ARFs, typified by ARF1, ARF5, and ARF6. ARF1 is the best characterized and localizes to the Golgi and ER. ARF5 localizes to late endosomes and may translocate to the plasma membrane under some circumstances. ARF6 localizes to the plasma membrane and possibly early endosomes. Localizations for the other ARFs are not well defined, and even the information reported for ARF1, ARF5, and ARF6 remains controversial. The mechanisms of action of the ARFs also differ. ARF1 and ARF5 are brefeldin A-sensitive and are largely cytosolic until agonist stimulation and subsequent GTP loading occurs. In contrast, ARF6 is brefeldin-insensitive, and the majority of it is plasma membrane-bound even in the absence of cellular stimulation.

We previously reported that PLD1 is activated by mammalian ARF1 (mARF1) more effectively than by yeast ARF2 (yARF2) (Fig. 3A, Ref. 20). We examined this response for PLD2Δ(1–308) and unexpectedly found that PLD2Δ(1–308) is more efficiently stimulated by yARF2 than by mARF1 (Fig. 3A). The preference is not readily attributable to the deletion of the amino terminus, since PLD1Δ(1–325) behaves similarly to wild-type PLD1, i.e. it responds better to mARF1. This finding prompted the question of the identity of the mammalian homologue to yARF2. To address this, we examined activation by mARF1, mARF5, and mARF6 and found that mARF6 was a relatively poor stimulator of both PLDs but that mARF5 stimulated PLD2Δ(1–308) more effectively than mARF1 did (Fig. 3B). This finding was examined in more precision by establishing a dose-response curve (Fig. 3C). We confirmed that although mARF1 and mARF5 stimulated both PLDs effectively, mARF5 consistently activated PLD2Δ(1–308) at a greater potency than mARF1 and possibly with greater efficacy as well. In contrast, mARF5 and mARF1 stimulation of PLD1 were not significantly different. mARF5 stimulated PLD1Δ(1–325) slightly more effectively than did mARF1 in three separate experiments, but the magnitude of the difference was variable and considerably smaller than was observed for PLD2Δ(1–308) (Fig. 3C and data not shown).

**Activation of PLD1 and PLD2Δ(1–308) by ARFs Occurs**

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**Fig. 4.** Identification of the regions of mARF1 and yARF2 that confer species specificity to PLD activation. A, COS-7 cell lysates overexpressing the PLD proteins listed were stimulated with 1 μM wild-type or mutant mARF1 or yARF2. Activities are presented relative to the stimulation observed for mARF1, after subtraction of background. The data were compiled from nine experiments. B, minimal regions identified as being involved in the species-specific stimulation of PLD1 and PLD2.
through Overlapping but Discrete Domains—The finding that mARF1 and yARF2 differentially stimulated PLD1 was used previously to define a central portion of mARF1 as the mARF1-specific region responsible for effective stimulation of PLD1 (20). We extended this type of analysis to PLD2 using the previously described mARF1/yARF2 chimeras and some additional ARF mutant proteins altered at amino acids differing in this region between mARF1 and yARF2 (Fig. 4).

The first set of mutant ARF proteins examined (set a in Fig. 4A) consisted of a series of swaps at species-specific amino acids in region I. None of the mutant proteins examined were altered for their relative capacity to stimulate PLD1 or PLD2 (1–308), suggesting that the amino terminus was not responsible for the species-specific activation. The next three groups of mutant proteins (sets b–d) consisted of swaps between regions I, II, and III. Set b demonstrated that the species-specific stimulation was not conferred by region I, confirming the results observed for the region I point mutants. Set c revealed that region III was not required for the mARF1-specific stimulation of PLD1 but does contribute to the yARF2-specific stimulation of PLD2 (1–308). Set d confirmed that the critical region for mARF1-mediated stimulation of PLD1 is region II and that both regions II and III contribute to the yARF2-specific stimulation of PLD2 (1–308). Finally, in set e, a series of swaps at species-specific amino acids in region II was examined for PLD1 stimulation. Only one site, amino acids 82 and 83, was found to be important for the differential stimulation. The critical regions are depicted in Fig. 4B.

Chimeric Analysis of PLD1/PLD2 chimeras—In an analogous manner, chimeric PLD1/PLD2 proteins were generated and assayed in an attempt to locate regions conferring isoform-specific activation properties for PLD1 and PLD2 (Fig. 5). Chimeras A and C consisted of substitutions of amino-terminal portions of PLD2 for the equivalent regions in PLD1. Although NH2-terminally truncated PLD1Δ(1–325) is enzymatically active (although refractory to PKC stimulation), chimeras A and C were essentially inactive. Chimera F exhibited decreased activity, although the ARF responsiveness retained was preferential for yARF2, suggesting that the species-specific interaction site for ARF may lie in the carboxyl terminus.

PLD2 is characterized by high basal activity in vitro, and this constitutive activity is largely lost when the first 308 amino acids are truncated. A chimeric protein in which the first 234 amino acids of PLD2 are substituted by the corresponding region of PLD1 (chimera B) behaves relatively similarly to wild-type PLD2, whereas substitution of the first 308 amino acids (chimera D) results in a protein that behaves relatively similarly to PLD2Δ(1–308). This finding suggests that the region that mediates the high basal activity of PLD2 is located between amino acids 235 and 308. The overall activity of chimera B is nonetheless decreased somewhat except for the PKC response. This suggests that the NH2-terminal PLD1 fragment may be conferring some negative regulation and PKC responsiveness to the chimera, which is consistent with the role proposed for this region (see the PLD1Δ(1–325) construct above). However, PKC responsiveness is not observed for chimera D, which lessens the support for this possibility. Chimera E, for which the carboxyl terminus of PLD1 was substituted for the corresponding region in PLD2 (the reciprocal to chimera F) exhibited only low levels of activity, although its relative stimulation by mARF1 was higher than by yARF2, consistent with the hypothesis that the species-specific ARF binding site is in the carboxyl terminus.

Finally, the loop region unique to PLD1 was inserted in PLD2 (chimera G). Perhaps surprisingly, the resulting protein is catalytically active and was relatively unperturbed by the insertion except for a moderate general decrease in activity. It was previously reported that the loop region empirically acts as
activity. Unlike the amino terminus, which tolerates peptide tags such as HA, green fluorescent protein, or His6 quite well (i.e. the resulting proteins remain enzymatically active), the carboxyl terminus is refractory. The addition of HA or other peptide tags inactivates PLD1.3 This suggested that a free-carboxyl terminus is required for enzymatic activity, as one possibility because it mediates membrane association, potentially through post-translational modification of the terminal amino acid, which is a conserved threonine. The possibility that a free carboxyl terminus was required to mediate membrane association was tested by fusing a Ras membrane localization peptide to the carboxyl terminus, which should block the PLD2 carboxyl terminus but result in the hybrid protein being transported to the membrane surface regardless. Analysis of the chimeric proteins revealed that the role of the conserved carboxyl terminus is unlikely to involve membrane localization because fusion of the Ras membrane localization tag to the carboxyl terminus inactivated PLD2 (Fig. 7). The possibility that the terminal amino acid (threonine) becomes modified was examined by mutating it to alanine, which is not capable of being modified in the same manner as threonine. Examination of this mutant PLD2 revealed that modification of the carboxyl terminus appears unlikely to be critically important, since PLD2-T933A exhibited essentially wild-type PLD2 activity (Fig. 7).

**FIG. 6. In vivo and Western analysis of chimeric PLD proteins.** A, in vivo assays were carried out as described previously and under “Experimental Procedures.” The results shown are representative of three separate experiments conducted, except for the activity for PLD1, which is higher than was normally observed (see Fig. 2C). PLD1Δ(505–621) lacks the PLD1-specific loop region as described previously.2 Control was pGEX-2B. B, Western analysis. Lysates were electrophoresed in SDS-polyacrylamide gel electrophoresis gels, and recombinant proteins were visualized using a monoclonal antibody to detect the HA epitope tag as described previously (7) and under “Experimental Procedures.” Predicted sizes were as follows: PLD1, 120–124 kDa; PLD2, 106 kDa; PLD1Δ(1–325), 84 kDa; PLD2Δ(1–308), 66 kDa. Breakdown or truncated products are observed for PLD2 and all chimeras. The monoclonal antibody nonspecifically detects 50- and 80-kDa cytoplasmic nonspecific proteins in COS-7 cell lysates. The latter coincides with the truncated but correctly sized protein observed for PLD1Δ(1–325).

Taken together, analysis of the chimeric PLDs provides some insight into the role of several regions of the PLD proteins but also reveals that the many of the functions potentially mediated by these regions are not readily transferable. For example, the amino terminus of PLD1 is required for response to PKC-α, but appending it to an amino-terminal truncated PLD2 does not confer PKC-α responsiveness to the hybrid PLD2. Similarly, appending the amino terminus of PLD2 to PLD1 does not increase the hybrid PLD1’s basal activity and in fact renders it inactive.

A Free Carboxyl Terminus Is Required for PLD2 Activity, but Not Because the COOH Terminus Undergoes Modification—As reported previously, the carboxyl terminus of PLD1 is relatively well conserved with PLD2 and is required for enzymatic activity.

**DISCUSSION**

Prior to the molecular cloning of PLD1 and PLD2, it was anticipated that there would be at least two different PLD isoforms (reviewed in Ref. 26). There was general agreement that PLD was activated by agonist stimulation, but several groups had reported that PLD activities originating from different subcellular compartments exhibited significantly different regulatory characteristics. Most frequently, such reports described Rho-selectively responsive or ARF-selectively responsive PLDs. With the cloning of PLD1 and PLD2, it became clear that PLD1 was unexpectedly capable of responding in vitro to all Rho and ARF family members examined as well as PKC-α (2). In vivo, however, PLD1 regulation is less well understood, since phorbol 12-myristate 13-acetate (which activates PKC-α) stimulates it effectively, but activated forms of ARF and Rho excite only minimal PLD1 activation (reviewed in Ref. 2). The in vitro constitutive activity reported for PLD2 was unexpected, since this type of PLD had not previously been detected in cellular fractions enriched for PLD activity.

It was accordingly proposed that PLD2 would be regulated differently in vivo. This idea was supported by the observation that there was a variable and very modest response to ARF by full-length PLD2 in vitro (~1–1.5-fold; e.g. Fig. 2B).

Since the original description of PLD2, several hypotheses have been developed to resolve the issue of in vitro constitutive activity versus presumed regulation in vivo. These included PLD2-specific inhibitors (15), sequestration of PLD2 by unstimulated tyrosine kinase receptors (16), and regulation of phosphatidylinositol 4,5-bisphosphate levels (27). We suggest here that PLD2 may be regulated by ARF family members in vivo either through the action of unknown factors that render it strongly ARF-dependent or by being truncated to an ARF-responsive isoform.

In a prior report, we described the finding that the amino terminus of PLD1 is required for PLD1 to respond to PKC-α. As part of the present work, we engineered the corresponding truncated PLD2 protein and found that it lost most of its basal activity and became ARF-responsive but not Rho- or PKC-responsive (Fig. 1). Since the Rho interaction site on PLD1

**TABLE**

| PLD Activity | ARF | Rho |
|--------------|-----|-----|
| PLD1         | Yes | Yes |
| PLD2         | No  | Yes |

A Free Carboxyl Terminus Is Required for PLD2 Activity, but Not Because the COOH Terminus Undergoes Modification—As reported previously, the carboxyl terminus of PLD1 is relatively well conserved with PLD2 and is required for enzymatic activity.
The ARF and PLD proteins are likely to interact through the two ARF switch regions (amino acids 41–55 and 70–80). Such sequences with the evolution of lowered basal activity and the capacity for effector stimulation.

Both PLDs, no specific types of interactions are ruled out by our findings. PLD1 is best activated by ARF1, consistent with the prior reports that both are located in perinuclear compartments (4, 25). PLD2 was previously reported to localize to the plasma membrane in serum-starved cells and to submembranous vesicles potentially part of an endocytic cycle after serum stimulation (4). PLD2 is not activated well by ARF6, which has been reported to localize to the plasma membrane (25), but it is activated well by ARF5, which may mediate several endocytic steps (30). Nonetheless, since all of the ARF proteins activate both PLDs, no specific types of interactions are ruled out by our findings.

The ARF and PLD proteins are likely to interact through the two ARF switch regions (amino acids 41–55 and 70–80). Such regions mediate effector actions for all small GTP-binding proteins. The species-specific activation of PLD1 and PLD2 by mARF1 and yARF2 does not appear to originate from species-specific loop sequences with the evolution of lowered basal activity and the capacity for effector stimulation.

PLD1 and PLD2 have been proposed to localize to different subcellular regions (4). The finding that PLD1 and PLD2 preferentially respond to different ARFs supports and extends this finding. PLD1 is best activated by ARF1, consistent with the prior reports that both are located in perinuclear compartments (4, 25). PLD2 was previously reported to localize to the plasma membrane in serum-starved cells and to submembranous vesicles potentially part of an endocytic cycle after serum stimulation (4). PLD2 is not activated well by ARF6, which has been reported to localize to the plasma membrane (25), but it is activated well by ARF5, which may mediate several endocytic steps (30). Nonetheless, since all of the ARF proteins activate both PLDs, no specific types of interactions are ruled out by our findings.

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specific amino acids in the ARF switch regions, since, with the exception of a single difference that does not alter the ARF responsiveness (P76S, Fig. 4A), the switch regions are conserved. Instead, a site adjacent to the second switch region (amino acids 82 and 83) appears to be critical and may cause a subtle conformational change in the switch:effector orientation. Although ARF5 was the most potent stimulator tested for PLD2, there may be other ARFs that are better. In addition to the three ARFs that were not tested, ARF-like proteins exist as well that also stimulate endogenous PLD (31, 32).

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