Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs

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The signaling enzyme phospholipase D1 (PLD1) facilitates membrane vesicle trafficking. Here, we explore how PLD1 subcellular localization is regulated via Phox homology (PX) and pleckstrin homology (PH) domains and a $PI4,5P_2$-binding site critical for its activation. PLD1 localized to perinuclear endosomes and Golgi in COS-7 cells, but on cellular stimulation, translocated to the plasma membrane in an activity-facilitated manner and then returned to the endosomes. The $PI4,5P_2$-interacting site sufficed to mediate outward translocation and association with the plasma membrane. However, in the absence of PX and PH domains, PLD1 was unable to return efficiently to the endosomes. The PX and PH domains appear to facilitate internalization at different steps. The PH domain drives PLD1 entry into lipid rafts, which we show to be a step critical for internalization. In contrast, the PX domain appears to mediate binding to PI5P, a lipid newly recognized to accumulate in endocytosing vesicles. Finally, we show that the PH domain–dependent translocation step, but not the PX domain, is required for PLD1 to function in regulated exocytosis in PC12 cells. We propose that PLD1 localization and function involves regulated and continual cycling through a succession of subcellular sites, mediated by successive combinations of membrane association interactions.

Introduction

Phospholipase D1 (PLD1)* is activated in a variety of cell types in response to hormone, neurotransmitter, and growth factor–stimulated receptor signaling (Liscovitch et al., 2000), and it hydrolyzes the membrane phospholipid phosphatidylcholine to generate phosphatidic acid (PA) and choline. PA has been proposed to function as a lipid signal that activates enzymes such as phosphatidylinositol 4-phosphate 5-kinase, which generates $PI4,5P_2$. It may also act as a lipid anchor for a growing number of proteins involved in membrane trafficking, as a physical regulator of membrane curvature, or become further metabolized to diacylglycerol and lysoPA. Although other functions have been proposed, cell biological roles for PLD1 most commonly involve regulation of vesicular trafficking. Such roles include promoting outward trafficking of secretory vesicles in neuroendocrine (Chen et al., 1997), adipocyte (Emoto et al., 2000), or mast cells (Brown et al., 1998; Choi et al., 2002), or the fusion of secretory granules into the plasma membrane (PM) during regulated exocytosis in neuroendocrine and neural cells (Humeau et al., 2001; Vitale et al., 2001).

PLD1 exhibits a variable pattern of subcellular steady-state localization that differs in different cell types. In COS-7 and HeLa cells, PLD1 is found in perinuclear structures including Golgi, early endosomes, late endosomes, and multi-vesicular bodies/lysosomes (Colley et al., 1997; Freyberg et al., 2001; Lucocq et al., 2001). A very small fraction of PLD1 is found on the PM in these cells; however, PLD1 primarily localizes to the PM in adrenal chromaffin and PC12 cells (Vitale et al., 2001). Under stimulatory conditions in mast cells and adipocytes, PLD1 colocalizes with secretory vesicles and

translocates with them to the PM (Brown et al., 1998; Emoto et al., 2000; Choi et al., 2002). This implies that PLD1 must then return from the PM to intracellular vesicles, and colocalization of PLD1 with internalized EGF receptor–containing endosomes has been described previously (Hughes and Parker, 2001). Together, these reports suggest that the localization of the PLD1 is not static and that regulated translocation and recycling may be crucial to its proper functioning.

To begin to explore this issue, we undertook to determine the mechanisms that target PLD1 to specific subcellular compartments. The mechanisms were anticipated to be complicated because PLD1 contains several potential membrane association domains or motifs, including a pleckstrin homology (PH) domain, a Phox homology (PX) domain, and a putative $PI4,5P_2$-interacting basic amino acid–rich motif (Frohman et al., 1999), all of which have been demonstrated to mediate membrane localization in other proteins through binding to lipid or protein targets (for review see Lemmon and Ferguson, 2000; Cockcroft and De Matteis, 2001; Sato et al., 2001). In present paper, we show that these domains appear to mediate distinct steps in the PLD1-cycling pathway in response to dynamic changes in the cells.

Results

PLD1 localizes to perinuclear vesicles in COS-7 cells and translocates to the PM on cellular stimulation in an activity-facilitated manner

A typical perinuclear vesicular localization pattern for PLD1 in resting cells as reported previously by us and others (Freyberg et al., 2001; Lucocq et al., 2001) is shown in Fig. 1 A. A subset of the vesicles colocalize with the transferrin receptor (TfR), which is found in sorting/recycling endosomes (Fig. 1 B); others colocalize with EEA1, an early endosomal marker, and some colocalization is also observed with GM130, a Golgi marker. Infrequently (unpublished data), a small amount of PLD1 is found on the PM. The relative distribution of wild-type PLD1 in serum-starved cells was determined by examining 300 cells. As shown in the left panel of Fig. 1 E, PLD1 was exclusively found in such perinuclear vesicles in >90% of the cells. PMA stimulation, which activates PKC, resulted in translocation of most or all of the PLD1 to the PM and peripheral vesicles (Fig. 1, C and C′) in the majority of cells (>80%, Fig. 1 E) within 2 h. By 4 h after stimulation, much of the PLD1 had returned to the perinuclear vesicles (Fig. 1, D and E), although some residual localization on the PM remained in some cells (Fig. 1 D′).

A similar pattern of localization was observed in serum-starved cells for PLD1-K898R, a catalytically inactive allele (Sung et al., 1997). However, substantially decreased translocation to the PM occurred with PMA stimulation (summarized in Fig. 1 E, right), indicating that the translocation is facilitated when PLD1 is capable of being activated. This is consistent with several prior reports that increases in PLD1 activity facilitate vesicular trafficking, whereas the catalytically inactive allele can act as a dominant-negative mutant (Chen et al., 1997; Brown et al., 1998; Emoto et al., 2000; Humeau et al., 2001; Vitale et al., 2001; Choi et al., 2002).
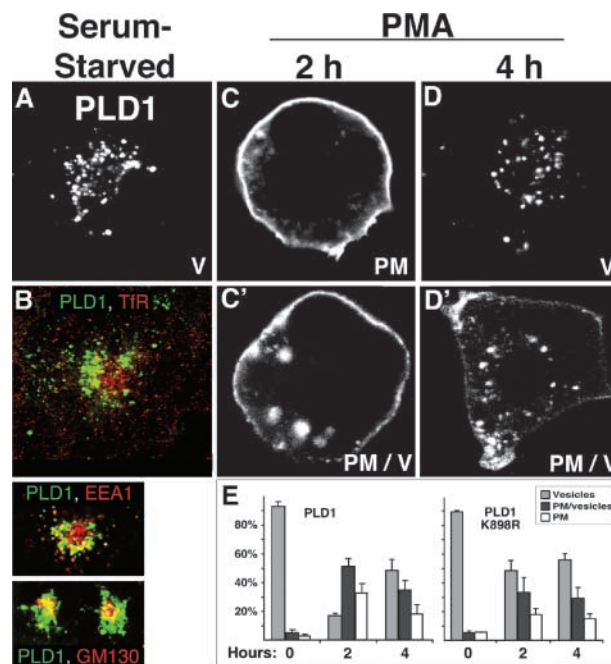

Figure 1. **PLD1 recycles between PM and intracellular vesicles in an activity-facilitated manner on PMA stimulation.** COS-7 cells were transiently transfected with an HA-tagged PLD1 expression plasmid. 36 h later, the cells were stimulated with PMA for varying periods of time (A, C, and D) and then fixed and immunostained using an anti-HA mAb. Images were captured using a confocal microscope (TCS SP2; Leica) using a green fluorophore-labeled secondary antibody. Colocalization with EEA1, GM130, and TfR (B) was performed using far-red as the second fluorophore. On stimulation by PMA, PLD1 translocates to the PM; shown is a 2-h time point (C). C′ and D′ show additional images of cells in which translocation or return was not complete at the respective time points. E depicts a tabulation of percent localization for 300 cells at each time point, including SDs that were determined by conducting the experiment three times. Vesicles (V), plasma membrane (PM), and intermediate cells (PM/V) were scored as indicated by the labels in the bottom right corner of each image.

Summary of constructs

To delineate the mechanisms through which PLD1 becomes targeted to different subcellular sites, we generated a series of mutant alleles (Fig. 2). We focused on the four regions most likely to mediate membrane association and specific subcellular targeting: a PX domain, a PH domain, a $PI4,5P_2$ binding motif, and the loop region of PLD1. The mutants were confirmed to be expressed at similar levels and at the expected sizes (unpublished data), and except for the isolated domains, were assayed for regulated activity in vitro using ARF1 as a stimulator. With one exception (PLD1 R691,695G, in which the $PI4,5P_2$-binding site is disrupted), all of the mutants were active at approximately wild-type levels. It should be noted that it is relatively easy to destabilize PLD1 through manipulations involving mutagenesis, resulting in inactive, presumably substantially misfolded, and mislocalized protein (Zhang et al., 1999; Du et al., 2000). A number of variations on the constructs shown in Fig. 2 were inactive and were discarded. Perhaps not surprisingly, the exact boundaries at which deletions were made turned out to be crucial to preserve enzymatic activity (and presumably,
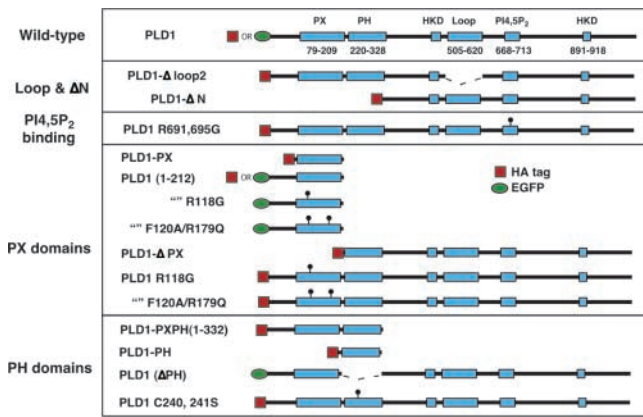
Figure 2. **Summary of the PLD1 constructs used in this paper.** The constructs were NH$_2$-terminally HA- or EGFP-tagged as indicated. See Results for details.

adequate folding). This issue could account for discrepancies between our results and a report on mutagenesis of the PLD1 PH domain in which the described mutations generated inactive alleles (Hodgkin et al., 2000).

A distinct structure in PLD1 consists of a central 138 amino acid "loop" region found in PLD1 (and *Drosophila* and *Caenorhabditis elegans* PLD), but not in the other mammalian isoform, PLD2 (Hammond et al., 1995; Colley et al., 1997). To examine this region for possible contributions to PLD1's pattern of localization, we characterized a mutant allele lacking it (PLD1(Δloop2)) that is expressed at wild-type levels and is fully enzymatically active (Sung et al., 1999b). However, it exhibited a wild-type localization pattern (unpublished data), suggesting that it is not involved in membrane localization.

A central basic amino acid–rich PI4,5P$_2$-interacting site is required and suffices to promote PLD1 localization to the PM after cellular stimulation

The PLD1 NH$_2$ terminus would be expected to play a role in localization because it contains both PX and PH domains, each of which have been shown to target many other proteins to membranes through binding to lipid or protein targets. Indeed, a PLD1 allele lacking the PX- and PH-containing NH$_2$ terminus (PLD1-ΔN) that is enzymatically active (Sung et al., 1999b) is cytosolic in quiescent cells (Fig. 3 A), demonstrating that the NH$_2$ terminus is required for localization to perinuclear membrane vesicles. However, on stimulation by PMA, dramatic recruitment to the PM was observed (in >85% of the cells). Moreover, once recruited to the PM, this mutant allele persistently localized there; no reentry into the cell was observed by 4 h after stimulation (Fig. 1, D and E). The first result indicates that the mechanism responsible for PM recruitment does not involve the PX or PH domain, leaving the potential PI4,5P$_2$-interacting site as the most likely candidate. The second result suggests that the PX or PH domain mediates internalization.

Previously, we demonstrated that an arginine/lysine-rich sequence found in the center of PLD2 (aa 554–575) and conserved in PLD1 bound vesicles containing PI4,5P$_2$ and was responsible for the activation of PLD2 observed in the

presence of PI4,5P$_2$ (Sciorra et al., 1999). On the other hand, Wakelam and colleagues reported that the PI4,5P$_2$-interacting and activating site in PLD1 lies in its NH$_2$-terminal PH domain (Hodgkin et al., 2000). Therefore, we set out to assess whether the PLD1 arginine/lysine-rich sequence (aa 691–712) is important for PI4,5P$_2$-mediated binding and activity, using an allele mutated at this site. We found that the mutant PLD1 allele, PLD1-R691G,R695G, exhibited only 5% of the wild-type PLD1 ARF1 simulated-response in a PLD in vitro assay. Similar results were observed using an in vivo PLD assay (unpublished data). Moreover, we found that PLD1-R691G,R695G no longer exhibits an increased affinity for PI4,5P$_2$-containing lipid vesicles (Fig. 3 B). In contrast, all of our PLD1 mutants lacking the PH domain are still active and are still PI4,5P$_2$-dependent (Fig. 2; also see Sung et al., 1999b). Together with the prior reports, we would conclude that PLD1, like PLD2, is activated by interaction with PI4,5P$_2$ at its central arginine/lysine-rich sequence rather than through its NH$_2$-terminal PH domain.

Next, we examined subcellular localization of the PI4,5P$_2$-noninteracting mutant PLD1 allele in vivo. The PLD1 R691G,R695G mutant exhibited a complicated pattern of localization: it still colocalized with wild-type PLD1 in peri-
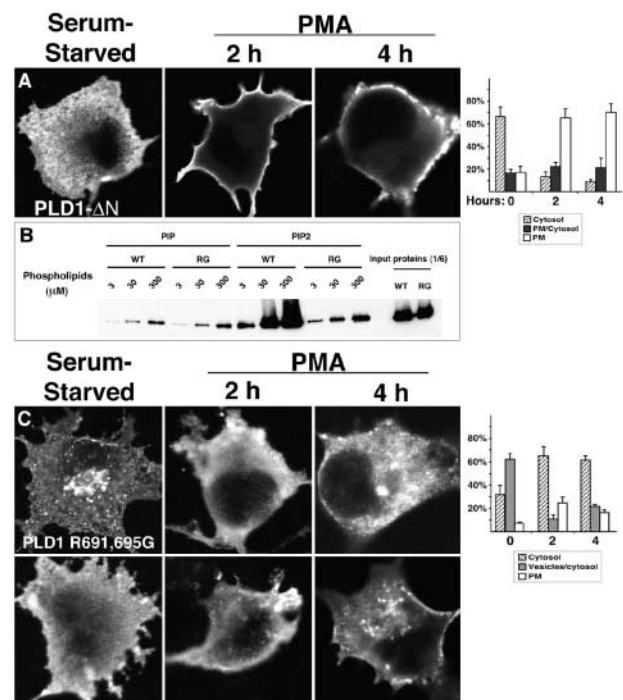

Figure 3. **The NH$_2$ terminus contains targeting signals for the endosomes and Golgi and is required for internalization, whereas the PI4,5P$_2$-interacting motif mediates recruitment to the PM.** (A and C) COS-7 cells were transiently transfected with deletion or mutated PLD1 constructs as shown in Fig. 2, and were processed as described in Fig. 1. In C, two examples are shown for each time point. (B) Characterization of the PLD1 PI4,5P$_2$-binding motif. Wild-type (WT) and R691G,R695G (RG) mutant PLD1 proteins purified from baculovirus-infected sf9 cells were mixed with different amounts of liposomes containing PC, PE, and 5% PIP or 5% PI4,5P$_2$. The vesicles were sedimented by centrifugation and the pellets were analyzed by Western blotting for cosedimentation of PLD1.

nuclear vesicles (Fig. 3 C, top serum-starved panel), suggesting that the preferred site of membrane localization remained unchanged. However, cytosolic localization was also observed and in some cells dominated (Fig. 3 C, bottom serum-starved panel), suggesting that PI4,5P$_2$-interactions contribute to the avidity of PLD1 perinuclear vesicular localization, although they are not strictly required. On PMA stimulation, little recruitment to the PM was observed; in fact, most of the protein relocated to the cytosol. This confirms that the PX and PH domains do not mediate PLD1 translocation to the PM under these circumstances, and that the PI4,5P$_2$-interacting site is critical.

The increased cytosolic localization of PLD1 R691G, R695G in COS-7 cells was also confirmed using cell fractionation by microcentrifugation. For wild-type PLD1, such centrifugation suffices to pellet all of the enzyme (Zhang et al., 1999). In contrast, part ($\sim$50%) of the PLD1-R691G, R695G protein was not pelleted under these conditions, indicating that a substantial portion of it is not in association with membranes (unpublished data). This loss of association does not ensue from its lack of activity because the catalytically inactive mutant allele PLD1-K898R is membrane associated similar to the wild-type protein (Zhang et al., 1999).

The PLD1 PX domain regulates internalization potentially through a PI5P-dependent mechanism

The PX domain was initially defined as an $\sim$120-amino acid conserved region present in the p40^{phox} and p47^{phox} subunits of NADPH oxidase (Ponting, 1996). Recent work from many groups has revealed that PX domains, which are now known to be present in more than 60 proteins, frequently bind PI3P and PI3,4P$_2$ and act as endosomal targeting domains (for review see Sato et al., 2001). Some PX domains have also been reported to bind PI4,5P$_2$ or protein targets.

The PX domain plays a relatively subtle role in PLD1 localization in serum-starved cells (Fig. 4 A) because an allele lacking the PX domain is still found in perinuclear vesicles. However, the nature of the vesicles targeted appears to be shifted because nearly uniform colocalization in sorting/recycling endosomes was observed with the TfR and the dominant-negative ARF6 mutant T27N (unpublished data; pattern of localization of TfR and ARF6-T27N discussed in D'Souza et al., 1995), but colocalization with GM130 or EEA1 was decreased (unpublished data). However, more obviously, the PLD1-ΔPX allele still underwent robust translocation to the PM after PMA stimulation, but did not undergo wild-type-like reentry (Fig. 4 A). Similar results were observed for point mutants (R118, F120/R179) in the PX domain at amino acid residues predicted to be required for interaction with phosphoinositide lipid anchors (based on the published structures of the p40 and p47 PX domains and sequence alignment of the entire set of known PX domains; for review see Sato et al., 2001). Together, these findings suggest that the PX domain is required in a phosphoinositide-dependent process for PLD1 to reenter COS-7 cells once it has become localized to the PM.

Classical PX domains such as p40 bind PI3P, and through this interaction localize to EEA1-containing endo-
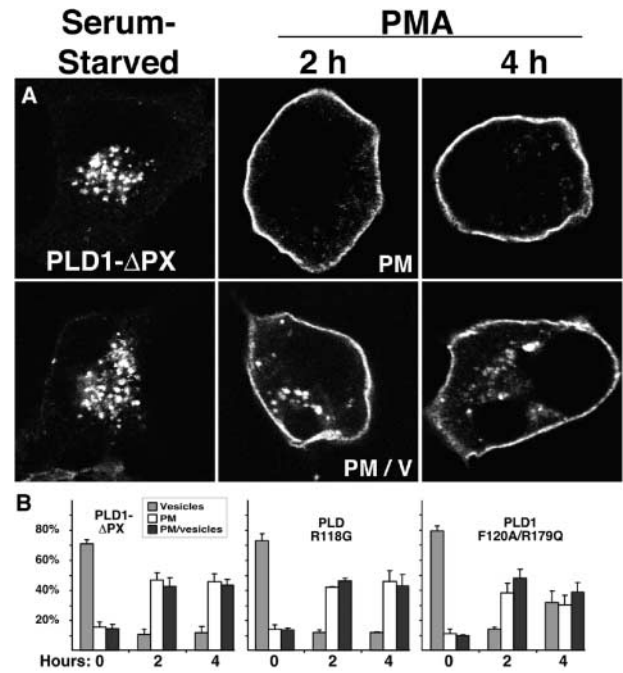

Figure 4. **The PX domain is required for PLD1 internalization potentially through phosphoinositide interactions, but is not required for localization to at least some types of endosomes.** COS-7 cells were transiently transfected with deletion or mutated PLD1 constructs as shown in Fig. 2, and were processed as described in Fig. 1. Two examples are shown for each time point to indicate how cells were scored.

somes (Kanai et al., 2001). This mechanism can be demonstrated in NIH3T3 cells using p40PX-EGFP, which localizes to endosomes in a discrete punctate pattern that is disrupted on addition of wortmannin, a specific inhibitor of PI3 kinases (Fig. 5 C). The isolated PLD1-PX domain localizes to cytosolic vesicles (Fig. 5 A), but colocalization with EEA1 was not observed, suggesting that interaction with PI3P does not underlie its targeting mechanism. Supporting this observation, addition of wortmannin to the transfected cells did not affect PLD1-PX domain localization (Fig. 5 C). Moreover, mutation of R118 did not alter localization in quiescent cells of either the isolated domain or the full-length protein (Fig. 5 B), suggesting that the targeting mechanism here is distinct from the potential phosphoinositide-interacting one that regulates PLD1 reentry (Fig. 4 B). Finally, examination of the binding specificity of the PLD1-PX domain expressed and purified from bacteria revealed a significant interaction with PI5P (Fig. 5 D), a PI that has recently been proposed to accumulate on endocytic vesicles from the hydrolysis of PI4,5P$_2$ (Terebiznik et al., 2002).

The PLD1 PH domain also mediates internalization

PH domains are well known to bind different kinds of phosphoinositides; a minority of the domains bind through strong and specific interactions; however, most of them characteristically bind through nonspecific low affinity interactions (Lemmon and Ferguson, 2000). In addition, some PH domains bind protein targets. Such interactions
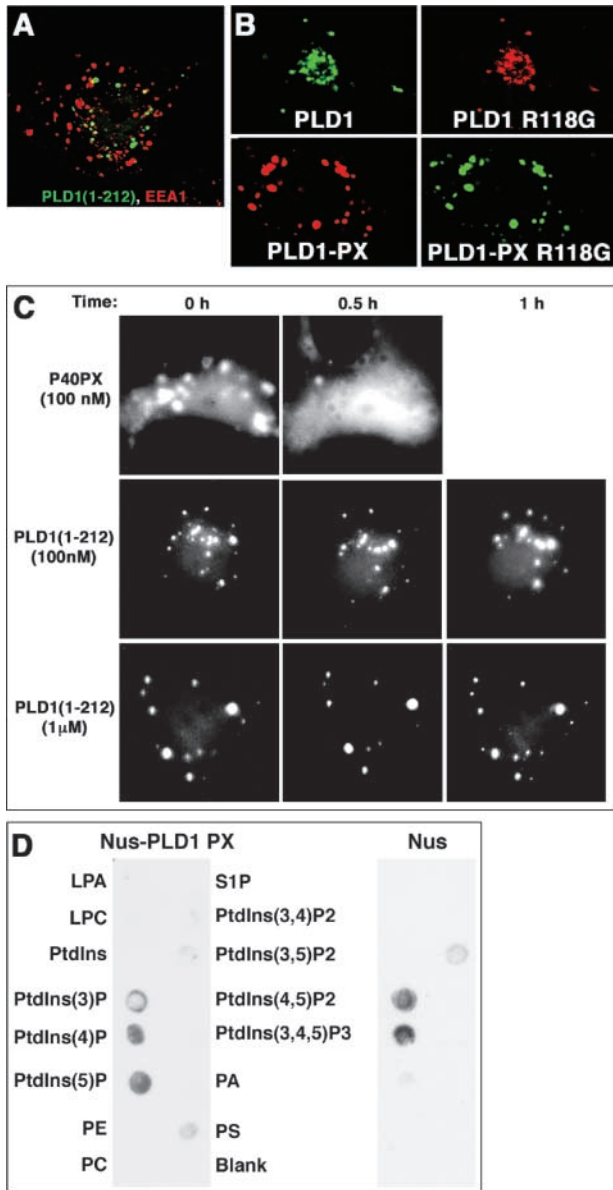
Figure 5. **The PX domain mediates endosomal association independent of PI3 kinase products or lipid interactions, but may facilitate reentry through interaction with PI5P**. (A and B) COS-7 cells were transiently transfected with wild-type or mutated full-length PLD1 or isolated PX domain constructs as shown in Fig. 2, and were processed as described in Fig. 1. (A) The isolated PLD1 PX domain localizes to cytosolic vesicles, but does not colocalize with EEA1 endosomes (or GM130, not depicted). Not depicted are similar results that were observed for the isolated domain and for the full NH$_2$ terminus (1–212, which contains an extra 70 amino acids, as shown in Fig. 2). (C) COS-7 cells transiently transfected with p40 or PLD1 EGFP-PX domain constructs were followed over time after the addition of varying concentrations of wortmannin. (D) Purified bacterial Nus protein or Nus–PLD1 PX domain fusion protein was used to probe a set of phospholipids spotted on nitrocellulose strips, after which the proteins were detected using an anti-Nus mAb.

sometimes serve to activate PH domain–containing proteins, but most often assist in directing their correct localization. As shown in Fig. 3 B, affinity for PI4,5P$_2$ does not appear to represent a major component of the role of the PLD1 PH domain.

A catalytically active PLD1-ΔPH allele exhibited cytosolic localization (Fig. 6, A and B) and exhibited some (but not complete) PMA-stimulated translocation to the PM followed by delayed reentry. The lack of robust PMA-elicited translocation (for example, in comparison to the PLD1-ΔN allele described in Fig. 3, which lacks even more sequence) suggests that the deletion of the PH domain may have affected the functionality of the other domains, despite the fact that catalytic activity was maintained.

Sugars et al. (1999) have reported that the PLD1 PH domain is palmitoylated at two cysteines (positions 240 and 241), and that the loss of this palmitoylation causes PLD1 to translocate from perinuclear vesicles to the PM. Although we would agree with this localization pattern in cells maintained in media containing 10% FCS (unpublished data), we also observed that much of the PLD1 C240S/C241S localized to the cytosol in serum-starved cells (Fig. 6 C). However, robust translocation to the PM again with extended
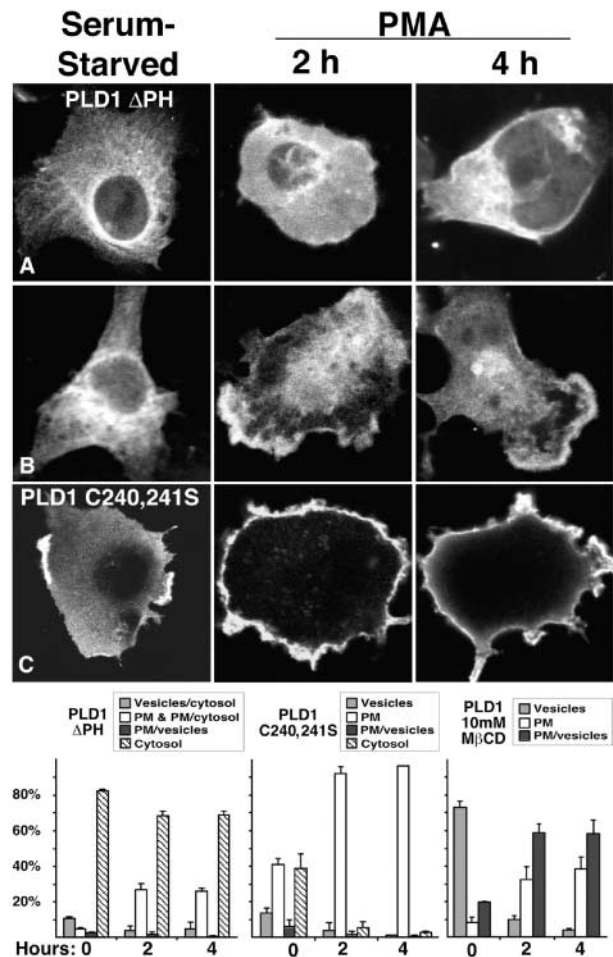

Figure 6. **The PLD1 PH domain targets endosomes in quiescent cells and mediates internalization from the PM through controlling entry into lipid rafts.** COS-7 cells were transiently transfected with deletion or mutated PLD1 constructs as shown in Fig. 2, and were processed as described in Fig. 1. (A and B) Two examples are shown for the deletion construct at each time point. Where indicated (rightmost quantification panel), methyl-β-cyclodextrin was added to the culture medium for 45 min and then thoroughly washed away before PMA stimulation.

persistence was observed after PMA stimulation. Zacharias et al. (2002) have reported that the addition of two or more palmitates to proteins drives their accumulation into lipid rafts; wild-type PLD1 can also be found there (Kim et al., 2000). Taking these observations together, we would propose that PLD1 can translocate to the PM even if lacking palmitoylation, but cannot enter into lipid rafts, and that entry into the rafts is a key step in the intracellular reentry pathway.

To test the importance of lipid rafts for PLD1 trafficking, we next examined whether preexposure of cells to methyl-β-cyclodextrin, an agent that disrupts lipid rafts, affected wild-type PLD1 internalization. As shown in the summary in Fig. 6 (right), in cells lacking lipid rafts, PLD1 localized normally to perinuclear vesicles and translocated to the PM with PMA stimulation, but was unable to cycle back to the intracellular vesicles with normal kinetics. Together, these findings demonstrate that entry of PLD1 into lipid rafts via palmitoylation is a critical step in its cycling pathway.

The isolated PLD1 PH domain associates with membranes weakly (Fig. 7). It localizes in resting cells to perinuclear membrane structures. With PMA, a very weak translocation is observed to the PM (Fig. 7), suggesting that the PH domain may contribute to a small degree to regulated translocation, although this is minor at best in the context of the full-length protein (as shown in Fig. 3).

The PH domain–mediated translocation step into rafts is required for PLD1 facilitation of regulated exocytosis

Previously, we reported that PLD1 is expressed in neuroendocrine cells and facilitates regulated exocytosis of secretory granules (Vitale et al., 2001). In the assay system used for those analyses, the PC12 cells used were cultured in 15% serum, and exocytosis was triggered by the elevation of potassium, which induces depolarization. PLD1 localized to the PM in these cells and increased exocytotic responses when overexpressed, whereas the inactive allele, PLD1-K898R, which localized similarly, inhibited exocytosis (Fig. 8; see also Vitale et al., 2001). We also reported that interaction with $PI4,5P_2$ was potentially required for the PM localization in nondepolarized cells because the PLD1-R691,695G mutant allele localized to the cytosol and did not alter the extent of exocytosis. Using some of the mutant alleles gener-
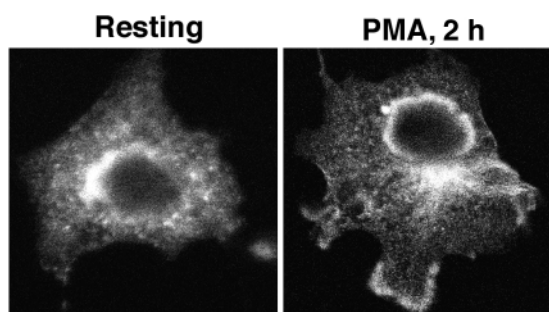

Figure 7. **The isolated PLD1 PH domain associates only weakly with internal and plasma membranes, both in resting cells and stimulated cells.** COS-7 cells were transfected with plasmids expressing the PH domain flanked by leader and trailing sequences.
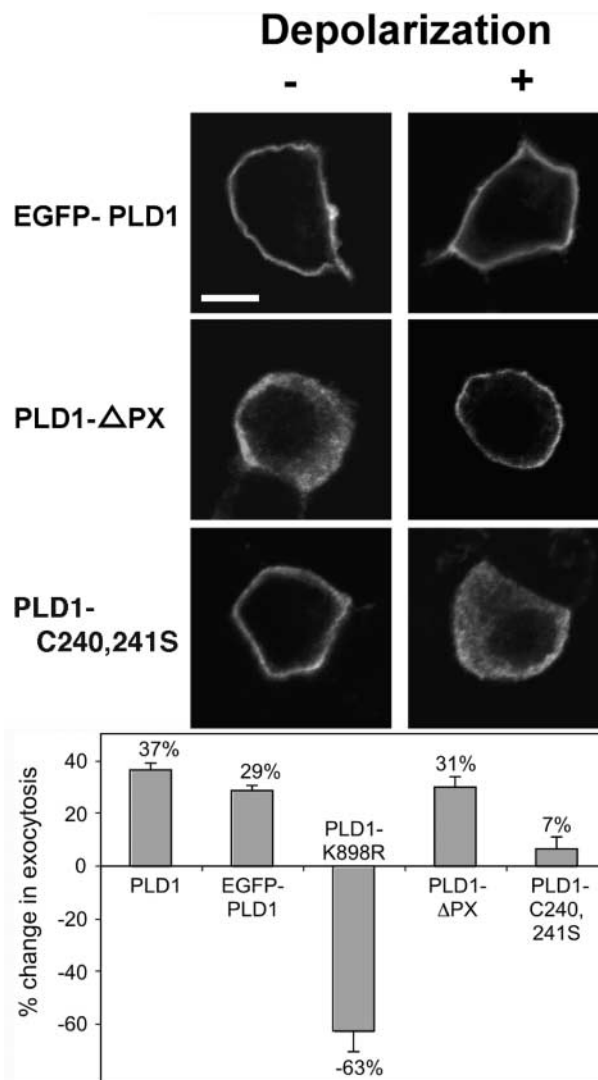

Figure 8. **PLD1 localization affects regulated exocytosis in PC12 cells.** PC12 cells were cotransfected with plasmids encoding hGH and tagged PLD1 alleles. 48 h after transfection, cells were incubated for 10 min in calcium-free Locke's solution (nondepolarized) or in 59 mM K^+ solution (depolarized). (Top) Cells were immunostained to visualize the PLD1 protein by confocal microscopy. Bar, 5 μm. (Bottom) Assay for regulated exocytosis. The total GH content in control and PLD1-expressing cells was in the range of 5 ng/well. The net K^+-evoked secretory response was obtained by subtracting the basal hGH release from the hGH release evoked by 59 mM K^+. In these experiments, basal release ranged from 5.0 to 5.7%. The net K^+-evoked GH release was 25.7% ± 0.4 from empty plasmid-transfected control cells, and, depending on the allele, ranged from 12.8 to 32.3% in PLD1-expressing cells. Finally, the net secretion under experimental conditions was divided by the net secretion in control cultures to estimate the percent change in regulated exocytosis. The experiments were performed twice in triplicate, and SEM is presented.

ated in this report, we now further examine in PC12 cells whether the different PLD1 membrane-association motifs control its subcellular distribution, and what the importance of PLD1 localization is in regulating exocytosis.

As shown in Fig. 8, PLD1 localizes to the PM both before and after depolarization. In contrast, the PLD1-ΔPX allele localizes to the cytoplasm before depolarization, revealing that it is a combination of $PI4,5P_2$ and PX domain interac-

tions that targets PLD1 to the PM in this setting. However, PLD1-ΔPX translocates to the PM and facilitates exocytosis on depolarization, demonstrating that the PX domain is no longer required for PM localization or function in secretagogue-stimulated cells. This could be because the $PI4,5P_2$ interaction is strengthened (due to $PI4,5P_2$ levels rising at the PM on depolarization). Alternatively, PLD1 may shift to a different subcompartment within the PM. The latter hypothesis is supported by the results shown for the PLD1-C240,241S allele. This allele is found at the PM in the non-depolarized state, demonstrating that in this setting, the PX and $PI4,5P_2$ interactions suffice in the absence of palmitoylation to promote PM localization. However, on depolarization, the allele falls into the cytoplasm, indicating that the PX and $PI4,5P_2$ interactions no longer suffice to retain PLD1 on the PM. Moreover, the lack of facilitation of exocytosis by this allele reveals that it becomes mislocalized without having the opportunity to mediate its normal functional role in this event. Together, these results confirm that a combination of three separate interaction mechanisms (PX-, PH-, and $PI4,5P_2$-interacting) regulates PLD1 localization to the PM under resting and different stimulatory conditions, and that this is important for its functional role.

Discussion

PLD1 cycles using a complex set of membrane determinants

Cycling of PLD1 from perinuclear locations to the PM and back may occur more commonly than had been appreciated. We and others had reported translocation of PLD1 in several specialized settings, such as insulin-stimulated adipocytes (Emoto et al., 2000) and degranulating mast cells (Choi et al., 2002), but in most reports, the localization has been described as static in the face of agonist stimulation. In establishing the system used for the experiments reported here, we found that culture in 10% serum desensitized COS-7 cells; robust translocation of PLD1 was best observed subsequent to prior serum starvation. In fact, this is the approach used to study Glut-4 glucose transporter translocation in adipocytes, and under similar conditions, we have observed regulated cycling of PLD1 in response to both receptor tyrosine kinase signaling (insulin receptor) and G protein–coupled receptor signaling (angiotensin II; unpublished data). The intracellular path taken by PLD1 is not clear. In the resting state, PLD1 is found in a complex set of vesicles including EEA1-containing endosomes, and TfR-, dominant-negative ARF6-containing sorting/recycling vesicles, suggesting that there may be both short and long routes through which PLD1 cycles, as has been found for other cycling transducers. PLD1 can also be found in the Golgi compartment, adding to this complexity. It is not presently clear which routes of trafficking are predominant.

Colocalization studies of PLD1 and Glut-4 containing storage vesicles or mast cell granules have suggested that the mechanism of PLD1 trafficking to the PM involves cotranslocation on the vesicles themselves. However, the trafficking step is not a passive one; in mast cells (Choi et al., 2002) and as shown here for COS-7 cells (Fig. 1), PLD1 activity promotes increased trafficking of the vesicles and thus the translocation/cycling of PLD1 is linked to its physiological functioning.

In this paper, we examine the localization of wild-type and mutant PLD1 alleles in different states of cellular activation in two different cell types. Three different domains or motifs (PX-, PH-, and $PI4,5P_2$-interacting) in PLD1 were shown to affect PLD1 localization and translocation at different points in these states.

$PI4,5P_2$ interaction through a central basic amino acid–rich motif

The observation that the allele lacking both the PX and PH domains (PLD1-ΔN, Fig. 3 A) is in the cytosol in serum-starved COS-7 cells but translocates to the PM on PMA stimulation demonstrates that (1) the PX and PH domains are required for localization to perinuclear vesicles in this state; (2) the $PI4,5P_2$-interacting site suffices for membrane association if $PI4,5P_2$ levels become sufficiently elevated, as happens broadly at the PM when COS-7 cells are artificially and powerfully stimulated by PMA. This motif also contributes to PLD1 interactions with perinuclear vesicles because a weakened association with these vesicles is observed even in resting cells when the $PI4,5P_2$-interacting site is crippled (Fig. 3 C); and (3) The PX- and PH-mediated interactions do not suffice to localize PLD1 to the PM because PMA-promoted attempted translocation of PLD1 to the PM largely fails (i.e., it redistributes into the cytoplasm) when the $PI4,5P_2$-interacting site is crippled (Fig. 3 C).

PC12 cells cultured in 15% serum could be in a state of modest activation in comparison to serum-starved COS-7 cells (although less so than PMA-stimulated COS-7 cells). Alternatively, there may be cell type–specific differences that affect how PLD1 localizes in each cell type. For PC12 cells as assayed here, the $PI4,5P_2$ interaction is critical (Vitale et al., 2001), but not sufficient (Fig. 8) for PM localization because the PX domain is also required. Interestingly, once depolarization occurs, the PX domain contribution is no longer required. A possible explanation is that depolarization leads to increased levels of $PI4,5P_2$ at the PM. Indeed, $PI4,5P_2$ located at the PM is important for exocytosis in mast and PC12 cells (Holz et al., 2000; Way et al., 2000). Moreover, it has been proposed that the docking and fusion sites for exocytosis are defined by $PI4,5P_2$-containing lipid rafts at the PM that allow structural and spatial organization of the secretory machinery (Chamberlain et al., 2001; Lang et al., 2001). PLD1 entry into these exocytotic sites as mediated by the palmitoylated PH domain may be required for the $PI4,5P_2$-interacting residues to have access to the sites at which $PI4,5P_2$ is accumulating. Because PA activates PI5K, which generates $PI4,5P_2$, the recruitment of PLD1 into rafts may lead to a $PI4,5P_2$/PLD1 positive feedback loop that facilitates exocytosis. These ideas are supported by the observation that PLD1 lacking palmitoylation (C240,241S) falls into the cytoplasm and fails to promote exocytosis on depolarization (Fig. 8). This suggests that the translocation into rafts is required for PLD to mediate its function in regulated exocytosis, and that the environment for PM association outside

of the rafts becomes less hospitable because either the PX-interacting protein or lipid becomes less accessible, or PI4,5P$_2$ levels diminish. In contrast, PMA stimulation of COS-7 cells leads to generalized increases in PI4,5P$_2$ across the PM, and thus entry into lipid rafts is not required for the PI4,5P$_2$-interacting site to promote PM localization of PLD1 in that setting.

PX domain interactions

Our findings suggest that the PX domain may mediate more than one type of association. In resting cells, the PX domain promotes association with cytosolic vesicles (Fig. 5). This association is not disrupted by mutation of amino acids predicted to interact with phospholipids (e.g., R118), nor does it depend on interactions with PI3 kinase products. This suggests that the interaction may be mediated through association with an unknown protein partner and is consistent with recent papers that have reported that the region encompassing the PLD1 PX domain may interact with specific protein targets such as α-actinin (Park et al., 2000), PKC (Sung et al., 1999b), and PKN (Oishi et al., 2001).

In contrast, the PX domain is also required for PLD1 internalization subsequent to cellular stimulation (Fig. 4), and in this setting, mutation of phospholipid-interacting sites do disrupt its function. It has been challenging to generate the PLD1-PX domain in a soluble form; many approaches that succeeded for PX domains from other proteins yielded only insoluble protein for the PLD1-PX domain (unpublished data). A soluble protein was generated in fusion with the bacterial Nus protein, and although the Nus protein itself exhibited binding to PI3P and PI4P precluding further assessment of these lipids, the PLD1–Nus fusion protein did promote specific binding to PI5P. PI5P has recently garnered attention as a lipid that accumulates during endocytosis as PI4,5P$_2$ is dephosphorylated on the 4 position (Terebiznik et al., 2002). It is tempting to propose that PLD1 associated with the PM through interaction between PI4,5P$_2$ and PLD1's PI4,5P$_2$-interacting motif shifts to vesicles undergoing internalization via its PX domain as the levels of PI4,5P$_2$ on the PM, and vesicles fall and the levels of PI5P on vesicles rise. There is precedence for the first aspect of this idea: generation of PI4,5P$_2$ at the PM is thought to recruit PH domain–containing proteins required for endocytosis (Lemmon and Ferguson, 2000); this PI4,5P$_2$ then becomes hydrolyzed once the endocytic vesicles are fully formed. This eliminates the recruiting signal, allowing the endocytic proteins to recycle back to the PM through diffusion or association with other lipid or protein targets traveling in the opposite direction. We would propose that PLD1 also becomes recruited to the sites of endocytosis through PI4,5P$_2$ elevation; but then instead of abandoning the endocytic vesicles as the PI4,5P$_2$ is hydrolyzed, it remains associated through PX domain–mediated association with PI5P.

Finally, the PX domain can undergo reversible phosphorylation at the PM (Kim et al., 2000). This, or interactions with cytoskeletal or signaling proteins (Sung et al., 1999b; Park et al., 2000; Oishi et al., 2001), may help regulate the translocation steps it affects during agonist signaling events.

PH domain and palmitoylation

Ktistakis and coworkers had reported that loss of palmitoylation at the C240/241 pair of cysteine residues in the PLD1 PH domain caused it to become retargeted from the perinuclear vesicle population to the PM, and suggested that regulated palmitoylation controlled its localization (Sugars et al., 1999). However, a paradoxical aspect of that model was that palmitate is generally added, not removed, at the PM, making it unclear how the regulation would take place. Here, we would propose instead an alternate model based on a recent observation that addition of two or more palmitates to proteins drives their accumulation in lipid rafts (Zacharias et al., 2002), in which wild-type PLD1 can also be found (Kim et al., 2000). In this model, PLD1 would be constitutively palmitoylated. On arrival to the PM, it would rapidly localize to lipid rafts and then reenter the cell. Accordingly, the C240/241 mutation would block PLD1 entry into rafts and its subsequent internalization.

Our findings in Fig. 6 C demonstrated that entry of PLD1 into lipid rafts via palmitoylation is a critical step in its internalization in COS-7 cells. Endocytosis from lipid rafts is generally considered to be clathrin-independent (Nichols, 2002); PLD1 may be restricted to this route of reentry, in contrast to PLD2, which has been shown to associate and enter with the insulin receptor (Rizzo et al., 1999, 2000) during its clathrin-dependent endocytosis (Ceresa et al., 1998).

Although we have discussed primarily the role of palmitoylation in this report, it is possible that the PH domain may mediate other interactions as well. It was recently reported that the oxysterol binding protein PH domain is targeted to the Golgi through interaction with PI4P and as well an ARF-dependent determinant, suggesting that its organelle-specific localization ensues from combinatorial signals (Levine and Munro, 2002).

The cycling steps we propose for PLD1 are summarized in Fig. 9. Key observations were similar in COS-7 and PC12, although there were some differences as well. Whether this reflects the different culture and stimulatory conditions used or whether it indicates that the PX, PH, and PI4,5P$_2$ associations combine to play out somewhat differently in different cell types remains to be determined. Further clarification and extension of the proposed model will come from identifying the relevant protein and/or lipid targets with which PLD1 interacts, and from following PLD1 trafficking in real time from selected compartments using photoactivatable alleles.

Materials and methods

General reagents

All phospholipids were purchased from Avanti Polar Lipids, Inc. PI4,5P$_2$ was isolated as described previously (Frohman et al., 2000). L–Dipalmitoyl phosphatidylcholine [choline-methyl-3H] ([3H]phosphatidylcholine) was obtained from American Radiolabeled Chemicals, Inc. All cell culture media (DME and Opti-MEM®-I) and LipofectAMINE™ Plus were from GIBCO BRL. Wortmannin and methyl-β-cyclodextrin were from Sigma-Aldrich. TLC plates were obtained from Fisher Scientific. SuperSignal® West Pico Trial Kit for detection of HRP was from Pierce Chemical Co. All other reagents were of analytical grade unless otherwise specified. Membranes with various phospholipids (PIP-strips) were from Echelon.

Antibodies

3F10 antibody (rat monoclonal anti-HA tag antibody) was from Roche. M2 antibody (mouse monoclonal anti-FLAG tag antibody) was from Sigma-Aldrich. EEA-1 and GM 130 were from BD Biosciences. Goat anti–mouse
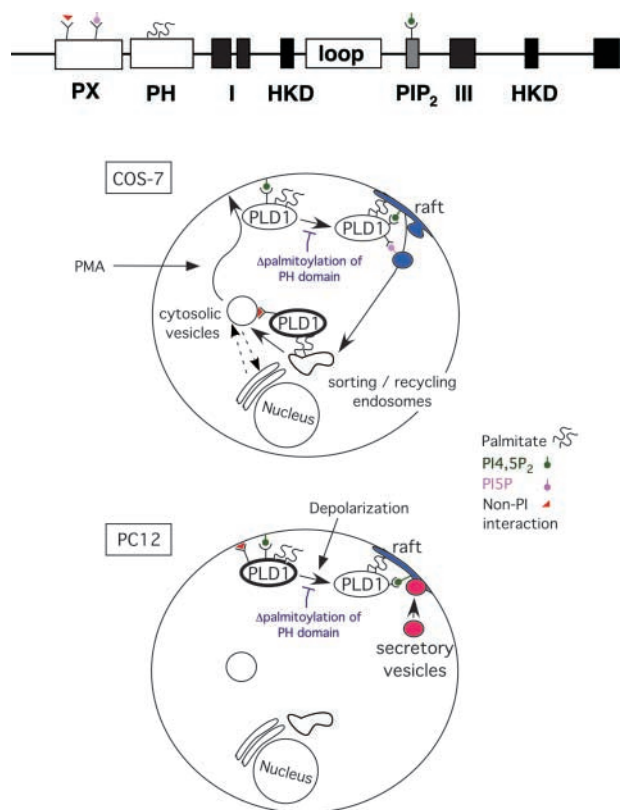
Figure 9. **A model for regulated cycling of PLD1.** See Discussion for details. In brief, in quiescent COS-7 cells, PLD1 localizes to a complex set of perinuclear and cytosolic vesicles (top schema, heavy black circle). This is mediated by a non-PI interaction by the PX domain, the palmitoylated PH domain, and weak interactions with $PI4,5P_2$ by the central basic amino acid–rich motif, although the different domains most likely preferentially target distinct subpopulations of vesicles. In PC12 cells, a combination of PX domain interactions and interactions with $PI4,5P_2$ suffice to recruit PLD1 to the PM (bottom schema, heavy black circle). With higher levels of stimulation, such as exposure to PMA for COS-7 cells, the $PI4,5P_2$ interaction alone suffices to promote PM association. However, to reenter the cell with normal kinetics, PLD1 needs to enter into lipid rafts, and for this, the PH domain to be palmitoylated. Once in rafts, the PX domain association with the PM ceases; but a new PI-dependent interaction takes place, potentially through the binding of PX to PI5P, which facilitates translocation of PLD1 to vesicles internalizing through endocytosis. In PC12 cells, secretagogue-evoked stimulation (depolarization) recruits PLD1 into sites of active exocytosis, which are found in rafts and are marked by increased levels of $PI4,5P_2$. Palmitoylation of the PH domain is similarly required for this recruitment, but once it happens, the PX domain interaction is no longer required for membrane association or for PLD1 functional promotion of exocytosis. How PLD1 returns to its original location remains to be determined.

IgG conjugated with Alexa® 488, 568, and 647 were from Molecular Probes, Inc. Goat anti–rat and anti–mouse IgG conjugated with HRP or Cy3 were from Jackson ImmunoResearch Laboratories.

Site-directed mutagenesis
Site-directed mutagenesis of expression plasmids was performed using the QuikChange® kit (Stratagene). Plasmids were sequenced to confirm the intended mutation and the integrity of the surrounding sequences for at least 500 bp.

Construction of plasmids
All constructs with NH_2-terminal appended HA tags were cloned into the XbaI and XmaI sites of pCGN (for wild-type PLD1, see Hammond et al.,

1995); those with NH_2-terminal appended GFP tags were cloned into the XhoI and XmaI sites of pEGFP-C1 (for wild-type PLD1, see Sung et al., 1999a); those with COOH-terminal appended GFP tags were cloned into the HindIII and XmaI sites of pEGFP-N1.

Generation of PLD1 proteins
The generation of viruses for wild-type and inactive (K898R) PLD1 have been described before (Du et al., 2000). Recombinant bacmids were prepared by transformation of DH10Bac cells with GluGlu-tagged PLD1 R691,695G in pFastBac™. Recombinant baculoviruses were amplified and propagated using standard procedures. Monolayer cultures of exponentially growing Sf9 cells were infected with baculoviruses at a multiplicity of 10 and were cultured for 48 h at 27°C. These proteins were purified by affinity chromatography using an immobilized anti-GluGlu mAb and were eluted using GluGlu peptide. The concentrations of the proteins were measured by Coomassie Plus-200 protein assay reagent (Pierce Chemical Co.).

Expression and purification of the PLD1 PX domain
The PLD1 PX domain (aa 77–212) was cloned into the SacI and HindIII sites of pET43.1a, which encodes Nus and 6xHis tags at its NH_2 terminus, and transformed into *Escherichia coli* BL21(DE3). Cells were grown at 37°C to A600 0.5–0.6 and induced with 0.5 mM isopropyl-D-thiogalactoside for 4 h at 30°C. Cells were lysed by lysozyme/sonication in extraction buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and protease inhibitor cocktail), and was then cleared by centrifugation. The supernatant was incubated with Ni^{2+}-nitrilotriacetic acid agarose beads (QIAGEN) at 4°C for 1 h. After several washes, the recombinant proteins were eluted from the beads with elution buffer (25 mM Tris-HCl, 150 mM NaCl, and 250 mM imidazole, pH 8.0).

Cell culture and transfection
COS-7 cells were maintained in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. For transfection, the cells were grown in 6-well plates or 35-mm dishes ($3–4 \times 10^5$ cells/dish), transfected with 1 µg of DNA/dish using LipofectAMINE™ Plus for 4 h, and then switched into complete DME for a further 24 h. For PMA stimulation, the COS-7 cells were serum starved overnight in DME medium containing 0.2% BSA, starting 20 h after transfection. The cells were then stimulated with 100 nM PMA for 2 or 4 h. PC12 cells grown on poly-D-lysine–coated glass coverslips were maintained in Locke's solution or stimulated with elevated K^+ (Locke's containing 59 mM KCl and 85 mM NaCl).

Western analysis
Protein samples or cell lysates from transfected COS-7 cells were mixed with equal volumes of $2\times$ loading buffer containing 8 M urea, separated by 8% SDS PAGE, transferred to nitrocellulose membrane, and detected using a rat anti-HA monoclonal (3F10) or a rabbit anti-PLD1 COOH terminus polyclonal antisera, followed by HRP-conjugated secondary antibodies. The immunoreactive bands were visualized with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co.).

Confocal and fluorescent microscopy
For live cell studies, COS-7 cells grown on 6-well plates were transiently transfected with pEGFP constructs. 20–24 h after transfection, the cells were treated with wortmannin for 0.5 or 1 h Images were taken before and after treatment on an inverted fluorescent microscope (Eclipse TS100; Nikon) using a digital camera (Coolpix 990; Nikon).

For all other images, COS-7 cells were cultured on coverslips and transfected. 20–24 h after transfection, the cells were fixed with 2% PFA for 10 min and stained. In brief, the cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% BSA and normal goat serum. The cells were then immunostained using primary antibodies against the specific proteins, followed by fluorescent dye–conjugated secondary antibodies. All experiments were performed at least three times with similar results.

Quantification of the localization of PLD1 alleles was performed using a $40\times$ dry lens with a fluorescent microscope (model BX 60; Olympus). All experiments were repeated at least three times. At least 300 cells were randomly selected and scored for localization for each sample. The results described present the mean and SD.

PC12 cells were fixed, permeabilized, and immunostained as described previously (Vitale et al., 2001). Stained cells were visualized using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.).

PLD activity and exocytosis assays
PLD activity assays were performed using the in vitro head-group release assay for a 30-min time period (Morris et al., 1997). Recombinant ARF1

proteins were purified and were activated using 50 µM GTPγS as described previously (Du et al., 2000). PC12 exocytosis assays were performed as described previously (Vitale et al., 2001). PC12 exocytosis was monitored using ectopically expressed human growth hormone (hGH) as a reporter. In brief, cells were cotransfected with pXGH5 together with a plasmid encoding PLD1 or a tagged allele. 48 h after transfection, cells were washed in Locke's solution and incubated for 10 min in Locke's solution or elevated K$^+$. Cell-free supernatant was collected to represent the secreted hGH fraction. Cells were scraped and lysed to obtain the nonsecreted fraction. hGH in each fraction was measured by radioimmunoassay (Vitale et al., 2001).

Liposome binding assay

The method has been described previously (Sciorra et al., 1999; Du et al., 2002). In brief, sucrose-loaded phospholipid vesicles containing equal molar amounts of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine, and 5% PI4,5P$_2$ were prepared and resuspended in buffer containing 100 mM KCl and 1 mM MOPS. PLD1 proteins were mixed with the vesicles in siliconized tubes and incubated on ice for 30 min. The vesicles were sedimented by centrifugation at 30,000 g for 1 h. The pelleted vesicles were resuspended in 100 µl MOPS buffer. The distribution of the wild-type and mutant PLD1 proteins in the supernatant and pellet was analyzed by Western blotting.

Protein-lipid overlay assay

Membranes spotted with assorted phospholipids (PIP-strips) were blocked in 3% fatty acid–free BSA in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h at RT. The membranes were then incubated overnight at 4°C in the same solution with 0.2 µg/ml Nus or Nus-tagged protein. Bound protein was detected using an anti-Nus mAb/HRP-conjugated goat anti–mouse secondary antibody.

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