Hierarchy of Membrane-targeting Signals of Phospholipase D1 Involving Lipid Modification of a Pleckstrin Homology Domain*

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The amino terminus of phospholipase D1 (PLD1) contains three potential membrane-interacting determinants: a phox homology (PX) domain, a pleckstrin homology (PH) domain and two adjacent cysteines at positions 240 and 241 within the PH domain that are fatty acylated in vivo. To understand how these determinants contribute to membrane localization, we have mutagenized critical residues of the PLD1 PH domain in the wild type or palmitate-free background in the intact protein, in a fragment that deletes the first 210 amino acids including the PX domain, and in the isolated PH domain. Mutants were expressed in COS-7 cells and examined for membrane residence, intracellular localization, palmitoylation, and catalytic activity. Our results are as follows. 1) Mutagenesis of critical residues of the PH domain results in redistribution of PLD1 from membranes to cytosol, independently of fatty acylation sites. Importantly, PH domain mutants in the wild type background showed greatly reduced fatty acylation, despite the presence of all relevant cysteines. 2) The isolated PH domain did not co-localize with PLD1 and was not palmitoylated. 3) The PX deletion mutant showed similar distribution and palmitoylation to the intact protein. Interestingly, PH domain mutants in this background showed significant palmitoylation and incomplete cytosolic redistribution. 4) PH domain mutants in the wild type or palmitate-free background maintained catalytic activity. We propose that membrane targeting of PLD1 involves a hierarchy of signals with a functional PH domain allowing fatty acylation leading to strong membrane binding. The PX domain may modulate function of the PH domain.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidycholine to generate membrane-bound phosphatidic acid and soluble choline. PLD activity is evident in most cell types, and it is thought to constitute an important component of signal transduction pathways that operate in the control of diverse cellular functions (1–3). In most mammalian cell types and tissues, a substantial portion of PLD activity is found associated with the membrane fraction under all conditions (4) although neither of the two mammalian PLD isoforms that have been identified, PLD1 and PLD2, contains a membrane-spanning domain (5, 6). Whereas PLD2 is mostly associated with the plasma membrane (5) PLD1 is bound to intracellular membranes of endocytic and secretory origin when its distribution is assessed by subcellular fractionation or by indirect immunofluorescence (5, 7, 8). It has also been reported that PLD1 localizes to the Golgi stacks (9). We are trying to understand the mechanisms for membrane targeting of PLD1.

Part of the requirement for targeting to internal membranes is fulfilled by palmitoylation of two adjacent cysteine residues at positions 240 and 241 near the amino terminus of PLD1 (10). However, unlike other proteins for which absence of palmitoylation results in cytosolic redistribution or miss-targeting to internal membranes (11), PLD1 redistributes from internal membranes to the plasma membrane upon removal by mutagenesis of its palmitoylation sites (10). One way to explain this result is to assume that PLD1 contains multiple membrane-targeting signals; removal of the palmitate sites may expose an additional signal that specifies plasma membrane localization. What is the nature of the second signal? In analogy with other proteins, it could be modification with a second type of fatty acid, for example myristoylation (11). However, neither the primary sequence of PLD1 nor labeling with radioactive myristate and immunoprecipitation provides any evidence for such a modification. Alternatively, the second signal could be an additional membrane-binding determinant. At least two such determinants exist in PLD1, a PH domain and a PX domain (2). The fatty acylation site on PLD1 is on a flexible loop of a putative PH domain that encompasses amino acids 220–330 of the protein (12, 13). Therefore, it is possible that removal of the fatty acid would still maintain the PH domain and that PLD1 would redistribute to the plasma membrane presumably as a reflection of the preference of its PH domain to binding a plasma membrane determinant (which could be a phosphoinositide) (10). Similar dynamics could be envisaged for the PLD1 PX domain, which is located in close proximity to the PH domain at its amino-terminal side (14). In light of recent reports that have described a preference of PX domains for binding phosphoinositides (15–18), it is possible that upon removal of the palmitates, the PX domain assumes a dominant role and is responsible for the redistribution of the protein.

In this work we have examined the relative contribution to cellular localization of PLD1 of its three membrane-targeting determinants, the palmitoylation sites, the PH domain, and the PX domain. We provide evidence by selected mutagenesis that the PLD1 PH domain in the intact protein is important both for membrane targeting and for fatty acylation. In contrast, the
isolated PH domain does not recapitulate any of the membrane localization characteristics of PLD1 and cannot therefore be studied in isolation. Finally, the smallest PLD1-derived fragment that recapitulates membrane localization, palmitoylation, and lipid binding of the intact protein is a deletion mutant missing the first 210 amino-terminal amino acids including the PX domain. Our results suggest that, under basal conditions, the PLD1 PH domain and its fatty acylation account to a significant extent for the localization of the protein on membranes. However, it is also clear that those two membrane-targeting signals are not independent but rather constitute a hierarchy, with the PH domain allowing PLD1 to bind to membranes and the subsequent (or concurrent) fatty acylation serving as a means to stabilize this interaction and to provide specificity. To our knowledge this is the first example of a dual membrane-targeting motif involving a PH domain and its fatty acylation state.

EXPERIMENTAL PROCEDURES

Techniques Previously Described—Antibodies, plasmids, and lipid mixtures for measuring PLD activity in immunoprecipitated samples have been reported previously (19). The labeling protocol for [3H]palmitate, [3H]myristate, or 35S-labeled TranSlable (all radiochemicals from Amersham Biosciences) has been described before (19). Mutagenesis was done in duplicate as described, and in all cases, both independent mutant plasmids were expressed and their proteins assayed in COS-7 cells (19). Fractionation of transfected COS cells was done using a steel Dounce as described before, and subcellular fractions were stored at −70 °C until use (19). Immunofluorescence microscopy was done as described recently (8).

Construction of a210 and PH1EFG Mutants—Fragments that initiate immediately before the PLD1 PH domain were amplified using polymerase chain reaction. The oligonucleotide CGCTCGAGCT-GTCTTTCATCCATGATTTGGGAC was used for 5′-HpaI, and 5′-Hpyrtratase, or 3′-S-labeled TranSlable (all radiochemicals from Amersham Biosciences) has been described before (19). Mutagenesis was done in duplicate as described, and in all cases, both independent mutant plasmids were expressed and their proteins assayed in COS-7 cells (19). Fractionation of transfected COS cells was done using a steel Dounce as described before, and subcellular fractions were stored at −70 °C until use (19). Immunofluorescence microscopy was done as described recently (8).

RESULTS

The PH Domain in the Intact Protein Is Essential for Membrane Localization and Fatty Acylation—PH domains from β-spectrin and phospholipase C-δ (PLCδ) crystallized in the presence of IP3 reveal two distinct ligand-binding arrangements: in the case of spectrin, IP3 lies on the surface of a positively charged region (20), whereas for PLCδ the binding site for IP3 resembles a deep pocket that contains positively charged amino acids (21). In the linear sequence, the positively charged region of spectrin is spatially distinguishable from that of PLCδ (Fig. 1). By comparing amino acid sequences we attempted to infer the arrangement of the putative lipid-binding pocket for the PLD1 PH domain in relation to that of spectrin or PLCδ. We found that it was impossible to distinguish between the two since PLD1 contains positively charged amino acids that could be involved in lipid binding at both positions (Fig. 1). We therefore mutagenized separately both regions of positively charged amino acids. In addition, we mutagenized the invariant tryptophan residue in the core of the a-helical region of the PH domain; for PLD1, which has two adjacent tryptophan residues in that position (Fig. 1), this meant a double mutation. All mutations were done in the wild type and in the palmitate-free (C240S/C241S) background and are summarized in Fig. 2. All mutants were expressed transiently in COS cells and examined for altered localization by indirect immunofluorescence. None of the mutants showed complete redistribution by immunofluorescence (data not shown), and their localization resembled to a large extent that of the PLD1 species from which they were derived. However, some subtle differences were apparent. Mutants that changed lysines 288 and 289 into alanines (modeled on the PH domain of spectrin) showed totally unaltered localization. Mutants that changed the invariant tryptophan (W318A/W319A) or those that changed arginine 252 and arginine 253 into alanines (modeled on the PH domain of PLCδ) showed partial redistribution into the cytosol. Interestingly, redistribution was stochastic but complete, i.e. a small percentage of cells (~10%) showed complete redistribution, whereas for the rest distribution was unaltered (data not shown). We reasoned from the above that the PH domain of PLD1 resembles more that of PLCδ, but to disturb it completely more than one mutation was required. We therefore mutagenized simultaneously the invariant tryptophans and the positively charged residues at positions 252 and 253 in the wild type and in the palmitate-free background.

When the resultant mutant proteins were expressed in COS-7 cells, they showed a dramatic redistribution into a cytosolic-like staining pattern that was evident in all transfected cells (Fig. 3, first two rows). Surprisingly, this redistribution was evident for the mutant derived from the wild type (Fig. 3, PH) as well as for that derived from the palmitate-free protein (Fig. 3, Palm-PH), i.e. it was independent of palmitoylation sites. We determined the localization of PLD1 and its relevant mutants in two additional cell lines to ensure that such altered localizations were not restricted to a specific cell type. In one set of experiments, the wild type PLD1, the palmitate-free...
C240S/C241S mutant, and both PH domain mutants were expressed in primary smooth muscle cells of low passage established in this laboratory. PLD1 in those cells was localized in a perinuclear punctate pattern, the palmitate-free mutant was largely detected on plasma membrane, and both PH domain mutants were cytosolic (Fig. 3, third row). Very similar distributions of the four proteins were seen in CHO cells, a well characterized immortal cell line (data not shown). We concluded from the above that the altered distributions of the mutants discussed here depend on cellular mechanisms that are common to many cell types.

The distribution of the PH domain mutants in comparison to the parental proteins was also determined biochemically. As we have reported before, wild type PLD1 and the palmitate-free mutant are largely membrane-bound after subcellular fractionation (Fig. 4) (10). In contrast, we found here that both PH domain mutants showed a significant and reproducible redistribution into the cytosolic fraction (Fig. 4, note percentages at the bottom of the gel). In addition, the electrophoretic mobility of the PH domain mutants was clearly different from that of the parental proteins in that the upper form normally seen with the wild type PLD1 was absent in the PH domain mutants (note PNS lanes in all four samples and also see Fig. 6). Zhang et al. have reported a fairly good correlation between membrane residence and the appearance of the upper band of PLD1 (22), and we think it likely that its absence from the PH domain mutants reflects their cytosolic redistribution.

Because redistribution into the cytosol was evident for both PH and Palm-PH mutants, we examined whether palmitoylation was affected once the PH domain was impaired in the wild type background. All three single PH domain mutants in the wild type background (K288A/K289A, K252A/R253A, W318A/W319A, see also Fig. 2) as well as the double PH domain mutants in both backgrounds were analyzed for incorporation of [35-S]methionine or [3H]palmitate after expression in COS-7 cells (Fig. 5A). The clear result was that the PH domain mutant in the wild type background (Fig. 5A, PH) showed greatly reduced palmitoylation. Equally clear was the level of palmitoylation of the single PH domain mutants; those that by immunofluorescence revealed partial redistribution (K252A/R253A, W318A/W319A) also showed reduced palmitoylation, whereas the mutant that was indistinguishable in distribution from the wild type protein (WT(K/A)2) was comparable in palmitoylation to wild type PLD1. As was expected, the mutant missing the palmitoylation sites (Palm-PH) did not show any palmitoylation.

It has been difficult, despite repeated attempts, to unambiguously identify the nature of the lipid label on PLD1 by mass spectrometry. A particular concern is the possibility that a catalytic intermediate with phosphatidic acid may be formed in vivo during catalysis, and this species would incorporate radio-label not as a result of bona fide palmitoylation but because of the label on the substrate phospholipid. To explore this possibility, COS-7 cells expressing the wild type protein and various
mutants were labeled in parallel with equal amounts of \(^{3}H\)palmitate or \(^{3}H\)myristate. Under these conditions, cellular phospholipids are labeled to the same extent, and equal amounts of the PLD product are formed (data not shown). Labeled proteins were immunoprecipitated, resolved by SDS-PAGE, and analyzed either by immunoblotting to ensure comparable expression or by autoradiography (Fig. 5B). As shown by immunoblotting, expression was very similar (Fig. 5B, upper panel). Labeling of PLD1 with \(^{3}H\)palmitate was easily detected, whereas the S911A and the C240S/C241S mutants had undetectable label as we have reported before (Fig. 5B, lower panel). For samples labeled with \(^{3}H\)myristate, we detected weak labeling of PLD1 (\(\sim 15\) times less than that seen with \(^{3}H\)palmitate) and nearly undetectable labeling of the S911A and C240S/C241S mutants (Fig. 5B, lower panel). We believe that this experiment provides additional support for the idea that the label on PLD1 is due to palmitoylation and not to formation of a PLD-phosphatidic acid covalent intermediate.

The PH Domain Is Not Required for Catalysis

It was of interest to determine whether the mutants with impaired PH domain described here were catalytically active. In preliminary experiments, the PH domain mutants appeared to have reduced catalytic activity in vitro following expression and immunoprecipitation. However, we noted that the efficiency of immunoprecipitation for these mutants was also reduced by about 2–3-fold in comparison to the parental protein, and it was difficult by varying antibody amounts to produce immunoprecipitates with equivalent amount of protein. A different strategy was to immunoprecipitate with equivalent amounts of antibody, quantify the bound protein by immunoblotting, and use equal amounts for the PLD1 assay. When such corrected immunoprecipitates were used (Fig. 6A, note increased IgG for PH and Palm-PH), we found that catalytic activity was unchanged for the PH domain mutants in comparison to the wild type protein (Fig. 6B).

The Isolated PH Domain Does Not Recapitulate Any of the Membrane Localization Characteristics of the Intact Protein

We attempted to study the membrane-binding characteristics of the PLD1 PH domain in isolation by expressing in COS-7 cells fusion proteins between the isolated PH domain and green fluorescent protein (GFP). Placing the isolated PH domain at either the amino or carboxyl terminus of GFP resulted in proteins whose distribution was entirely distinct from that of the full-length protein. The PH-GFP protein was detected in a fine punctate distribution that did not co-localize with any endogenous organelle markers (data not shown). Furthermore, introducing mutations that have strong effects on the distribution of the intact protein (for example C240S/C241S or K252A/R253A/W318A/W319A, see Fig. 2) had no effects on the distributions of the GFP fusion constructs (data not shown). Finally, none of the GFP fusion proteins became palmitoylated (data not shown). We concluded from the above that it is not possible to study the PLD1 PH domain in isolation.

A Mutant Missing the First 210 Amino Acids Is the Smallest PLD1-derived Fragment That Recapitulates Membrane Localization and Fatty Acylation

In work reported previously we noted that it was not possible to detect fatty acylation of various PLD1-derived fragments expressed separately (19). In addition, expression of these fragments in isolation showed that none co-localized with the intact protein. Together with the results reported above with the isolated PH domain, it is clear that the various structural elements of PLD1 are difficult to study in isolation, although catalytic activity can be restored if

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Fig. 2. Mutants discussed in this work. The wild type PLD1 gene codes for a protein of 1074 amino acids. In the linear representation of the protein, regions corresponding to a PKC-interacting domain, the PX and PH domains, four regions of high conservation among PLD family members (I-IV) and the region implicated in PI(4,5)P2-regulated catalysis are boxed (see also Ref. 2). All point mutations within the PH domain and the palmitoylated region are listed, and mutants that are abbreviated in the text are indicated. Also shown schematically are the two fragments of PLD1 used in this work (Δ210 and PH\(_{\text{PLD1}}\)-GFP) for which we also created the corresponding point mutations.

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2 J. M. Sugars, S. Cellek, M. Manifava, J. Coadwell, and N. T. Ktistakis, unpublished results.
N- and C-terminal fragments are co-expressed (23). Nevertheless, because a functional PH domain seems to be critical for membrane localization, we investigated whether it also represented the minimal targeting determinant that could direct the rest of the protein to the proper subcellular compartment(s). We therefore constructed a mutant PLD1 that is missing the first 210 amino acids (including the PX domain), thus initiating immediately before the PH domain (Δ210, see Fig. 2), and studied its subcellular distribution. PLD1 owes its punctate distribution to its presence in both the intermediate endoplasmic reticulum to Golgi compartment and in early endosomes as revealed by counterstaining cells that express PLD1 with antibodies to the endogenous KDEL receptor (KDEL) and the early endosome antigen 1 (EEA1, respectively (Ref. 8)). Side by side comparisons of the wild type PLD1 and the Δ210 deletion mutant, showed similar accumulation of the two proteins in EEA1- and KDELR-positive compartments (Fig. 7). We noted that in some cells the Δ210 mutant showed reduced punctate

![Immunofluorescence staining of PLD1 and mutants](image-url)

**Fig. 3. Immunofluorescence staining of PLD1 and mutants.** First two rows, COS-7 cells were transfected with plasmids encoding PLD1, Palm, PH or Palm-PH as indicated and 40 h after transfection were fixed and stained with PLD1 antibodies made against N- or C-terminal-derived peptides. Shown are representative examples of many experiments. Notice that PLD1 is on internal punctate structures, Palm is on plasma membrane as reported before (10), whereas both PH and Palm-PH show a similar diffuse distribution, characteristic of cytosolic staining. Third row, primary smooth muscle cells were transfected with plasmids encoding PLD1 or the indicated mutants using LipofectAMINE. 28 h after transfection, the cells were fixed and stained with PLD1 antibodies. Notice that the overall distributions of the four proteins are similar to what was seen for COS-7 cells. The bar represents 10 μm. Note that smooth muscle cells are much larger than CHO cells.
distribution, but it was not possible to quantitate this difference.

In parallel experiments, we examined the fatty acylation of the Δ210 protein and various mutants that, in addition to the deletion, also contain amino acid substitutions in the PH domain or of the fatty acylation sites (see Fig. 2 for details). Labeling with [35S]methionine or [3H]palmitate revealed that the Δ210-derived mutants recapitulate the palmitate incorpo-

**FIG. 4.** Subcellular fractionation of PLD1, Palm, and their corresponding PH domain mutants. For each plasmid, 10 15-cm plates of COS-7 cells were transfected to produce 0.25 ml of cell pellet for homogenization. After homogenization, a portion of the postnuclear supernatant (PNS) was set aside, and the rest was centrifuged at 100,000 × g to produce a cytosolic (C) and a membrane (M) fraction. Relative percentages of the PLD1-related band(s) for each sample were estimated using the NIH Image 1.61 program (developed at the National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image/) and are indicated at the bottom of the photograph. The absence of upper band for both PH mutants is very reproducible.

**FIG. 5.** Relative incorporation of [3H]palmitate and [3H]myristate for PLD1 and the mutants discussed in this work. A, COS-7 cells were transfected with plasmids encoding PLD1 or the indicated mutants, and 40 h after transfection the samples were labeled with [35S]methionine or [3H]palmitate as indicated. Labeling with [35S]methionine was for 2 h, whereas labeling with [3H]palmitate was for 4 h. At the end of labeling, samples were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. B, COS-7 cells transfected with a control plasmid (−) or with plasmids encoding PLD1, S911A, or Palm were labeled for 4 h with 250 μCi/ml of [3H]palmitate or [3H]myristate as indicated. After labeling and immunoprecipitation, samples were resolved by SDS-PAGE in duplicate gels. One gel was blotted and probed with PLD1 antibodies (immunoblot), whereas the other gel was enhanced, dried, and exposed to film for autoradiography (autoradiograph). Shown for the autoradiograph is a 52-day exposure.
ration of their full-length counterparts (Fig. 8A). For example, the Δ210 protein contains similar amounts of palmitate label as the full-length protein (Fig. 8A, compare lanes 1 and 2), whereas Δ210 mutants with impaired palmitate sites show no palmitoylation (Fig. 8A, lanes 3 and 5). In addition, a mutant that changes serine 911 to alanine, which has been shown by us not to be labeled with palmitate (Fig. 5, lane 2) (19), is similarly palmitate-free in the Δ210 background (Fig. 8A, lane 6). We noted however a single exception in the palmitate labeling of the Δ210-derived mutants: the PH domain mutant in the Δ210 background had considerably higher palmitate label than its wild type-derived counterpart (compare lanes 1 and 2 in Fig. 8A with lanes 1 and 6 in Fig. 5). In addition, side by side comparisons of the distribution of the four proteins after transfection into COS-7 cells showed that, whereas the PH mutant in the wild type background was devoid of any punctate staining, the same mutant in the Δ210 background showed some residual immunoreactivity in punctate structures (Fig. 8B). Thus, although the Δ210 mutant can be considered the smallest PLD1 fragment that faithfully recapitulates membrane localization and fatty acylation, the missing sequence, including the PX domain, may have a role to play in modulating the function of the PH domain in the intact protein.

**DISCUSSION**

PLD1 is a peripheral membrane protein that exhibits tight association with membranes without containing a membrane-spanning domain (24). In this work we have analyzed the mechanisms that allow such tight association by concentrating on three potential membrane-interacting determinants contained within the first 300 N-terminal amino acids of PLD1: a dual palmitoylation site, a PH domain, and a PX domain (Fig. 2). A mutagenesis strategy identified limited amino acid changes within the PH domain that resulted in redistribution of the protein from the membranes into the cytosol. However, we were unable to determine the relative contribution of the palmitoylation sites on membrane binding independently of the PH domain since we found that palmitoylation was significantly suppressed in the mutants with an impaired PH domain. Based on this we propose that the PLD1 PH domain and its palmitoylation constitute a hierarchy of membrane-targeting signals. The simplest view is that a functional PH domain is required for initial membrane targeting. Subsequent (or at least concurrent) palmitoylation stabilizes the PLD1-membrane interaction, and it also ensures that the protein is bound to the appropriate membrane. Once PLD1 is palmitoylated, this modification probably becomes the dominant contributor to membrane binding, overriding any other targeting determinants. The contribution of the PX domain to this mechanism must be minimal based on our observation that the Δ210 deletion mutant, which is missing the entire PX domain, exhibits normal levels of palmitoylation and normal subcellular localization on membranes of endocytic and secretory origin (Figs. 7 and 8).

Two recent reports have described results that are relevant to this work. Hodgkin et al. have proposed that the isolated PLD1 PH domain binds PI(4,5)P₂ and, in the intact protein, is required for catalytic activity and membrane localization (25). Our experiments with the isolated PH domain suggested that it does not recapitulate any of the properties of the intact protein in terms of localization or fatty acylation, and we did not examine its lipid-binding properties further. We were also unable to show any effects of PH domain mutations on catalytic activity, even for mutants that, in agreement to those described by Hodgkin et al. were redistributed to the cytosol and must therefore contain significant disruptions of the PH domain. Although phosphatidylinositol 4,5-bisphosphate, is absolutely required for PLD1 activity (26, 27), a phosphoinositide-interacting site required for catalysis was recently identified in a region encompassing amino acids 668–712 of the protein, far removed from the PH domain (28). We consider it likely that
the PLD1 PH domain discussed here is not responsible for PI(4,5)P2-dependent stimulation of hydrolysis, in agreement with previous deletion analyses that did not assign catalytic significance to this region of PLD1 (29–31). Palmitoylation of PLD1 has also been investigated by Xie et al. who reported that its absence weakens the PLD1-membrane interaction (32). In agreement with Xie et al., we have also found that palmitate-free PLD1 is extracted from membranes with detergents much more readily than the wild type protein (data not shown). Xie et al. have also reported that palmitoylation requires the association of N- and C-terminal sequences of PLD1 in trans when the two halves of the protein are co-expressed from different plasmids. As speculated by Xie et al., this result could also explain the puzzling but consistent observation that the S911A mutant of PLD1 is not palmitoylated (19) since they find that the S911A mutation disrupts interdomain association (32). In this view, N- and C-terminal domain association in the intact protein may be sufficiently disrupted by the S911A mutation to lead to defects in palmitoylation. Our results differ from those of Xie et al. in that we were able to show that the Δ210 deletion mutant maintains normal palmitoylation levels (Fig. 8A), whereas a Δ168 deletion mutant in rat PLD1 was reported to be devoid of palmitate labeling (32). This is a noteworthy difference since in our view the Δ210 deletion mutant contains the minimum of determinants required for proper localization under basal conditions. We think it unlikely that the difference is due to the fact that we use human PLD1, whereas Xie et al. investigate the rat enzyme. A more likely explanation may lie in the choice of the N-terminal starting sequence for these deletion mutants. We chose to end the deletion immediately upstream of the PH domain, whereas Xie et al. constructed their deletion within the PLD1 PX domain. Given the requirements for interdomain association, we consider it likely that the Δ168 mutant may disrupt not only the PX domain but also the folding of the entire protein such that palmitoylation is lost.

It is interesting that the PH mutant in the Δ210 background contains significantly more palmitate label than the equivalent

**Fig. 7.** Localization of PLD1 and the Δ210 mutant in compartments of endocytic and secretory origin. COS cells transfected with plasmids encoding PLD1 or the Δ210 mutant were fixed and stained with rabbit polyclonal antibodies to PLD1 and mouse monoclonal antibodies to EEA1 or KDEL as indicated. Note that the KDEL samples were treated with 5 mg/ml of brefeldin A for 30 min before fixing to redistribute the KDELR more to the intermediate compartment. Secondary antibodies were fluorescein isothiocyanate-coupled goat anti-rabbit and rhodamine isothio-coupled goat anti-mouse.
mutant in the intact protein, and it also exhibits some residual punctate distribution (Fig. 8). This result strengthens the correlation between palmitate labeling and localization on internal membranes for PLD1, but we do not understand its cause. It is possible that in the deletion mutant a region outside of the PH domain assumes a more important role in membrane targeting and/or fatty acylation. In general, we suspect on the basis of extensive mutagenesis studies on PLD1 undertaken by many groups that regions outside the PH domain can contribute significantly to membrane targeting, either directly or indirectly by influencing the overall structure of the protein. For example, mutations within the PI(4,5)P2-binding region affect membrane binding (33), and in addition, C'-terminal sequences can contribute to endosomal localization (34). Although palmitoylation state was not determined for either of the above mutants, it is possible that it was affected and that it in turn affected membrane binding.

PH domains were originally implicated in membrane targeting because in some members of families of peripheral membrane proteins they appeared to substitute for fatty acylation sites that were evident in related members of the same family (35). Similarly, in some members of related families, PX domains have been found to replace PH domains (36). PLD1 is the first, but perhaps not the only, example of a peripheral mem-

**Fig. 8.** Palmitoylation and localization of Δ210-derived mutants. A, COS-7 cells were transfected with plasmids encoding PLD1, the Δ210 mutant, or the indicated Δ210-derived mutants. 40 h after transfection the samples were labeled for 2 h with [35S]methionine or for 4 h with [3H]palmitate as indicated. At the end of labeling, samples were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. B, COS-7 cells transfected with plasmids encoding PLD1, the Δ210 mutant, or the corresponding PH mutants in the two backgrounds were stained for immunofluorescence with antibodies to the C terminus of PLD1. Note that the PH mutant in the Δ210 background maintains some punctate staining.
brane protein that contains all three membrane-binding determinants. We believe that this complex array of domains reflects the possibility that PLD1 may function in (and must be targeted to) multiple cellular locations, under basal and signal-dependent conditions.

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