We have reported previously that phospholipase D1 (PLD1) is labeled specifically with [3H]palmitate following transient expression and immunoprecipitation and that this modification appeared important both for membrane localization and catalytic activity. In this work we identify by mutagenesis that the acylation sites on PLD1 are cysteine residues 240 and 241, with the cysteine at position 241 accounting for most but not all of the modification. Replacement of both cysteine residues with either serines or alanines resulted in a mutant protein that contained undetectable [3H]palmitate. In comparison with the wild type protein, the double mutant showed reduced catalytic activity in vitro, whereas its activity in vivo was unchanged. In addition, the localization of the double mutant was altered in comparison with the wild type protein, whereas wild type PLD1 is primarily on intracellular membranes and on punctate structures, the double mutant was on plasma membrane. Because cysteines 240 and 241 lie within a putative pleckstrin homology domain of PLD1, it is likely that fatty acylation on these residues modulates the function of the PLD1 pleckstrin homology domain.

Two classes of PLD genes have been identified in mammalian cells (1, 2). PLD1 hydrolyses phosphatidylcholine to generate phosphatidic acid and choline. Hydrolysis is stimulated in vivo and in vitro by ADP-ribosylation factor (Arf), Rho, and protein kinase C, and it requires phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (3, 4). PLD1 is found primarily on intracellular membranes where it may regulate the formation of transport intermediates (5). In contrast, PLD2, which has identical hydrolytic activity, is not stimulated greatly by any proteins, and it only requires PIP$_2$ for activity. PLD2 is localized primarily on the plasma membrane (1). The sequence elements which account for the differential localization of the two PLD genes have not been established.

It is equally unknown by what mechanism(s) the PLD proteins become membrane bound. They contain neither a transmembrane domain nor a canonical signal sequence for lipid modification, yet, at least for PLD1, more than 90% of the protein is found with the membrane fraction after fractionation.

We have reported recently (6) that PLD1 is fatty acylated as shown by labeling of PLD1-expressing cells with [3H]palmitate and immunoprecipitation. A catalytically inactive point mutant (S911A) did not show palmitate labeling, and it was partially redistributed to the cytosol. In addition, treatment of PLD1 with hydroxylamine under conditions that remove the palmitate from the protein resulted in inactive protein in vitro. These results led us to propose that fatty acylation of PLD1 may be important both for catalytic activity and for membrane localization. In this work, we have identified by mutagenesis the two cysteine residues on PLD1 that become labeled with palmitate. The catalytic properties and subcellular localization of the double mutant devoid of palmitate point to a role of fatty acylation in intracellular membrane targeting and activity of PLD1.

**EXPERIMENTAL PROCEDURES**

Antibodies and PLD1 expression plasmids used in this work were as reported previously (6). Similarly, transfection into COS-7 cells, labeling with Tran35S-label or with [3H]palmitate, immunoprecipitation and PLD in vitro assay using as a substrate liposomes with [14C]phosphatidylcholine were exactly as reported before (6).

To measure PLD1-induced activity in vivo following transfection of COS-7 cells, we modified a protocol reported by Bi et al. (7). COS-7 cells were transfected on 6-well plates on day 0. On day 1 in the evening, they were washed three times with serum-free Dulbecco's modified Eagle's medium and [3H]palmitate label (1.5 ml/well containing 100 μCi [3H]palmitate) was added overnight. The label was made by drying completely the palmitate solution (prepared in ethanol) under a stream of nitrogen and then resuspending in dialyzed serum with vortexing for 5 min before adding the appropriate volume of Dulbecco's modified Eagle's medium to make up the serum amount to 10%. In some experiments, ethanol was added at this stage. On day 2, the label was removed, and fresh medium containing 3% ethanol was added to the plates for 30 min. At the end of 30 min, cellular lipids were extracted and resolved by thin layer chromatography exactly as described before (7).

Molecular modeling of the putative PLD1 pleckstrin homology (PH) domain on known crystal structures of other PH domains was done as follows. The general alignment of Steed et al. (8) was refined using the PFAM pleckstrin homology domain alignment. The invariant tryptophan residue at position 319 of PLD1 and the N-terminal 50 residues were used to anchor this alignment. Two PH domain sequences from Bruton's tyrosine kinase (BTK) and pleckstrin that had associated structural information were compared with PLD1 as being the closest in sequence. Using the INSIGHT/DISCOVER/HOMOLOGY package of programs on an Indigo II computer from Silicon Graphics, a structural model was produced of PLD1 in comparison with BTK with special emphasis on the N-terminal region that contains the palmitoylated residues of PLD1. This alignment was centered on two regions of conserved sequences (boxed in red in Fig. 7), and it suggested that the palmitoylated cysteines of PLD1 were at the center of an inserted loop of variable length in the full PH domain structural model. The program HOMOLOGY was then used to produce a loop of the desired length and amino acid composition between the two anchored N-terminal regions. On this calculated loop, the palmitate residues were drawn to scale and were added to the appropriate cysteines.

**RESULTS AND DISCUSSION**

Labeling of PLD1 with [3H]palmitate was sensitive to neutral hydroxylamine treatment after electrophoresis, suggesting that the fatty acid is linked to the protein via a thioester bond...
on a cysteine residue (6). Because the amino acid sequence of PLD1 does not contain a readily identifiable palmitoylation site, we decided to mutate sequentially all the cysteine residues to identify the site of fatty acylation. There are twelve cysteine residues on PLD1 dispersed throughout the 1074-amino acid sequence (Fig. 1). Interestingly, none fall within the 4 regions that are conserved between mammalian PLDs and PLD enzymes from other species (labeled CRI, CRII, and HKD in Fig. 1). In the first round of mutagenesis, cysteines at positions 79, 240, 403, 650, 787, 876, and 1004 were changed one by one to serines. These mutant proteins were expressed in COS-7 cells and immunoprecipitated following labeling of the cells either with [35S]methionine or with [3H]palmitate (Fig. 2A, the C787S mutant not shown). With the exception of the catalytically inactive S911A mutant, which contains no palmitate label as we reported before, all of the other mutants contained palmitate label. We noted however that, whereas the majority of the mutants showed a good correspondence between palmitate and methionine label, the mutant that changes cysteine 240 into serine showed reduced palmitate label (Fig. 2A). This residue falls within a region of PLD1 that contains two additional cysteines (PGLNCCGQGRACYR, Fig. 1 boxed region) at positions 241 and 247. We hypothesized that palmitoylation on PLD1 may occur on more than one cysteine residues that are in close proximity as is the case for other palmitoylated proteins (9), and we decided to mutate the additional cysteines in the region either singly or in combination. Analysis of these mutants by parallel [3H]palmitate- and [35S]methionine-labeling revealed that the majority of the palmitate label was on cysteine 241 and that the double mutant C240S/C241S contained no palmitate label (Fig. 2B, results for C247S not shown). In this experiment, we also analyzed for palmitate labeling an additional catalytically inactive mutant (Fig. 2B, K898R) (10). The K898R mutant contained less than the wild type but detectable label, indicating that absence of palmitoylation is not a general property of all catalytically inactive mutants of PLD1 (see also below). Because the palmitoylated sites were revealed in this series of experiments as cysteine residues 240 and 241, we chose not to mutate the remaining cysteine residues at positions 284, 310, 851, and 959 of PLD1.

It was of interest to determine the properties of the non-palmitoylated PLD1 mutant in comparison with the wild type protein in terms of cellular localization and activity. When expressed transiently in COS-7 cells, the wild type PLD1 protein is seen by immunofluorescence on perinuclear regions and on numerous punctate structures (Fig. 3A, two top panels). The nonpalmitoylated double mutant showed a very different distribution: it was absent from perinuclear sites and from punctate structures, but it was instead primarily localized to the plasma membrane (Fig. 3A, two bottom panels). Localization of
the double mutant was so dominant on the plasma membrane, that it was consistently difficult in the cells expressing it to discern the location of the nucleus. To eliminate the possibility that this altered localization was somehow caused by the presence of the two serines in place of the cysteine residues, we also mutated residues 240 and 241 into alanines. The resulting double mutant (C240A/C241A) was also devoid of palmitate label, and showed identical localization to the C240S/C241S mutant (results not shown). To eliminate the possibility that this very different localization of the palmitate-free double mutants was somehow caused by artifacts of the expression levels and growth conditions, we have examined their distribution in comparison with the wild type protein under a variety of conditions, especially varying the length of transfection and/or the use of serum after the end of transfection. The double mutants were consistently altered in their localization compared with the wild type PLD1 and were in fact always enriched on the plasma membrane (results not shown). We analyzed the distribution of wild type PLD1 and of various mutants by subcellular fractionation (Fig. 3B). Under conditions where the majority of PLD1 was recovered with the membrane fraction, there was a small increase in the proportion of all three mutants (C240S, C241S, C240S/C241S) recovered in the cytosolic pool (Fig. 3B, compare lanes 2 and 3 with lanes 5, 6, 8, 9, 11, and 12). However, all three mutants showed a substantial proportion (65% for the C240S/C241S) still with the membrane fraction. We concluded from these data that fatty acylation of PLD1 contributes very strongly to its localization on intracellular membranes, but it cannot account completely for the localization of PLD1 on membranes in general.

We examined the hydrolytic activity of the nonpalmitoylated PLD1 mutants in comparison with the wild type protein both in vivo and in vitro. COS-7 cells contain relatively high endogenous PLD activity, which can interfere with the detection of PLD activity that is specific for transiently expressed proteins. When activity was measured under standard assay conditions following [3H]palmitate labeling overnight and subsequent challenge with 3% ethanol, there was a 2–3-fold increase in formation of the PLD-specific phosphatidylethanol product between control cells and those overexpressing PLD1 (Fig. 4A, compare lane 3 to lane 4). However, if a small amount of ethanol (0.25–0.5%) was deliberately included during palmitate labeling, we observed that a much cleaner difference was evident in phosphatidylethanol formation attributable to the presence of transiently expressed PLD1, even in the absence of additional ethanol stimulation at the end of labeling (Fig. 4A, compare lane 5 to lane 6). In fact, under those conditions of labeling, subsequent challenge with ethanol did not appreciably increase but rather decreased the difference between control samples and those that contained overexpressed PLD1 (Fig. 4A, compare lane 7 to lane 8). We used both conditions to...
FIG. 6. Conservation of cysteine residues 240 and 241 between PLD1 and PLD2. The region containing the relevant cysteines from several PLD1 and PLD2 genes is shown. The number on the left refers to the first amino acid of the region shown. The numbers in parentheses are accession numbers for the various genes. Letters preceding the PLD gene refer to the organism from which the gene was isolated (h, human; r, rat; m, mouse; c, chinese hamster).

FIG. 7. Fatty acylation of PLD1 within a putative PH domain. The region of PLD1 encompassing amino acids 220 to 320 was aligned to the PH domains of BTK and pleckstrin (PLS) at the level of amino acid sequence (bottom panel). Based on the N-terminal conserved regions of this alignment (boxes in red in bottom panel), the region of PLD1 containing the palmitoylated cysteines was modeled on the known crystal structures of BTK (top panel). In BTK, the analogous region is a loop 6-amino acids long (colored orange in the model to correspond to the amino acids shown at the bottom panel). The same region in PLD1 containing the palmitates is an extended loop (colored yellow, notice the bifurcation in the ribbon diagram) and the palmitates are drawn to scale (purple for palmitates on red for cysteines). Also highlighted in the crystal structures are residues that are known in BTK to bind phosphoinositides (colored cyan and magenta to correspond to the amino acid designations in the bottom panel). We emphasize for PLD1 that the exact geometry of the loop region is fairly arbitrary. However, because the points on which the loop is anchored have been modeled on the known crystal structure of BTK, the spatial relation of the palmitates to the lipid binding region is not without basis.

measure PLD1 and mutant activity in vivo. We found that, in comparison with the wild type protein, the C2408S/C2418S mutant showed activity reduced by 50–80% (Fig. 4B). Its activity however was not totally eliminated, as was the case for both catalytically inactive point mutants (Fig. 4B, samples S911A and K689R). The differences in activity between the various alleles of PLD1 are reproducible (n = 4), and they do not reflect variations in their expression levels (Fig. 4C).

A different result was obtained when activity was measured in vitro (Fig. 5). We did not detect any differences in basal or in Arf-stimulated activity of the double mutant in comparison with the wild type protein, under conditions in which the S911A point mutant was indeed catalytically inactive (Fig. 5A). The mutant replacing the cysteine residues with alamines was equally active under those conditions (Fig. 5B, first six lanes). More importantly, the nonpalmitoylated mutant was as sensitive to pretreatment with neutral hydroxylamine as was the wild type protein (Fig. 5B, last eight lanes). This last result proves that our earlier suggestion, that hydroxylamine eliminates PLD1 activity by removing the palmitate from the protein, was incorrect, despite the fact that reduction in activity and palmitate removal were dependent to the same extent on hydroxylamine concentration and length of treatment (6). We can only speculate that hydroxylamine may remove another prosthetic group from PLD1 to render the resultant protein catalytically inactive. In this context we note recent work from Dixon and colleagues (11) showing that the Nuc endonuclease, which belongs to the PLD superfamily, uses a hydroxylamine-sensitive phosphohistidine intermediate for nucleophilic attack during catalysis.

In several respects, the nonpalmitoylated PLD1 that was obtained in this work resembles PLD2: it had a plasma membrane localization, and it was not active in vivo (1). However two important points suggest that the difference between PLD1 and PLD2 is not simply a reflection of their palmitoylated state: first, the nonpalmitoylated PLD1 had low basal activity and exhibited Arf-dependent stimulation in our assays making it different in properties than PLD2. Second, inspection of the relevant sequences in PLD1 and PLD2 shows that both proteins contain the relevant cysteine residues (Fig. 6). We suggest two possibilities based on our data, which may be relevant in explaining the altered properties of PLD1 and PLD2. In terms of in vivo activity, it is possible that the nonpalmitoylated mutant PLD1 by virtue of relocating to the plasma membrane encounters the same inhibitor(s) responsible for maintaining PLD2 inactive (12). In terms of localization, it is possible that PLD1 gains access to intracellular membranes by a mechanism not related to palmitoylation and once there it becomes palmitoylated and is maintained intracellularly. This would imply that the enzyme responsible for PLD1 palmitoylation resides on intracellular membranes, in analogy to palmitoylation of other proteins in an early Golgi compartment (13), and it is consistent with our observations that fragments of PLD1 and the S911A mutant did not reach the plasma membrane even in the presence of the relevant cysteine residues (Fig. 6). We suggest two possibilities based on our data, which may be relevant in explaining the altered properties of PLD1 and PLD2 in vivo activity, it is possible that the nonpalmitoylated mutant PLD1 by virtue of relocating to the plasma membrane encounters the same inhibitor(s) responsible for maintaining PLD2 inactive (12). In terms of localization, it is possible that PLD1 gains access to intracellular membranes by a mechanism not related to palmitoylation and once there it becomes palmitoylated and is maintained intracellularly. This would imply that the enzyme responsible for PLD1 palmitoylation resides on intracellular membranes, in analogy to palmitoylation of other proteins in an early Golgi compartment (13), and it is consistent with our observations that fragments of PLD1 and the S911A mutant did not reach the plasma membrane even in the presence of the relevant cysteine residues (Fig. 6).

Fatty acylation of signaling proteins is a positive signal for plasma membrane targeting in most cases (9, 14), and our data on PLD1 showing that absence of palmitoylation results in plasma membrane targeting appear contradictory to this general rule. Although we cannot resolve this discrepancy with certainty, we suggest an explanation based on the region of PLD1 containing the fatty acylation sites. It has been noted by Steed et al. (8) that the region of PLD1 encompassing residues 220 to 330 contains a PH-like domain. One function of PH domains is to mediate, at least in part, binding to specialized regions of plasma membrane rich in phosphoinositides, espe-
cially PIP$_2$ and PIP$_3$ (15, 16). The PH domain region of PLD1 was modeled on the known structure of other PH domains, and the palmitate residues (drawn to scale) were added to the structure at cysteine residues 240 and 241 (Fig. 7, palmitates in purple). It is apparent from this model that the palmitate groups are on a loop region between two conserved stretches of the PH domain and could potentially interact with the region of the PH domain responsible for binding phosphoinositides (residues colored cyan and magenta in Fig. 7). Whether this interaction would result in positive or negative effects on the lipid binding cannot be ascertained from the structure. Based on our results we would suggest that the palmitates may obscure the lipid binding region of the PH domain, and, upon palmitate removal, the PH domain may be better exposed and bind phosphoinositides at the plasma membrane. The regulated exposure of the PH domain depending on the palmitoylated state of the loop region may constitute a novel mechanism of regulation of PH domains, and, in the case of PLD1, may contribute to the appearance of PLD1 on the plasma membrane upon certain conditions. This hypothesis can be tested by selected mutagenesis of the PLD1 PH domain and by establishing the lipid-binding characteristics of this domain with or without the fatty acid modification.

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REFERENCES

1. Colley, W., Sung, T. C., Roll, R., Hammond, S. M., Altshuller, Y. M., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) *Curr. Biol.* 7, 191–201
2. Hammond, S. M., Altshuller, Y. M., Sung, T. C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) *J. Biol. Chem.* 270, 29640–29643
3. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) *Annu. Rev. Biochem.* 66, 457–509
4. Exton, J. H. (1997) *J. Biol. Chem.* 272, 15579–15582
5. Ktistakis, N. T. (1998) *Bioessays* 20, 495–504
6. Maniavana, M., Sugars, J., and Ktistakis, N. T. (1999) *J. Biol. Chem.* 274, 1072–1077
7. Bi, K., Roth, M. G., and Ktistakis, N. T. (1997) *Curr. Biol.* 7, 301–306
8. Steed, P. M., Clark, K. L., Boyar, W. C., and Lasala, D. J. (1998) *FASEB J.* 12, 1309–1317
9. Dunphy, J. T., and Linder, M. E. (1998) *Biochim. Biophys. Acta* 1436, 245–261
10. Sung, T. C., Roper, R. L., Zhang, Y., Rudge, S. A., Temel, R., Hammond, S. M., Morris, A. J., Moss, B., Engebrecht, J., and Frohman, M. A. (1997) *EMBO J.* 16, 4519–4530
11. Gottlin, E. B., Rudolph, A. E., Zhao, Y., Matthews, H. R., and Dixon, J. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 9202–9207
12. Sung, T. C., Altshuller, Y. M., Morris, A. J., and Frohman, M. A. (1999) *J. Biol. Chem.* 274, 494–502
13. Schlesinger, M. J., Veit, M., and Schmidt, M. F. G. (1993) in *Lipid Modifications of Proteins* (Schlesinger, M. J., ed), pp. 2–19, CRC Press, Inc., Boca Raton, FL
14. Towler, D. A., Gordon, J. I., Adams, S. P., and Glaser, L. (1988) *Annu. Rev. Biochem.* 57, 69–99
15. Lemmon, M. A., Falasca, M., Ferguson, K. M., and Schlesinger, J. (1997) *Trends Cell Biol.* 7, 237–242
16. Shaw, G. (1996) *Bioessays* 18, 35–46