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Dimerization of Phospholipase D Isozymes

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Two mammalian phospholipase D (PLD) isozymes (PLD1 and PLD2) have been reported. In this study, we differentially tagged these isozymes with enhanced green fluorescent protein (EGFP-rPLD1 and EGFP-rPLD2) or Xpress peptide epitope (Xpress-rPLD1 and Xpress-rPLD2) to examine the association between these isozymes. Overexpressed EGFP-rPLD1 coimmunoprecipitated with Xpress-rPLD1 using anti-Xpress antibody. However, the coimmunoprecipitation was independent of the activity of rPLD1. Xpress-rPLD2 also bound to EGFP-rPLD1 although the binding was less efficient than observed with Xpress-rPLD1. The association between rPLD2 and rPLD1 was confirmed by coimmunoprecipitation of EGFP-rPLD2 with Xpress-rPLD1. EGFP-rPLD2 also bound to Xpress-rPLD2 as shown by coimmunoprecipitation. Immunofluorescence staining of COS-7 cells coexpressing EGFP-rPLDs and Xpress-rPLDs showed that the PLD isozymes colocalized in the perinuclear and plasma membrane regions, suggesting that they could associate in a cellular setting. These results suggest that rPLD1 and rPLD2 can exist as homodimers and can form heterodimers. © 2002 Elsevier Science

Key Words: **phospholipase D; isozyme; dimer; localization.**

Phospholipase D (PLD) is a ubiquitous enzyme expressed in bacteria, fungi, plants, and mammals (1). The PLD superfamily has been defined by a common motif named HKD which contains the sequence $HX(X)_4D$ (2). Two PLD isozymes (PLD1 and PLD2) have been isolated from mammalian species. These isozymes share several common domains (2, 3) and have two HKD motifs. PLD is activated *in vivo* by phorbol esters, G protein-mediated agonists and various growth factors (40). Signals transmitted by receptors to these agents regulate PLD activity via protein kinase C (PKC) and/or members of the Rho family of small GTPases. In contrast to PLD1, PLD2 exhibits high basal activity, but is not activated by PKC, Rho, or ADP-ribosylation factors *in vitro* (5).

Both HKD motifs are essential for PLD catalytic activity, as shown by mutagenesis (6) and it has been suggested that they dimerize to form the catalytic site. This model is supported by structural analysis of a dimer of nuc, a bacterial endonuclease that contains a single HKD motif (7). The HKD motifs have also been shown to be essential for the association between N- and C-terminal halves of the enzyme (8). When the N-terminal half of PLD containing one HKD domain is coexpressed with the C-terminal half with the other HKD domain, the two halves associate together to reconstruct PLD activity, but this does not occur with mutations in the HKD domains (8). These observations are consistent with the occurrence of intramolecular dimerization of the two HKD domains of PLD to produce a catalytically active enzyme. However, it also seemed possible that PLD could undergo intermolecular dimerization. More specifically this was explored in order to test if dimerization could explain why expression of inactive forms of PLD in cells results in suppression of endogenous PLD activity (6, 9–12). The present report indicates that PLD1 and PLD2 can undergo homodimerization and heterodimerization, as revealed by coimmunoprecipitation studies in COS-7 cells.

EXPERIMENTAL PROCEDURES

Materials. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. The transfection reagent FuGENE6 was from Roche Molecular Biochemicals and the pcDNA EpiTag vector system, and monoclonal antibodies against to Xpress and V5epitope tags were from Invitrogen. Protein A-sepharose and anti-mouse and anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase were from Amersham-Pharmacia Biotech. The pEGFP-C3 vector system and anti-GFP antibody conjugated with horseradish peroxidase were from Clontech. Texas-Red conjugated antibody against to mouse IgG was from Molecular Probes. The PLD2 antiserum was kindly provided by Dr. Sylvain Bourgoin (Université Laval) and the PLD1 antiserum was prepared in rabbits using the C-terminal dodecapeptide of rPLD1.

Plasmid construction. The vector constructs expressing Xpress-tagged rPLD1 (Xpress rPLD1) and EGFP-tagged rPLD1-V5 (EGFP-

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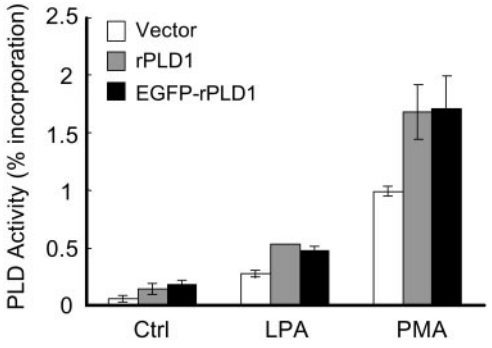

FIG. 1. Activation of EGFP-rPLD1 by LPA and PMA. COS-7 cells were transfected with pcDNA4C vector, rPLD1 or EGFP-rPLD1, and the PLD activity was measured with and without stimulation by 5 μM LPA or 100 nM PMA. PLD activity was expressed as the percentage of total lipid radioactivity incorporated into PtdBuOH. Data are representative of two independent experiments performed in triplicate.

rPLD1-V5) were prepared as previously described [8]. EGFP-tagged rPLD1 (EGFP-rPLD1) was created by the same method for EGFP-rPLD1-V5 using a rPLD1 expressing construct subcloned in pcDNA3.1(+). Xpress-rPLD2 created by cloning into pcDNA3.1 EpiTag vector from a rat cDNA library was a gift of Z. Xie (Vanderbilt University). The protein-coding region of rPLD2 was subcloned into pEGFP-C3 vector from the Xpress-rPLD2 construct by using KpnI and XbaI.

Cell culture and transfection. COS-7 cells were purchased from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.3 mg/ml L-glutamine at 37°C in a humidified atmosphere of air-CO_2 (19:1). The constructs expressing tagged or untagged rPLD1 and rPLD2 were transfected into COS-7 cells using FuGENE6 according to the manufacturer's instructions.

Measurement of PLD activity. COS-7 cells were plated on 6-well plates and transfected as described above. Cells were starved in DMEM containing 0.5% bovine serum albumin for 24 h and labeled with 1 μCi/ml of [9,10-3H]myristic acid. The formation of [3H]phosphatidylbutanol in the presence of 0.3% butan-1-ol was measured as previously described (9).

Immunofluorescence microscopy. Xpress-rPLD was coexpressed with EGFP-rPLD in COS-7 cells for 24 h. Cells were fixed with 3.7% formaldehyde in PBS, and permeabilized with 0.5% Triton X-100 in PBS. Xpress-rPLD was immunolabeled using anti-Xpress antibody and anti-mouse IgG antibody conjugated with Texas-Red. Fluorescence-labeled cells were observed with a Zeiss LSM 410 confocal laser scanning inverted microscope.

Immunoprecipitation. Tagged rPLDs were transfected into COS-7 cells plated on 100 mm culture plates. Cells were washed twice with PBS and lysed in 1 ml of lysis buffer containing 50 mM Tris (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, and protease inhibitor cocktail. The Xpress-tagged rPLD1 or rPLD2 was precipitated with anti-Xpress antibody and protein A-sepharose. The proteins in the immunoprecipitates were analyzed by Western blotting.

RESULTS

Coimmunoprecipitation of EGFP-rPLD1 and Xpress-rPLD1. rPLD1 was tagged at its N-terminus with EGFP since tagging at the C-terminus inactivates the enzyme (13). Figure 1 shows that there was no difference in catalytic activity between EGFP-rPLD1 and intact rPLD1, and that both LPA and PMA activated EGFP-rPLD1 as effectively as wild-type rPLD1. Similar results were found with rPLD1 tagged at its N-terminus with Xpress (data not shown). When Xpress-rPLD1 was coexpressed with EGFP-rPLD1 in COS-7 cells and the lysates immunoprecipitated using anti-Xpress antibody, EGFP-rPLD1 was precipitated together with Xpress-rPLD1, as detected by Western blotting with anti-PLD1 antibody (lane 3, Fig. 2A and lanes 1 and 2, Fig. 3). Both figures show that the amounts of EGFP-rPLD1 and Xpress-rPLD1 in the immunoprecipitates were similar, despite the much greater expression of EGFP-rPLD1 in the lysates. EGFP-rPLD1 was not precipitated by Xpress-rPLD1 antibody in the absence of Xpress-rPLD1 (lane 1, Fig. 2A). Figure 2B shows that the coimmunoprecipitation was not due to a nonspecific affinity between EGFP and Xpress-rPLD1 since EGFP alone was not coimmunoprecipitated even in the presence of Xpress-rPLD1. These results suggest that rPLD1 can dimerize.

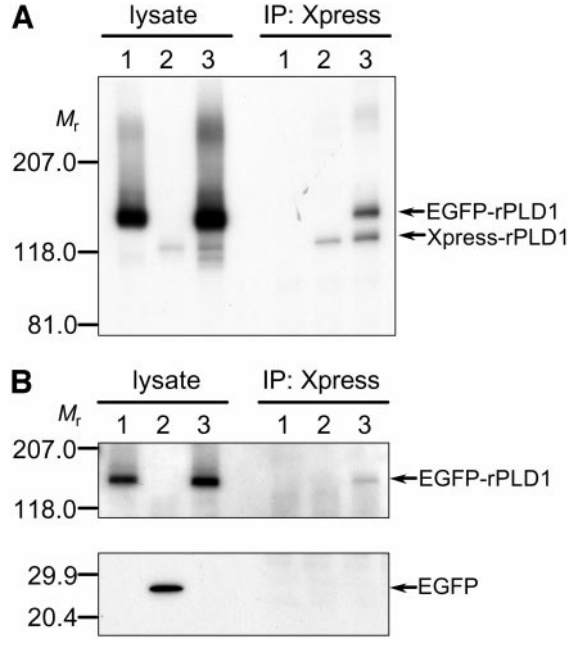

FIG. 2. Coimmunoprecipitation of EGFP-rPLD1 and Xpress-rPLD1 using anti-Xpress antibody. COS-7 cells were cotransfected with EGFP-rPLD1 and pcDNA4C vector (lane 1), pEGFP-C3 vector and Xpress-rPLD1 (lane 2), or EGFP-rPLD1 and Xpress-rPLD1 (lane 3). After 24 h, cells were lysed and immunoprecipitated with anti-Xpress antibody. (A) The lysates and the immunoprecipitates were separated on 8% Tris-glycine polyacrylamide gels and visualized by Western blotting using anti-rPLD1 antibody. (B) The lysates and the immunoprecipitates were separated on 8–16% Tris-glycine gradient polyacrylamide gels and visualized by Western blotting using anti-GFP antibody.

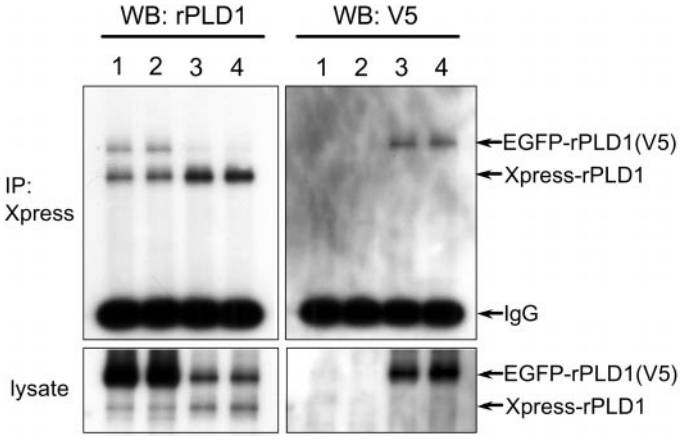

FIG. 3. Dimerization is independent of the activity of rPLD1. After 24 h, coexpression of EGFP-rPLD1 (lanes 1 and 2) or EGFP-rPLD1-V5 (lanes 3 and 4) with Xpress-rPLD1, cells were starved for 18 h. Xpress-rPLD1 was immunoprecipitated using anti-Xpress antibody with (lanes 2 and 4) or without (lanes 1 and 3) treatment with 100 nM PMA for 15 min. The lysates and immunoprecipitates were separated on 8% Tris-glycine polyacrylamide gels and visualized by Western blotting with anti-rPLD1 or anti-V5 antibody.

Dimerization of rPLD1 is not dependent on its activity. To examine if dimerization is influenced by the activity of rPLD1, we compared dimer formation before and after activation by PMA. However, no difference in dimerization was detected (lanes 1 and 2, respectively, Fig. 3). This result shows that the dimer exists regardless of PLD activation. We also tested if dimerization occurred between Xpress-rPLD1 and an inactive mutant of rPLD1. EGFP-rPLD1-V5, which is totally inactive, bound to Xpress-rPLD1 as efficiently as EGFP-rPLD1 (lanes 3 and 4, Fig. 3). PMA treatment did not alter this binding also. EGFP-rPLD1-V5 includes additional amino acids at its C-terminus and is poorly detected by the anti-rPLD1 antibody. For this reason, Western blotting using anti-V5 antibody was used to demonstrate the presence of EGFP-rPLD1-V5 in the immunoprecipitate (right panels of Fig. 3).

PLD1 can heterodimerize with PLD2. The dimerization between rPLD1 and rPLD2 was examined since the two isozymes share many conserved domains. As shown in Fig. 4, we detected coimmunoprecipitation of EGFP-rPLD1 with Xpress-rPLD2 (lane 5 in the middle panel of Fig. 4), and the band intensity of EGFP-rPLD1 was similar to that coprecipitated with Xpress-rPLD1 (lane 3). However, the amount of Xpress-rPLD2 in the immunoprecipitate was much higher than that of Xpress-rPLD1 (see Western blotting results using Xpress-antibody in the bottom panel, Fig. 4). These results indicate that the association between rPLD1 and rPLD2 occurs, but is less efficient than that between rPLD1 and rPLD1.

The association between the two PLD isozymes was confirmed in the reverse way using EGFP-rPLD2. Both Xpress-rPLD2 and EGFP-rPLD2 showed high basal activity and were significantly activated by PMA (Fig. 5A). When EGFP-rPLD2 was co-expressed with Xpress-rPLD1 or with Xpress-rPLD2, it was coimmunoprecipitated with both isozymes by anti-Xpress antibody (Fig. 5B).

Overexpressed rPLD1 and rPLD2 colocalize in COS-7 cells. To determine if the association between the isozymes could occur in intact cells, cellular localization of the coexpressed isozymes was explored in COS-7 cells. As shown in Fig. 6A, no difference in cellular location could be detected between Xpress-rPLD1 and EGFP-rPLD1 when they were coexpressed in the cells (upper panels, Fig. 6A). Similar results were obtained with differentially tagged rPLD2 (lower panels, Fig. 6A). Both PLDs were localized to the perinuclear region and to a variable extent in the plasma membrane, but the distribution of PLD2 was more dispersed than PLD1. These data also indicate that the addition of EGFP (27 kDa molecular mass) to rPLD1 or rPLD2 did not alter its cellular localization. Figure 6B shows the colocalization of rPLD1 and rPLD2. When Xpress-tagged rPLD1 or rPLD2 was coexpressed with the other isozyme tagged with EGFP, they were colocalized in the perinuclear region. These results suggest that rPLD1 and rPLD2 can interact with each other in certain cellular settings.

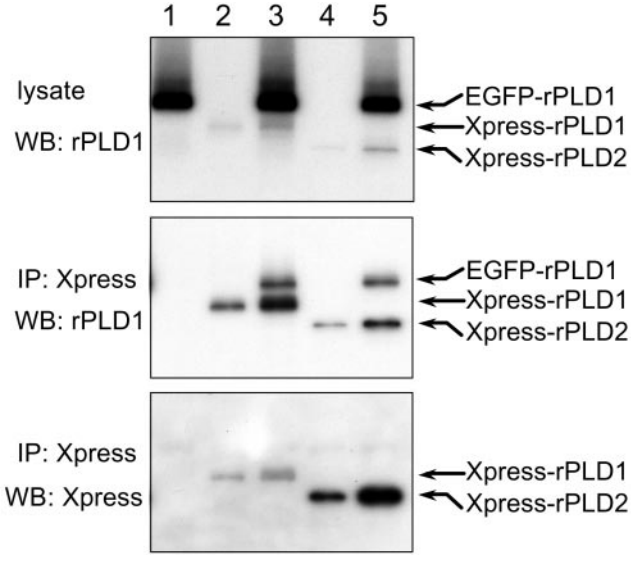

FIG. 4. EGFP-rPLD1 can associate with Xpress-rPLD2. COS-7 cells were cotransfected with a combination of EGFP-rPLD1 and pcDNA4C vector (lanes 1), pEGFP-C3 vector and Xpress-rPLD1 (lane 2), EGFP-rPLD1 and Xpress-rPLD1 (lane 3), pEGFP-C3 vector and Xpress-rPLD2 (lane 4), or EGFP-rPLD1 and Xpress-rPLD2 (lane 5). The lysates and the immunoprecipitates obtained using anti-Xpress antibody were visualized by Western blotting with anti-rPLD1 or anti-Xpress antibody.

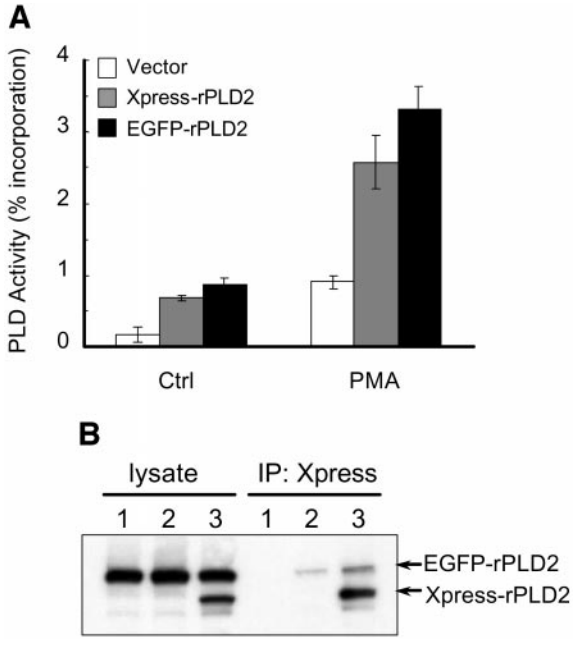

FIG. 5. The activities of Xpress-rPLD1 and EGFP-rPLD2 and the association between these. (A) COS-7 cells were transfected with pcDNA vector, Xpress-rPLD2, or EGFP-rPLD2, and the PLD activity was measured with and without stimulation by 100 nM PMA. PLD activity was expressed as the percentage of total lipid radioactivity incorporated into PtdBuOH. Data are means from three independent experiments. (B) EGFP-rPLD2 was cotransfected with pcDNA4C vector (lane 1), Xpress-rPLD1 (lane 2), or Xpress-rPLD2 (lane 3) into COS-7 cells. The lysates and the immunoprecipitates obtained using anti-Xpress antibody were visualized by Western blotting using anti-PLD2 antibody.

DISCUSSION

The enzymatic properties of mammalian PLD have been intensively investigated (1). The linear domain structures of PLD1 and PLD2 have also been defined by sequence-homology comparisons and mutagenesis studies (2). However, the three dimensional structure of mammalian PLD is unknown. It has been suggested that the two HKD domains dimerize to form the catalytic domain, based on structural information of a bacterial endonuclease containing a single HDK motif (7). Since association is observed when the two halves of PLD1 are coexpressed in cells, but not when they are added together *in vitro* (8), HKD dimerization probably occurs during folding of the enzyme after it is synthesized on the ribosome. Although the HKD dimer (catalytic site) is obviously accessible to the phospholipid substrate, it seemly unlikely to be involved in the intermolecular dimerization observed in the present study. This is because the HKD domains in each PLD molecule would be dimerized, making residues in these motifs less available for interaction with another PLD molecule. Furthermore, if intermolecular dimerization involved the HKD motifs it would be expected to interfere with catalysis.

The coimmunoprecipitation of differentially tagged rPLD1 molecules shown in Fig. 2A and Fig. 3 implies that there was an actual association between two rPLD1 molecules. The amount of EGFP-rPLD1 immunoprecipitated was similar to that of Xpress-rPLD1 although the actual expression level of EGFP-rPLD1 was much higher than Xpress-rPLD1. The results also showed that the immunoprecipitation of EGFP-rPLD1 was totally dependent on the presence of Xpress-rPLD1. The fact that almost equal amounts of EGFP-rPLD1 and Xpress-rPLD1 were found in the precipitates suggests that the interaction involved dimerization rather than a higher order of polymerization.

The activity of rPLD1 is highly regulated by PKC and other stimuli (4). However, activation of the enzyme by PMA did not influence the dimerization. Dimerization of rPLD1 was also observed in serum-starved conditions and with a totally inactive PLD mutant (Fig. 3). These observations suggests that the activity of the enzyme does not control its dimerization.

rPLD2 also coimmunoprecipitated with rPLD1 (Fig. 4) suggesting the formation of a heterodimer. However, the interaction did not appear to be as strong as that between EGFP-rPLD1 and Xpress-rPLD1. This probably reflects the differences in the amino acid sequences of the two PLD isozymes. EGFP-rPLD2 and Xpress-rPLD2 also associated (Fig. 5).

Colocalization of EGFP-rPLD1 with Xpress-rPLD1 and of EGFP-rPLD2 with Xpress-rPLD2 was observed when they were overexpressed in COS-7 cells, indicating that the large EGFP tag did not disturb the localization of these isozymes. rPLD1 was principally located in the perinuclear region whereas rPLD2 was more diffusely distributed (Fig. 6). However, it must be noted that these findings on the expressed isozymes may not relate to the location of endogenous PLD1 or PLD2 in these cells. Interestingly, when tagged rPLD1 and rPLD2 were coexpressed, a significant colocalization in the perinuclear region was observed. These findings suggest, but certainly do not prove, that the two isozymes might interact in this region. Most studies have localized PLD1 to the perinuclear region, but have differed on whether or not the association is with the Golgi, or whether the enzyme is also associated with the plasma membrane (5, 9, 12, 14–19). Overexpressed tagged PLD2 is usually found at the plasma membrane (5, 14, 18, 29), but there is evidence that PLD2 and PLD1 also associate with the actin cytoskeleton (20, 21).

The present study does not define the domain(s) in PLD involved in the intermolecular interaction. A possibility is the PX domain, which is located in the N-terminus of both PLD1 and PLD2 and is thought to be involved in protein-protein interactions as well as binding to 3-phosphoinositides (2, 22). In the absence of other known protein-protein interaction motifs, mu-

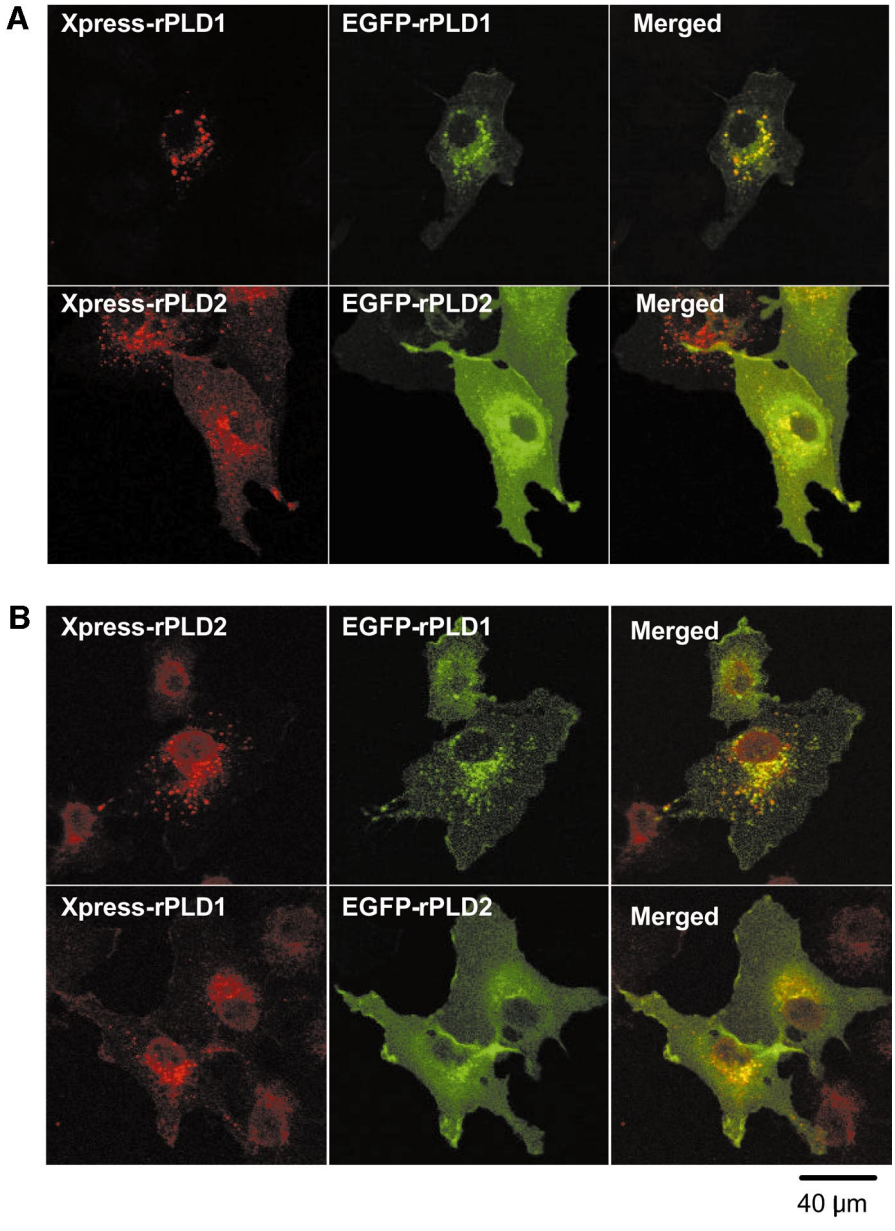

FIG. 6. Cellular localization of overexpressed PLDs. (A) COS-7 cells were cotransfected with a combination of Xpress-rPLD1 and EGFP-rPLD1 (upper panels) or Xpress-rPLD2 and EGFP-rPLD2 (lower panels). The cells were fixed and permeabilized as described under Experimental Procedures, and the Xpress-tagged PLDs were visualized using anti-Xpress antibody and anti-mouse IgG antibody conjugated with Texas Red (red color). The fluorescence images of Xpress and EGFP were merged to compare the localization. (B) Xpress-rPLD1 or Xpress-rPLD2 was co-expressed with EGFP-rPLD2 or EGFP-rPLD21, respectively. The cells were treated and the PLD visualized as described in A.

tagenesis studies will be required to define the sequence(s) involved in the dimerization.

An important issue is the significance of the dimerization. One possibility is that the catalytic activity of the dimer is different from that of the monomer. As noted above, dimerization was not dependent on the activity of the enzyme since PMA strongly activated the enzyme in the absence of a detectable change in dimerization, and dimerization was observed in unstimulated cells. However, these findings do not address the issue of an activity change with dimerization.

Another possibility is that the dimers are located at a different cellular site(s) than the monomers. This could occur because the dimer occludes certain domains that specify localization of the monomer. It is of interest to note that coexpressed rPLD1 and rPLD2 were colocalized to the perinuclear region, whereas rPLD2 was more diffusely distributed (Fig. 6).

Finally, the studies could explain why the expression of catalytically inactive forms of PLD1 and PLD2 can inhibit cellular PLD activity (6, 9–12). By dimerizing with the endogenous enzyme, these mutant forms could inhibit its activity. This could occur because the inactive monomer prevents activation of the wild-type monomer by its regulators (PIP_2, ARF, Rho, PKC) or impedes it access to its membrane substrate. Other mechanisms of interaction between the active and inactive enzymes are possible.

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REFERENCES

1. Exton, J. H. (1998) Phospholipase D. *Biochim. Biophys. Acta* **1436,** 105–115.
2. Frohman, M. A., Sung, T.-C., and Morris, A. J. (1999) Mammalian phospholipase D structure and regulation. *Biochim. Biophys. Acta* **1439,** 175–186.
3. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) Phospholipase D: Molecular and cell biology of a novel gene family. *Biochem. J.* **345,** 401–415.
4. Exton, J. H. (1999) Regulation of phospholipase D. *Biochim. Biophys. Acta* **1439,** 121–133.
5. Colley, W. C., Sung, T.-C, Roll, R., Jenco, J., Hammond, S. M., Altshuller, Y., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7,** 191–201.
6. Sung, T. C., Roper, R., Zhang, Y., Rudge, S. A., Temel, R., Hammond, S. M., Morris, A. J., Moss, B., Engebrecht, J., and Frohman M. A. (1997) Mutagenesis of phospholipase D defines a superfamily including a *trans*-Golgi viral protein required for poxvirus pathogenicity. *EMBO J.* **16,** 4519–4539.
7. Stuckey, J. A., and Dixon, J. E. (1999) Crystal structure of a phospholipase D family member. *Nature Struct. Biol.* **6,** 278–284.
8. Xie, Z., Ho, W.-T., and Exton, J. H. (2000) Association of the N- and C-terminal domains of phospholipase D. Contribution of the conserved HKD motifs to the interaction and the requirement of the association for Ser/Thr phosphorylation of the enzyme. *J. Biol. Chem.* **275,** 24962–24969.
9. Kam, Y., and Exton, J. H. (2001) Phospholipase D activity is required for actin stress fiber formation in fibroblasts. *Mol. Cell. Biol.* **21,** 4055–4066.
10. Zhang, Y., Altshuller, Y. M., Hammond, S. M., Hayes, F., Morris, A. J., and Frohman, M. A. (1999) Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C. *EMBO J.* **18,** 6339–6348.
11. Rizzo, M. A., Shome, K., Vasudevan, C., Stolz, D. B., Sung, T.-C., Frohman, M. A., Watkins, S. C., and Romero, G. (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* **274,** 1131–1139.
12. Vitale, N., Caumont, A.-S., Chasserot-Golaz, S., Du, G., Wu, S., Sciorra, V. A., Morris, A. J., Frohman, M. A., and Bader, M.-F. (2001) Phospholipase D1: A key factor for the exocytotic machinery in neuroendocrine cells. *EMBO J.* **20,** 2424–2434.
13. Xie, Z., Ho, W.-T., and Exton, J. H. (2000) Conserved amino acids at the C-terminus of rat phospholipase D1 are essential for enzymatic activity. *Eur. J. Biochem.* **267,** 7138–7146.
14. Liscovitch, M., Czarny, M., Fiucci, G., Lavie, Y., and Tang, X. (1999) Localization and possible functions of phospholipase D isozymes. *Biochim. Biophys. Acta* **1439,** 245–263.
15. Brown, F. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson, N. T., Solari, R., and Wakelam, M. J. O. (1998) Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. *Curr. Biol.* **8,** 835–838.
16. Kim, Y., Kim, J.-E., Lee, S. D., Lee, T. G., Kim, J. H., Park, J. B., Han, J. M., Jang, S. K., Suh, P.-G., and Ryu, S. H. (1999) Phospholipase D1 is located and activated by protein kinase Cα in the plasma membrane in 3Y1 fibroblast cell. *Biochim. Biophys. Acta* **1436,** 319–330.
17. Sugars, J. M., Cellek, S., Manifava, M., Coadwell, J., and Ktistakis, N. T. (1999) Fatty acylation of phospholipase D1 on cysteine residues 240 and 241 determines localization on intracellular membranes. *J. Biol. Chem.* **274,** 30023–30027.
18. Emoto, M., Klarlund, J. K., Waters, S. B., Hu, V., Buxton, J. M., Chawla, A., and Czech, M. P. (2000) A role for phospholipase D in GLUT4 glucose transporter translocation. *J. Biol. Chem.* **275,** 7144–7151.
19. Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M., and Shields, D. (2001) Intracellular localization of phospholipase D1 in mammalian cells. *Mol. Biol. Cell.* **12,** 943–955.
20. Lee, S., Park, J. B., Kim, J. H., Kim, Y., Kim, J. H., Shin, K. J., Lee, J. S., Ha, S. H., Suh, P.-G, and Ryu, S. H. (2001) Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* **276,** 28252–28260.
21. Iyer, S. S., and Kusner, D. J. (1999) Association of phospholipase D activity with the detergent-insoluble cytoskeleton of U937 promonocytic leukocytes. *J. Biol. Chem.* **274,** 2350–2359.
22. Wishart, M. J., Taylor, G. S., and Dixon, J. E. (2001) Phoxy lipids: Revealing PX domains as phosphoinositide binding modules. *Cell* **105,** 817–820.