

Regulation of Expression of Phospholipase D1 and D2 by PEA-15, a Novel Protein That Interacts with Them*

Received for publication, April 18, 2000, and in revised form, July 19, 2000
Published, JBC Papers in Press, August 3, 2000, DOI 10.1074/jbc.M003329200

Yue Zhang[‡], Olga Redina[‡], Yelena M. Altshuller[‡], Masakazu Yamazaki[§], Joe Ramos^{||},
Herve Chneiweiss^{**}, Yasunori Kanaho[§], and Michael A. Frohman[‡] ^{‡‡}

From the [‡]Department of Pharmacology and the Institute for Cell and Developmental Biology, SUNY at Stony Brook, Stony Brook, New York 11794-8651, the [§]Department of Pharmacology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan, the ^{||}Department of Vascular Biology, The Scripps Research Institute, La Jolla, California 92037, and the ^{**}Institut National de la Sante et de la Recherche Medicale U114 and Chaire de Neuropharmacologie, Collège de France, 75231 Paris Cedex 05, France

Phospholipase D (PLD), a signal-transducing membrane-associated enzyme, is implicated in diverse processes including apoptosis, ERK activation, and glucose transport. Prior studies have identified specific PLD activators and repressors that directly regulate its enzymatic activity. Using two-hybrid screens, we have identified PEA-15 as a PLD interactor that unexpectedly functions to alter its level of expression. PEA-15 is a widely expressed death effector domain-containing phosphoprotein involved in signal transduction, apoptosis, ERK activation, and glucose transport. The PLD1-interacting site on PEA-15 consists of part of the death effector domain plus additional C-terminal flanking sequences, whereas the PEA-15-interacting site on PLD1 overlaps the previously identified RhoA-interacting site. PEA-15 did not affect basal or stimulated *in vitro* PLD1 enzymatic activation. However, co-expression of PEA-15 increased levels of PLD1 activity. This increased activation correlated with higher PLD1 protein expression levels, as marked by faster accumulation and longer persistence of PLD1 when PEA-15 was present. PEA-15 similarly increased protein expressions level of PLD2 and co-immunoprecipitated with it. These results suggest that PEA-15 may stabilize PLD or act as a PLD chaperone. The common involvement of PEA-15 and PLD in apoptosis, ERK activation, and glucose transport additionally suggests functional significance.

lies all activate PLD through direct stimulation. There are also inhibitors that target PLD directly through interaction or through hydrolysis of phosphatidylinositol 4,5-bisphosphate, its required co-factor (reviewed in Ref. 1). The fact that these activators and inhibitors regulate very important cellular functions has implied that PLD will also play important roles. These roles are just starting to be elucidated and include glucose transport (3), regulation of the actin cytoskeleton and membrane ruffling (4), secretion (5), and apoptosis (reviewed in Ref. 6). Little is known, though, about the regulation of other aspects of PLD cell biology, for example, how its subcellular localization is determined, if it translocates upon activation in some circumstances, why and how it is regulated on a transcriptional level in many cell types, and whether there are additional mechanisms such as phosphorylation or other post-translational mechanisms that are also important. Many of these processes involve protein-protein interactions that can be explored using the yeast two-hybrid system.

An interaction between RhoA and the C terminus has already been reported (7, 8). In addition, the structure of the PLD superfamily indicates that the mammalian PLDs are likely to participate in several types of protein-protein interactions that as of yet wait to be defined. For example, mammalian PLD1 and PLD2 and the *Caenorhabditis elegans*, *Drosophila*, and yeast PLDs all encode N-terminal plox and pleckstrin homology domains that are not required for PLD activity (9). Such domains can mediate protein-protein interactions and represent good candidate regions to regulate PLD subcellular localization (3, 10, 11).

In this study, we describe the result of a two-hybrid screen that identified a novel PLD1-interacting protein that appears to regulate PLD expression levels rather than enzymatic activity and which physically links PLD to sites of apoptosis and glucose transport.

EXPERIMENTAL PROCEDURES

General Reagents—[³H]Dipalmitoyl phosphatidylcholine [choline-methyl-³H] ([³H]phosphatidylcholine) was obtained from PerkinElmer Life Sciences and palmitic acid [9,10-³H(N)] from American Radiolabeled Chemicals, Inc. (St. Louis, MO). GTP γ S and anti-HA monoclonal antibody (clone 12CA5 or 3F10) were purchased from Roche Molecular Biochemicals. Anti-FLAG monoclonal antibody (M2) or agarose beads conjugated with M2 were from Sigma. Anti-mouse or rat IgG from goat conjugated with horseradish peroxidase was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alexa 488 conjugated anti-mouse and Alexa 576-conjugated anti-rabbit antibodies were obtained from Molecular Probes (Eugene, OR). TLC Plates (LK5DF silica Gel 150A or K6 silica gel 60A) were obtained from Fisher. All cell culture media (*i.e.* Dulbecco's modified Eagle's medium, Opti-MEM-1) and LipofectAMINE PLUS were from Life Technologies, Inc. Phosphatidyl-

Phospholipase D (PLD)¹ catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid and choline (reviewed in Ref. 1). PLD activity and the production of phosphatidic acid have been implicated in signal transduction, membrane vesicular trafficking, cytoskeletal reorganization, and cell proliferation (reviewed in Ref. 2). Signal transduction cascades leading to the activation of PLD have been the target of recent intensive investigation. A subset of classical protein kinase C (PKC) isoform family members and members of the ADP-ribosylation factor (ARF) and Rho small G-protein fami-

* This work was supported by National Institutes of Health Grant GM54813 (to M. A. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^{‡‡} To whom correspondence should be addressed. Tel.: 631-632-1476; Fax: 631-444-3218; E-mail: michael@pharm.sunysb.edu.

¹ The abbreviations used are: PLD, phospholipase D; PKC, protein kinase C; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; HA, hemagglutinin; DED, death effector domain; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; ARF, ADP-ribosylation factor.

inositol 4,5-bisphosphate and phorbol 12-myristate 13-acetate (PMA) were from Sigma, and all other lipids were from Avanti Polar Lipids (Alabaster, AL). All other reagents were of analytical grade unless otherwise specified.

Plasmids and Yeast Strains—Fragments of human PLD1b (using PLD1a numbering system for consistency) were fused C-terminal to a LexA DNA binding domain in the yeast shuttle plasmid pBTM116-Ade2-KN. The yeast shuttle plasmid pBTM116 was described previously (12) and was modified to generate pBTM116-Ade2-KN, which includes the Ade2 gene inserted at the unique *PvuII* site using linkers in the order *KpnI-NotI-Ade2-NotI-KpnI* (available upon request from M. A. Frohman). Sequences in the vicinity of the 5' junction of the PLD1 fragments were confirmed by sequencing. Wild type and mutant PLD1 and PLD2 coding sequences in the mammalian expression vector pCGN, which contains a cytomegalovirus promoter and encodes an N-terminal appended HA epitope tag, were described previously (7, 11, 13). Full-length PEA-15 (phosphoprotein enriched in astrocytes, 15 kDa) was amplified with primer A (5'-AGCTCTAGAATGGCAGAGT-ACGGAACCTCC-3') and primer B (5'-TCGGCCCGGGCTTGTTCAGGCCTTCTTGG-3') from an E10.5 mouse embryo cDNA pool, digested with the oligonucleotide *XbaI* and *XbaI* sites, and ligated into pCGN and pBluescript (Stratagene). The point mutation S104A in the PEA-15 pCGN expression plasmid was introduced using the QuikChange site-directed mutagenesis kit as per manufacturer's recommendations (Stratagene). The yeast strains L40 and AMR70 (used for the two-hybrid analyses) were a gift from Dr. Rolf Sternglanz. Stan Hollenberg kindly provided the two-hybrid cDNA library (12). Yeast two-hybrid analyses were performed according to general protocols (12).

Purification of His₆-tagged PEA-15 Protein—Full-length PEA-15 protein was produced in the bacteria BL-21 as a fusion protein with a His₆ tag at the N terminus after isopropyl-1-thio-β-D-galactopyranoside induction. Large scale PEA-15 was purified using a Zinc column (TALON metal affinity Resin) according to the manufacturer's protocol (CLONTECH, Palo Alto, CA). The PEA-15 was then dialyzed into phosphate-buffered saline and adjusted to a concentration of about 50 ng/μl for use in the *in vitro* PLD activity assay.

Cell Culture and Transfections—COS-7 cells and HEK293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) in a humidified atmosphere containing 5% CO₂ at 37 °C. Transient transfections using LipofectAMINE PLUS were carried out as described previously (11). Optimal PLD1 activation and expression as a function of co-transfection with PEA-15 was observed when the plasmids were transfected in a 10:1 (PLD1:PEA-15) ratio.

PLD Activity Assays—PLD activity assays were carried out using the *in vitro* head group release assay and the *in vivo* transphosphatidylation assay (14) as described previously (11). Recombinant ARF1, RhoA, and PKC-α were purified and activated using 50 μM GTPγS or 100 nM PMA as described previously (11, 15). ATP was not present in the assay when PKC was used except where noted.

Western Analysis and Immunofluorescent Staining—Both were performed according to published methods (9). Proteins were visualized by Western blotting analysis using monoclonal antibody 3F10 (rat anti-HA) and horseradish peroxidase-conjugated secondary antibodies (Roche Molecular Biochemicals). The signals were visualized using ECL reagents (Amersham Pharmacia Biotech). The intracellular localizations of PLD1 and PEA-15 were determined via indirect immunofluorescent microscopy using protocols previously published (16).

Co-immunoprecipitation—PEA-15 was generated as a GST fusion protein attached to glutathione beads, and FLAG-D4 in phosphate-buffered saline was prepared as described previously (8, 17). 50 ng of FLAG-D4 and equal molar amounts of the GST fusion proteins were mixed in phosphate-buffered saline at 4 °C for 2 h. The beads were then washed extensively (more than five times) with phosphate-buffered saline. Proteins were eluted by incubating in sample buffer containing urea and subjected to Western analysis.

To assess *in vivo* interactions, pCGN-PEA-15 and pFLAG-PLD1 or -PLD2 were co-expressed in two 35-mm plates of COS-7 cells. Cells were lysed in 100 μl of buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1× proteinase inhibitor mixture (Roche Molecular Biochemicals), following which the supernatants were added to 100 μl of 10% anti-FLAG M2 antibody affinity resin (3.3 mg protein/ml gel, Kodak) that had been washed into the same buffer. The mixture was rotated for 4 h at 4 °C. The beads were then washed five times with 1 ml of washing buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100. Proteins were eluted in 1× sample buffer, and one-quarter of the total elute was subjected to Western analysis to detect the HA tag fused in frame to PEA-15.

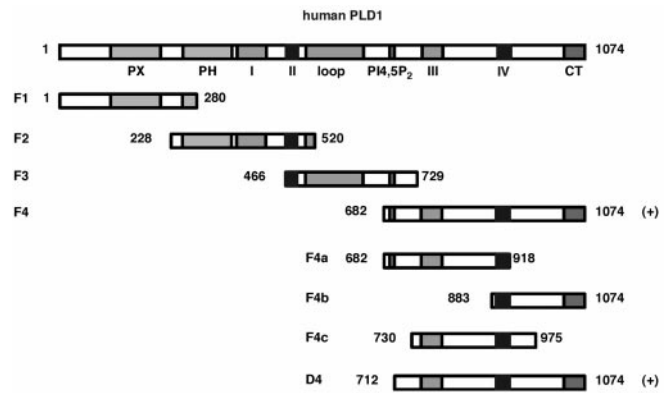


FIG. 1. Domain structure of PLD1 and fragments used in the two-hybrid study. PLD1 contains well established conserved domains as described in detail in the text. Dark shaded boxes indicate the spilt catalytic domains. Light shaded boxes indicate conserved regions found in eukaryotic PLDs. Regions I–IV are also found in prokaryotic PLDs. *F* and *D* numbers denote PLD1 fragments used in this study. The numbers flanking each fragment indicate the amino acid at which the fragment initiates or terminates. (+) indicates successful interaction with PEA-15.

RESULTS

Identification of PEA-15 as a PLD1-interacting Protein—Mammalian PLD1 contains N-terminal phox and pleckstrin homology domains followed by four conserved regions (I–IV) that are found in all PLD family members. Conserved regions II and III are separated by a unique and seemingly dispensable “loop” region and by the phosphatidylinositol 4,5-bisphosphate interacting site. There is an additionally conserved C-terminal region that is critical for enzymatic activity (Fig. 1 and Ref. 1). To accomplish the two-hybrid screen, PLD1 was divided into four fragments (F1–F4) encompassing different domains that overlapped in an attempt to preserve the correct three-dimensional structure of each domain (Fig. 1). These fragments were used as baits against a mouse embryonic cDNA library, and 2.8×10^6 to 9×10^6 colonies were recovered from each screen (Table I). This effort should have resulted in a fairly complete sampling because the library used contained about 3×10^6 individual clones that statistically represented each cDNA 10–30 times (12). Specificity was assessed by determining whether the candidates recovered failed to interact with a battery of negative controls.

For fragments F1 and F2, specific interacting partners were not identified. A single specific candidate that interacted with F3 was found to encode a partial clone of pyruvate kinase. Of the 27 clones that interacted specifically with fragment F4, three encoded a partial sequence of a mouse homolog of the human KIAA0661 protein (GenBank™ accession number AB014561) whose function is unknown. Two encoded a “Ariadne” family member (GenBank™ accession number AJ130977) that is best known for its RING finger domain (a zinc-binding motif); however, the region that mediated the interaction did not contain the RING finger. One encoded a RNA binding protein (GenBank™ accession number X79233). Given the lack of obvious significance of these clones in the context of PLD biology, they were not further pursued. The remaining 21 clones encoded partial cDNA sequences for a gene previously cloned and denoted as PEA-15 (Fig. 2 and Ref. 18). PEA-15 is a widely expressed PKC-regulated 15-kDa protein involved in signal transduction, apoptosis, and regulation of glucose transport via Glut1 and Glut4 (17, 19–22). Given the proposed roles for PLD in signal transduction (reviewed in Ref. 1), apoptosis (reviewed in Ref. 6), and regulation of Glut4 translocation (3) and its regulation by PKC, PEA-15 appeared

TABLE I
Results of the yeast two-hybrid library screen

	F1	F2	F3	F4
Total transformants	2.8×10^6	3.2×10^6	9×10^6	4.6×10^6
His ⁺ colonies	64	72	80	107
β -Gal ⁺ (blue colonies)	15	13	18	30
Cured of bait; β -Gal ⁻ and His ⁻	7	1	9	28
His ⁺ and β -Gal ⁺ subsequent to retransformation	0	0	1	27
Interacted specifically with bait ^a			1	27

^a Negative controls for specificity included LexA, LexA-Lamin, LexA-Topoisomerase1, LexA-Topoisomerase3, LexA-Spo14, and LexA-F1, LexA-F2, and LexA-F3.

to be an intriguing interacting partner of potential biological significance.

To verify the interaction in the two-hybrid system, we swapped the bait (PLD1-F4) and the target (PEA-15) on the two hybrid vectors and found that the resulting pair of fusion proteins was still capable of interacting (data not shown). This eliminated the possibility that the interaction depended on a specific fusion protein-induced conformation. We also tested the ability of the full-length coding region of PEA-15 to interact with PLD1-F4. A successful interaction occurred as evidenced by a quick development of blue color in the β -galactosidase filter assay; however, single clones did not appear in the growth assay on plates lacking histidine (data not shown). This may indicate that the fusion protein was not stable or that there was a weaker interaction between full-length PEA-15 and PLD1-F4 in the two-hybrid system, because in the yeast strain used there are only four binding sites in the promoter region of the HIS3 reporter, whereas eight such sites drive expression of the LacZ gene (12). Collectively, these data demonstrated that PEA-15 and PLD1-F4 interact specifically in the two-hybrid system.

Both the DED Domain and the PKC Substrate Site of PEA-15 Are Required for Its Interaction with PLD1-F4—Because multiple overlapping PEA-15 clones were identified in the two-hybrid screen, we were immediately able to localize the PLD1 interacting domain. The shortest overlapping region among the 21 clones was denoted as the minimum interacting domain (Fig. 2, *double underlined region*) and confirmed using polymerase chain reaction to amplify this region followed by two-hybrid analysis (data not shown). PEA-15 contains an N-terminal DED and a *bona fide* protein kinase C phosphorylation site followed by a calcium/calmodulin-dependent kinase II site in the C terminus (Fig. 2). The DED domain (as solved for FADD (Fas-associated death domain-containing protein)) contains six anti-parallel, amphipathic α -helices and one β -turn connecting the fourth and fifth helices (Fig. 2 and Ref. 23). The PLD1-F4 interacting domain of PEA-15 starts with the β -turn and includes the last two DED helices and the PKC substrate site.

Interactions identified in the two-hybrid system have to be validated using additional methods. To accomplish this, a slightly smaller PLD1 fragment that also interacted with PEA-15 in yeast (D4, shown in Fig. 1) was expressed as a FLAG-tagged protein in bacteria and purified according to a protocol previously published (8). GST-PEA-15 or GST fused to PEA-15 protein fragments or to Ral (as a negative control) were generated (Fig. 3A), purified, and used *in vitro* to attempt to pull down FLAG-PLD1-D4. Because of the similar size of FLAG-D4 and GST-PEA-15, the D4 was detected using Western blotting visualization. As shown in Fig. 3B, whereas the full-length PEA-15 interacts with PLD1-D4 successfully, nei-

ther the N-terminal half (amino acids 1–80) nor the C-terminal half (amino acids 81–130) does. The small G-protein Ral was reported previously to associate directly with PLD1 (24); here we found that GST-Ral does not interact with PLD1-D4 under conditions that succeed for PEA-15 (Fig. 3B). This *in vitro* biochemical approach confirms the specificity of the PEA-15 interaction with D4 and additionally demonstrates that neither the DED nor the C terminus of PEA-15 alone suffice to mediate the interaction.

The PEA-15 and RhoA-interacting Sites on PLD1 Are Not Identical—We previously reported that the PLD1-F4/D4 region interacts with the activated form of RhoA (7, 8). Because PEA-15 might compete with RhoA for PLD1 interaction if the sites overlapped, we accordingly sought to determine whether the sites could be differentiated. To address this question, we first tried to identify the shortest region of F4/D4 that specifically interacts with either RhoA or PEA-15. F4 was fractionated into three smaller overlapping pieces (F4a-F4c; Fig. 1), again encompassing different conserved domains. However, none of these smaller fragments retained the ability to interact with RhoA^{Val-14} or PEA-15 (data not shown). It is possible that the interactions involve multiple, spatially separated contact points or that the smaller pieces did not fold correctly in yeast.

In a second approach, we utilized 16 PLD1 alleles (pentapeptide insertion mutants, or PIMs; Ref. 11), each characterized by a single but spatially distinct five-amino acid insertion into the D4 region, to attempt to map the interaction site(s). The alleles were assessed for their ability to interact with PEA-15 and RhoA^{Val-14} in the yeast two-hybrid system. As shown in Table II, some of the mutants interacted with both RhoA and PEA-15, indicating that the insertions did not disrupt the regions required for interaction and accordingly shrinking the potential region of interaction to amino acids 730–1030, simplistically assuming that each PIM induces a local disruption limited to 20–30 residues in each direction. In the instances where the alleles interacted with neither target protein, the insertions eliminated both binding sites, although the sizes of the structural disruptions induced are not known. Of greatest interest was the finding that although PIM751 interacts with RhoA^{Val-14} with wild type efficiency, it does not interact with PEA-15. This indicated that the mutant PIM751 F4 fragment folded correctly at a gross level because its RhoA^{Val-14} interacting site was intact and therefore that a local perturbation induced by the PIM insertion had eliminated the PEA-15 interaction site. This indicates that the PEA-15 and RhoA^{Val-14} PLD1 interacting sites are distinct at least in part. Amino acid 751 is located in the third PLD conserved region. This region is critical for activity and has been proposed to interact with the choline headgroup of the phosphatidylcholine substrate (7).

PLD1 and PEA-15 Interact *in Vivo*—With the demonstration that the two proteins interact specifically *in vitro*, we set out to examine the interaction *in vivo*. PEA-15 was originally identified as a phosphoprotein in astrocytes (18). Astrocytes have previously been described as a cell type that exhibits elevated PLD activity upon agonist (*e.g.* endothelin) stimulation (25). However, because there is no well characterized astrocytic cell line and PEA-15 is actually expressed in many tissues and because existing anti-PLD1 antisera cannot detect endogenous PLD1 in most cell types, we elected to pursue this question using COS-7 cells transiently co-transfected with HA-tagged PEA-15 (or HA-tagged-RhoA^{Val-14}) and FLAG-tagged PLD1 expression plasmids. Transfected cells were lysed and the FLAG fusion proteins immunoprecipitated using a monoclonal antibody, following which the co-immunoprecipitated HA-tagged PEA-15 (or HA-tagged-RhoA^{Val-14}) was detected using Western blot analysis. As shown in Fig. 4A, FLAG-tagged-PLD1-D4

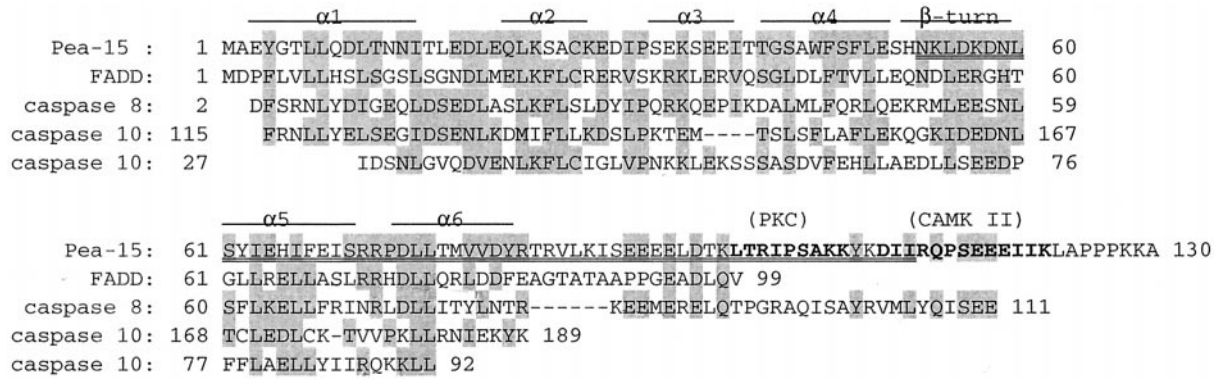


FIG. 2. The DED sequences of PEA-15 aligned with those of other DED-containing proteins and the region in it that interacts with PLD1. The sequence of PEA-15 is shown compared with other DED containing proteins. Identical and similar amino acid residues are shaded. The DED structure ($\alpha 1$ – $\alpha 6$) of PEA-15 is predicted based on sequence homology with FADD. *Double-underlined sequence*, PLD1 interacting site; *first bold region*, PKC substrate site (containing Ser-104); *second bold region*, substrate site for calcium/calmodulin-dependent kinase II. GenBank™ accession numbers: PEA-15 (X86694); FADD/Mort1 (U43184 and U50406); caspase 8/MACH/Mach5/FLICE (AF102146, X98177, and AF009620); and caspase 10 (AF111344).

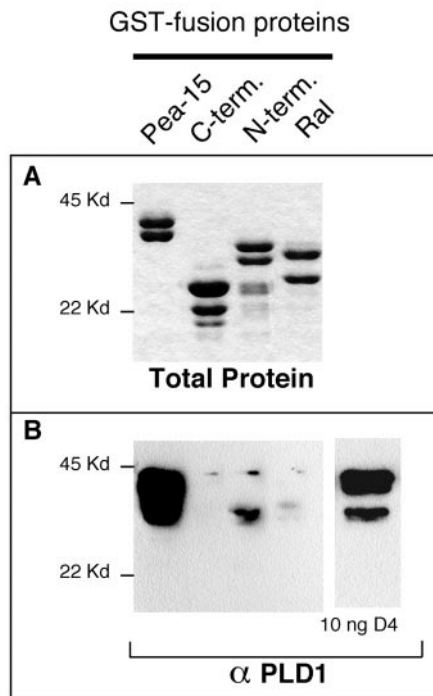


FIG. 3. PEA-15 and PLD1-D4 interact *in vitro*. Equal molar amounts of GST fusion proteins (attached to agarose beads) and FLAG-PLD1-D4 were mixed, incubated, washed extensively, resuspended in loading buffer containing urea, and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis. A, Coomassie Brilliant Blue staining demonstrating that equivalent amounts of GST fusion proteins were used. B, Western blot visualization (*left*) of the PLD1-F4 pulled down by the fusion proteins and (*right*) of FLAG-PLD1-D4 showing that the tagged protein migrated as a doublet because of limited proteolysis, which was also observed for the GST fusion proteins. An experiment representative of three is shown.

PIM751 interacted with RhoA^{Val-14} under conditions in which wild type FLAG-tagged-PLD1-D4 interacts with RhoA^{Val-14} (8), whereas the PIMs that did not interact with RhoA^{Val-14} in the two-hybrid assay also failed to interact with it in this *in vivo* assay (Table II). In contrast, whereas PEA-15 co-immunoprecipitated efficiently with the wild type D4 fragment (Fig. 4B), it did not co-immunoprecipitate with PIM751. This demonstrates that PLD1 and PEA-15 interact *in vivo*, that the interaction is specific, and that the RhoA and PEA-15 binding sites on PLD1 are distinct at least in part.

Finally, we also documented that interaction does occur be-

TABLE II
 PLD1-PIM751 interacts with RhoA^{Val-14} but not PEA-15.

PLD1 F4 allele	RhoA ^{Val-14} interaction ^a	PEA-15 interaction ^a	PLD activity ^b
			% of wild type
Wild type	+	+	100
699	+	+	0
705	+	+	8
751	+	–	0
759	+	–/+	23
796	+	+	3
810	–	–	37
840	+	+	9
858	+	+	1
875	–	–	2
958	–	–	0
1027	+/-	–	60
1035	–	–	26
1053	+	+	4
1068	+	+	12
1071	+	+	0

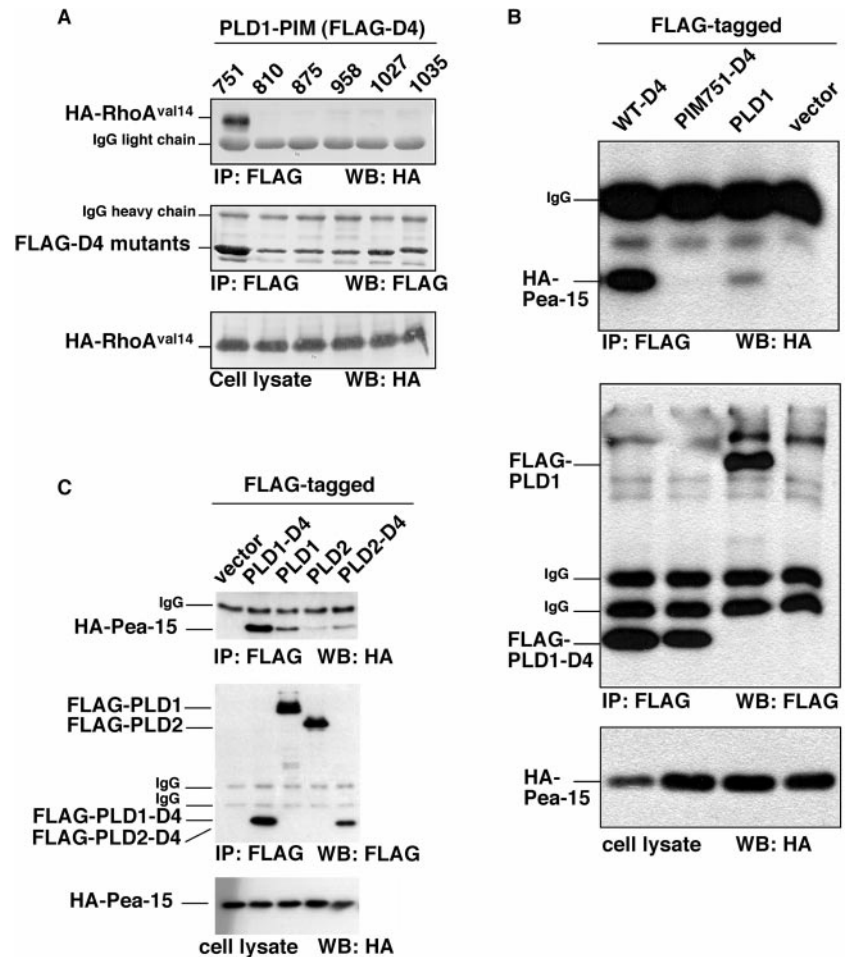
^a Scored by β -gal filter assay and growth on plates lacking histidine.

^b As reported in Ref. 11.

tween full-length PLD1 and PEA-15 *in vivo* (Fig. 4B, top panel), which was difficult to demonstrate using the two-hybrid or GST approaches for technical reasons. The amount of PEA-15 associating with full-length PLD1 was substantially less than that immunoprecipitated by the D4 fragment, even after taking into account that a smaller molar amount of the full-length protein was expressed. In addition, the PEA-15 that associated with the full-length PLD1 represented only a small percentage of the total PEA-15 protein expressed in the cell. Possibilities for this are addressed under “Discussion.” We also examined the interaction of PEA-15 with PLD2 or the equivalent PLD2-D4 fragment using this approach and found that they also successfully co-immunoprecipitated (Fig. 4C).

PEA-15 and PLD1 Subcellular Localization Patterns Partially Overlap—To begin addressing the potential significance of the PLD1/PEA-15 interaction, we examined the PEA-15 subcellular localization in COS-7 cells. When transiently expressed in cells, PLD1 is membrane-associated (9). During fractionation, when cell lysates are subject to high speed centrifugation (30,000 $\times g$), PLD1 is located in the pellet, where most of the membrane components segregate (11). In contrast, PEA-15 is in the soluble fraction after such treatment, presumably in the cytosol (18). If the two proteins always interact, then it is possible that when expressed together, one of them will translocate to a different fraction. Or, on the other hand, if the interaction is transient, as is characteristic of the activation

FIG. 4. Distinct interactions between PLD alleles and Rho/PEA-15. FLAG-PLD1 (full length, D4, or PIM-D4 s) or PLD2 (full-length or D4) and HA-Rho^{Val-14} or HA-PEA-15 were transiently co-expressed in COS-7. The cells were lysed and subjected to immunoprecipitation using anti-FLAG antibody-conjugated agarose beads. Proteins bound to the beads were eluted and electrophoresed for Western blot analysis. *A, top panel*, D4 fragments from different PLD1 PIM mutants were assessed for their ability to interact with RhoA^{Val-14}. The *middle panel* demonstrates that all of the D4 PIM alleles were expressed and immunoprecipitated efficiently. The *bottom panel* demonstrates that Rho was expressed equally well in each sample. *B, top panel*, full-length PLD1 and wild type and PIM751 D4 were assessed for their ability to interact with PEA-15 (see text for details). The *middle panel* demonstrates the D4 or full-length PLD1 immunoprecipitation by the M2 antibody. The *bottom panel* indicates the amount of PEA-15 present in the cell lysates. *C, top panel*, FLAG-tagged PLD1/2 and their D4 fragments were assessed for their ability to interact with PEA-15. The *middle panel* demonstrates the tagged PLD proteins immunoprecipitated by the M2 antibody. The *bottom panel* indicates that similar amounts of PEA-15 were present in each cell lysate. *IP*, immunoprecipitating target; *WB*, Western blot target. An experiment representative of four is shown.



of PLD1 by its stimulators, then the fractionation profile would remain unchanged. We found that the latter is the case. When PLD1 and PEA-15 were expressed together, no obvious changes were observed for either protein after fractionation (data not shown). This result suggests that the interaction is transient, although it does not rule out a weak interaction. This is consistent with the previous finding that only a small percentage of PEA-15 was pulled down by full-length PLD1 (Fig. 4B).

It was also possible that the interaction is regulated by PKC phosphorylation *in vivo* because the PLD1-interacting domain of PEA-15 contains a PKC substrate site. To investigate this possibility, we also examined the protein localization in fractionation experiments before and after the cells were treated with PMA, which activates PKC directly. Again, there were no obvious localization changes for either PLD1 or PEA-15 (data not shown). Similarly, there were no significant changes when the serine 104 residue in the PKC substrate site was mutated to alanine (data not shown). Collectively, these results suggest a transient or weak interaction. In addition, the result implied that PEA-15 might not be directly involved in the activation of PLD1, because the factors that stimulate PLD1, such as ARF, Rho, and PKC, all translocate to the membrane where PLD1 is, after becoming activated or after cells are stimulated with PMA.

Finally, we examined the intracellular localization of both proteins by indirect fluorescent microscopy. When expressed in COS-7 cells alone, PEA-15 exhibits a mesh-work pattern resembling the endoplasmic reticulum and is observed in some cases on what appears to be cytoskeletal thin filaments (Fig. 5A). The intracellular localization of PLD1 comes into two

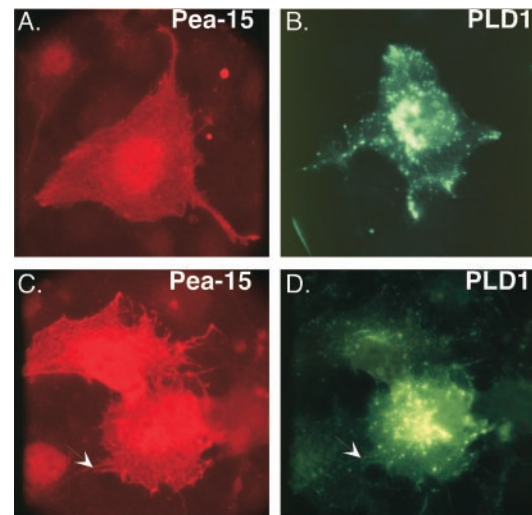


FIG. 5. Intracellular expression pattern of PEA-15 and PLD1 partially overlaps. COS-7 cells were transfected with FLAG-tagged PLD1, HA-tagged PEA-15 or a combination of both. PEA-15 was detected using a polyclonal rabbit anti-PEA-15 antiserum (17) and PLD1 was detected by the monoclonal M2 anti-FLAG antibody. *A*, PEA-15; *B*, PLD1; *C* and *D*, co-transfected PEA-15 and PLD1. Arrows indicate overlapping sites in membrane vesicles and filamentous structures. Representative cells are shown.

predominant patterns. When the protein expression level is low to moderate, it is observed on numerous moderately sized membrane vesicles in the perinuclear region (Fig. 5B and Refs. 3, 10, and 16). However, when the protein levels are high, a faint

meshwork/filamentous structure is observed superimposed over the perinuclear vesicular pattern (Fig. 5D). When PEA-15 and PLD1 are co-expressed, both proteins can occasionally be found in the perinuclear vesicles (Fig. 5, C and D, arrows); but more often, both are localized in the filamentous network. In summary, the two intracellular localization patterns partially overlap, consistent with a weak or transient interaction.

PEA-15 Does Not Affect *In Vitro* or *In Vivo* PLD1 Activation but Increases PLD1 Protein Levels when Co-expressed—We next examined the most obvious possibility, that PEA-15 is an activator of PLD1. Histidine-tagged PEA-15 was expressed and purified from bacteria and used to stimulate partially purified PLD1 at up to a 30-fold molar excess of PEA-15. However, no significant activation of PLD was observed (data not shown), suggesting that PEA-15 does not stimulate PLD1 directly. We similarly investigated the possibility that PEA-15 may interfere with PLD1 activation. This was an especially important question to address for RhoA, because its PLD1 interaction site potentially overlaps with that of PEA-15. Increasing amounts of PEA-15 were combined with standard concentrations of the activators ARF1, Rac1, and PKC (Rac1 belongs to the Rho family and has been demonstrated to activate PLD1 similarly to RhoA and using the same binding site; Ref. 15). At up to a 30-fold molar excess of PEA-15, no significant differences in PLD activation were observed (data not shown). This indicates that PEA-15 does not interfere with PLD1 activation as induced by ARF, Rho, or PKC family members.

PEA-15 is a PKC substrate, and its properties could change upon phosphorylation (18). We ruled out this possibility by supplementing ATP in the *in vitro* assay when both PEA-15 and PKC were present. Again, there were no significant changes in PLD1 activity (data not shown). These results collectively suggest that PEA-15 does not regulate the *in vitro* activity of PLD1.

To begin to address the possibility that histidine-tagged PEA-15 produced from bacteria lacks critical post-translational modifications, we co-expressed PEA-15 with PLD1 in mammalian cells and examined its activation in cell lysates using the *in vitro* assay. Surprisingly, an increase in PLD1 activity was observed after stimulation of the lysate PLD1 with ARF or Rho (Fig. 6A), and similar increases were observed *in vivo* after PMA stimulation of cells co-expressing PLD1 and PEA-15 (Fig. 6B).

However, when protein expression levels were examined using Western blot analysis, it became clear that the levels of PLD1 protein had been increased by the co-expression of PEA-15 (Fig. 6C), suggesting that PEA-15 was altering the absolute amount of PLD1 protein present rather than affecting its activity in a direct manner. These findings suggested the unexpected possibility that PEA-15 might function as a chaperone to facilitate (PLD1) protein folding or that it might inhibit PLD1 degradation through an unknown mechanism. To explore these possibilities, we followed the protein expression levels of PLD1 after transient transfection of the PLD1 cDNA expression plasmid. In the absence of PEA-15, the PLD1 protein levels increased during the first 24 h and then decreased during the following 2 days (Fig. 7, first and third rows, fifth set of lanes). However, in the presence of PEA-15, PLD1 proteins appeared more rapidly during the first 24 h (Fig. 7, first row, fifth set of lanes), accumulated to a higher level, and persisted longer (Fig. 7, third row, sixth set of lanes). The mechanism does not appear to involve PLD catalytic activity because expression of an inactive allele, PLD1-K898R (7), was similarly increased by PEA-15 (Fig. 7, third and fourth sets of lanes), and the phenomenon may be a general one for PLD, because an increase in PLD2 expression levels was additionally observed (Fig. 7, first and second sets of lanes).

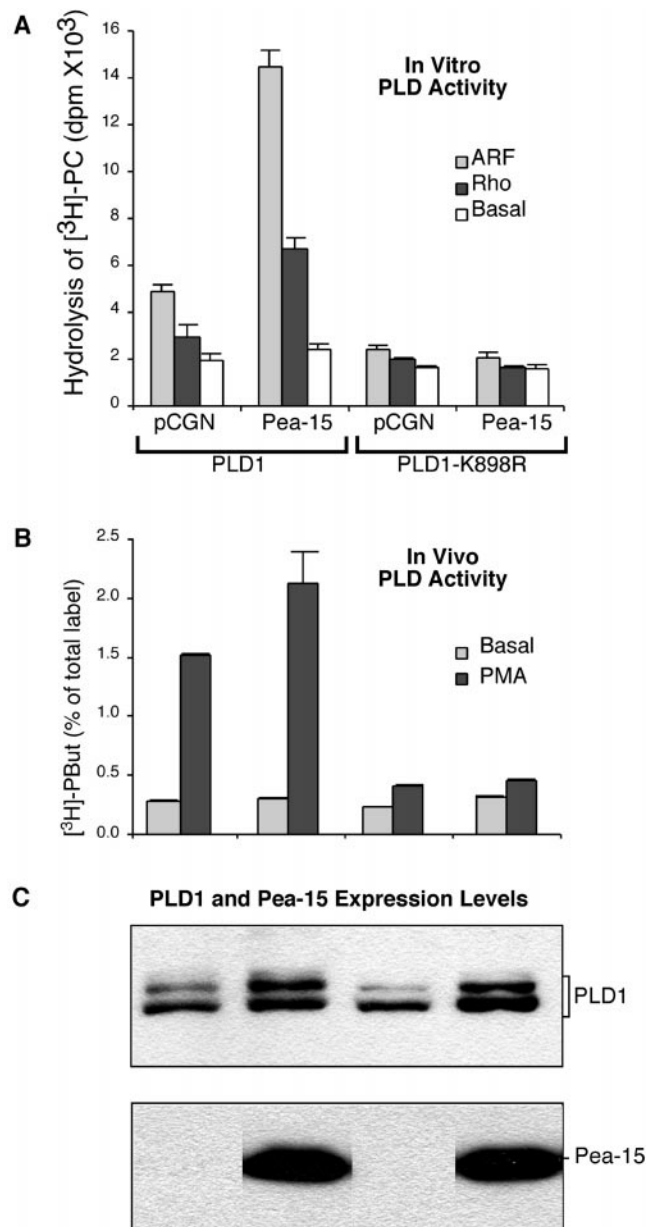
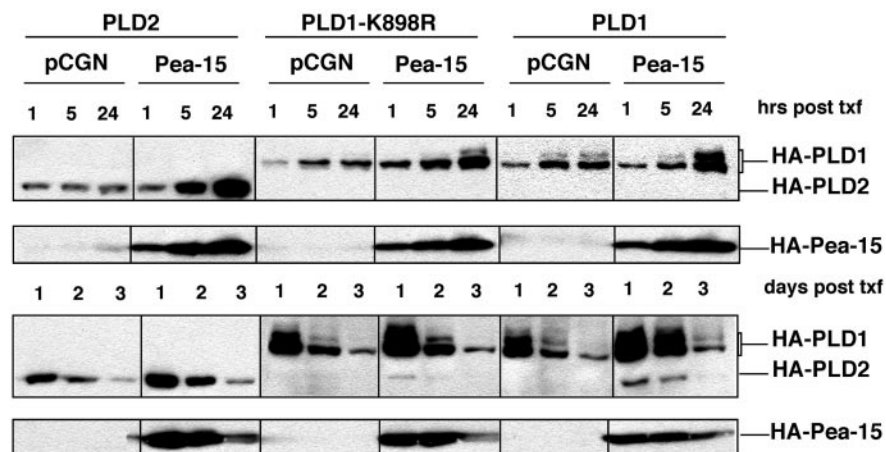


FIG. 6. Co-expression of PEA-15 increases PLD activity by increasing PLD protein levels. PLD1 and PEA-15 were co-expressed in COS-7 cells in 35-mm tissue culture dishes using the combinations of plasmids indicated. A, cell lysates were prepared by sonication in a volume of 60 μ l. PLD activity in 10- μ l aliquots of the lysates was quantitated *in vitro* using the choline head group release assay in the absence (basal) or presence of activators (GTP γ S loaded-ARF1 or RhoA). An experiment representative of three is shown. B, PLD activity as a function of co-expression with PEA-15 was measured using the transphosphatidyl transfer reaction. Transfected COS-7 cells in 35-mm tissue culture dishes were labeled with [3 H]palmitic acid for 24 h and stimulated in the presence of 0.3% 1-butanol without (basal) or with 100 nM PMA for 30 min. An experiment representative of three is shown. C, 10- μ l aliquots of the cell lysates used in A were assessed for PLD1 and PEA-15 protein expression levels.

DISCUSSION

Using a Yeast Two-hybrid Library Screen to Search for PLD1-interacting Proteins—An outcome anticipated for this search might have been to recover cDNAs encoding ARF, Rho, and protein kinase C, because these PLD1 activators are abundant and have been shown to stimulate PLD1 through direct interaction with it (reviewed in Ref. 1). However, these proteins interact efficiently with PLD1 only when in the activated conformation, as mediated through GTP loading for ARF and Rho

FIG. 7. Time course of PEA-15-induced increases in PLD protein expression levels. COS-7 cells were cotransfected with HA-PLD and HA-PEA-15 as shown. Cultures were lysed, and 10- μ l aliquots of the cell lysates assessed by Western blot analysis at the times indicated after addition of serum to the transfected cells (see "Experimental Procedures"). *A*, 1, 5, or 24 h post-transfection. *B*, 1, 2, or 3 days post-transfection. An experiment representative of three is shown.



and through stimulation by diacyl glycerol and Ca^{2+} for PKC. This has been physically demonstrated most conclusively for Rho, in that the GTP-loaded form interacts with PLD1 in the yeast two-hybrid assay but the GDP-loaded form does not (7, 8). Accordingly, the two-hybrid system would appear to be limited to identifying constitutive interacting partners for highly regulated enzymes such as PLD1. PEA-15 seems to satisfy this criteria. Although PEA-15 is regulated in the sense that it has a *bona fide* PKC phosphorylation site within the PLD1-minimal interacting domain that becomes phosphorylated when PKC is activated, phosphorylation of the targeted residue, Ser-104, is not required for the PLD1 interaction because a mutant PEA-15 allele incapable of being phosphorylated (S104A) interacts with PLD1 with wild type efficiency (data not shown).

It is curious that Ral was not identified as a PLD1-interacting protein (either through the screen or in the GST pull-down experiment, Fig. 3), because it was previously reported that Ral binds to PLD1 in a GTP-independent manner (24). This may signify that the interaction reported was not a direct one.

Finally, proteins interacting with the N-terminal phox and pleckstrin homology domains were not recovered, which was surprising, because protein-protein interactions have been demonstrated for both types of motifs (reviewed in Refs. 26 and 27). This suggests that the hypothetical protein(s) that these regions bind may need to be in an active conformation, as is the case for the PLD activators, or bound to membrane surfaces, which could not be efficiently achieved in the yeast two-hybrid system. Alternatively, it is possible that the N-terminal domain did not fold correctly as a fusion protein in the yeast cells. Supporting the possibility that correct conformation of the N terminus may occur only in the context of the full-length protein, alterations to the N terminus can result in inactive enzyme (11), even though it is not intrinsically required for enzymatic activity (9). This suggests that there are long range conformational changes transmitted by interactions between the N terminus and the remainder of the protein that take place when PLD is activated rather than individual domains that function autonomously.

PEA-15, a Novel PLD-1-interacting Protein—The PEA-15/PLD1 interaction appears to be a genuine one, based on 1) identification of 21 individual, nonidentical clones in the two-hybrid search; 2) interaction after switching the positions of PLD1 and PEA-15 on the respective two-hybrid vectors; 3) GST pull-downs; 4) *in vivo* co-immunoprecipitation; and 5) intriguing biological relevance.

Formation of a functional PLD1-interacting site on PEA-15 requires the C terminus and part of the DED. DEDs (defined originally for FADD and caspase 8) mediate hetero-associations with other DED-containing proteins to promote apoptosis

(28). Oligomerization of the FADD and caspase 8 DEDs results in caspase 8 undergoing auto-processing, which initiates apoptosis (28). PEA-15 complexes with both FADD and with caspase 8 through DED/DED interactions (22), altering the pro- and anti-apoptotic balance (20, 22, 29). Accordingly, if binding of PLD1 to PEA-15 blocks its interaction with other DED-containing proteins, then PLD1, FADD, and the caspases may all be competing for interaction with PEA-15. Alternatively, binding of PLD1 to PEA-15 might not inhibit its interaction with FADD and caspase 8 (*e.g.* the physical site of interaction for PLD1 could be solely in the C terminus of PEA-15). If that is the case, then PEA-15 might recruit PLD1 into the complexes that initiate apoptosis through simultaneous interaction with FADD/caspase 8 and PLD1. This hypothesis is attractive because PEA-15 and PLD1 have both been proposed to play anti-apoptotic roles (6, 20, 22), and this newly identified interaction provides a potential mechanism that would link PLD1 to apoptotic sites of action through PEA-15. Moreover, as shown in Fig. 2, caspase 8 (but not FADD) has sequence similarity with PEA-15 that extends beyond the DED domain through the length of the C-terminal PLD1-interacting region. This raises the possibility that caspase 8 and/or related family members might also exhibit affinity for PLD1 or PLD2.

Finally, PEA-15 may recruit PLD to other receptor complexes through the same mechanism. PLD2 and PEA-15 co-immunoprecipitate with the EGF and insulin receptors (30),² suggesting that PEA-15 and PLD2 are associated in this setting. Moreover, both PEA-15 (31) and PLD2 (32) are present in ERK mitogen-activated protein kinase signaling complexes. Although this does not prove direct interaction, it provides supportive evidence for the hypothesis that PEA-15 is involved in the association of PLD1 and PLD2 with other types of receptor complexes.

Overlap of the RhoA and PEA-15 Binding Sites on PLD1—The interaction of both RhoA and PEA-15 with the same C-terminal fragment of PLD1 and nearly the same set of PIMs suggests that the binding sites are likely to overlap. Analysis underway of a large set of PLD1 alleles with point mutations has revealed that about 40% of the PLD1-D4 alleles that no longer interact with RhoA still interact with PEA-15,³ again suggesting that the sites are distinct but may overlap. This result raises the possibility that PEA-15 and RhoA may compete for binding to PLD1 and hence PEA-15 could act as a modifier of Rho stimulation of PLD1, but we think this is not likely to be the case. First, we were unable to inhibit RhoA

² Y. Zhang, and M. A. Frohman, unpublished data.

³ G. Du, Y. M. Altshuler, Y. Kim, J. M. Han, S. H. Ryu, A. J. Morris, and M. A. Frohman (2000) *Mol. Biol. Cell*, in press.

stimulation of PLD1 *in vitro*, using even large excesses of PEA-15. Second, the two-hybrid interaction between Rho and PLD1 is stronger than between PEA-15 and PLD1 (data not shown), suggesting that RhoA has a higher affinity for PLD1 than PEA-15. Third, the subcellular site(s) of interaction for RhoA and PLD1 may differ from that for PEA-15 and PLD1.

We previously reported that the C-terminal 362 amino acid residues of PLD1 interact with RhoA^{Val-14} (8) but were not able to identify the interaction site with any greater resolution. The data presented in Table II rule out large parts of the D4 fragment, because only insertions at amino acid 810 and between amino acids 875 and 1035 disrupted the RhoA-PLD1 interaction. At a first level of analysis, this suggests that the RhoA interacting domain might consist of these regions. However, PEA-15 also failed to interact with these PIMs, suggesting as above either that the PEA-15 binding site overlaps the RhoA binding site or that there were extended structural disruptions induced for these PIMs (when expressed as a fusion protein in yeast) rather than localized ones. Several of the PIMs (810, 1027, and 1035) were at least partly enzymatically active but not selectively Rho-nonresponsive when expressed as intact proteins in COS-7 cells (Table II and Ref. 11), indicating that they did not disrupt the *bona fide* Rho interaction site. Two of the PIMs (875, 958), however, were inactive, which means that an interaction of Rho at these sites cannot be ruled out. This results in the restricted hypothesis that the RhoA site is situated roughly between amino acids 865 and 1000, simplistically assuming that each PIM induces a local disruption limited to 20–30 residues in each direction.

Regulation of PLD1 by PEA-15—Based on our *in vitro* and *in vivo* analyses, PEA-15 does not appear to stimulate PLD1 directly nor does it inhibit it. Elevated levels of PLD1 activity were observed *in vitro* and *in vivo* after co-expression with PEA-15, but these correlated with elevated levels of PLD protein, leading to the conclusion that PEA-15 was affecting the accumulation or degradation rates of PLD1 rather than its activity *per se*. How might this occur? One potential mechanism might be indirect. PEA-15 has been shown to inhibit apoptosis (20, 22). Supraphysiologically elevated levels of PLD activity are deleterious for cells,² and PEA-15 could be acting on our transiently transfected cells to rescue the cells expressing the highest levels of PLD1, which would hypothetically have otherwise died before being assayed. We do not favor this explanation for several reasons. First, it does not provide a rationale for the direct interaction between PLD1 and PEA-15 that led to this study. Second, increased levels of protein expression were also observed for PLD1-K898R, an inactive allele of PLD1 (7), essentially ruling out a role for PEA-15 in opposing the consequences of elevated PLD activity through suppression of apoptosis in our assay system.

Several direct mechanisms are also possible. PEA-15 interacts with and co-localizes with PLD1 and PLD2 *in vivo* and could act to stabilize it, accordingly decreasing its rate of degradation. Alternatively, a novel hypothesis would be that PEA-15 might function as a chaperone. A number of observations support this possibility. Only a small portion of the PEA-15 is in steady-state association with PLD1 after co-expression (Figs. 4B and 5) as indicated by the subcellular localization and co-IP results, suggesting that the interaction is transient. Furthermore, PEA-15 has an acidic PI, has a small size of 15 kDa, and contains amphipathic α -helices, all of which are characteristic of the pathogenic bacterial protein-specific chaperones that stabilize proteins secreted into the mammalian cytoplasm (33). PEA-15 could function as a (non-endoplasmic reticulum) chaperone to interact with the C-terminal region of PLD to facilitate its folding (or transport), following

which dissociation would occur. Protein that does not fold correctly is targeted for quick elimination; hence, the presence of PEA-15 could lead to higher levels of steady-state PLD1 expression. Interestingly, the protein levels of Fanconi anemia complementation group A protein (FANCA) become similarly increased as a consequence of overexpression of sorting nexin A, a protein that has been proposed to play a role in receptor trafficking between organelles (34).

Potential Nonapoptotic Biological Significance of the PLD1/PEA-15 Interaction—PEA-15 and PLD1 exhibit subcellular and cellular co-localization and are activated through common pathways (e.g. PKC). A good example of the cellular co-localization is in astrocytes, the cell type in which PEA-15 was first identified and is found in abundance (18). PLD1 and PLD2 are found in astrocytes,⁴ and astrocytes generate most of the central nervous system PLD response to signaling agonists such as endothelin (25). However, the significance of the interaction in this circumstance is not clear.

An intriguing setting in which the potential relationship would be more functionally obvious involves glucose transport. PEA-15 was recently identified as a protein overexpressed in type II diabetes (21). Moreover, normal skeletal muscle cells made to overexpress PEA-15 exhibit a similar phenotype that appears to ensue from altered subcellular localization of the Glut1 and Glut4 glucose transporters. PLD1 and PLD2 have been linked at several levels to insulin signaling and glucose transport including at the receptor level. Most relevant, however, is a very recent report that PLD1 and Glut4 co-localize and that increasing PLD activity promotes translocation of Glut4 to the plasma membrane (3). Because PEA-15 overexpression reportedly decreases Glut4 translocation to the plasma membrane, one interpretation might be that the excess PEA-15 sequestered or diverted PLD1 away from the Glut4 pathway. There are other scenarios, though, that would be equally relevant to the abnormal cellular physiology of type II diabetes.

Acknowledgments—We thank Dr. J. Engebrecht and R. Sternglanz for extensive assistance with the yeast two-hybrid system, G. Du for allowing us to describe data not shown, and A. Morris and members of the laboratory for discussion and critical readings of the manuscript.

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