Molecular Heterogeneity of Phospholipase D (PLD)

CLONING OF PLDγ AND REGULATION OF PLANT PLDγ, -β, AND -α BY POLYPHOSPHOINOSITIDES AND CALCIUM

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Phospholipase D (PLD) has emerged as an important enzyme involved in signal transduction, vesicle trafficking, and membrane metabolism. This report describes the cloning and expression of a new Arabidopsis PLD cDNA, designated PLDγ, and the regulation of PLDγ, -β, and -α by phosphatidylinositol 4,5-bisphosphate (PIPγ) and Ca2++. The PLDγ cDNA is 3.3 kilobases in length and codes for an 855-amino acid protein of 95,462 Da with a pl of 6.9. PLDγ shares a 66% amino acid sequence identity with PLDb, but only a 41% identity with PLDa. A potential N-terminal myristoylation site is found in PLDγ, but not in PLDa and -β. Catalytically active PLDγ was expressed in Escherichia coli, and its activity requires polyphosphoinositides. Both PLDγ and -β are most active at μM Ca2+ concentrations, whereas the optimal PLDa activity requires mM Ca2+ concentrations. Binding studies showed that the PLDs bind PIPγ in the order of PLDγ > PLDβ > PLDa. This binding ability correlates with the degree of conservation of a basic PIPγ-binding motif located near the putative catalytic site. The binding of [3H]PIPγ was saturable and could be competitively decreased by addition of unlabeled PIP. Neomycin inhibited the activities of PLDγ and -β, but not PLDa. These results demonstrate that PLD is encoded by a heterogeneous gene family and that direct polyphosphoinositide binding is required for the activities of PLDγ and -β, but not PLDa. The different structural and biochemical properties suggest that PLDa, -β, and -γ are regulated differently and may mediate unique cellular functions.

Still lacking. Calcium has been thought to be a regulator of plant PLD, but the requirement for nonphysiological mM levels of Ca2+ by the PLDs previously purified from various plants has made Ca2+ regulation an enigma. Recent advances in the molecular understanding of PLD have brought new insights into the control and cellular roles of PLD. An intracellular PLD was first cloned from castor bean (3), and the cloning of PLDs has since been reported from Arabidopsis (4, 5), rice, maize (6), yeast (7), human (8), and mouse (9). Analysis of the PLD sequences has led to the identification of probable catalytic and regulatory domains of PLD (10, 11). Two PLDs with distinct regulatory properties have been cloned from Arabidopsis: one is the conventional μM Ca2+-requiring PLD, designated PLDa, and the other, named PLDb, requires mM Ca2+ and polyphosphoinositides for activity (4, 5, 12).

The polyphosphoinositide activation of plant PLDs is a property shared by PLDs cloned from mammals and yeast (8, 9, 13). This property has been proposed to be physiologically important because, in addition to being precursors for signaling messengers, polyphosphoinositides themselves can also modulate the functions of proteins through direct binding. Such an interaction is believed to either recruit or activate proteins essential for signaling or membrane trafficking pathways, thus allowing coordinated regulation of different cellular processes (14). However, the mechanism by which polyphosphoinositides affect PLD activities has not been elucidated. Direct phosphatidylinositol 4,5-bisphosphate (PIPγ) binding has not been demonstrated previously for PLDs from any source.

In this report, we first describe the cloning and molecular analysis of a new Arabidopsis PLD, PLDγ, whose activity is regulated by polyphosphoinositides. To delineate the mechanisms of the polyphosphoinositide modulation of PLDs, we then provide evidence indicating that plant PLDs directly bind PIPγ and that this binding is required for the activities of PLDγ and -β, but not PLDa. Additionally, the regulation by Ca2+ and the domain structures of plant PLDγ, -β, and -α are compared.

EXPERIMENTAL PROCEDURES

Lipid Materials—Dipalmitoylglycerol-3-phosphoinositol-2-[3H]inositol 4,5-bisphosphate and dipalmitoylglycerol-3-phospho[methyl-3H]choline were products of Amersham Corp. PC from soybean and PIPγ from bovine brain were purchased from Sigma and American Radiolabeled Chemicals (St. Louis, MO), respectively. Other lipids were obtained from Avanti Polar Lipids.

Cloning and Sequencing of the PLDγ cDNA—An expression sequence-tagged cDNA clone, ~1 kilobase in length, was identified as a putative incomplete new Arabidopsis PLD cDNA by searching the GenBank™ Data Bank against the cloned PLD sequences. A full-length PLDγ cDNA clone was isolated from a ZAPII cDNA library, constructed from 3–6-kilobase mRNA isolated from hypocotyls of 3-day-old Arabidopsis seedlings (15). The library was screened using the expression sequence-tagged cDNA clone as a probe with hybridization conducted at 65 °C. The subsequent DNA manipulation of the positive clones, sequencing, and sequence analysis were based on previously described procedures (3). The final sequence was determined from both

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Expression of PLD cDNAs in Escherichia coli—The expression of the PLDα cDNA was performed in two constructs: pBluescript SK(−) and the BamHI-XhoI site of pGEX-2T (Pharmacia Biotech). Prior to ligation into the expression plasmid, the cDNA was amplified by PCR from Arabidopsis genomic DNA using primers encoding the glutathione S-transferase (GST) fusion protein. Oligonucleotides with an added BamHI site were used as primers to amplify a 2.9-kilobase fragment by polymerase chain reaction. The 5′-end primer corresponded to nucleotides 342–360 of the PLDα cDNA. After BamHI digestion, the insert was ligated into pBluescript SK or pGEX-2T, and the recombinant plasmids were transformed into E. coli JM109. The expression of PLDα was induced using the same procedure as described for PLDs and β (3, 5). The cells were lysed by sonication in the resuspension buffer, and cell debris was removed by centrifugation at 10,000 × g for 5 min. Protein content in the supernatant was measured by the Bradford method according to the manufacturer’s instructions (Bio-Rad). Aliquots of the supernatant were assayed for PLD activity or stored at −80 °C until use.

In addition to the GST-PLDα fusion protein, GST-PLDδ and GST-PLDβ were also used in this study. The construct of GST-PLDα was reported previously (3), and the GST-PLDβ fusion protein was generated by excising the PLDβ cDNA insert in pBluescript SK (5) with EcoRI digestion and then ligating it into pGEX-2T. The procedures for induction and protein harvest were the same as those described for the pBluescript constructs (5), except that 0.2 mM isopropyl-1-thio-

β-D-galactopyranoside was used for induction.

Purification of GST Fusion Proteins—GST-PLDα, GST-PLDβ, GST-PLDγ, and GST-PLDδ were purified as described (16). Briefly, induced bacteria were pelleted and rinsed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2 mM EDTA by centrifugation at 3000 × g for 10 min. The pellet was resuspended in rinsing buffer containing 2 μg/ml antipain, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 0.5 mg/ml lysozyme and was incubated on ice for 15 min. Dithiothreitol was then added to a final concentration of 5 mM, and the samples were briefly sonicated on ice. To solubilize the expressed protein, N-laurylsarcosine was added to 1.5% (v/v), and then the samples were vortexed and centrifuged at 10,000 × g for 5 min. The supernatant was transferred to a new tube, and Triton X-100 was added to a final concentration of 4% (v/v). Samples of GST-PLDα, GST-PLDβ, and GST-PLDδ with equal amounts of GST activity were then absorbed to swollen glutathioneagarose beads (50%, v/v, overnight at 4 °C). The beads were rinsed with 15 bed volumes of phosphate-buffered saline. Both the GST activity and protein content of the rinsed beads were measured. GST activity was measured using 1-chloro-2,4-dinitrobenzene (5) as described by the manufacturer’s instructions (Pharmacia Biotech Inc.) and calculated in units of A405 units/min per milliliter. Protein content was determined by the Bradford method using purified GST fusion proteins eluted from the glutathione beads with 10 mM glutathione.

Phospholipid Binding—Phospholipid binding by the GST-PLD proteins and GST was performed according to a method adapted from Daus, Biskup and Subotf (17). Stock vesicles of PIP2 were prepared by mixing 0.9 μCi of [3H]PIP2 with 1.0 μmol of unlabeled PIP2 in chloroform, and the solvent was evaporated under a stream of N2. The phospholipids were dispersed in 0.5 ml of H2O by sonication at room temperature. The fusion proteins attached to the glutathione-agarose beads (30-μl wet volume) were incubated with 20 nmol of [3H]PIP2 vesicles in buffer (50 mM MES (pH 7.0), 0.5 mM MgCl2, 80 mM KCl, 0.4 mM lipid vesicles, and 30 μg of pBluescript SK-expressed protein in a total volume of 100 μl). CaCl2 was added to the reactions at the concentrations noted below. Lipid vesicles contained 35 nmol of PE, 3 nmol of PIP2, and 2 nmol of PC. PLD- mediated hydrolysis of PC was measured using [14C]glycero-3-phosphocholine or dipalmitoylglycero-3-phospho-[methyl-3H]choline as substrate. The reaction was initiated by addition of substrate and incubated at 30 °C for 30 min in a shaking water bath. Choline, PA, and phosphatidylethanol- nol produced in the PLD reaction were separated and quantitated as described previously (12). In the phospholipid activation experiments, PIP2 was replaced with 3 nmol of PE, PA, PG, PS, PI, or PIP. In the Ca2+ dependence experiments, varying amounts of Ca2+ were added to the reaction mixture, and the free Ca2+ was determined using arsenazo III, a Ca2+-sensitive dye (18), in reconstituted reaction mixtures. Control assays were performed using 30 μg of protein from lyzed bacteria harboring the pBluescript SK(−) plasmid without a PLD cDNA insert. The background activity from bacteria was very low (5) and was subtracted from the activity of the samples containing PLDs.

Assaying PLD Activity in the Presence of PIP2—The PIP2-dependent PLD assay (see Fig. 3, B and C) used a reaction mixture of 100 mM MES (pH 6.5), 0.5 mM SDS, 1% (v/v) ethanol, 30 μg of protein, and 0.4 mM PC (egg yolk) containing dipalmitoylglycero-3-phospho[methyl-3H]choline. CaCl2 was added to reactions as noted below. The substrate preparation, reaction conditions, and separation of products were performed as described previously (3). Release of [3H]choline into the aqueous phase was quantitated by scintillation counting. Control assays used 30 μg of protein from lyzed bacteria harboring the pBluescript SK(−) plasmid without a PLD cDNA insert. The background PLD activity was negligible (3) and was subtracted from the activity of the samples containing the recombinant PLDs.

DNA Isolation and Southern Blotting—Genomic DNA was isolated from Arabidopsis leaves and digested with different restriction enzymes. Full-length cDNAs of PLDα, β, and γ were used as probes to hybridize the digested DNA at 65 °C under the previously described conditions (3).

Protein Extracts from Arabidopsis—PLDα-deficient plants were generated by introducing a PLDα antisense cDNA to Arabidopsis (12). Microsomal membranes were obtained from leaves of the PLDα antisense gene-suppressed and wild-type plants and then extracted with 0.44 M KCl following a described method (12). A previous study showed that most of the PIP2-dependent PLD activity from the antisense plants was recovered in the salt extract (12). The salt-extracted PLD was assayed in the presence of varying concentrations of neomycin.

RESULTS

Cloning of the PLDγ cDNA and Sequence Comparison with Other PLDs—An Arabidopsis expression sequence-tagged cDNA was identified as a putative, new PLD cDNA by searching the GenBank™ Data Bank against the plant PLD cDNAs. This cDNA, a 1 kilobase in length, was used as a probe to screen a ZAP Arabidopsis cDNA library constructed using 3–6-kilobase mRNA from hypocotyls of 3-day-old seedlings (15). A full-length PLD γ cDNA, designated PLDγ, was identified and sequenced from both strands. The PLDγ cDNA is composed of 3234 nucleotides, with the longest open reading frame from nucleotides 312 to 2876, and imparts a deduced protein sequence of 855 amino acids (Fig. 1). The PLDγ polypeptide has a calculated molecular mass of 95,462 Da and a pI of 6.0, and PLDα has 968 amino acids with a pI of 6.0, and PLDβ has 986 amino acids and a pI of 7.9.

The amino acid and nucleotide sequences of PLDα and γ are more closely related to PLDβ than to PLDα from Arabidopsis. PLDγ has a 66% amino acid identity to PLDβ and an overall similarity of 81%. In contrast, PLDγ displays only 41% identity and 60% overall similarity to PLDα from Arabidopsis. The C-terminal regions of the three PLDs share a higher degree of amino
acid sequence similarities than their N-terminal residues. Phylogenetic alignments of PLD sequences from various sources show that PLD\(\gamma\) and \(-\beta\) form a cluster that is divergent from PLD\(\alpha\)s cloned from Arabidopsis, castor bean, rice, and maize. PLD\(\alpha\)s from different plant species share 75–90% amino acid sequence identity. PLD\(\gamma\) and \(-\beta\) are more closely related than PLD\(\alpha\)s to the PLD\(\alpha\)s cloned from human and yeast.

**Domain Organization of PLDs**—Sequence analysis indicates that PLD\(\gamma\), \(-\beta\), and \(-\alpha\) share some common structural motifs and differ in others (Fig. 1). PLD\(\gamma\) possesses a myristoylation consensus sequence (19), MGXXXS, that is not present in PLD\(\alpha\)s or \(-\beta\). PLD\(\gamma\) contains a Ca\(^{2+}\)/phospholipid-binding C2 domain near its N terminus, and this domain is conserved in all cloned plant PLDs, but not in yeast and human PLDs. C2 domains have been identified in a number of proteins involved in signal transduction and membrane trafficking and are important in Ca\(^{2+}\)-regulated binding to phospholipids (10, 20). Ca\(^{2+}\)-binding is coordinated by four to five amino acid residues provided by bipartite loops within the C2 domain (21, 22). PLD\(\gamma\), like PLD\(\beta\), conserves all of the calcium-coordinating acidic amino acids, whereas two of the acidic residues in the C2 domain of PLD\(\alpha\)s are substituted by either positively charged or neutral amino acids, indicating a loss of affinity for Ca\(^{2+}\) in PLD\(\alpha\)s. Like all PLDs cloned from plants, yeast, and human, PLD\(\gamma\) possesses two HXXXXXD motifs (Fig. 1). It has been hypothesized that the conserved histidine, lysine, and asparagine residues act as a catalytic triad responsible for the formation or hydrolysis of phosphoester bonds (11).

**Fig. 1. Amino acid sequence alignment and domain structures of Arabidopsis PLD\(\gamma\), \(-\beta\), and \(-\alpha\)**—The C2 sequences underlined were aligned according to the structure of rat synaptotagmin (10, 21). The boldface residues show the positions corresponding to the Ca\(^{2+}\)-binding acidic residues in the C2 domain of synaptotagmin. Two HKD/phos-}

Molecular Heterogeneity of Phospholipase D in Plants

PLD\(\gamma\), \(-\beta\), and \(-\alpha\) are activated by polyphosphoinositides (Ref. 5 and results below). A motif rich in basic amino acids ((KR)(KR)XXX(K/R)XXX(RK/R)) has been found to be responsible for phospholipase D activity in proteins such as the actin-severing proteins gelsolin and villin and phospholipase C (23–25). This motif (RXXXXKRR) and an inverted sequence (RKXXXXXK) are present in PLD\(\alpha\)s near the C-terminal domain of the C2 domain. Three of these four conserved basic residues are conserved in PLD\(\gamma\), whereas some are replaced by acidic residues (Fig. 1).

**Distinct Genes for PLD\(\gamma\), \(-\beta\), and \(-\alpha\)**—PLD\(\gamma\) is encoded by a single gene distinct from those of PLD\(\alpha\)s cloned from plants, yeast, and human, PLD\(\gamma\) possesses the least conservation of amino acids among those which are replaced by acidic residues (Fig. 1).

**Fig. 2**—To verify that the cloned cDNA encodes a PLD, protein from this cDNA was produced in E. coli using pBluescript SK as an expression vector. After isopropyl-1-thio-D-galactopyranoside induction, this protein was recognized by antiserum generated against a synthetic peptide that PLD\(\gamma\) shares with PLD\(\beta\), but not with PLD\(\alpha\)s, detergent extracts of E. coli—
shared by PLDβ (Fig. 3B). On the other hand, PLDα was active under such conditions (Fig. 3B). When these PLDs were assayed in the presence of 50 μM Ca²⁺, SDS, and PC vesicles, none of them was active (Fig. 3C). This result indicated that the lack of PLDβ and γ activities in the conventional PLD assay was due to inhibition by mM Ca²⁺. On the other hand, the lack of PLDα activity at 50 μM Ca²⁺ was due to its requirement for higher Ca²⁺ concentrations (Fig. 4).

Ca²⁺ Dependence of PLDγ, -β, and -α—To compare the effect of Ca²⁺ on different PLDs, PLDγ, -β, and -α activities were measured in the presence of varying amounts of Ca²⁺. Compared with PLDβ and -α, PLDγ displayed an ~2-fold higher basal activity in the absence of Ca²⁺, and it showed the smallest activation in response to Ca²⁺ (Fig. 4). PLDγ activity peaked at four times its basal level as Ca²⁺ was increased to ~50 μM. As Ca²⁺ concentrations increased above 50 μM, PLDγ activity decreased and showed a near complete inhibition at 20 mM Ca²⁺. PLDγ exhibited an optimal activation over a broader range of Ca²⁺ concentrations than PLDγ, and its activity at 50 μM Ca²⁺ was ~10-fold higher than its basal activity. In addition, PLDβ activity was not as completely inhibited by mM levels of Ca²⁺. PLDα, in striking contrast to PLDγ and -β, displayed little activity at μM Ca²⁺ concentrations, but showed a marked increase in activity as Ca²⁺ concentrations increased to the mM range.

The present Ca²⁺ dependence study used an unbuffered system in which the free Ca²⁺ was determined using the Ca²⁺-sensitive dye arachidonic acid III in reconstructed reaction mixtures. In this system, the Ca²⁺ optimum of PLDβ (50 μM) was higher than that (0.5–1 μM) reported in a previous study (5), in which the free Ca²⁺ was calculated according to a Ca²⁺-EGTA buffer system. When the free Ca²⁺ level in the buffered system was evaluated using arachidonic acid III, it was found that the presence of EDTA in the enzyme sample buffer interfered with the Ca²⁺ buffering system, particularly at the low μM Ca²⁺ range. This interference resulted in a shift of the PLDβ Ca²⁺ optimum to an apparently lower free Ca²⁺ concentration.

Activation of PLDs by Polyphosphoinositides—The inability of PLDγ to hydrolyze PC in PC-only vesicles and its activity in the presence of mixed vesicles (Fig. 3) suggested that PLDγ might require polyphosphoinositides for activity. To examine this possibility, PLDγ was assayed with mixed lipid vesicles, PC/PEX (2:35:3 mole ratio), where PE and PC were kept constant, and X was PIP₂, PIP, PI, PG, PA, PE, or PS. The highest PLDγ activity was found in the PIP₂-containing vesicles. PIP gave ~70% of the PIP₂-stimulated activity, whereas PI, PS, PG, and PA showed much less of a stimulatory effect (Fig. 5A). PLDγ showed a significant activation in the presence of the polyphosphoinositides PIP and PIP₂, but not any other phospholipid. PLDα, which has been well documented to be capable of hydrolyzing PC without any additional phospholipids (Fig. 3B), was also stimulated by PIP₂ and PIP at a suboptimal Ca²⁺ concentration (5 mM) and in the absence of the detergent SDS.

The mode of PIP₂ stimulation of the PLDs was tested by examining their ability to be inhibited by neomycin, which is a high affin...
The effects of neomycin on these PLDs is that PIP2 stimulates the total PIP2 added for GST-PLD. Showed a decreased PIP2 binding ability, indicating that this PE, PA, PG, PS, PI, PIP, or PIP2. Binding was saturable. Scatchard plot analysis showed that the absence or presence of Ca2+ than PLD-.

-XPLD assays were composed of PC/PEX (5:87.5:7.5 mol%). PLD- consisted of PC/PE/PIP2 (5:87.5:7.5 mol%). PLD- showed a 2-fold more PIP2 than PLD-. Addition of 100 mM Ca2+ stimulated the activities for reversible membrane association (26–28). It is believed that the hydrophobic nature of myristate allows it to associate directly with membrane lipids, but there are proteins, such as mammalian cAMP-dependent kinase, in which myristoylation has no membrane-targeting function, but is instead important for structural stabilization (29). In some cases, it has been reported that myristate interacts with a specific membrane protein, thus mediating a targeted protein-protein interaction (30). The putative myristoylated N-terminal glycine is -30 amino acids away from the first B-sheet strand in the C2 domain of PLDγ. Since myristoylation and C2 domains of many proteins are involved in protein-membrane interactions and since the putative myristoylation site and C2 domain of PLDγ are very close together, it is possible that a covalently linked myristate and the C2 domain coordinate the association of PLDγ with membranes. Similar myristoylation consensus sequences have been observed in other plant proteins such as certain Ca2+-dependent protein kinases (31). However, this type of covalent modification is not well understood in plants. It is known that for myristoylation to occur by the yeast N-myristoyltransferase, the initiator methionine preceding the targeted glycine residue has to be removed (19). The MGGXXS sequence in PLDγ is 12 amino acids downstream of the first methionine in the continuous open reading frame, but the true initiation site for PLDγ remains to be experimentally determined. Efforts are underway to determine whether PLDγ is myristoylated and, if so, what the functional consequences of this covalent modification are.

The results have demonstrated clearly that the activities of PLDγ, -γ, and -α are regulated differently by Ca2+ and polyphosphoinositides. PIP2 is a proposed regulator of PLDs from plants (5, 12), animals (8, 9), and yeast (13), but the mode of this PIP2 effect is unclear. This study has provided evidence that plant PLDs bind PIP2 and that this binding is required for

![Polyphosphoinositide stimulation of PLD activities](image-url)
Molecular Heterogeneity of Phospholipase D in Plants

PLDβ and γ activities. This conclusion is supported by several lines of evidence. A direct indication is the association of radioactive PIP_2 with these PLDs (Fig. 6). That inhibition of PIP_2 binding by neomycin or mM Ca^{2+} also inhibits PLDβ and γ activities (Figs. 4 and 5) provides correlative evidence for this conclusion. The sensitivity of PIP_2 binding by PLDβ and γ to cationic molecules such as neomycin and Ca^{2+} suggests that these molecules compete with the basic amino acids of the polyphosphoinositide-binding motif. The requirement for Ca^{2+} and direct PIP_2 binding for the activity of PLDγ and β suggests that Ca^{2+} may serve as both a positive and negative regulator of these enzymes. At concentrations at or below 100 μM, Ca^{2+} is a positive regulator of PLDγ and β activities. Above 100 μM, Ca^{2+} inhibits the binding of PIP_2 to PLDγ and β, thus negatively regulating their activities.

The presence of basic polyphosphoinositide-binding motifs near the putative catalytic sites (Fig. 1) may provide a structural basis for the observed PIP_2 binding by the PLDs. The extent of PIP_2 binding exhibited by the different PLDs appears to be correlated with the degree of amino acid conservation in the putative PIP_2-binding motifs. The PIP_2-binding sequence is also present in the PLDs cloned from human (amino acids 549-556) and yeast (1421 to 1428), both of which are also stimulated by PIP_2 (8, 13). Direct evidence for PIP_2 binding by this basic motif comes from a recently solved crystal structure of phospholipase Cβ. This binding motif in phospholipase Cβ stretches from amino acids 434 to 441 as the sequence KILLKGKK (24). Lysines 438 and 440 serve as direct ligands to phosphate groups at the 4- and 5-positions of inositol 1,4,5-trisphosphate (22). For phospholipases C, the basic binding motif is part of their catalytic sites because PIP_2 is a substrate. On the other hand, work in this laboratory has shown that PLDγ, β, and α do not hydrolyze PIP_2 and thus, the binding of PIP_2 by PLDs is part of PLD regulation and activation. A detailed mechanism for the role of PIP_2 binding in PLD-mediated hydrolysis remains to be elucidated.

In addition to direct binding, polyphosphoinositides may also enhance PLD activity by an alteration of substrate vesicle or membrane structure. This is evident from the PIP_2 stimulation of PLDα activity. It has been well documented that PLDα is most active in the presence of mM Ca^{2+} and detergents, and this activity is independent of polyphosphoinositides (3, 32). At suboptimal Ca^{2+} (5 mM) and with no detergent, PLDα is stimulated significantly by PIP_2. This stimulation does not require direct PIP_2 binding because it is insensitive to neomycin (Fig. 5B) and is not correlated with the extent of PIP_2 bound to PLDα (Fig. 6A). Although PLDα binds PIP_2 at lower Ca^{2+} concentrations, this binding is almost absent at 5 mM Ca^{2+} (Fig. 6A).

K. Pappan and X. Wang, unpublished results.

liposomes in the presence of 1 mM EGTA or 0.1 or 5 mM Ca^{2+}. B, PIP_2 concentration dependence of PIP_2 binding by GST-PLD fusion proteins. C, competition of [H]PIP_2 with unlabeled PIP_2. Twenty nmol of [H]PIP_2 was incubated with 25 μl of GST-PLD fusion proteins attached to glutathione beads in the presence of 1 mM EGTA, and varying amounts of unlabeled PIP_2 were then added. Background PIP_2 binding by GST was small and was subtracted from the PIP_2 binding values for the GST-PLD fusion proteins. D, lipid binding specificity by GST-PLD fusion proteins. The same specific radioactivity of 20 nmol of [H]PIP_2 or [H]PC was incubated with 25 μl of GST-PLD fusion proteins attached to glutathione beads in the presence of 1 mM EGTA. GST-PLDα, GST-PLDβ, GST-PLDγ, and GST were affinity-purified using glutathione-agarose. Phospholipid binding was determined by scintillation counting after extensive rinsing of the beads. The amount of PIP_2 bound to the GST-PLD fusion proteins is expressed based on the same GST activity measured as ΔA_{540}/min. Values are means ± S.E. of three experiments.
whereas PLDα activity increases at this Ca\textsuperscript{2+} concentration (Fig. 4). One explanation for these results is that there are different locations for the PIP\textsubscript{γ} binding sites on the PLDs. One is the polyphosphoinositide-binding motif near the C termini of PLDβ and -γ, which is absent in PLDα. PIP\textsubscript{γ} binding at this site may be required for catalysis. The other is the C2 domain, which is known to bind acidic phospholipids (20, 21). PIP\textsubscript{2} binding at this less specific site may be involved in regulating the membrane association of all three PLDs (20). Furthermore, PLDα contains a basic amino acid motif in the middle of its C2 domain, stretching from amino acids 58 to 66 as the sequence KARVGRTRK (Fig. 1).

The present results also suggest that, in addition to PIP\textsubscript{2}, other inositol-containing lipids may be involved in PLD regulation. All three plant PLDs are stimulated by PIP, albeit to a lesser extent than by PIP\textsubscript{2} (Fig. 5A). The potential exists for inositol phospholipids to serve as a key point of intersection for several phospholipid signaling pathways. For instance, in addition to being an activator of PLD, PIP\textsubscript{2} is a substrate for PLC-5-kinase (33). Such an activation may have a dual signaling effect by providing an activator of PLD and a substrate for PLC.

Prior findings from this laboratory have demonstrated that PLD is the polyphosphoinositide-binding motif near the C termini of PLDα, PLDβ, and -γ, which binds at this less specific site may be involved in regulating the membrane association of all three PLDs (20). Furthermore, PLDα contains a basic amino acid motif in the middle of its C2 domain, stretching from amino acids 58 to 66 as the sequence KARVGRTRK (Fig. 1).

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