Activation of Plant Phospholipase Dβ by Phosphatidylinositol 4,5-Bisphosphate: Characterization of Binding Site and Mode of Action†

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ABSTRACT: Hydrolysis of phospholipids by plant phospholipase Dβ (PLDβ) requires phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. Here we show that PLDβ is stimulated by different polyphosphoinositides, among which PI(4,5)P_2 is most effective. On the basis of amino acid sequence analysis, PI(4,5)P_2 binding assay, and protein engineering studies, we have identified in the catalytic region of PLDβ a new PI(4,5)-P_2 binding region (PBR1), which is conserved in eukaryotic PLDs. PBR1 is a second domain besides the previously characterized N-terminal C2 domain of PLDβ which also binds PI(4,5)P_2. Submillimolar levels of calcium ions, while inhibiting PI(4,5)P_2 binding by the C2 domain, enhanced the affinity of PBR1 for that phosphoinositide. Substrate binding by PLDβ was promoted by PI(4,5)P_2-bound PBR1. Isolated, recombinant PBR1 bound PI(4,5)P_2 specifically and in a saturable manner. Deletion of PBR1 from PLDβ or mutation of the conserved basic amino acid residues in PBR1 (K437G/K440G) abolished the enzymatic activity. Circular dichroism spectroscopy revealed a conformational change caused by PI(4,5)P_2 binding to the catalytic region of PLD. The conformational change apparently helps in the recruitment of the substrate to the active site of the enzyme. The results taken together allow us to describe an anchorage-scooting model for the synergistic activation of PLDβ by PI(4,5)P_2 and Ca^{2+}.

Activation of phospholipase D (PLD)[1] (EC 3.1.4.4) is involved in many cellular processes, including signal transduction, vesicular trafficking, cytoskeletal rearrangements, hormone action, and stress responses (*1*−*5* and references cited therein). To date, several PLD activators have been identified. These include ADP-ribosylation factor, Rho, and protein kinase C, Ca^{2+}, and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. Whereas the proteinaceous factors synergistically activate mammalian PLDs (*1*, *2*, *6*, *7*), all characterized PLDs in plants require Ca^{2+} for activity (*4*, *8*, *9*). PI(4,5)P_2 is the common lipid activator shared by PLDs from plants, animals, and yeast (*1*−*4*).

A trace phospholipid, PI(4,5)P_2 participates in many cellular functions (*10*). In addition to being the substrate for generating inositol 1,4,5-trisphosphate and diacylglycerol, PI(4,5)P_2 serves as a membrane attachment site for proteins with pleckstrin homology (PH) domains and is required for membrane-trafficking events. Furthermore, it modulates cytoskeletal dynamics by interacting with many actin binding proteins, including gelsolin (*11*), cortexicillin I (*12*), and α actinin (*13*). Recently, PI(4,5)P_2 has been proposed to play a critical role in the activation of PLD (*1*, *2*, *6*, *7*). Arrest of PI(4,5)P_2 synthesis by blocking of PI 4-kinase and PI 4-phosphate 5-kinase decreases PLD activity in mammalian cells (*14*). The in vitro activity of plant PLDβ requires PI(4,5)P_2, and replacement of PI(4,5)P_2 by other phospholipids, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), leads to loss of enzymatic activity (*15*). Similarly, neomycin, a strong PI(4,5)P_2 chelator, significantly diminishes PLD activity (*15*).

PLDβ contains a C2 domain at its N-terminus (PLDβ-C2) and a catalytic region (PLDβ-cat) at its C-terminus, which encompasses two HxKxxxxD motifs (*15*; Figure 1A). Earlier studies have shown that the N-terminal C2 domain of PLDβ binds PI(4,5)P_2 in the absence of Ca^{2+} and the binding is inhibited by the metal ion (*16*). However, it is unknown as to whether the C2 domain is the only region that binds PI(4,5)P_2. Clusters of basic residues of the kinds, RxKxxxxRR and RKxRxxxxR, have been implicated in PI-(4,5)P_2 binding by gelsolin, villin, and PLCδ (*17*, *18*). Thus, on this basis, two putative PI(4,5)P_2 binding regions (PBR2) were proposed near the second HKD motif of PLDβ (*15*; Figure 1A), pending experimental verification. Recent studies on mammalian and yeast PLDs show that mutation of basic residues within a small segment between the two catalytic domains, the first and second HxKxxxxD motifs, represses

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[1] Abbreviations: PLD, phospholipase D; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol 3-monophosphate; PI(4)P, phosphatidylinositol 4-monophosphate; PI(5)P, phosphatidylinositol 5-monophosphate; PI(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; GST, glutathione *S*-transferase; IPTG, isopropyl 1-thio-β-galactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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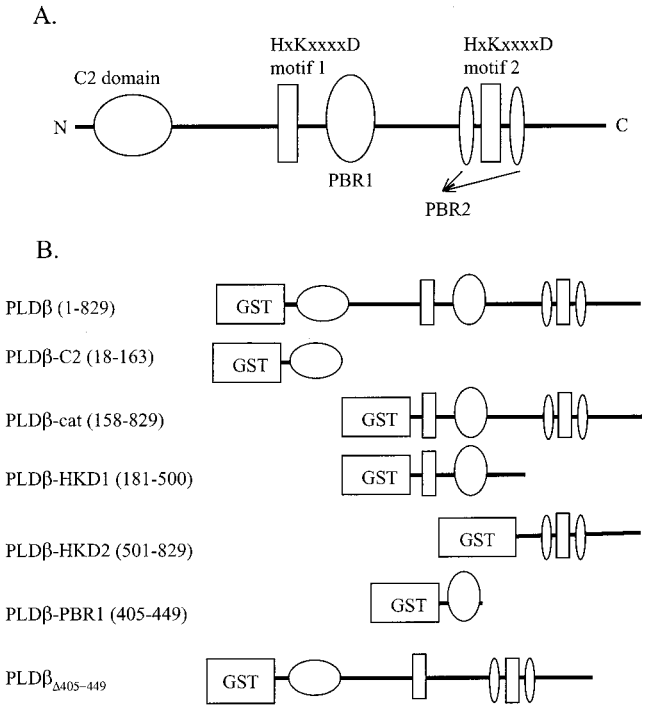

FIGURE 1: Schematic diagram of the domain structure of PLDβ and its GST fusion proteins. (A) Domain structure of full-length PLDβ showing different motifs represented by different shapes. The C2 domain is a calcium and PC binding motif (*16*), the HxKxxxxD stands for the catalytic site, and PBR denotes the PI(4,5)P_2 binding region. PBR2, previously suggested as a putative PI(4,5)P_2 binding site (*15*), is presently found to bind weakly, if at all, the phosphoinositide. PBR1, selected on the basis of its sequence homology to the proposed PI(4,5)P_2 binding region in mouse PLD2 (*19*), is the lipid-activator binding domain characterized in this work. (B) Schematic representations of GST-fused PLDβ and its mutants. Full-length or different isolated regions of PLDβ were fused to the C-terminal end of GST, as indicated by abbreviations with the numbers of starting and ending residues given within parentheses.

PLD activity (*19*). That fragment (Figure 2) has consequently been proposed as the putative PI(4,5)P_2 binding region (*19*). Multiple amino acid sequence alignment of PLDs from plants, animals, and yeast reveals conservation of some of the basic residues of this PI(4,5)P_2 binding motif in plant PLDβ (Figure 2). However, the Arg residues in the RX_3R motif identified in mammalian and yeast PLDs (*19*) are replaced by Tyr and Thr in plant PLDβ and, thus, cannot account for the PI(4,5)P_2 requirement by PLDβ. In addition, it is unknown whether this region is capable of binding PI(4,5)P_2 and serves as an independent binding motif. Furthermore, it is not yet clear as to how the phospholipid activator interacts with and stimulates the enzyme.

Here we show that PLDβ is activated not only by PI(4,5)P_2 but also by PI(4)P and other polyphosphoinositides, PI(3,4)P_2, PI(3,5)P_2, and PI(3,4,5)P_3, although most stimulation occurs with PI(4,5)P_2. On the basis of multiple amino acid sequence alignment, we have identified in PLDβ a new PI(4,5)P_2 binding region, designated as PBR1, near the first HxKxxxxD motif and demonstrated by mutagenesis studies that PBR1 is critical to the enzymatic activity. Furthermore, we have established that PBR1 is an independent structural unit capable of binding PI(4,5)P_2 and identified the essential amino acid residues involved in PI(4,5)P_2 binding, which is modulated by Ca^{2+}. The collective results suggest an anchorage-scooting model for the synergistic activation of PLDβ by Ca^{2+} and PI(4,5)P_2, according to which the membrane-bound enzyme, upon activation, grasps its substrate without dissociating from the surface.

MATERIALS AND METHODS

Lipid Materials. Nonradioactive polyphosphoinositides were purchased from CellSignals (Lexington, KY). Other phospholipids were obtained from Sigma (St. Louis, MO). Radioactively labeled PC, PI, PS, and PI(4,5)P2 were products of Amersham Life Sciences (Arlington, IL).

Construction of Expression Plasmids of PLDβ and Its Mutants. Nine GST fusion proteins, GST−PLDβ (1−829), GST−PLDβ-cat (181−829), GST−PLDβ-C2 (18−163), GST−PLDβ-HKD1 (181−500), GST−PLDβ-HKD2 (501−829), GST−PLDβ-PBR1 (405−449), GST−PLD$\beta_{K437G/K440G}$, GST−PLD$\beta_{K446E/K448S}$, and GST−PLD$\beta_{\Delta405-449}$, and a 6His-tagged PLDβ-HKD1 were used in this study. Their schematic domain structures are given in Figure 1B. GST−PLDβ and GST−PLDβ-C2 constructs were reported previously (*15*, *16*). PLDβ-cat is a PLDβ variant in which the C2 domain is deleted. A DNA fragment encoding the region of PLDβ-cat was generated by polymerase chain reaction (PCR) using the PLDβ cDNA in pBluescript SK as a DNA template, T7 primer as 3′ primer, and a synthetic oligonucleotide as 5′ primer, which includes an *Eco*RI restriction site at its 5′ end. The PCR-amplified DNA fragment was digested with *Eco*RI restriction enzyme and ligated directly into the pGEX-2T vector (Pharmacia). The DNA fragment encoding PLDβ-HKD1, in which the first HxKxxxxD motif is included, was obtained by digestion of plasmid DNA of pGEX-2T-PLDβ-cat with *Bam*HI. The DNA fragment was then ligated into the pGEX-4T vector (Pharmacia), the same vector as pGEX-2T except that it contains more multiple cloning sites. The same DNA fragment was also ligated into pET28a (Novagen) to construct the plasmid expressing 6His-tagged PLDβ-HKD1. For the construction of GST−PLDβ-HKD2, which contains the second HxKxxxxD motif, a DNA fragment encoding this region was amplified by PCR. An *Eco*RI restriction site was added to the 5′ primer, and a *Xho*I restriction site was incorporated into the 3′ primer. The PCR product was digested with *Eco*RI and *Xho*I restriction enzymes and then ligated into the pGEX-4T vector. The same procedure was used to construct the expression plasmid encoding GST−PLDβ-PBR1. Site-directed and deletion mutagenesis was performed using a sequential PCR procedure, as described previously (*20*). PLD$\beta_{\Delta405-449}$ is a deletion mutant lacking PBR1. Two conserved basic residues were mutated in PLD$\beta_{K437G/K440G}$ and two nonconserved basic residues in PLD$\beta_{K446E/K448S}$. DNA sequencing was performed to confirm all of the mutations (DNA sequencing facility, Iowa State University, Ames, IA). All constructs were transformed into *Escherichia coli* BL21 for the expression of GST fusion proteins or into *E. coli* BL21(DE3) for the expression of poly-His-fused proteins.

Expression and Purification of Recombinant Fusion Proteins. The *E. coli* BL21 or *E. coli* BL21(DE3) containing the plasmid that encoded one of the fusion proteins was grown at 37 °C to an absorbance of about 1.0 at 600 nm and induced by 0.2 mM isopropyl 1-thio-β-galactopyranoside (IPTG) in the case of the GST fusion proteins or by 1 mM

Proteins		PI(4,5)P_2-Binding Region Sequences	Organism	GenBank Number
Animal PLDs	PLD2	533 ETTP**R**MPWRDVGVVVHGVAARDLARHFIQ**R**WNFT**K**TT**K**A----RYKT 575	*Mus musculus*	U87577
	PLD2	533 ETTP**R**MPWRDVGVVVHGVAARDLARHFIQ**R**WNFT**K**TI**K**A----RYKI 575	*Rattus norvegicus*	AB003172
	PLD2	533 ETTP**R**MPWRDVGVVVHGLPARDLARHFIQ**R**WNFT**K**TT**K**A----KYKT 575	*Homo sapiens*	AF038441
	PLD1	671 YSTP**R**MPWHDIGSVVHGKAARDVARHFIQ**R**WNFT**K**IM**K**P----KYRS 713	*Mus musculus*	AF083497
	PLD1	633 YSTP**R**IPWHDIGSVLHGKAARDVARHFIQ**R**WNFT**K**IM**K**P----KYRS 675	*Cricetulus griseus*	U94995
	PLD1	671 YSTP**R**MPWHDIASAVHGKAARDVARHFIQ**R**WNFT**K**IM**K**S----KYRS 713	*Homo sapiens*	U38545
	PLD1a	671 YSTP**R**MPWHDIGSVVHGKAARDVARHFIQ**R**WNFT**K**IM**K**A----KYRS 713	*Rattus norvegicus*	AB003170
	PLD	860 TTTP**R**MPWHDVGLCVVGTSARDVARHFIQ**R**WNAM**K**LE**K**L----RDNT 902	*Drosophila melanogaster*	AF228314
Plant PLDs	PLDβ	409 SGCP**R**EPWHDLHSKIDGPAAYDVLTNFEE**R**W--L**K**AA**K**PSGIK**K**F**K**L 449	*Arabidopsis thaliana*	U84568
	PLDγ	427 DDGP**R**EPWHDLHSKIDGPAAYDVLANFEE**R**W--M**K**AS**K**LAELGNENT 471	*Arabidopsis thaliana*	AF027408
	PLDδ	424 KA-P**R**QPWHDLHCRIDGPAAYDVLINFEQ**R**W--R**K**AT**R**W---KEFSL 464	*Arabidopsis thaliana*	AF322228
	PLD	410 VGCP**R**EPWHDMHSKIDGPAAYDVLVNFEE**R**W--L**K**AA**K**PHGLKKLKK 452	*Gossypium hirsutum*	AF159139
	PLDβ1	426 TGCP**R**EPWHDLHSRIEGPAAYDVLTNFEE**R**W--L**K**AS**K**RHGLQKMKA 467	*Lycopersicon esculentum*	AY013255
	PLDβ2	475 AGCP**R**EPWHDLHSKIDGPAAYDVLTNFEE**R**W--L**K**AS**K**PHGIRKLKT 519	*Lycopersicon esculentum*	AY013256
	PLDγ	185 TGCP**R**EPWHDLHSRIEGPAAYDVLTNFEE**R**W--L**K**AS**K**RHGLQKMKA 227	*Nicotiana tabacum*	AF19564
	PLDα	397 KGCP**R**EPWHDIHSRLEGPIAWDVMYNFEQ**R**W--S**K**QGG----KEILV 437	*Arabidopsis thaliana*	U36381
	PLDα1	397 KGGP**R**EPWHDIHSRLEGPIAWDVLFNFEQ**R**W--R**K**QGG----KDILV 437	*Lycopersicon esculentum*	AY013252
	PLD1	395 KGGP**R**EPWHDIHSRLEGPIAWDVLYNFEQ**R**W--S**K**QGG----KDILV 435	*Brassica oleracea*	AF090445
	PLD	399 KGGP**R**EPWHDIHSRVEGPIAWDVLFNFEQ**R**W--R**K**QGG----KDILA 439	*Vigna unguiculata*	U92656
	PLD	400 KGGP**R**EPWHDIHSRLEGPIAWDVLYNFEQ**R**W--R**K**QGG----KDLLV 440	*Zea mays*	D73410
	PLD	396 KGGP**R**EPWHDIHSRLEGPVAWDVLFNFEQ**R**W--R**K**QGG----KDILL 436	*Pimpinella brachycarpa*	U96438
	PLD1	396 KGGP**R**EPWHDIHSRLEGPIAWDVLFNFEQ**R**W--K**K**QGG----KDVLL 436	*Craterostigma plantagineum*	AJ133001
	PLD2	396 KGGP**R**EPWHDIHSRLEGPIAWDVLFNFEQ**R**W--K**K**QAG----RDLLI 436	*Craterostigma plantagineum*	AJ133000
	PLD	396 KGGP**R**EPWHDIHSRLEGPIAWDVLFNFEQ**R**W--R**K**QGG----KDLLI 436	*Ricinus communis*	L33686
	PLDα1	397 KGGP**R**EPWHDIHSRLEGPIAWDVLFNFEQ**R**W--R**K**QGG----KDVLL 437	*Lycopersicon esculentum*	AY013252
	PLD	421 SYGP**R**QPWHDLHCKIEGPAAFDILTNFEQ**R**W--R**K**AT**K**WKVNLKKVA 465	*Oryza sativa*	AF271358
Yeast PLDs	PLD	865 KVIP**R**MPWHDVQMMTLGEPARDLARHFVQ**R**WNYLLRA**K**RPSRLTPLL 911	*Saccharomyces cerevisiae*	Z28256
	PLD	840 NIVP**R**MPWHDVHMYTCGQTARDLSRHFVQ**R**WNYLIRQ**K**RPSRLTPLL 886	*Candida albicans*	AB010810
	PLD1	719 SAVP**R**MGWHDVSMCIIGQPARDAARHFVQ**R**WNYLIQC**K**KPARKTPLL 755	*Schizosaccharomyces pombe*	Z50142

FIGURE 2: Conserved putative PI(4,5)P_2 binding region in various eukaryotic PLD isoforms. The multiple sequence alignment was performed by comparing the PI(4,5)P_2 binding segment proposed for mouse PLD2 (*19*) with the primary structures of other PLDs from various organisms, using the Blast program (National Library of Medicine, Bethesda, MD). The RX_3R motifs identified for PI(4,5)P_2 binding in mammalian and yeast enzymes are boxed. Conserved Arg and Lys residues are boldfaced. Amino acid residues of plant PLDβ subjected to mutagenesis in the present work are underlined.

IPTG in the case of 6His fusion proteins at 25 °C for 4 h. The induced cells were harvested and lysed by sonication. The GST fusion proteins were purified as described previously (*21*) and stored at 4 °C. The 6His-tagged PLDβ-HKD1 was found to be in the inclusion body fraction. It was purified and refolded according to a previously described procedure (*16*). Briefly, the particulate pellet of the His-tagged protein isolated from 1 L of culture was solubilized in a binding buffer (20 mM Tris-HCl, 0.5 M NaCl, and 5 mM imidazole at pH 8.0) containing 8 M urea. After centrifugation, the supernatant was passed through a 10 mL Ni^{2+}-charged His-resin column equilibrated with the binding buffer. The column was thoroughly washed with a buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, and 8 M urea at pH 8.0. Proteins bound to the resin were eluted with a 500 mM imidazole buffer. Urea in the denatured recombinant protein was diluted by adding the solution dropwise into a stirring buffer containing 50 mM Tris-HCl (pH 7.5) and 2 M urea at 4 °C. The solution was kept at 4 °C for 1 h and incubated at room temperature for 1 h. The protein solution was dialyzed against 50 mM Tris-HCl, pH 7.5, overnight at 4 °C and then concentrated by means of a negative-pressure dialysis system (Spectrum).

Phospholipid Binding Assay. A similar method as described previously was used to determine PI(4,5)P_2 binding and PC binding characteristics of the proteins of interest (*22*). The phospholipid vesicles used to examine PI(4,5)P_2 binding were made of 0.4 mM PI(4,5)P_2 and 0.4 μCi/mL ^{3}H-labeled PI(4,5)P_2 (dipalmitoylglycero-3-phospho[*inositol*-2-^{3}H]inositol 4,5-bisphosphate, DuPont). Purified GST fusion proteins bound to glutathione−agarose were suspended in a binding buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and either 1 mM EGTA or 100 μM Ca^{2+}. Fifty microliters of 20% agarose beads was mixed with an equal volume of PI(4,5)P_2 and incubated for 30 min at room temperature. The beads were spun down and washed with 1 mL of the corresponding binding buffer three times. PI(4,5)P_2 bound to the protein was measured by scintillation counts. The background binding was determined from PI(4,5)P_2 bound to GST−agarose beads. All experiments were repeated three times. For the determination of PC bound to the GST fusion proteins in the presence of different amounts of PI(4,5)P_2, a given GST fusion protein bound to glutathione−agarose was suspended in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 100 μM Ca^{2+} and incubated with a series of phospholipid vesicles containing PC, PE, and PI(4,5)P_2. The molar ratio of PC was fixed at 5%, and the molar ratios of PE and PI(4,5)P_2 were changed in steps from 95% to 75% and from 0% to 20%, respectively. The lipid binding was determined by measuring the binding of [^{3}H]PC (dipalmitoylglycero-3-phospho[*methyl*-^{3}H]choline) to the recombinant fusion protein.

PLD Activity Assay. PLD activity was measured according to a slightly modified version of a previously described procedure (*23*). A certain amount of the protein was mixed with a reaction buffer containing 100 mM MES (pH 7.0),

100 μM $CaCl_2$, 1 mM $MgCl_2$, 80 mM KCl, and 0.4 mM phospholipid vesicles to a total volume of 100 μL. To test the activation of PLDβ by different polyphosphoinositides, the lipid vesicles were made of PE/PX/PC in the molar ratio of 85:10:5, where PX is PE, PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(3,5)P_2, or PI(3,4,5)P_3. To assay the enzymatic activity in the presence of different amounts of PI(4,5)P_2, lipid vesicles made up of PE, PI(4,5)P_2, and PC were used. The molar ratio of PC was fixed at 5%, and the molar ratios of PE and PI(4,5)P_2 were changed in steps from 95% to 75% and from 0% to 20%, respectively. The hydrolysis of PC was monitored using dipalmitoylglycero-3-phospho[*methyl*-^{3}H]choline as a tracer. The amount of [^{3}H]-choline released into the aqueous phase was quantified by scintillation counting.

GST Activity Assay. A slightly modified version of the published procedure (*24*) was used: a 10 μL protein sample was mixed with 10 μL of 100 mM 1-chloro-2,4-dinitrobenzene (CNDB), 10 μL of 100 mM glutathione, and 970 μL of reaction buffer (0.1 mM potassium phosphate buffer, pH 7.0), and the absorbance at 340 nm (A_{340}) was monitored and recorded for 2 min using a Hitachi U-2000 spectrophotometer. The unit of GST activity was calculated as ΔA_{340} min^{-1} mL^{-1} of sample.

CD Spectroscopy. The CD spectrum of PLDβ-HKD1 was recorded with a Jasco J-720 spectropolarimeter. PLDβ-HKD1 was suspended in 50 mM Tris-HCl, pH 7.5, containing 0.2 mM PI or PI(4,5)P_2 in a 0.1 cm cell. The sample was scanned 32 times at room temperature. A reference spectrum was recorded under the same condition with the same solution, but without the protein, and was subtracted from the protein spectrum. A program supplied with the instrument was used to calculate the molar ellipticity. The molar concentration of PLDβ-HKD1 was determined using the ϵ_{280} value of 55910 M^{-1} cm^{-1}, calculated by the GCG program (University of Wisconsin) on the basis of the amino acid sequence and the calculated molecular mass of 36355 Da.

RESULTS

Activation of PLDβ by Polyphosphoinositides. Previous studies showed that PI(4,5)P_2 was required for PLDβ activation (*16*, *23*). To determine whether other polyphosphoinositides also activate PLDβ, we employed in the activity assay different phospholipid vesicles composed of PC, PE, and PX in the molar ratio of 5:85:10, where PX was PE, PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, or PI(3,4,5)P_3. The results reveal that while PI has almost no effect, the other phosphatidylinositol derivatives show considerable stimulation effects (Figure 3). However, PI(4,5)P_2 is most effective. The decreasing order of activation efficacy of other compounds is PI(3,4,5)P_3 > PI(3,4)P_2 > PI(4)P > PI(3,5)P_2 > PI(3)P or PI(5)P. PI(4)P appears to have a 4-fold effect as that of PI(3)P or PI(5)P.

Multiple PI(4,5)P_2 Binding Regions of PLDβ with Distinct Binding Properties. To map the regions of PLDβ that interact with PI(4,5)P_2, we made several GST fusion proteins that contained the full-length PLDβ or parts of PLDβ (Figure 1). PLDβ comprises a C2 domain at its N-terminus (PLDβ-C2) and a catalytic region (PLDβ-cat) at its C-terminus, which encompasses two HxKxxxxD motifs (Figure 1A). As suggested by the multiple amino acid sequence alignment

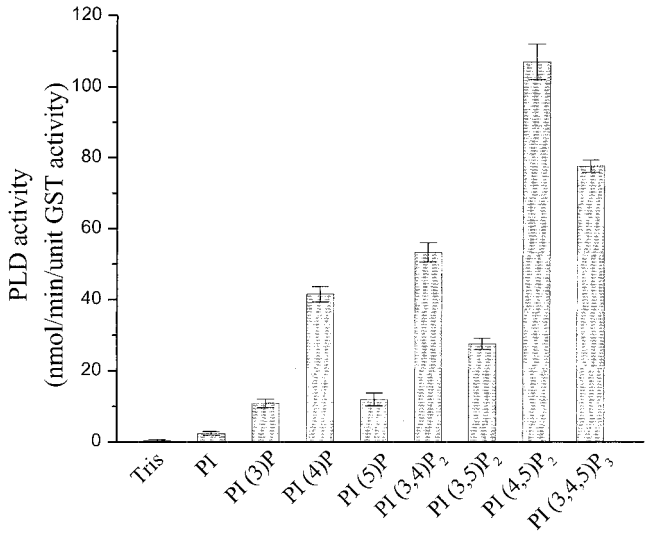

FIGURE 3: Activation of PLDβ by polyphosphoinositides. The effect of polyphosphoinositides on recombinant plant PLDβ was tested using 0.4 mM lipid vesicles composed of 85 mol % of PE, 5 mol % of PC and [^{3}H]PC, and 10 mol % of PX, where PX was PE, PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, and PI(3,4,5)-P_3. The activities were expressed as nanomoles of PC hydrolyzed per minute per unit GST activity of GST−PLDβ. The background scintillation count obtained with GST alone was subtracted from that obtained with GST−PLDβ. Values are means ± SE of three experiments.

with the bacterial endonuclease, the catalytic region of PLD can be divided into two segments, each containing an HxKxxxxD motif (*25*, *26*). These are termed PLDβ-HKD1 and PLDβ-HKD2 (Figure 1B). The region corresponding to the putative PI(4,5)P_2 binding region proposed for mammalian and yeast PLD isoenzymes (Figure 2) is identified as PBR1 and occurs near the first HKD motif of PLDβ (Figure 1). The previously proposed PI(4,5)P_2 binding motifs, RxKxxxxRR and RKxRxxxxR, occur near the second HxKxxxxD (*15*) and are denoted as PBR2 for convenience (Figure 1A).

These PLDβ variants were assayed for PI(4,5)P_2 binding (Figure 4). The full-length enzyme and the isolated C2 domain bound PI(4,5)P_2, as observed earlier (*16*). In addition, PLDβ-cat and PLDβ-HKD1, but not PLDβ-HKD2, appreciably bound PI(4,5)P_2. More important is the finding that PLDβ-PBR1, the isolated PBR1, binds PI(4,5)P_2 nearly to the same extent as does PLDβ-cat. This suggests that PBR1 is an independent domain capable of interacting with PI(4,5)P_2. Ca^{2+} at 0.1 mM level promotes PI(4,5)P_2 binding of PLDβ and PLDβ-cat but inhibits that of PLDβ-C2 (Figure 4). The metal ion has no significant effect on PI(4,5)P_2 binding of PLDβ-HKD1 and PLDβ-PBR1.

Characterization of the PI(4,5)P_2 Binding Domain of PLDβ. PLDβ-PBR1, which corresponds to residues 405−449 of PLDβ, is highly conserved in eukaryotic PLDs (Figure 2), thus suggesting important functions for this region. The binding of PI(4,5)P_2 by PLDβ-PBR1 is found to be dose-dependent and saturable (Figure 5A), thus indicating a specific interaction between the phospholipid activator and the protein. The half-maximum binding occurs at about 70 μM and saturation at about 200 μM PI(4,5)P_2. To examine the phospholipid specificity of PLDβ-PBR1, the binding of [^{3}H]PC, [^{3}H]PI, [^{14}C]PS, and [^{3}H]PI(4,5)P_2 to PLDβ-PBR1 was examined and the specific amount of each phospholipid

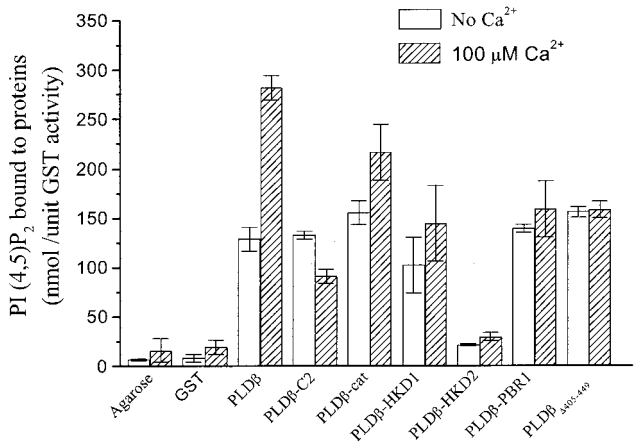

FIGURE 4: Mapping of PI(4,5)P_2 binding regions of PLDβ. Bead-bound GST fusion proteins were incubated with a final concentration of 0.2 mM PI(4,5)P_2 vesicles containing [^{3}H]PI(4,5)P_2 as a tracer, in the presence of either 1 mM EGTA or 100 μM Ca^{2+}. GST bound to the beads was used to determine the background binding, which was deducted from the protein binding data. The PI(4,5)P_2 binding was measured by scintillation counting, and the specific amount of bound PI(4,5)P_2 was calculated and expressed as nanomoles per unit GST activity. Values are means ± SE of three experiments.

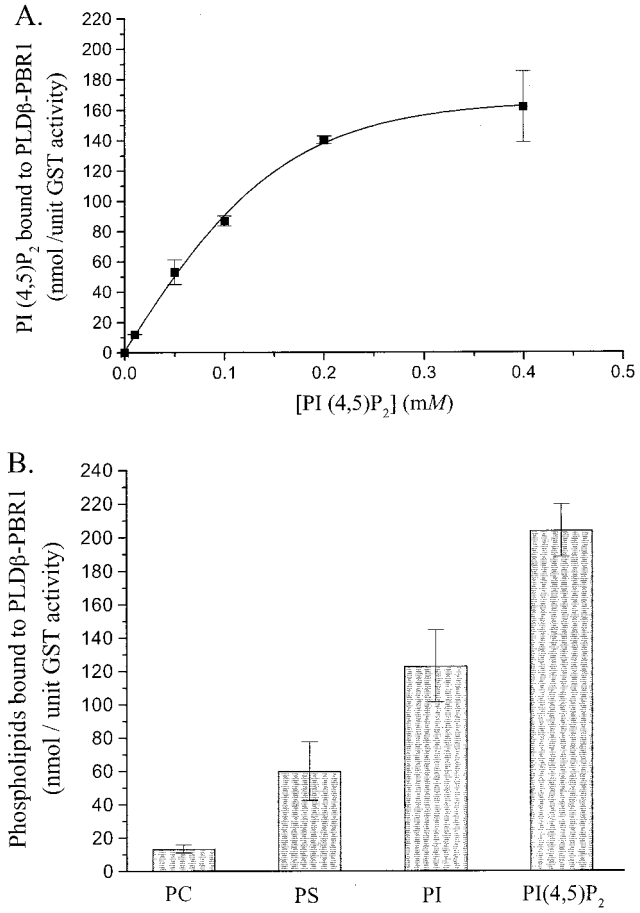

FIGURE 5: Characterization of PI(4,5)P_2 binding to PLDβ−PBR1. (A) Dose-dependent PI(4,5)P_2 binding by PLDβ−PBR1. The line drawn through the data points represents the nonlinear least squares fit. (B) Phospholipid binding specificity of PLDβ−PBR1. Phospholipid binding data were obtained by scintillation counting, using ^{3}H as a tracer, as described in the caption to Figure 4.

bound to the protein calculated (Figure 5B). Thus, it is found that PLDβ-PBR1 has the highest efficacy for PI(4,5)P_2. It also binds PI, but only with half as much efficacy. While PS binding by PLDβ-PBR1 is low, PC binding is negligible.

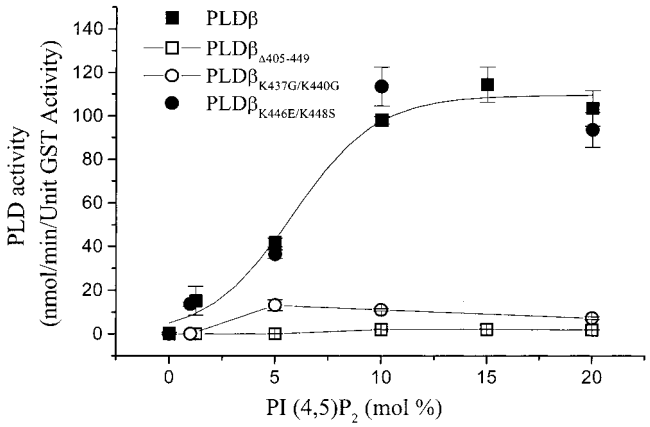

FIGURE 6: PI(4,5)P_2-dependent PLD activities of PLDβ and its PBR1 mutants. PLDβ, PLD$\beta_{\Delta405-449}$, PLD$\beta_{K437G/K440G}$, and PLD$\beta_{K446E/K448S}$ were expressed as recombinant GST fusion proteins. The hydrolysis of PC by these proteins was tested with phospholipid vesicles consisting of 95−75 mol % of PE, 0−20 mol % of PI(4,5)P_2, and 5 mol % of PC. The activities were expressed as nanomoles of PC hydrolyzed per minute per unit GST activity of GST-fused PLDβ or its mutants. The background hydrolysis by GST alone was subtracted from the hydrolysis by GST fusion proteins. Values are means ± SE of three experiments. The line drawn through the data points of PLDβ represents the nonlinear least squares fit. Data points corresponding to PLD$\beta_{\Delta405-449}$ and PLD$\beta_{K437G/K440G}$ are connected by lines to guide the eye.

PLDβ−PBD1 Is Essential for PLD Activity. The evidence that PBR1 alone is sufficient for the specific and saturable binding of PI(4,5)P_2 (Figure 5A) suggests that this region may play a critical role in PLD activation by PI(4,5)P_2. To verify this hypothesis, a deletion mutant that lacks PBR1, PLD$\beta_{\Delta405-449}$, was constructed and expressed as a GST fusion protein. This deletion mutant completely loses PLD activity (Figure 6) and Ca^{2+}-stimulated binding of PI(4,5)P_2 (Figure 4). In the absence of Ca^{2+}, the mutant and wild-type PLDβ bind similar levels of PI(4,5)P_2 (Figure 6), and this binding by the deletion mutant is expected because the C2 domain interacts with this lipid (Figure 4).

To define the residues involved in the PI(4,5)P_2 activation of PLD, two site-directed mutants, GST−PLD$\beta_{K437G/K440G}$ and GST−PLD$\beta_{K446E/K448S}$, were made, and their PLD activities were assessed in the presence of varying amounts of PI(4,5)P_2 (Figure 6). The residues of PLD$\beta_{K437G/K440G}$ were chosen because the Lys residues are conserved in PLDβ and PLDγ that require PI(4,5)P_2 for activity (*8*). In contrast, the PI(4,5)P_2-independent PLDα (8) has only one of the Lys residues. Gly was substituted for Lys because it is found in PLDα (Figure 2). The two adjacent Lys residues K446/K448 are not conserved in PLDγ, PLDδ, or PLDα (Figure 2). The substitutions of PLD$\beta_{K446E/K448S}$ were made because the Glu and Ser residues are found in PLDδ (Figure 2) that exhibits only a conditional requirement of PI(4,5)P_2 for activity (*9*). PLDβ displays a PI(4,5)P_2-dependent activity with the optimal value reached at about 10 mol % of PI(4,5)P_2 (Figure 6). PLD$\beta_{K437G/K440G}$ retains about 10% activity of the wild-type enzyme. In contrast, PLD$\beta_{K446E/K448S}$, in which two nonconserved lysine residues are replaced, retains essentially unaltered enzyme function.

PI(4,5)P_2 Stimulates PC Binding of the Catalytic Region of PLDβ. A possible function of PI(4,5)P_2 as a cofactor of

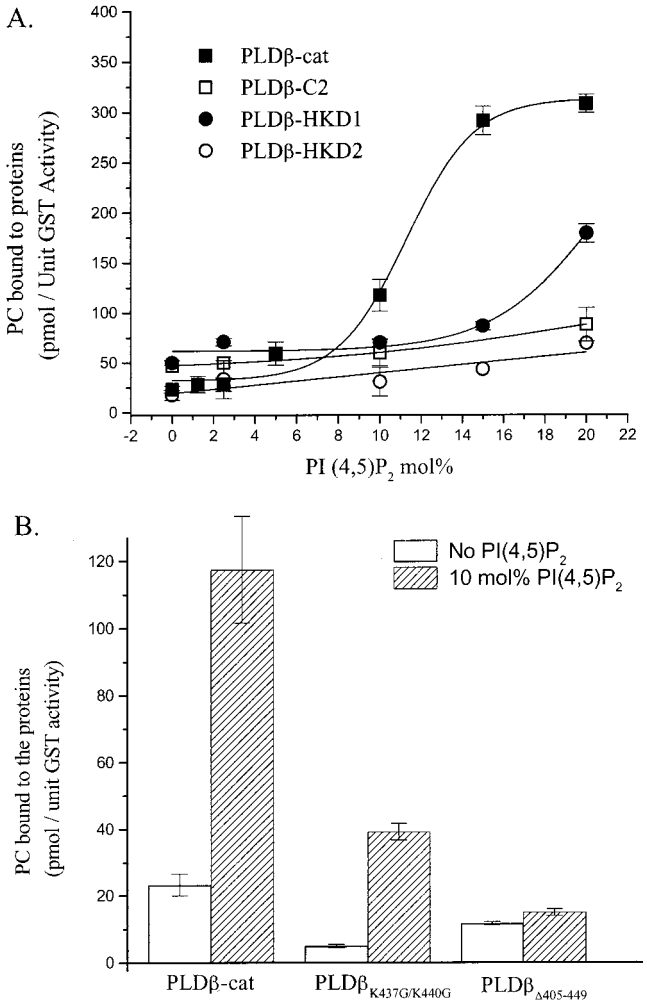

FIGURE 7: PI(4,5)P_2-dependent PC binding by PLDβ and its variants. (A) PI(4,5)P_2-dependent PC binding to different regions of PLDβ. Glutathione−agarose bead-bound PLDβ-cat, PLDβ-C2, PLDβ-HKD1, or PLDβ-HKD2 was mixed with phospholipid vesicles made up of PE/PI(4,5)P_2/PC and ^{3}H-labeled PC. In all of the lipid vesicles tested, the molar percentage of PC was set at 5 mol %, while the molar percentage of PE was changed from 95 to 75 mol % and the molar percentage of PI(4,5)P_2 was correspondingly changed from 0 to 20 mol %. PC binding was measured from scintillation counts. After the background binding was subtracted, the specific amount of protein-bound PC was calculated and expressed as picomoles per unit GST activity. Values are means ± SE of three experiments. (B) PI(4,5)P_2-stimulated PC binding to PLDβ-cat, PLD$\beta_{K437G/K440G}$, and PLD$\beta_{\Delta405-449}$. PC binding to PLDβ-cat, PLD$\beta_{K437G/K440G}$, or PLD$\beta_{\Delta405-449}$ was assayed in the absence or presence of 10 mol % of PI(4,5)P_2, using the same procedure as described in panel A. Values are means ± SE of three experiments. Lines drawn through data points are least squares fits.

PLDβ is to facilitate substrate binding. To test this possibility, the same phospholipid vesicles as used in PLD activity assay were mixed with purified GST−PLDβ-cat, GST−PLDβ-HKD1, GST−PLDβ-HKD2, or GST−PLDβ-C2 bound to glutathione−agarose. The PC binding capacity was examined at different molar ratios of PI(4,5)P_2. The PC bound to GST alone was used as a reference. In the absence of PI(4,5)P_2, PLDβ-cat, PLDβ-HKD1, PLDβ-HKD2, and PLDβ-C2 bound low levels of PC (Figure 7A). The PC binding capacity of PLDβ-cat increased with PI(4,5)P_2 concentration. The binding was saturated when the PI(4,5)P_2 level reached about 15 mol %, and the saturation level of PC binding was about 10 times the level at 0 mol % of PI(4,5)P_2. The stimulation

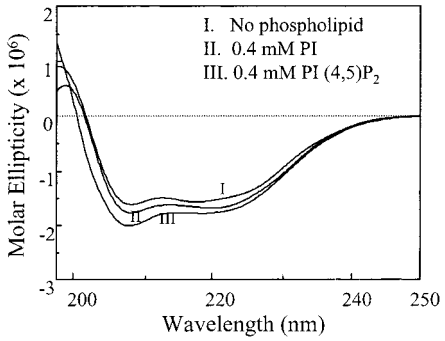

FIGURE 8: CD spectra of PLDβ-HKD1 in the absence or presence of PI or PI(4,5)P_2. Purified 6His-PLDβ-HKD1 was suspended in the buffer only or buffer containing either 0.4 mM PI or 0.4 mM PI(4,5)P_2. CD spectra were recorded at room temperature, and the molar ellipticity was calculated after subtraction of reference spectra, which were recorded under the same conditions with the solution containing no protein.

effect of PI(4,5)P_2 was less on PLDβ-HKD1 than on PLDβ-cat. This is not surprising because deletion of the HKD2 region would likely disrupt the substrate binding site, which is formed by both HxKxxxxD motifs (*25*, *26*). PI(4,5)P_2 had a negligible effect on PC binding of PLDβ-HKD2 and PLDβ-C2. The PI(4,5)P_2-stimulated PC binding of PLD$\beta_{K437G/K440S}$ and PLD$\beta_{\Delta405-449}$ was also tested in the presence of 10 mol % of PI(4,5)P_2 (Figure 7B). Deletion of PBR1 abolished the phospholipid stimulation effect, and mutation of critical basic residues in this region significantly diminished it.

To determine if PI(4,5)P_2 binding caused a conformational change in the catalytic region which would result in enhanced substrate (PC) binding, CD spectra were compared of 6His-tagged PLDβ-HKD1 with and without PI(4,5)P_2 and PI (Figure 8). It is deduced that the phosphoinositide binding induces conformational changes. The magnitude of the spectral changes appears to be smaller in the case of PI binding, consistent with the decreased binding capacity of isolated PBR1 for PI relative to PI(4,5)P_2 (Figure 8).

DISCUSSION

The present study demonstrates that PLDβ prefers PI(4,5)P_2 over PI(3,4)P_2 and PI(3,5)P_2 as an activator because of specific binding and interactions, in which the 4-phosphate group appears to play a dominant role. Recent structural studies on other PI(4,5)P_2 binding proteins, epsin (*27*), adapter protein 180 (*28*), and clathrin assembly lymphoid myeloid leukemia protein (*28*), show that salt bridges are formed between Lys residues and the lipid phosphate groups, thus providing major forces for maintaining the protein−phosphoinositide complex.

In addition to the N-terminal C2 domain, whose PI(4,5)P_2 binding property we have previously characterized (*16*), we have now identified a novel PI(4,5)P_2 binding motif, PBR1, in PLDβ. While this 45-residue region contains many basic side chains (Lys, Arg, and His) and bears a strong sequence homology with a number of other eukaryotic PLDs (Figure 2), it nonetheless exhibits some distinct features: The key residues of PBR1 involved in binding make the pattern KX_2K, unlike the RX_3R motif identified in mammalian and yeast PLDs (*19*). These phospholipid-binding Arg residues are replaced by Tyr and Thr in plant PLDβ. Recent structural studies of the epsin NH_2-terminal homology (ENTH) domain

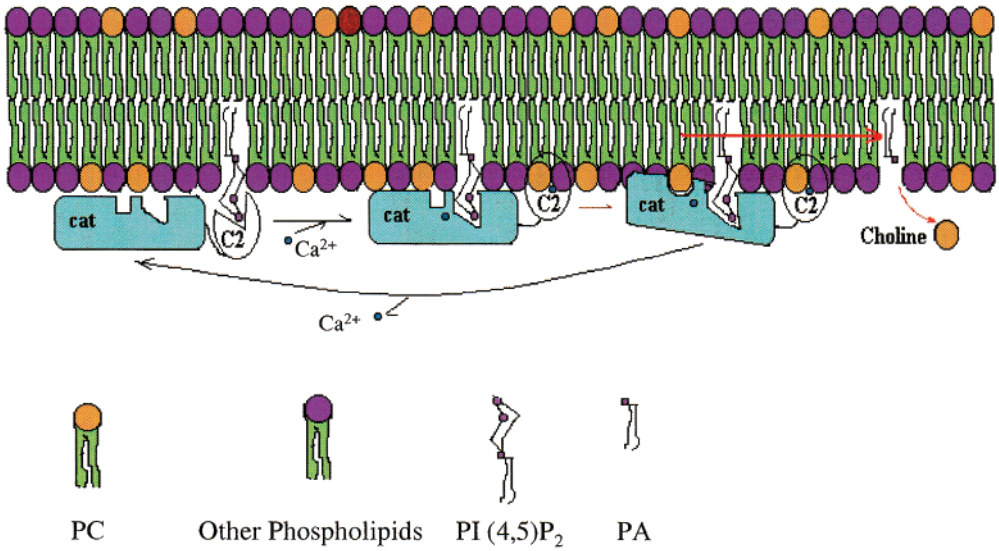

FIGURE 9: Anchorage-scooting model of activation of PLDβ by Ca^{2+} and PI(4,5)P_2. PLDβ remains bound to the membrane. Normally, it attaches to the membrane because of specific interactions between its N-terminal C2 domain and PI(4,5)P_2. External stimuli elevate the level of cytosolic Ca^{2+}, which interacts with the C2 domain and induces conformational changes; consequently, PI(4,5)P_2 is released from the C2 domain, which then binds PC. The catalytic region also binds Ca^{2+} and increases its PI(4,5)P_2 affinity. Binding of PI(4,5)P2 to PBR1 within the catalytic region induces a conformational change, which facilitates substrate binding at the active site. By this scooting mechanism, PLDβ can reach its substrate without dissociation from the membrane.

of endocytic proteins of AP180 and epsin families reveal that three Lys residues and one His residue of the protein electrostatically interact with the phosphate groups of PI(4,5)P_2 (*27*, *28*). However, the PI(4,5)P_2 binding motif found in these proteins is of the type KX_9KX(K/R)(H/Y). The presently characterized motif in plant PLDβ is also different from (K/R)X_4KX(K/R)(K/R), the one found in phosphoinositide binding cytoskeletal proteins, gelsolin, vilin, cofilin, and profilin (*10*). It is noteworthy that PLDβ-HKD2 only weakly binds PI(4,5)P_2, if at all, although it contains potentially favorable binding sequences, RX_4KXRR and RKXRX_4R, both identified as PBR2 in Figure 1A. The studies reported here provide strong evidence that PBR1 is a structurally independent PI(4,5)P_2 binding domain in PLDs: deletion of PBR1 or mutation of its conserved Lys residues leads to loss of catalytic activity. Planned structural studies will elucidate the physical basis for the specificity and strength of PBR1−PI(4,5)P_2 interactions.

Previously, we have shown that submillimolar levels of Ca^{2+} enhance substrate (PC) binding, but inhibit PI(4,5)P_2 binding, of PLDβ-C2 (*16*). Now we find that a similar level of Ca^{2+} improves PI(4,5)P_2 binding capacities of PLDβ and PLDβ-cat (Figure 4). In addition, deletion of the PBR1 region results in a complete loss of the Ca^{2+} stimulation (Figure 4), suggesting that PBR1 is involved in the Ca^{2+}-stimulated PI(4,5)P_2 binding. However, isolated PBR1 did not display Ca^{2+}-stimulated PI(4,5)P_2 binding (Figure 4). This apparent discrepancy could mean that the accessibility of PBR1 to lipids in intact PLDβ is impeded in the absence of Ca^{2+} and that increases in Ca^{2+} induce conformational changes of PLDβ that permit PBR1 accessible to lipids and thus PI(4,5)P_2 binding. Support for Ca^{2+}-induced conformational changes has been obtained in our previous studies involving Ca^{2+} and PLDβ-C2 interactions (*16*). These results strongly suggest that Ca^{2+} and PI(4,5)P_2 function as synergistic activators. The PI(4,5)P_2 binding site in PLDβ-cat is located near the first catalytic site (HxKxxxD). It is most likely that PI(4,5)P_2-bound PLDβ-cat undergoes a conformational change that leads to better substrate binding and hence enhanced enzyme activity. Consistent with this are the CD spectral changes caused by the addition of PI(4,5)P_2 to PLDβ-HKD1, the deletion mutant that includes only the first catalytic site (Figure 8).

The present investigation suggests a mechanism of activation of PLDβ by PI(4,5)P_2 that is consistent with the model expounded for a number of lipid-activated enzymes (*29*), such as diacylglycerol kinase (*30*), phosphatidylinositol 4-phosphate kinase (*31*), protein kinase C (*32*), and phospholipase C-δ1 (*33*). According to this surface-dependent model, which is applicable to both activation and inhibition (*29*), the lipid activator specifically binds to the enzyme during the interfacial activation step and favors substrate binding by means of conformational changes, thereby triggering the catalysis. On the basis of our previous and present work, we describe an anchorage-scooting model (*33*) for the activation of plant PLDβ by PI(4,5)P_2 in concert with Ca^{2+} (Figure 9). The essence of this model is that the membrane-bound enzyme, upon binding an activator, undergoes a conformational change and grabs its substrate without separating from the surface. Thus, in the resting state of low cytosolic Ca^{2+} levels, PLDβ is anchored to the membrane because of specific interactions between its N-terminal C2 domain and PI(4,5)P_2 (*16*; Figure 4). In response to appropriate stimuli, the concentration of cytosolic Ca^{2+} increases, which leads to Ca^{2+} binding by the C2 domain of PLDβ and concomitant conformational changes (*16*). As a consequence, PI(4,5)P_2 binding by the C2 domain is weakened, but that by PBR1 located near the first catalytic site is strengthened. Another effect is the enhanced PC (substrate) binding by the C2 domain (*16*). Ca^{2+} also binds to the catalytic region of PLDβ[2] and increases its affinity for PI(4,5)P_2. PI(4,5)P_2 binding by PBR1 in the catalytic region induces a conformational change (Figure 8), which facilitates

[2] K. Pappan, L. Zheng, R. Krishnamoorthi, and X. Wang, unpublished results.

substrate binding at the active site (Figure 7). Thus, by this scooting mechanism, PLDβ can reach its substrate without dissociation from the membrane. Also consistent with this is the observation that all PI(4,5)P_2-dependent PLD activities in *Arabidopsis* are associated with the membrane (*23*). The proposed mechanism suggests that changing levels of Ca^{2+} and PI(4,5)P_2 synergistically activate PLDβ in the cell.

REFERENCES

1. Jones, D., Morgan, C., and Cockcroft, S. (1999) *Biochim. Biophys. Acta 1439*, 229−244.
2. Frohman, M. A., Sung, T. C., and Morris, A. J. (1999) *Biochim. Biophys. Acta 1439*, 175−186.
3. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) *Biochem. J. 345*, 401−415.
4. Wang, X. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 52*, 211−231.
5. Munnik, T. (2001) *Trends Plant Sci. 6*, 227−233.
6. Exton, J. H. (1999) *Biochim. Biophys. Acta 1439*, 121−133.
7. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. (1997) *J. Biol. Chem. 272*, 3860−3868.
8. Qin, W., Pappan, K., and Wang, X. (1997) *J. Biol. Chem. 272*, 28267−28273.
9. Wang, C., and Wang, X. (2001) *Plant Physiol. 127*, 1102−1112.
10. Martin, T. F. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 231−264.
11. Jost, M., Simpson, F., Kavran, J. M., Lemmon, M. A., and Schmid, S. L. (1998) *Curr. Biol. 8*, 1399−402.
12. Desrivieres, S., Cooke, F. T., Parker, P. J., and Hall, M. N. (1998) *J. Biol. Chem. 273*, 15787−15793.
13. Janmey, P. A., and Stossel, T. P. (1989) *J. Biol. Chem. 264*, 4825−4831.
14. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) *J. Biol. Chem. 270*, 5130−5135.
15. Pappan, K., Qin, W., Dyer, J. H., Zheng, L., and Wang, X. (1997) *J. Biol. Chem. 272*, 7055−7061.
16. Zheng, L., Krishnamoorthi, R., Zolkiewski, M., and Wang, X (2000) *J. Biol. Chem 275*, 19700−19706.
17. Janmey, P. A., Lamb, J., Allen, P. G., and Matsudaira, P. T. (1992) *J. Biol. Chem. 267*, 11818−11823.
18. Yu, F.-X., Sun, H.-Q., Janmey, P. A., and Yin, H. L. (1992) *J. Biol. Chem. 267*, 14616−14621.
19. Sciorra, V. A., Rudge, S. A., Prestwich, G. D., Frohman, M. A., Engebrecht, J., and Morris, A. J. (1999) *EMBO J. 18*, 5911−5921.
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) *Current Protocols in Molecular Biology*, pp 8.51−8.59, John Wiley, New York.
21. Frangioni, J. V., and Neel, B. G. (1993) *Anal. Biochem. 210*, 179−187.
22. Davletov, B. A., and Südhof, T. C. (1993) *J. Biol. Chem. 268*, 26386-26390.
23. Pappan, K., Zheng, S., and Wang, X. (1997) *J. Biol. Chem. 272*, 7048−7054.
24. Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) *J. Biol. Chem. 249*, 7130−7139.
25. Stuckey, J. A., and Dixon, J. E. (1999) *Nat. Struct. Biol. 6*, 278−284.
26. Xie, Z., Ho, W. T., and Exton, J. H. (2000) *J. Biol. Chem. 275*, 24962−24969.
27. Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001) *Science 291*, 1047−1050.
28. Ford, M. G. J., Pearse, B. M. F., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R., and McMahon, H. T. (2001) *Science 291*, 1051−1055.
29. Carman, G. M., Deems, R. A., and Dennis, E. A. (1995) *J. Biol. Chem. 270*, 18711−18714.
30. Hjelmstad, R. H., and Bell, R. M. (1991) *Biochemistry 30*, 1731−1740.
31. Moritz, A., De Graan, P. N., Gispen, W. H., and Wirtz, K. W. (1992) *J. Biol. Chem. 267*, 7207−7210.
32. Bell, R. M., and Burns, D. J. (1991) *J. Biol. Chem. 266*, 4661−4664.
33. Cifuentes, M. E., Honkanen, L., and Rebecchi, M. J. (1993) *J. Biol. Chem. 268*, 11586−11593.

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