 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)P2]. Here we show that PLDβ is stimulated by different polyphosphoinositides, among which PI(4,5)P2 is most effective. On the basis of amino acid sequence analysis, PI(4,5)P2 binding assay, and protein engineering studies, we have identified in the catalytic region of PLDβ a new PI(4,5)-P3 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)P2. Submillimolar levels of calcium ions, while inhibiting PI(4,5)P2 binding by the C2 domain, enhanced the affinity of PBR1 for that phosphoinositide. Substrate binding by PLDβ was promoted by PI(4,5)P2-bound PBR1. Isolated, recombinant PBR1 bound PI(4,5)P2 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)P2 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)P2 and Ca2++. PLDβ 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), cortactin (12), and α-actinin (13). Recently, PI(4,5)P2 has been proposed to play a critical role in the activation of PLD (1, 2, 6, 7). Arrest of PLD(4,5)P2 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)P2, and replacement of PI(4,5)P2 by other phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), leads to loss of enzymatic activity (15). Similarly, neomycin, a strong PI(4,5)P2 chelator, significantly diminishes PLD activity (15).

PLDβ contains a C1 domain at its N-terminus (PLDβ-C1) 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 C1 domain of PLDβ binds PI(4,5)P2 in the absence of Ca2+ 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)P2. Clusters of basic residues of the known, RxxKxxxxRR and RKxRxxxxR, have been implicated in PI(4,5)P2 binding by gelsolin, villin, and PLCβ (17, 18). Thus, on this basis, two putative PI(4,5)P2 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...
anchorage-scotting 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 PI, PS, and PI(4,5)P_2 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–PLDβK437G/K440G, GST–PLDβK449E/K449G, and GST–PLDβK449E/K449G, 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 EcoRI restriction site at its 5′ end. The PCR-amplified DNA fragment was digested with EcoRI 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 BamHI. 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 EcoRI restriction site was added to the 5′ primer, and a XhoI restriction site was incorporated into the 3′ primer. The PCR product was digested with EcoRI and XhoI 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). PLDβK405–449 is a deletion mutant lacking PBR1. Two conserved basic residues were mutated in PLDβK437G/K440G and two nonconserved basic residues in PLDβK449E/K449G. 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-galactosidase (IPTG) in the case of the GST fusion proteins or by 1 mM
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 PLDâHKD1 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 Ni²⁺-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)P₂ 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 RX₃RX motifs identified for PI(4,5)P₂ 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.

**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 Tris-HCl (pH 7.5), 200 mM NaCl, and either 1 mM EGTA or 100 μM Ca²⁺. Fifty microliters of 20% agarose beads was mixed with an equal volume of PI(4,5)P₂ 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)P₂ bound to the protein was measured by scintillation counts. The background binding was determined from PI(4,5)P₂ 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)P₂, a given GST fusion protein bound to glutathione-agarose was suspended in a buffer containing 50 mM Tris-HCl, 200 mM NaCl, and either 1 mM EGTA or 100 μM Ca²⁺ and incubated with a series of phospholipid vesicles containing PC, PE, and PI(4,5)P₂. The molar ratio of PC was fixed at 5%, and the molar ratios of PE and PI(4,5)P₂ were changed in steps from 95% to 75% and from 0% to 20%, respectively. The lipid binding was determined by measuring the binding of [³H]PC (dipalmitoylglycerol-3-phospho[methyl-³H]choline) to the recombinant fusion protein.
100 μM CaCl₂, 1 mM MgCl₂, 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)P₂, PI(3,5)P₂, PI(5,3)P₂, or PI(3,4,5)P₃. To assay the enzymatic activity in the presence of different amounts of PI(4,5)P₂, lipid vesicles made up of PE, PI(4,5)P₂, and PC were used. The molar ratio of PC was fixed at 5%, and the molar ratios of PE and PI(4,5)P₂ were changed in steps from 95% to 75% and from 0% to 20%, respectively. The hydrolysis of PC was monitored using dipalmitoylglycerol-3-phospho[methyl-³H]choline as a tracer. The amount of [³H]-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 (CNDNB), 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 (A₃₄₀) was monitored and recorded for 2 min using a Hitachi U-2000 spectrophotometer. The unit of GST activity was calculated as ΔA₃₄₀ 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 PE (3,4,5)P₃ 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 ε₂₈₀ 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)P₂ 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)P₂, PI(3,5)P₂, PI(5,3)P₂, or PI(3,4,5)P₃. The results reveal that while PI has almost no effect, the other phosphatidylinositol derivatives show considerable stimulation effects (Figure 3). However, PI(4,5)P₂ is most effective. The decreasing order of activation efficacy of other compounds is PI(3,4,5)P₃ > PI(4,3)P > PI(4,5)P > PI(3,5)P > PI(3,4)P > PI(5,3)P > PI(5)P. PI(4,5)P₃ appears to have a 4-fold effect as that of PI or PE.

Multiple PI(4,5)P₂ Binding Regions of PLDβ with Distinct Binding Properties. To map the regions of PLDβ that interact with PI(4,5)P₂, 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-terminal (PLDβ-C2) and a catalytic region (PLDβ-cat) at its C-terminal, which encompasses two HxKxxxxD motifs (Figure 1A). As suggested by the multiple amino acid sequence alignment 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)P₂ 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)P₂ binding motifs, RxKxxxRR and RKxRxRxxR, occur near the second HxKxxxxD (15) and are denoted as PBR2 for convenience (Figure 1A).

These PLDβ variants were assayed for PI(4,5)P₂ binding (Figure 4). The full-length enzyme and the isolated C2 domain bound PI(4,5)P₂, as observed earlier (16). In addition, PLDβ-cat and PLDβ-HKD1, but not PLDβ-HKD2, appreciably bound PI(4,5)P₂. More important is the finding that PLDβ-PBR1, the isolated PBR1, binds PI(4,5)P₂ nearly to the same extent as does PLDβ-cat. This suggests that PBR1 is an independent domain capable of interacting with PI(4,5)P₂. Ca²⁺ at 0.1 mM level promotes PI(4,5)P₂ 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)P₂ binding of PLDβ-HKD1 and PLDβ-PBR1.

Characterization of the PI(4,5)P₂ 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)P₂ 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)P₂. To examine the phospholipid specificity of PLDβ-PBR1, the binding of [³H]PC, [³H]PI, [¹⁴C]PS, and [³H]PI(4,5)P₂ to PLDβ-PBR1 was examined and the specific amount of each phospholipid...
bound to the protein calculated (Figure 5B). Thus, it is found that PLDβ-PBR1 has the highest efficacy for PI(4,5)P2. It also binds PI, but only with half as much efficacy. While PS binding by PLDβ-PBR1 is low, PC binding is negligible.

**PLDβ-PBD1 Is Essential for PLD Activity**

The evidence that PBR1 alone is sufficient for the specific and saturable binding of PI(4,5)P2 (Figure 5A) suggests that this region may play a critical role in PLD activation by PI(4,5)P2. To verify this hypothesis, a deletion mutant that lacks PBR1, PLDβ405-449, was constructed and expressed as a GST fusion protein. This deletion mutant completely loses PLD activity (Figure 6) and Ca2+ -stimulated binding of PI(4,5)P2 (Figure 4). In the absence of Ca2+, the mutant and wild-type PLD bind similar levels of PI(4,5)P2 (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)P2 activation of PLD, two site-directed mutants, GST-PLDβK437G/K440G and GST-PLDβK446E/K448S, were made, and their PLD activities were assessed in the presence of varying amounts of PI(4,5)P2 (Figure 6). The residues of PLDβK437G/K440G were chosen because the Lys residues are conserved in PLDβ and PLDα, which require PI(4,5)P2 for activity (8). In contrast, the PI(4,5)P2-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 PLDβ, PLDγ, PLDδ, or PLDε (Figure 2). The substitutions of PLDβK446E/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)P2 for activity (9). PLDβ displays a PI(4,5)P2-dependent activity with the optimal value reached at about 10 mol % of PI(4,5)P2 (Figure 6). PLDβK446E/K448S retains about 10% activity of the wild-type enzyme. In contrast, PLDβK446E/K448S, in which two nonconserved lysine residues are replaced, retains essentially unaltered enzyme function.

**PI(4,5)P2 Stimulates PC Binding of the Catalytic Region of PLDβ**

A possible function of PI(4,5)P2 as a cofactor of PLDβ, PLDβK405-449, PLDβK437G/K440G, and PLDβK446E/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)P2, 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 mean ± SE of three experiments. The line drawn through the data points of PLDβ represents the nonlinear least squares fit. Data points corresponding to PLDβK405-449 and PLDβK446E/K448S are connected by lines to guide the eye.
Plant PLD\(\beta\) Activation by PLD(4,5)P\(_2\)

**Figure 7**: PI(4,5)P\(_2\)-dependent PC binding by PLD\(\beta\) and its variants.
(A) PLD(4,5)P\(_2\)-dependent PC binding to different regions of PLD\(\beta\). Glutathione–agarose bead-bound PLD\(\beta\)-cat, PLD\(\beta\)-C2, PLD\(\beta\)-HKD1, or PLD\(\beta\)-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 Pl(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) PLD(4,5)P\(_2\)-stimulated PC binding to PLD\(\beta\)-cat, PLD\(\beta\)-K437GK440G, and PLD\(\beta\)-K437GK440G. PC binding to PLD\(\beta\)-cat, PLD\(\beta\)-K437GK440G, or PLD\(\beta\)-K437GK440G was assayed in the absence or presence of 10 mol \% of Pl(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.

**Figure 8**: CD spectra of PLD\(\beta\)-HKD1 in the absence or presence of PI or Pl(4,5)P\(_2\). Purified 6His-PLD\(\beta\)-HKD1 was suspended in the buffer only or buffer containing either 0.4 mM PI or 0.4 mM Pl(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.

**DISCUSSION**

The present study demonstrates that PLD\(\beta\) prefers Pl(4,5)P\(_2\) over Pl(3,4)P\(_2\) and Pl(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 Pl(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 Pl(4,5)P\(_2\) binding property we have previously characterized (16), we have now identified a novel Pl(4,5)P\(_2\) binding motif, PBR1, in PLD\(\beta\). 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 of the motif different from the RX\(_2\)K motif identified in mammalian and yeast PLDs (19). These phospholipid-binding Arg residues are replaced by Tyr and Thr in plant PLD\(\beta\). Recent structural studies of the epsin NH\(_2\)-terminal homology (ENTH) domain...
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)P₂ (27, 28). However, the PI(4,5)P₂ binding motif found in these proteins is of the type KX₆KX(K/R)(H/Y).

The presently characterized motif in plant PLDα is also different from (K/R)X₄KX(K/R)(K/R), the one found in the C2 domain and induces conformational changes; consequently, PI(4,5)P₂ is released from the C2 domain, which then binds PC. The catalytic region also binds Ca²⁺ and increases its PI(4,5)P₂ 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, PLDα can reach its substrate without dissociation from the membrane.

Figure 9: Anchorage-scooting model of activation of PLDβ by Ca²⁺ and PI(4,5)P₂. 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)P₂. External stimuli elevate the level of cytosolic Ca²⁺, which interacts with the C2 domain and induces conformational changes; consequently, PI(4,5)P₂ is released from the C2 domain, which then binds PC. The catalytic region also binds Ca²⁺ and increases its PI(4,5)P₂ 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.

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)P₂ to PLDβ-HKD1, the deletion mutant that includes only the first catalytic site (Figure 8).
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)P₂-dependent PLD activities in Arabidopsis are associated with the membrane (23). The proposed mechanism suggests that changing levels of Ca²⁺ and PI(4,5)P₂ synergistically activate PLDβ in the cell.

REFERENCES