A Novel Phospholipase D of Arabidopsis That Is Activated by Oleic Acid and Associated with the Plasma Membrane[1]

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Oleate-dependent phospholipase D (PLD; EC 3.1.4.4) has been reported in animal systems, but its molecular nature is unkown. Multiple PLDs have been characterized in plants, but none of the previously cloned *PLD*s exhibits the oleate-activated activity. Here, we describe the biochemical and molecular identification and characterization of an oleate-activated PLD in Arabidopsis. This PLD, designated *PLDδ*, was associated tightly with the plasma membrane, and its level of expression was higher in old leaves, stems, flowers, and roots than in young leaves and siliques. A cDNA encoding the oleate-activated PLD was identified, and catalytically active PLDδ was expressed from its cDNA in *Escherichia coli*. PLDδ was activated by free oleic acid in a dose-dependent manner, with the optimal concentration being 0.5 mM. Other unsaturated fatty acids, linoleic and linolenic acids, were less effective than oleic acid, whereas the saturated fatty acids, stearic and palmitic acids, were totally ineffective. Phosphatidylinositol 4,5-bisphosphate stimulated PLDδ to a lesser extent than oleate. Mutation at arginine (Arg)-611 led to a differential loss of the phosphatidylinositol 4,5-bisphosphate-stimulated activity of PLDδ, indicating that separate sites mediate the oleate regulation of PLDδ. Oleate stimulated PLDδ's binding to phosphatidylcholine. Mutation at Arg-399 resulted in a decrease in oleate binding by PLDδ and a loss of PLDδ activity. However, this mutation bound similar levels of phosphatidylcholine as wild type, suggesting that Arg-399 is not required for PC binding. These results provide the molecular information on oleate-activated PLD and also suggest a mechanism for the oleate stimulation of this enzyme.

Phospholipid hydrolysis occurs in response to various cellular and environmental cues (for review, see Chapman, 1998; Wang 2000). Such hydrolysis can be involved in many cellular processes through roles in generating signal messengers, membrane remodeling, and/or lipid degradation. Phospholipases are key enzymes catalyzing the initial step of lipid hydrolysis. Phospholipase D (PLD; EC 3.1.4.4), which hydrolyzes phospholipids into a water-soluble head group and phosphatidic acid, recently has been linked to various cellular processes in plants. PLD is involved in abscisic acid- and ethylene-promoted senescence (Fan et al., 1997), wound-induced accumulation of unsaturated fatty acids and jasmonic acid (Wang et al., 2000; Zien et al., 2001), stomatal movement (Jacob, et al., 1999), plant water loss (Sang et al., 2001b), seed germination (Ritchie and Gilroy, 1998), and reactive oxygen generation (Sang et al., 2001a). In addition, activation of PLD occurs in various other cellular responses, including those related to pathogen elicitation (van der Luit et al., 2000), ethylene production (Ryu et al., 1997), osmotic stresses (Munnik et al., 2000), and nodulation (Den Hartog et al., 2001).

The diverse cellular functions of PLD indicate that this enzyme is subjected to complex regulation in the cell. PLD is a family of heterogenous enzymes that are grouped into several distinct types based on their biochemical and sequence properties (for review, see Wang, 2000). Three types of PLDs, *PLDα*, *PLDβ*, and *PLDγ*, have been characterized in Arabidopsis. PLDα is the most common plant PLD and is independent of phosphatidylinositol 4,5-bisphosphate (PIP_2) for activity when assayed at millimolar concentrations of Ca^{2+}. In contrast, the recently identified PLDβ and PLDγ are PIP_2 dependent and are most active at micromolar levels of Ca^{2+} (Qin et al., 1997). The PIP_2 requirement is a property shared by cloned PLDs from animal cells, and mammalian PLD activities are divided into two major types based on the requirements for lipids and G-proteins (Liscovitch et al., 2000). One type of PLD is dependent on PIP_2 and stimulated by ADP-ribosylation factor. Two PLD genes, *PLD1* and *PLD2*, have been characterized extensively in mammalian cells, and both gave the PIP_2-dependent and ADP-ribosylation factor-stimulated activities (Hammond et al., 1995, 1997; Kodaki and Yamashita, 1997; Lopez et al., 1998; Liscovitch et al., 2000).

The other type of mammalian PLD is activated by oleate and usually is referred to as oleate-dependent PLD (Liscovitch et al., 2000). The oleate-activated PLD is tightly membrane bound and requires low

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millimolar concentrations of oleate for optimal activity. At the optimal concentrations, oleate inhibits both rat and human PLD1 and PLD2 expressed from their respective cDNAs (Kodaki and Yamashita, 1997; Lopez et al., 1998). In addition, the oleate-activated PLD has been separated physically and biochemically from PLD1 and PLD2 in a number of animal tissues and cell types (Massenburg et al., 1994; Okamura and Yamashita, 1994). One recent report shows that the activity of recombinant human PLD2 can be stimulated equally well by the unsaturated fatty acids oleate, linoleate, and arachidonate. However, this stimulation requires oleate concentrations much lower than those characterized for the oleate-activated PLD, with the optimal oleate concentration being about 20 μM (Kim et al., 1999). Therefore, the common oleate-dependent *PLD* has not been cloned, and molecular nature for the oleate activation is unknown in animal cells (Liscovitch et al., 2000).

In plants, none of the previously cloned *PLD*s has the oleate-activated activity, nor has the occurrence of such PLD activity been reported in any tissue. In this study, we identified and characterized an oleate-activated PLD in Arabidopsis that is associated tightly with the plasma membrane. These results also provide the molecular and mechanistic information on the oleate activation of PLD.

RESULTS

Identification of Oleate-Activated PLD in Arabidopsis

To determine whether the oleate-activated PLD occurs in plants, soluble and microsomal fractions of Arabidopsis were measured for the oleate-activated activity using phosphatidylcholine (PC) vesicles in the presence of 50 μM Ca^{2+}. High levels of oleate-activated PLD activity were detected in the microsomal fraction, but not in the soluble fraction (Fig. 1A). Without oleate, however, no PC hydrolysis occurred in either fraction (Fig. 1A). As a control, PLDα activity was measured and found to be present in both soluble and microsomal fractions (Fig. 1A), and this distribution was consistent with previous observations (Wang et al., 2000). The assay conditions for the oleate-activated PLD activity were distinctly different from those defined for PLDα, PLDβ, and PLDγ. PLDα was active on PC-only vesicles in the presence of millimolar concentrations of Ca^{2+} (Wang et al., 1994; Qin et al., 1997), whereas the hydrolysis of PC by PLDβ and PLDγ required polyphosphoinositides and mixed-lipid vesicles containing mostly phosphatidylethanolamine (Pappan et al., 1997; Qin et al., 1997). To further establish that the oleate-activated PLD activity did not result from the cloned PLDs, PLDα, PLDβ, and PLDγ were expressed in *E. coli* and assayed for oleate-activated PLD activity. Although those bacterially expressed PLDs were highly active under their own respective assay conditions as reported previously (Qin et al., 1997), none of them displayed the oleate-activated PLD activity found in plants (Fig. 1B).

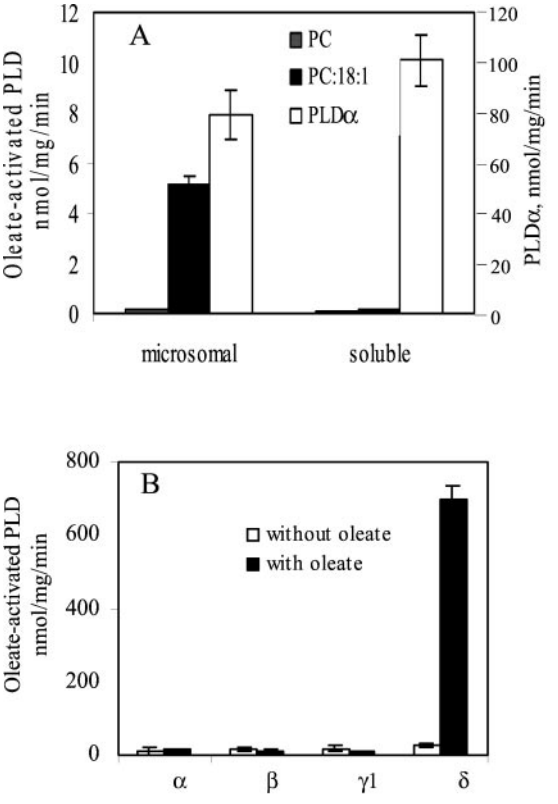

Figure 1. Identification of oleate-activated PLD activity in membrane fractions of Arabidopsis. A, Distribution of oleate-activated PLD activity (PC:18:1, black bar) and the millimolar Ca^{2+}-requiring PLDα activity (white bar) in microsomal and soluble fractions of Arabidopsis leaves. PC (striped bar) was the PLD activity assayed under the same condition as that of the oleate-activated PLD except that oleate was omitted. Soluble fraction was the supernatant after 100,000*g* centrifugation, and microsomal fraction was from the pellet after 100,000*g* centrifugation of the 6,000*g* supernatant. B, Oleate-activated PLD activities of PLDα, β, γ, and δ that are expressed in *Escherichia coli* from the cDNAs of Arabidopsis *PLD*β, γ*1*, and δ and castor bean *PLD*α. The assay conditions for the two types of PLD activities were described in "Materials and Methods." Values are means ± SE of two experiments, each done in triplicates.

Cloning and Sequence Characteristics of *PLDδ*

The inability to detect oleate-activated PLD activity with the expressed PLDα, PLDβ, and PLDγ indicated that the membrane-associated oleate-activated PLD activity resulted from a new, yet uncharacterized *PLD* gene. Searching Arabidopsis databases identified several putative *PLD* expressed sequence tag (EST) clones with distinct sequence differences from the characterized *PLD*α, *PLD*β, and *PLD*γ. Inserts in these clones were sequenced from both ends, and one EST clone, H6C4T7, was found to contain a 5′-translation initiation codon ATG, an in-frame stop codon, and a poly(A) tail, indicating that a full length of the coding region was obtained.

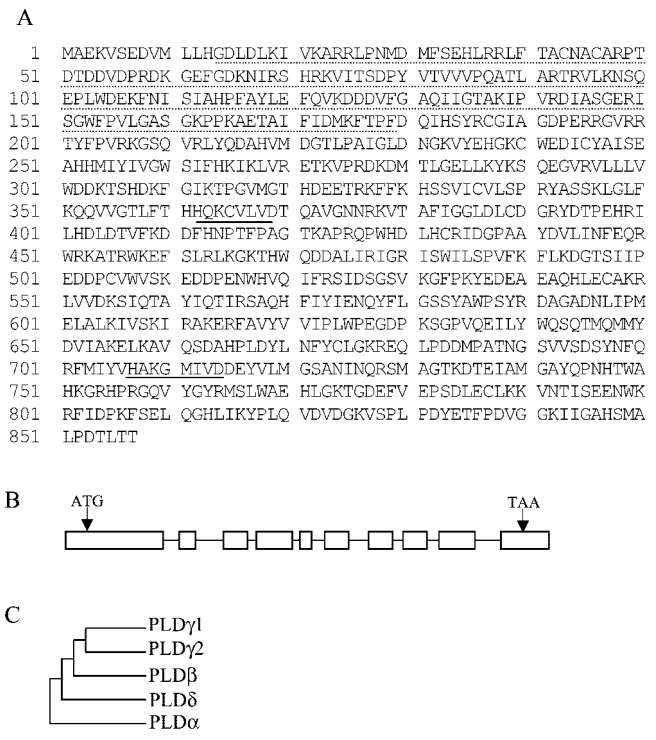

Figure 2. Sequence characteristics of *PLDδ*. A, Amino acid sequence of *PLDδ* (AF322228). Amino acid residues underlined correspond to the C2 domain (dotted line) and the duplicated HKD motifs (black line). B, Gene structure of PLDδ. Boxes mark exons, and lines between the exons represent introns. The translation initiation and stop codons in respective exons are indicated with arrows. C, Comparison of amino acid sequences based on the cloned *PLDs* from Arabidopsis. The dendrogram of the clustering relationship was generated using the PILEUP program from the University of Wisconsin Genetics Computer Group.

The cDNA consisted of 2,787 nucleotides with an open reading frame from nucleotides 16 to 2,589, encompassing 857 amino acids (Fig. 2A; AF322228). The deduced amino acid sequence of this protein was 42%, 53%, and 49% identical to those of Arabidopsis *PLDα*, *β*, and *γ*, respectively (Fig. 2C). The predicted protein had a calculated molecular mass of 97,778 D and a pI of 7.15.

This *PLD*, designated *PLDδ*, had the domain features common to the other cloned plant PLDs. It contained two HxKxxxxD motifs that were conserved in all cloned *PLD*s and form catalytic triads responsible for the hydrolysis of phosphoester bonds (Fig. 2A). It also contained a Ca^{2+}/phospholipid binding (C2) domain near the N terminus, and the acidic residues in the three Ca^{2+}-binding loops are conserved (Zheng et al., 2000). The cDNA sequence matched to the sequence of the first 10 exons of a putative gene (accession no. 7270513) on chromosome 4 revealed from the Arabidopsis genome sequencing projects (Fig. 2B). The sequences of nine of the 10 annotated exons were 100% identical to that of the cDNA except exon 2. Comparison between the *PLDδ* cDNA and genomic sequences showed that the 5′-splicing site for exon 2 was 33 nucleotides upstream of the proposed site in the database. In addition, the putative gene had six additional exons toward the 3′ end that are likely an annotation artifact. The overall gene structure of *PLDδ* was similar to structures of *PLDβ* and *γ* that also have 10 exons, whereas *PLDα* has four exons (Wang, 2000).

Production of Oleate-Activated PLDδ and Specific Antibodies

To verify that the cloned cDNA encodes a PLD, the cDNA was expressed in *E. coli* using the pGEX-4T-1 vector that produces glutathione *S*-transferase (GST) fusion proteins. After induction with isopropyl-1-thio-*β*-galactopyranoside, the bacterial lysates were analyzed for the presence of expressed protein. Coomassie Blue staining of an SDS-PAGE gel detected a distinct protein band of approximately 124 kD in cells containing the GST-PLD vector, but not in the control cells with an empty vector (Fig. 3A). The expressed protein was purified to apparent homogeneity using glutathione-agarose beads. The expres-

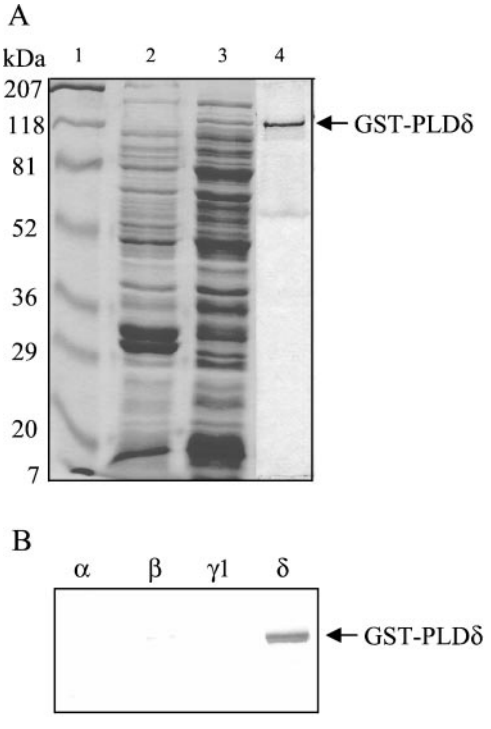

Figure 3. Purification and immunoblotting of PLDδ. A, Coomassie Blue staining of an 8% (w/v) SDS-PAGE gel for PLDδ expressed in *E. coli*. Lane 1, M_r markers; lane 2, bacterial lysate with an empty vector; lane 3, bacterial lysate with the PLDδ expression vector; lane 4, PLDδ purified after glutathione-agarose affinity chromatography. B, Immunoblotting of PLDs with a PLDδ-specific antibody. Bacterially expressed PLD*α*, *β*, *γ*1, and *δ* (20 μg lane^{-1}) were separated by an 8% (w/v) SDS-PAGE. The blot was incubated with a PLDδ antibody (1:1,000 dilution) and then a second antibody conjugated to an alkaline phosphatase. PLD antibody complexes were made visible by staining the alkaline phosphatase activity.

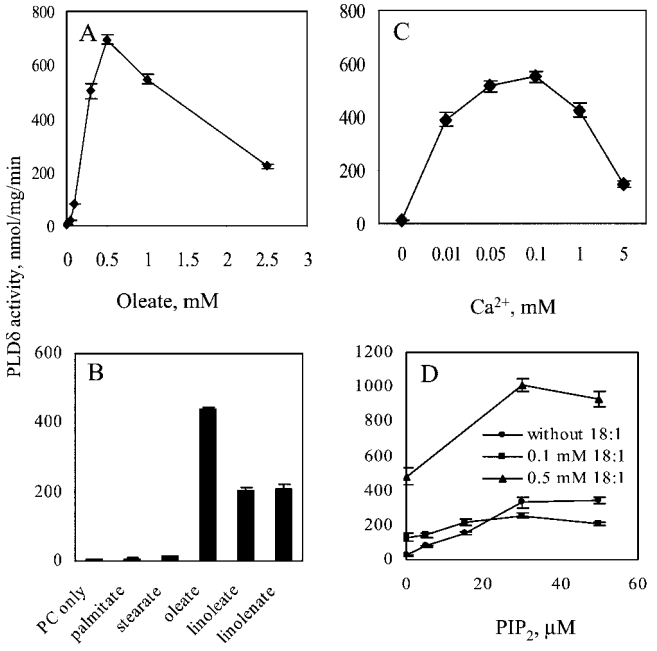

Figure 4. Effects of free fatty acids, PIP_2, and Ca^{2+} on PLDδ activity. A, PLDδ activity as a function of changing oleic acid concentrations. B, PLDδ activity as affected by unsaturated and saturated fatty acids. The same fatty acid concentrations (0.5 mM) were used in these assays. C, Dependence of PLDδ on Ca^{2+} concentrations. Lipid vesicles contained 0.125 mM PC and 0.5 mM oleic acid. D, Effect of PIP_2 on PLDδ activity in the absence of oleic acid and the presence of 0.1 mM and 0.5 mM oleic acid. PLDδ was expressed in *E. coli* and affinity purified. The purified PLDδ and 0.125 mM PC vesicles were used in all the assays. Values were means ± SE of three experiments.

sion of *PLDδ* was confirmed further by immunoblotting using antibody raised against a synthetic peptide corresponding to the 16 C-terminal amino acids of this protein (Fig. 3B). A discrete band of PLDδ was detected by PLDδ antibody, but the same antibody did not react with PLDα, PLDβ, or PLDγ. These results demonstrate that PLDδ is expressed in *E. coli* and that the PLDδ antibody specifically recognizes PLDδ. Under the same condition used for measuring the oleate-activated PLD activity found in plant microsomes, the purified PLDδ displayed oleate-activated PLD activity (Fig. 1B). After removal of the GST fusion, PLDδ yielded the same oleate-activated activity (data not shown).

Catalytic Properties of Oleate-Activated PLDδ

The purified PLDδ showed no activity toward PC in the absence of oleic acid, but became highly active in the presence of oleic acid. The oleate activation of PLDδ was dose dependent, with the maximal stimulation being at 0.5 mM (Fig. 4A). Other unsaturated fatty acids, such as linoleic and linolenic acids, at 0.5 mM increased the PLD activity to a lesser extent (50%) than oleic acid (Fig. 4B). In contrast, the saturated fatty acids, palmitic and stearic acids, had no stimulatory effect. These results indicated that the oleate stimulation was not caused by oleate acting as a nonspecific surfactant in the assay. Ca^{2+} was required for the oleate-activated PLD activity, and the optimal Ca^{2+} concentration was approximately 100 μM (Fig. 4C). With the optimal concentrations of

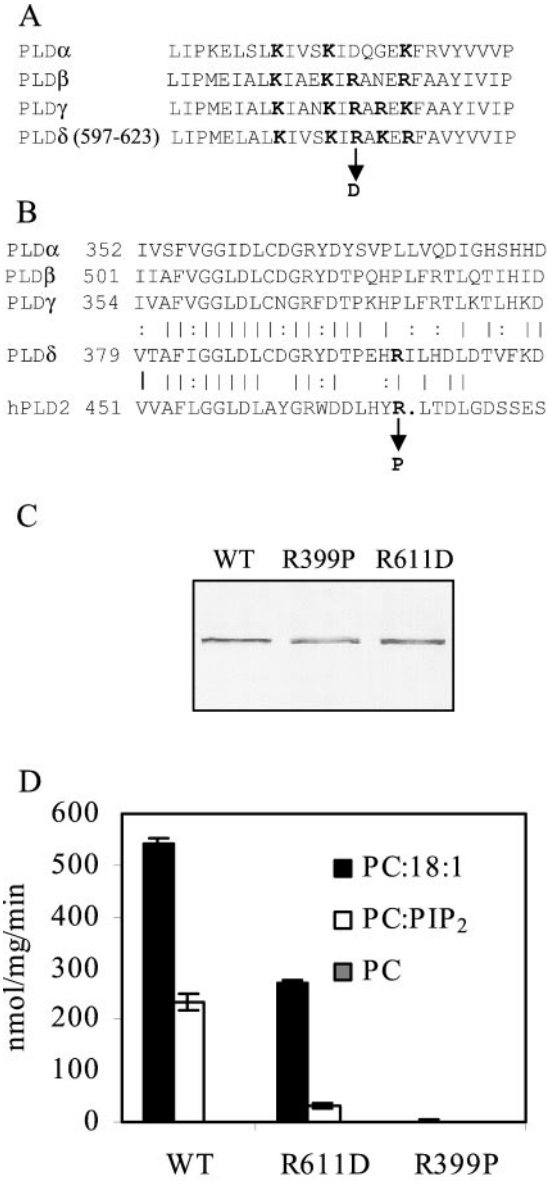

Figure 5. Site-specific mutagenesis of *PLDδ* and the effect on PLDδ activities stimulated by PIP_2 and oleic acid. A, Sequence alignment of a polybasic region in Arabidopsis PLDα, β, γ, and δ and mutation of Arg-611 to Asp, the residue found in PLDα. B, Sequence alignment of a region in Arabidopsis PLDα, β, γ, and δ with human PLD2 and mutation of Arg-399 to Pro, the residue found in PLDβ and γ. C, Immunoblot of wild-type PLDδ and mutated proteins R399P and R611D with PLDδ antibodies. The proteins were expressed in *E. coli* and purified with a reduced glutathione column. The same amounts of proteins were loaded to each lane. D, Oleate- and PIP_2-stimulated PLD activities of wild-type (WT) and mutated PLDδ proteins. PC refers to the PLD activity when no oleate or PIP_2 was included in the assay.

oleate and Ca^{2+}, PLDδ was most active at pH between 6.0 and 7.0 (data not shown).

The effect of PIP_2 on PLDδ activity was examined also because PIP_2 is a required factor for activities of plant PLDβ and PLDγ, mammalian PLD1 and 2, and yeast PLD1. Inclusion of PIP_2 in PC vesicles stimulated PLDδ activity, with the optimal concentration being approximately 30 μM (Fig. 4D). PIP_2 was not as effective as oleic acid, and the optimal stimulation of PLDδ by PIP_2 was about 50% of that by oleic acid. This PIP_2 stimulation of PLDδ was distinctly different from that of previously characterized PIP_2-dependent PLDβ and PLDγ, whose activities required the presence of high proportions of phosphatidyethanolamine in the substrate vesicles (Pappan et al., 1998). When optimal concentrations of oleate (500 μM) and PIP_2 (30 μM) were included in substrate vesicles, oleate and PIP_2 had an additive effect on PLDδ. It is interesting that at a suboptimal concentration of oleate (100 μM), PIP_2 showed only a slight stimulatory effect on PLDδ (Fig. 4D).

Motifs Involved in PIP_2 or Oleate Stimulation of PLDδ

To gain insights into the mechanism for the activation by PIP_2 and oleate, the sequence of PLDδ was compared with those of other PLDs, followed by site-specific mutagenesis and functional analysis. Motifs involved in the lipid regulation of PLDδ can be divided into two major regions. One is the N-terminal C2 domain that has been shown to bind PIP_2 in PLDα and β (Zheng et al., 2000). The other is the catalytic region, which contains the two HKD motifs and lies in the C-terminal two-thirds of the protein (Fig. 2A). The PIP_2-depedent PLDβ contains two polybasic motifs (K/RxxxxK/RxK/RK/R) that have been shown to interact with PIP_2 in other proteins (for review, see Martin, 1998). In contrast, PLDδ does not contain the two motifs, a property shared by PLDα (Qin et al., 1997). The PIP_2-binding motif recently identified in mammalian PLD (Sciorra et al., 1999) is not found in PLDδ. Our analysis of PLDβ has shown that other motifs in the catalytic regions are involved also in PIP_2 binding (L. Zheng and X. Wang, unpublished data). Therefore, a region of PLDδ with polybasic residues that are conserved in the PIP_2-requiring PLDγ and β, but divergent from PLDα (Fig. 5A), was chosen to test its involvement in the requirements for PIP_2 and oleate. The Arg-611 residue was changed to Asp, the residue found in PLDα (Fig. 5A). The mutated protein was expressed and purified in the same way as wild-type PLDδ (Fig. 5C). The R611D mutant lost more than 80% of PIP_2-stimulated activity, but approximately 50% of the oleate-stimulated activity (Fig. 5D). This differential loss suggested that R611 was involved in the PIP_2 regulation and that other sites were required for the oleate stimulation of this PLD. Like wild-type PLDδ, the mutated enzyme showed no PC-hydrolyzing activity in the absence of PIP_2 or oleate (Fig. 5D).

To identify the regions involved in the oleate stimulation of PLDδ, we compared the sequences of PLDδ with other plant PLDs and mammalian PLD2 and looked for the regions of PLDδ that might show amino acid sequence identities to PLD2 more than to other plant PLDs. Such comparison was made because the activity of recombinant human PLD2 could be stimulated by oleate (Kim et al., 1999), whereas the other plant PLDs could not (Fig. 1B). The conserved residues between PLDδ and PLD2 might be involved in the oleate stimulation although the oleate stimulation of PLD2 differed in many aspects from the mammalian common oleate-dependent PLD (Liscovitch et al., 2000). One such region was identified and located approximately 30 amino acid residues after the first HKD motif. In particular, Arg-399 of PLDδ was conserved in all PLD2s, but not in the other plant PLDs (Fig. 5B). To test its function, Arg-399 was mutated to Pro, the residue found in PLDβ and γ (Fig. 5B). The mutated protein was expressed and purified equally well as wild-type PLDδ and the mutant R611D (Fig. 5C). However, unlike R611D, the mutant R399P lost all PLDδ activities (Fig. 5D).

To investigate how the mutation abrogated PLDδ activity, we measured the binding of oleate by PLDδ and the mutants. R399P bound reproducibly less oleate than wild-type PLDδ whereas the mutation at Arg-611 had no affect on the oleate binding (Fig. 6A). The decrease in R399P was approximately 30% in vesicles containing the equal molar PC and oleate. Keeping the PC concentration constant, a decrease in the PC:oleate mol ratio to 1:0.5 slightly lowered the oleate binding in all three proteins, but R399P still bound less oleic acid than wild type and R611D (Fig. 6A). Then, we examined the effect of oleate on the PLDδ's binding to its substrate PC and whether the mutations affected PC binding. Without oleic acid, no PC binding to PLDδ was detected, but inclusion of oleate stimulated greatly the PC binding (Fig. 6B). In contrast, substitution of oleate with stearate produced no stimulation in PC binding, indicating that the stimulation of PC binding is specific to oleate. However, the mutant R399P bound similar levels of PC as wild type (Fig. 6B), suggesting that although Arg-399 is one of the sites involved in the oleate binding, the binding of oleate to Arg-399 is not required for PC binding. The oleate-promoted binding of PC by PLDδ may be one of the forces to associate this enzyme to cellular membranes and its substrates.

Subcellular Distribution and Membrane Association of PLDδ

To determine the membrane association of PLDδ in plant tissues, Arabidopsis leaves were fractionated into soluble and microsomal fractions, which then were immunoblotted with the PLDδ-specific antibody. PLDδ was detected only in the membrane fractions (Fig. 7A). This localization of PLDδ protein is in

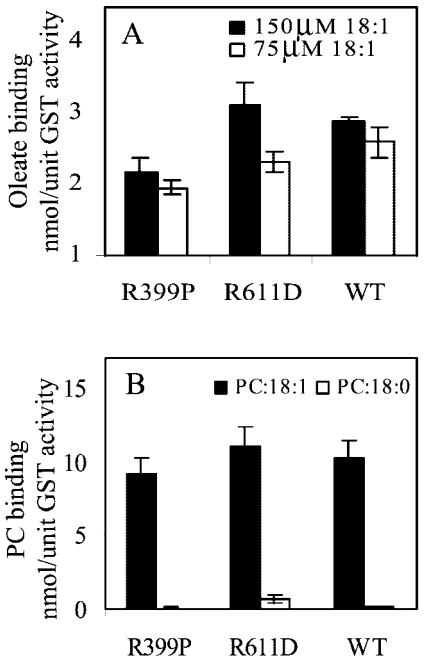

Figure 6. Binding of oleic acid and PC by wild-type and mutated PLDδ proteins. A, Oleate binding. Glutathione-agarose beads bound with PLDδ, R399P, or R611D were mixed with vesicles containing PC and ^{3}H-labeled oleate in 1:1 or 1:0.5 mol ratios at 25°C. The beads were washed with buffer three times, and scintillation counts were determined. B, PC binding in the presence of oleic acid or stearic acid. PC binding was determined using vesicles of ^{3}H-labeled PC and oleic or stearic acid. Background binding of oleate or PC by GST beads was subtracted from the oleate or PC binding values for the GST-PLD fusion. Bound oleate or PC was quantified as nmol per min per unit of GST activity. Values were means ± SE of six measurements.

agreement with the distribution of the oleate-activated PLD activity (Fig. 1A). In contrast, PLDα activity and protein were associated with both microsomal and soluble fractions (Figs. 1A and 7A). To examine the nature of the membrane association, the microsomal membranes were washed with 0.44 M KCl in the presence of EDTA, which should have removed most proteins peripherally associated with membranes. A majority of PLDα was removed from the membrane fraction after the salt treatment (Fig. 7A). In contrast, PLDδ remained associated with membranes, and no PLDδ protein was detected in the salt-solublized fraction (Fig. 7A). Treatment of microsomal membranes with 1% (w/v) Triton X-100 completely solubilized PLDδ. The immunoblotting result was consistent with that of the distribution of the oleate-activated PLD activity in the various membrane fractions (Fig. 7, A and B). This agreement between the immunoblotting and activity assays confirmed that the oleate-activated PLD activity in Arabidopsis membranes resulted from PLDδ. It also indicated that the lack of oleate-activated activity in the soluble fraction was not due to the presence of inhibitory factors in the fraction.

To identify the specific membrane that PLDδ is associated with, subcellular fractions were prepared from fully expanded Arabidopsis leaves, and the identity and purity of each fraction were determined by assaying activities of appropriate marker enzymes and reported previously (Fan et al., 1999). In brief, the plasma membrane fraction showed the highest activity of its marker enzyme, vanadate-sensitive ATPase, and little activity for the other enzymes tested. The intracellular membrane fraction had the highest activity of cytochrome c reductase, a marker

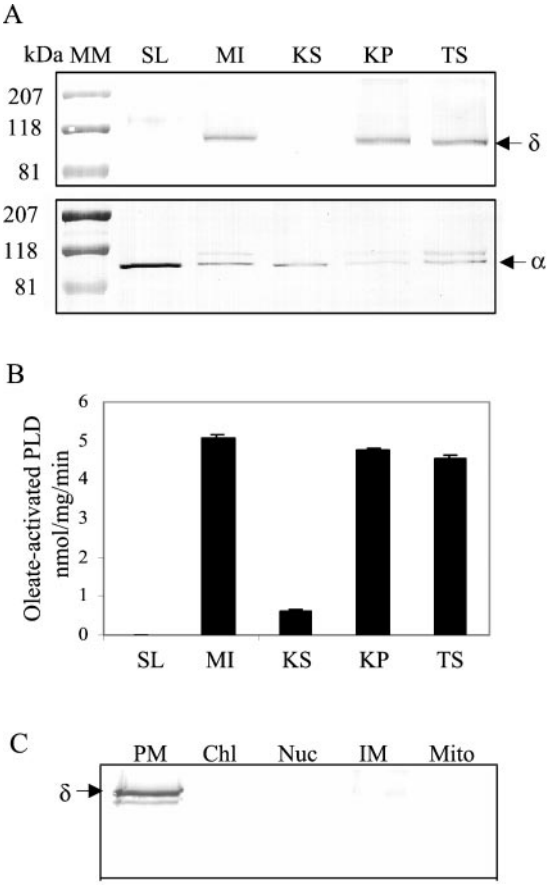

Figure 7. Membrane association and subcellular distribution of PLDδ in Arabidopsis leaves. A, Immunoblotting of PLDδ (upper) and PLDα (lower) in soluble and microsomal fractions. Equal amounts of proteins (10 μg lane^{-1}) were loaded and separated by 8% (w/v) SDS-PAGE. PLDδ was made visible by alkaline phosphatase after blotting with affinity-purified PLDδ and PLDα antibodies, respectively. The lane labels are: MM, M_r markers; SL, soluble proteins (100,000*g* supernatant); MI, microsomal protein (the pellet of 100,000*g* centrifugation of the 6,000*g* supernatant); KS, microsomal proteins solubilized by 0.44 M KCl; KP, microsomal pellet that was not solubilized by KCl; TS, 1% (w/v) Triton X-100-solubilized microsomal proteins. B, Oleate-activated PLD activity in the fractions corresponding to A. C, Immunoblotting of PLDδ in subcellular fractions. The plasma membrane (PM) and intracellular membranes (IM) were isolated by a two-phase partitioning method. Chloroplasts (Chl), mitochondria (Mito), and nuclei (Nuc) were isolated by Percoll gradient centrifugation. Proteins were separated by 8% (w/v) SDS-PAGE and made visible by alkaline phosphatase.

enzyme of ER, indicating enrichment of ER in this fraction. The mitochondrial fraction displayed high activities of the mitochondrial marker enzymes, cytochrome c oxidase and fumarase. Chloroplasts and nuclei showed very few enzymatic activities that are characteristic of other organelles. Immunoblotting of those fractions revealed that PLDδ was associated with the plasma membrane, and no discrete PLDδ protein band was detected in intracellular membranes, mitochondria, chloroplasts, or nuclei (Fig. 7C). In contrast, using the same subcellular fractions, PLDα was detected in the plasma membrane, intracellular membrane, and mitochondrial fractions as shown in previous studies (Fan et al., 1999), whereas most PLDγ was associated with intracellular membranes and lesser amounts with in the plasma membrane, nuclei, and mitochondria (Fan et al., 1999).

Tissue Expression of *PLDδ*

PLDδ protein was detectable in all tissues examined, and its amounts relative to the total proteins in roots, flowers, and stems were higher than those in leaves and siliques (Fig. 8A). The amount of PLDδ was greater in senescent than young leaves. Oleate-activated PLD activity in these tissues gave the same pattern of distribution as the PLDδ protein (data not shown). The RNA blotting with a *PLDδ* gene-specific probe showed that the mRNA levels of *PLDδ* were much higher in old leaves, stems, flowers, and roots than in young leaves and siliques (Fig. 8, B and C).

DISCUSSION

The present study has identified a new type of plant PLD activity that is activated by oleic acid. This activity is different from the previously characterized PLD activities, the common one stimulated by high-millimolar Ca^{2+} concentrations and the recently identified one requiring PIP_2 (Qin et al., 1997). In addition, the oleate-activated PLD is associated with a membrane fraction, whereas the other two types of PLDs occur in both soluble and membrane fractions (Fan et al., 1999; Wang et al., 2000). The association of oleate-activated PLD with the plasma membrane is also in contrast to the intracellular distribution of the other PLDs; both common and PIP_2-requring PLDs were found in intracellular membrane fractions (Fan et al., 1999). Furthermore, the expression of cloned PLDs has confirmed that the newly identified PLDδ has the oleate-activated PLD activity, whereas PLDα, β, and γ have no such activity. These results demonstrate that only PLDδ possesses the oleate-activated PLD activity in plants. Occurrence of the distinctly different types of PLD activities indicates that multiple PLDs in the cell are subjected to different mechanisms of regulation.

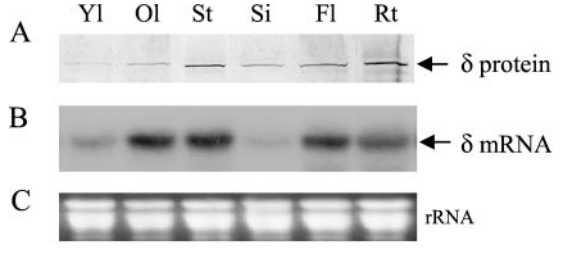

Figure 8. Tissue distribution and expression of *PLDδ* in Arabidopsis. A, Immunoblotting of PLDδ extracted from various tissues from 6-week-old Arabidopsis plants. Equal amounts (10 μg lane^{-1}) of total protein extracts were loaded and separated by 8% (w/v) SDS-PAGE. PLDδ was made visible by alkaline phosphatase after blotting with affinity-purified PLDδ antibody. B, Autoradiography of PLDδ transcript on an RNA gel blot. Total RNA (10 μg lane^{-1}) from different tissues was used. C, rRNA used to indicate the equal loading. Yl, Young leaf (not fully expanded, top leaves on plants); Ol, old leaf (bottom leaves of flowering plants with yellowing at the tip); St, stem; Si, silique; Fl, flower; Rt, root.

Results of this study also provide insights into the mechanism by which oleate stimulates PLDδ activity. One effect of oleate is to promote the binding of PLDδ to PC, and this effect is specific because stearate has no stimulatory effect. In addition, separate regions on PLDδ are involved potentially in the PLDδ stimulation by oleate and PIP_2 because mutation at Arg-611 resulted in a differential loss of PIP_2-stimulated PLDδ activity. Furthermore, this study has indicated that Arg-399 is critical to the oleate regulation of PLD; mutation of this residue decreased oleate binding by PLDδ and resulted in an inactive PLDδ. The positively charged Arg could serve as a binding site for the negatively charged oleate. However, the mutation at Arg-399 did not abolish completely oleate binding, suggesting that the other amino acid residues in this and/or other region(s) are involved also in the oleate binding. The presence of multiple oleate-interacting sites is also consistent with the PC-binding result, which showed that oleate promoted the binding of PLDδ to PC, but the mutation at Arg-399 did not affect the oleate-stimulated PC binding. It is possible that the oleate-mediated PC binding occurs predominantly through the C2 domain at the near N terminus of PLDδ. The C2 domain of PLDδ is similar to that of PLDβ that has been shown to act as a phospholipid binding module (Zheng et al., 2000). The PC binding to the C2 domain could be involved in the enzyme's association with membranes, whereas the interaction of oleate at Arg-399 might modulate the enzymatic hydrolysis of PC. However, it is unlikely that Arg-399 is involved directly in PLD catalysis because this residue is not conserved in the other plant PLD isoforms (Fig. 5B). In addition to direct binding, part of the oleate effect might occur through producing a suitable surrounding for optimal recognition of PLDδ for its substrates. Further studies are necessary to define the mechanism by which PLDδ interacts with oleate and the structural basis for the oleate activation.

The oleate-activated PLD is associated tightly with the plasma membranes, and this location places the oleate-activated PLD at a vital junction in signal transduction and cell regulation. Oleic acid and other unsaturated fatty acids are thought to serve as mediators in signal transduction. In addition to activating PLD, oleic acid regulates the activity of various proteins, including protein kinase C (Shinomura et al., 1991; Khan et al., 1992), Ca^{2+}-calmodulin-dependent kinase (Piomelli et al., 1989), secretory chloride channels (Hwang et al., 1990), guanylate cyclase (Gerzer et al., 1986), and phospholipase Cγ1 (Hwang et al., 1996). In human T cell leukemia Jurkat T cells, oleate-activated PLD increased drastically during apoptosis, and this increase was associated with elevated levels of unesterified fatty acids in the cell (Kasai et al., 1998). These changes are consistent with a notion that induction of apoptosis increases cellular free fatty acids, which then activate oleate-activated PLD. This suggests a role of PLD in the survival and apoptosis of mammalian cells (Nakashima and Nozawa, 1999).

In plants, most current studies on the roles of fatty acids in cell signaling have been centered around polyunsaturated fatty acids, particularly linolenic acid (Farmer et al., 1998). The identification of PLDδ as a target of oleate indicates the significance of oleate in cell regulation. It is known that free unsaturated fatty acids, including oleate, increase during defense responses, such as pathogen elicitation (Kirsch et al., 1997) and wounding (Ryu and Wang, 1998). In particular, sharp and transient increases in oleate were observed in parsley cells treated with fungal peptide elicitors, whereas the level of stearate remained unchanged. In contrast, these cells showed a decrease in free linoleate, and a slower but steady increase in linolenate (Kirsch et al., 1997). The present studies have shown that the protein and mRNA levels of oleate-activated PLDδ are higher in old than young leaves. Increases in free fatty acids are characteristic of leaf senescence (Hong et al., 2000). These raise interesting questions of the role of PLDδ in leaf development and senescence. It would be of interest in future studies to examine whether the oleate-activated PLD plays a role in cell death associated with senescence and defense responses. Therefore, results of this study will facilitate understanding not only of the cellular regulation and functions of the PLD family, but also of the roles of oleate in cell regulation.

Another Arabidopsis PLDδ cDNA has been reported recently (Katagiri et al., 2001), which differs from this characterized cDNA by having 33 more nucleotides and thus encodes a protein of 868, instead of 857, amino acids. It is possible that the same *PLDδ* gene gives rise to two different transcripts through alternative splicing of the exon 2. Therefore, we propose to designate the 868-amino acid form PLDδa and the 857 one PLDδb to distinguish the two PLDδ proteins and transcripts. Whether PLDδa has the same biochemical properties as those determined for PLDδb awaits further investigation. Gene expression results suggest that *PLDδ* may play a role in PA accumulation in plant response to dehydration (Katagiri et al., 2001).

MATERIALS AND METHODS

Subcellular Fractionation and Identification

Arabidopsis plants (ecotype Columbia) were grown in growth chambers (Wang et al., 2000). Fully expanded leaves were homogenized and centrifuged at 6,000*g* for 10 min at 4°C to remove tissue debris as described previously (Fan et al., 1999). The supernatant was centrifuged at 100,000*g* for 60 min at 4°C to obtain the soluble and microsomal fractions. To determine the type of membrane association, the microsomal pellet was resuspended in a solution (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol) containing 0.44 M KCl. The resuspension was centrifuged at 100,000*g* for 60 min at 4°C, and the resulting supernatants contained proteins dissociated from membranes by the salt wash. The pellet from the 0.44 M KCl wash was solubilized with 1% (w/v) Triton X-100 and centrifuged again at 100,000*g* for 60 min at 4°C.

Subcellular membrane fractionation and marker enzyme assays were performed as described previously (Fan et al., 1999). In brief, to separate the plasma membrane from intracellular membranes, total membranes were separated using an aqueous polymer two-phase system consisting of 6.4% (w/w) dextran T500 and 6.4% (w/v) PEG3350. Chloroplasts, mitochondria, and nuclei were isolated using discontinuous Percoll gradients according to established procedures. Purity and identity of the various fractions were determined by measuring marker enzymes. The markers for the plasma membrane and endoplasmic reticulum were vanadate-sensitive ATPase and cytochrome c reductase, respectively, whereas cytochrome c oxidase and fumarase were used as the mitochondrial marker enzymes. Identities of the chloroplast and nuclear fractions were confirmed by microscopic observation, and their purity was judged by the levels of the activities of marker enzymes characteristic of other organelles and membranes.

PLD Activity Assays

Oleate-activated PLD activity was determined based on a published assay with some modification (Banno et al., 1997). In brief, a typical reaction contained 0.15 mM egg yolk PC mixed with dipalmitoylglycerol-3-phospho-[*methyl*-^{3}H]choline (0.02 μCi, 84 Ci mmol^{-1}), 0.6 mM oleic acid, and an assay buffer consisting of 100 mM MES (4-morpholineethanesulfonic acid), pH 7.0, 2 mM $MgCl_2$, 80 mM KCl, and 100 μM $CaCl_2$. To prepare the reaction mixture, oleic acid and PC in chloroform were mixed and dried under a stream of N_2, and the lipid was emulsified in the assay buffer by sonication at room temperature. The reaction was initiated by addition of protein and incubated at

30°C for 30 min in a water bath with shaking. The reaction was stopped by adding 1 mL of chloroform:methanol (2:1, v/v) and 100 μL of 2 M KCl. After vortexing and centrifugation at 12,000*g* for 5 min, a 300-μL aliquot of the aqueous phase was mixed with 3 mL of scintillation fluid, and the release of ^{3}H choline was measured by scintillation counting. The conditions for assaying the PIP_2-stimulated PLD activity were the same as those for the oleate-stimulated PLD except that lipid vesicles contained 150 μM PC (0.02 μCi ^{3}H-PC reaction^{-1}) and 30 μM 77 PIP_2 or different concentrations of PIP_2 as indicated. The assay of PLDα activity in the presence of 50 mM Ca^{2+} and PC-only vesicles was performed according to a method described previously (Wang et al., 1994).

Sequencing and Site-Directed Mutagenesis of PLD cDNAs

EST clones for putative *PLD*s were identified by searching databases against known *PLD* sequences. These clones were obtained from the Arabidopsis Resource Center (Ohio State University, Columbus). Inserts in these clones were sequenced from both ends, and one cDNA clone, H6C4T7, was found to be a full-length cDNA of a putative Arabidopsis *PLD*, designed *PLD*δ. The insert was sequenced fully from both strands.

Mutation in *PLD*δ was generated via two rounds of PCR amplification with the wild-type *PLD*δ cDNA as a template. The first round PCR for the mutation R611D (Arg-611 to Asp) produced two fragments. One was amplified using a forward primer 5′-ACGCGTCGACTCATGGCGGA G A AAGTATC-3′ (primer I, with added *Sal*I site) and a reverse prime 5′-CTTAGC**ATC**GATTTTGCTAACAATCTTTAGTGCCA-3′, and the other used a forward prime 5′-GTTAGCAAAATC**GAT**GCTAAGGAAAGATTTGCCGT-3′ and a reverse prime 5′-ACGCGTCGACTTACGTGGTTAAAGTGTCAG-3′ (primer II, with added *Sal*I site). The two fragments had a 21-bp overlap at which the mutation (shown in bold in the primers) was introduced. These fragments were combined to serve as templates and primers in the first cycle of the second round PCR, allowing the overlap to extend. The primers I and II were added then as flanking primers to amplify the full-length, mutated product. In a similar manner, the mutation R399P (Arg-399 to Pro) was achieved using the prime I and a prime 5′-ATCGTGGAGTAT**CGG**ATGCTCAGGTGTGTCA-TAAC-3′, and a prime 5′-GAGCAT**CCG**ATACTCCACGATCTTGACACGTATT-3′ and the prime II for the first round PCR amplification. The final mutated product was completed using the primes I and II. The mutations were verified by sequencing.

Expression of *PLD*δ and Its Mutants in *Escherichia coli*

cDNA fragments (2.6 kb) of wild type and mutated *PLD*δ were amplified by PCR using the primers I and II and digested with *Sal*I. They were ligated into the pGEX-4T-1 vector (Amersham-Pharmacia Biotech, Piscataway, NJ) that produced a GST fusion at the N terminus. The recombinant plasmids were transformed into *E. coli* JM 109 and then into BL21 (Promega, Madison, WI). All cell cultures were grown in Luria-Bertani medium with 50 mg L^{-1} of ampicillin. The transformed cells were grown at 37°C to an absorbance of approximately 1.0 at 600 nm. Five milliliters of the cells was transferred into a 1-L flask with 200 mL of Luria-Bertani medium containing 0.1 mM isopropyl-1-thio-β-galactopyranoside and incubated overnight at room temperature. Then the cells were harvested and lysed by sonication in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, and 1 mM EDTA. Cell debris was removed by centrifugation at 10,000*g* for 5 min. The supernatant with GST fusion protein was incubated then with swollen glutathione Sepharose 4B beads prewashed with phosphate-buffered saline buffer containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3) with gentle agitation at room temperature for 0.5 to 1 h. Proteins bound to the beads were eluted with a buffer of 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. The protein concentration was estimated by using a dye-binding protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The expression and purification of plant PLDα, β, and γ1 in *E. coli* were described previously (Qin et al., 1997).

Lipid-Binding Assays

To prepare lipid-binding vesicles, PC from egg yolk was mixed with oleate or stearate in chloroform and dried under a stream of N_2. The mixture was emulsified by sonication in a binding buffer (100 mM MES, pH 7.0, 2 mM $MgCl_2$, and 80 mM KCl) at room temperature. Dipalmitoylglycerol-3-phospho[*methyl*-^{3}H]choline (0.02 μCi, 84 Ci mmol^{-1}) and oleic acid[9,10]-^{3}H(N)] (0.05 μCi, 15 Ci mmol^{-1}) were used to detect PC and 18:1 binding, respectively. GST fusion proteins bound to glutathione beads were incubated in 100 μL of the binding buffer containing 0.15 mM PC and 0.15 mM oleate or stearate for 20 min at room temperature with gentle agitation. The binding mixture was centrifuged at 500*g* for 2 min to pellet the glutathione beads. Unbound lipids were removed by washing the beads three times with 1 mL of the binding buffer. Lipids bound to GST-PLD beads were quantified by scintillation counting. Oleate or PC bound to GST beads were used to determine background binding, which was subtracted from the oleate or PC-binding values for the GST-PLD fusion. Bound oleate or PC was expressed as nmol per unit of GST activity.

Generation of PLD-Specific Antibody, SDS-PAGE, and Immunoblotting

A peptide was synthesized that consisted of a Cys and 16 other amino acids corresponding to the C terminus of PLDδ. The peptide was conjugated to keyhole limpet hemocyanin and used as an antigen to raise antibodies in rabbits (Pappan et al., 1997). For SDS-PAGE analysis, protein extracts were separated by an 8% (w/v) gel and transferred onto polyvinylidene difluoride filters. The membranes were blotted with PLDδ antibody, followed by

incubation with a second antibody conjugated to alkaline phosphatase as described previously (Fan et al., 1999).

RNA Isolation and Blotting

Total RNA was isolated from different tissues of Arabidopsis plants with a cetyltrimethylammonium bromide extraction method (Wang et al., 2000). Equal amounts of total RNA (10 μg) were separated by 1% (w/v) formaldehyde agarose denaturing gel electrophoresis and transferred to nylon membranes. A *PLD*δ-specific probe was labeled with [α-^{32}P]dCTP by random priming. The hybridization, washing, and visualization were performed as described previously (Wang et al., 1999).

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