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Arabidopsis Phospholipase Dα1 Interacts with the Heterotrimeric G-protein α-Subunit through a Motif Analogous to the DRY Motif in G-protein-coupled Receptors*

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Phospholipase D (PLD) and heterotrimeric G-protein both play important, diverse roles in cellular regulation and signal transduction. Here we have determined the physical interaction between plant PLD and the only canonical α-subunit (Gα) of the G-protein in *Arabidopsis thaliana* and the molecular basis for the interaction. PLDα1 expressed in either *Escherichia coli* or *Arabidopsis* was co-precipitated with Gα. PLDα1 contains a sequence motif analogous to the Gα-interacting DRY motif normally conserved in G-protein-coupled receptors. Mutation of the central Lys residue PLD_{K564A} of this motif abolished the PLDα1-Gα binding, whereas mutation of the two flanking residues PLD_{E563A} and PLD_{F565A} decreased the binding. Addition of Gα to PLDα1 inhibited PLDα1 activity, whereas the PLD_{K564A} mutation that disrupted the Gα-PLDα1 binding abolished the inhibition. GTP relieved the Gα inhibition of PLDα1 activity and also inhibited the binding between PLDα1 and Gα. Meanwhile, the PLDα1-Gα interaction stimulated the intrinsic GTPase activity of Gα. Therefore, these results have demonstrated the direct binding between Gα and PLDα1, identified the DRY motif on PLDα1 as the site for the interaction, and indicated that the interaction modulates reciprocally the activities of PLDα1 and Gα.

Phospholipase D (PLD),[1] which hydrolyzes phospholipids to phosphatidic acid and a head group, plays diverse roles in cellular metabolism and regulation. Plant PLD comprises a family of enzymes with different regulatory properties (1). Several *Arabidopsis* PLDs have been shown to display different requirements for Ca^{2+}, polyphosphoinositides, and free fatty acids as well as varied substrate selectivity. *Arabidopsis* has at least 12 PLDs, of which PLDα1 is most prevalent and responsible for the common plant PLD activity (1, 2). PLDα1 produces a majority of the phosphatic acid under several stress conditions, such as freezing and wounding (3, 4). Suppression of PLDα1 delayed abscisic acid (ABA)-promoted senescence (5), decreased wound-induced accumulation of jasmonic acid (4) and reactive oxygen generation (6), and increased freezing tolerance and water loss (3, 7). These results show that the common plant PLD has multifaceted functions, including roles in metabolism and cell signaling, dependent on the nature and severity of the stress conditions.

The Gα subunit of heterotrimeric G-proteins plays an important role in signal transduction. In animal systems, Gα interacts with the upstream transmembrane G-protein-coupled receptors (GPCRs) and with the β-subunit. The binding of a ligand to a cognate receptor promotes the exchange of GDP for GTP on Gα, and the GTP-bound Gα activates the downstream effector proteins (8). In addition, Gα may interact with non-receptor proteins to mediate signaling (9). Mammalian cells contain a number of Gαs that mediate many distinctive cellular functions (8). In contrast, the number of Gαs is very limited in plants; *Arabidopsis* has only a single canonical Gα gene, *GPA1* (10, 11). *Arabidopsis* Gα-null mutants are impaired in several cellular processes, including cell division, certain ABA signaling steps in guard cells, and germination behaviors in response to glucose and hormones (12–14). These changes indicate that Gα is involved in multiple cellular processes in plants and thus may interact with multiple effector proteins for different functions. The only protein reported to interact with Gα in *Arabidopsis* is the cupin-domain protein AtPirin, which interacts with a CCAAT box-binding transcriptional factor (15). The mechanism by which plant Gα interacts with its targets and the function of the interaction are unknown.

A role of G-proteins in regulating plant PLD has been proposed recently. Most of the studies have involved the use of potential G-protein activators and inhibitors, such as toxins, nonhydrolyzable guanine nucleotide analogues, and alcohols (16, 17). In barley aleurone cells, a PLDα-like activity has been suggested to be associated with a G-protein on the plasma membrane to mediate ABA signaling (17). Co-incubation of bacterially expressed tobacco PLDα with Gα decreased the PLDα activity (18). It has also been shown that ablation of either Gα or PLDα1 in *Arabidopsis* affects ABA-mediated stomatal movement and increases plant water loss (7, 12). These observations raise intriguing questions of whether PLDα1 and Gα directly interact with one another and, if so, what the molecular bases are for the interaction. Here we show that PLDα1 binds to Gα through a motif analogous to the DRY motif present in many GPCRs and that the binding modulates the activity of PLDα1 and Gα.

EXPERIMENTAL PROCEDURES

Expression and Purification of Active Plant PLDα in Escherichia coli—Arabidopsis PLDα1 cDNA was cloned previously in pBlue SK (19). The 2.4-kb PLDα1 cDNA was amplified by polymerase chain reaction and cloned into the pGEM T-easy vector. The forward and reverse primers were 5′-GC**GGATCC**ATGGCGCAGCATCTGTTGCACG and

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[1] The abbreviations used are: PLD, phospholipase D; ABA, abscisic acid; GPCR, G-protein-coupled receptor; Gα, α-subunit of heterotrimeric G-protein; BSA, bovine serum albumin; GST, glutathione *S*-transferase; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; GDPβS, guanyl-5′-yl thiophosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GAP, GTPase-activating protein.

5′-CG**GAGCTC**TTAGGTTGTAAGGATTGGAGGC, respectively, and the bold letters mark the inserted BamHI and SacI sites. The PLDα1 cDNA insert was digested with BamHI and SacI and ligated into the pET28(+)a vector to produce PLDα1 with 6 histidine residues fused at the N terminus. The recombinant plasmid was transformed into *E. coli* BL21(DE3). Expression of PLDα1 was induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature for 12 h. After induction, bacteria were precipitated and lysed by sonication in phosphate-buffered saline plus 2 mM phenylmethanesulfonyl fluoride. Bacterial lysate was centrifuged at 12,000 $\times$ *g* for 10 min. The resulting supernatant was incubated with Ni-affinity agarose beads for 1 h. The beads were pelleted by centrifugation at 500 $\times$ *g* at 4 °C and washed three times with a washing buffer containing 20 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole at pH 8.0. PLDα1 bound to the resin was eluted with an elution buffer with 1 M imidazole, Tris-HCl, and 0.5 M NaCl. In some cases, PLDα1 bound to resins was used directly for assaying PLDα1 activity. Concentration of the purified PLDα1 was measured by the Bradford method using a Bio-Rad kit with bovine serum albumin (BSA) as a standard. The protein was stored in 20% glycerol at −20 °C until use.

Expression and Purification of Active Plant Gα in E. coli—An *Arabidopsis* Gα cDNA clone was generously provided by Dr. Hong Ma (Pennsylvania State University, University Park, PA) (20). The 1.3-kb Gα cDNA was amplified by PCR and cloned into the pGEM T-easy vector. The forward and reverse primers were 5′-**GAATTC**ATGGGCTTACTCTGCAGTAGAA and 5′-**CTCGAG**TCATAAAAGGCCAGCCTCCAGTA, respectively, and the bold letters mark the inserted EcoRI and XhoI sites. The cDNA insert was digested with XhoI and EcoRI and ligated into pGEX-4T to produce Gα with glutathione *S*-transferase (GST) fused at the N terminus. Junction of the GST-GPA1 fusion and full-length cDNA of Gα was confirmed by sequencing. The recombinant plasmid was transformed into *E. coli* BL21(DE3) to express the GST-fused Gα according to the procedure described previously (21).

Site-directed Mutagenesis of the DRY Motif in PLDα1—Mutagenesis of the three codons in the putative DRY motif of PLDα1 was performed using the QuikChange XL site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The three complementary reverse primers for mutating E563A, K564A, and F565A were as follows: 5′-GATTGAGAAAGGA**GCG**AAGTTCAGGGTCTATGTTGTGG, 5′-GATTGAGAAAGGAGAG**GCG**TTCAGGGTCTATGTTGTGG, and 5′-GAGAAAGGAGAGAAG**GCC**AGGGTCTATGTTGTGG, respectively. The wild-type PLDα1 cDNA in pET28(+)a served as the PCR template. The mutant clones were verified by DNA sequencing and were then transformed into BL21(DE3) for protein expression. Expression and purification of the mutated PLDα1 proteins in *E. coli* were performed with the same procedure as that for the original PLDα1.

Protein Extraction from Arabidopsis—Fully expanded leaves of *Arabidopsis thaliana* (Columbia) plants were frozen with liquid N_2 and homogenized with a buffer containing 50 mM Tris-HCl (pH 7.5), 80 mM KCl, 2 mM EDTA, 5 mM dithiothreitol, and 2 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 10,000 $\times$ *g* at 4 °C for 15 min, and the resultant supernatant was used as protein extracts for co-precipitation. Protein concentration in the supernatant was measured by the Bradford method using a Bio-Rad kit with BSA as a standard.

PLDα Pulldown by GST-Gα-Agarose Beads and Immunoblotting—Purified GST-Gα-agarose beads were incubated with plant protein extracts; bacterially expressed PLDα1; or PLDα1 mutants in a co-precipitation buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, and protease inhibitors (5 μg each of aprotinin, leupeptin, and antipain) in a total volume of 150 μl. After incubation at 4 °C for 3 h, beads were pelleted by centrifugation at 500 $\times$ *g* and washed three times with the co-precipitation buffer containing 0.01% Triton X-100 at 4 °C. Pulldown beads were assayed for PLDα1 activity and/or were subjected to 10% SDS-PAGE, followed by immunoblotting with antibodies specific to PLDα1. PLDα1 polyclonal antibodies were raised in rabbit against the 13 C-terminal amino acid residues as described previously (5). PLD bands on blots were visualized by staining alkaline phosphatase activity conjugated to a second antibody (goat against rabbit immunoglobulin). The procedures for SDS-PAGE, protein blotting, and band detection were described previously (5).

PLDα1 Activity Assays—PLD activity was assayed by using 1,2-dipalmitoyl-3-phosphatidyl-[*methyl*-^{3}H]choline as a substrate. Two different methods were used. One is the common plant PLD activity assay

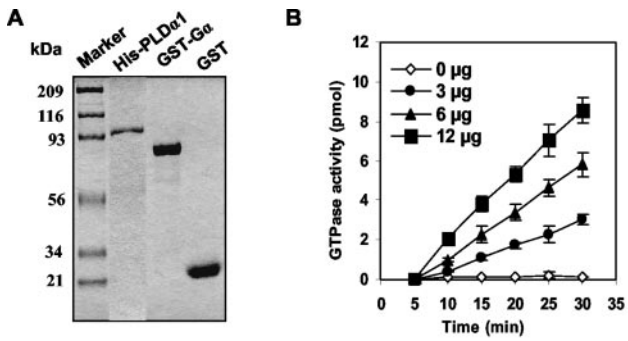

FIG. 1. **Expression and purification of *Arabidopsis* Gα and PLDα1 in *E. coli*.** *A*, purified GST-Gα, His-PLDα1, and GST resolved by a 10% SDS-PAGE gel and stained with Coomassie Blue. GST and His tag were fused to the N terminus of *Arabidopsis* Gα and PLDα1, respectively. *B*, GTPase activity of expressed Gα. Hydrolysis of GTP was assayed by measuring P_i release as a function of time and amounts of purified GST-Gα.

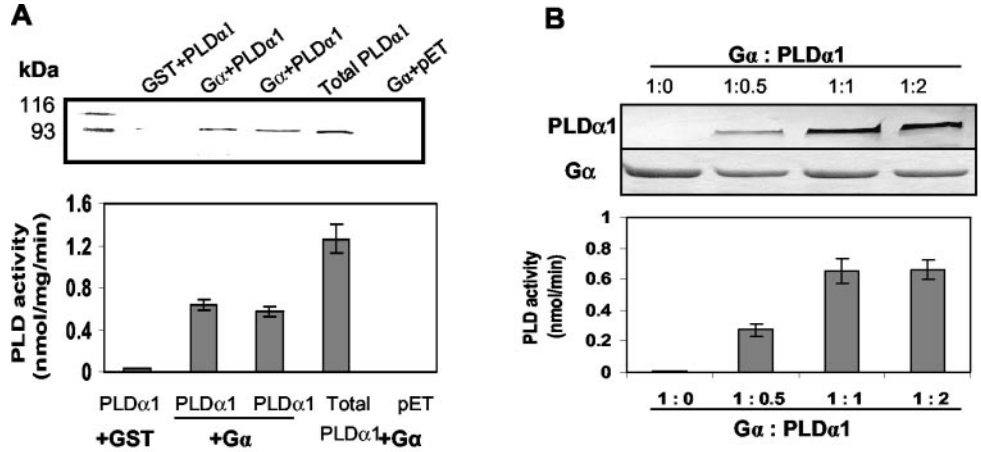

FIG. 2. **Binding of Gα with PLDα1 expressed in *E. coli*.** *A*, co-precipitation of PLDα1 with GST-Gα. *Top*, immunoblotting of PLDα1 that was co-precipitated with GST-Gα. GST-Gα (0.05 μmol) was used to precipitate bacterially expressed His-PLDα1 (0.15 μmol). *GST+PLDα1*, PLDα1 precipitated with GST-bound beads (molar equivalent to the GST-Gα-bound beads). *Total PLDα1*, the starting PLDα used for precipitation. *Gα+pET*, bacterial lysate harboring an empty pET vector precipitated by Gα. *Bottom*, PLDα1 activity in the co-precipitates corresponding to those in the immunoblot *above*. PLD activity was expressed as nmol of choline released/min/mg of starting proteins. *B*, effect of different molar ratios of Gα to PLDα1 on the binding. Purified GST-Gα (0.05 μmol) was mixed with 0, 0.025, 0.05, and 0.1 μmol of purified His-PLDα1 in a binding buffer followed by pulldown with glutathione beads. *Top*, immunoblotting of PLDα1 that was co-precipitated with GST-Gα. *Middle*, Coomassie Blue staining of GST-Gα in the precipitates resolved by 10% SDS-PAGE. *Bottom*, PLDα1 activity in the Gα precipitates, expressed as nmol of choline released/min from the same amount of precipitates.

involving the use of phosphatidylcholine (PC), 25 mM Ca^{2+}, and 0.3 mM SDS (22). This assay is economical and specific for PLDα1 and thus was used for most PLDα1 assays unless otherwise stated. When the effect of Gα binding on PLD activity was measured, however, the presence of SDS and high levels of Ca^{2+} might have interfered with the PLD-Gα interaction. Thus, a second PLD assay was used, which contained mixed lipid vesicles composed of 3.6 μmol of phosphatidylethanolamine, 0.32 μmol of PIP_2, and 0.22 μmol of PC in the presence of 100 μM $CaCl_2$. This assay, referred to as the PC-PIP_2 method, was performed according to a procedure described previously (23).

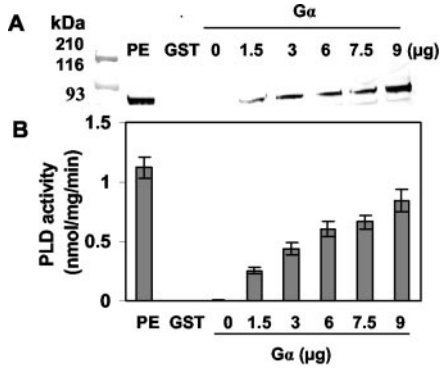

FIG. 3. **Binding of Gα with PLDα1 from *Arabidopsis* leaf extracts.** *A*, immunoblotting of PLDα1 co-precipitated with Gα. Increasing amounts of purified GST-Gα that were bound to beads were added to plant extracts (*PE*; 200 μg/reaction) to precipitate PLDα1. As a control, GST-bound beads (*GST*) were added to plant extracts and precipitated in the same manner. Precipitates pulled down with GST-Gα were subjected to 8% SDS-PAGE followed by blotting with PLDα1 antibody. The PLDα1 band was made visible by staining alkaline phosphatase activity conjugated to a second antibody. *B*, PLDα1 activity in the GST-Gα precipitates corresponding to those in *panel A*. PLD activity was expressed as nmol of choline released/min/mg of starting plant proteins.

Assay of GTPase Activity of E. coli-expressed GPA1—GTPase activity assays were performed using the EnzChek® phosphate assay kit (Molecular Probes, Eugene, OR). The rate of phosphate released from GTP by Gα was monitored spectrophotometrically at $A_{360\ nm}$ according to the manufacturer's instructions. Briefly, the assay mixture contained different amounts of GST-Gα, 10 μl of 0.2 mM GTP or GTP at indicated concentrations, 0.2 ml of 2-amino-6-mercapto-7-methylpurine ribonucleotide, 10 μl (1 unit) of purine nucleotide phosphorylase, and 0.72 ml of reaction buffer (50 mM Tris-HCl, pH 7.6, and 10 mM NaCl) in a total volume of 1 ml in a cuvette. After mixing, the reaction was started with addition of 10 mM $MgCl_2$. Absorbance changes were recorded every 5 min. The amount of phosphate released from GTP was calculated based on a standard curve that was determined with known amounts of KPO_4 in the same manner as described above.

RESULTS

Gα Binds to PLDα1 Expressed in E. coli and Arabidopsis—Gα and PLDα1 were expressed in *E. coli* as GST-fused and His-tagged proteins, respectively (Fig. 1*A*). Both PLDα1 and Gα are catalytically active as indicated by the presence of their respective activities (Figs. 1*B* and 2*A*). When bacterial lysate harboring His-PLDα1 was incubated with the lysate containing GST-Gα, PLDα1 was pulled down together with GST-Gα by

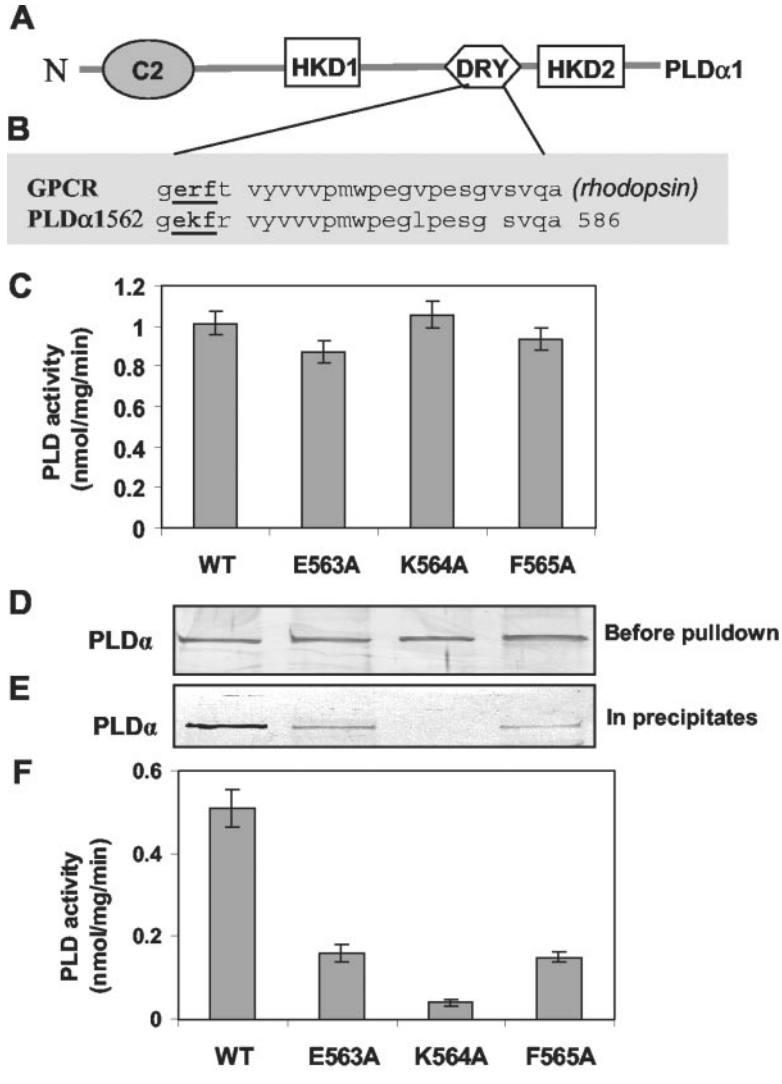

FIG. 4. **The DRY motif of PLDα1 and the amino acid residues involved in the PLDα1 binding to Gα.** *A*, schematic presentation of various domain structures of PLDα1. C2 is a Ca^{2+}-dependent phospholipids-binding fold. HKD1 and HKD2 are the duplicated catalytic motifs. *B*, alignment of the DRY motif sequences between PLDα1 and the chicken GPCR rhodopsin. *C*, PLDα1 activity in wild type (*WT*) and the DRY motif mutants E563A, K564A, and F565A. Proteins expressed and purified from *E. coli* were assayed for PLD activity. *D*, immunoblotting of wild-type and mutated PLDαs with PLDα1 antibodies before Gα precipitation with glutathione beads. *E*, immunoblotting of wild-type and mutated PLDαs in Gα precipitates from the same amounts of starting PLDα proteins (0.2 μmol; see *panel D*). *F*, PLD activity in the Gα precipitates, expressed as nmol of choline released/min/mg of starting PLDα proteins.

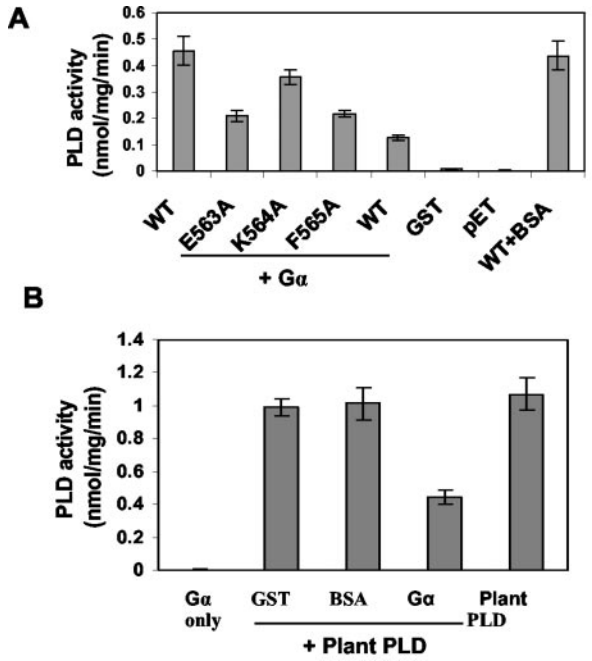

FIG. 5. **Effect of Gα on PLDα1 activity.** *A*, effect of Gα on the PLD activity of wild-type (*WT*) PLDα1 and mutated PLDα1s expressed in *E. coli*. Purified PLDα1s were co-incubated with GST-Gα or BSA (0.05 μmol each) for 30 min. As controls, equivalent molar amounts of GST-bound beads (*GST*) and His tag-bound beads (*pET*) were assayed for PLDα1 activity. *B*, effect of Gα on PLDα1 activity in plant extracts. GST-Gα beads (0.05 μmol of GST-Gα) or the same molar amounts of GST beads and BSA were added to plant protein extracts (200 μg). PLD activity was assayed using the PC-PIP_2 method.

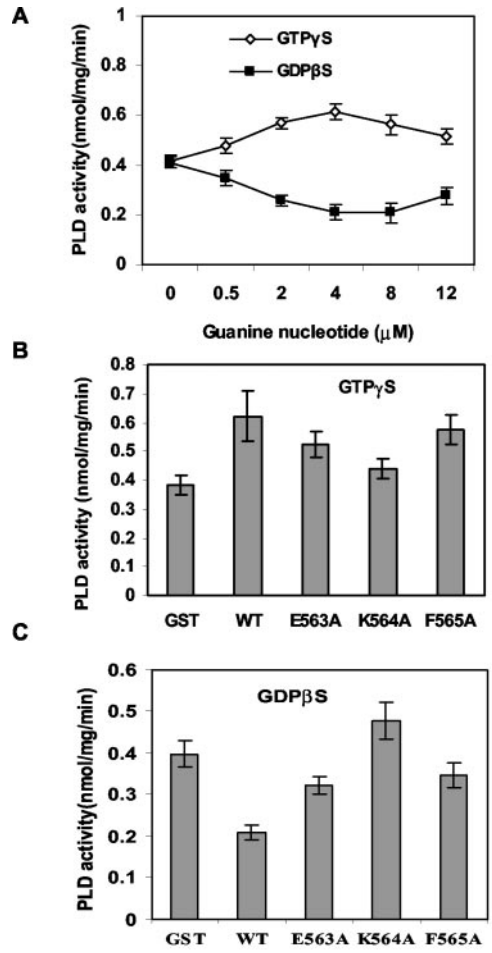

FIG. 6. **Effects of guanine nucleotide analogues on PLDα1 activity.** *A*, dose-dependent increase and decrease of PLDα1 activity by GTPγS and GDPβS, respectively, in the presence of Gα. *B*, GTPγS (5 μM) stimulation of PLD activity of bacterially expressed PLDα1 and its mutants. *WT*, wild-type. *C*, GDPβS (5 μM) inhibition of PLD activity of PLDα and its DRY motif mutants. Equal molar amounts of bacterially expressed, purified PLDα1 and Gα (0.17 μmol each) were co-incubated in a binding buffer for 30 min before the assays. PLD activity was assayed using the PC-PIP_2 method.

glutathione beads (Fig. 2*A*). The presence of PLDα1 in the precipitates was measured by immunoblotting with PLDα1-specific antibodies and assaying PLDα1 activity (Fig. 2*A*). PLDα1 was not pulled down with GST, indicating that the co-precipitation resulted from the presence of Gα but not GST or the beads. To estimate the stoichiometry of the interaction, Gα and PLDα1 were purified and co-incubated at different molar ratios (Fig. 2*B*). Increasing the ratio of PLDα1 to Gα from 0.5 to 1 led to a doubling of the PLD co-precipitated with Gα, whereas an increase in the molar ratio to 2:1 did not lead to a further increase in PLDα complexed with Gα. These results indicate that PLDα1 binds to Gα in a 1:1 ratio.

To demonstrate the association of Gα with native PLDα1, the purified Gα was incubated with *Arabidopsis* leaf protein extracts followed by precipitation with glutathione beads. Native PLDα1 was co-precipitated with Gα as measured by immunoblotting and PLDα1-specific activity assays (Fig. 3). As a control, GST beads gave negligible binding to PLDα1 from plant extracts. The amount of PLDα1 co-precipitated with Gα increased as the amount of Gα was increased (Fig. 3), indicating that the Gα and PLDα1 binding is dose-dependent.

PLDα1 Contains a DRY-like Motif That Is Responsible for Its Binding to Gα—To determine the molecular basis for the PLDα1 and Gα interaction, the PLDα1 sequence was analyzed for the presence of sequences with similarity to known G-protein-interacting motifs. At amino acid residues 562–586, the sequence of PLDα1 is highly similar to the DRY motif that has been found in more than 200 GPCRs (Fig. 4, *A* and *B*). This motif in GPCRs is located at the cytosolic juncture of the third transmembrane domain and is thought to associate with Gα of heterotrimeric G-proteins (24, 25). The DRY motif consists of a core triplet of amino acids, D-R-Y, and also a highly conserved hydrophobic region, VYVVV, located immediately downstream. The hydrophobic region is completely conserved in PLDα1 (Fig. 4*B*) and is conservatively modified in eight other *Arabidopsis* PLDs. The corresponding triplet amino acids in PLDα1 are conservatively substituted to EKF, which lies between the two duplicated, catalytic HKD motifs (Fig. 4*A*). Conservative substitutions of the DRY amino acid residues are allowable. For example, receptors in chicken and bullfrog both exhibit E-R-F (Fig. 4*B*).

The EKF residues were mutated individually to Ala to determine the involvement of each of the residues in Gα binding. All of the three mutated PLDα1s displayed the PLD activity comparable with that of wild-type PLD (Fig. 4*C*), indicating that changing any of the residues to Ala results in no major alteration of PLD catalytic activity. GST-Gα was then co-incubated with the same amounts of wild-type and mutated PLDα1s (Fig. 4*D*), followed by precipitation of Gα with glutathione beads. Virtually no $PLD\alpha1_{K564A}$ was co-precipitated as measured by the lack of PLDα1 protein (Fig. 4*E*) and activity (Fig. 4*F*) in the Gα precipitates. The amount of mutated PLD_{E563A} and PLD_{F565A} co-purified with Gα was more than 3-fold lower than that of wild-type PLDα1 (Fig. 4, *E* and *F*). These results indicate that the residue Lys^{564} is essential for PLDα1 to interact with Gα, whereas the flanking Glu^{563} and Phe^{565} are important for enhancing this interaction.

Association of Gα with PLDα1 Decreases PLDα1 Activity—To test the effect of the Gα binding on PLDα1 activity, purified Gα

was co-incubated with wild-type and mutated PLDα1s at approximately a 1:1 molar ratio, followed by assaying PLDα1 activity (Fig. 5). Addition of Gα decreased the wild-type PLDα1 activity by more than 3-fold, whereas addition of the same amount of an unrelated protein, BSA, had no effect, indicating that the Gα inhibition is not a nonspecific protein effect (Fig. 5*A*). The Gα inhibition of $PLD\alpha1_{E563A}$ or $PLD\alpha1_{K565A}$ was not as strong as that of wild-type PLDα1, and the lower degree of inhibition is consistent with the decreased Gα binding exhibited by these mutated PLDs. Gα had the least inhibitory effect on $PLD\alpha1_{K564A}$ (Fig. 5*A*), which exhibited very little binding to Gα (Fig. 4, *E* and *F*). The extent of Gα inhibition of PLDα1 is conversely correlated with the binding ability of the various PLDα1s to Gα, indicating that the binding is required for the Gα inhibition of PLDα1 activity.

The Gα inhibition of PLD activity was also observed with proteins from *Arabidopsis* (Fig. 5*B*). When purified GST-Gα was co-incubated with *Arabidopsis* leaf protein extracts, Gα decreased ~60% of PLDα1 activity, whereas co-incubation with the same molar amount of GST or BSA had no effect on PLD activity. This result indicates that the inhibition is caused by Gα, not by GST or agarose beads present in the solution. The extent of inhibition for the leaf PLD was smaller than that of the bacterially expressed PLDα1, which could be caused by having other PLDs in plant extracts, as all *Arabidopsis* PLDs are active under the PC-PIP_2 assay conditions (1, 2). In addition, because the molar amount of PLDα1 in plant extracts was difficult to determine, the presence of excessive PLDα1 could also contribute to less inhibition of PLD by Gα.

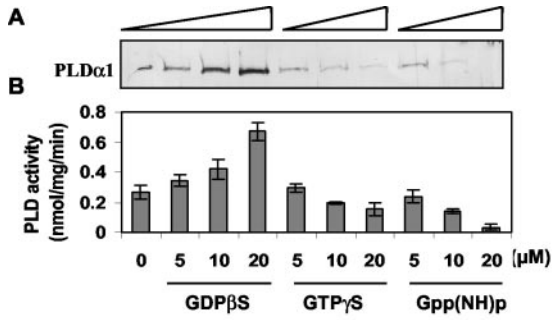

FIG. 7. **Effects of guanine nucleotide analogues on the Gα binding to PLDα from *Arabidopsis*.** *A*, immunoblotting of *Arabidopsis* PLDα1 co-precipitated with GST-Gα in the presence of 0, 5, 10, and 20 μM GDPβS, GTPγS, or Gpp(NH)p. *Arabidopsis* leaf extracts (50 μg of proteins from 10,000 × *g* supernatant) were co-incubated with or without the nucleotide analogues followed by precipitation with GST-Gα beads (0.05 μmol of GST-Gα). A portion of the precipitates was subjected to 10% SDS-PAGE and immunoblotting, and another was assayed for PLD activity. *B*, PLDα1 activity in the GST-Gα precipitates. PLD activity was expressed as nmol of choline released/min/mg of starting plant proteins.

Guanine Nucleotides Affect the Gα-PLDα1 Binding and PLDα1 Activity—Gα can bind GDP and GTP; it is GDP-bound in the resting state, whereas an exchange of GDP for GTP is associated with the activation of Gα. To determine whether guanine nucleotides affect the Gα-PLDα1 interaction, purified PLDα1 and Gα were co-incubated at a 1:1 molar ratio in the presence of different concentrations of the hydrolysis-resistant analogues GDPβS and GTPγS. GDPβS gave a dose-dependent inhibition, whereas GTPγS yielded a dose-dependent stimulation of PLDα1 activity (Fig. 6*A*). When GDPβS or GTPγS was added to the DRY motif mutants and Gα complexes, the activity of $PLD\alpha1_{K564A}$ was not affected significantly (Fig. 6, *B* and *C*; GST *versus* K564A). Compared with that of wild-type PLDα1 and $PLD\alpha1_{K564A}$, GDPβS and GTPγS had an intermediate inhibitory and stimulatory effect, respectively, on the activity of $PLD\alpha1_{E563A}$ and $PLD\alpha1_{F565A}$ (Fig. 6, *B* and *C*; wild-type (*WT*) *versus* E563A and F565A). Binding results showed that $PLD\alpha1_{K564A}$ was unable to bind to Gα and that $PLD\alpha1_{E563A}$ and $PLD\alpha1_{F565A}$ had decreased binding (Fig. 4*E*). Thus, these nucleotide effects on PLD activity are in agreement with the Gα binding abilities of these PLDs, indicating that guanine nucleotides modulate PLD activity through their effects on the Gα and PLDα1 binding.

The effect of guanine nucleotides on the Gα-PLDα1 binding was further determined using *Arabidopsis* leaf extracts (Fig. 7). Consistent with the results shown in Figs. 1–6, Gα was able to bind to PLDα1 without any added guanine nucleotide. Adding GDPβS to plant protein extracts increased the amounts of PLDα1 co-purified with Gα as measured by immunoblotting and assaying PLDα1 activity in the co-precipitates (Fig. 7). Conversely, GTPγS decreased the Gα-PLDα1 binding in a dose-dependent manner. The inhibition of Gα-PLDα1 binding by Gpp(NH)p, another guanosine triphosphate nucleotide analogue, was even greater than that by GTPγS; almost no PLDα1 was co-precipitated with Gα at 20 μM Gpp(NH)p. This difference could be attributed to the possibility that the Gpp(NH)p is more stable than GTPγS (Fig. 7). These results indicate that the binding of GTP to Gα, thus the activation of Gα, decreases the interaction between Gα and PLDα1, whereas Gα in the resting, presumably GDP-bound state binds to PLD.

PLDα1 Binding Stimulates GTPase Activity—To determine potential reciprocal effects of the Gα-PLDα1 interaction, the intrinsic GTPase activity of Gα was determined in the presence of wild-type PLDα1 or the non-Gα-binding mutant $PLD\alpha1_{K564A}$. Wild-type PLDα1 increased the GTPase activity by about 35%, whereas $PLD\alpha1_{K564A}$ had no stimulatory effect on the GTPase activity (Fig. 8*A*). This result indicates that binding of PLDα to Gα is required for the PLDα1 stimulation of Gα-mediated hydrolysis of GTP. The kinetics of the GTPase activity was assessed by varying the substrate GTP concentrations in the presence or absence of PLDα1 (Fig. 8*B*). The

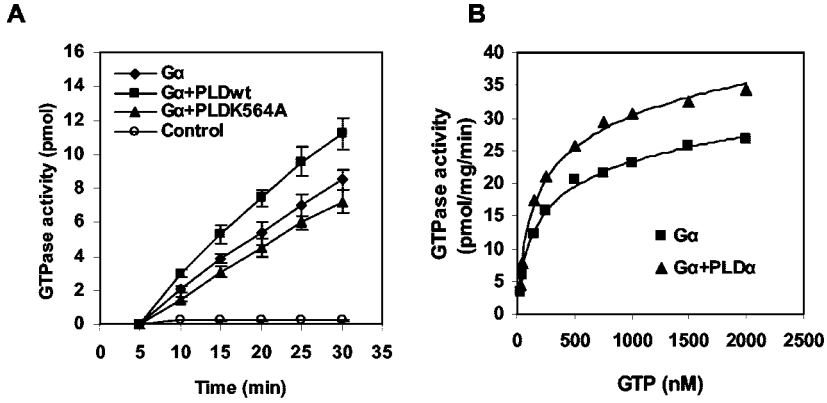

FIG. 8. **PLDα1 stimulation of the GTPase activity of Gα.** *A*, intrinsic GTPase activity of Gα as a function of incubation time and as affected by wild-type PLDα1 (*PLDwt*) and by the non-Gα-binding $PLD\alpha1_{K564A}$ (*PLDK564A*). *B*, GTPase activity of Gα as a function of increasing the substrate GTP concentrations in the presence or absence of wild-type PLDα1. Equal molar amounts of bacterially expressed, purified PLDα1 and Gα (0.17 μmol each) were co-incubated for the assays. Hydrolysis of GTP was assayed by measuring P_i release by GST-Gα. The control was the reaction with GST-Gα in the absence of GTP.

Michaelis constant K_m and the maximal rate V_{max} were derived by the Lineweaver-Burk plot. With or without PLDα1, the K_m for GTP was 182 nM. The V_{max} was 27.0 and 36.4 pmol/mg of protein/min in the presence or absence of PLDα1, respectively. These data suggest that the PLDα1 promotes the turnover number of the GTPase activity without affecting the affinity of Gα for GTP.

DISCUSSION

Results of this study provide evidence for the direct physical interaction between PLDα1 and Gα. Moreover, this study has identified the specific DRY motif on PLDα1 responsible for the binding (Fig. 3). The DRY motif is present in PLDα-related PLDs from different plant species (26), indicating that this Gα regulation is conserved for the common plant PLD. The DRY motif is normally found on GPCRs and is important in coupling the receptor and G-protein activation, as mutation of this motif results in a variety of modified phenotypes, including constitutive, moderate, and null receptor activities (24, 25). This raises the question of whether the DRY motif on plant PLDs competes with plant GPCRs from the receptor-Gα interaction. However, almost nothing is known about the coupling of Gα to GPCR in plants (10, 11). The only reported putative *Arabidopsis* GPCR is GCR1 (27), but its direct interaction with Gα has yet to be demonstrated. In addition, our sequence analysis indicates that *Arabidopsis* GCR1 does not contain the DRY motif. It has also been stated that the loss-of-function mutant of GCR1 (*gcr1*) showed no shared phenotypes with that of Gα (*gpa1*) and that Gα might not be coupled to GCR1 in the cell (11). Thus, the study on the role of the PLD-Gα interaction in the receptor-Gα interaction awaits identification of a receptor interacting with Gα in plants.

It is unlikely that PLDα1 itself serves as a canonical Gα-coupled receptor protein because of its lack of transmembrane domains. Rather, PLDα1 may function as an intracellular regulator of G-proteins. A number of regulator proteins have been identified in other organisms, which include GTPase-activating protein (GAP) and guanine nucleotide exchange factors (8, 9). However, proteins with such functions have not been reported in plants (10, 11). The present results indicate that PLDα1 has some GAP activity and that the GAP activity requires the Gα-PLDα1 binding (Fig. 8*A*). The GTPase-activating effect of PLDα1 is relatively small, and this could be due to the absence of an activated GPCR that is required for the function of some GAPs (28). Kinetic analysis suggests that PLDα1 stimulates GTPase activity via increasing the turnover number without affecting the GTP affinity to the enzyme (Fig. 8*B*). One way of increasing turnover is to promote the exchange between GDP and GTP. In addition, other modes of regulation of Gα by PLDα1 should also be considered. PLDα1 has been proposed to translocate between cytosol to membranes in plants (1, 4), and it might be possible that the PLDα1-Gα binding modulates the intracellular location and thus the activity of G-protein.

The effect of the PLDα1-Gα interaction is reciprocal; while it stimulates the GTPase activity of Gα, the interaction inhibits PLDα1 activity (Fig. 5). Results from the mutagenesis of the DRY motif have indicated that the direct binding is required for the effect on both Gα and PLDα1. Whereas the DRY motif on PLDα1 is essential to the binding, the state of Gα activation also determines the PLDα1-Gα interaction. PLDα1 is able to bind to Gα in the resting or GDP-bound state but is unable to bind GTP-bound Gα. A previous study with bacterially expressed tobacco Gα and PLDα also indicates that GTPγS can decrease the Gα inhibition of PLDα activity (18). The dissociation of Gα from PLDα1 is likely to result from a conformational change of Gα triggered by the exchange of GDP for GTP and the activation of Gα.

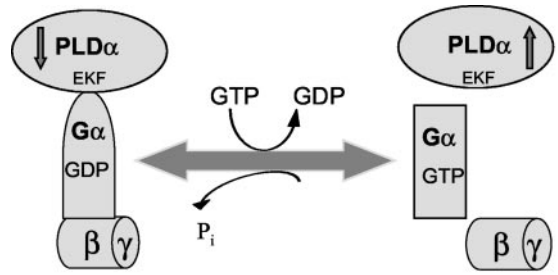

FIG. 9. **A proposed model depicting the PLDα1-Gα binding and the functions of the interaction.** PLDα1 binds to the resting state, GDP-bound Gα through the EKF residues in the DRY motif. The PLDα1-Gα binding decreases PLDα1 activity, and GTP decreases the binding and restores PLDα1 activity. Reciprocally, the PLD binding to Gα promotes GTPase activity, possibly via promoting the exchange of GDP for GTP and also the dissociation of G$\beta\gamma$ from Gα. The binding of GTP to and the activation of Gα promote dissociation of PLDα1-Gα complexes. Thus, the association and dissociation between PLD and Gα form a positive loop to stimulate the function of both PLD and G-proteins. The *upward* and *downward arrows* next to PLD indicate a decrease and increase in activity, respectively. The scale of drawing is not proportional to the size of the proteins.

One intriguing question raised from the PLDα1-Gα interaction is whether the PLDα1-Gα interaction modulates the association of Gα with G$\beta\gamma$. According to the mammalian heterotrimeric G-protein paradigm, Gα in the GDP-bound resting state associates with G$\beta\gamma$, whereas Gα in the GTP-bound activated state dissociates from G$\beta\gamma$ (8). Some activators of G-protein signaling proteins identified in other organisms have been shown to bind to the resting or GDP-bound form of Gα to release G$\beta\gamma$ (28, 29). The present results demonstrate that PLDα1-Gα interaction occurs with or without added GDP, and thus, PLDα1 binds to the resting state, GDP-bound Gα. One canonical Gβ gene and two Gγ genes have been identified in *Arabidopsis* (30, 31). The association has been demonstrated for Gβ and Gγ subunits (31), but not for Gα and G$\beta\gamma$. Further studies will be needed to identify the condition under which Gα interacts with G$\beta\gamma$ and then to determine whether the PLDα1 binding regulates the association and/or dissociation between Gα with G$\beta\gamma$. Possibilities remain also that the association of Gα with G$\beta\gamma$ may affect the Gα-PLDα1 interaction.

Another question relevant to the understanding of the PLD-Gα interaction is the specificity of the Gα interaction with various PLDs. Except for two PLDζs and PLDγ2, nine of the 12 PLDs in *Arabidopsis* have the DRY motif-like sequences (data not shown) and thus can potentially interact with Gα. Different patterns of temporal and spatial expression and distribution have been noted for some PLDs (32–34), which could be important determinants for the Gα interaction with different PLDs in different cells and timing. In addition, individual PLDs exhibit different requirements for Ca^{2+}, PIP_2, free fatty acids, and lipid environments for activity (1, 33, 35, 36). Thus, the other cellular factors might also modulate the PLD-Gα interaction and specificity. It should be noted that analyses of PLDα1-depleted (7) and Gα-null (12) *Arabidopsis* have shown some shared phenotypic alterations, such as retardation of stomatal movement and increasing plant water loss. It is likely that the PLDα1-Gα interaction documented in this study underlies a structural and molecular basis for their involvement in specific plant signaling pathways.

Based on "Results" and "Discussion," a working model is proposed for the role and further investigation of the PLDα1-Gα interaction (Fig. 9). PLDα1 binds to the resting state or GDP-bound Gα through the DRY motif. The PLDα1-Gα binding hinders PLDα1 activity and may also promote dissociation of G$\beta\gamma$ from Gα. In addition, the PLDα1-Gα binding

stimulates GTPase activity, possibly via promoting the exchange of GDP for GTP. At the same time, the binding of GTP to Gα promotes dissociation of the PLDα1-Gα complexes and thus restores PLDα1 activity. The released PLDα1 will be able to bind to GDP-bound Gα again, and another cycle begins. Thus, the association and dissociation between PLD and Gα would form a positive loop to stimulate the activity of PLDα1 and Gα, and this dynamic process would regulate reciprocally the cellular function of both PLD and G-proteins. Further studies on elucidating this novel signaling process and identifying upstream regulators and downstream effectors of the interaction will facilitate the understanding of the cellular roles of PLDs and heterotrimeric G-protein in plant signal transduction.

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Note Added in Proof—A Gα-interacting regulator of G-protein signaling (RGS) protein, designated AtRGS1, has been identified recently in *Arabidopsis*. AtRGS1 has a predicted structure similar to a GPCR and an RGS box with GTPase-accelerating activity (37).

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Arabidopsis Phospholipase Dα1 Interacts with the Heterotrimeric G-protein α -Subunit through a Motif Analogous to the DRY Motif in G-protein-coupled Receptors

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