

Phosphatidylinositol (3,4,5)-trisphosphate specifically interacts with the phox homology domain of phospholipase D1 and stimulates its activity

Jun Sung Lee¹, Jong Hyun Kim¹, Il Ho Jang¹, Hyeon Soo Kim¹, Jung Min Han¹, Andrius Kazlauskas², Hitoshi Yagisawa^{1,3}, Pann-Ghill Suh¹ and Sung Ho Ryu^{1,*}

¹Division of Molecular and Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea

²Schepens Eye Research Institute, Harvard Medical School, 20 Staniford Street, Boston, MA 02114, USA

³Graduate School of Life Science, University of Hyogo, Harima Science Garden City, Hyogo 678-1297, Japan

*Author for correspondence (e-mail: sungho@postech.ac.kr)

Accepted 28 June 2005

Journal of Cell Science 118, 4405-4413 Published by The Company of Biologists 2005
doi:10.1242/jcs.02564

Summary

Phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline, plays key roles in cellular signal transduction by mediating extracellular stimuli including hormones, growth factors, neurotransmitters, cytokines and extracellular matrix molecules. The molecular mechanisms by which domains regulate the activity of PLD – especially the phox homology (PX) domain – have not been fully elucidated. In this study, we have examined the properties of the PX domains of PLD1 and PLD2 in terms of phosphoinositide binding and PLD activity regulation. Interestingly, the PX domain of PLD1, but not that of PLD2, was found to specifically interact with phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P₃). We found that mutation of the conserved arginine at position 179 of the PLD1 PX domain to lysine or to alanine (R179A or R179K, respectively) disrupts PtdIns(3,4,5)P₃ binding. In NIH-3T3 cells, the EGFP-PLD1 PX wild-type domain, but not the two mutants, localized to the plasma membrane after 5-minute treatment with platelet-derived growth factor

(PDGF). The enzymatic activity of PLD1 was stimulated by adding PtdIns(3,4,5)P₃ in vitro. Treatment with PDGF resulted in the significant increase of PLD1 activity and phosphorylation of the downstream extracellular signal-regulated kinases (ERKs), which was blocked by pre-treatment of HEK 293 cells with phosphoinositide 3-kinase (PI3K) inhibitor after the endogenous PLD2 had been depleted by siRNA specific for PLD2. Nevertheless, both PLD1 mutants (which cannot interact with PtdIns(3,4,5)P₃) did not respond to treatment with PDGF. Moreover, PLD1 was activated in HepG2 cells stably expressing the Y40/51 mutant of PDGF receptor that is required for the binding with PI3K. Our results suggest that the PLD1 PX domain enables PLD1 to mediate signal transduction via ERK1/2 by providing a direct binding site for PtdIns(3,4,5)P₃ and by activating PLD1.

Key words: PX domain, PLD, Phosphoinositides, ERK phosphorylation, PDGF

Introduction

Phospholipase D (PLD) mediates various cellular signaling events, including cytoskeletal rearrangement, vesicle trafficking, and proliferation by modulating membrane compositions and/or by producing phosphatidic acid (Cross et al., 1996; Frohman et al., 1999; Jones et al., 1999; Rizzo et al., 1999). Phosphatidic acid, the product of PLD action, is a well known second messenger and is implicated in various regulatory roles in signaling molecules (Frohman and Morris, 1999). The activity regulation of PLD is achieved by phosphorylation (Kim et al., 1999; Lee et al., 1997) and binding with small molecular weight guanine nucleotide binding proteins (SMGs) such as ADP-ribosylation factor (ARF), Rho, Rac and Cdc42 (Hammond et al., 1997; Yamazaki et al., 1999). However, little is known about the details of the molecular mechanism and the roles of domains in the regulation of PLD.

Phosphoinositide (PI) functions in diverse cellular processes

(Martin, 1998; Odorizzi et al., 2000). The D-*myo*-inositol head-group of PtdIns contains five hydroxyl groups (at positions 2, 3, 4, 5 and 6), three of which (3, 4, and 5) are known to be reversible phosphorylation targets to yield singly, doubly or triply phosphorylated phosphatidylinositide (PtdIns) derivatives (PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂, and PtdIns(3,4,5)P₃) of different combinations. The activities of specific PtdIns are regulated by controlling their cellular levels, which is achieved by complex networks of proteins that regulate their synthesis (kinases) and degradation (phosphatases and lipases) (De Camilli et al., 1996; Fruman et al., 1998; Rameh and Cantley, 1999; Vanhaesebroeck et al., 2001). Of these, PtdIns(3,4,5)P₃ is synthesized when the mammalian class I phosphatidylinositide 3-kinase (PI3K) is recruited to the plasma membrane after the activation of a cell-surface-receptor kinase. The binding of PtdIns(3,4,5)P₃ to its effector phosphoinositide-dependent kinase-1 (PDK-1) (Stokoe et al., 1997) mediates the

translocation of PDK-1 to a place where it can phosphorylate and activate Akt (protein kinase B, PKB) (Stephens et al., 1998), which results in the increased phosphorylation of several substrates including glycogen synthase kinase-3 (GSK-3), p70s6k and 4E-BP1 (Scott et al., 1998). These, in turn, activate diverse metabolic pathways and are necessary for cell survival (Stephens et al., 1998). However, PtdIns-specific cellular functions, especially PtdIns(3,4,5) P_3 -induced molecular activation and signaling events, are not fully understood.

The Phox homology (PX) domain is named after its presence in the p40^{phox} and p47^{phox} subunits of the NADPH oxidase complex and has an approximately ~140 amino-acid-long motif (Ponting, 1996). PX domains are found in a range of proteins associated with membranes such as PLD, sorting nexins and yeast Vam7p, and contain a proline-rich motif, which enables them to recognize the Src homology 3 (SH3) domain (Jang et al., 2003; Kay et al., 2000; Ponting, 1996). Moreover, recent studies have shown that, specific interactions between PX domains and phosphoinositides play a crucial role in the localization and enzymatic regulation of PX-domain-containing proteins, and have identified the PX domain as a phosphoinositide-binding module involved in cellular signal transduction (Ago et al., 2001; Cheever et al., 2001; Ellson et al., 2001; Kanai et al., 2001; Xu et al., 2001; Zhan et al., 2002).

PLD1 and PLD2 isozymes share several structural domains including the N-terminally localized PX domain (Frohman et al., 1999; Ponting, 1996). Recently, Du et al. suggested that the PX domain of PLD1 plays a role in the re-entry of PLD1 into perinuclear vesicles after its PMA-stimulated translocation to the plasma membrane (Du et al., 2003). However, the molecular characteristics of the PX domain of PLD, especially its role in the regulation of enzymatic activity, remains unclear. To check the phosphoinositide-binding ability of the PLD PX domain and to characterize the functional role of this interaction, we investigated the lipid affinity of PLD. Here, we show that the PLD1 PX domain specifically binds to PtdIns(3,4,5) P_3 , which is critical in PLD1 activity regulation.

Materials and Methods

Chemicals and reagents

Phosphoinositides were purchased from Echelon Biosciences Inc. (Salt Lake City, UT). The enhanced chemiluminescence kit (ECL system), dipalmitoyl-phosphatidyl [methyl-³H]choline, and glutathione sepharose 4B were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Dipalmitoyl-phosphatidylcholine (dipalmitoyl-PC), dioleoyl-phosphatidylethanolamine (dioleoyl-PE) was purchased from Sigma (St Louis, MO). Water soluble inositolphosphates were a generous gift from Sung Ki Chung (Pohang University of Science and Technology, Korea). Silica gel 60 TLC plates and Triton X-100 were acquired from EM Science (Gibbstown, NJ). Dulbecco's modified Eagle's medium (DMEM) and LipofectAMINE were purchased from Invitrogen (Carlsbad, CA). Bovine calf serum was obtained from HyClone (Logan, UT). Horseradish peroxidase-conjugated goat anti-rabbit IgG, anti-mouse IgG, IgM, and IgA were purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). LY 294002 and porcine platelet-derived growth factor (PDGF) were purchased from Calbiochem (San Diego, CA). Antibody against phosphorylated extracellular signal-regulated kinases (ERKs) was from Cell Signaling (Beverly, MA). Antibody against the C-terminal region of PLD was prepared as previously described (Lee et al., 1997).

Cell culture

NIH-3T3, HEK 293 and HepG2 cells stably expressing mutant PDGFR β receptor Y40/51 (Bae et al., 2000; Valius and Kazlauskas, 1993) were cultured at 37°C in a humidified 5% CO₂ atmosphere in high glucose DMEM supplemented with 10% fetal bovine serum.

Generation of plasmids and recombinant proteins

Mutants PLD1(R179K) or PLD1(R179A) were generated by point mutating the arginine residue in wild-type PLD1 at position 179 to lysine or alanine, respectively, by using the splice-overlap extension method (Ho et al., 1989). Briefly, PLD1(R179K) was obtained by using forward primer: 5'-G TTC TTT GGC AAG AGG CAA C-3' and reverse primer: 5'-G TTG CCT CTT GCC AAA GAA C-3'. A similar strategy was used to generate PLD1(R179A), this time by using forward primer: 5'-G TTC TTT GGC GCG AGG CAA C-3' and reverse primer: 5'-G TTG CCT CGC GCC AAA GAA C-3'. The resulting products were cloned into the pCDNA3.1 vector (Invitrogen). Sequences of the PLD1 PX domain (amino acids 76-196) of wild type, and mutants (R179K and R179A) were amplified by polymerase chain reaction (PCR) and subcloned into vector pGEX4T1 (Amersham Pharmacia Biotech) or pEGFP (enhanced green fluorescent protein)-C1 (pEGFP-C1, Clontech) vector to generate glutathione-S-transferase (GST)-PLD1 PX or EGFP-PLD1 PX constructs, respectively. To purify the recombinant proteins, *Escherichia coli* BL21 cells were transformed with individual expression vectors encoding GST fusion protein and grown at 37°C to an OD₆₀₀ of 0.8. Fusion proteins were then induced by incubating the cells in the presence of 100 μ M isopropyl- β -D-thiogalactopyranoside for 4 hours at 25°C. After harvesting the cells, the fusion proteins were purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

Liposome-binding assay

Phospholipid-vesicles composed of 53 μ M phosphatidylethanolamine (PE), 3.3 μ M phosphatidylcholine, and 4.6 μ M phosphoinositides (PtdIns) were incubated with 10 ng of the purified GST-PLD PX domains in 150 μ l buffer A (50 mM Hepes-NaOH pH 7.5, 3 mM MgCl₂, 2 mM CaCl₂, 3 mM EGTA, and 80 mM KCl) as previously described (Kim et al., 1998). After incubation at 37°C for 15 minutes, the reaction mixtures were centrifuged at 300,000 *g* for 30 minutes in a TL-100 ultracentrifuge (Beckman). Supernatants and pellets were then subjected to SDS-PAGE and immunoblotted using anti-GST antibody. In competition analysis, water soluble inositolphosphates were added to incubation mixtures.

In vitro PLD activity assays

PLD activity was determined by measuring choline release from phosphatidylcholine as previously described (Kim et al., 1999) with a minor modification. The enzyme sources in this study were obtained from HEK 293 cells. Cells transfected with PLD1, PLD1(R179K), and PLD1(R179A) were harvested with buffer A. After sonication, 1 μ g of lysate was used per assay.

In vivo PLD activity assays

PLD activity analysis was performed as previously described (Kim et al., 1999). Briefly, full-length PLD1 wild-type, PLD1(R179K) or PLD2 genes were transiently expressed. Transfection was carried out with LipofectAMINE, according to the manufacturer's instructions. In HEK 293 cells, depletion of endogenous PLD2 was performed using small interference RNA (siRNA) for PLD2, as described previously (Kim et al., 2005). After starving the cells for 24 hours, they were incubated with 10 μ Ci [³H]myristic acid for 3 hours. PLD activity was determined using the transphosphatidylation reaction in

the presence of 0.4% 1-butanol. Lipids were extracted and separated by Silica Gel 60 thin-layer chromatography (volume ratio of chloroform: methanol: acetic acid, 90:10:10), and the amounts of labeled phosphatidylbutanol and total lipid were determined using a Fuji BAS-2000 image analyzer (Fuji Film).

Immunocytochemistry and confocal imaging

NIH-3T3 cells grown on cover slips were transfected with pEGFP-PLD1 PX wild type, pEGFP-PLD1 PX (R179K) or pEGFP-PLD1 PX (R179A). After 24 hours of serum starvation, cells were treated with PDGF (25 ng/ml) and fixed with 4% (w/v) paraformaldehyde for 30 minutes. The slides were mounted and signals were visualized by confocal laser scanning microscopy (Zeiss LSM510, Germany).

Fractionation of cells

NIH-3T3 cells were washed twice with cold phosphate-buffered saline (PBS) and scraped with buffer B (PBS containing 0.25 M sucrose and a protease-inhibitor mixture). The cell suspension was then homogenized using a Dounce homogenizer and centrifuged at 3000 g for 15 minutes, and the supernatant was further centrifuged at 100,000 g for 30 minutes. The resulting supernatants (cytosol fraction) and pellets (membrane fraction) were subjected to SDS-PAGE and marked as Cyt and Mem, respectively.

Results

The PLD1 PX domain binds specifically to PtdIns(3,4,5)P₃. PLD contains a N-terminal PX domain, which can bind phosphoinositide to modulate its localization and activity. Fig. 1A shows the sequence alignment of part of the PX domain of several PX-domain-containing proteins. The PX domains of PLD have high homology with those of other proteins. To determine whether the PLD-PX domain interacts with phosphoinositides, purified GST-PLD1 PX and GST-PLD2 PX domains were incubated with liposomes containing a range of phosphoinositides. As shown in Fig. 1B, the PLD1 PX domain, but not the PLD2 PX domain, selectively bound to PtdIns(3,4,5)P₃. The PLD1 has a conserved Arg (R179), crucial for phosphoinositide binding (Ago et al., 2001; Fruman et al., 1999), whereas PLD2 has a Lys residue at the corresponding residue. Therefore, we mutated R179 of PLD1 to Lys (R179K) or Ala (R179A) and, as expected, both GST-PLD1 PX(R179K) and GST-PLD1 PX(R179A) failed to interact with PtdIns(3,4,5)P₃. We further characterized the PtdIns(3,4,5)P₃ selectivity of PLD1 PX by inositolphosphate competition analysis. Fig. 1C shows that the interaction between PLD1 PX and PtdIns(3,4,5)P₃-containing liposome

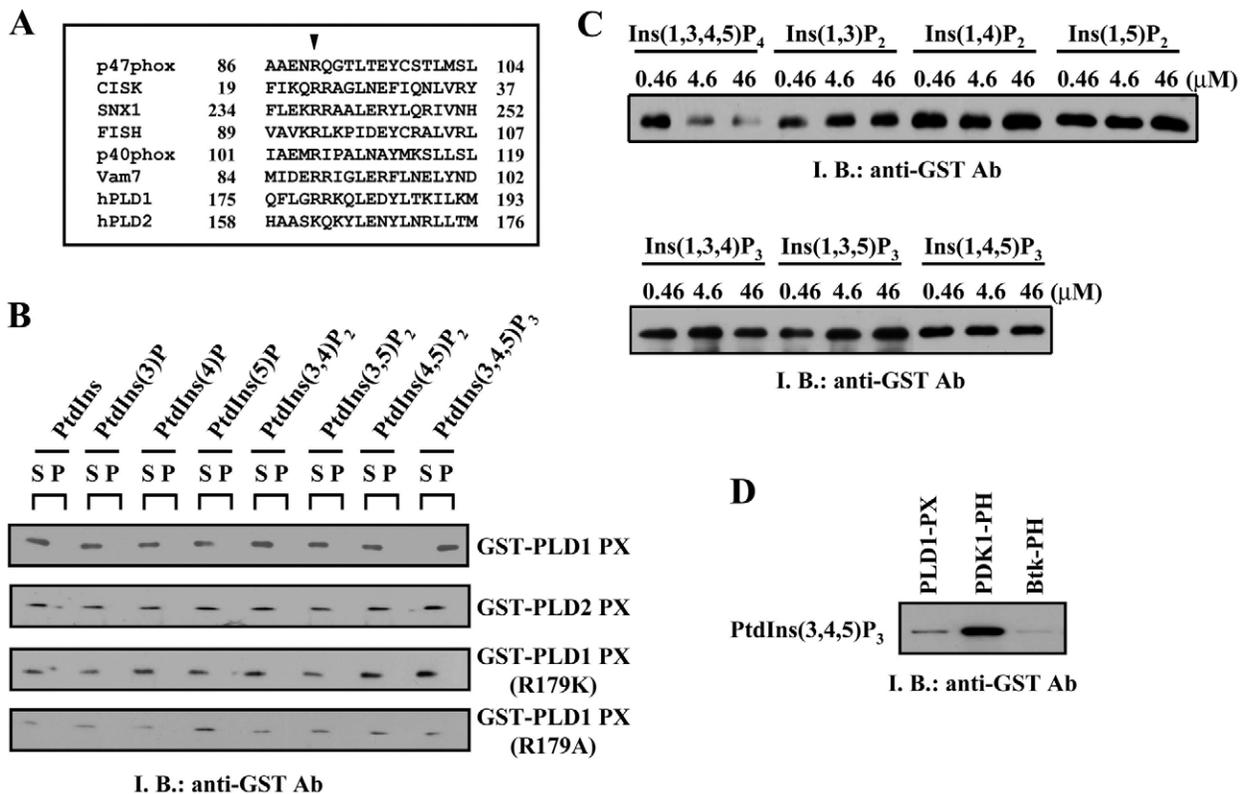


Fig. 1. The PX domain of PLD1 specifically binds to PtdIns(3,4,5)P₃. (A) The sequence alignment of regions from several PX domains. The conserved Arg residue mutated in this study is marked as ▼. (B) Specificities of PLD1 PX, PLD1 PX(R179K), PLD1 PX(R179A) and PLD2 for a range of phosphatidylinositols were analyzed by liposome-binding assay. Binding of GST-PLD PX domains to liposomes containing PE (phosphatidylethanolamine), PC (phosphatidylcholine) and indicated PtdIns (phosphatidylinositol) were analyzed. Liposomes (see Materials and Methods) were incubated with 10 ng of purified GST-PLD PX for 15 minutes at 37°C. The reaction mixtures were then centrifuged and the resulting supernatant (S) and pellets (P) were subjected to SDS-PAGE. Western blotting was performed with anti-GST antibody. A representative result of three individual experiments is shown. (C) The selectivity of PtdIns(3,4,5)P₃ for the PLD1 PX domain was confirmed by competition analysis. Water-soluble inositolphosphates were added to the reaction mixture as described in (B). (D) The affinity of PLD1 PX for PtdIns(3,4,5)P₃ was compared with those of PH domains from Btk and PDK-1.

was competed by the addition of $\text{Ins}(1,3,4,5)P_4$ in a dose-dependent manner but that it was not affected by any other inositolphosphate. Next, we checked the affinity between the PLD1 PX domain and $\text{PtdIns}(3,4,5)P_3$ by comparing it with the pleckstrin homology (PH) domain of PDK-1 and Bruton's tyrosine kinase (BTK) that have a K_d value for $\text{PtdIns}(3,4,5)P_3$ of 1.6 nM and 40 nM, respectively (Currie et al., 1999). The liposome-binding analysis with $\text{PtdIns}(3,4,5)P_3$ -containing vesicles showed that, for $\text{PtdIns}(3,4,5)P_3$, the PLD1 PX domain has a higher affinity than the Btk PH domain but a lower affinity than PDK-1 PH domain (Fig. 1D). Taken together, these results suggest that the PLD1 PX domain can mediate $\text{PtdIns}(3,4,5)P_3$ interaction and that the conserved Arg179 is crucial for this interaction.

The PLD1 PX domain interacts with $\text{PtdIns}(3,4,5)P_3$ in NIH-3T3 cells

PI3K is known to catalyze the phosphorylation of the D-3 position of the inositol ring of phosphoinositides and to prefer

$\text{PtdIns}(4,5)P_2$ as its substrate, resulting in a high level of $\text{PtdIns}(3,4,5)P_3$ in cells (Auger et al., 1989; Vanhaesebroeck et al., 2001). With the obtained knowledge that PLD1PX has a high affinity for $\text{PtdIns}(3,4,5)P_3$ in vitro, we wanted to examine whether this interaction occurs also in vivo. Therefore, we generated EGFP-PLD1 PX constructs. pEGFP-PLD1 PX wild type, pEGFP-PLD1 PX(R179K) or pEGFP-PLD1 PX(R179A) were expressed in NIH-3T3 cells. After 24 hours of serum starvation, we observed a diffused pattern of the wild type and both mutants in the cytosol (Fig. 2A). Interestingly, after a 5-minute treatment with PDGF, EGFP-PLD1 PX as well as Bruton's tyrosine kinase (Btk) PH domain, which specifically recognizes $\text{PtdIns}(3,4,5)P_3$, were localized in plasma membrane. The R179K and R179A mutants, however, which have no affinity to $\text{PtdIns}(3,4,5)P_3$ in vitro, did not change their intracellular localization in the presence of PDGF. Moreover, when cells were fractionated into cytosolic (Cyt) and membrane (Mem) fractions, only the wild-type PLD1 PX domain showed membrane localization after PDGF treatment (Fig. 2B). To further determine whether the plasma membrane localization of PLD1 PX was mediated by interaction with $\text{PtdIns}(3,4,5)P_3$, we examined the effect of LY 294002, a PI3K-specific inhibitor. When cells were pre-treated with LY 294002, a PDGF-induced plasma membrane localization of PLD1 PX did not occur (Fig. 3A). This was further confirmed by the cell-fractionation studies (Fig. 3B). Taken together, these data show that the PLD1 PX domain can interact with $\text{PtdIns}(3,4,5)P_3$ in vivo, resulting in its localization in the plasma membrane in NIH-3T3 cells.

$\text{PtdIns}(3,4,5)P_3$ activates PLD1 in vitro

We found that the PLD1 PX domain serves as a

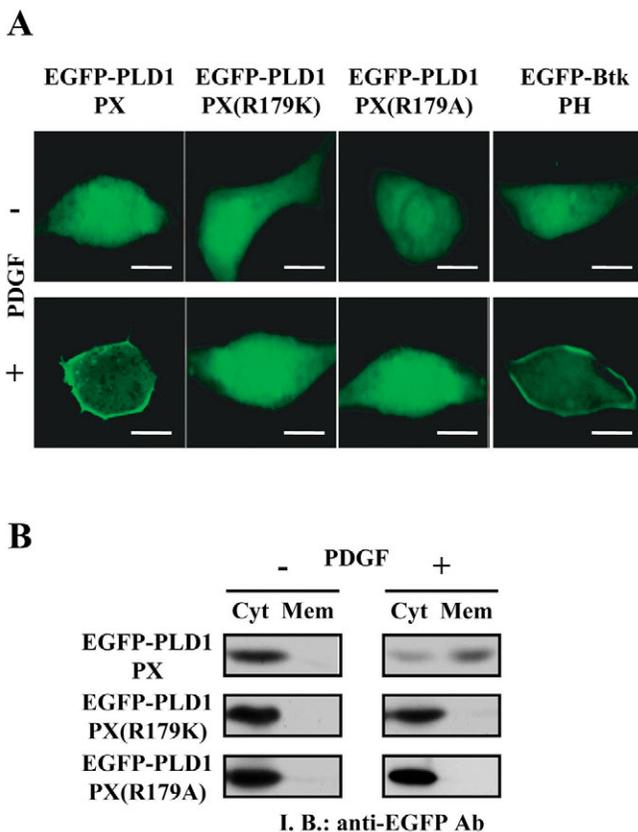


Fig. 2. The PLD1 PX domain interacts with $\text{PtdIns}(3,4,5)P_3$ in NIH-3T3 cells. NIH-3T3 cells grown on cover slips were transfected with pEGFP-PLD1 PX, pEGFP-PLD1 PX(R179K), pEGFP-PLD1 PX(R179A), or pEGFP-Btk PH. (A) After 24 hours of serum starvation, cells were treated with PDGF (25 ng/ml). After 5 minutes, cells were fixed and the localization of PLD1 PX domains was visualized by confocal microscopy. Bars, 10 μm . (B) Cells as described in (A) were cultured in dishes and fractionated into cytosolic (Cyt) and membrane (Mem) fractions. Western blotting was performed with anti-EGFP antibody and show that only the wild-type PLD1 PX domain showed membrane localization after PDGF treatment.

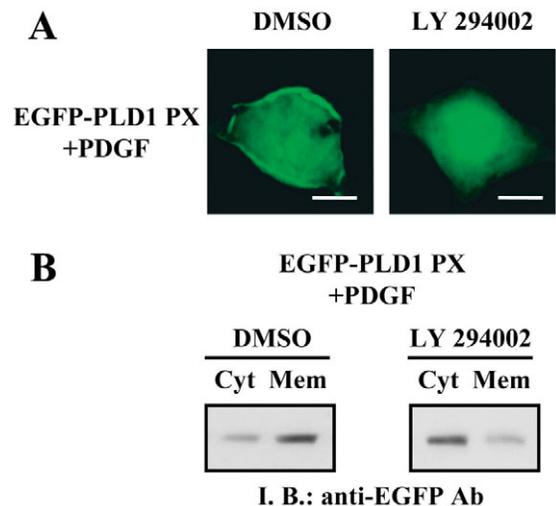


Fig. 3. Inhibition of PI3K attenuates the plasma membrane localization of PLD1 PX. NIH-3T3 cells transfected with pEGFP-PLD1 PX were serum starved for 24 hours. PDGF (25 ng/ml) was applied for 5 minutes, after the pre-incubation of cells with DMSO or 50 μM of the PI3K inhibitor LY 294002, for 20 minutes at 37°C. (A) Subcellular PLD1 PX localization was visualized by confocal microscopy. Bars, 10 μm . (B) Biochemical fractionation study showing localization of PLD1 PX in the cytosol (Cyt) or the plasma membrane (Mem), in the absence or presence of LY 294002.

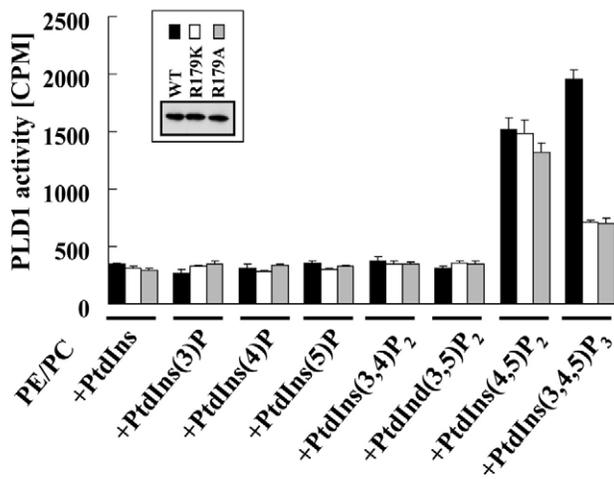
PtdIns(3,4,5) P_3 -binding module. Thus we investigated whether the interaction of PtdIns(3,4,5) P_3 with the PLD1 PX domain plays a regulatory role in the enzymatic activity of PLD. In vitro, PLD activity was measured as described in Materials and Methods, using phosphoinositide-containing lipid vesicles as a substrate. Homogenates of HEK 293 cells transfected with wild-type PLD1, R179K PLD1 or R179A PLD1 were used as an enzyme source. As shown in Fig. 4A, PtdIns(4,5) P_2 , which was previously reported to stimulate PLD (Kim et al., 1998; Sciorra et al., 1999), enhanced the activities of PLD1 wild type, R179K and R179A. Addition of ARF and GTP γ S further stimulated the PtdIns(4,5) P_2 -induced activation of wild-type PLD1 and the two mutants R179K and R179A PLD1 (data not shown), indicating that both mutants can respond to ARF, a well known activator of PLD1 (Hammond et al., 1997). Interestingly, PtdIns(3,4,5) P_3 strongly stimulated wild-type

PLD1 activity when compared with PLD1(R179K) or PLD1(R179A). Moreover, as shown in Fig. 4B, PtdIns(3,4,5) P_3 -induced PLD1 activation occurs in a dose-dependent manner. These results indicate that PtdIns(3,4,5) P_3 interacts directly with the PX domain of PLD1 and stimulates its activity in vitro.

PtdIns(3,4,5) P_3 -induced PLD1 activation promotes ERK phosphorylation

Next, we checked whether the interaction of PtdIns(3,4,5) P_3 with PLD1 PX is involved in PLD1 activity in cells. To prevent background effects caused by PLD2, we depleted endogenous PLD2 with siRNA in HEK 293 cells, and investigated PLD1 activity by measuring the amount of phosphatidylbutanol that had accumulated within 2 minutes after PDGF treatment. As shown, activity of PLD1 dramatically rose in the presence of PDGF, an effect that was prevented by the pre-treatment of LY

A



B

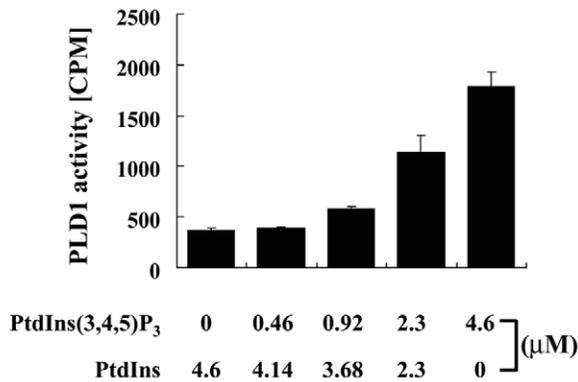
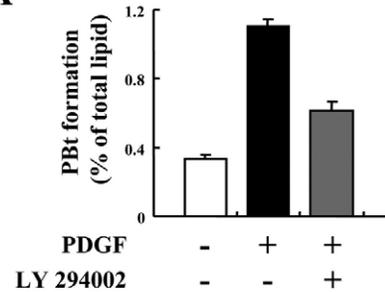


Fig. 4. PtdIns(3,4,5) P_3 activates PLD1 in vitro. (A) The effects of a range of phosphatidylinositols on the in-vitro activity of PLD1 were analyzed as described in Materials and Methods. Liposomes, reconstituted with phosphatidylethanolamine (PE), phosphatidylcholine (PC) and the indicated phosphatidylinositols, were used as substrates. Error bars indicate standard deviations. Data are representative of three independent experiments. Immunoblot in the inset indicates that the amount of PLD1 preparation used in this study were similar. (B) The dose-dependency of stimulation was checked using liposomes containing PE, PC, PtdIns and PtdIns(3,4,5) P_3 . The sum of the concentrations of PtdIns and PtdIns(3,4,5) P_3 was maintained at 4.6 μ M.

A



B

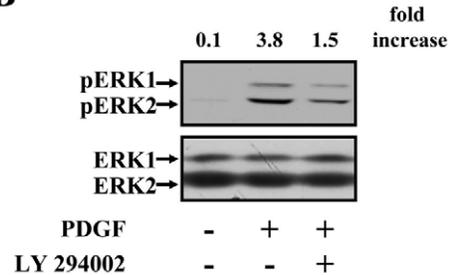


Fig. 5. PtdIns(3,4,5) P_3 -induced activation of PLD1 promotes ERK phosphorylation. (A) 5×10^5 HEK 293 cells depleted of endogenous PLD2, were transfected with 1 μ g of wild-type PLD1 DNA. 24 hours after transfection, cells were starved for 24 hours followed by an incubation with [3 H]myristic acid for 3 hours. Phosphatidylbutanol (Pbt) accumulation was measured in the presence of 0.4% butanol after 2 minutes. Cells were incubated with LY 294002 (50 μ M) or DMSO for 20 minutes before PDGF treatment. Error bars indicate standard deviations. Data are representative of three separate experiments. To check expression levels of PLD, the same amounts of cell lysates (20 μ g) were subjected to SDS-PAGE and then immunoblotted with anti-C-terminal PLD antibody (data not shown). (B) Activation of ERK1 and ERK2 induced by the stimulation of PLD were compared. (Upper panel) The phosphorylation status of ERK1/2 was analyzed by western blotting with an anti-phosphorylated-ERK antibody. (Lower panel) Expression levels of ERK1/2. The fold-increases of ERK2 phosphorylation were calculated from the ratio of phosphorylated ERK2 to unphosphorylated ERK2 (pERK2:ERK2) determined by densitometric analysis.

294002 (Fig. 5A). Previous reports have suggested that phosphatidic acid generated by PLD, mediates Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase (MAPK) pathway (Rizzo et al., 1999; Rizzo et al., 2000). Thus, we checked whether PtdIns(3,4,5) P_3 -induced PLD1 activity results in MAPK activation. As shown in Fig. 5B, high levels of phosphorylated ERK1 and ERK2 were observed after PDGF treatment. Pre-treatment with the PI3K-specific inhibitor LY 294002, however, blocked this PDGF-induced ERK1/2 phosphorylation, which correlates well with the activity profiles of PLD1. ERK2 phosphorylation is expressed as fold increase of phosphorylated ERK2 divided by total ERK2 as determined by densitometry analysis (Fig. 5B, upper immunoblots). These results indicate that generation of PtdIns(3,4,5) P_3 induces PLD1 stimulation and promotes ERK activation in HEK 293 cells.

Interaction with PtdIns(3,4,5) P_3 is crucial for the PDGF-induced PLD1 activation

We next addressed the association of PtdIns(3,4,5) P_3 with PLD1 PX in context of the enzymatic activity of PLD1. Since PDGF-induced PLD activation has been assigned to SMGs (Shome et al., 1998; Voss et al., 1999) and phospholipase C- γ 1 (PLC γ 1) (Lee et al., 1994; Yeo et al., 1994), we used HepG2 cells that stably expressed the mutant PDGF receptor Y40/51 PDGF β R. This mutant receptor can recruit PI3K but not the Ras GTPase-activating protein (Ras GAP), the Src homology 2 (SH2)-domain-containing protein-tyrosine phosphatase-2 (SHP-2) and PLC γ 1 (Bae et al., 2000; Valius and Kazlauskas, 1993). In contrast to PLD1(R179K) and PLD2, stimulation with PDGF resulted in a threefold increase of activity of endogenous and wild-type PLD1, as shown in Fig. 6. Furthermore, inhibition of PI3K by pre-incubating the

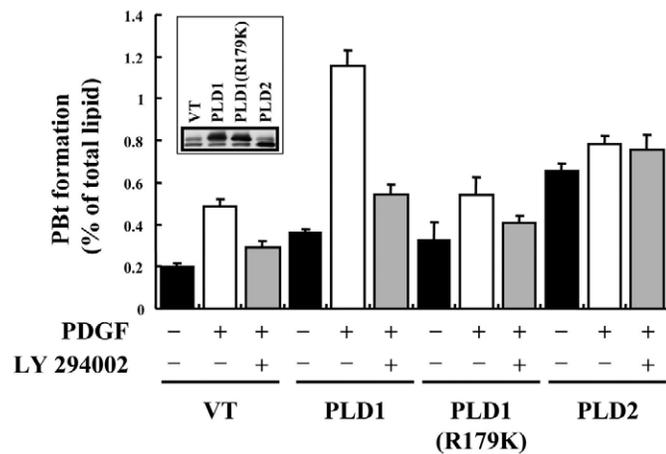


Fig. 6. Interaction with PtdIns(3,4,5) P_3 is crucial for PDGF-induced activation of PLD1. HepG2 cells stably expressing mutant Y40/51 PDGF β R were transiently transfected as indicated. PLD activity was measured for 2 minutes in the presence of PDGF (25 ng/ml). To inhibit PI3K, cells were treated with 50 μ M LY 294002 for 20 minutes before treatment with PDGF. Data are representative of three separate experiments. Error bars indicate standard deviations. Immunoblots in the inset show that the expression levels of PLD were similar.

cells with LY 294002 prevented the activation of PLD1 by PDGF. These results indicate that the PI3K pathway is involved in PDGF-mediated PLD1 activation, and that the interaction between PtdIns(3,4,5) P_3 and the PLD1 PX domain plays a crucial role in the activation of PLD1 in vivo.

Discussion

It is becoming clear that specific interactions of phosphoinositide with the PX domain are crucial for the cellular function of host proteins. In this study, we investigated whether the PLD PX domain can bind to specific phosphoinositides and the meaning of this interaction. Our findings show that PtdIns(3,4,5) P_3 has affinity for the PLD1 PX domain and enhances the enzymatic activity of PLD1.

The downstream molecular mechanisms that mediate the suggested function of PI3K in growth-factor-induced signal transduction such as cell survival, vesicle trafficking and cytoskeletal rearrangement (Downward, 1997; Fruman et al., 1998; Martin, 1998; Rameh and Cantley, 1999; Toker and Cantley, 1997) are not fully understood. To elucidate how PI3K acts, several laboratories have tried to identify PtdIns(3,4,5) P_3 -binding proteins (Fukui et al., 1998; Vanhaesebroeck et al., 2001). PDK-1 is a well known PtdIns(3,4,5) P_3 -binding protein that translocates to the plasma membrane after binding to PtdIns(3,4,5) P_3 through its PH domain, thus regulating the metabolism and cell-survival-related roles of PI3K by phosphorylating Akt. In addition, SWAP-70, a PtdIns(3,4,5) P_3 -dependent guanine nucleotide exchange factor (GEF) for Rac, can regulate membrane ruffling induced by epidermal growth factor (EGF) or PDGF (Shinohara et al., 2002). Although it was reported that the inhibition of PI3K activity results in the downregulation of PLD activity (Emoto et al., 2000; Kozawa et al., 1997; Standaert et al., 1996), the mechanism involved is unknown. Our finding that the PLD1 PX domain has affinity for PtdIns(3,4,5) P_3 is an important clue. It provides an explanation for the previously suggested molecular mechanism that PLD is involved in insulin-like growth factor I (IGF-I)-induced ERK activation in Chinese hamster ovary (CHO) cells (Banno et al., 2003). Furthermore, we found that PLD1 can mediate the activation of the MAPK pathway by interacting with PtdIns(3,4,5) P_3 , the product of PI3K activity (Fig. 5). Taken together, we suggest that PLD1 is a novel PtdIns(3,4,5) P_3 -binding protein that can mediate PI3K signaling.

PtdIns(4,5) P_2 has been considered to be a major regulator of PLD1 in enzymatic activity and cellular localization by binding to the PH domain of PLD1 (Hodgkin et al., 2000). Although PtdIns(3,4,5) P_3 has also been reported to activate PLD1 in vitro, the mechanism involved is unclear. For example, PtdIns(3,4,5) P_3 activated PLD1, purified from insect (Sf9) cells, in the presence of ARF (Hammond et al., 1997; Min et al., 1998) or Rac1 (Hodgkin et al., 2000). Under our assay conditions, we found that PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 stimulated the activity of wild-type PLD1 almost equally when GTP γ S and ARF were added (data not shown), which is in agreement with Min et al. (Min et al., 1998). In the absence of ARF, however, activation of PLD1 by PtdIns(3,4,5) P_3 was about 1.3-fold stronger than that by PtdIns(4,5) P_2 (Fig. 4). Differences between the activation potentials of PtdIns(4,5) P_2

in the presence or absence of ARF might be due to its abilities to directly bind to ARF (Randazzo, 1997) and to stimulate GDP dissociation and GTP binding of ARF (Terui et al., 1994). In conclusion, we suggest that the PX domain of PLD1 plays a role in the PtdIns(3,4,5) P_3 -dependent activation of PLD1 in vitro. We also demonstrate for the first time that PLD1 can be activated by PtdIns(3,4,5) P_3 through direct interaction in cells (Figs 5 and 6).

In the present study, we observed that the activation of PLD1 in vitro and in vivo depends on the interaction of PLD1 PX with PtdIns(3,4,5) P_3 . Another example of the PX-phosphoinositide-interaction-dependent enzymatic activation was reported for cytokine-independent survival kinase (CISK) (Virbasius et al., 2001). This study suggested that binding of the CISK PX domain to PtdIns(3) P is required for its localization to endosomes and for the activation of CISK protein kinase by IGF-I or EGF. These results suggest a new feature of the PX domains not only for the translocation but also for the activation of PX-domain-containing proteins. Furthermore, in addition to its phosphoinositide-binding pocket, PLD1 PX has a phosphorylation site for protein kinase C (PKC) and can thus be phosphorylated, which enables PLD1 to be activated upon agonist stimulation (Kim et al., 2000). Therefore, the PX domain of PLD1 is probably a center for fine-tuning and modulating enzymatic activity and localization of PLD1.

An NMR structural study of the p47^{phox} PX domain showed that, binding of the SH3 domain to the PxxP motif caused a shift in the NMR signals of residues within its phosphoinositide-binding pocket (Hiroaki et al., 2001), and that the lipid binding also causes a shift of residues within the PxxP motif (Cheever et al., 2001). This suggests that the binding properties of these two distinct motifs (the phosphoinositide-binding pocket and the PxxP motif) in the PX domain are allosterically regulated by each other. Recently, we reported that the PX domains of PLD interact with the SH3 domain of PLC γ 1 through their PxxP motifs, and that this interaction is crucial for EGF-induced PLC γ 1 activation and intracellular Ca²⁺ mobilization (Jang et al., 2003). Taken together, the PLD PX domains might be multiple-binding modules and might have multifunctionality in order to coordinate signaling from receptors. It is not clear, however, whether protein-binding to the PLD1 PX domain alters the nature of the lipid-PX interaction or vice versa. So it would be interesting to characterize the affinity and the activity profiles of the PLD1 PX domain by using a combination of binding proteins and PtdIns(3,4,5) P_3 .

PDGF stimulates a number of cellular responses following its binding to the specific cell surface receptor and the activation of intrinsic tyrosine kinase (Ullrich and Schlessinger, 1990; Williams, 1989). Although the main upstream signals of PDGF-induced PLD activation have been ascribed to the ARF, Rho (Shome et al., 1998), Ral, PKC and PLC γ (Lee et al., 1994; Voss et al., 1999; Yeo et al., 1994) pathways, no direct evidence concerning the isotype-specific activation mechanism of PLD1 by PDGF has been available. In this study, we identified PI3K as a novel signaling molecule that specifically activates PLD1 but not PLD2 upon PDGF stimulation (Fig. 6). Thus, PDGF-induced PLD1 activation can be mediated by PI3K as well as SMGs and by the PLC γ -PKC pathway for a fine-tuned regulation in cells.

PLD1 localizes in diverse subcellular membrane structures, including the endoplasmic reticulum (ER), Golgi complex, endosomes, lysosomes and in the plasma membrane (Brown et al., 1998; Jones et al., 1999; Kim et al., 1999; Roth et al., 1999; Toda et al., 1999). In this study, we found that PtdIns(3,4,5) P_3 binds to the PX domain of PLD1 and induces its localization in plasma membrane. In case of wild-type PLD1, regulation of localization is performed by complex mechanisms. Hodgkin et al. reported that the deletion mutant of PLD1 (Δ PH-PLD1), which lacked the PH domain, showed a punctate distribution in IIC9 cells (Hodgkin et al., 2000). In addition, we previously reported that palmitoylation is important for the localization of PLD1 in caveolin-enriched membranes (CEM) (Han et al., 2002). Thus it is thought that the determination of PLD1 localization is achieved by the coordinated effect of lipid modification, PX-PI(3,4,5) P_3 -binding, interaction of PH domain and polybasic motif-PI(4,5) P_2 , as well as, presumably, by association with binding proteins in the membrane. Furthermore, considering that the full-length PLD1(R179K) mutant, which cannot bind to PI(3,4,5) P_3 , still showed a localization pattern similar to the one of wild type PLD1 (data now shown), we speculate that the combination of palmitoylation and PI(4,5) P_2 -binding is a major determinant for PLD1 to localize in the plasma membrane. After that, binding of PLD1 to PX-PI(3,4,5) P_3 occurs, thereby generating additional linkage to the plasma membrane. Nevertheless, there remains a discrepancy in terms of the localization of full-length PLD1. Recently, Du et al. showed that the PX domain of PLD1 participates in the recycling of PLD1 into perinuclear vesicles after PMA stimulation of COS-7 cells (Du et al., 2003), thereby identifying PLD1 PX as a recycling regulatory domain. In NIH-3T3 cells, however, we observed no dramatic effects of LY 294002 in the localization of wild-type PLD1 (data not shown). To clarify this issue, extensive studies concerning cell type specificity, experimental conditions, mutant comparisons and lipid modifications will be necessary.

This work was supported by the program of the National Research Laboratory and the 21st Frontier Proteome Research of the Ministry of Science and Technology in the Republic of Korea.

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