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Novel Functions of the Phospholipase D2-Phox Homology Domain in Protein Kinase Cζ Activation†

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It has been established that protein kinase Cζ (PKCζ) participates in diverse signaling pathways and cellular functions in a wide variety of cells, exhibiting properties relevant to cellular survival and proliferation. Currently, however, the regulation mechanism of PKCζ remains elusive. Here, for the first time, we determine that phospholipase D2 (PLD2) enhances PKCζ activity through direct interaction in a lipase activity-independent manner. This interaction of the PLD2-Phox homology (PX) domain with the PKCζ-kinase domain also induces the activation loop phosphorylation of PKCζ and downstream signal stimulation, as measured by p70 S6 kinase phosphorylation. Furthermore, only the PLD2-PX domain directly stimulates PKCζ activity in vitro, and it is necessary for the formation of the ternary complex with phosphoinositide-dependent kinase 1 and PKCζ. The mutant that substitutes the triple lysine residues (Lys^{101}, Lys^{102}, and Lys^{103}) within the PLD2-PX domain with alanine abolishes interaction with the PKCζ-kinase domain and activation of PKCζ. Moreover, breast cancer cell viability is significantly affected by PLD2 silencing. Taken together, these results suggest that the PLD2-mediated PKCζ activation is induced by its PX domain performing both direct activation of PKCζ and assistance of activation loop phosphorylation. Furthermore, we find it is an important factor in the survival of breast cancer cells.

Protein kinase C (PKC) has been implicated in many cellular key functions, such as cell proliferation, survival, and migration (2, 40, 44). The PKC family is subclassified into three groups—classical, novel, and atypical PKC—according to differences in the lipid activation profile (42). It has been established that the phosphorylation and activation of atypical PKCζ, especially, is an important factor in the survival of cancer cells (21, 41). The phosphorylation of PKCζ is one of the main mechanisms for regulating its activity. Recently, it has been reported that moderate activation of PKCζ is mediated through activation loop phosphorylation by phosphoinositide-dependent kinase 1 (PDK-1), followed by a subsequent autophosphorylation (8, 38, 59). PKCζ is also stimulated by the interaction of acidic lipids, including phosphatidic acid (PA) and phosphoinositides. Due to its structural uniqueness, PKCζ is insensitive to second messengers, such as Ca^{2+} or diacylglycerol (DAG), known to be potent activators of the other families (46). Therefore, the activation of PKCζ may be expected to rely on a peculiar mechanism, which is perhaps regulated by many cellular proteins. However, the exact protein-protein interactions intrinsic to the regulation mechanism of PKCζ remain largely unclear.

Phospholipase D (PLD) exists as a membrane-bound protein and is widely distributed in a variety of cells. It hydrolyzes phosphatidylcholine to generate choline and PA as a response to diverse stimuli. In many cancer cells, the abnormal overexpression of PLD is associated with the promotion of mitogenesis, oncogenic transformation, and cell proliferation and the suppression of apoptosis (7, 10, 15). PLD activity is most commonly controlled by several regulators, such as PKC or small G protein (ARF, Rho, and RalA), in the presence of phosphatidylinositol 4,5-bisphosphate (PIP2) (5, 57, 58, 60). To date, two phosphatidylcholine-specific mammalian isoforms of PLD, PLD1 and PLD2, have been isolated and characterized (9, 22, 23). PLD1 is localized mainly in the Golgi apparatus and perinuclear vesicle regions in multiple cell types (13, 18, 58), whereas PLD2 is primarily located in the plasma membrane (12, 19). The differences in the localization of these PLDs can provide important clues suggesting their specific roles in various situations and cell types (12). PLD also has specific domains, such as the Phox (PX) and the pleckstrin homology (PH) domains. Although it is known that the PX and PH domains are mediated by protein-protein and protein-lipid interactions, the exact roles of these domains remain unclear and debatable. Recently, our group had reported that both the activation and phosphorylation of PLD1 are regulated by PKCα in phorbol myristate acetate-treated COS-7 cells (32) and that PLD2 activity is also stimulated by PKCδ in the neuronal cell (24). Other research had also reported the interrelationships between PLD and PKC isoforms in a variety of cell types (1, 3, 17, 25, 37, 45, 49, 51). So, the regulation mechanisms of PLD by PKC are relatively well known, but the details of PKC regulation mechanisms, regarding the PLD, are still an enigma.

In this study, we determined that PLD2 directly interacts with PKCζ, regardless of lipase activity, and suggested that the PLD2-PX domain can play an important role as an activator of PKCζ both through direct stimulation and modulating the activation loop phosphorylation of PKCζ.

MATERIALS AND METHODS

Materials. An enhanced chemiluminescence kit (ECL system) and glutathione Sepharose 4B were purchased from Amersham Pharmacia Biotech (Bucking-

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hamshire, U.K.). Myelin basic protein and protease inhibitor cocktail were from Sigma. Rabbit polyclonal anti-phospho Thr410 PKCζ was kindly provided by Alex Toker (Harvard Medical School, Boston, Mass.) and purchased from Cell Signaling (Beverly, Mass.). Anti-actin antibody was from ICN Pharmaceuticals (Costa Mesa, Calif.). Polyclonal anti-phospho Thr421 and Ser424 p70 S6 kinase were purchased from Cell Signaling. Rabbit polyclonal PKCζ was from Santa Cruz. Anti-PKCα and anti-PKCδ monoclonal antibodies were purchased from Transduction Laboratories (Lexington, Ky.). Human recombinant PKCζ, myristoylated PKCζ pseudosubstrate inhibitor, and β-octylglucopyranoside were from Calbiochem (San Diego, Calif.). Human recombinant PKCζ is phosphorylated at the threonine 410 residue and has a biologically active ability. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgA, IgM, and IgG were from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). [γ-^{32}P]ATP and [^{3}H]myristic acid were from Perkin-Elmer Life Sciences (Boston, Mass.). Immobilized protein A was from Pierce and Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies, Inc. (Gaithersburg, Md.). Polyclonal antibody for the recognition of PLD1 and PLD2 was generated as described previously (50).

Purification of recombinant PLD from Sf9 cells. Hexahistidine (His_6)-tagged PLD was expressed in Sf9 cells and purified by chelating Sepharose affinity column chromatography, as described previously (31).

Cell culture. COS-7 cells were maintained in DMEM supplemented with 10% (vol/vol) bovine calf serum, at 37°C, in a humidified, CO_2-controlled (5%) incubator. HEK (human embryonic kidney 293, MDA-MB 468, and Hs 578T cells were cultured under the same conditions, but with DMEM containing 10% (vol/vol) fetal bovine serum. As described previously, these cells were transfected using Lipofectamine (Life Technologies, Inc.) for the transient expression of the indicated plasmids and small interfering RNA (siRNA).

siRNA sequences. The siRNA of three independent 21-nucleotide sequences corresponding to human PLD2 sequences (nucleotides 703 to 723, AAGAGG UGGCUGGUGGUGAAG; nucleotides 1234 to 1254, AACAGUGGCUAUA GCAAGAGG; and nucleotides 1966 to 1986, AAGGUGGGCGAUGAGAUU GUG) and a human PLD1 sequence (nucleotides 1454 to 1474, AAGGUGGG ACGACAAUGAGCA) were purchased from Dharmacon Research Inc. (Lafayette, Colo.). The PLD2 siRNA was used in the mixtures of three independent sequences for efficient silencing. Results of a BLAST search of all siRNA sequences revealed no significant homology to any other sequences in the database program.

Construction and preparation of GST fusion proteins. Glutathione *S*-transferase (GST) fusion proteins of PLD2 and PKCζ were generated as previously described (30). To further construct the PX and PH domains and F1-1, -2, -3, -4, -5, -6, and -6M, the N-terminal region (amino acids 1 to 314) of human PLD2 was used as a template. These mutants of PLD2 were amplified by PCR using specific primers, digested with restriction enzymes EcoRI and XhoI, and ligated into pGEX-4T1 vector (Amersham Pharmacia Biotech). PKCζ deletion mutants such as ζF1, ζF2, ζF3, and ζF4, and ζF3-C1, -C2, -C3, -N1, -N2, -N3, -C2-1, and -C2-2 were also subcloned by the same methods as previously described. *Eschericha coli* BL21 cells were transformed with individual expression vectors encoding the GST fusion proteins and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C for 4 h. After harvesting the cells, GST fusion proteins of PLD2 and PKCζ were purified, as previously described. These GST fusion proteins were purified by standard methods, by using glutathione Sepharose 4B (30). PLD2-K3A was PCR amplified using forward primer 5′-GG GGT ACC ATG ACG GCG ACC CCT GAG AGC-3′, reverse primer 5′-TGG ACA ACC GCG GCG GCA TAC CGT CAT-3′, forward primer 5′-ATG ACG GTA TGC CGC CGC GGT TGT CCA-3′, and reverse primer 5′-GC TCT AGA CTA TGT CCA CAC TTC TAG GGG-3′, digested with restriction enzymes KpnI and XbaI, and subcloned into mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, Calif.).

Generation of PLD2-WT and PLD2-K3A addback plasmids. One of three independent 21-nucleotide sequences (siRNA) corresponding to human PLD2 sequences is nucleotides 703 to 723: AAGAGGTGGCTGGTGGTGAAG. Three residues of human PLD2 cDNA are substituted to AAGAGATGGCTA GTAGTGAAG for addback mutants of PLD2-WT and -K3A. These mutations are silencing mutations. This gene is subcloned into mammalian expression vector pcDNA3.1 (Invitrogen) and digested with restriction enzymes KpnI and XbaI. These mutations are confirmed through nucleotide sequence analysis.

In vitro binding analysis. In vitro binding between all of the GST fusion proteins and PLD2 was performed in PLD assay buffer (50 mM HEPES-NaOH, pH 7.4, 3 mM EGTA, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 80 mM KCl), containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail, at 4°C for 1.5 h. After a brief centrifugation, the precipitated complexes were washed three times in the same buffer before being loaded onto a polyacrylamide gel (30).

Coimmunoprecipitation. COS-7 cells were transfected in combination with the indicated plasmids. These cultured cells were harvested and lysed with PLD assay buffer containing 1% Triton X-100, 1% cholic acid, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. After a brief sonication, the lysates were centrifuged at 100,000 × *g* for 30 min, and the supernatants (2 mg) were incubated with anti-PLD or anti-HA antibody-conjugated protein A-Sepharose for 4 h. After a brief centrifugation, the coimmunoprecipitated complexes were washed three times with the same buffer before being loaded onto a polyacrylamide gel.

Immunoblot analysis. Immunoblot analysis was performed as previously described (29). In brief, proteins were denatured by boiling at 95°C for 5 min in a Laemmli sample buffer. The denatured proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking in TTBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% skimmed milk powder, the membranes were incubated with individual monoclonal or polyclonal antibodies and subsequently reincubated with either anti-mouse or anti-rabbit IgG, as required, coupled with horseradish peroxidase. Detection was performed using an enhanced chemiluminescence kit, according to the manufacturer's instructions. Immunoblot intensity was determined using a Fuji BAS-2000 image analyzer (Fuji, Tokyo, Japan).

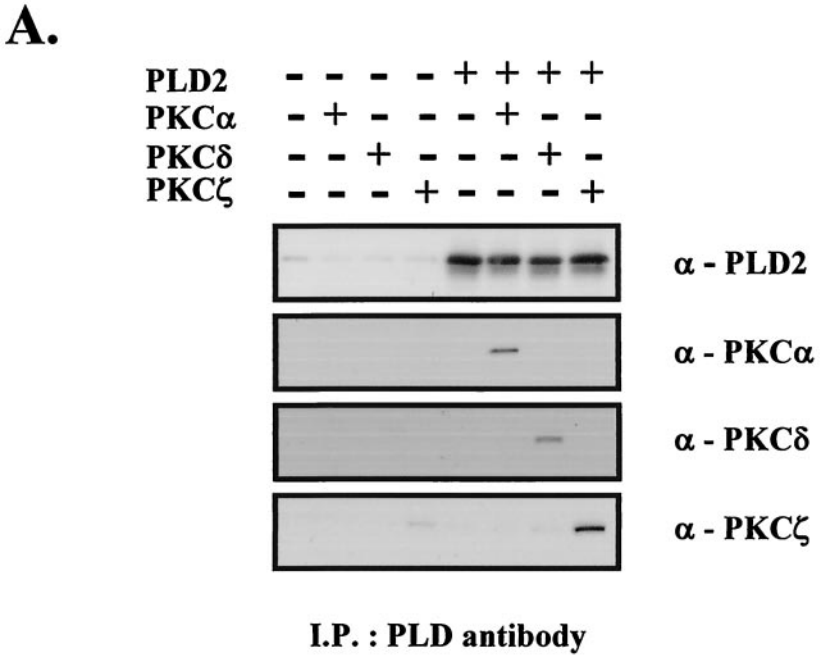
Measurement of PLD activity in cells. PLD activity was assayed by measuring the formation of phosphatidylbutanol, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol, as previously described (36). In brief, the cells were labeled with [^{3}H]myristic acid (10 μCi/ml) for 4 h and then washed twice with DMEM. The labeled cells were incubated with 0.4% 1-butanol for 5 min, harvested in 0.8 ml of methanol and 1 M NaCl (1:1), and mixed with 0.4 ml of chloroform. After vortexing, the tubes were centrifuged at 15,000 ×*g* for 1 min, the organic phase was dried, and the lipids were separated by a Silica Gel 60 TLC plate, which was then developed with ethyl acetate-trimethylpentane-acetic acid (9/5/2, vol/vol/vol). The amount of [^{3}H]phosphatidylbutanol formed was expressed as a percentage of the total ^{3}H-lipid, to account for differences in cell labeling efficiency.

PKCζ kinase assay. PKCζ activity was measured by the aforementioned protocol, with minor modifications (6). In brief, PKCζ was incubated with myelin basic protein (2 μg) at 37°C for 30 min in kinase buffer (50 mM Tris-HCl, pH 7.5, 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM phenylmethylsulfonyl fluoride, 4 μg of phosphatidylserine, 5 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP [3,000 Ci/mmol]), and an in vitro phosphorylation assay was performed. This reaction was terminated by the addition of 15 μl of 2× SDS sample buffer and boiling of the samples for 5 min. The sample was analyzed by SDS-PAGE and exposed to photographic film for autoradiography.

MTT assay in the breast cancer cells. The MTT assay was used as a crude measurement of cell viability (16). MTT is a yellow tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] which is converted by metabolically active cells into a colored water-insoluble formazan salt. The cells were seeded at 5×10^4/ml (100 μl/well) in 96-well plates, grown for 36 h, and depleted with serum for indicated times. MTT reagent (5 mg/ml in phosphate-buffered saline) was then added to the cells (10 μl/well), and the cultures were incubated at 37°C for 2 h. The reaction was stopped by the addition of 70 μl of isopropanol (including 0.2% HCl), and the tetrazolium crystals were dissolved at room temperature. The samples were measured by a Biotrak II Reader at a test wavelength of 450 nm.

RESULTS

PKCζ interacts with PLD2. Our group had previously published results suggesting that the activation and phosphorylation of PLD1 are regulated by PKCα in phorbol myristate acetate-treated COS-7 cells (32) and that PLD2 activity is also controlled by PKCδ in PC12 cells (24). To delineate the specificity of interactions between PLD2 and PKC isozymes, we attempted to examine the physical associations between PLD2 and PKC isozymes in COS-7 cells. As shown in Fig. 1A, we observed that three PKC isozymes, PKCα, -δ, and -ζ, interact with PLD2 in a fashion similar to their respective interaction potencies. Furthermore, PLD2 activity is upregulated by PKCα- or PKCδ-overexpressed cells, but its activity is unaf-



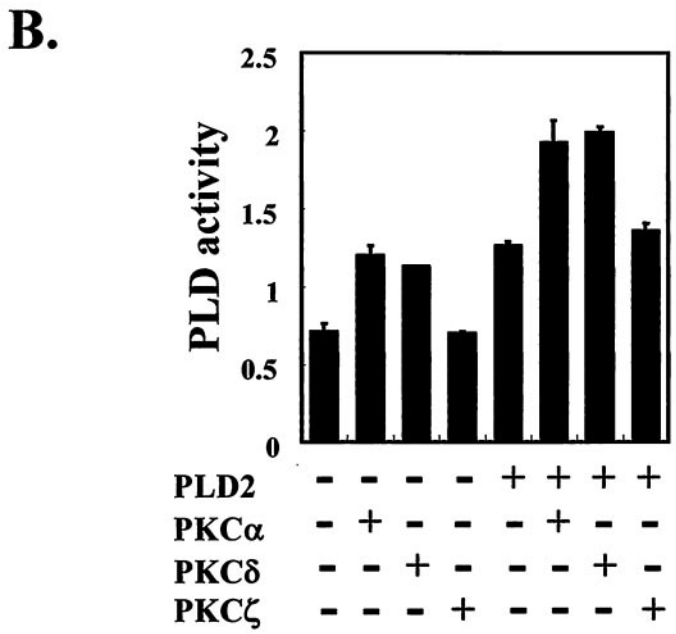


FIG. 1. The effects of representative PKC isoforms, including α, δ, and ζ, on PLD2 interaction and activity. COS-7 cells transiently transfected with indicated constructs were cultured for 36 h. A. The cells were lysed with buffer containing 1% Triton X-100 and 1% cholic acid. The extracts were immunoprecipitated (I.P.) with anti-PLD antibody. After a brief centrifugation and washing, the precipitates were subjected to SDS-PAGE and immunoblotted with anti-PLD2 and anti-PKCα, -δ, and -ζ antibodies. B. The cells were labeled with [^{3}H]myristic acid for 4 h. The measurement of [^{3}H]phosphatidylbutanol formation was quantified as described in Materials and Methods. The data represent one of two independent experiments.

fected by PKCζ in the same cells (Fig. 1B). We also obtained similar results in HEK 293 cells (data not shown). These results demonstrated that PLD2 associates with PKCζ, resulting in the formation of a physical complex.

PLD2 specifically interacts with PKCζ in a lipase activity-independent manner. In the next phase of the experiment, we further confirmed the specificity of PLD isozymes on PKCζ binding. As shown in Fig. 2A, PLD2, rather than PLD1,

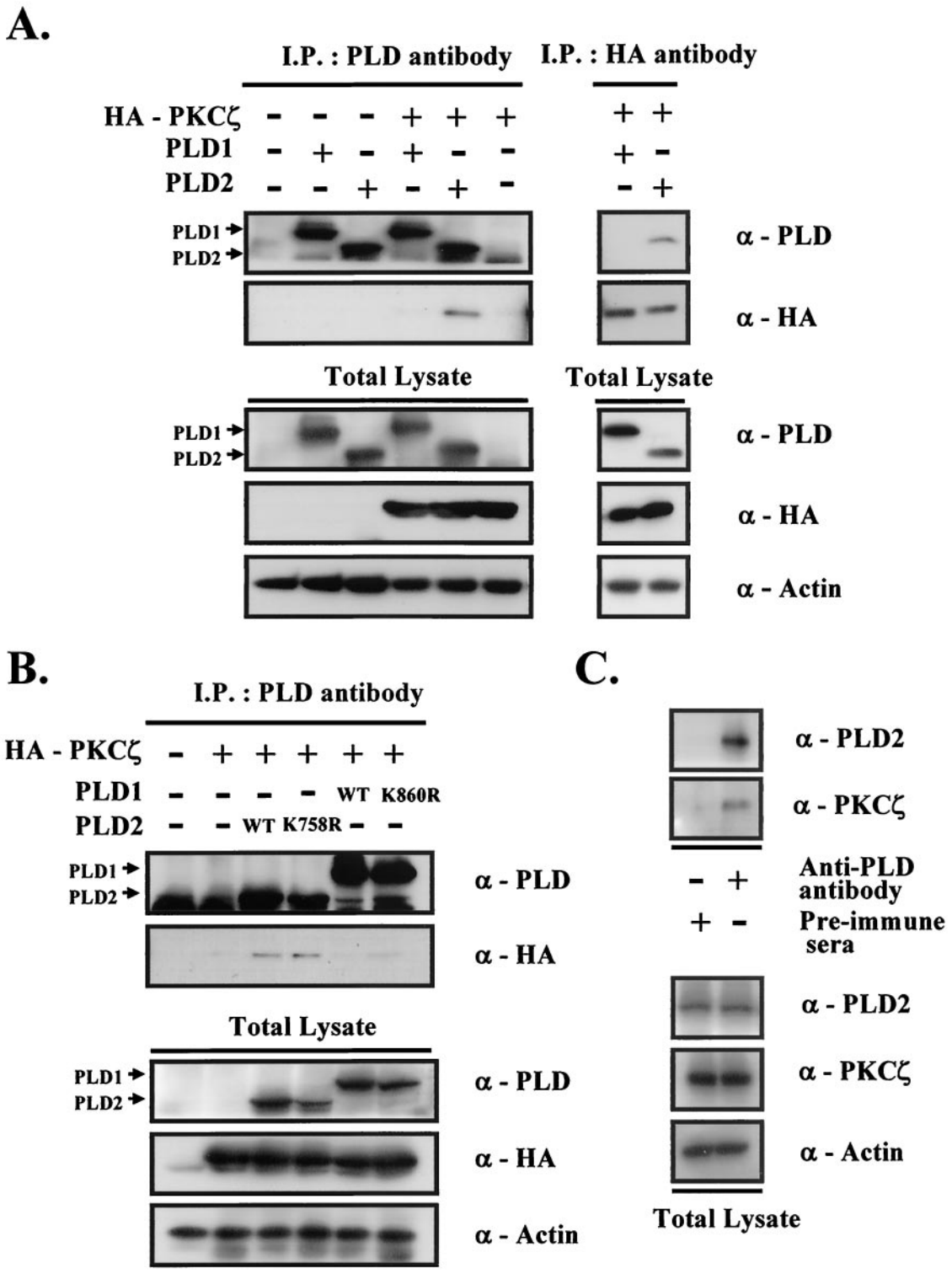


FIG. 2. The effects of PLD isozymes and lipase activity on PKCζ interaction. A. COS-7 cells transiently transfected with indicated constructs were grown for 36 h. The cell lysates were solubilized with buffer containing 1% Triton X-100 and 1% cholic acid. The extracts were immunoprecipitated (I.P.) with anti-PLD antibody or antihemagglutinin (HA) antibody. The complexes were briefly washed with the same buffer, subjected to SDS-PAGE, and immunoblot analyzed with anti-PLD antibody and anti-HA antibody. B. COS-7 cells were transfected with indicated plasmids, cultured, and harvested with lysis buffer. The extracts were immunoprecipitated with anti-PLD antibody, and the precipitates were subjected to immunoblot analysis with the indicated antibodies. C. COS-7 cells (2 mg) growing in media supplemented with 10% bovine calf serum were lysed with buffer containing 1% Triton X-100 and 1% cholic acid. Immunoprecipitation with the indicated antibodies was performed as described in Material and Methods and 40 μg of cell lysate was loaded for the total lysate blot.

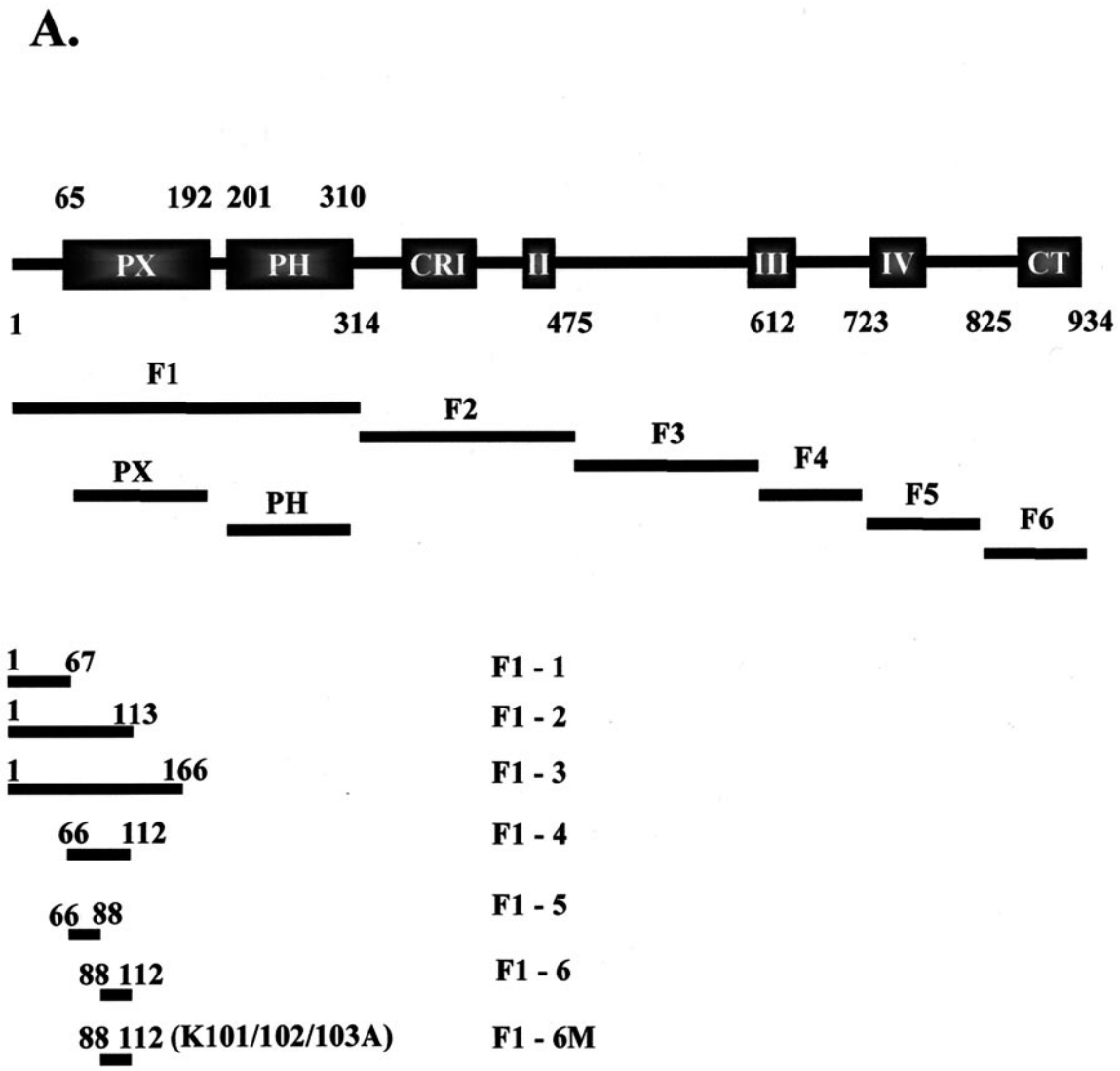


FIG. 3. The requirement of the triple lysine residues within the PLD2-PX domain for PKCζ interaction. A. Primary structure and individual domains of PLD2 used in this study. GST fusion proteins containing individual different domains of PLD2 were expressed in bacteria and purified by glutathione Sepharose beads. The individual fragments of PLD2 were followed by F1 (amino acids [a.a.] 1 to 314), F2 (a.a. 315 to 475), F3 (a.a. 476 to 612), F4 (a.a. 613 to 723), F5 (a.a. 724 to 825), F6 (a.a. 826 to 934), PX domain (a.a. 65 to 192), and PH domain (a.a. 201 to 310). F1-1 (a.a. 1 to 67), F1-2 (a.a. 1 to 113), F1-3 (a.a. 1 to 166), F1-4 (a.a. 66 to 112), F1-5 (a.a. 66 to 88), F1-6 (a.a. 88 to 112), and F1-6M (a.a. 88 to 112; K101/102/103A). B, C, D, and E. GST fusion proteins containing individual fragments of PLD2 were incubated with COS-7 cell lysates as a source of PKCζ for 1.5 h. After brief centrifugation and washing, the precipitates were subjected to SDS-PAGE and analyzed with anti-PKCζ antibody. The details of the experiment are described in Materials and Methods. The total amount of GST fusion proteins used in this experiment was shown by Ponceau S staining. I.B., immunoblot.

strongly interacts with PKCζ. To investigate the involvement of PLD lipase activity in the interactions between proteins, we transfected the indicated cDNA encoding plasmids in COS-7 cells. As shown in Fig. 2B, the PLD2 lipase inactive mutant (PLD2-K758R) as well as the PLD2 wild type (PLD2-WT) interact with PKCζ, regardless of lipase activity. Furthermore, as shown in Fig. 2C, we also determined that endogenous PLD2 as a major isoform in COS-7 cells is coimmunoprecipitated with endogenous PKCζ. These results demonstrated that the specific interaction between PLD2 and PKCζ occurs in a lipase activity-independent manner.

The triple lysine residues within the PLD2-PX domain are required for interaction with PKCζ. We also confirmed that the purified PLD2 directly interacts with PKCζ in vitro (data not shown). To determine the PKCζ-binding region in PLD2, we generated GST fusion fragments of PLD2. The schematic diagram and the individual GST fusion fragments of PLD2 used in this study are depicted in Fig. 3A. We performed in vitro binding analysis with GST fusion fragments of PLD2. The N-terminal region (amino acids 1 to 314) of PLD2 is required for binding to PKCζ (Fig. 3B). In the N-terminal region, the PX domain is necessary for interaction with PKCζ (Fig. 3C).

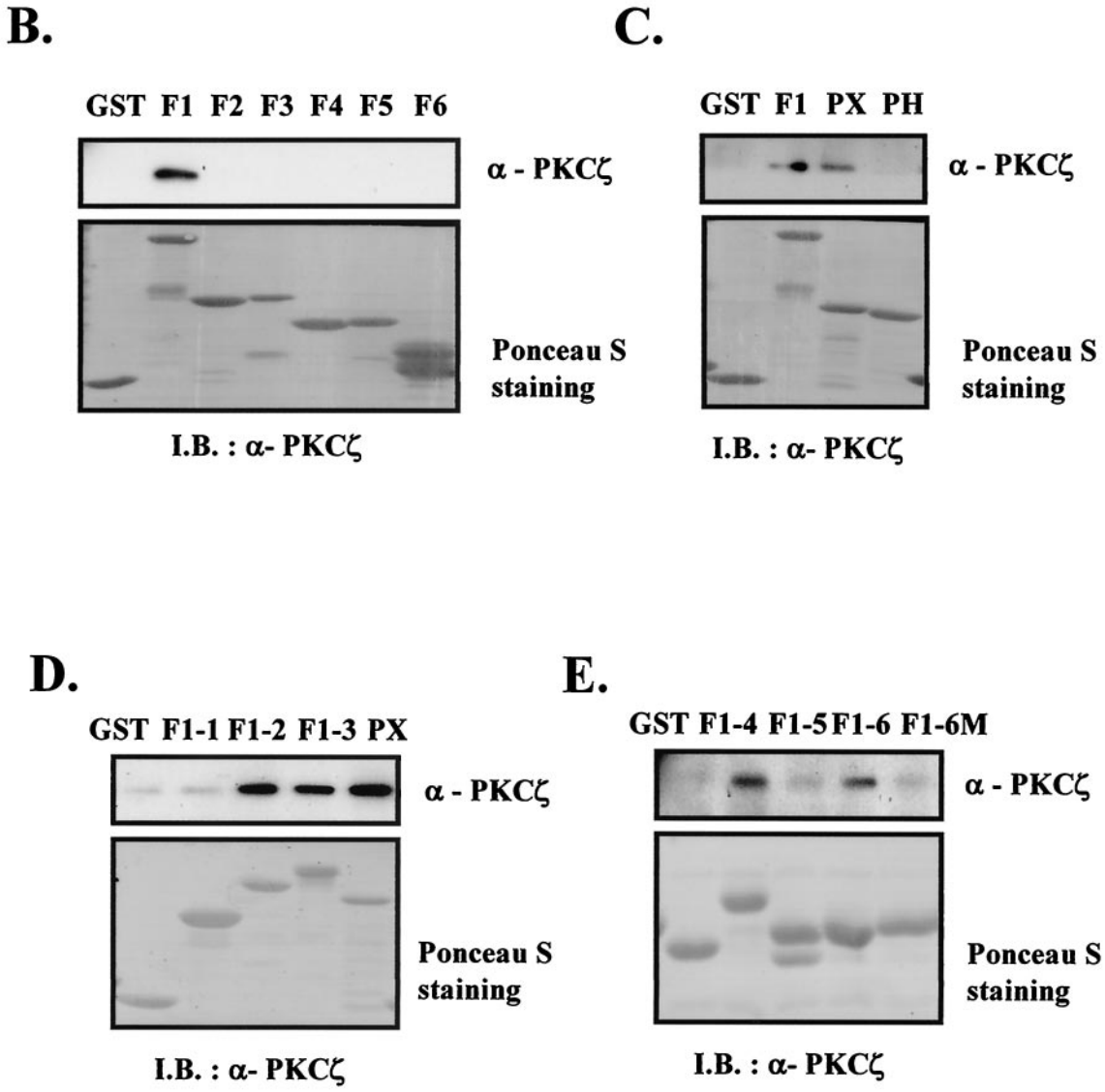


FIG. 3—*Continued*.

We generated serial deletion mutants of the PX domain to do fine mapping of the binding site. It is critical for the amino acid 68 to 113 region derived from serial deletion mutants of the PLD2-PX domain and, especially, for the amino acid 88 to 112 region to interact with PKCζ. This was verified through detailed binding analysis (Fig. 3D and E). It has been reported that the positive- or negative-charge residues of PKCζ interacting proteins are important for the associations between both proteins (43, 53). On the basis of previous reports, we generated a mutant in the triple lysine residues (Lys^{101}, Lys^{102}, and Lys^{103}) within the PLD2-PX domain and also confirmed that these residues are critical in the interaction with PKCζ (Fig. 3E). Furthermore, we generated a PLD2 mutant, in which the triple lysine residues were substituted with alanine (PLD2-K101/102/103A; PLD2-K3A). Coimmunoprecipitation binding analysis showed that the PLD2-K3A mutant abolishes interaction with endogenous PKCζ in COS-7 cells (see Fig. S1 in the supplemental material). Taken together, these results led us to the conclusion that the triple lysine residues within the PLD2-PX domain are responsible for association with PKCζ.

The PKCζ-kinase domain is responsible for direct interaction with PLD2. PKCζ is comprised of three distinctive domains, including Phox and Bem1p (PB1), C1, and the serine/threonine kinase domains. The GST fusion fragments of PKCζ used in this study are shown in Fig. 4A. We prepared GST fusion fragments of PKCζ and performed GST pull-down assays. As shown in Fig. 4B, the PKCζ-kinase domain interacts exclusively with PLD2 purified from Sf9 cells and not with the PB1 and C1 domains. To specifically verify the potential interaction region, we generated serial deletion mutants of the PKCζ-kinase domain and carried out GST pull-down assays. As shown in Fig. 4C and D, the amino acid 348 to 370 region within the PKCζ-kinase domain is responsible for interaction with PLD2. Taken together, these results suggest that the amino acid 348 to 370 region within the PKCζ-kinase domain is required for binding to PLD2.

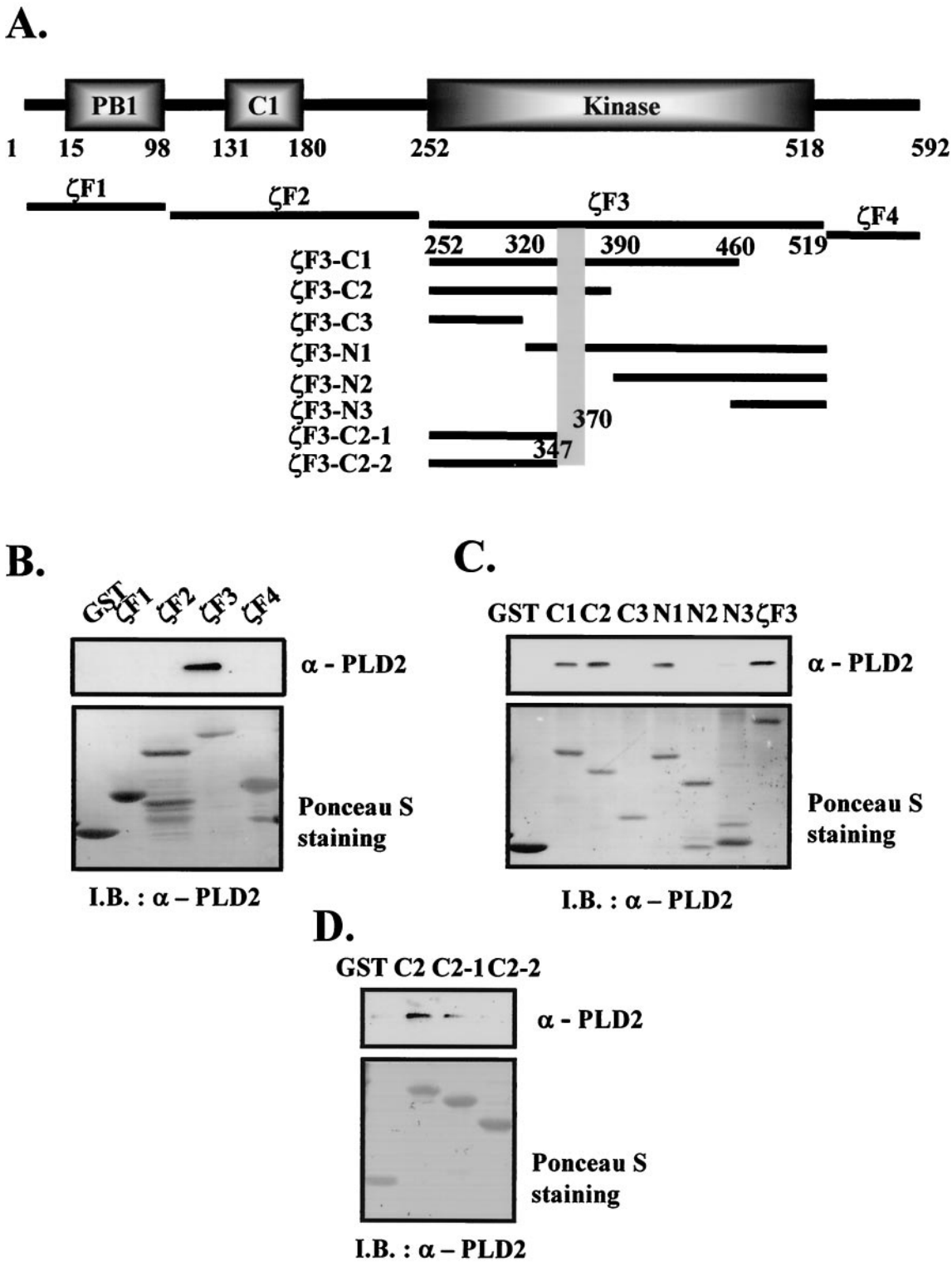


FIG. 4. The interaction of PLD2 with the amino acid 348 to 370 region within the PKCζ-kinase domain. A. Primary structure and individual domains of PKCζ used in this study. GST fusion proteins containing distinctive domains of PKCζ were expressed in *E. coli* and purified by glutathione Sepharose beads. The individual fragments of PKCζ were followed by ζF1 (amino acids [a.a.] 1 to 98), ζF2 (a.a. 99 to 251), ζF3 (a.a. 252 to 519), ζF4 (a.a. 520 to 592), ζF3-C1 (a.a. 252 to 460), ζF3-C2 (a.a. 252 to 390), ζF3-C3 (a.a. 252 to 320), ζF3-N1 (a.a. 320 to 519), ζF3-N2 (a.a. 390 to 519), ζF3-N3 (a.a. 460 to 519), ζF3-C2-1 (a.a. 252 to 370), and ζF3-C2-2 (a.a 252 to 347). B, C, and D. GST fusion proteins containing PKCζ fragments were incubated with recombinant PLD2 purified from Sf9 cells. After brief centrifugation and washing, glutathione bead complexes were subjected to SDS-PAGE and analyzed by immunoblotting (I.B.) with anti-PLD2 antibody. The total amount of GST fusion proteins used in this experiment was shown by Ponceau S staining.

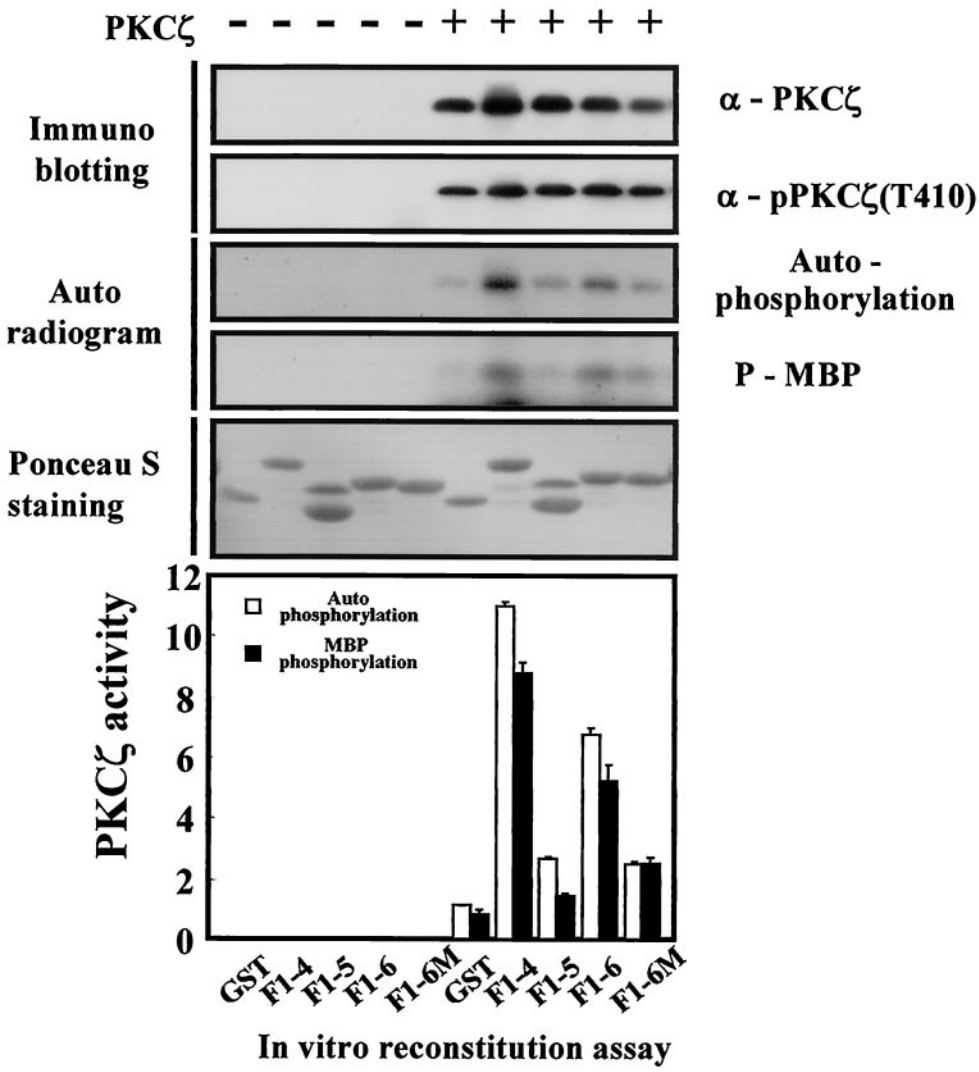


FIG. 5. The effects of the specific binding motif within the PLD2-PX domain on PKCζ activity. The activation loop-phosphorylated PKCζ was incubated with specific fragments within the PLD2-PX domain, such as F1-4, F1-5, F1-6, and F1-6M in kinase buffer (50 mM Tris-HCl, pH 7.5, 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM phenylmethylsulfonyl fluoride, 4 μg of phosphatidylserine, 5 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP [3,000 Ci/mmol]) for 30 min, and an in vitro phosphorylation assay was performed. This reaction was terminated by the addition of SDS sample buffer, and the precipitates were subjected to SDS-PAGE and exposed to photographic film for autoradiography. The data represent one of three independent experiments. The detailed procedures are described in Materials and Methods.

The F1-6 fragment directly stimulates PKCζ activity in vitro. Considering the findings that the triple lysine residues within the PLD2-PX domain directly interact with the PKCζ-kinase domain (Fig. 3 and 4), we examined the kinase activity of PKCζ to substantially characterize the meaning of the interactions between the two proteins. We performed in vitro phosphorylation assays with maltose binding protein (MBP) and phosphorylated PKCζ. As shown in Fig. 5, the F1-4 fragment corresponding to the amino acid 66 to 112 region within the PLD2-PX domain exhibits an approximate 10-fold increase in autophosphorylation and displays an approximate eightfold increase in MBP phosphorylation and, interestingly, the F1-6 fragment corresponding to the amino acid 88 to 112 region induces about a sixfold increase in PKCζ activity. However, the F1-5 and F1-6M fragments, which are deficient in binding ability, result in a little less than a twofold increase in PKCζ activity. The obvious conclusion is that PKCζ activity is more efficiently stimulated by F1-6 than by F1-6M. These results suggest that the amino acid 88 to 112 region within the PLD2-PX domain has the ability to directly stimulate PKCζ activity.

PLD2 protein significantly stimulates PKCζ activity in cells. Because the PLD2-PX domain can directly stimulate PKCζ activity in vitro (Fig. 5), we attempted to verify whether the PLD2-mediated PKCζ activation induces the propagation of downstream signaling flow in cells. It has been reported that PKCζ induces p70 S6 kinase activation in murine prostate cancer cells such as TRAMP (21). Actually we observed that activation of p70 S6 kinase is potentiated in PKCζ-WT adenovirus-infected COS-7 cells and decreased in PKCζ-DN-expressing cells under similar conditions without changing the PLD2 expression (see Fig. S2 in the supplemental material).

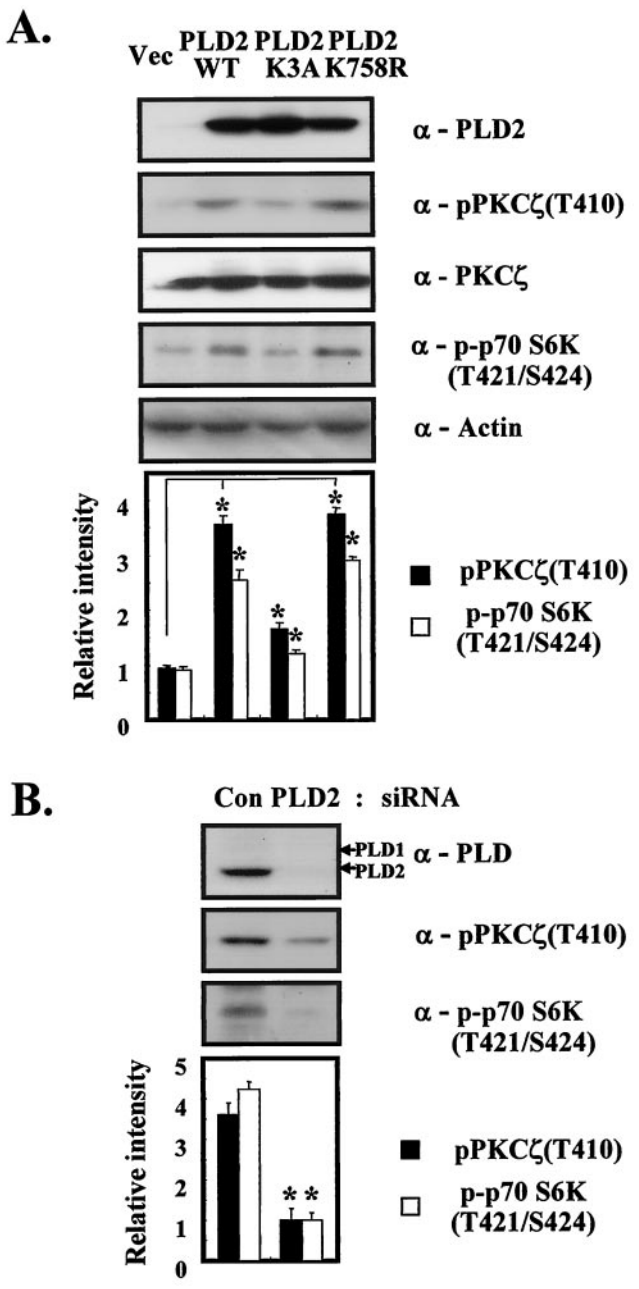


FIG. 6. The effects of PLD protein on activation of PKCζ and p70S6K. A and B. COS-7 cells were transfected with the indicated plasmids encoding various PLD2 constructs or siRNA for control (luciferase) and PLD2, cultured for 36 h, starved in serum for 12 h, and then lysed with buffer containing 0.1% Triton X-100 for 30 min on ice. The cell lysates, removed from the crude nuclear fraction using centrifugation, were analyzed by immunoblotting with indicated antibodies. The *n*-fold increase is expressed as the percentage of the indicated antibodies' intensity above the basal level. Results are expressed as average values ± standard errors of three independent experiments. *, P of <0.05 compared with vector (Vec) or control (Con) siRNA-transfected cells.

As shown in Fig. 6A, the activation of p70 S6 kinase is enhanced in PLD2-WT- and PLD2-K758R-overexpressed COS-7 cells, but PLD2-K3A had no effect on p70 S6 kinase activation in comparison with vector-transfected cells. Furthermore, when endogenous PLD2 is depleted by siRNA, the activation of p70 S6 kinase is lessened in comparison with that of control siRNA for luciferase (Fig. 6B). Interestingly, as shown in Fig. 6A, we observed that the activation loop phosphorylation (pT410) of endogenous PKCζ was significantly enhanced in PLD2-WT and -K758R cells but not PLD2-K3A-transfected cells. When endogenous PLD2 expression was silenced with PLD2 siRNA, the activation loop phosphorylation of PKCζ decreased dramatically (Fig. 6B). Taken together, these results suggest that the activation and phosphorylation of PKCζ might be regulated by PLD2 protein, in a correlative fashion with p70 S6 kinase activation.

The ternary complex including PDK-1, PLD2, and PKCζ is formed in COS-7 cells. Because the activity of PDK-1 phosphorylating Akt has been unaffected by the extent of PLD2 expression, indicating that PLD2 may not directly affect PDK-1 activity (see Fig. S3 in the supplemental material), we examined whether PLD2 exists in the physical complex of PDK-1 and PKCζ, hence increasing the activation loop phosphorylation of PKCζ. As shown in Fig. 7A, PLD2-K758R as well as PLD2-WT existed in the ternary complex with PDK-1 and PKCζ. However, PLD2-K3A did not exist in this complex. PLD2-K3A bound with PDK-1 but not PKCζ, and PLD2-K3A had no effect on the complex arising from the interaction of PKCζ with PDK-1. Since the PLD2-PX domain was involved in direct interaction with PKCζ (Fig. 3C), we examined whether it was sufficient for ternary complex formation and enhanced activation loop phosphorylation of PKCζ. As shown in the Fig. 7B, surprisingly, the PLD2-PX domain was found to exist in a physical complex with PKCζ and PDK-1 and to enhance activation loop phosphorylation of PKCζ. But the PLD2-PX-K3A domain did not form the physical ternary complex with PKCζ and PDK-1 and had no effect on binding of PKCζ to PDK-1. Taken together, these results suggested that the PLD2-PX domain also plays the role of a critical cofactor, helping the activation loop phosphorylation of PKCζ in a ternary complex with PKCζ and PDK-1.

Cell viability is affected by PLD2 silencing in breast cancer cells. Recent reports have demonstrated that the regulation of PKCζ activity is relevant to cell survival and apoptosis in breast cancer cells (41) and also that PLD2 is highly overexpressed in a wide variety of human cancer cells (48, 60, 61, 63). Therefore, we examined whether the activation of PKCζ is controlled by PLD2 expression. In Fig. 8A and B, we demonstrated that activation loop phosphorylation and activity of PKCζ decreased dramatically under PLD2 silencing conditions in breast cancer cells containing Hs 578T and MDA-MB 468 cells. The activation of p70 S6 kinase also significantly decreased under the same circumstances with a concomitant correlation with the attenuation of PKCζ activation loop phosphorylation. The PDK-1 activity was unaffected by PLD silencing in breast cancer cells (see Fig. S4 in the supplemental material). As shown in Fig. 8C and D, cell viability is attenuated in PLD2-silenced breast cancer cells with a correlation to cells treated with PKCζ inhibitors in a time-dependent manner. Control and PLD1-silenced cells exhibited significantly less effects on cell viability than did PLD2-silenced cells. Furthermore, as shown in Fig. 8E and F, it was also determined that the cell viability was recovered by PLD2-WT (addback plasmids) and PLD2-WT-PX but not by PLD2-K3A (addback

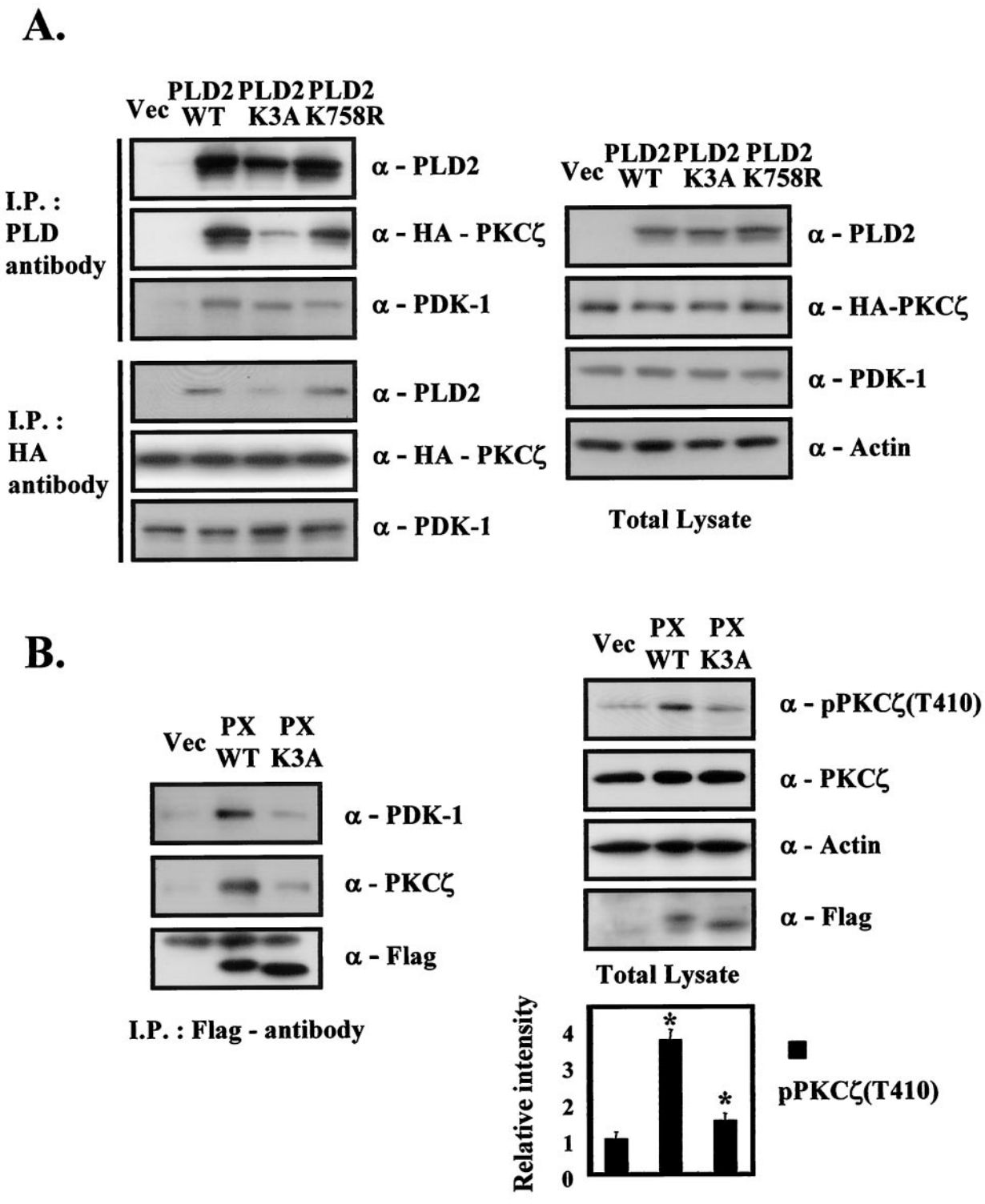


FIG. 7. The formation of ternary complex including PDK-1, PLD2, and PKCζ. A. COS-7 cells were transiently transfected with various PLD2 constructs, grown for 48 h, depleted with serum for 12 h, and then lysed with buffer containing 1% Triton X-100 and 1% cholic acid. The extracts, immunoprecipitated (I.P.) with anti-PLD antibody or antihemagglutinin (HA) antibody, were analyzed by immunoblotting with indicated antibodies. Anti-actin antibody was used with normalized control. B. COS-7 cells were transfected with the indicated plasmids encoding vector (Vec), Flag-tagged PLD2-PX-WT, and Flag-tagged PLD2-PX-K3A, cultured for 36 h, depleted with serum for 12 h, and then lysed with buffer containing 0.1% Triton X-100 for 30 min on ice. The cell lysates, removed from the crude nuclear fraction using centrifugation, were immunoprecipitated with anti-Flag antibody. The precipitated complex was analyzed with the indicated antibodies. The total lysates were also analyzed by immunoblotting with the indicated antibodies. The *n*-fold increase is expressed as a percentage of the indicated antibodies' intensity above the basal level. The experiment represents one of three independent experiments. *, P of <0.05 compared with vector-transfected cells.

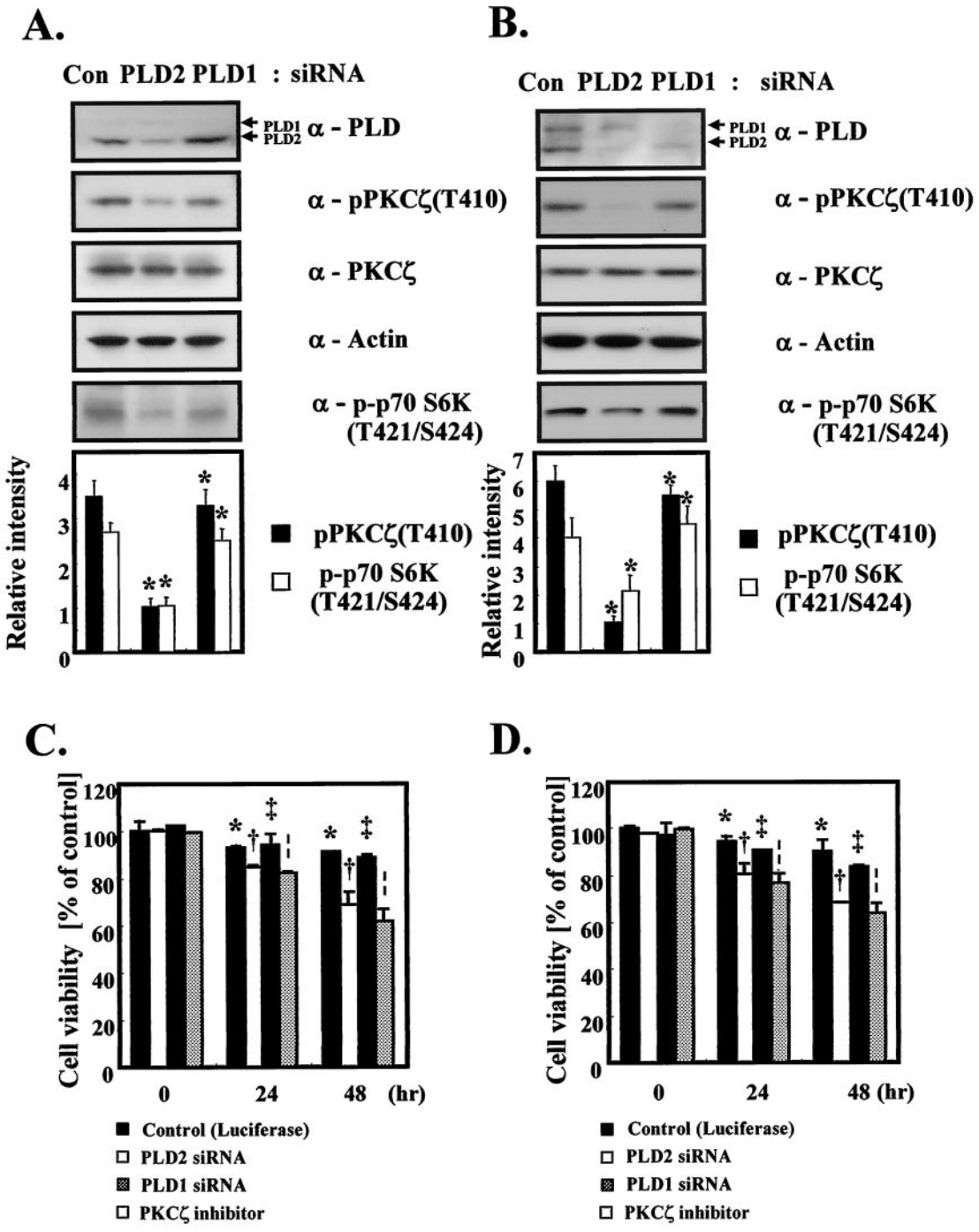


FIG. 8. The effects of PLD silencing on cell viability in breast cancer cells. A and B. Hs 578T cells and MDA-MB 468 cells were transfected with siRNA for control (Con), PLD1, and PLD2. The details of the procedures are described in Materials and Methods. In brief, the cells were cultured for 48 h, depleted with serum for 12 h, and then lysed with buffer containing 0.1% Triton X-100, for 30 min on ice. The cell lysates, removed from the crude nuclear fraction by using centrifugation, were analyzed by immunoblotting with the indicated antibodies. The *n*-fold increase is expressed as the percentage of the indicated antibodies' intensity above the basal level. The data represent one of three independent experiments. *, P of <0.05 compared with control siRNA-transfected cells. C and D. Hs 578T cells and MDA-MB 468 cells were transfected with siRNA for control, PLD2, and PLD1 and treated with the myristoylated PKCζ pseudosubstrate inhibitor (50 μM). After serum depletion for the indicated time points, the cells were washed, lysed with isopropanol including 0.2% HCl, and measured at a wavelength of 450 nm. *, P of <0.05 compared with control siRNA-transfected cells; † or ‡, P of <0.05 versus PLD2 or PLD1 siRNA-transfected cells; ¦, P of <0.05 versus PKCζ inhibitor-treated cells. E and F. Hs 578T (panel E) and MDA-MB 468 (panel F) cells were transfected with the indicated siRNA and plasmids. The siRNA used in the experiments was nucleotides 703 to 723, AAGAGGTGGCTGGTGGTGAAG, for addback of PLD2-WT or -K3A (addback plasmids). After serum depletion for 48 h, the cells were washed, lysed with isopropanol including 0.2% HCl, and measured at a wavelength of 450 nm. *, P of <0.05 compared with control siRNA-transfected cells; †, P of <0.05 versus PLD2 siRNA-transfected cells; ‡, P of <0.05 versus PLD2 siRNA + PLD2-WT addback; ¦, P of <0.05 versus PLD2 siRNA + PLD2-K3A addback; ¶, P of <0.05 versus PLD2 siRNA + PLD2-WT-PX; §, P of <0.05 versus PLD2 siRNA + PLD2-K3A-PX.

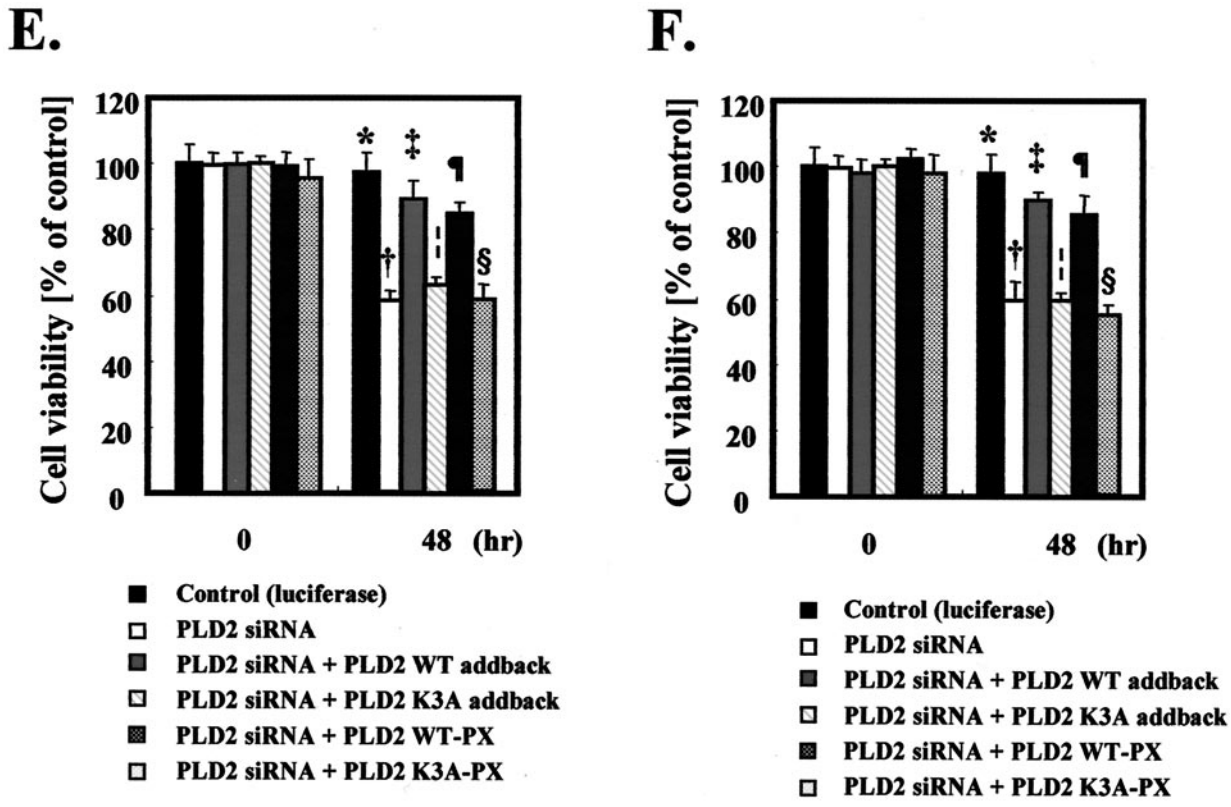


FIG. 8—*Continued*.

plasmids) and PLD2-K3A-PX. These results suggest that PLD2-mediated PKCζ activation is important for the survival of breast cancer cells.

DISCUSSION

PLD has a large role in the activity of lipid hydrolysis, and until now there have been many reports that its activity is elaborately controlled by many cellular regulators in diverse cell systems (20, 22, 26, 31, 33, 34, 35, 37, 50). In contrast, little is known about the details of mechanisms in the regulation of other proteins by PLD through direct binding. In this study, we suggest that the PLD2-PX domain directly binds to PKCζ, simultaneously performing dual roles—one as a direct activator for PKCζ, and the other as a cofactor increasing the activation loop phosphorylation of PKCζ (Fig. 9). Furthermore, PLD2-mediated PKCζ activation is also implicated in cancer cell viability.

The conventional PKC activation mechanisms are well understood. Mechanisms have been established for sequential phosphorylation, the recruiting of proper localization, and the exchanging of binding proteins (47). It is generally understood that synthesized PKC is translocated into the membrane and phosphorylated by PDK-1 and that phosphorylated PKC is regulated by Ca^{2+} or DAG generated by stimuli, culminating in the release of specific domains from the substrate binding cavity (61). In contrast, since PKCζ is insensitive to second messengers such as Ca^{2+} and DAG in its PB1 and C1 domains, it has been theorized that its activity is primarily regulated by protein-protein interaction. However, the activation mechanisms and cellular regulators of PKCζ remain unclear. Here, our data demonstrated that, regardless of lipase activity, PLD2 enhances the activation of PKCζ by increasing the activation loop phosphorylation of PKCζ under basal conditions. Also, the activation loop-phosphorylated PKCζ is stimulated by direct binding of PLD2 (Fig. 5). Moreover, PLD2-mediated PKCζ activation induces the phosphorylation of p70 S6 kinase, resulting in downstream signal flow (Fig. 6). The regulation of PKCζ activity by PLD2 results from the signaling complex composed of PDK-1 and PKCζ, not from the upregulation of PDK-1 activity as upstream kinase (Fig. 7A; also see Fig. S3 in the supplemental material). Furthermore, PLD2-WT, like PLD2-K3A, has no effect on binding with PDK-1 or on the binding of PKCζ with PDK-1. According to previous reports, when PKCζ is translocated into the membrane, bound to PDK-1, and then phosphorylated, PLD2 as a membrane-bound protein can assist the activation loop phosphorylation of PKCζ (Fig. 6). In this study, we suggest PLD2 as a novel protein cofactor for full and complete activation of PKCζ.

PKCζ phosphorylates p70 S6 kinase. Furthermore, it is known that p70 S6 kinase is activated through multiple-site phosphorylation. Another kinase for p70 S6 kinase is known to be mTOR, which is rapamycin sensitive and a target of PA (4, 14). mTOR phosphorylates p70 S6 kinase at the Thr^{389} residue in a PA-dependent manner, but PKCζ phosphorylates p70 S6 kinase at the Thr^{421} and Ser^{424} residues regardless of PA because, as shown in Fig. 6A, the PLD2-K758R lipase-inactive mutant abolishes generation of PA and also PLD2-WT in-

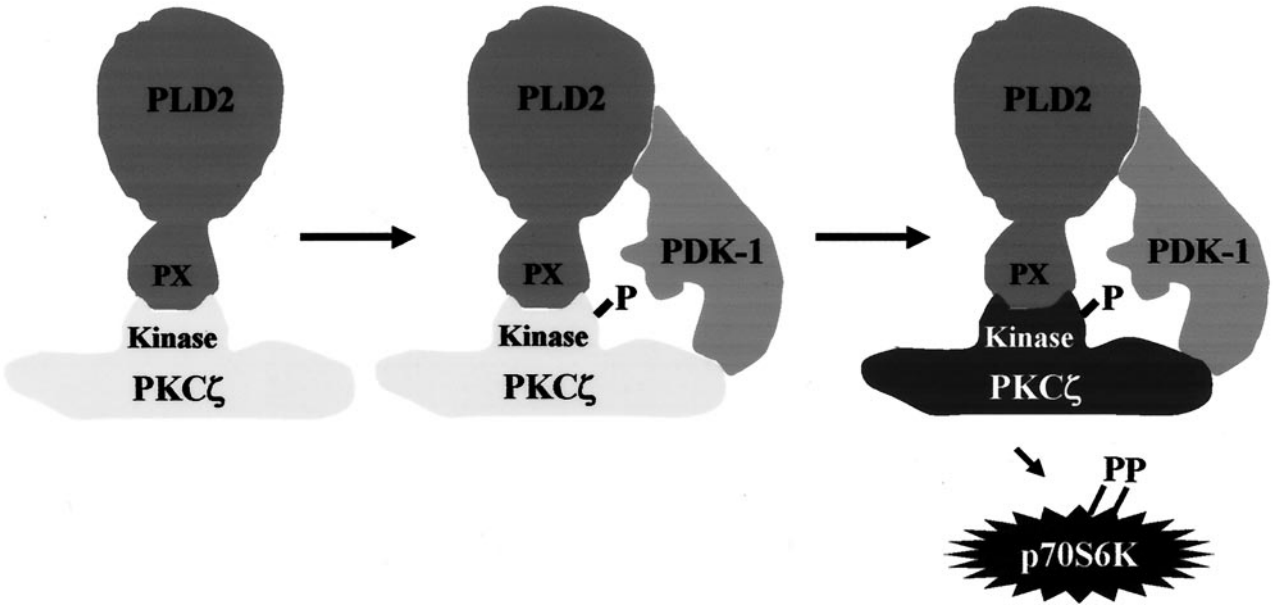


FIG. 9. Proposed model of PKCζ activation by PLD2. The PLD2-PX domain directly interacts with the PKCζ-kinase domain. Ultimately, PLD2 is a ternary complex of PKCζ with PDK-1 and its PX domain assists the activation loop phosphorylation of PKCζ by PDK-1. The activation loop-phosphorylated PKCζ is fully stimulated through direct interaction of PLD2-PX domain. The completely activated PKCζ (black) upregulates downstream signals, such as p70 S6 kinase.

creases phosphorylation of p70 S6 kinase. So, PLD2 interaction-dependent PKCζ activation could stimulate p70 S6 kinase. Accordingly, p70 S6 kinase activation is differently regulated by two kinases such as PKCζ and mTOR. Moreover, PLD2-mediated PKCζ, rather than mTOR activated by PA, phosphorylates p70 S6 kinase at the Thr^{421} and Ser^{424} residues.

PKCζ activity is regulated by dual mechanisms—the direct activation of PLD2 in vitro and assistance of the activation loop phosphorylation of PKCζ (Fig. 5 and 6). PLD2 stimulates PKCζ activity through direct interaction with the PKCζ-kinase domain (Fig. 4). The distinctive domains of PKCζ, including PB1, C1, and the kinase domain, are known to mediate protein-protein or protein-lipid interactions. It is known that the specific domains of PKCζ are important for interacting with several binding proteins such as Par6, Par3/ASIP, Cdc42, and mammalian Lgl, involved in cell polarity (28, 39, 52), and p62/ZIP, MEK5, and p70 S6 kinase, implicated in the signal transduction pathway (11, 52, 54, 56). Among the interacting proteins, Par3 exhibits the greatest affinity for the kinase domain, but whether its interaction regulates kinase activity remains unclear (39). In this study, we determine that PLD2 interacts with the amino acid 348 to 370 region within the PKCζ-kinase domain, subsequently regulating PKCζ activity. A recent report has predicted this region, by multiple sequence alignment under secondary structure modeling, to be located in the α-E helix within the C-lobe side of the kinase domain (64). In the case of cyclin-dependent kinase (CDK), the activation loop has been found to be structurally coupled with the α-C helix, stabilizing its inactive conformation. The direct interaction of cyclin with the α-C helix of CDK induces an alteration in the orientation of the α-C helix and eventually results in activation loop phosphorylation by CDK-activating kinase (27, 55). We suggested that PLD2, interacting with the amino acid 348 to 370 region corresponding to the α-E helix, can facilitate activation loop phosphorylation, due to a conformational change of PKCζ by interactions with the neighboring phosphorylation site. This region can also hold the possible potential motif providing the direct binding site for the PLD2-PX domain, necessary for the full activation of PKCζ. Surprisingly, the PLD2-PX domain is only sufficient for the signaling complex with PKCζ and PDK-1 and enhances the activation loop phosphorylation of PKCζ (Fig. 7B). The PLD2-PX domain also contains an activation motif of PKCζ, indicating the stimulation of activation loop-phosphorylated PKCζ activity through direct interaction. The autophosphorylation of PKCζ was also enhanced by the PLD2-PX domain and then activated PKCζ increased the phosphorylation of MBP (Fig. 5). Therefore, we suggest that PKCζ activation by PLD2 binding may be a new mechanism for kinase activation. Furthermore, as shown in Fig. S6 in the supplemental material, it is suggested that PKCζ phosphorylation is attenuated under the basal condition in PLD2-silenced cells, and the signal-dependent PKCζ phosphorylation also is decreased in PLD2-silenced cells. In addition, the signal-dependent PKCζ phosphorylation occurs as the basal PKCζ phosphorylation is reduced by PLD2 silencing. Therefore, it was found that PLD2 is essential for basal PKCζ activation. The issue of PLD2-PKCζ signaling may require further characterization.

PLD2 has two distinctive domains including the PX and PH domains. Their exact roles have not yet been well defined. Recently, our group suggested that one of the roles of the PLD2-PX domain is the exertion of an anchoring function, supporting the thesis that PLD2 functions as an adaptor in the redistribution of PLC-γ1 to the membrane region in epidermal growth factor signaling (26). We also theorized that it may play a secondary role, in the provision of the docking sites regulat-

ing PLD2 activity with its interacting protein, such as Munc18-1 (33). Here, we suggest that another of its functions is to serve as a novel direct activator for PKCζ, since the PLD2-PX domain can enhance stimulation and activation loop phosphorylation of PKCζ (Fig. 5 and 7B). The triple lysine residues within the PLD2-PX domain are especially responsible for PKCζ activation and interaction. However, the PLD2-K3A mutant abolishes interactions with PKCζ and inhibits its mediated PKCζ activation (Fig. 3 and 5). Although it cannot be affirmed that these mutations have no effects on the conformation of the PLD2-PX domain, it does not change the expression level, subcellular localization, or catalytic properties of PLD2 in vitro (data not shown). These results suggest that the effect of these mutations on PLD2 structure, if any, is neither dramatic nor significant in PLD2 function, except for its interaction with PKCζ. Taken together, we suggest that the PLD2-PX domain, as one of a collection of multiple binding modules, executes a myriad of functional roles in various cellular contexts.

PLD2 is known to be highly overexpressed in a wide variety of human cancer cells (48, 62, 63, 65), but it is unclear what the role of PLD might be in cancer cells. Furthermore, there are several reports in which the regulation of PKCζ, and p70 S6 kinase activity, is implicated in cell survival and apoptosis in prostate and breast cancer cells (21, 41). As shown in Fig. 8, we determined that the PLD2-mediated PKCζ activation is important for cell viability after the induction of cell death as a serum-depleted condition. Furthermore, we demonstrated that the effects of PLD2 silencing on the viability of breast cancer cells are nonadditive in PKCζ inhibitor-treated cells for supporting functional significance (see Fig. S5A and B in the supplemental material). We performed PKCζ rescue experiments to reveal the linking between PLD2 and PKCζ after silencing with PLD2 siRNA (see Fig. S5C and D in the supplemental material). As the amounts of PKCζ were increased, the cell viability was not significantly rescued under the PLD2 silencing condition. The reason that cell survival was not completely recovered is possibly the result of the silencing of PLD2, which acts as either a cofactor or activator for PKCζ. Therefore, PKCζ activation is dependent on PLD2 signaling. Therefore, we suggested that PLD2-mediated PKCζ activation is essential for the survival of breast cancer cells. Considering whether these tumor cells, if any, exist in either serum-exhausted or nutrient-deficient conditions in the angiogenesis process, we suggested that the PLD2-mediated PKCζ activation may be important in the survival of breast cancer cells. Accordingly, the issue of physical interaction may be applied to unravel some problems in cancer cells and necessary to further characterize the uncertain details of the signal mechanisms.

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