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Phosphorylation of Phospholipase C-δ_1 Regulates its Enzymatic Activity

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ABSTRACT

Phosphorylation of phospholipase C-δ_1 (PLC-δ_1) in vitro and in vivo was investigated. Of the serine/threonine kinases tested, protein kinase C (PKC) phosphorylated the serine residue(s) of bacterially expressed PLC-δ_1 most potently. It was also demonstrated that PLC-δ_1 directly bound PKC-α via its pleckstrin homology (PH) domain. Using deletion mutants of PLC-δ_1 and synthetic peptides, Ser35 in the PH domain was defined as the PKC mediated in vitro phosphorylation site of PLC-δ_1. In vitro phosphorylation of PLC-δ_1 by PKC stimulated [^{3}H]PtdIns(4,5)P_2 hydrolyzing activity and [^{3}H]Ins(1,4,5)P_3-binding of the PLC-δ_1. On the other hand, endogenous PLC-δ_1 was constitutively phosphorylated and phosphoamino acid analysis revealed that major phosphorylation sites were threonine residues in quiescent cells. The phosphorylation level and the species of phosphoamino acid were not changed by various stimuli such as PMA, EGF, NGF, and forskolin. Using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, we determined that Thr209 of PLC-δ_1 is one of the constitutively phosphorylated sites in quiescent cells. The PLC activity was potentiated when constitutively phosphorylated PLC-δ_1 was dephosphorylated by endogenous phosphatase(s) in vitro. Additionally, coexpression with PKC-α reduced serine phosphorylation of PLC-δ_1 detected by an anti-phosphoserine antibody and PLC-δ_1-dependent basal production of inositol phosphates in NIH-3T3 cells, suggesting PKC-α activates phosphatase or inactivates another kinase involved in PLC-δ_1 serine phosphorylation to modulate the PLC-δ_1 activity in vivo. Taken together, these results suggest that PLC-δ_1 has multiple phosphorylation sites and phosphorylation status of PLC-δ_1 regulates its activity positively or negatively depends on the phosphorylation sites. J. Cell. Biochem. 108: 638–650, 2009.

KEY WORDS: PH DOMAIN; PHOSPHOLIPASE C-δ_1; PROTEIN KINASE C; PHOSPHORYLATION; PHOSPHATASE

Phosphatidylinositol-specific phospholipase C (PI-PLC[‡]) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to produce two second messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DG), involved in intracellular Ca^{2+} mobilization and protein kinase C (PKC) activation, respectively [Nishizuka, 1992; Berridge, 1993]. At present, at least 13 PLC

Abbreviations used: PI-PLC, phosphatidylinositol-specific phospholipase C; PH, pleckstrin homology; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; DG, diacylglycerol; PKA, cAMP-dependent kinase; PKC, protein kinase C; PKG, cGMP-dependent kinase; CaM-PK-2, calmodulin-dependent protein kinase 2; CK, casein kinase; ERK, extracellular signal-related kinase; RhoGAP, Rho GTPase activating protein; GST, glutathione *S*-transferase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

Makoto Fujii and Kye Sook Yi contributed equally to this work.

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isozymes have been identified, divided into 6 main families, PLC-β, γ, δ, ε, ζ, and η [Rhee and Bae, 1997; Rebecchi and Pentyala, 2000; Bunney and Katan, 2006; Cockcroft, 2006; Swann et al., 2006; Yagisawa et al., 2006; Suh et al., 2008]. Of six PLC families, it is thought that PLC-δ is, in evolutionary terms, the most basal form in mammalian somatic cells. Nevertheless, the activation mechanism and physiological function of the δ isoform family has not been completely resolved. It has been reported that PLC-δ_1 is activated by capacitative Ca^{2+} entry following the activation of PLC-β in bradykinin stimulated PC12 cells [Kim et al., 1999]. There are several binding partners which regulate PLC-δ_1 positively or negatively, such as p122/Rho GTPase activating protein (RhoGAP), Ghα/Transglutaminase II, Ral, and calmodulin [Homma and Emori, 1995; Feng et al., 1996; Sidhu et al., 2005]. Studies using PLC-δ family gene knockout mice have identified that PLC-δ_1 is involved in the determination of skin stem cell lineage and that PLC-δ_4 is an essential factor for the zona pellucida-induced acrosome reaction in sperm [Fukami et al., 2001, 2003; Nakamura et al., 2003]. Although PLC-δ_3 knockout mice showed no obvious abnormalities, PLC-δ_1/δ_3 double knockout mice exhibited embryonic lethality resulting from a defect in trophoblast development [Nakamura et al., 2005].

Phosphorylation of the PI-PLC isoforms has been investigated in vivo and in vitro assay systems. Phosphorylation of PLC-β isoforms by cAMP-dependent kinase (PKA) and/or PKC attenuated their activation by heterotrimeric G protein [Liu and Simon, 1996; Ali et al., 1997; Yue et al., 1998, 2000]. The extracellular signal-related kinase (ERK) dependent phosphorylation of PLC-β_1 plays a critical role in the activation of nuclear phosphoinositide hydrolysis and is also crucial to the mitogenic action of insulin-like growth factor I (IGF-I) [Xu et al., 2001]. Tyrosine phosphorylation of PLC-γ isoforms by receptor or non-receptor tyrosine kinases has been extensively studied, and the precise phosphorylation mechanisms have been revealed [Rhee and Bae, 1997; Rebecchi and Pentyala, 2000; Bae et al., 2002]. It is now believed that the phosphorylation of PLC-γ plays a critical role in many signaling systems involving phosphoinositide turnover and intracellular Ca^{2+} mobilization.

In contrast, information regarding the phosphorylation of δ type PLC is limited. A few reports have dealt with the phosphorylation of PLC-δ [Kim et al., 1989; Meisenhelder et al., 1989; Ryu et al., 1990] but the regulation mechanisms and the physiological significance of the phosphorylation remain to be clarified. Additionally, we do not have any information about the phosphorylation site(s) for PLC-δ_1 or any other δ isoforms. Neither do we know about the kinase(s) and phosphatase(s) associated with these isoforms. It is possible that an extracellular stimulus other than those examined in the previous studies or the cell cycle progression regulates the phosphorylation status of the PLC-δ isoforms through which its enzymatic activity and localization are controlled. In order to obtain further insights into the regulation of the δ isoforms and their physiological functions, it is important to investigate the phosphorylation of the most ubiquitous δ isoform, PLC-δ_1.

In the current study, we describe in vitro and in vivo phosphorylation of PLC-δ_1. We demonstrated that PLC-δ_1 binds to PKC-α via its pleckstrin homology (PH) domain. A potential phosphorylation site in this domain was determined to be serine 35. In vitro phosphorylation of PLC-δ_1 by PKC stimulated its enzymatic activity and ligand binding. Endogenous PLC-δ_1 was constitutively phosphorylated and the major phosphorylation sites were threonine residues including threonine 209. The dephosphorylation of constitutive phosphorylation at threonine residues increased PLC activity. Additionally, overexpression of PKC-α reduced serine phosphorylation of PLC-δ_1 and inhibited intracellular PLC activity. We conclude that PLC-δ_1 has multiple phosphorylation sites and is regulated by various kinases and phosphatases including PKC-α in the cells.

MATERIALS AND METHODS

CHEMICALS

[^{32}P]orthophosphate (5.55 GBq/ml) and *myo*-D-[^{3}H]Ins(1,4,5)P_3 (777 GBq/mmol) were obtained from NEN Life Science Products. Non-radioactive Ins(1,4,5)P_3, [inositol-2-^{3}H]PtdIns(4,5)P_2 (37 GBq/mmol), myo-[2-^{3}H]inositol (696 GBq/mmol, 37 MBq/ml), and [γ-^{32}P]ATP (370 MBq/ml) were from GE Healthcare. PKC (from rat brain), phosphatidylserine, 1,2-dioleoyl-*sn*-glycerol, phorbol 12-myristate 13-acetate (PMA), epidermal growth factor (EGF), nerve growth factor (NGF), and forskolin were purchased from Sigma. F-moc amino acids were purchased from Peptide Institute Incorporation. Thin layer chromatography (TLC) plates were obtained from MERCK. An anti-hemagglutinin (HA) monoclonal antibody and anti-PKC-α polyclonal antibody were kindly given by Dr. W. Cho (University of Illinois, U.S.A.), and anti-phosphothreonine monoclonal antibody was obtained from Cell Signaling. An anti-phospho-threonine polyclonal antibody and an anti-phospho-serine polyclonal antibody were purchased from Zymed. An anti-PLC-δ_1 monoclonal antibody (Z78) [Suh et al., 1988], and anti-PLC-δ_1 polyclonal antibody have been described elsewhere [Fujii et al., 1999].

CONSTRUCTION OF THE EXPRESSION PLASMID AND ITS DELETION MUTANTS

Plasmids for truncation mutants expressing the N-terminal 140 residues, pGST/PLC-δ_1 Δ141–756 were constructed from pGST3/PLC-δ_1: a *Sal*I/*Pst*I (blunt ended) fragment of pGST3/PLC-δ_1 was inserted into the *Sal*I/*Hin*dIII (blunt ended) site of pGST4. Three GST-fused N-terminal deletion mutants (Δ1–23, Δ1–53, Δ1–134) were a kind gift from Dr. M. Katan (The Institute of Cancer Research, U.K.) [Ellis et al., 1993]. The construction of other PLC-δ_1 deletion mutants has been described previously [Yagisawa et al., 1994]. The PH domain of PLC-β_1 (17–138), β_2 (12–133), β_3 (1–121), β_4 (1–120), γ_1 (27–142), γ_2 (20–131), δ_2 (bovine δ_4: 17–124), δ_3 (11–119), and δ_4 (17–124) were amplified by PCR and subcloned into the pGEX vector to express the PH domains as GST-fusion proteins. The expression and purification of the GST–PLC fusion protein and its mutants have been described previously [Yagisawa et al., 1994, 1998].

Wild-type rat PLC-δ_1 cDNA was introduced into the *Xho*I sites of the mammalian expression vector pcDNA3.0-HA. To construct the PLC-δ_1 deletion mutants that lack either the PH (Δ21–130), EF (Δ133–281), or C2 domain (Δ630–720), corresponding regions were amplified by PCR and cloned into pcDNA3.0-HA. The Thr209 was substituted with Ala by site-directed mutagenesis using pcDNA/HA-PLC-δ_1 as a template. For PKC isozymes, full-length cDNA was also introduced into pcDNA3.0-HA.

PHOSPHORYLATION OF RECOMBINANT PLC-δ_1 BY PKC IN VITRO

GST–PLC-δ_1 or its mutant (1 μg) was incubated for 30 min in 15 μl of phosphorylation reaction mixture (20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 20 μg/ml 1,2-dioleoyl-*sn*-glycerol) containing 200 μM [γ-^{32}P]ATP (0.5–1.0 μCi/tube) and 3.75 U PKC at 30°C. Note that 1 U of PKC is defined as the enzyme activity which catalyzes the transfer of 1 pmol phosphate from ATP to histone H1 in 1 min at 30°C. Reaction products were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then detected by autoradiography using Bio-Image analyzer BAS 2000 (Fuji Film Co. Ltd).

PHOSPHOAMINO ACID ANALYSIS

Phosphoamino acid analysis was carried out as described previously [Meisenhelder and Hunter, 1991]. Briefly, [^{32}P]-labeled PLC-δ_1 was eluted from an SDS–PAGE gel using elution buffer (50 mM NH_4HCO_3, 0.1% SDS, 2% 2-mercaptoethanol) at 37°C for 10–12 h. The eluted proteins were precipitated with 2 volumes of acetone at −20°C for 2 h and then pelleted by centrifugation, and washed three times with 500 μl of cold (−20°C) acetone/ethanol (1:1). Proteins were dissolved in 100 μl of 6 N HCl and hydrolyzed in a 98°C oven for 3 h. The supernatant was dehydrated using a vacuum concentrator. The pellet was resuspended in 5 μl of a pH 1.9 buffer (2.2% formic acid, 7.8% CH_3COOH) containing a 1/10 volume of the phosphoamino acid standard mixture (1 mg/ml phosphoserine, 1 mg/ml phospho-threonine, 1 mg/ml phosphotyrosine) and then subjected to two-dimensional electrophoresis. The first dimension was run in a pH 1.9 buffer for 60 min at 500 V, after which the plate was dried, and the second dimension was run in a pH 3.5 buffer (5% CH_3COOH, 0.5% pyridine) for 60 min at 500 V. After the plate was dried, it was sprayed with ninhydrin to visualize the standards and then subjected to autoradiography.

CELL CULTURE AND TRANSFECTION

C6 and COS7 cells were grown in DMEM containing 10% fetal bovine serum (FBS). NRK cells and MDCK cells stably expressing GFP-PLC-δ_1 [Fujii et al., 1999] were grown in DMEM containing 5% FBS. PC12 cells were grown in DMEM containing 5% FBS and 10% horse serum. NIH-3T3/neo and NIH-3T3/PLC-δ_1 cells were maintained in DMEM containing 10% FBS plus 50 μg/ml G418. Each medium was supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were maintained at 37°C in a humidified CO_2 incubator. COS7 cells (1×10^6) were seeded in 100 mm dish and transfected using lipofectamine (Invitrogen) after 24 h. The cells were incubated for 36 h and then lyzed in lysis buffer for immunoprecipitation or immunoblot analysis.

IMMUNOPRECIPITATION AND IN VITRO BINDING ASSAY

Cells were lyzed in lysis buffer (30 mM Tris–HCl (pH 7.5), 150 mM NaCl, 6 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.25 mM EGTA, 1% Triton X-100). Cell lysate (500 μg to 2 mg protein) was incubated with the indicated antibodies immobilized onto protein A-agarose for 3 h at 4°C. The immune complexes were washed three times with lysis buffer and then subjected to SDS–PAGE and immunoblot analysis using the indicated antibodies. An in vitro binding experiment was carried out using 100 ng of purified PKC-α and 500 ng of purified PLC-δ_1. PKC-α and PLC-δ_1 were incubated in lysis buffer for 1 h at 4°C and immunoprecipitated with an anti-PLC-δ_1 monoclonal antibody.

GST PULL-DOWN ASSAY

Rat brain was homogenized in lysis buffer and centrifuged at 100,000*g* for 1 h at 4°C. The supernatant (1 mg) was incubated with 5 μg of GST-fusion proteins immobilized on glutathione–Sepharose 4B (Amersham Life Science) for 3 h at 4°C. The beads were washed three times with lysis buffer and then subjected to SDS–PAGE and immunoblot analysis using the indicated antibodies.

PEPTIDE PREPARATION

A peptide corresponding to residues 30–43 of rat PLC-δ_1 (PLC-δ_1 30–43) and peptides with the same sequence except that the serine residue(s) were substituted with alanine: S33A, S34A, S35A, or SSS33–35AAA (Fig. 3C) were synthesized using the F-moc (*N*-(9-fluorenyl)methoxycarbonyl) cleavage method on an Applied Biosystems 431A peptide synthesizer as described previously [Yagisawa et al., 1994].

PHOSPHORYLATION OF THE PLC-δ_1 30–43 PEPTIDE AND RELATED PEPTIDES BY PKC

Peptide phosphorylation by PKC was evaluated according to the method employed in the PKC enzyme assay system (Amersham Life Science). Briefly, 0.42 mM peptide was incubated at 30°C in 30 μl of a reaction mixture containing 30 mM Tris–HCl (pH 7.5), 60 mM $MgCl_2$, 10 mM calcium acetate, 0.25 mg/ml phosphatidylserine, 20 μg/ml PMA, 25 mM dithiothreitol, 1 mM [γ-^{32}P]ATP (0.2 μCi/tube), and 12.5 U PKC. At the indicated times, the reaction was terminated by the addition of 10 μl of 300 mM H_3PO_4, then the sample (40 μl) was spotted on the peptide binding paper (Whatman P81 cation exchange paper). After drying, the paper was washed three times with 75 mM H_3PO_4 to remove non-specific radioactivity. After drying, the paper was subjected to liquid scintillation counting.

PtdIns(4,5)P_2-PLC ASSAY AND [^{3}H]Ins(1,4,5)P_3 BINDING ASSAY

Phosphorylation of GST–PLC-δ_1 for PLC assay or [^{3}H]Ins(1,4,5)P_3-binding assay was carried out as follows. GST–PLC-δ_1 (0.5 μg) was incubated with 300 U of PKC in 100 μl of the reaction mixture for 120 min, then an aliquot (2–20 μl) containing 10–100 ng of GST–PLC-δ_1 was subjected to PtdIns(4,5)P_2-PLC assay. GST–PLC-δ_1 (1 μg) was incubated with 300 U of PKC in 50 μl of the reaction mixture for 120 min, then the whole reaction mixture was subjected to [^{3}H]Ins(1,4,5)P_3-binding assay. PtdIns(4,5)P_2-PLC assay and [^{3}H]Ins(1,4,5)P_3-binding assay were carried out as described elsewhere [Yagisawa et al., 1994, 1998].

MALDI-TOF MASS SPECTROMETRY

PLC-δ_1 was immunoprecipitated with an anti-PLC-δ_1 monoclonal antibody (Z78) from C6 cells and then digested by trypsin. The peptide fragments of PLC-δ_1 were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as described previously [Kim et al., 2002].

[^{32}P]LABELING OF INTACT CELLS AND DEPHOSPHORYLATION OF PLC-δ_1 BY INTRINSIC PHOSPHATASES

Semi-confluent cells were washed three times with 2 ml of phosphate-free Krebs–Ringer bicarbonate buffer (20 mM HEPES-NaOH (pH 7.4), 118 mM NaCl, 4.75 mM KCl, 1.2 mM $MgCl_2$, 0.26 mM $CaCl_2$, 25 mM $NaHCO_3$). Cells were then incubated in 2 ml of phosphate-free DMEM containing 0.2% dialyzed FBS and 200 μCi of [^{32}P]orthophosphate for 2 h at 37°C. Each stimulant was added to the plate at the indicated concentration and for the indicated time. Cells were then washed twice with ice-cold phosphate-free Krebs–Ringer bicarbonate buffer, and extracted with 1 ml of ice-cold 1% NP-40-TNE buffer (10 mM Tris–HCl (pH 7.8), 1% NP-40, 150 mM NaCl, 4 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 50 U/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.1 mM benzamidine) containing phosphatase inhibitors (10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM Na_3VO_4). The cell lysates were subject to immunoprecipitation using an anti-PLC-δ_1 polyclonal antibody. The immune complex was washed three times with 1 ml of ice-cold 1% NP-40-TNE buffer and was subjected to SDS–PAGE. Phosphorylated proteins were detected by autoradiography. To detect the dephosphorylation of PLC-δ_1 by intrinsic phosphatases, metabolically radiolabeled PC12 cells were extracted with ice-cold 0.1% NP-40-TNE buffer (same as 1% NP-40-TNE buffer except that the NP-40 concentration was reduced to 0.1%) with or without phosphatase inhibitors. The extracts were incubated for the indicated time at 37°C, and then subjected to immunoprecipitation.

MEASUREMENT OF TOTAL INOSITOL PHOSPHATES

NIH-3T3/neo or NIH-3T3/PLC-δ_1 cell lines were seeded at 2×10^5 cells/well in 6-well plates and infected with adenovirus expressing PKC-α. After 24 h, cells were labeled with 1 μCi/ml *myo*-[2-^{3}H]inositol in inositol-free DMEM for 24 h. The cells were washed and incubated with 20 mM LiCl for 40 min in inositol-free DMEM, then lyzed by adding 0.4 ml of ice-cold 5% $HClO_4$. After sonication, the extracts were centrifuged, diluted 1:5 with distilled water and applied to a Dowex AG 1-X8 anion exchange column (Bio-Rad). The column was washed extensively with 4 ml distilled water and with 10 ml washing buffer (0.06 M ammonium formate, 5 mM sodium tetraborate). Total inositol phosphate was eluted from the column using elution buffer (1 M ammonium formate, 0.1 M formic acid) and quantified by liquid scintillation counting.

RESULTS

PHOSPHORYLATION OF GST/PLC-δ_1 IN VITRO

A motif search of the PLC-δ_1 primary sequence demonstrated that the enzyme contains a number of potential phosphorylation sites. To investigate the possibility of whether PLC-δ_1 is phosphorylated by canonical kinases in vitro, recombinant PLC-δ_1 (GST/PLC-δ_1) was incubated with calmodulin-dependent protein kinase 2 (CaM-PK-2), casein kinase (CK), cdc2 kinase, cGMP-dependent kinase (PKG), PKA, or PKC. Of these kinases, PKC, PKG, and PKA (CGA kinases) phosphorylated GST/PLC-δ_1 (Fig. 1A) while CaM-PK-2, CK, and cdc2 kinases did not (data not shown). Since GST alone was not phosphorylated at all, the phosphorylation site is present in PLC-δ_1 (Fig. 3B). Interestingly, PKC phosphorylated only serine residues in PLC-δ_1 (Fig. 1B).

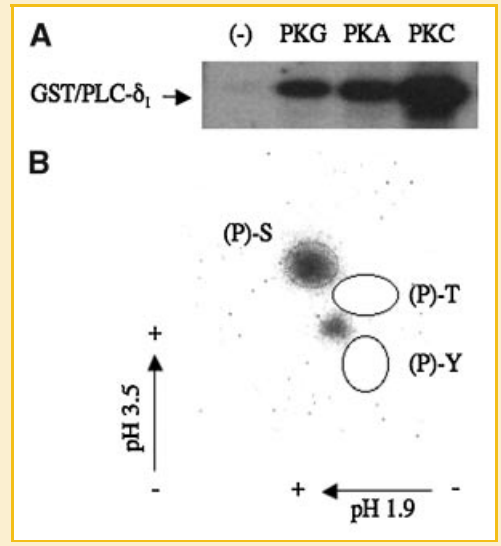

Fig. 1. Phosphorylation of PLC-δ_1 in vitro. A: GST/PLC-δ_1 (1 μg) was incubated with or without the indicated kinases in the presence of [γ-^{32}P]ATP and subjected to SDS–PAGE. The phosphorylation bands (arrow) were detected by autoradiography. B: The phosphoamino acid analysis of GST/PLC-δ_1 phosphorylated by PKC (rat brain) was carried out as described in Materials and Methods Section. (P)-S, (P)-T, and (P)-Y denote the positions of the standard phosphoamino acids phospho-serine, phospho-threonine, and phospho-tyrosine, respectively. The running directions are indicated by arrows. The result represents one of the three independent experiments. Other experiments gave similar results.

PLC-δ_1 INTERACTS WITH PKC-α

Because PKC phosphorylated GST/PLC-δ_1 most potently, we focused on relation between PKC and PLC-δ_1. First, we examined whether PLC-δ_1 binds to PKC isozymes [Ohno and Nishizuka, 2002; Shirai and Saito, 2002; Corbalan-Garcia and Gomez-Fernandez, 2006]. HA-tagged PKC-α, βII, δ, ε, or ζ was transfected with PLC-δ_1 into COS7 cells and then PLC-δ_1 was immunoprecipitated with an anti-PLC-δ_1 monoclonal antibody. Only PKC-α and βII, which belong to conventional PKC, were co-precipitated with PLC-δ_1 (Fig. 2A). This interaction was PLC-δ_1 specific. Endogenous PKC-α was not co-precipitated with transfected PLC-β_1 or γ_1 (Fig. 2B). To examine whether this interaction is direct, purified PLC-δ_1 and PKC-α were pre-incubated and then immunoprecipitated using an anti-PLC-δ_1 monoclonal antibody. Purified PKC-α was precipitated by anti-PLC-δ_1 antibody only in the presence of purified PLC-δ_1 (Fig. 2C).

PKC-α BINDS TO THE PH DOMAIN OF PLC-δ_1

To determine the PKC-α binding site on PLC-δ_1, we constructed PLC-δ_1 deletion mutants lacking the PH, EF, or C2 domain. Each mutant was transfected into COS7 cells and then immunoprecipitated with an HA antibody. ΔEF or ΔC2 domain precipitated PKC-α as full-length PLC-δ_1 did, but ΔPH did not (Fig. 2D). The binding specificity of PKC-α on the PH domain was examined using GST

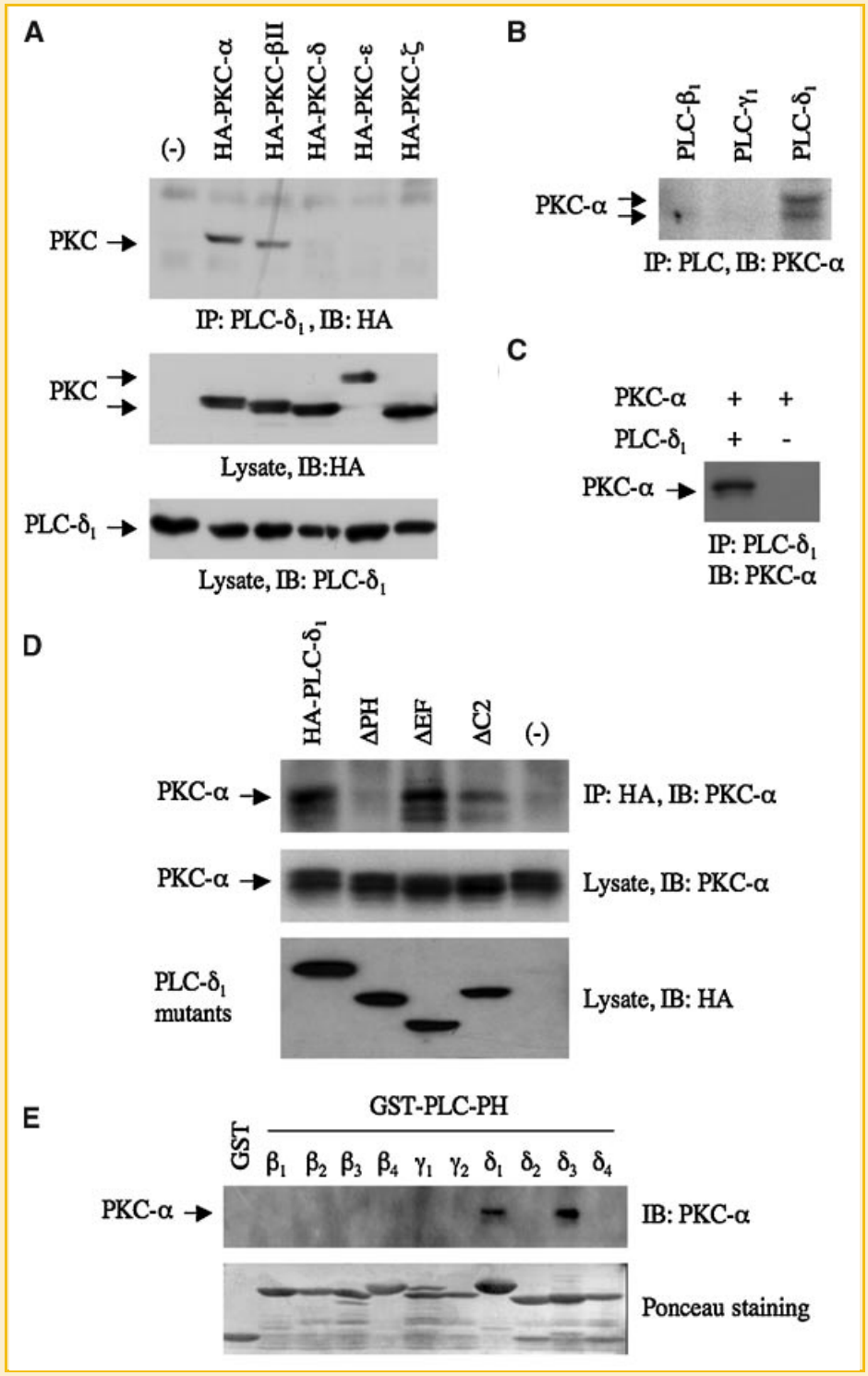

Fig. 2. Interaction between PLC and PKC-α. A: PLC-δ_1 was co-transfected with the indicated HA-PKC isozymes into COS7 cells. PLC-δ_1 was immunoprecipitated with an anti-PLC-δ_1 monoclonal antibody (Z78) and then subjected to immunoblot analysis using an anti-HA monoclonal antibody. The top panel demonstrates that PKC-α shows the highest affinity for PLC-δ_1. The bottom two panels show the immunoblot using an anti-HA monoclonal antibody and an anti-PLC-δ_1 monoclonal antibody to confirm similar expression levels of PKC isozymes and PLC-δ_1, respectively. B: PLC-β_1, γ_1, or δ_1 were transfected into COS7 cells. Each PLC isozyme was immunoprecipitated and subjected to immunoblot using polyclonal anti-PKC-α antibody. C: Purified PKC-α was incubated with or without purified PLC-δ_1 and then PLC-δ_1 was immunoprecipitated with an anti-PLC-δ_1 monoclonal antibody. Co-precipitated PKC-α was detected by an anti-PKC-α polyclonal antibody. D: PLC-δ_1 or its deletion mutants were transfected into COS7 cells (see Materials and Methods Section for detail). Immunoprecipitation and immunoblot were performed. The bottom two panels show the immunoblot that confirms the expression of endogenous PKC-α and transfected PLC-δ_1 (or mutants), respectively. E: The GST-fused PH domain of indicated PLC isozymes (see Materials and Methods Section for detail) immobilized onto glutathione–Sepharose 4B beads were incubated with rat brain lysates. Precipitated PKC-α was detected by an anti-PKC-α polyclonal antibody. The bottom panel shows Ponceau S-staining of the membrane to show the relative amount of each GST–PH protein. The result represents one of the three independent experiments. Other experiments gave similar results.

pull-down assay. The PH domains of 10 different PLC isozymes were expressed as GST-fusion proteins and used for the assay. Of the 10 different PH domains, only PLC-δ_1 and δ_3 were able to pull-down PKC-α (Fig. 2E). Recently, it was reported that PLC-δ_2 and δ_4 are from the same gene of different species [Irino et al., 2004]. These results indicate that PKC-α binds to PLC-δ_1 and δ_3 through the PH domain.

DETERMINATION OF THE PLC-δ_1 PHOSPHORYLATION SITE(S) IN VITRO

After mapping the PKC-α binding site to the PH domain of PLC-δ_1, we next determined the phosphorylation site of PLC-δ_1 by PKC in vitro. Several GST fused deletion mutants (Fig. 3A) were incubated with PKC and their phosphorylation was observed. As shown in Figure 3B, N-terminal 23 amino acids deletion (Δ1–23) did not affect the phosphorylation level, while partial deletion of the PH domain (PLC-δ_1 Δ1–57) decreased the phosphorylation level and complete deletion of the PH domain (PLC-δ_1 Δ1–223) abolished PLC-δ_1 phosphorylation. Conversely, a construct expressing the PH domain alone (PLC-δ_1 Δ141–756) was significantly phosphorylated. To rule out the possibility that GST modulated the ternary structure of PLC-δ_1, therefore PKC was able to phosphorylate PLC-δ_1, we cleaved GST using thrombin and then phosphorylated the PLC-δ_1 by PKC. As shown in Figure 3B, PKC phosphorylated the PLC-δ_1 to the same extent as GST/PLC-δ_1. Taken together, these data suggest that the N-terminal portion of the PH domain (residues 24–56) contains

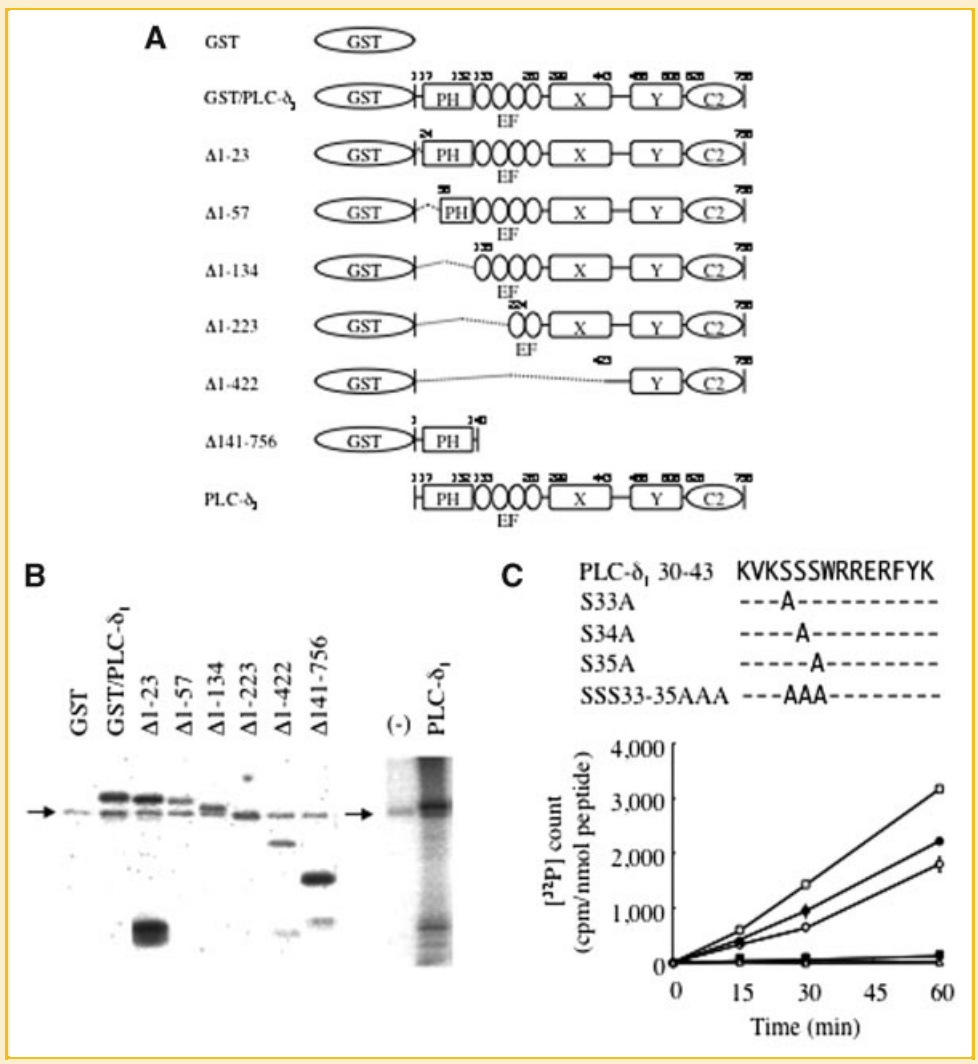

Fig. 3. Phosphorylation of GST–PLC-δ_1, the PLC-δ_1 30–43 peptide, and their mutants by PKC. A: Schematic representation of GST/PLC-δ_1 and its deletion mutants. PH, EF, X, Y, and C2 in the PLC-δ_1 denote the pleckstrin homology domain, an EF hand motif, catalytic X and Y domains, and a C2 domain, respectively. Numbers above figures refer to the first and the terminal amino acid numbers of each domain or construct. B, left: GST/PLC-δ_1 and its deletion mutants were phosphorylated by PKC in vitro. Right: PLC-δ_1 was phosphorylated by PKC in vitro. The PKC phosphorylation assay without substrate (−) was also performed to detect the autophosphorylation band of PKC. The phosphorylation bands were detected by autoradiography. The position of autophosphorylated PKC is marked by arrows. The result represents one of the three independent experiments. C, top: A schematic representation of the PLC-δ_1 30–43 peptide and mutant peptides with same sequence expect that the serine residue(s) were substituted with alanine. Bottom: Each peptide was incubated with PKC. At the indicated times, the reaction was terminated and then subjected to liquid scintillation counting. 30–43 (open circle), S33A (filled circle), S34A (open square), S35A (filled square), or SSS33–35AAA (open triangle). Values were the means ± SE of the two experiments carried out in duplicate.

the PKC phosphorylation site(s). Interestingly, a construct (PLC-δ_1 Δ1–422) that contains two conserved domains, Y and C2, was phosphorylated by PKC even though it does not have the PH domain. Presumably new phosphorylation site(s) in the Y or C2 domain appeared resulting from deletion of most of the X region.

PKC PHOSPHORYLATES THE PLC-δ_1 30–43 PEPTIDE

Within amino acid residues 24–56 of PLC-δ_1, ^{32}KSSSWR fitted into the consensus PKC recognition sequence [Kennelly and Krebs, 1991; Nishikawa et al., 1997], BXS/T*XXB, or BXXS/T*XB where B stands for either K or R, and S/T* stands for S or T that can accept phosphorylation. Both Ser34 and Ser35 were potential phosphorylation sites. To define the exact phosphorylation site, amino acid 30–43 was synthesized as peptides and each serine residues were substituted to alanine (S33A, S34A, S35A, or SSS33–35AAA) (Fig. 3C). Each peptide was incubated with PKC and peptides S33A and S34A were phosphorylated to the same extent as PLC-δ_1 30–43, whereas peptides S35A and SSS33–35AAA were not phosphorylated by PKC at all (Fig. 3C). These data suggest that Ser35 is one of the phosphorylation sites of PLC-δ_1 by PKC in vitro. Based on the amino acid sequence alignment, the corresponding PKC phosphorylation site, Thr74, is conserved in mouse PLC-δ_3.

EFFECT OF PKC ON PLC-δ_1 ACTIVITY AND Ins(1,4,5)P_3 BINDING

It is also important to know the biochemical implication of phosphorylation of the Ser35 on PLC-δ_1 by PKC, since Ser35 is located in the first variable loop in the PLC-δ_1 PH domain (Fig. 7A) [Ferguson et al., 1995], and is thought to play an important role in accommodating its ligand, the head group of PtdIns(4,5)P_2 or Ins(1,4,5)P_3. We incubated PLC-δ_1 with PKC in the presence of ATP, and then the mixture was subjected to PLC assay using [^{3}H]PtdIns(4,5)P_2 as a substrate. As shown in Figure 4A, the enzymatic activity showed a threefold increase after phosphorylation. To determine if this effect reflects a change in ligand binding, we examined [^{3}H]Ins(1,4,5)P_3 binding of phosphorylated and non-phosphorylated PLC-δ_1. The bound [^{3}H]Ins(1,4,5)P_3 increased to about sixfold in phosphorylated PLC-δ_1 (Fig. 4B). These results indicate that the phosphorylation caused by PKC introduces positive regulation of PLC-δ_1 in vitro.

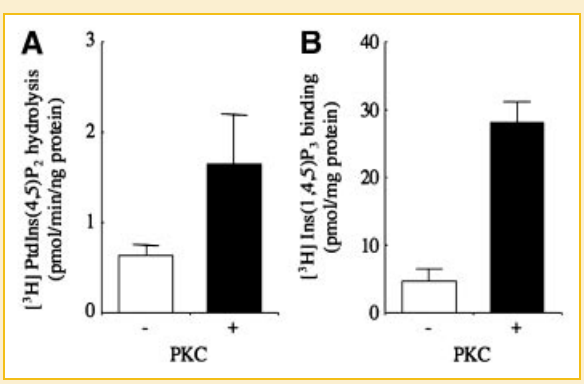

Fig. 4. Effect of PKC phosphorylation on PLC activity and Ins(1,4,5)P_3 binding. A,B: GST/PLC-δ_1 was incubated with (+) or without (−) 300 U of PKC (rat brain) in the reaction mixture with 200 μM ATP as described in Materials and Methods Section. Then an aliquot was subjected to PtdIns(4,5)P_2-PLC assay (A) or [^{3}H]Ins(1,4,5)P_3-binding assay (B). Values were the means ± SE of the two experiments carried out in duplicate.

PHOSPHORYLATION OF ENDOGENOUS PLC-δ_1

In early studies, although phosphorylated PLC-δ was isolated from cells showing that PLC-δ can be phosphorylated, no further phosphorylation was observed in vivo by extracellular stimuli, such as PMA, EGF, PDGF, or forskolin [Kim et al., 1989; Meisenhelder et al., 1989; Ryu et al., 1990]. We repeated this experiment using a polyclonal antibody specifically recognizes PLC-δ_1. As shown in Figure 5A, phosphorylated PLC-δ_1 was extracted from quiescent PC12 cells which were metabolically labeled with [^{32}P]orthophosphate. Consistent with previous reports [Kim et al., 1989; Meisenhelder et al., 1989; Ryu et al., 1990], there was no notable change in PLC-δ_1 phosphorylation by various stimulants (Fig. 5A). Similar results were obtained from NRK cells and NIH-3T3/PLC-δ_1 (data not shown). Hypo-osmotic stress, which induced translocation of PLC-δ_1 in MDCK cells [Fujii et al., 1999], had no effect on the phosphorylation of PLC-δ_1 (data not shown). Unlike in vitro phosphorylation by PKC, endogenous PLC-δ_1 was phosphorylated on threonine residue(s) (Fig. 5B). We were not able to detect phospho-serine residue(s) using phosphoamino acid analysis, but the serine phosphorylation of endogenous PLC-δ_1 was detected using an anti-phospho-serine antibody (Fig. 6C). These results indicate that PLC-δ_1 is constitutively phosphorylated and that threonine is the major phosphorylation site in vivo.

IDENTIFICATION OF Thr209 AS A PHOSPHORYLATION SITE OF ENDOGENOUS PLC-δ_1

Since there is no significant effect of external stimuli on the phosphorylation status of endogenous PLC-δ_1, we identified the basal phosphorylation site of endogenous PLC-δ_1 by using MALDI-TOF mass spectrometry. The m/z values, 1232.5972, 1248.5775, and 1312.5892, matched to unmodified, oxidized, and phosphorylated peptide fragment of PLC-δ_1, ^{207}MLTQRAEIDR, respectively (Fig. 5C). These data suggest that this peptide fragment contains the phosphorylation site. The amino acid sequence, ^{208}LTQR, fits into a consensus sequence XS/T*XB recognized by PKC [Kennelly and Krebs, 1991; Nishikawa et al., 1997], where B stands for either K or R, and S/T* stands for S or T that can accept phosphorylation. This data strongly suggests that Thr209 is one of the threonine phosphorylation sites in PLC-δ_1 (Fig. 5C). To confirm this result, we introduced a Thr209 to Ala mutation and the basal phosphorylation level was monitored using an anti-phospho-threonine antibody. The phosphorylation level of PLC-δ_1 Thr209Ala was decreased ~30% (Fig. 5D). The result suggests that PLC-δ_1 is phosphorylated on multiple sites and Thr209 is one of the sites.

EFFECT OF DEPHOSPHORYLATION OF ENDOGENOUS PLC-δ_1 ON ITS ACTIVITY

Since no stimulant changed the phosphorylation status of endogenous PLC-δ_1, we investigated whether the constitutive

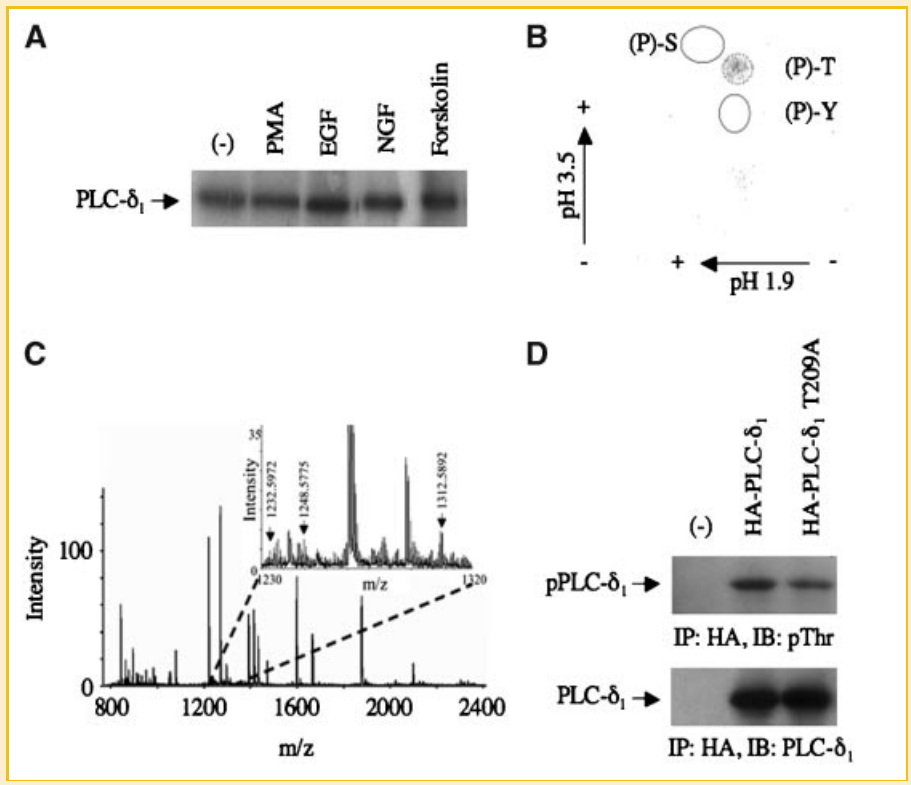

Fig. 5. Phosphorylation of endogenous PLC-δ_1. A: PC12 cells were metabolically radiolabeled with 200 μCi/ml [^{32}P]orthophosphate for 2 h and then were unstimulated (–), stimulated with 100 ng/ml PMA for 30 min, 50 ng/ml EGF for 5 min, 50 ng/ml NGF for 5 min, or 1 μM forskolin for 5 min. PLC-δ_1 was immunoprecipitated with an anti-PLC-δ_1 polyclonal antibody. Phosphorylated PLC-δ_1 was detected by autoradiography. B: Phosphorylated PLC-δ_1 extracted from unstimulated PC12 cells was subjected to phosphoamino acid analysis. C: PLC-δ_1 was immunoprecipitated with an anti-PLC-δ_1 monoclonal antibody from C6 cells and then digested with trypsin. The peptide fragments of PLC-δ_1 were subjected to MALDI-TOF mass spectrometry. D: HA–PLC-δ_1 or Thr209Ala mutant were transfected into COS7 cells and immunoprecipitated with an anti-HA monoclonal antibody. The precipitated products were subjected to immunoblot using an anti-phospho-Thr monoclonal antibody (pThr) (top) and an anti-PLC-δ_1 monoclonal antibody (bottom), respectively. The result represents one of the three independent experiments. Other experiments gave similar results.

phosphorylation of PLC-δ_1 was affected by endogenous phosphatase(s). Metabolically labeled PC12 cells were extracted in a mild extraction buffer with or without broad-specific phosphatase inhibitors. Cell extracts were immunoprecipitated with an anti-PLC-δ_1 antibody. The immune complexes were subjected to SDS–PAGE and analyzed by autoradiography. As shown in Figure 6A, phosphorylation level of the 85-kDa band decreased time-dependently (by 85% after 120 min) in the absence of phosphatase inhibitors, whereas the addition of phosphatase inhibitors blocked this dephosphorylation. These results suggest that the constitutive phosphorylation sites in native PLC-δ_1 are target sites of endogenous phosphatase(s).

To investigate the effect of dephosphorylation of endogenous PLC-δ_1 on the enzymatic activity, we carried out PLC assay using PLC-δ_1 immune complexes. Endogenous PLC-δ_1 was isolated using anti-PLC-δ_1 antibody and dephosphorylated by endogenous phosphatase in vitro. When PLC activity assay was performed using the immune complexes, the enzymatic activity was increased ~30% after dephosphorylation (Fig. 6B). These data suggest that the constitutive phosphorylation of PLC-δ_1 negatively regulate its activity.

PKC-α DECREASES SERINE PHOSPHORYLATION OF PLC-δ_1 IN VIVO

Finally, we investigated the effect of PKC on the phosphorylation of PLC-δ_1 in vivo. For this experiment, we overexpressed the PKC-α into NIH-3T3/neo or NIH-3T3/PLC-δ_1 cells and then phosphorylation of PLC-δ_1 was detected using an anti-phospho-threonine or an anti-phospho-serine antibody. The overexpression of PKC-α unexpectedly decreased the serine phosphorylation of PLC-δ_1 about 20% (Fig. 6C). The threonine phosphorylation of PLC-δ_1 was not affected by PKC-α (Fig. 6C).

PKC-α SUPPRESSES PLC-δ_1-DEPENDENT INOSITOL PHOSPHATE PRODUCTION IN VIVO

Since overexpression of PKC-α decreased serine phosphorylation of PLC-δ_1, we investigated the effect of PKC-α overexpression on PLC-δ_1 activity in vivo. To address this, NIH-3T3/neo or NIH-3T3/PLC-δ_1 were infected with different pfu of adenoviruses expressing PKC-α. After labeling with [^{3}H]inositol for 24 h, total inositol phosphates were measured using the AG 1-X8 anion exchange column system. Normally, the generation of basal inositol phosphates was higher in NIH-3T3/PLC-δ_1 cells than NIH-3T3/neo cells (Fig. 6D). This basal inositol phosphates generation decreased as the expression level of

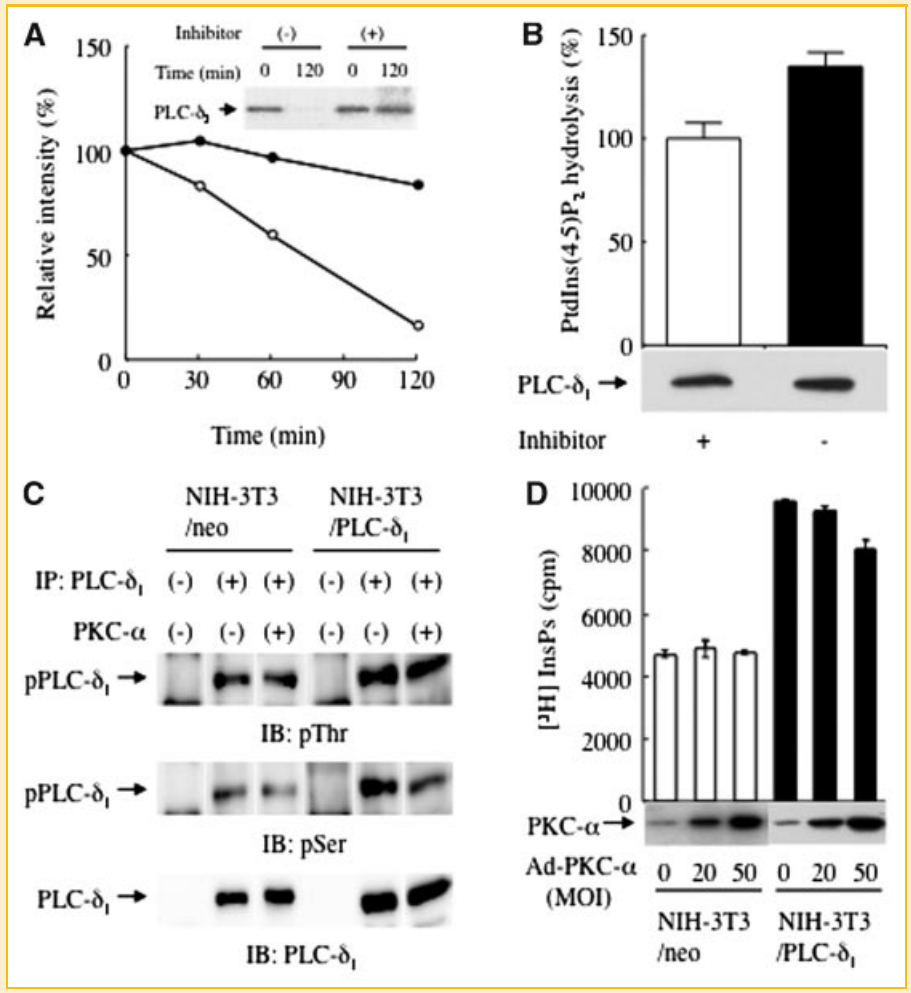

Fig. 6. Relationship between the basal phosphorylation level of PLC-δ_1 and PLC activity. A: PC12 cells were metabolically radiolabeled with 200 μCi/ml [^{32}P]orthophosphate for 2 h and then extracted by 0.1% NP-40-TNE buffer with (+) or without (−) phosphatase inhibitors. The extracts were then incubated for the indicated time at 37°C. After immunoprecipitation using an anti-PLC-δ_1 polyclonal antibody followed by SDS-PAGE, the phosphorylation level of PLC-δ_1 was determined by autoradiography. Time-dependent changes in the phosphorylation level of endogenous PLC-δ_1 with (closed circle) or without (open circle) phosphatase inhibitors are shown. Inset shows an autoradiogram of phosphorylated PLC-δ_1 at time 0 or 120 min after incubation at 37°C. The data represent one of the three independent experiments. B: PC12 cells were extracted with 0.1% NP-40-TNE buffer with (+) or without (−) phosphatase inhibitors, and the extracts were incubated for 2 h at 37°C. After immunoprecipitation, products were subjected to PLC assay. Values were the means ± SE of the two experiments carried out in duplicate. Immunoblotting of the precipitated products using an anti-PLC-δ_1 polyclonal antibody is shown in the lower panel to confirm the protein amount. C: NIH-3T3/neo cells or NIH-3T3/PLC-δ_1 cells were transfected with PKC-α and then cell lysates were subjected to immunoprecipitation using an anti-PLC-δ_1 polyclonal antibody. The immunocomplexes were solved by SDS-PAGE and analyzed by immunoblotting using an anti-phospho-Thr polyclonal antibody (pThr) (top), an anti-phospho-Ser polyclonal antibody (pSer) (middle) or an anti-PLC-δ_1 polyclonal antibody (bottom). D: NIH-3T3/neo cells or NIH-3T3/PLC-δ_1 cells were infected with the indicated pfu of adenoviruses expressing PKC-α and labeled with 1 μCi/ml [^{3}H]inositol for 24 h. After 40 min incubation with 20 mM LiCl, cells were collected, extracted, and total [^{3}H]inositol phosphates were sampled and measured using an AG 1-X8 anion exchange column. Values were the means ± SE of the two experiments carried out in duplicate. The bottom panel shows the expression level of PKC-α.

PKC-α increased in NIH-3T3/PLC-δ_1 cells, whereas NIH-3T3/neo cells remained unchanged (Fig. 6D). Taken together, these results suggest that PKC induces dephosphoylation of serine residue of PLC-δ_1 by unknown mechanism and that PKC suppresses PLC-δ_1 activity in the cells.

DISCUSSION

The PH domain is a protein module that functions as a phosphoinositide/inositolpolyphosphate-binding motif, in addition to its interaction with various proteins [Hirata et al., 1998; Lemmon and Ferguson, 1998, 2000; Cozier et al., 2004]. There are several reports concerning phosphorylation of PH proteins by PKC. For example, pleckstrin, which has two PH domains, is phosphorylated by PKC in vitro at two serine residues, Ser113 and Ser117, in the N-terminal PH domain [Craig and Harley, 1996]. Furthermore, this phosphorylation is essential for membrane targeting or cytoskeletal distribution of pleckstrin [Brumell et al., 1997]. In another case, the Tec family tyrosine kinases Btk and Emt, both participate in B-cell maturation, are associate with several PKC isoforms (α, βI, βII, ε, ζ, and θ) via their PH domains in vivo and in vitro [Yao et al., 1994, 1997;

Kawakami et al., 1995]. Phosphorylation of Btk by purified PKC (α, β, and γ) down-regulates its tyrosine kinase activity, and PKC-specific inhibitors potentiate the tyrosine phosphorylation of Btk induced by FcεRI cross-linking in vivo [Yao et al., 1994]. Conversely, co-expression of Emt and a Ca^{2+}-dependent PKC isoform (α, βI, or βII) in COS7 cells results in phosphorylation of Emt, and this phosphorylation activates the tyrosine autophosphorylation activity of Emt. It has been also reported that RACK (the receptor for activated C-kinase) binds to the PH domain of β-spectrin or dynamin-1 depending on the activation state of PKC [Rodriguez et al., 1999]. These results suggest that several proteins can be phosphorylated in their PH domains by PKC isoforms and that PKC phosphorylation regulates their function such as enzymatic activity and/or subcellular localization.

In this article, we demonstrated that PLC-δ_1 bound to PKC-α or -βII (Fig. 2A–C) and was phosphorylated by PKC in vitro (Figs. 1A and 3B). The PKC-binding site was defined as the PH domain of PLC-δ_1 (Fig. 2D). This interaction was conserved in PLC-δ_3 but not in the PLC-$\delta_{2/4}$ isoform (Fig. 2E). The Ser35 in the PH domain of PLC-δ_1 was one of the PKC phosphorylation sites in vitro (Fig. 3). PLC-δ_1 phosphorylation by PKC increased the enzymatic activity and [^{3}H]Ins(1,4,5)P_3 binding of PLC-δ_1 in vitro (Fig. 4). These data clearly indicate that PLC-δ_1 binds conventional PKC via its PH domain and its activity is regulated by PKC related phosphorylation similar to other PH domain-containing proteins.

While potential PKC phosphorylation site(s) in vitro were revealed to be serine residues, endogenous PLC-δ_1 was mainly phosphorylated on threonine residues and relatively small levels of phosphorylation occurred on serine residues in quiescent PC12 and NIH3T3 cells (Figs. 5B and 6C). Dephosphorylation of this basal phosphorylation by endogenous phosphatase(s) (Fig. 6A) increased the PLC-δ_1 activity in PC12 cells (Fig. 6B). Interestingly, overexpression of PKC-α decreased phosphorylation of PLC-δ_1 at serine residues but not threonine residues (Fig. 6C) and also suppressed PLC-δ_1-dependent basal inositol polyphosphate production in NIH-3T3/PLC-δ_1 cells (Fig. 6D). These results combined together suggest that the constitutive phosphorylation of PLC-δ_1 at threonine residues confers a potential negative regulation and that overexpression of PKC-α also endows negative regulation by dephosphorylation of phospho-serine(s) in vivo.

What is the relationship between our phosphorylation data obtained from in vitro and in vivo experiments, regarding residue(s) (serine vs. threonine) to be phosphorylated and the effect on the PLC activity? As mentioned above, when it binds directly to isolated unmodified PLC-δ_1, PKC-α phosphorylates serine residues of PLC-δ_1 (Fig. 1B) including Ser35 (Fig. 3) and this phosphorylation enhanced the enzymatic activity of PLC-δ_1 in vitro (Fig. 4A). Dephosphorylation of endogenous PLC-δ_1 especially on its serine residues could therefore cause suppression of the enzymatic activity. In fact, overexpression of PKC-α induced dephosphorylation of PLC-δ_1 at serine residues (Fig. 6C: the middle panel) that correlates with suppression of the enzymatic activity in vivo (Fig. 6D). On the other hand, a majority of the constitutive phosphorylation of PLC-δ_1, which occurred on threonine residues including Thr209, was not significantly affected by activation with various stimulants (Fig. 5A) or overexpression of PKC-α (Fig. 6C: the top panel) and the phosphorylation of PLC-δ_1 at threonine residues suppressed PLC-δ_1 activity (Fig. 6A,B). One speculative explanation is that the phosphorylation at Thr209 functions as constitutive negative regulator and that unidentified stimuli induce dephosphorylation of PLC-δ_1 Thr209 and subsequent phosphorylation on other kinase acceptor site(s) including Ser35 accomplishes full activation of PLC-δ_1. The physiological relevance of the Ser35 phosphorylation and of the constitutive threonine phosphorylation, which presumably occurs at multiple sites, should be clarified in the future. Furthermore, it is also important to identify the kinase(s) and phosphatase(s) that involved in PLC-δ_1 phosphorylation and dephosphorylation, respectively. Although PKC was a potent kinase for phosphorylation of PLC-δ_1, other kinases such as PKG or PKA also phosphorylated PLC-δ_1 in vitro (Fig. 1A). It is possible that various kinases and phosphatases regulate PLC-δ_1 activity by modulating phosphorylation in vivo. Additionally, it is possible that the phosphorylation/dephosphorylation of PLC-δ_1 at multiple sites will induce the conformational change of PLC-δ_1 and would confer an allosteric effect on its activity as discussed below.

The PLC-δ_1 PH domain and PLC-δ_1 without the PH domain were co-crystallized with Ins(1,4,5)P_3 independently and three-dimensional structures were obtained from X-ray crystallographic studies [Ferguson et al., 1995; Essen et al., 1996, 1997]. These structural studies suggest that the positively charged amino acids in the PH domain form hydrogen bonds with the phosphate groups of Ins(1,4,5)P_3. Ser35 is situated in the loop between the β1 and β2 strands (the β1/β2 loop), one of the three important ligand-binding loops (Fig. 7A). In general, phosphorylation brings a large steric and electrostatic disturbance near the phosphorylation site. Since the side chain of Ser35 is positioned in such a way that it faces the ligand-binding pocket of the PH domain, Ser35 phosphorylation was predicted to hinder the ligand binding. Contrary to our expectation, however, phosphorylated enzyme showed apparent increases both in ligand binding and in enzymatic activity (Fig. 4).

It is plausible that the introduction of a bulky negatively charged molecular cluster causes a dramatic change to the structure of the PH domain so that the affinity for the ligand increases, allowing the rest of the enzyme to interact with membranes without changing its conformation. Alternatively, it is possible that phosphorylation of Ser35 induces a conformational change in the PH domain that has an allosteric effect on the rest of the molecule, especially in the catalytic domain, resulting in efficient hydrolysis of the substrate.

Although Thr209 was identified as a potential in vivo phosphorylation site, its function is still unclear. Since Thr209 is situated in the first EF hand motif that is far from the catalytic core (Fig. 7B), phosphorylation status of this site may not directly influence enzymatic activity. Nevertheless, Thr209 may modulate the PLC-δ_1 function by modifying Ca^{2+} binding affinity of the EF hand.

It is possible that phosphorylation of PLC-δ_1 regulates its subcellular distribution and local activation as reported in other PH domain-containing proteins such as pleckstrin and DG kinase δ_1 (DGKδ_1) [Brumell et al., 1997; Imai et al., 2004]. It was reported that PLC-β_1 was phosphorylated by ERK in the nucleus. This phosphorylation plays a critical role in the activation of the nuclear phosphoinositide hydrolysis and mitogenic action of IGF-I [Xu

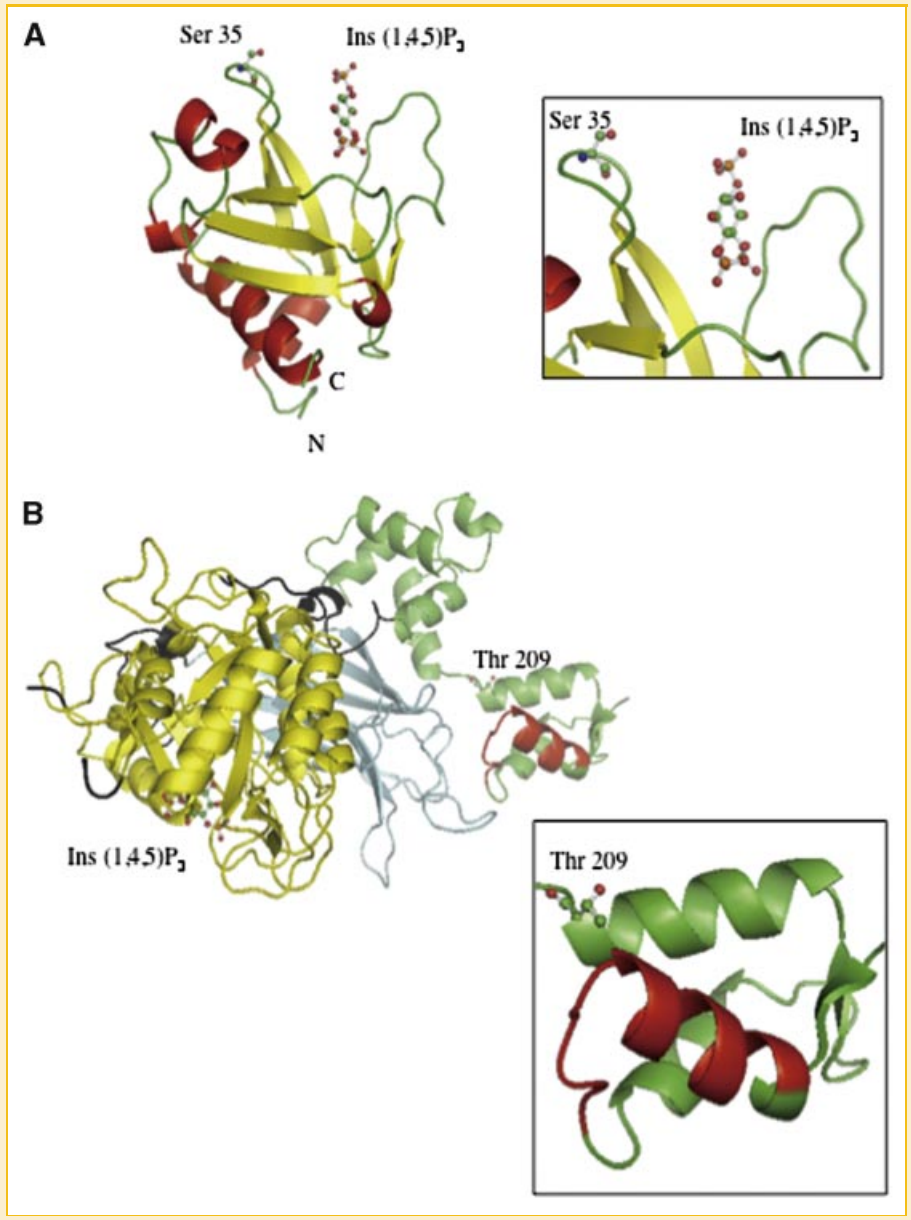

Fig. 7. Positions of Ser35, the estimated phosphorylation site by PKC, in the PLC-δ_1 PH domain and of Thr209, the constitutively phosphorylated residue in the EF hand motif. The 3D ribbon models for the PLC-δ_1 PH domain and the core structure were depicted by MacPyMOL software using protein data base 1MAI and 1DJX, respectively [Ferguson et al., 1995; Essen et al., 1997]. A: A model of the PLC-δ_1 PH domain with Ins(1,4,5)P_3. The α-helices and β-sheets are shown in red and yellow, respectively. Ins(1,4,5)P_3 and Ser35 are depicted by the ball and stick structure. Inset shows a magnified view of the ligand site and Ser35. B: A model of the PLC-δ_1 catalytic domain with Ins(1,4,5)P_3. The EF hand domain, the NES sequence, catalytic X–Y domain, and the C2 domain are shown in green, red, yellow, and cyan color, respectively. Ins(1,4,5)P_3 and Thr209 are depicted by ball and stick structure. Inset shows a magnified view of the NES sequence and Thr209. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2001]. Likewise, there is a possibility that the phosphorylation of PLC-δ_1 occurs in restricted subcellular compartments such as in the nucleus, since PLC-δ_1 shuttles between the cytoplasm and the nucleus using its nuclear export signal (NES) sequence and nuclear localization signal-like sequence [Yamaga et al., 1999; Okada et al., 2002, 2005]. Interestingly, Thr209 is localized near the NES sequence (amino acid 164–177) (Fig. 7B) [Yamaga et al., 1999], therefore it is possible that the phosphorylation at Thr209 affects the subcellular localization of PLC-δ_1.

In conclusion, we showed that PLC-δ_1 activity is regulated by phosphorylation and dephosphorylation. PKC-α was one of the kinases that modulate PLC-δ_1 phosphorylation. Ser35 and Thr209 were identified as potential phosphorylation sites. It is important to elucidate the physiological relevance of the Ser35 phosphorylation by PKC and the constitutive phosphorylation on Thr209. PKC-α mediated phosphorylation can at least partly regulate the functional responsiveness probably by changing the structure and localization of the enzyme. Use of PKC-α gene knockdown and/or ectopic

expression of catalytically active PKC-α would be the next step to examine the functional relevance of PLC-δ_1 phosphorylation.

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