Phosphorylation and Activation of Phospholipase D1 by Protein Kinase C in Vivo: Determination of Multiple Phosphorylation Sites†

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Received March 12, 1999; Revised Manuscript Received May 21, 1999

ABSTRACT: Protein kinase C (PKC) is an important regulator of phospholipase D1 (PLD1). Currently there is some controversy about a phosphorylation-dependent or -independent mechanism of the activation of PLD1 by PKC. To solve this problem, we examined whether PLD1 is phosphorylated by PKC in vivo. For the first time, we have now identified multiple basal phophopeptides and multiple phorbol myristate acetate (PMA) induced phosphopeptides of endogenous PLD1 in 3Y1 cells as well as of transiently expressed PLD1 in COS-7 cells. Down regulation or inhibition of PKC greatly attenuated the PMA-induced phosphorylation as well as the activation of PLD1. In the presence of PMA, purified PLD1 from rat brain was also found to be phosphorylated by PKCα in vitro at multiple sites generating seven distinct tryptic phosphopeptides. Four phosphopeptides generated in vivo and in vitro correlated well with each other, suggesting direct phosphorylation of PLD1 by PKCα in the cells. Serine 2, threonine 147, and serine 561 were identified as phosphorylation sites, and by mutation of these residues to alanine these residues were proven to be specific phosphorylation sites in vivo. Interestingly, threonine 147 is located in the PX domain and serine 561 is in the negative regulatory "loop" region of PLD1. Mutation of serine 2, threonine 147, or serine 561 significantly reduced PMA-induced PLD1 activity. These results strongly suggest that phosphorylation plays a pivotal role in PLD1 regulation in vivo.

Phospholipase D (PLD)[1] hydrolyzes phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. PA can be metabolized to diacylglycerol (DAG) by PA phosphohydrolase. PA and DAG are involved in receptor-mediated intracellular signal transduction, secretion, cytoskeletal reorganization, and respiratory burst (*1*, *2*).

It has been suggested for a long time that protein kinase C (PKC) is a major mediator of PLD activation by various agonists such as PDGF, EGF, thrombin, bradykinin, angiotensin II, and vasopressin (*3*−*6*). PLD becomes activated in various cells in response to phorbol ester, an activator of PKC, or in response to receptor agonists which induce phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (*3*, *4*). PKC inhibitors or down regulation of PKC block phorbol myristate acetate (PMA) or agonist-induced activation of PLD in many cell types (*3*, *4*). Although PKC regulates a variety of cellular functions via phosphorylation of target proteins, in vitro studies with cell membranes or partially purified PLD have indicated that PKCα and PKC*β* can directly activate the enzyme in an ATP-independent manner (*7*−*9*). However, it is possible that the in vivo stimulation of PLD may require a phosphorylation-dependent process, since inhibitors of the catalytic activity of PKC such as Ro-31-8220, bisindolymaleimide, H7, and chelerythrine usually diminish agonist-induced PLD activity in many cells (*3*−*6*, *10*, *11*). Evidences of ATP-dependent activation of PLD by PKC in cell-free preparations have been reported (*12*, *13*).

Recently, two mammalian PLD isoforms, PLD1 and PLD2, have been cloned and isolated from human, rat, and mouse cDNA libraries (*14*−*19*). PLD1a, a 124 kDa protein, is specific for PC and can be activated by PKC, ADP-ribosylation factor (ARF), and Rho A in vitro (*14*, *20*). PLD1b has been identified as a shorter splice variant of PLD1a, lacking 38 amino acids while retaining properties similar to PLD1a (*20*). PLD2 is constitutively active, but it is only poorly activated by PKCα in vitro (*19*). In COS-7 cells, the activity of recombinantly expressed PLD1 was enhanced by PMA treatment (*15*, *19*) while PLD2 was constitutively active and insensitive to PMA treatment (*19*). Therefore, one could conclude that PLD1 is responsible for that part of PLD activity that is induced by PMA treatment of cells. However, it remained unclear whether PLD1 becomes phosphorylated upon PMA treatment. We report

† This work was supported in part by a program of the Korea Science and Engineering Foundation (98-0403-08-03-5), the "97 Good Health R & D Project" of the Ministry of Health & Welfare, and the Center for Cell Signaling Research in the Republic of Korea.

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[1] Abbreviations: PLD, phospholipase D; PMA, phorbol myristate acetate; PKC, protein kinase C; PC, phosphatidylcholine; PA, phosphatidic acid; DAG, diacylglycerol; ARF, ADP-ribosylation factor; PIP_2, phosphatidyl inositol 4,5-bisphosphate; PEt, phosphatidylethanol; Sf9, *Spodoptera frugiperda*; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization.

10.1021/bi990579h CCC: $18.00
Published on Web 07/20/1999

here the PMA-dependent phosphorylation of endogenous PLD1 in rat embryo fibroblastic 3Y1 cells as well as of transiently expressed PLD1b in COS-7 cells. The phosphorylated sites were determined, and the importance of the phosphorylation at the sites is being discussed. We suggest that phosphorylation at these sites is critical to the regulatory mechanism.

EXPERIMENTAL PROCEDURES

Materials. PMA and TPCK-trypsin were purchased from Sigma (St. Louis, MO). PMSF, leupeptin, pepstatin A, aprotinin, and FuGene were obtained from Boehringer Mannheim (Mannheim, Germany). [^{3}H]Orthophosphate and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Dupont NEN (Boston, MA). [^{3}H]Myristic acid (54 Ci/mmol) was obtained from Amersham International (Buckinghamshire, U.K.). Silica gel 60 thin-layer chromatography (TLC) plates and cellulose TLC plates were from MERCK (Darmstadt, Germany). Immobilized protein A was obtained from PIERCE (Rockford, IL). Dulbecco's modified Eagle's medium, low glucose Dulbecco's modified Eagle's medium, phosphate-free Dulbecco's modified Eagle's medium, TC-100, bacterial alkaline phosphatase (150 units), and LipofectAMINE were purchased from GIBCO-BRL (Grand Island, NY). Bovine calf serum and fetal calf serum were obtained from HyClone (Logan, UT). Ro-31-8220 and GF 109203X (bisindolymaleimide) were obtained from Calbiochem (San Diego, CA). Sequencing grade trypsin was obtained from Promega (Madison, WI). PD-10 prepacked columns, Hitrap Q cartridge, and Phenyl Superose FPLC column were obtained from Pharmacia (Uppsala, Sweden). His-Bind resin was purchased from Novagen (Madison, WI).

Cell Culture. Rat embryonic fibroblast 3Y1 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in low glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. COS-7 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in high glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. *Spodoptera frugiperda* (Sf9) cells were cultured at 27 °C in TC-100 medium supplemented with 10% fetal calf serum.

In Vivo Assay of PLD. Labeling in 3Y1 cells was done as described previously (*5*, *21*) with some modification. Initially, 8×10^5 3Y1 cells/60 mm dish were washed twice with serum-free medium. To down-regulate PKC, the cells were treated with 100 nM PMA in serum-free medium. After 48 h of serum starvation, the cells were incubated with 3 mL of serum-free medium containing 9 μCi [^{3}H]myristic acid for 3 h. After the cell monolayers were washed twice with PBS, the cells were treated with or without 10 μM GF 109203X for 15 min followed by 100 nM PMA in the presence of 1.5% ethanol for 5 min as indicated in the figure legends. At the end of the incubations, lipids were extracted and separated with on a Silica gel 60 TLC plate. The radioactivity associated with phosphatidylethanol was determined by liquid scintillation counting after scraping the samples from the TLC plate. For PLD assay in COS-7 cells, the PLD1 or its various mutants were transiently overexpressed in 5×10^5 cells/60 mm dish. Transfection was done with LipofectAMINE according to the manufacturer's instructions. At 24 h after transfection, the cells were starved for 24 h and then incubated with 9 μCi [^{3}H]myristic acid for 3 h. The cells were treated with 100 nM PMA in the presence of 1.5% ethanol for 15 min.

In Vitro Assay of PLD with Mixed Lipid Vesicles. PLD activity was measured by choline release from PC essentially as described previously (*21*).

Phosphorylation of PLD1 in Intact Cells after Treatment with PMA. Initially, 1×10^7 3Y1 cells/150 mm dish were incubated with 3 mCi [^{3}H]orthophosphate in 15 mL of phosphate-free Dulbecco's modified Eagles medium for 3 h at 37 °C. After the cells were washed twice with serum-free medium, they were then treated with 100 nM PMA for 5 min as indicated in the figure legends. Next, 2.5×10^6 COS-7 cells/100-mm dish were transfected with 5 μg of pcDNA3.1 expression vectors containing rPLD1b cDNA obtained from Dr. John H. Exton at Vanderbilt University (*15*). For down regulation of PKC, the cells were treated with 100 nM PMA in serum-free medium 24 h after transfection. At 48 h after transfection, the cells were washed twice with serum-free medium. The cells were then exposed to 1.5 mCi [^{3}H]-orthophosphate in 7 mL phosphate-free Dulbecco's modified Eagles medium for 3 h at 37 °C. After the cells were washed twice with serum-free medium, they were then treated with 10 μM GF 109203X or 10 μM Ro-31-8220 for 15 min followed by 100 nM PMA for 5 min as indicated in the figure legends. The cells were washed with cold PBS and lysed in 1 mL of lysis buffer A (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, and 10 mM NaCl, 1% Triton X-100, and 1% sodium cholate) containing protease inhibitors (0.5 mM PMSF, 1 μg/mL leupeptin, and 5 μg/mL aprotinin) and phosphatase inhibitors (30 mM NaF, 1 mM Na_3VO_4, and 30 mM $Na_4O_7P_2$). After centrifugation (12000*g* for 15 min), equal amounts of soluble extract were incubated with 2 μg of anti-C-terminal of PLD1 antibody (*22*) and 25 μL of resin volume of immobilized protein A. Immunoprecipitation was done as described previously (*5*). The immunoprecipitated proteins were separated in 8% SDS−polyacrylamide gels according to Laemmli (*23*). The dried gel was then allowed to expose a photographic film for autoradiography.

Two-Dimensional Phosphopeptide Mapping. Two-dimensional phosphopeptide mapping was performed as described previously (*24*). We examined by Western blot that the same amount of PLD1 always immunoprecipitated from equal amounts of the soluble extracts. Therefore, phosphopeptide maps using the immunoprecipitated PLD1 could be used to compare quantitative differences. ^{32}P-Labeled PLD1 was excised from dehydrated SDS−polyacrylamide gels, after which it was eluted from the gel, precipitated with trichloroacetic acid, washed with absolute ethanol, oxidized with performic acid, and digested with TPCK-treated trypsin. The tryptic digest was lyophilized, and the phosphopeptides were then dissolved in 10 μL of pH 1.9 buffer (88% formic acid and acetic acid in deionized water at a ratio 25:78:897, v/v/v) and subjected to cellulose thin-layer chromatography. Electrophoresis in the first dimension was performed at 1000 V for 30 min in the pH 1.9 buffer using a HTLE 7000 electrophoresis system (C. B. S. Scientific Company, Del Mar, CA). The plate was subjected to ascending chromatography in phospho-chromatography buffer (*n*-butanol, pyridine, acetic acid, deionized water at a ratio 15:10:3:12, v/v/v/v). The plate was then mounted for autoradiography.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was performed essentially as described previously (*24*). Phosphopeptides were hydrolyzed in 200 μL of 6 N HCl, at 110 °C for 1 h. Hydrolysis was stopped by the addition of 200 μL of distilled H_2O, and the samples were centrifuged. The supernatants were lyophilized. After thin-layer electrophoresis using the HTLE 7000 electrophoresis system according to the manufacturer's instructions, the standards were visualized with ninhydrin, and the phosphoamino acid content of PLD1 was analyzed by autoradiography.

Purification of PLD1 from Rat Brain. Pure rat brain PLD1 was obtained by immunoaffinity column chromatography as described previously (*22*).

Expression and Purification of Rat PLD1b. cDNA of rat PLD1b was subcloned into pVL1393 (Invitrogen, NV Leek, The Netherlands). Sf9 cells were then cotransfected with recombinant pVL1393/PLD1b and BaculoGold (Pharmingen, San Diego, CA) using the FuGene transfection reagent. Recombinant baculovirus was amplified following the instruction manual (Parmingen). Monolayers of Sf9 cells (2×10^7 cells/150 mm dish, 30 dishes in all) were lysed and sonicated in 30 mL of lysis buffer B (20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 300 mM NaCl, 0.2 mM DTT, and 1% β-octylglucopyranoside) containing protease inhibitors (0.5 mM PMSF, 1 μg/mL leupeptin, and 5 μg/mL aprotinin). The lysates were centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was incubated with 1.5 mL of Ni^{2+}-bound His-Bind resin at 4 °C overnight with constant agitation. The resin was washed with 10 column volumes of buffer B containing 1 M NaCl. Bound protein was eluted sequentially with 20 mM, 50 mM, 80 mM, and 200 mM imidazole in 7.5 mL of buffer B. The fractions eluted with 200 mM imidazole buffer were pooled and injected onto four PD-10 gel-filtration columns equilibrated with buffer B. The fractions were then analyzed by SDS−PAGE and Western blotting. Pure PLD1b was obtained in 160 μg yield.

*Expression and Purification of PKC*α. PKCα was expressed in baculovirus (a kind gift from Dr. Wonhwa Cho, University of Illinois) infected Sf9 cells and purified as described (*25*) with some modifications. Monolayers of Sf9 cells (2×10^7 cells/150 mm dish, 10 dishes of cells) were infected at a multiplicity of infection of 10. PKCα was purified by Hitrap Q followed by Phenyl Superose FPLC.

*In Vitro Phosphorylation of PLD1 by PKC*α. PLD1 (100 ng) purified from rat brain was incubated with 15 ng of purified recombinant PKCα in phosphorylation buffer (30 mM Tris/HCl, pH 7.0, 6 mM Mg-acetate, 0.25 mM EGTA, 0.4 mM $CaCl_2$ 0.12 mM ATP, 2 μCi [γ-^{32}P]ATP (3000 Ci/mmol) in the presence of 1 μM PMA for 15 min. The reaction mixture was then electrophoresed through an 8% SDS−polyacrylamide gel. The dried gel was exposed to photographic film for autoradiography.

Separation of Tryptic Peptides by High-Performance Liquid Chromatography (HPLC). Purified recombinant PLD1 (80 μg) was incubated with 120 μg of purified recombinant PKCα in phosphorylation buffer containing 1 μM PMA for 1 h. The radiolabeled PLD1 was submitted to SDS−PAGE, and the proteins were transferred to nitrocellulose membrane. The radioactive PLD1 band was excised and digested overnight with trypsin (Promega, sequencing grade) using two additions of the enzyme in a ratio of 1:10 (wt/wt, enzyme:substrate). The digest was submitted to HPLC (79916OD-552, 5 μm, 100 × 2.1 mm, Hewlett-Packard) at a flow rate of 0.1 mL/min and eluted with a linear gradient solution ranging from 100% solvent A (0.01% CF_3COOH in distilled water) to 50% solvent B (0.01% CF_3COOH in CH_3CN) over 84 min and then to 100% solvent B over 18 min. Peptides were detected by monitoring UV absorbance at 210 nm. Radioactivity was monitored by Cerenkov counting.

Mass Spectrometry. The peak fractions of the elute containing radioactive peptides were completely lyophilized and resuspended in pH 1.9 buffer. The phosphopeptides were then further purified by two-dimensional phosphopeptide chromatography. The phosphopeptides were scraped from the cellulose plates and eluted with distilled water. The eluted phosphopeptides were completely lyophilized, resuspended in 10 μL of 25 mM NH_4HCO_3 buffer, pH 7.9, and then divided into two aliquots. The samples were then incubated either without or with 1 μL of bacterial alkaline phophatase at 37 °C for 1 h. The peptides were then lyophilized and resuspended in 5 μL of distilled water. A 1 μL aliquot was analyzed by a Voyager DE time-of-flight (TOF) mass spectrometer (Perceptive Biosystems, Inc., Framingham, MA) in the Korea Basic Science Institute. Matrix-assisted laser desorption/ionization (MALDI) was performed with cyano-4-hydroxycinnamic acid as the matrix. A precision of at least ±0.02% was achieved by using delayed extraction and calibration of the mass scale with three internal peptide standards. Sequence searches were performed with Peptide Mass Database program.

Plasmid Construction and Mutagenesis. Rat PLD1b cDNA was isolated and subcloned into pCDNA3.1 mammalian expression vector. Site-directed mutagenesis was performed by using the splice-overlap extension method (*26*). Briefly, the S2A mutation was obtained by using forward primer 5′-GG GGT ACC ATG GCA CTA AGA AGT GAG GCC CGG GTG AAC-3′/reverse primer 5′-GCT CTA GAT TAA GTC CAA ACC TCC ATG GGC AC-3′. From the PCR product, KpnI/XbaI fragment was cloned into pCDNA3.1. A similar strategy was used to generate T147A, S561A, and S588A mutations using the following oligonucleotides: forward primer 5′-GGG GTA CCC TGT CAG AAG TTA GAA TGT CAC-3′/reverse primer 5′-GAC ATT TTG TCT TCT AAA GGC ATG TCT TTT GGT-3′ (T147A) and forward primer 5′-C ACC AAA AGA CAT GCC TTT AGA AGA CAA AAT GTC-3′ (T147A)/reverse primer 5′-GCT CTA GAT TAA GTC CAA ACC TCC ATG GGC AC-3′, and forward primer 5′-GGG GTA CCC TGT CAG AAG TTA GAA TGT CAC-3′/reverse primer 5′-GCG ATA GAG GGC AAA TTT GGA GAA TTT CC-3′ (S561A) and forward primer 5′-GG AAA TTC TCC AAA TTT GCC CTC TAT CGC-3′ (S561A)/reverse primer 5′-GCT CTA GAT TAA GTC CAA ACC TCC ATG GGC AC-3′, and forward primer 5′-GGG GTA CCC TGT CAG AAG TTA GAA TGT CAC-3′/reverse primer 5′-C ACT TCG GAT GGC GCC GGT GTT GG-3′ (S588A) and forward primer 5′-CC AAC ACC GGC GCC ATC CGA AGT G-3′ (S588A)/reverse primer 5′-GCT CTA GAT TAA GTC CAA ACC TCC ATG GGC AC-3′. Next, the T147A, S561A, and S588A mutant DNAs were amplified using forward primer 5′-GGG GTA CCC TGT CAG AAG TTA GAA TGT CAC-3′ and reverse primer 5′-GCT CTA GAT TAA GTC CAA ACC TCC ATG GGC AC-3′. KpnI/XbaI fragments from the larger PCR

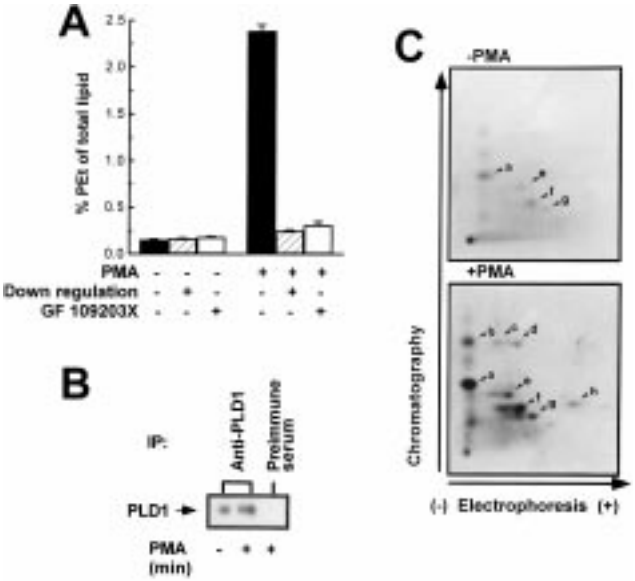

FIGURE 1: PMA-dependent activation and phosphorylation of endogenous PLD1 in 3Y1 cells. (A) PLD activity was measured in 3Y1 cells. For down regulation of PKC, the cells were exposed to 100 nM PMA in serum-free medium for 24 h after 24 h of serum starvation. The cells were incubated with 3 mL of serum-free medium containing [^{3}H]myristic acid for 3 h. The cells were then treated with 10 μM GF109203X for 15 min. Then 100 nM PMA was added in the presence of 1.5% ethanol for 5 min. Bars represent the range of duplicate determinations. (B) After the cells were loaded with [^{32}P]orthophosphate for 3 h, the cells were treated with 100 nM PMA for 5 min. Protein extracts (2 mg) were then incubated with anti-PLD1 antibody or preimmune serum. Immunoprecipitated proteins were separated by 8% SDS−PAGE, and the gel was dried. The gel was then allowed to expose a photographic film for autoradiography. (C) The phosphorylated PLD1 was excised and subjected to two-dimensional phosphopeptide mapping. Phosphopeptides were indicated by arrowheads and labeled as described in the Results section. The data are representative of three separate experiments.

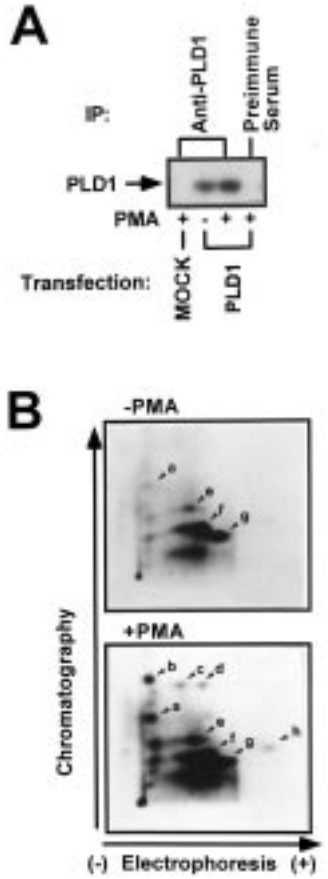

FIGURE 2: PMA-dependent phosphorylation of transiently expressed PLD1b in COS-7 cells. (A) COS-7 cells were transfected with vector or rat PLD1b cDNA. After loading with [^{32}P]orthophosphate for 3 h, the cells were treated with 100 nM PMA for 5 min. Protein extracts (1 mg) were then incubated with anti-PLD1 antibody or preimmune serum. The immunoprecipitated proteins were then separated by 8% SDS−PAGE. The dried gel was then mounted to expose a photographic film for autoradiography. (B) The phosphorylated PLD1 was excised, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping. Phosphopeptides are indicated by arrowheads and labeled as described in the Results section. The data are representative of three separate experiments.

products were cloned into pCDNA3.1. All mutations were verified by sequence analysis.

RESULTS

PMA-Dependent Phosphorylation of PLD1. PLD activity was judged by the accumulation of phosphatidyl ethanol (PEt), a stable product of transphosphatidylation of PLD. Treatment of rat embryonic 3Y1 cells with PMA greatly increased the activity of PLD. Down regulation of PKC by long-term treatment with PMA or inhibition of the kinase activity of PKC by preincubation with GF109203X attenuated the PMA-induced PLD activity (Figure 1A). Next, we tested PMA-dependent phosphorylation of PLD1 in 3Y1 cells. Endogenous PLD1 became phophorylated already during incubation with [^{32}P]orthophosphate (basal phosphorylation). It then continued to become even more phosphorylated after PMA treatment (Figure 1B). Phosphorylation of endogenous PLD1 after PMA treatment was also observed in Madin-Darby canine kidney cells (data not shown). We then eluted ^{32}P-labeled PLD1 species from a gel, digested it extensively with trypsin, and subjected it to two-dimensional phosphopeptide mapping. Multiple phosphopeptides could be resolved from phosphorylated PLD1 after PMA treatment as well as before. Eight phosphopeptides, which are indicated by arrowheads and designated a−h in Figure 1C, were increased or generated de novo after PMA treatment. To confirm the PMA-induced phosphorylation of PLD1, we transiently expressed rat PLD1b in COS-7 cells. Here PLD1 was also basally phosphorylated and became further phosphorylated after PMA treatment (Figure 2A). The basal phosphorylation also occurred in the COS-7 cells after serum deprivation for 24 h (data not shown). Two-dimensional phosphopeptide analysis of the phosphorylated PLD1 revealed similarity in the pattern of the maps between the transfected COS-7 cells and the 3Y1 both before and after PMA treatment (Figure 2B). However, the ratio of basal phosphopeptides (e, f and g in Figure 2B) to PMA-induced phosphopeptides was much higher in the COS-7 cells than in the 3Y1 cells.

PMA-Dependent Phosphorylation of PLD1 Is Directly Mediated by PKC. To examine whether it was PKC that mediated the PMA-dependent phosphorylation of PLD1, we down-regulated PKC by long-term treatment of the cells with PMA or by pretreatment of the cells with Ro-31-8220 or GF109203X, both of which act as competitive inhibitors of the ATP-binding site on PKC (Figure 3). The results are

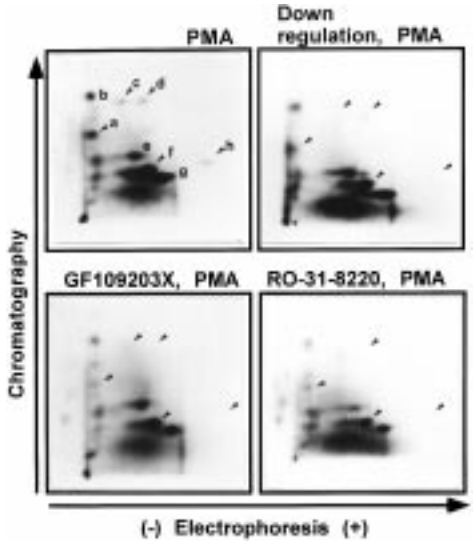

FIGURE 3: Effect of down regulation and inhibition of PKC on PMA-dependent phosphorylation of PLD1. COS-7 cells were transfected with rat PLD1b cDNA. To down-regulate PKC, 100 nM PMA was applied for 24 h. After loading with [^{32}P]orthophosphate for 3 h, the cells were pretreated with 10 μM GF109203X or 10 μM Ro-31-8220 for 15 min and then treated with 100 nM PMA for 5 min. The PLD1 was immunoprecipitated from 1 mg amounts of protein extracts and separated by 8% SDS−PAGE. The band of phosphorylated PLD1 was excised and digested with trypsin. The tryptic digest was subjected to two-dimensional phosphopeptide mapping. The arrowheads indicate phosphopeptides that appear significantly reduced after down regulation of PKCα or after pretreatment with PKC inhibitors. Spot "e" was used as an internal control to normalize comparisons. The data are representative of two separate experiments.

Table 1: Effect of Down Regulation and Inhibition of PKC on the PMA-Induced[a] PLD1 Phosphorylation

	relative intensity[b]			
spots		DR[c]	GF[c]	RO[c]
a	++	−	−	−
b	++	++	++	++
c	+	−	−	+
d	+	−	−	−
e	4+	4+	4+	4+
f	10+	8+	8+	8+
g	8+	8+	8+	8+
h	+	−	−	−

[a] PMA (100 nM) was treated for 5 min. [b] Relative intensity based on the summarized results of densitometric quantitation from two separate experiments. Spot "e" was used as an internal control to normalize the relative intensity of spots. [c] DR: down regulation of PKC, GF: preincubation of GF 109203X, RO: Ro-31-8220.

summarized in Table 1. At least five PMA-induced phosphopeptides (a, c, d, f, and h: arrowheads in Figure 3) were significantly reduced or had disappeared upon down regulation of PKC or treatment with PKC inhibitors. These results indicate that the PMA-induced phosphorylation of PLD1 is indeed mediated by PKC.

Previously we had observed a PMA-dependent association of PLD1 with PKCα in NIH-3T3 fibroblast cells and COS-7 cells transiently expressing PLD1 (*22*). PMA-dependent association of PLD1 with PKCα was also found in a caveolin-enriched membrane fraction (*27*). This led us to examine whether the PMA-induced phosphorylation of PLD1 was directly mediated by PKCα. We found that while purified PKCα could autophosphorylate, it did not phosphorylate purified PLD1 from rat brain in the absence of PMA (Figure 4A). On the other hand, in the presence of PMA, PKCα became even more autophosphorylated, and it also phosphorylated PLD1 (Figure 4A). The two-dimensional phosphopeptide map of in vitro phosphorylated PLD1 revealed seven distinct phosphopeptides (designated as 1−7 in Figure 4B). Among those seven only four (1, 2, 4, 7) overlapped the in vivo generated phosphopeptides (a, d, f, h) (Figure 4B). These results, therefore, indicate that these four phosphopeptides (1/a, 2/d, 4/f, and 7/h) are generated directly by PKC.

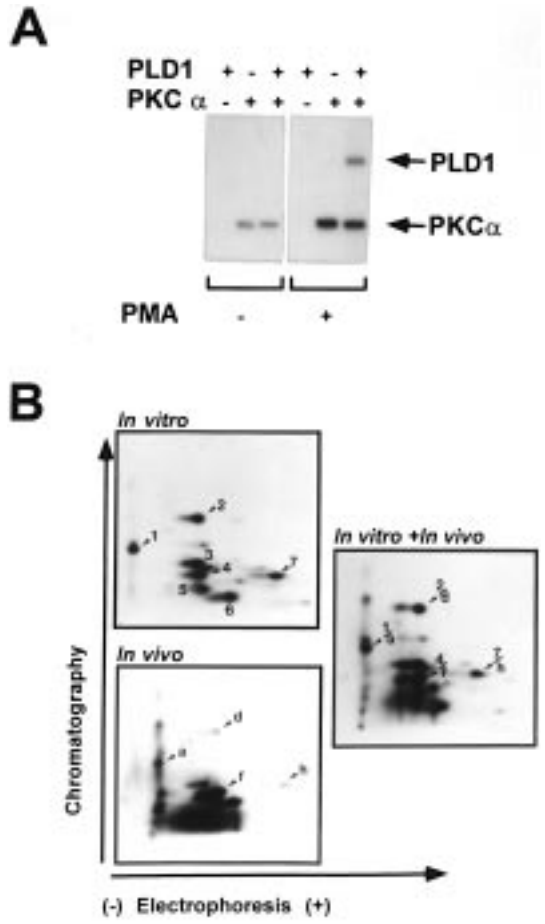

FIGURE 4: Comparison of the phosphopeptides of PLD1 phosphorylated in vitro and in vivo. (A) Immunoaffinity purified PLD1 (100 ng) of rat brain and/or 15 ng of PKCα were incubated in phosphorylation buffer in the absence or presence of 1 μM PMA for 15 min as indicated. (B) The in vitro phosphorylated rat brain PLD1 and the phosphorylated rat PLD1b obtained from COS-7 cells after a 5 min treatment with PMA were digested with trypsin. Each tryptic digest or the mixture of the two tryptic digests underwent two-dimensional phosphopeptide mapping. The arrowheads indicate overlapping phosphopeptides. The data are representative of three separate experiments.

N-Terminal Six-Histidine Tag on PLD1 Blocked Phosphorylation of Serine 2. In an effort to get a sufficient amount of PLD1 to be able to pin down the phosphorylation sites of PLD1, we expressed rat PLD1b as a histidine tagged fusion protein in Sf9 cells using recombinant baculovirus. The phosphorylation experiments with recombinant rat $(His)_6$-PLD1b revealed that one phosphopeptide had disappeared from the phosphopeptide map when compared to the map derived from phosphorylated PLD1 from rat brain (Figure 5A). The N-terminal histidine tag seemed to hinder the

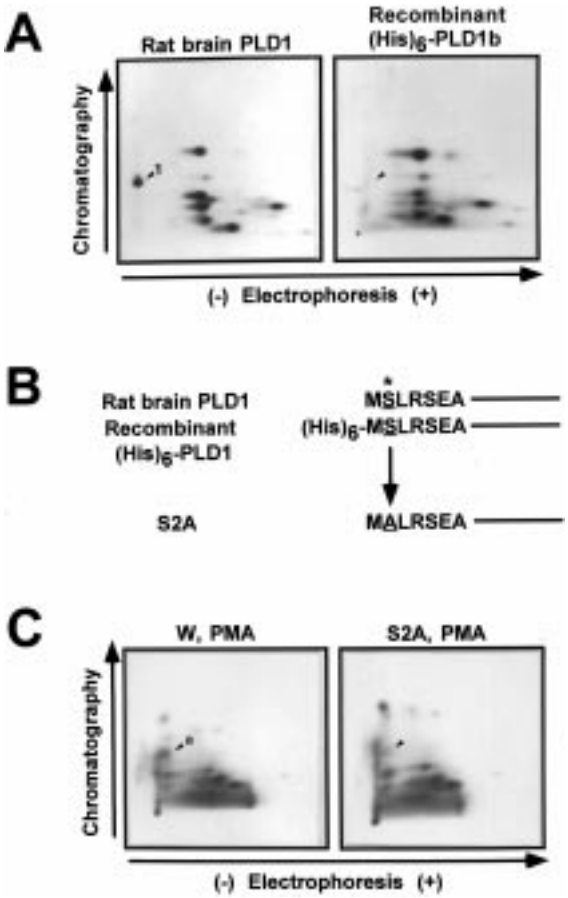

FIGURE 5: N-Terminal $(His)_6$ tag of PLD1 blocks the phosphorylation of serine 2. (A) Comparison of the in vitro phosphopeptide maps of rat brain PLD1 and recombinant $(His)_6$-PLD1b. An arrowhead indicates the position of spot 1. (B) Rationale for site-directed mutagenesis of serine 2 to alanine. (C) COS-7 cells were transfected with rat PLD1b (W) or S2A (S2A). After treatment with PMA for 5 min, the phosphorylated PLD1 was extracted and subjected to two-dimensional phosphopeptide analysis. The arrowhead indicates the position of spot "a". The data are representative of two separate experiments.

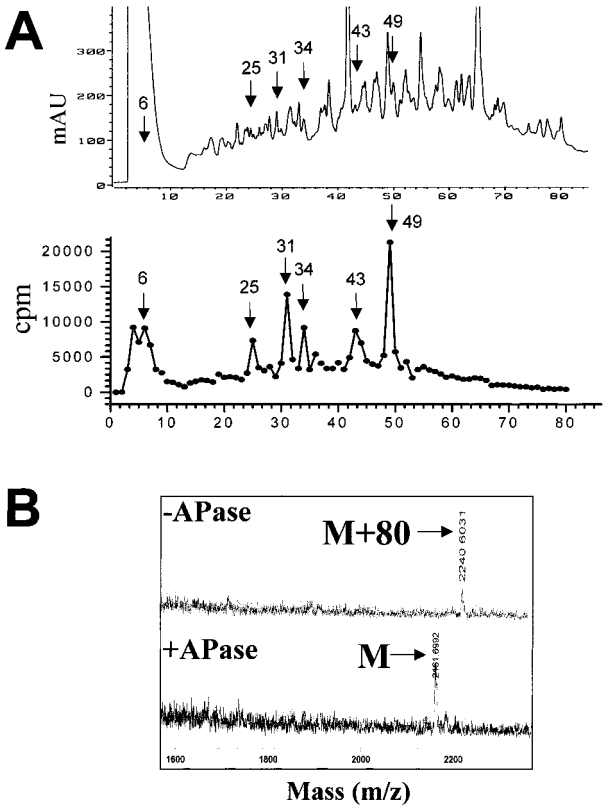

FIGURE 6: Determination of PLD1 phosphorylation sites. (A) Tryptic digests derived from 80 μg of phosphorylated PLD1 were injected into a C18 microbore reverse phase HPLC system. The eluted peptide peaks (upper profile) correlating with six major peaks of radioactivity (lower profile) are marked by arrows. The fraction numbers marked by arrows are 6, 25, 31, 34, 43, and 49. (B) A representative illustration showing the mass analysis of phosphopeptides in fraction 43 by MALDI mass spectrometry. The sequence of the phosphopeptide was identified based on the value of the mass. The results of the mass analysis are summarized in Table 2.

phosphorylation of one site (spot 1). Serine at position 2 is the only site with a consensus sequence for phosphorylation by PKC (SXR) up to position 92 (Figure 5B). When we mutated the serine at position 2 to alanine (S2A), one phosphopeptide (spot a) was absent from the map of in vivo phosphorylated S2A when compared to the wild type map (Figure 5C). This suggests that serine 2 is an in vivo site (spot a) for phosphorylation by PKC.

Sequence Identification of Phosphopeptides Recovered from Reverse Phase HPLC. To identify other phosphorylation sites, PKCα phosphorylated rat PLD1b was digested with trypsin and the tryptic digest was injected onto the HPLC column. The HPLC profiles of the tryptic peptides (absorbance at 210 nm) and the phosphopeptides (cpm) are shown in Figure 6A. Six major peaks of radioactivity were seen (fraction 6, 25, 31, 34, 43, and 49 indicated by arrows in Figure 6A). These peak fractions were further analyzed and purified by two-dimensional phosphopeptide mapping. The phosphopeptides in peak fractions 6, 25, 31, 34, 43, and 49 were correlated with spot 6, 4/f, 7/h, 3, 5, and 2/d, respectively (Table 2). The phosphoamino acid analysis of the phosphopeptide spots revealed that all phosphorylated residues are serine residues except spot 7/h which is threonine (Table 2).

The purified phosphopeptides eluted from the TLC plates were divided into two portions, and one was treated with alkaline phosphatase. We identified a peak with *m*/*z M* plus ~80 mass units which shifted to *m*/*z M* upon treatment with alkaline phophatase as illustrated in Figure 6B. The results are summarized in Table 2. The MALDI mass spectrometry spectrum of peak 31, peak 43, and peak 49 displayed a peak at *m*/*z* 640, 2240, and 765, respectively. Each peak shifted to *m*/*z* 560, 2161, and 685, respectively, upon treatment with alkaline phophatase, which is in accordance with the removal of a phosphate group from each peptide. Searching the Peptide Mass Database, we identified three tryptic peptides of PLD1: 146HTFL149 (*m*/*z* 560), 570NLHNSDSISSVDSASNTGSIR590 (*m*/*z* 2161), and 560FSLYR564 (*m*/*z* 685). Threonine 147, serine 561, and serine 588 were identified as the phosphorylation sites in these peptides by looking for a consensus sequence for phosphorylation by PKC (RXT, SXR, and KXS). We then mutated these residues to alanine (T147A, S561A, and S588A, respectively). Phosphorylation experiments with COS-7 cells expressing these mutants revealed that threonine 147 and serine 561 are in vivo sites for phosphorylation by PKC (data not shown). It is unlikely that the mutation at threonine 147 or serine 561 disrupts PKC

Table 2: Phosphopeptides Identified in the Tryptic Digest of PKC−Phosphorylated Rat PLD1

spots	peak[a]	P-AA[b]	$M+$ (~80)[c]	M[d]	sequence	site
1 (a[e])		P-Ser				serine 2[f]
2 (d)	49	P-Ser	765	685	560FSLYR564	serine 561
3	34	P-Ser				n.d.[g]
4 (f)	25	P-Ser				n.d.
5	43	P-Ser	2240	2161	570NLHNSDSISSVDSASNTGSIR590	serine 588
6	6	P-Ser				n.d.
7 (h)	31	P-Thr	640	560	146HTFL149	threonine 147

[a] Corresponding spots could be found in the two-dimensional phosphopeptide map of the peak fraction of radioactivity. [b] Phosphoamino acid was determined as described in Experimental Procedures. [c] *m/z* value in the absence of alkaline phosphatase. [d] *m/z* value in the presence of alkaline phosphatase. [e] Corresponding spots in vivo. [f] The site was determined in Figure 5. [g] Determination of mass failed in this method.

binding to PLD1, since T147A or S561A is still phosphorylated to a level similar to that for the wild type at serine 2 and 561, or threonine 147 and serine 2, respectively, by the treatment of PMA (data not shown). We could not determine the mass of the phosphopeptides in peak 6, 25, and 34 by the method described above.

PMA-Induced PLD1 Activity Is Reduced by Mutation of Serine 2, Threonine147, or Serine 561. PLD1 activity was measured in COS-7 cells transiently expressing wild type PLD1 or mutant PLD1 (Figure 7). Mutation of phosphorylation sites in vivo significantly reduced PLD1 activity after PMA stimulation (Figure 7A). However, there were no changes in the basal level of PLD1 activity (in the absence of PMA stimulation) with the mutant PLDs. To check whether the mutants had altered catalytic activity, we used the cell homogenates in a reconstitution assay with purified recombinant ARF and GTPγS (Figure 7B). Substitution of serine 2, threonine 147, or serine 561 did not interfere with GTPγS-dependent stimulation of PLD1 activity in vitro by ARF, suggesting that the mutated PLDs are still capable of full activity and the PLD structure is not grossly affected by the mutations. In all cases, the protein expression levels in the transfected PLD1 mutants were comparable to the level of the wild type PLD1 (Figure 7C). These results indicate that PKC phosphorylation is critical for PMA-induced PLD1 activity in vivo.

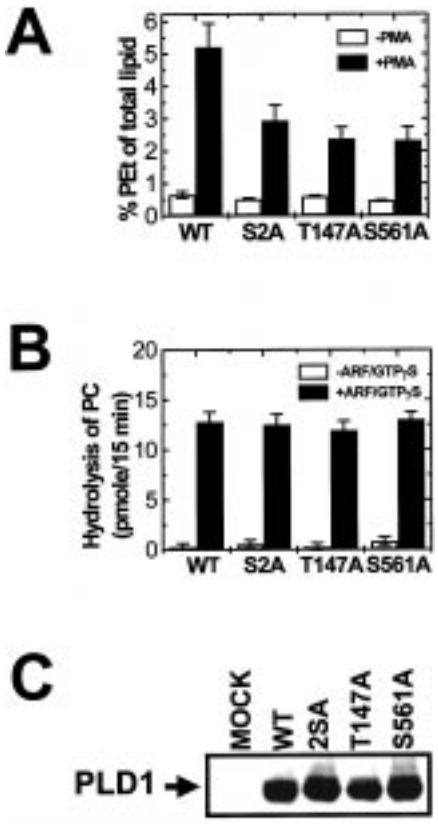

FIGURE 7: PMA-induced PLD1 activity is reduced by mutation of serine 2, threonine 147, or serine 561. COS-7 cells were transfected with vector (MOCK), wild type PLD1b (WT), S2A, T147A, or S561A. Twenty-four hours after transfection, the cells were starved for 24 h prior to incubation with [^{3}H]myristic acid. (A) After labeling with [^{3}H]myristic acid for 3 h, PEt accumulation was measured in the presence of 100 nM PMA and 1.5% ethanol for 15 min. The values were corrected for the background activities observed in cells transfected with vector (pCDNA3.1) and stimulated under the same conditions. PEt production is corrected for the expression level of PLD1. The data are a combined result summarized from four separate experiments. Bars represent the range of duplicate determinations. (B) In vitro PLD activity was measured with mixed lipid vesicles. Cell homogenates (0.2 μg) were assayed for PLD activity in the absence or in the presence of 1 μM purified recombinant ARF and 10 μM GTPγS. The values were corrected for the background activities observed in cells transfected with pCDNA3.1. PLD activity is corrected for the expression level of PLD1. The data are a combined result summarized from three separate experiments. Bars represent the range of duplicate determinations. (C) Cell homogenates (20 μg) were subjected to immunoblot analysis with anti-PLD1 antibody. The data are representative of more than four separate experiments.

DISCUSSION

Although PKC is a major activator of PLD in response to various agonists, the activation mechanism operating in vivo was still unknown. Thus, it became an important issue to determine whether PLD is phosphorylated directly by PKC in vivo.

In this paper we report the identification of multiple in vivo phosphorylation sites for PKCα on PLD1. PLD activity was stimulated by PMA treatment, and this was accompanied by phosphorylation of PLD1 (Figure 1). PMA-dependent activation and phosphorylation of PLD1 could be blocked by inhibiting PKC (Figure 1 and Figure 3). Although the phosphorylations could not be linked simply and directly to PLD activation because of basal and mutiple phosphorylations, phosphorylation seems to somehow play an important role in the regulation of PLD1 activity. In fact, the experiments with mutants in which these phosphorylation sites were substituted with alanine proved the importance of the phosphorylation at these sites. S2A, T147A, and S561A showed significantly reduced PMA-dependent PLD1 activity (Figure 7). However, the mutation of any single phosphorylation site did not completely abolish the PMA-induced PLD1 activity, implying that a combined phosphorylation at these sites might be important for the activation.

The identified in vivo PKCα phosphorylation sites on PLD1 are localized in specific regions of PLD1. A recent molecular analysis of PLD1 revealed that the N-terminal 168 amino acids respond to PMA in vivo (*28*). Sung et al. have shown that the amino terminal 325 amino acids are required for activation of PLD1 by PKCα in vitro and for the PMA dependency in vivo (*29*). Two of the determined phosphorylation sites, serine 2 and threonine 147, reside in this amino terminal region. Interestingly, threonine 147 is in the PX domain. It has been proposed that PX domains may possess *Src* homology 3 (SH3) domain-binding functions, since the majority of PX domains contain a polyproline motif (*30*). Phosphorylation of threonine 147 may, thus, regulate the interaction of PLD1 with yet unknown regulatory component-(s). Another phosphorylation site, serine 561, is located in the important region of PLD1 named the "loop". The amino acid sequence in this region (amino acids 505−620 in human PLD1a) is unique to PLD1, and it has been proposed that it serves as an effector regulatory region (*29*). Deletion of this region increases the basal activity of PLD1 more than 3-fold over the control. It is reasonable to guess that phosphorylation of serine 561 may participate in positive regulation of PLD1.

The pattern of the phosphorylation maps of PLD1 are complex. The phosphorylation spots can be categorized into three groups. The first group (a, d, f, h) contains phosphorylation sopts generated by PKC directly. These spots can be increased by PMA treatment, diminished by preincubation with PKC inhibitors, or by down regulation of PKC, and they correlate well with the phosphorylation spots generated by PKCα in vitro. The second category comprises the spots of basal phosphorylation (e, f, g) which have already occurred before treatment with PMA and are insensitive to the PKC inhibitors and down regulation of PKC. Spot f represents a basal phosphorylation before treatment with PMA which can be further increased by PMA treatment (Figure 1C and Figure 2B). However, only the PMA-induced portion of spot f is diminished by preincubation with PKC inhibitors or by down regulation of PKC. It is possible that two distinct phosphopeptides, a basal phosphopeptide and a PKC-phosphorylated peptide, might comigrate in the two-dimensional analysis. The third group (b, c) comprises PMA-dependent phosphorylation spots which do not correlate with phosphorylation spots generated by PKCα in vitro. The second and third group may be generated by other kinases. This complex pattern of phosphorylation implies that many signals may converge on PLD1 and result in its phosphorylation.

Phosphorylation-dependent regulation of PLD1 by PKC is a very important issue, yet it is far from clear. In this report, for the first time, we show evidence that PLD1 can be phosphorylated by PKC within cells, and we determined three important phosphorylation sites for PKC on PLD1 in vivo. These results prove the phosphorylation-dependent regulation of PLD1 by PKC.

ACKNOWLEDGMENT

We thank Dr. John H. Exton at Vanderbilt University for the cDNA of rat PLD1b, Dr. Wonhwa Cho at the University of Illinois for the PKCα-expressing baculovirus, and Ms. Gisela Hoschek for proofreading this manuscript.

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BI990579H