N-Benzyladriamycin-14-Valerate (AD198) Induces Apoptosis through Protein Kinase C-δ–Induced Phosphorylation of Phospholipid Scramblase 3

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Abstract

Phospholipid scramblase 3 (PLS3) is an enzyme that plays a critical role in mitochondrial morphology, functions, and apoptotic response. During apoptosis, activated protein kinase C-δ (PKC-δ) translocates to mitochondria and phosphorylates PLS3. Here, we utilize an extranuclear-targeted anthracycline *N*-benzyladriamycin-14-valerate (AD198), a PKC-δ activator, to investigate the mechanism of PLS3 phosphorylation by PKC-δ. Overexpression of PLS3 enhanced, whereas down-regulation of PLS3 by small interfering RNA decreased, the sensitivity of AD198-induced apoptosis. Overexpression of PKC-δ, but not the kinase-defective PKC-δ, and AD198 treatment enhanced threonine phosphorylation of PLS3. The phosphorylated threonine was mapped to Thr^{21} of PLS3. Mutation of Thr^{21} to alanine did not affect mitochondrial localization of PLS3 but abolished threonine phosphorylation by PKC-δ *in vitro* and AD198-induced PLS3 phosphorylation *in vivo*. Expression of PLS3(T21A) in cells could not enhance AD198-induced apoptosis compared with expression of the wild-type PLS3. Using benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone and cyclosporine A, we also showed that AD198-induced PLS3 phosphorylation occurs upstream of caspase activation and independent of mitochondrial permeability transition. These studies establish that AD198-activated PKC-δ induces phosphorylation of mitochondrial PLS3 at Thr^{21} and that PLS3 is a critical downstream effector of PKC-δ in AD198-induced apoptosis. (Cancer Res 2005; 65(21): 10016-23)

Introduction

Phospholipid scramblases (PLS) are enzymes responsible for bidirectional movement of phospholipids between two lipid compartments (1, 2). Four PLS family members have been identified by genome analysis (3). PLS1 is in the plasma membrane and can translocate to nucleus through the importin pathway (4, 5). PLS2 is in the nucleus but is less well characterized (6). PLS3 is present in mitochondria (7, 8). Our laboratory previously reported that PLS3 is a critical regulator of mitochondrial structure, respiratory function, and distribution of cardiolipin (7). Overexpression of wild-type PLS3 enhanced sensitivity to UV- and truncated Bid (tBid)–induced apoptosis and increased the amount of cardiolipin in the mitochondrial outer membrane. In contrast, mitochondria with expression of an inactive PLS3 mutant that abolished the calcium-binding motif had lower amounts of cytochrome *c* and cardiolipin and exhibited densely packed cristae. Consequently, their respiratory function, determined by oxygen consumption, was significantly compromised (7). Consistent with the respiratory defect in cells with suppressed PLS3 function, mice with targeted deletion of PLS3 had insulin resistance, glucose intolerance, and dyslipidemia that lead to aberrant accumulation of abdominal fat (9).

Protein kinase C (PKC), a family of phospholipid-dependent serine/threonine protein kinases, regulates a wide variety of cellular functions, including cell proliferation, differentiation, and cell death (10, 11). PKC-δ, a member of the novel PKC subfamily, is actively involved in apoptosis in a stimulus- and tissue-specific manner. PKC-δ regulates the expression and function of apoptosis-related proteins and is itself a target of caspases (12, 13). Both activation and intracellular distribution of PKC-δ have a significant effect on apoptosis. In response to apoptotic stimuli, the full-length or the catalytic fragment of PKC-δ translocates to distinct subcellular organelles, which include nucleus, mitochondria, Golgi complex, endoplasmic reticulum, and plasma membrane (12, 14–19). In each organelle, PKC-δ phosphorylates different substrates to induce various downstream events that eventually lead to cell death (8). One nuclear substrate is Rad9, which forms the 9-1-1 complex with Hus1 and Rad1 to regulate DNA damage response (18, 20). A substrate in the plasma membrane is PLS1 (21). Based on the observation that PLS1 is a substrate for PKC-δ, we investigated and established that PLS3 is a physiologic target of PKC-δ when PKC-δ translocates to mitochondria (8). After UV irradiation, PKC-δ physically interacted with and phosphorylated PLS3 with high affinity. Cells expressing wild-type PLS3 (HeLa-PLS3) became apoptotic upon phorbol ester stimulation, whereas the control cells did not. Expression of a mitochondrion-targeted PKC-δ enhanced apoptosis more prominently in HeLa-PLS3 cells than control HeLa cells and HeLa cells expressing an inactive PLS3 mutant (8).

With the establishment of PKC-δ as a death-promoting kinase, a potential strategy to induce apoptosis is to develop an activator of PKC-δ (13). This has been achieved by the development of the extranuclear-targeted anthracycline derivatives, *N*-benzyladriamycin-14-valerate (AD198) and *N*-benzyladriamycin-14-pivalate (AD445; refs. 22, 23). In contrast to the commonly used doxorubicin, AD198 has a weak effect in DNA binding and is a poor inhibitor of topoisomerase II (24–26). However, the experimental antitumor activity of AD198 is superior to that of doxorubicin, suggesting a distinct mechanism of cytotoxicity (24, 26, 27). AD198 rapidly accumulates in the cytoplasm and is able to circumvent resistance

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due to expression of multidrug resistance protein (23, 28, 29). Moreover, AD198 can override the antiapoptotic function of Bcl-2 and circumvent both nuclear factor-κB– and Bcl-xL–mediated resistance (25, 30). The C1b regulatory domain of PKC was shown to be the molecular target of AD198 (24, 26). AD198 promotes rapid translocation and activation of PKC-δ to mitochondria, which leads to cytochrome *c* release and caspase activation (26, 27). In the current study, we investigate the interaction of PLS3 and PKC-δ in response to AD198 and show that PLS3 is phosphorylated at Thr^{21} by PKC-δ.

Materials and Methods

Materials. The cDNA encoding full-length PLS3 was cloned into the QIAexpress pQE30 vector (Qiagen, Valencia, CA) to tag PLS3 with six consecutive histidine residues (6× His tag) at the NH_2 terminus (pQE-PLS3). A point mutant PLS3(T21A) was generated using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). As there were two serine residues generated at the cloning region of pQE30, one before and one after the consecutive histidine residues, these two serines were mutated to alanine to abrogate possible false phosphorylation. Mammalian expression vectors for His-tagged PLS3 and PLS3(T21A) were construed with pCMV vector. Mammalian expression vectors for wild-type (pHA-PKC-δ) and kinase-defective PKC-δ (pHA-PKC-δ KD, K376R) were kindly provided by Dr. Jae-Won Soh (Herbert Irving Comprehensive Cancer Center, Columbia University, NY). Mammalian PKC-δ small interfering RNA (siRNA) expression plasmid (pKD-PKC-δ-v3) was from Upstate (Lake Placid, NY). The polyclonal antibody to PLS3 was raised in rabbits against full-length recombinant PLS3 (Proteintech Group, Inc., Chicago, IL). The first 50-amino-acid fragment of PLS3 was made as His-tagged protein similar to full-length PLS3. The monoclonal antibody against PKC-δ was obtained from BD Biosciences (Palo Alto, CA). Monoclonal antibodies to phosphothreonine and β-actin were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). The polyclonal antibody to voltage-dependent anion channel was obtained from Affinity BioReagents (Golden, CO). Secondary antimouse or antirabbit antibodies conjugated with horseradish peroxidase, and protein G Sepharose beads were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Recombinant human PKC-δ enzyme was purchased from Calbiochem Biosciences (La Jolla, CA). [γ-^{32}P]ATP was from Life and Analytical Sciences (Boston, MA). siRNA against PLS3 and a random sequence siRNA were from Qiagen. Benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (Z-VAD) was from ICN Pharmaceuticals, Inc. (Aurora, OH) and cyclosporine A was from Sigma-Aldrich. MitoTracker Green was from Molecular Probes, Inc. (Eugene, OR). AD198 was provided by Dr. Mervyn Israel (Department of Pharmacology, University of Tennessee Health Science Center, Memphis, TN).

Cell culture, transfection, and treatment. HeLa cells were cultured in DMEM containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and penicillin (100 units/mL)-streptomycin (100 μg/mL) at 37°C in a humidified 5% CO_2 atmosphere. HeLa cells at 90% confluence were transfected with different mammalian expression vectors using LipofectAMINE 2000 according to the protocol of the manufacturer (Invitrogen, Inc., Carlsbad, CA). At 24 or 48 hours after transfection, cells were treated with 1:100 dilution of AD198 that was dissolved in DMSO. For down-regulation of PLS3, HeLa cells at 50% confluence were transfected with siRNA against PLS3 or a scrambled control. At 48 hours, cells were treated with AD198 for 16 hours and harvested for flow cytometry. The whole cell lysate was extracted for analysis with Western blotting.

Preparation of recombinant phospholipid scramblase 3 proteins. His-tagged PLS3 proteins was generated with *Escherichia coli* strain M15 [pREP4] containing the pQE-PLS3 or PLS3(T21A) after induction with 1 mmol/L isopropyl-β-thiogalactoside. Bacteria were lysed in a buffer containing 100 mmol/L NaH_2PO_4, 10 mmol/L Tris-HCl, and 8 mol/L urea (pH 8.0). His-tagged proteins were purified on nickel-nitrilotriacetic acid affinity beads (Ni beads) and washed extensively with the same buffer (pH 6.3). Bound proteins were eluted in the same buffer (pH 4.5) as described by the manufacturer (Qiagen). The purity of the protein was examined by gel electrophoresis, followed by Coomassie blue staining.

Pulldown of His-tagged proteins from transfected HeLa cells. Transfected HeLa cells were washed with ice-cold PBS and incubated in the lysis buffer [50 mmol/L NaH_2PO_4, 500 mmol/L NaCl, 20 mmol/L imidazole, 1% Triton X-100, 20 mmol/L 2-mercaptoethanol (pH 8.0)] for 5 minutes and sonicated briefly on ice. The lysates were centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant was saved and the protein concentration was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to same concentrations for each sample. The supernatants were incubated with Ni beads on a shaker for 2 hours at 4°C. Beads were then pelleted at 800 × *g* for 2 minutes at 4°C and washed with ice-cold wash buffer [50 mmol/L NaH_2PO_4, 500 mmol/L NaCl, 20 mmol/L imidazole, 1% Triton X-100 (pH 8.0)] five times. Beads were resuspended in SDS sample buffer and analyzed by Western blotting.

Preparation of whole cell lysates and subcellular fractionation. HeLa cells were washed with ice-cold PBS, lysed with ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride] for 5 minutes and sonicated briefly on ice. Cell lysates were centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatants were saved as the whole cell lysates and used for further immunoprecipitation or added into the SDS sample buffer for Western blotting. Subcellular fractionation was done as described (8).

Western blot analysis. Equal amounts of protein were analyzed by 10% SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Monoclonal PKC-δ, phosphothreonine, and β-actin and polyclonal voltage-dependent anion channel antibodies were used at 1:1,000 and polyclonal PLS3 antibody was used at 1:2,000 for immunoblotting. Secondary antimouse or antirabbit antibodies conjugated with horseradish peroxidase were used at 1:2,000 and incubated with the membrane for 1 hour at room temperature. After washing thrice with TBS-T (1× TBS and 0.1% Tween 20), blots were developed with enhanced chemiluminescence reagents (Pierce, Rockford, IL).

Immunoprecipitation. Whole cell lysates were incubated with 1.25 μg/mL PKC-δ antibody at 4°C for 2 hours and then protein G Sepharose beads were added for an additional 2 hours. After washing extensively with radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl (pH 8.0), 1% NP40, 0.2% deoxycholate, and 120 mmol/L NaCl], the pellets were resuspended in SDS sample buffer and subjected to Western blotting.

Analysis of apoptosis and determination of the transmembrane potential in mitochondria. Cell death was quantified by propidium iodide staining, followed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ). HeLa cells were fixed in 0.5 mL cold 70% ethanol at −20°C overnight. Cell pellet were resuspended in 300 μL PBS containing 0.025 mg/mL propidium iodide, 0.05% Triton X-100, 0.1 mg/mL RNase A, and incubated at room temperature for 30 minutes. The DNA content was evaluated by FACScan. For mitochondrial potential analysis, HeLa cells were incubated with MitoTracker Green at 37°C for 20 minutes. The cells were collected and washed with PBS and analyzed by FACScan.

***In vitro* phosphorylation assay.** *In vitro* phosphorylation was done in a total volume of 60 μL reaction mixture. Recombinant protein was diluted to <0.2 mol/L urea immediately before phosphorylation assays. The phosphorylation mixture contained 0.1 μg recombinant human PKC-δ enzyme, 20 mmol/L HEPES buffer (pH 7.4), 4 mmol/L $MnCl_2$, 4 mmol/L $MgCl_2$, 50 μmol/L ATP, 20 μCi [γ-^{32}P]ATP, and 1 μg recombinant PLS3 protein. The reaction mixture was incubated at room temperature for 20 minutes and terminated by adding 20 μL of 4× SDS sample buffer. The phosphorylated products were separated by SDS-PAGE, electrotransferred to the Immobilon-P membrane, and exposed by autoradiography.

Results

AD198-induced apoptosis is protein kinase C-δ dependent. Based on the report that AD198 activated PKC-δ (26, 27), we first examined whether AD198-induced apoptosis was suppressed by down-regulation of PKC-δ. HeLa cells were transfected with pKD-PKC-δ-v3 plasmid, which expresses PKC-δ siRNA to down-regulate

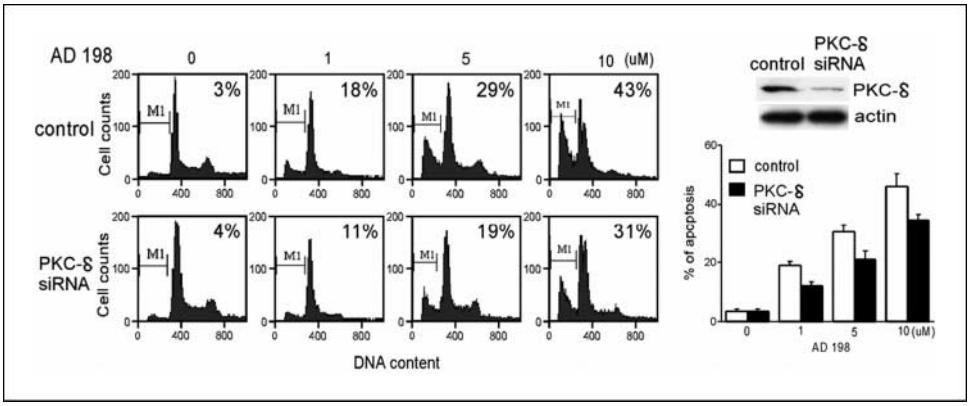

Figure 1. Down-regulation of PKC-δ decreases AD198-induced apoptosis. HeLa cells were transfected with mammalian PKC-δ siRNA expression plasmid pKD-PKC-δ-v3 or a control vector. Whole cell lysates were harvested at 48 hours for Western blotting. For apoptosis assays, HeLa cells at 90% confluence were transfected with pKD-PKC-δ-v3 plasmid for 48 hours. Cells were then treated with AD198 at 0, 1, 5, and 10 μmol/L for 16 hours. Cells were harvested and the apoptotic population was determined by propidium iodide staining. The sub-G_1 population was marked by M1 gate and used to represent the apoptotic population. *Columns,* mean from three independent experiments; *bars,* SD.

PKC-δ (Fig. 1). Cells were then treated with AD198 at 0, 1, 5, and 10 μmol/L for 16 hours. In all concentrations of AD198, less apoptosis was detected when PKC-δ was down-regulated by siRNA (Fig. 1), confirming that AD198-induced HeLa cells apoptosis is PKC-δ dependent.

Overexpression of phospholipid scramblase 3 enhances AD198-induced apoptosis. Because AD198-induced apoptosis is PKC-δ dependent, we evaluated whether PLS3, a mitochondrial substrate of PKC-δ, was involved in AD198-induced apoptosis. HeLa cells were transfected with the vector to overexpress wild-type PLS3. Cells were then treated with AD198 at various concentrations for 16 hours. At three different concentrations of AD198, HeLa cells with overexpression of PLS3 were more sensitive to AD198-induced apoptosis than those without PLS3 overexpression (Fig. 2*A*). These data indicate that overexpression of PLS3 sensitized HeLa cells to AD198-induced apoptosis.

We then used siRNA against PLS3 to down-regulate PLS3 (Fig. 2*B*). Flow cytometry showed that AD198-induced apoptosis was suppressed in HeLa cells transfected with siRNA against PLS3 compared with HeLa cells transfected with scrambled siRNA control (Fig. 2*B*). This finding again supports that AD198-induced apoptosis is mediated by a PLS3-dependent pathway.

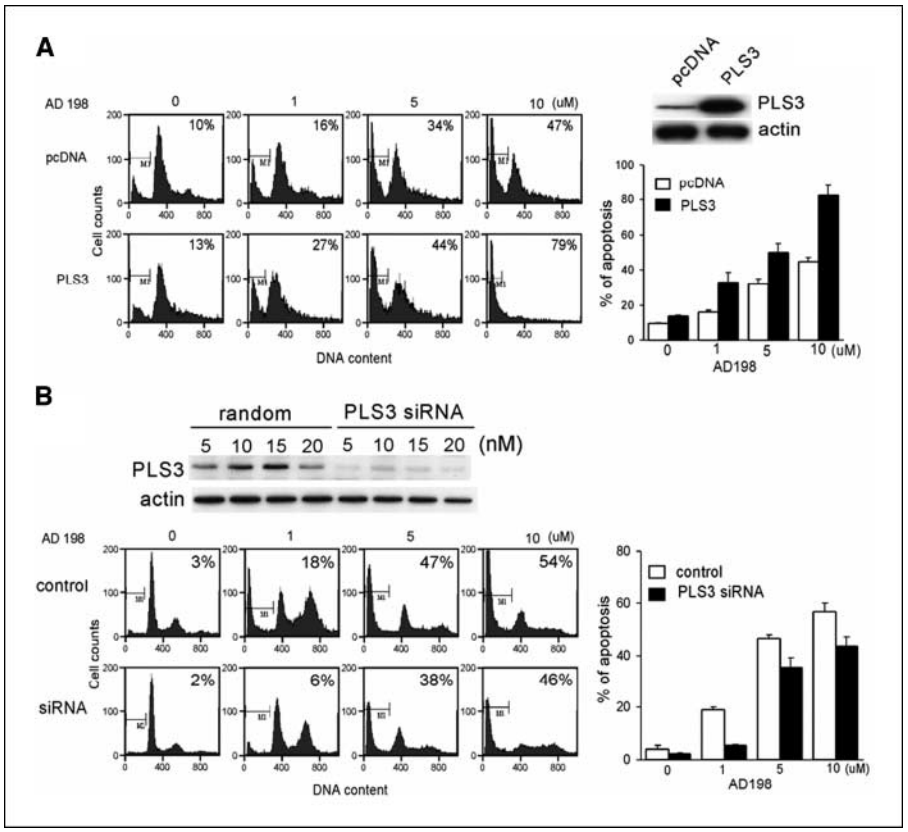

Figure 2. Modulation of AD198-induced apoptosis by PLS3. *A,* overexpression of PLS3 enhanced AD198-induced apoptosis. HeLa cells at 90% confluence were transfected with pcDNA control vector or PLS3-expression vector for 24 hours. The expression of PLS3 was shown by Western blot. Cells were then incubated with AD198 at 0, 1, 5, and 10 μmol/L. Cells were harvested at 16 hours after AD198 exposure and the apoptotic population was determined by staining with propidium iodide. The sub-G_1 population was marked by M1 gate and used to represent the apoptotic population. *B,* down-regulation of PLS3 by siRNA. HeLa-PLS3 cells were transfected with siRNA against PLS3 or random control siRNA at 5, 10, 15, and 20 nmol/L. Whole cell lysates were harvested at 48 hours for Western blotting. Down-regulation of PLS3 abolished AD198-induced apoptosis. HeLa cells at 50% confluence were transfected with 20 nmol/L siRNA against PLS3 or a random control siRNA for 48 hours. Cells were then treated with AD198 at 0, 1, 5, and 10 μmol/L for 16 hours followed by staining with propidium iodide. The apoptotic sub-G_1 population is marked as M1 gate and the percentages are indicated. *Columns,* mean from three independent experiments; *bars,* SD.

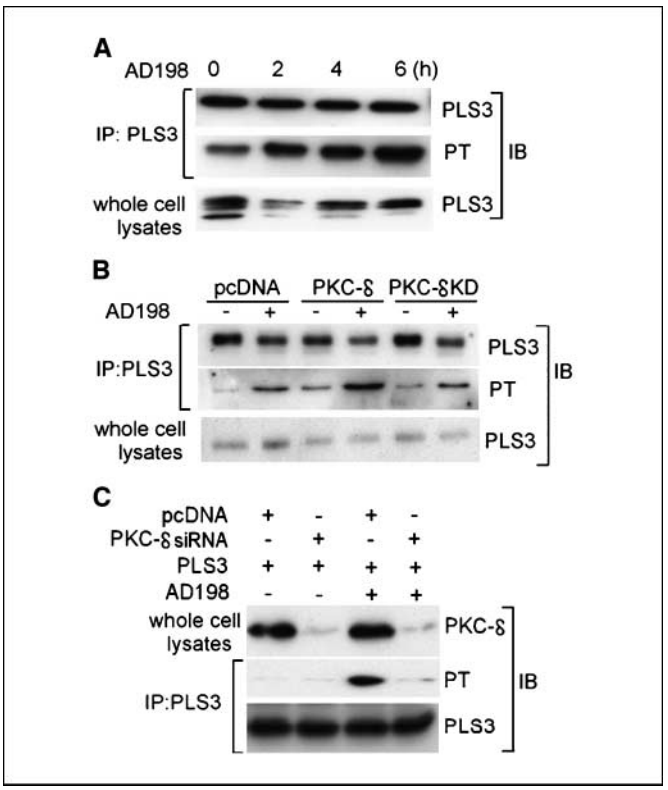

Figure 3. AD198 induces PLS3 phosphorylation at threonine. *A,* HeLa cells were transfected with the His-tagged PLS3 expression vector and treated with AD198 for 0, 2, 4, and 6 hours. Whole cell lysates were incubated with Ni affinity beads to pull down His-tagged PLS3. The pulldown samples were analyzed by Western blotting with antibodies against phosphothreonine and PLS3. *B,* HeLa cells were transfected with the His-tagged PLS3 vector combined with the pcDNA empty vector, PKC-δ, or kinase-defective PKC-δ (*PKC-δKD*) vectors. Cells were then incubated with or without AD198 (5 μmol/L) for 2 hours. Whole cell lysates were incubated with Ni beads to pull down His-tagged PLS3, and analyzed with the antibodies against phosphothreonine and PLS3. *C,* down-regulation of PKC-δ suppresses AD198-induced PLS3 phosphorylation. HeLa cells were transfected with various combinations of pcDNA, pKD-PKC-δ-v3, and pCMV-His-PLS3 as indicated. Cells were treated with or without AD198 for 16 hours. Whole cell lysates were probed with PKC-δ antibody, and PLS3 pulled down by Ni beads was probed with phosphothreonine antibody.

AD198 induces threonine phosphorylation of phospholipid scramblase 3 by protein kinase C-δ. Previously, our laboratory showed that UV treatment induced translocation of PKC-δ to mitochondria and phosphorylation of PLS3, and that phosphorylated PLS3 can be recognized by antiphosphothreonine antibody but not by antiphosphoserine antibody (8). We investigated whether AD198 can also induce PLS3 phosphorylation by activating PKC-δ. HeLa cells were transfected with the His-tagged PLS3 vector and treated with AD198 for 0, 2, 4, and 6 hours. His-tagged PLS3 was pulled down with Ni beads, and phosphorylation of PLS3 was evaluated by Western blotting by using phosphothreonine antibody. In untreated cells, there was a baseline phosphorylation of PLS3 at threonine, and threonine phosphorylation steadily increased after AD198 treatment for at least 6 hours (Fig. 3*A*). The same method of Ni beads pulldown was then used to examine the effect of PKC-δ overexpression. We cotransfected His-tagged PLS3 along with PKC-δ or kinase-defective PKC-δ into HeLa cells and then treated cells with AD198. Immonoblotting of His-tagged PLS3 with the phosphothreonine antibody confirmed that AD198 treatment enhanced PLS3 phosphorylation at threonine, and that overexpression of PKC-δ further enhanced this process but the kinase-defective PKC-δ did not (Fig. 3*B*). The presence of PLS3 phosphorylation in cells transfected with kinase-defective PKC-δ indicated that the endogenous PKC-δ was not completely suppressed. When PKC-δ was down-regulated by siRNA, phosphorylation of PLS3 at threonine after AD198 treatment was also suppressed (Fig. 3*C*), indicating that PKC-δ is the kinase activated by AD198 to induce PLS3 phosphorylation. Examination of the interaction between PLS3 and PKC-δ after AD198 treatment showed that PKC-δ was pulled down with PLS3 in cells cotransfected with PKC-δ. However, the interaction between PLS3 and kinase-defective PKC-δ was much weaker and did not increase after AD198 treatment (Fig. 4*A*). Similar results were obtained by immunoprecipitation of PKC-δ. In control cells, the immunoprecipitate of endogenous PKC-δ contained PLS3, which increased after cells were treated with AD198. Overexpression of PKC-δ increased the amount of PLS3 in the immunoprecipitate of PKC-δ regardless of AD198 treatment (Fig. 4*B*). These studies, combined with the results from Ni beads pulldown, confirmed the association between PLS3 and PKC-δ. The interaction between PLS3 and PKC-δ occurs before PLS3 phosphorylation and phosphorylation may stabilize the interaction between PLS3 and PKC-δ. Immunoprecipitation with PKC-δ antibody in lysates from HeLa-PLS3 cells expressing kinase-defective PKC-δ revealed no interaction between PLS3 and kinase-defective PKC-δ (Fig. 4*B*, *last two lanes*). In this immunoprecipitate, endogenous PKC-δ was far less abundant than the kinase-defective PKC-δ, which explains the complete lack of PLS3 in this immunoprecipitate.

Protein kinase C-δ phosphorylates phospholipid scramblase 3 at Thr[21]. Because PLS3 is a high-affinity substrate of PKC-δ (8), we investigated the site of phosphorylation in PLS3. We did *in vitro* phosphorylation of full-length and the 50-amino-acid NH_2-terminal fragment of PLS3. Phosphorylation was seen in both full-length

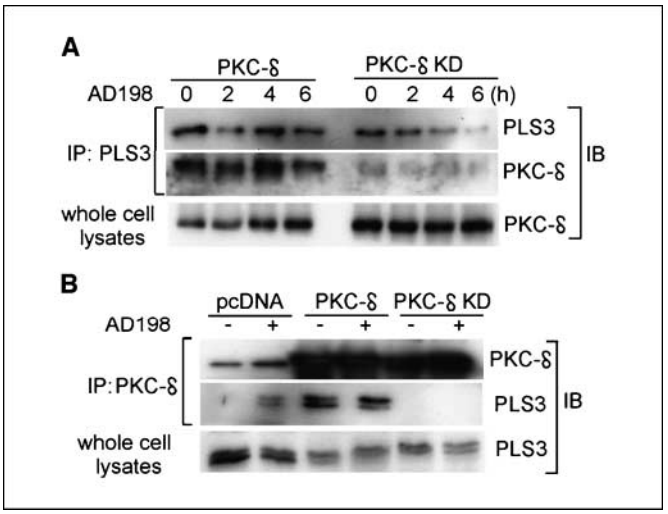

Figure 4. PLS3 interacts with wild-type PKC-δ but not with the inactive kinase-defective PKC-δ mutant. *A,* HeLa cells expressing His-tagged PLS3 were transfected with PKC-δ or kinase-defective PKC-δ vectors. They were then incubated with AD198 for 0, 2, 4, and 6 hours. Whole cell lysates were incubated with Ni beads to pull down His-tagged PLS3 and probed with antibodies against PLS3 and PKC-δ. *B,* HeLa-PLS3 cells transfected with pcDNA, PKC-δ, or kinase-defective PKC-δ vectors were harvested before and 2 hours after AD198 treatment. PKC-δ was immunoprecipitated from whole cell lysates and analyzed with antibodies against PKC-δ and PLS3. Western blotting of whole cell lysates revealed that the level of PLS3 expression was roughly similar before and after AD198 treatment.

PLS3 and its NH_2-terminal fragment (Fig. 5*A*), which allowed us to use point mutation to map the phosphorylation sites. Examination of the sequence of the first 50 amino acids of PLS3 revealed only a single threonine as the candidate phosphorylation site. We thus mutated Thr^{21} to alanine by site-directed mutagenesis, and generated recombinant PLS3(T21A) protein similar to wild-type PLS3 (Fig. 5*B*). *In vitro* phosphorylation revealed that mutation at Thr^{21} nearly eliminated PLS3 phosphorylation by PKC-δ (Fig. 5*B*), indicating that Thr^{21} is the site of phosphorylation in PLS3 by PKC-δ *in vitro*.

With the establishment of Thr^{21} as the site of PLS3 phosphorylation by PKC-δ *in vitro*, we transfected the mammalian expression vector pCMV-6His-PLS3(T21A) into HeLa cells to investigate the effect of T21A mutation *in vivo*. Subcellular fractionation of cells transfected with wild-type PLS3 or PLS3(T21A) revealed that mutation of this threonine residue did not affect mitochondrial targeting of PLS3 (Fig. 5*C*). After pulling down His-tagged PLS3(T21A) by Ni beads, the PLS3(T21A) mutant could not be recognized by phosphothreonine antibody even after AD198 treatment (Fig. 5*D*). In contrast, wild-type PLS3 was recognized by the same phosphothreonine antibody and the signal increased after AD198 treatment. This finding confirmed that Thr^{21} is the primary site of PLS3 phosphorylation induced by AD198. Next, we studied whether T21A mutation affected the interaction between PLS3 and PKC-δ by cotransfection with PKC-δ. As shown in Fig. 5*E*, the His-tagged PLS3 pulled down by Ni beads contained PKC-δ, but PLS3(T21A) was less effective in binding PKC-δ compared with wild-type PLS3 before AD198 treatment, although the amount of PLS3(T21A) was higher by blotting the same blot with PLS3 antibody (Fig. 5*E*, *last two lanes*). Upon incubation with AD198, there was increased association of PLS3 and PKC-δ for wild-type PLS3 versus mutant PLS3(T21A). The reciprocal immunoprecipitation with the PKC-δ antibody showed a similar finding. Cells treated with AD198 had more PLS3 present in the PKC-δ immunoprecipitate. The PLS3(T21A) mutant has a weaker interaction with PKC-δ (Fig. 5*F*). These results indicate that the interaction between PLS3 and PKC-δ was compromised in PLS3(T21A), and, therefore, AD198-induced association between PLS3 and PKC-δ requires phosphorylation of Thr^{21} in PLS3.

PLS3(T21A) does not enhance AD198-induced apoptosis. If PLS3 phosphorylation at Thr^{21} is critical for the apoptotic effect of AD198-activated PKC-δ, we predicted that overexpression of PLS3(T21A) would not be able to enhance AD198-induced apoptosis similar to the wild-type PLS3 (Fig. 2*A*). To test this possibility, we treated HeLa cells transfected with vectors to express wild-type PLS3 or PLS3(T21A) with AD198, and analyzed apoptosis by propidium iodide staining and flow cytometry. Wild-type PLS3 increased the percentage of AD198-induced apoptosis from 7% to 31%, whereas cells expressing PLS3(T21A) had the same degree of apoptosis as the control cells after AD198 treatment (Fig. 6). We conclude that AD198-induced PKC-δ activation and phosphorylation of PLS3 at Thr^{21} are critical to AD198-induced apoptosis.

AD198-induced phospholipid scramblase 3 phosphorylation is an upstream event of caspase activation and independent of mitochondrial permeability transition. Finally, we investigated

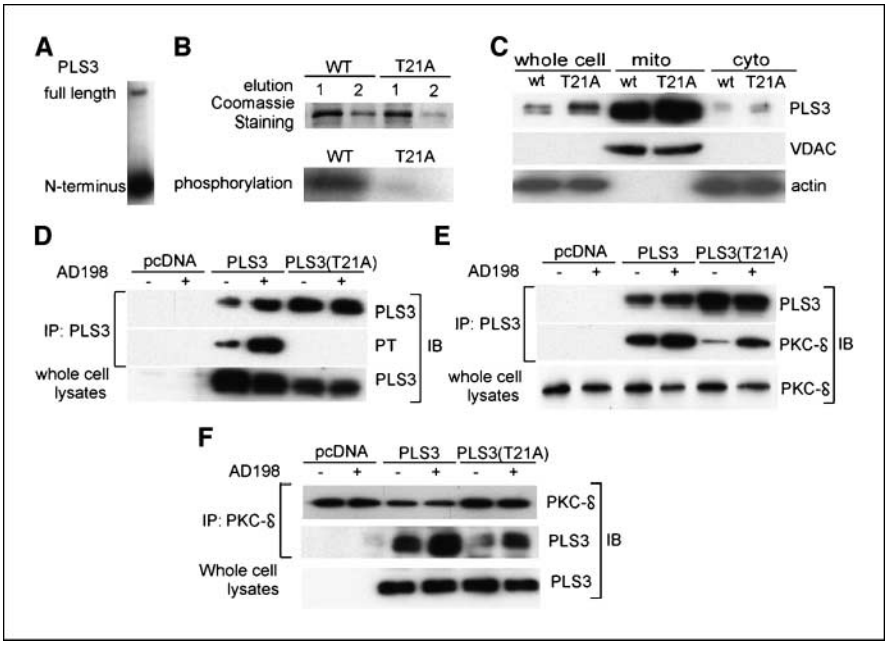

Figure 5. Identification of Thr^{21} as the phosphorylation site of PLS3 induced by PKC-δ. *A, in vitro* phosphorylation of recombinant PLS3 by PKC-δ. Equal amounts of recombinant PLS3 and a 6 kDa NH_2-terminal fragment of PLS3 were mixed together and phosphorylated by purified PKC-δ in the presence of [γ-^{32}P]ATP. The phosphorylated sample was analyzed with SDS gel and exposed by autoradiography. *B,* PLS3 phosphorylation by PKC-δ is suppressed by T21A mutation in PLS3. Recombinant His-tagged PLS3 (wild type) and T21A mutant was isolated by Ni beads from two elutions and analyzed with SDS gel. *Top,* yield of each protein in the two-step elution by Coomassie staining. *Bottom, in vitro* phosphorylation of equal amounts of PLS3 protein (*elution 1*) by recombinant PKC-δ. *C,* subcellular fraction of cells transfected with PLS3 or PLS3(T21A) expression vectors. *D,* T21A mutation abolished *in vivo* PLS3 phosphorylation after AD198 treatment. HeLa cells were transfected with the control vector or vectors expressing His-tagged PLS3 or PLS3(T21A). His-tagged PLS3 protein was pulled down by Ni beads from cells with or without AD198 treatment. Whole cell lysates and pulldown samples by Ni beads were analyzed with Western blotting using antibodies against PLS3 and phosphothreonine. *E,* interaction of PLS3(T21A) with PKC-δ. HeLa cells were cotransfected with the PKC-δ vector and pcDNA, PLS3, or PLS3(T21A) vector. Whole cell lysates were incubated with Ni beads to pull down PLS3. The pulldown samples were analyzed with Western blotting using antibodies against PLS3 and PKC-δ. *F,* HeLa cells were cotransfected as in (*E*). Whole cell lysates were immunoprecipitated with PKC-δ antibody. Whole cell lysates and immunoprecipitates were analyzed by Western blotting with the antibody against PLS3.

how PLS3 phosphorylation was positioned in the apoptosis pathway. HeLa cells were treated with a pancaspase inhibitor Z-VAD followed by AD198 treatment. Z-VAD could not block AD198-induced mitochondrial permeability transition but suppressed AD198-induced apoptosis (Fig. 7*A-C*). We also tested whether PLS3 was phosphorylated in the presence of Z-VAD by pulling down His-tagged PLS3 with Ni beads and probed with phosphothreonine antibody. Threonine phosphorylation of PLS3 after AD198 treatment was not affected by Z-VAD (Fig. 7*D*), suggesting that AD198-induced PLS3 phosphorylation occurs at the upstream of caspase activation. Similar studies were also done to examine whether AD198-induced PLS3 phosphorylation is upstream or downstream of mitochondrial permeability transition. When the mitochondrial permeability transition pore complex was blocked by cyclosporine A, AD198-induced loss of mitochondrial potential and PLS3 phosphorylation were not affected (Fig. 7*A-D*), indicating that PLS3 phosphorylation occurs independent of mitochondrial permeability transition.

Discussion

PKC-δ plays an important role in the process of cell death by translocating to different organelles to induce apoptosis. Identification of substrates in each organelle is essential for understanding the mechanism of PKC-δ–induced apoptosis. The critical substrates identified thus far include Rad9 in the nucleus (18) and PLS1 in the plasma membrane (21). Identification of the physiologic substrates in mitochondria is especially important because mitochondria are the integrators of apoptosis. We have shown that PLS3 is a substrate of PKC-δ upon translocation to mitochondria (8). In the current study, we utilized an extranuclear-targeted anthracycline derivative AD198, which binds to the C1b regulatory domain of PKC similar to phorbol ester (24), to activate PKC-δ and to investigate the effect of PLS3 in PKC-δ–induced apoptosis. We found that overexpression of PLS3 enhanced the sensitivity of cells to AD198-induced apoptosis. In contrast, down-regulation of PLS3 by siRNA resulted in decreased apoptosis by AD198. These findings confirmed that PLS3 is a downstream effector of PKC-δ in mitochondria when PKC-δ is activated by AD198 and translocates to mitochondria.

To investigate the mechanism of mitochondrial damage induced by PKC-δ phosphorylated PLS3, we determined that AD198-activated PKC-δ induced phosphorylation of PLS3 at threonine. The phosphorylated threonine was mapped to residue 21. This residue was confirmed to be the phosphorylated threonine *in vivo* because mutation to alanine prevented its recognition by the phosphothreonine antibody (Fig. 5*D*) despite the fact that the PLS3(T21A) mutant was still capable, although weaker, of interacting with PKC-δ (Fig. 5*E* and *F*). Phosphorylation may not be required for the interaction between PLS3 and PKC-δ as interaction was observed before cells were treated with AD198 (Figs. 4 and 5*E* and *F*). Phosphorylation of PLS3 by PKC-δ may stabilize the interaction between the two proteins. Cells overexpressing the PLS3(T21A) mutant, although still undergoing apoptosis after treatment with AD198, were comparable with cells expressing the empty vector. This observation was in contrast with the enhanced sensitivity to AD198 when cells were transfected with the vector to overexpress wild-type PLS3 (Fig. 6).

The PKC-δ–induced PLS3 phosphorylation is apparently an early event in AD198-induced apoptosis. The presence of caspase inhibitor Z-VAD suppresses AD198-induced apoptosis, but cannot prevent PLS3 phosphorylation. Thus, PKC-δ–induced PLS3 phosphorylation is an upstream event of caspase in the apoptotic pathway. When cyclosporine A was added with AD198, mitochondrial potential was still disrupted by AD198 and apoptosis occurred. AD198-induced PLS3 phosphorylation was not affected by cyclosporine A either. This finding indicated that PLS3 phosphorylation by PKC-δ is independent of mitochondrial permeability transition.

How does PLS3 induce mitochondrial damage? Previous data suggest that overexpression of PLS3 increases the amount of cardiolipin in the mitochondrial outer membrane, which can potentially enhance the targeting of tBid to mitochondria (8). The fact that mitochondria with high expression of PLS3 were more sensitive to tBid-induced second mitochondria-derived activator of caspases or cytochrome *c* release (8) supported this theory. The conformation of tBid in mitochondria has been examined in detail by two independent groups. Both concluded that tBid does not insert into mitochondrial membranes but has its α-helices nearly parallel to the membrane surface (31, 32). Because cardiolipin is synthesized in the mitochondrial inner membrane, PLS3 could be involved in the process of translocating cardiolipin to the mitochondrial surface. Understanding the mechanism of PLS3 activation will be critical to understanding how tBid targeting is

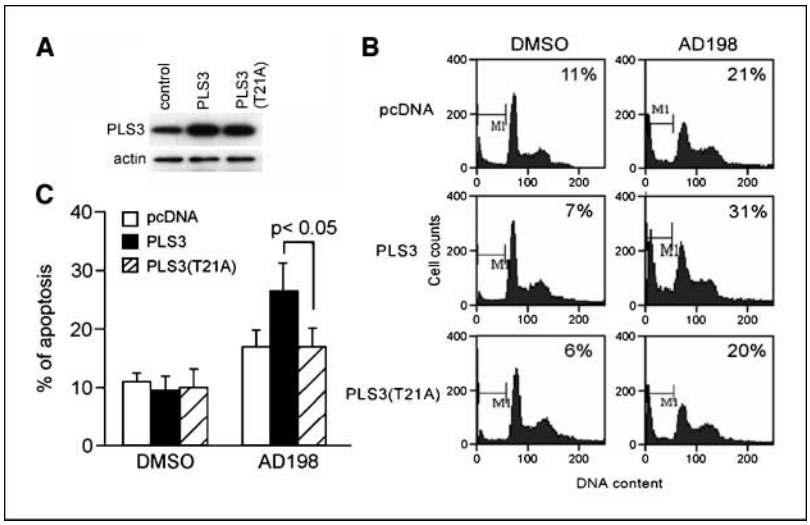

Figure 6. PLS3(T21A) does not enhance AD198-induced apoptosis like the wild-type PLS3. *A,* HeLa cells were transfected with the empty vector or vectors expressing PLS3 or PLS3(T21A). Cells were harvested for Western blotting with antibodies against PLS3 and actin. *B,* cells then treated with DMSO or 5 μmol/L AD198 for 16 hours followed by flow cytometry analysis with propidium iodide staining. The representatives of flow cytometry analysis were shown. The sub-G_0 apoptotic population was determined and indicated in each panel. *C,* histogram to show the average of apoptotic population in control cells and cells transfected with wild-type PLS3 or PLS3(T21A). Results are the averages of three independent experiments. Statistical significance ($P < 0.05$) was achieved by the paired *t* test between wild-type PLS3 versus control or PLS3(T21A).

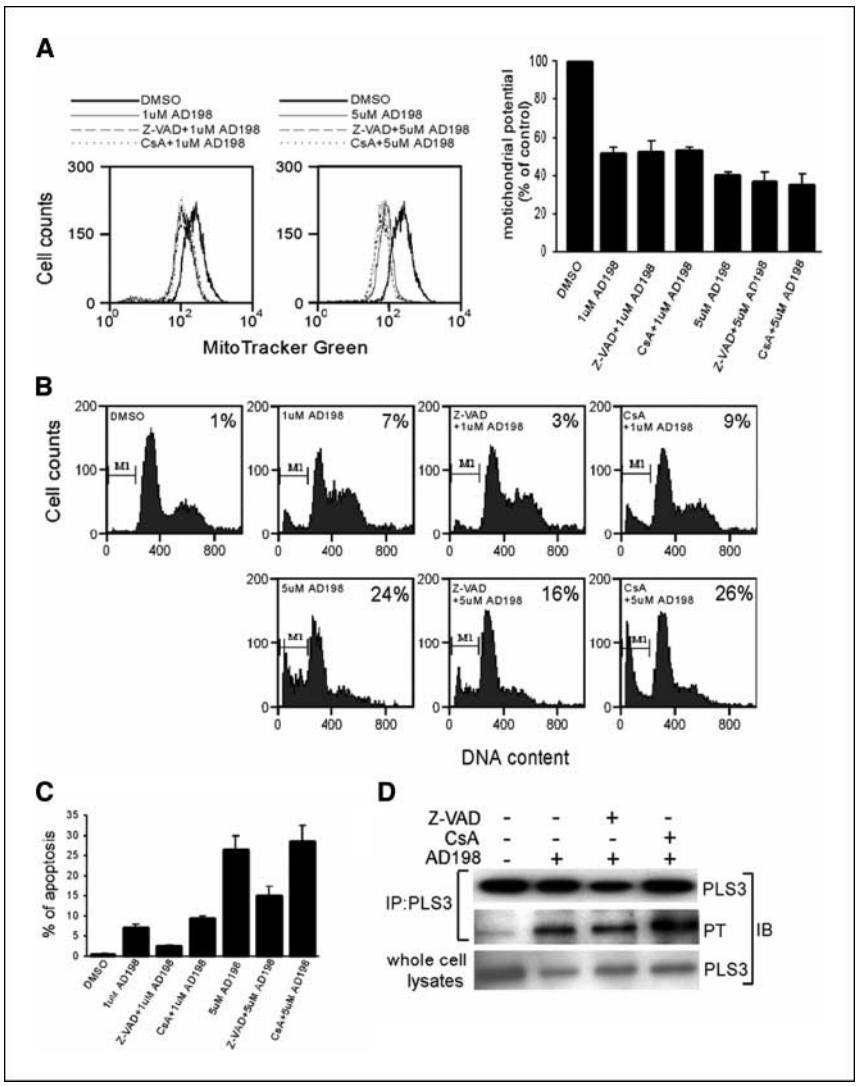

Figure 7. AD198-induced PLS3 phosphorylation is an upstream event of caspase activation and independent of mitochondrial permeability transition. *A,* Z-VAD and cyclosporine A (*CsA*) could not block AD198-induced loss of mitochondrial potential. HeLa cells were treated with 50 μmol/L Z-VAD or 5 μmol/L cyclosporine A for 30 minutes, and then AD198 was added at 0, 1 or 5 μmol/L for 16 hours. Cells were incubated with MitoTracker Green at 37°C for 20 minutes followed by flow cytometry analysis. *Columns,* mean from three independent experiments; *bars,* SD. *B,* Z-VAD, but not cyclosporine A, inhibits AD198-induced apoptosis. HeLa cells were treated as in (*A*) and collected for the apoptosis study with propidium iodide staining. *C,* percentage of apoptosis from three independent experiments in (*B*). *Columns,* mean; *bars,* SD. *D,* Z-VAD or cyclosporine A could not suppress AD198-induced PLS3 phosphorylation. HeLa cells were transfected with the vector expressing His-tagged PLS3. Cells were treated with AD198 (5 μmol/L) along with Z-VAD or cyclosporine A for 2 hours and harvested for Ni bead pulldown. Whole cell lysates and pulldown samples were probed with antibodies against phosphothreonine and PLS3.

regulated. Phosphorylation of PLS3 at Thr^{21} could lead to further PLS3 activation during apoptosis.

The major advantage of using AD198 to investigate PLS3-mediated mitochondrial damage is that AD198 induces translocation of the activated PKC-δ to mitochondria. This is in contrast to other apoptotic agents, such as H_2O_2, UV, γ-irradiation, or other chemotherapeutic agents, which induce PKC-δ activation and translocation to other organelles in addition to mitochondria (12, 13). Nuclear-targeted PKC-δ can phosphorylate Rad9, which is a critical mediator of the apoptotic process. Using an extranuclear-targeted anthracycline like AD198 will eliminate the contribution from nuclear-targeted PKC-δ. With the central role of mitochondria in apoptosis, the exclusive mitochondrial effect of AD198 is considered an advantage in directly inducing apoptosis. The fact that AD198 overcomes resistance due to Bcl-2 overexpression (25, 30) is particularly attractive because Bcl-2/Bcl-xL overexpression is a common mechanism of drug resistance in clinical application of chemotherapeutic agents. Further development of novel strategies to manipulate the PKC-δ/PLS3 pathway in mitochondria is a promising way to develop novel therapy for cancer.

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