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Phosphorylation of Mitochondrial Phospholipid Scramblase 3 by Protein Kinase C-δ Induces Its Activation and Facilitates Mitochondrial Targeting of tBid

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Abstract Phospholipid scramblase 3 (PLS3) is a member of the phospholipid scramblase family present in mitochondria. PLS3 plays an important role in regulation of mitochondrial morphology, respiratory function, and apoptotic responses. PLS3 is phosphorylated by PKC-δ at Thr21 and is the mitochondrial target of PKC-δ-induced apoptosis. Cells with overexpression of PLS3, but not the phosphoinhibitory mutant PLS3(T21A), are more susceptible to apoptosis induced by AD198, an extranuclear targeted anthracycline that activates PKC-δ. Here we report that the phosphomimetic mutant of PLS3(T21D) by itself can induce apoptosis in HeLa cells. Using proteoliposomes with addition of pyrene-labeled phosphatidylcholine (PC) at the outer leaflet, we measured the lipid flip-flop activity of PLS3 and its phosphorylation mutant. PLS3(T21D) is more potent than wild-type PLS3 or PLS3(T21A) to transfer pyrene-PC from the outer leaflet to the inner leaflet of liposomes. Based on our previous finding that PLS3 enhances tBid-induced mitochondrial damages, we tested the hypothesis that PLS3 enhances cardiolipin translocation to mitochondrial surface and facilitates tBid targeting. Fluorescein-labeled tBid(G94E) was used as a probe to quantify cardiolipin on the surface of mitochondria. Mitochondria from cells treated with AD198 or cells expressing PLS3(T21D) had a higher level of tBid-binding capacity than control cells or cells expressing wild-type PLS3. These findings indicate that phosphorylation of PLS3 by PKC-δ induces PLS3 activation to facilitate mitochondrial targeting of tBid and apoptosis. J. Cell. Biochem. 101: 1210–1221, 2007.

Key words: mitochondria; protein kinase C-δ; phosphorylation; phospholipid scramblase 3; AD198; apoptosis

Programmed cell death or apoptosis is a fundamental feature of many biological processes [Evan and Littlewood, 1998; Green and Evan, 2002]. It is characterized by distinct biochemical and morphological changes in cells, including nuclear fragmentation, chromatin condensation, and membrane blebbing [Kerr et al., 1972]. The extrinsic pathway of apoptosis is activated by death domain receptors, forming a death-induced signaling complex (DISC) to transduce cell death signals to the nucleus and mitochondria [Krammer, 2000; Baud and Karin, 2001]. During apoptosis, the plasma membrane itself, although maintaining its integrity, exhibits phospholipid translocation. Phosphatidylserine (PS) is translocated from the inner to outer leaflet of the plasma membrane to serve as a signal for recognition and clearance of apoptotic cells by macrophages

Abbreviations used: AD198, N-benzyladriamycin-14-valerate; DMSO, dimethyl sulfoxide; CL, cardiolipin; PLS3, phospholipid scramblase 3; PKC-δ, protein kinase C-δ; VDAC, voltage-dependent anion channel; FITC, fluorescein isothiocyanate; PI, propidium iodide; pyrene-PC, pyrene-labeled phosphatidylcholine.

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[Fadok et al., 1992]. This process is a complicated event regulated by at least three enzymes, phospholipid scramblase (PLS), aminophospholipid translocase (flippase) and floppase, which are responsible for phospholipid translocation in the inward or outward direction [Bevers et al., 1998, 1999].

In the intrinsic apoptotic pathway, mitochondria are the most important organelles, and the integrators of apoptosis [Wang, 2001]. Many critical mediators of apoptosis are either localized in or translocated to mitochondria to regulate the release of various apoptogenic factors, such as cytochrome c [Li et al., 1997], SMAC [Du et al., 2000; Verhagen et al., 2000], endonuclease G [Li et al., 2001; Parrish et al., 2001], and apoptosis-inducing factor (AIF) [Susin et al., 1999], to induce various downstream apoptotic responses. In addition to protein factors, changes in mitochondrial lipids were also recognized [McMillin and Dowhan, 2002; Cristea and Degli Esposti, 2004]. One distinct component of mitochondrial lipids is cardiolipin (CL), which is a phospholipid synthesized and localized exclusively in mitochondria [Schlame et al., 2000]. Changes in CL have been demonstrated in early steps of apoptosis, such as translocating to the outer leaflet of the mitochondrial inner membrane [Garcia Fernandez et al., 2002], and eventually to the surface of plasma membrane [Sorice et al., 2004]. CL is also responsible for recruitment of pro-apoptotic proteins tBid to mitochondria [Lutter et al., 2000] and to activate Bax and induce the release of apoptogenic proteins [Luo et al., 1998; Desagher et al., 1999; Eskes et al., 2000; Korsmeyer et al., 2000]. In mitochondria, CL forms a supramolecular complex with tBid and Bax [Kuwana et al., 2002]. If CL was depleted in cells expressing the temperature-sensitive mutant of the CL synthesis enzyme, Bid could not be targeted to mitochondria [Lutter et al., 2000]. Interaction of tBid with CL suppresses ADP-stimulated mitochondrial respiration and reduces ATP synthesis [Gonzalvez et al., 2005a, 2005b]. These tBid-induced damages lead to production of reactive oxygen species and oxidation of mitochondrial membrane components [Ding et al., 2004]. One enzyme that may play a role in regulating CL localization and tBid targeting to mitochondria is phospholipid scramblase 3 (PLS3), which plays important roles in mitochondrial morphology, respiratory function and apoptotic response [Liu et al., 2003b]. Our previous study showed that cells with overexpression of PLS3 were more sensitive to UV and TNF-α-induced apoptosis, whereas cells expressing an inactive PLS3 mutant were not. Overexpression of PLS3 increases the amount of CL in the mitochondrial outer membrane, which may potentially enhance the targeting of tBid to mitochondria to induce cytochrome c and SMAC release [Liu et al., 2003b].

Regulation of PLS3 activity could be mediated by post-translational phosphorylation [Liu et al., 2003a]. We established that PLS3 is a physiological target of PKC-δ-induced apoptosis in mitochondria. PKC-δ can physically interact with and phosphorylate PLS3 with a high affinity [Liu et al., 2003a]. Mutation of Thr21 in PLS3 to alanine abolished phosphorylation of PLS3 by PKC-δ in vitro and diminished the interaction between PLS3 and PKC-δ. A PKC-δ activator, N-benzyladriamycin-14-valerate (AD198), induced PLS3 phosphorylation in vivo. Expression of wild type PLS3, but not the PLS3(T21A) mutant, significantly enhanced AD198-induced apoptosis. These studies suggest that phosphorylation of PLS3 at Thr21 by PKC-δ may regulate PLS3 activity, but it has not been demonstrated that phosphorylation at Thr21 site directly lead to PLS3 activation [He et al., 2005].

In this study, we address this issue to investigate whether phosphorylation of PLS3 at Thr21 leads to PLS3 activation. Thr21 of PLS3 was mutated to aspartic acid (D) to mimic phosphorylation of PLS3. The activity of PLS3 and its mutant was measured in vitro with proteoliposomes, in which PLS3-dependent phospholipid flip-flop was determined by translocation of pyrene-PC from the outer leaflet to the inner leaflet [Muller et al., 2000; Epand et al., 2003]. The in vivo effect of PLS3 and PLS3(T21D) was investigated by their ability to induce apoptosis and to increase the amount of CL, which is represented by tBid-binding capacity, on the mitochondrial surface. Our results confirm that PLS3 is activated by PKC-δ-induced phosphorylation at Thr21, which plays an important role in apoptosis.

MATERIALS AND METHODS

Materials

PLS3(T21A) and (T21D) mutants were generated using the Quick Change site-directed

mutagenesis kit (Stratagene, La Jolla, CA). His-tagged PLS3 or mutants were cloned into the QIAexpress pQE30 vector (Qiagen, Valencia, CA) (pQE-PLS3) for generation of recombinant proteins as described. Mammalian expression vectors for His-tagged PLS3 and PLS3(T21D) were constructed with pcDNA3.1 vector respectively. The siRNAs to downregulate PLS3 and scrambled control were from Qiagen as described previously. The cDNAs encoding PLS3* and PLS3(T21D)* contain an additional single base mutation G99(GGC) to G99(GGA) at the center of PLS3-siRNA corresponding region so that the expression of the transgenes is not affected by PLS3 siRNA and can be used for rescuing. Mammalian PKC-δ siRNA expression plasmid (pKD-PKC-δ-v3) was from Upstate (Lake Placid, NY). PLS3 polyclonal antibody was raised in rabbits against full-length recombinant PLS3 (Proteintech Group, Inc., Chicago, IL). PKC-δ monoclonal antibody was obtained from BD Biosciences (Palo Alto, CA). β-actin monoclonal antibody was obtained from Sigma-Aldrich, Inc. (St. Louis, MO). The polyclonal antibody against voltage-dependent anion channel (VDAC) was obtained from Affinity BioReagents (Golden, CO). Secondary antibodies conjugated with horseradish peroxidase were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). AD198 was provided by Dr. Mervyn Israel (Department of Pharmacology, the University of Tennessee Health Science Center) [Harstrick et al., 1995]. Recombinant tBid(G94E) proteins was generated and purified as described [Liu et al., 2004] and labeled with fluorescein isothiocyanate (FITC), FITC-tBid(G94E), in a carbonate-bicarbonate (1,000 μg protein: 45 μg FITC, pH 9.0) and purified through Sephadex G25 gel filtration column (Amersham Pharmacia Biotech, Piscataway, NJ). 10-N-nonyl acridine orange (NAO) was purchased from Molecular Probes (Eugene, OR). 1-lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine (pyrene-PC) was purchased from Avanti Polar Lipids (Alabaster, AL).

Cell Culture, Transfection, Selection and Treatment

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C. HeLa cells at 90% confluence were transfected with different mammalian expression vectors or siRNA using Lipofectamin 2000 according to the manufacturer's protocol (Invitrogen, Inc., Carlsbad, CA). At 48 h after transfection, cells were incubated with 5 μM AD198 or dimethyl sulfoxide (DMSO) for 4 h or 16 h. After transfection, cells were selected with 1 mg/ml G418 for 2 week. After corresponding mock-transfected cells were killed by G418, the concentration of G418 was decreased to 0.5 mg/ml and maintained for another 2 weeks. The whole cell lysates or subcellular fractions were extracted for Western blotting analysis [Liu et al., 2003a]. Transfection control was performed with pEGFP-C1 (Clontech, Mountain View, CA), which expresses green fluorescence protein (GFP) to test transfection efficiency.

Generation of Recombinant PLS3 Proteins

His-tagged PLS3 proteins were produced with *E. coli* (M15 [pREP4]) transformed with pQE-PLS3. Bacteria were induced with 1 mM isopropyl-β-thiogalactoside (IPTG) for 4 h before harvest. Bacterial pellets were resuspended and stirred gently in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0 for 1 h at room temperature. After centrifugation, the supernatants were mixed with nickel-nitrilotriacetic acid affinity beads (Ni-beads) to pull down the His-tagged protein. The beads were washed twice with 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3. Bound proteins were renatured using a linear 7 M–0.1 M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris-HCl, pH 7.4. The renatured proteins were then eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0 as described by the manufacturer (Qiagen) and dialyzed in 50 mM Tris, 0.2 mM EGTA, 1 mM $MgCl_2$, 120 mM NaCl, 0.5% Triton X-100, 5% Na cholate hydrate, pH 7.4.

Preparation of Whole Cell Lysates and Subcellular Fractionation

HeLa cells were harvested with the lysis buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) on ice for 5 min and sonicated briefly. The lysates were centrifuged at 10,000*g* for 10 min at 4°C and the supernatants were saved as the whole cell lysates for Western blotting. Subcellular fractionation was performed as described [Liu et al.,

2003a] and isolated mitochondria were used for tBid(G94E)-binding analysis.

Western Blotting Analysis

Samples were separated by 10% SDS–PAGE and electrotransferred to the Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was blotted with 1:1,000 primary antibody overnight at 4°C and 1:2,000 secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL) were used to develop the blots.

Analysis of Apoptosis

HeLa cells were harvested and fixed in 70% ethanol at −20°C overnight. Cells were then washed with PBS and stained with 0.025 mg/ml propidium iodide (PI), 0.05% Triton X-100, 0.1 mg/ml RNase A for 30 min at room temperature. The DNA content was analyzed by FACScan (Becton-Dickinson), and the subG_0/G_1 population was used to represent apoptosis.

Analysis of the tBid-Binding Capacity of Mitochondria

Isolated mitochondria were incubated with FITC-tBid(G94E) in 50 μl reaction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 3 mM KH_2PO_4, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.5% mg/ml BSA, PH 7.4) for 30 min at room temperature. Mitochondria were pelleted at 10,000*g* and washed extensively with the reaction buffer to remove unbound FITC-tBid(G94E) and the fluorescence intensity was quantified with Bio-Tek microplate reader.

Lipid Flip-Flop Analysis

Recombinant Bid was produced as glutathione-S-transferase fusion protein in BL21 bacteria and purified by glutathione Sepharose 4B affinity beads. tBid was produced by cleavage with full-length Bid with recombinant caspase 8 (Calbiochem, San Diego, CA) before usage. Some of the initial transbilayer diffusion studies were carried out with tBid kindly provided to us by Jean-Claude Martinou (University of Geneva, Switzerland). Pyrene-PC was incorporated into the outer monolayer of preformed PLS3-containing proteoliposomes so that it was 10% of the lipids in the outer monolayer or 5% of the total lipids. Proteoliposomes with PLS3 were prepared by detergent dialysis, as previously described (manuscript in review). The rate of transbilayer diffusion of pyrene-10-PC was measured as previously described [Muller et al., 2000; Epand et al., 2003]. Excitation was 344 nm. When flip-flop occurs, there is a reduction in the excimer emission because of dilution of the probe from one monolayer to two. The ratio of the excimer (I_e, emission at 476 nm) to the monomer emission (I_m, emission at 397 nm) was measured as a function of time before and after the addition of ionomycin, 2 mM $CaCl_2$ and/or apoptotic proteins. The initial ratio at time 0 was set to 1. The fluorescence experiments were carried out in 2 ml silanized glass cuvettes at 37°C with PLS3 proteoliposomes at different lipid-to-protein ratios, and a lipid concentration of 25 μM. After addition of a freshly prepared solution of probe to the liposomes, the excimer/monomer emission ratio was monitored prior to addition of proteins or calcium until a plateau was reached. Measurements were done with two independent preparations. Values of the ratio I_e/I_m were normalized to that of the proteoliposomes alone and then averaged.

RESULTS

Phosphomimetic Mutant of PLS3 Induces Apoptosis

In our previous report, we showed that overexpression of PLS3 enhanced AD198-induced apoptosis and mapped Thr21 as phosphorylation site of PLS3 by PKC-δ in AD198-induced apoptosis [He et al., 2005]. In order to investigate the effect of PLS3 phosphorylation, we mutated Thr21 to the phosphomimetic residue Asp to examine whether phosphorylation of PLS3 could induce apoptosis in HeLa cells without AD198 treatment. HeLa cells were transfected with wild-type PLS3 or the phosphomimetic PLS3(T21D) mutant along with the pEGFP-C1 vector. Western blotting of the whole cell lysates revealed that the levels of PLS3 and PLS3(T21D) were equal (Fig. 1a). Cells were then treated with 5 μM AD198 or DMSO for 16 h. Apoptosis was analyzed with PI staining and flow cytometry. The GFP-positive cells were gated and the percentages of apoptosis were analyzed in the GFP-positive population. Without AD198 treatment, PLS3(T21D) induced apoptosis in 18% of HeLa cells, which is significantly higher than cells transfected with pcDNA control or wild-type PLS3 ($P < 0.05$) (Fig. 1b, c). When cells were treated with

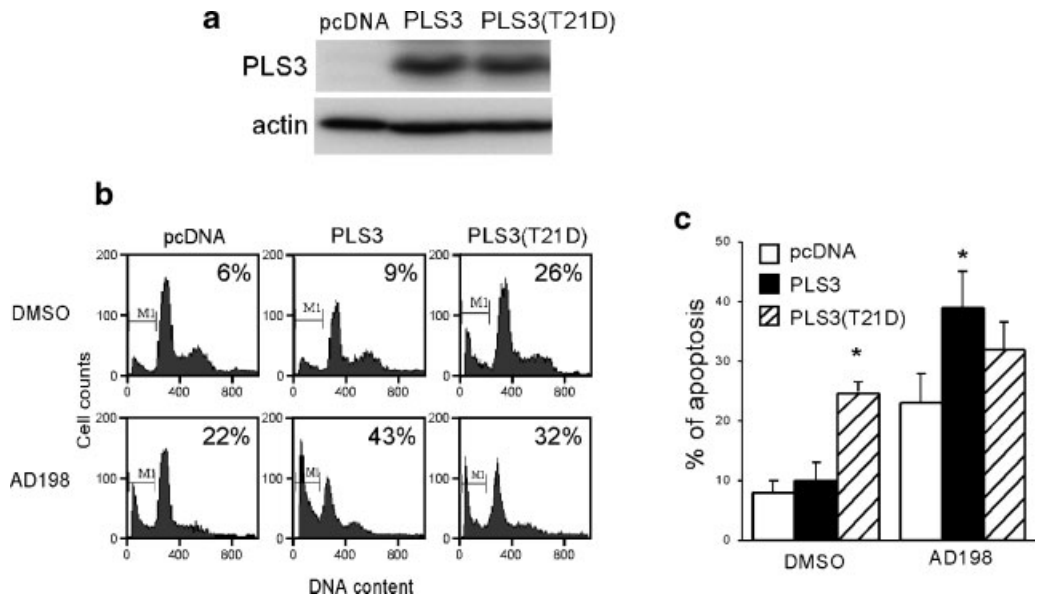

Fig. 1. Induction of apoptosis by phosphomimetic PLS3. **a**: Expression of PLS3 or PLS3(T21D). HeLa cells at 90% confluence were transfected with pcDNA control vector, PLS3- or PLS3(T21D)-expression vector for 48 h. Whole cell lysates were harvested for Western blotting using PLS3 antibody. β-actin was used as loading control. **b**: Determination of apoptosis by flow cytometry analysis. HeLa cells were transfected as in (a) and then treated with 5 μM AD198 or DMSO for 16 h. Cells were harvested and stained with PI. The apoptotic population was determined by flow cytometry. The subG$_1$ population was marked by M1 gate and used to represent the apoptotic population. **c**: Statistic analysis. The means ± standard deviations of apoptotic population were calculated from three independent experiments as in (b). Statistical significance ($P < 0.05$) was achieved by the paired *t*-test between PLS3(T21D) versus pcDNA control or wild-type PLS3 in DMSO groups, and between wild-type PLS3 versus pcDNA control or PLS3(T21D) in AD198 treated groups(indicated by *).

AD198, similar to what was described in our previous study, cells expressing the wild-type PLS3 had a dramatically enhanced apoptosis (9% to 43%) compared with the HeLa-control cells (6% to 22%). In contrast, cells transfected with PLS3(T21D) had only a modest increase of apoptosis after AD198 treatment (26% to 32%) (Fig. 1b, c). This finding suggests that phosphomimetic mutant PLS3(T21D) by itself can induce apoptosis, and that phosphorylation of PLS3 at Thr21 is critical to AD198-induced apoptosis.

Downregulation of PKC-δ Cannot Suppress Apoptosis Induced by Phosphomimetic PLS3(T21D)

Since PLS3 is a physiologic target of PKC-δ and phosphorylated at Thr21 by PKC-δ [He et al., 2005], we examined whether downregulation of PKC-δ can inhibit apoptosis induced by the phosphomimetic PLS3(T21D) mutant. HeLa cells were cotransfected with vectors to express PKC-δ siRNA and wild-type PLS3 or PLS3(T21D) individually or simultaneously. After 48 h, Western blot analysis of whole cell lysates from transfected cells was performed. The expression of wild-type PLS3 and PLS3(T21D) was similar and the expression of PKC-δ was decreased to less than 10% of normal by PKC-δ siRNA (Fig. 2a). Cells were then treated with 5 μM AD198 or DMSO for 16 h and harvested for apoptosis analysis. Similar to the result of Figure 1, PLS3(T21D) induced 18% apoptosis without AD198 treatment. Downregulation of PKC-δ by siRNA did not affect PLS3(T21D)-induced apoptosis, whereas downregulation of PKC-δ with siRNA inhibited the enhanced apoptosis induced by wild-type PLS3 (Fig. 2b, c). These results further support that PLS3 is a critical downstream effector of PKC-δ.

PLS3 Activity Is Enhanced by PKC-δ Phosphorylation

Next we investigated whether phosphorylation of PLS3 by PKC-δ leads to PLS3 activation. Using proteoliposomes with a similar composition to the mitochondrial outer membrane, we developed a phospholipid flip-flop assay to measure the activity of PLS3. Similar to other members of the scramblase family, PLS3 has the capability of promoting transbilayer lipid diffusion that is calcium-dependent. When the

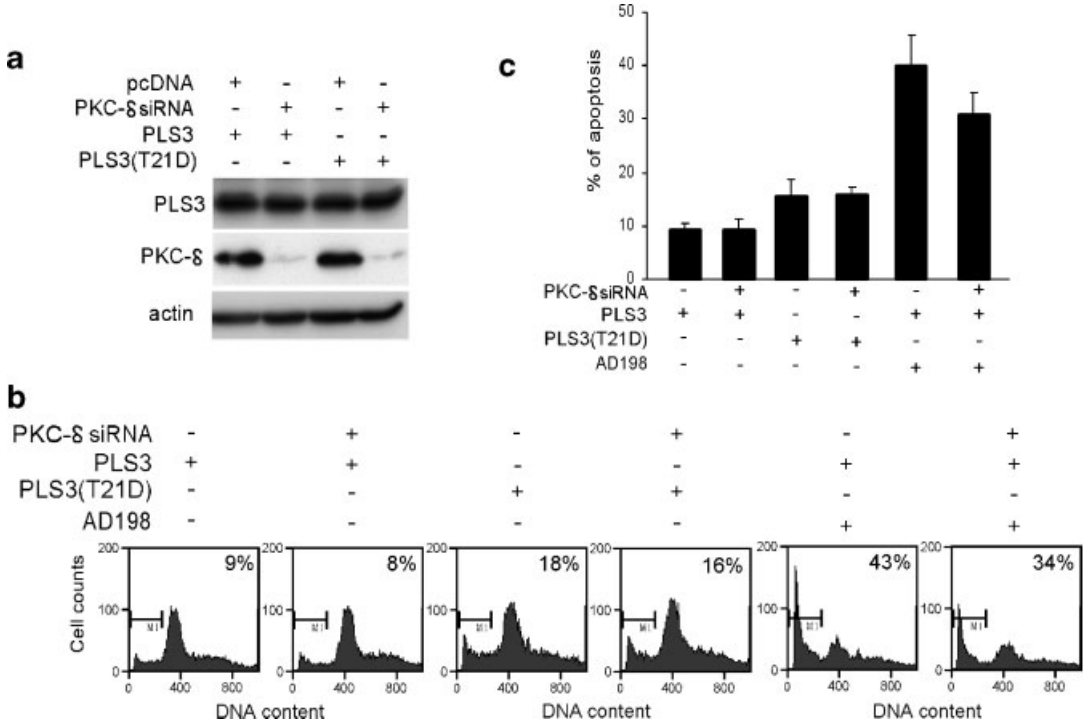

Fig. 2. Down-regulation of PKC-δ cannot inhibit apoptosis induced by phosphomimetic PLS3. **a**: Protein expression by Western blotting. HeLa cells were co-transfected with PKC-δ siRNA or pcDNA control vector and wild-type PLS3 or PLS3(T21D) vector at 90% confluence for 48 h. The down-regulation of PKC-δ and overexpression of wild-type PLS3 or PLS3(T21D) were examined by Western blotting of the whole cell lysates. **b**: Determination of apoptosis by flow cytometry analysis. HeLa cells were transfected as in (a) and then incubated with or 5 μM AD198 or DMSO. Cells were harvested at 16 h later and the apoptotic population was determined by PI staining followed by flow cytometry analysis. The $subG_1$ population was marked by M1 gate and used to represent the apoptotic population. **c**: Statistic analysis. The histogram represents the means ± standard deviations from three independent experiments as in (b).

highly conserved calcium-binding motif is mutated, the PLS3-induced phospholipid flip-flop activity is abolished. Using this assay to study the required cofactors of PLS3, we demonstrated that tBid is another essential cofactor for PLS3 activity (Liu and Lee, manuscript submitted). We used the same system to investigate whether PLS3 activity is modulated by phosphorylation at Thr21. First, we made proteoliposomes containing the same amounts of recombinant PLS3 or PLS3(T21D). Pyrene-labeled phosphatidylcholine (pyrene-PC) was added asymmetrically to the outer leaflet of the proteoliposomes as a probe. The rate of lipid flip-flop, determined by the decrease in the ratio of excimer to monomer fluorescence emission (I_e/I_m), was monitored continuously before and after sequential addition of calcium and tBid. We first compared PLS3 with PLS3 after it was phosphorylated by recombinant PKC-δ in vitro using this assay. However, a high background was detected due to the buffer used in the in vitro phosphorylation reaction and the results are inconclusive (data not shown). We thus switched to recombinant PLS3(T21D) to avoid the in vitro phosphorylation reaction. As shown in Figure 3, the decrease in the I_e/I_m ratio was much more rapid and significant in PLS3(T21D) proteoliposomes than that in wild-type PLS3 proteoliposomes, confirming that the phosphomimetic mutant of PLS3 has a stronger activity than unphosphorylated PLS3. Testing the proteoliposomes reconstituted with PLS3(T21A) revealed a similar activity to that of wild-type PLS3 (Fig. 3), indicating that the phosphoinhibitory mutation did not block the activity of PLS3. We conclude that phosphorylation at Thr21 is a mechanism of PLS3 activation, in which PKC-δ induces PLS3 activation by phosphorylation at Thr21.

Activation of PLS3 Increases tBid-Binding Capacity on the Mitochondrial Surface

We then examined the downstream event in mitochondria after PLS3 was activated by PKC-δ. Our previous quantification of CL in the

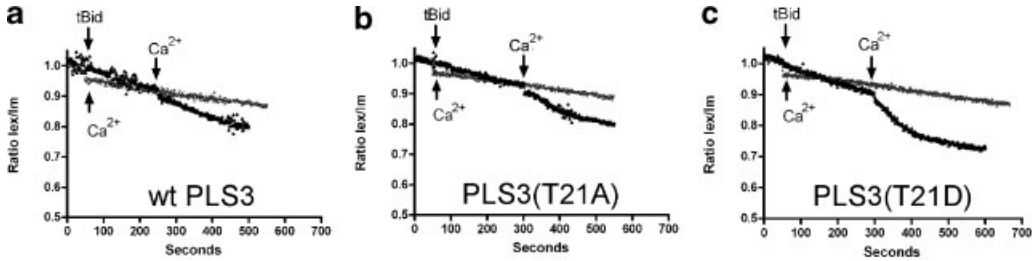

Fig. 3. Quantification of PLS3 activity by the lipid flip-flop assay. Proteoliposomes were reconstituted with equal amount of (**a**) PLS3, (**b**) PLS3(T21A), or (**c**) PLS3(T21D) and analyzed with the lipid flip-flop assay. Pyrene-PC was used as the probe to quantify the rate of flip-flop. The ratio of excimer and monomer(Ie/Im) was monitored after adding either 4 mM Ca^{2+} or 80 nM tBid, followed by 4 mM Ca^{2+}. The decrease of Ie/Im ratio indicates the occurrence of lipid flip-flop. Grey curves had only Ca^{2+} added at 50 s. Black curves had tBid added at 50 s and Ca^{2+} added at 250 s.

mitochondrial inner and outer membranes showed that cells with overexpression of PLS3 had an increase of CL, whereas cells expressing an dominant negative mutant of PLS3 had a decrease of CL in the mitochondrial outer membrane [Liu et al., 2003b]. During apoptosis, CL is an essential component for translocation of tBid to mitochondria [Lutter et al., 2000]. If the amount of CL on the mitochondrial surface increases, tBid targeting is anticipated to be enhanced. This theory is a potential explanation as to why mitochondria with overexpression of PLS3 are more susceptible to tBid-induced cytochrome c release. Based on this rationale, we investigated whether cells treated with AD198, which activates PKC-δ to phosphorylate PLS3, had the same response as PLS3 overexpression, that is, increased amount of tBid-binding capacity on the mitochondrial surface, which include CL, monolysocardiolipin [Esposti et al., 2003] or other tBid-binding partner [Grinberg et al., 2005]. We made a fluorescent probe, FITC-tBid(G94E) for this study. The G94E mutation was incorporated to eliminate the apoptotic ability of tBid by destroying the BH3 domain [Luo et al., 1998]. HeLa cells were treated with 5 μM AD198 or DMSO as control for 4 h. Mitochondria were isolated from cells, and incubated with FITC-tBid(G94E). Mitochondria from AD198-treated cells had a significantly higher tBid-binding capacity than those from DMSO-treated control cells (Fig. 4a, b). Given our previous study of AD198-induced PLS3 phosphorylation at the same time point [He et al., 2005], phosphorylation of PLS 3 by PKC-δ may also increase the tBid-binding capacity. However, a concern of this interpretation is that mitochondria from AD198-treated cells may lose their mitochondrial membrane integrity and allow the FITC-tBid(G94E) probe to get access to the inner membrane CL. To test this possibility, we selected HeLa cells that were transfected with the control vector, PLS3 or PLS3(T21D) expressing vector with G418 and successfully obtained stable cell lines. Subcellular fractionation and Western blotting showed that PLS3 and PLS3(T21D) were overexpressed in the selected clones and correctly localized in mitochondria (Fig. 4c). There was no detectable cytochrome c released from mitochondria expressing PLS3(T21D) (not shown), suggesting the maintenance of the integrity of the mitochondrial membrane. Mitochondria from the established stable cells were then used to test the tBid-binding capacity. As shown in Figure 4d, e, the mitochondria from HeLa-PLS3 cells bound more FITC-tBid(G94E) than those from HeLa-control cells, and mitochondria from HeLa-PLS3(T21D) had the highest tBid-binding capacity on the mitochondrial surface. This finding provides an evidence that PLS3(T21D) is more active than unphosphorylated PLS3.

Rescuing AD198-Induced Apoptosis by Expression of PLS3 Transgenes

In our previous report, we used siRNA to downregulate endogenous PLS3 and demonstrated that PLS3 was required for AD198-induced apoptosis. With the establishment of PLS3(T21D) as a more active form of PLS3, we examined whether expression of PLS3 or PLS3(T21D) transgenes could rescue AD198-induced apoptosis when endogenous PLS3 is suppressed by siRNA. Such experiments help to eliminate any potential problem induced by transfected siRNA oligos. To allow expression of PLS3 or PLS3(T21D) transgene under the circumstance of co-transfection with PLS3 siRNA, we generated PLS3* and PLS3(T21D)* by mutating the codon of Gly99 from GGC to

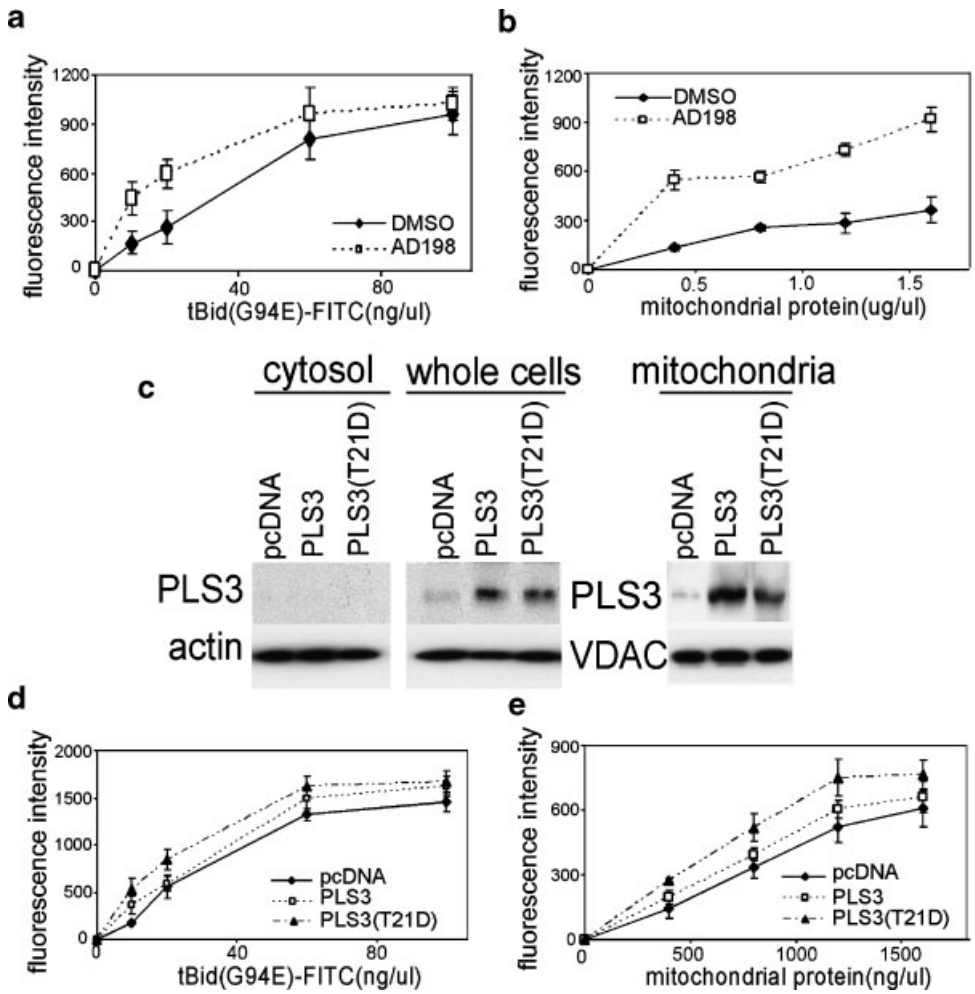

Fig. 4. Increased tBid-binding capacity by AD198 or PLS3 phosphorylation. **a**: Increased tBid-binding capacity by AD198. Mitochondria were isolated from HeLa cells treated with 5 μM AD198 or DMSO as control for 4 h. Mitochondria (50 μg by protein) were incubated with 0, 10, 20, 60, and 100 ng/μl FITC-tBid(G94E) in total volume 50 μl for 15 min followed by washing to remove unbounded probes. The fluorescent intensity was quantified with Bio-Tek microplate reader. Error bars indicate standard deviations of three independent experiments. **b**: Dose response with mitochondria. Various amounts of mitochondria in (a) were incubated with 10 ng/μl tBid(G94E)-FITC as in (a). The fluorescent intensity of the washed mitochondria was quantified with Bio-Tek microplate reader. **c**: Expression of PLS3 or PLS3(T21D) in selected HeLa cells. HeLa cells were transfected with pcDNA-, PLS3-, or PLS3(T21D)- expression vector and selected with G418. The stable clones were subjected to subcellular fractionation for Western blotting analysis using PLS3 antibody. VADC and β-actin were used as loading control. **d**: Increased tBid-binding capacity by overexpression of PLS3 or PLS3(T21D). Mitochondria were isolated from cells in (c). The tBid-binding capacity of the mitochondria from each cells were analyzed as in (a). **e**: Dose response with mitochondria. Mitochondria were isolated from cells in (c) and analyzed as in (b).

GGA, which is localized at the center of PLS3-siRNA sequence, and does not change the primary amino acid sequence of their products. Hence PLS3 siRNA only recognized and suppressed regular PLS3 or PLS3(T21D) but not PLS3* and PLS3(T21D)*. Western blotting of the transfected HeLa cell lysates confirmed our prediction (Fig. 5a). We then examined their sensitivity to AD198-induced apoptosis. Cells were treated with 5 μM AD198 or DMSO for 16 h. Apoptosis was analyzed by PI staining. Transfection efficiency evaluated by pEGFP-C1 vector showed that 60–70% of cells are EGFP-positive in all transfection. Consistent with the result in Figure 1, PLS3(T21D) by itself could induce small degree of apoptosis without AD198 treatment, and this effect is suppressed by PLS3 siRNA as the expression of PLS3(T21D) was downregulated (Fig. 5b). If cells expressed PLS3(T21D)*, same degree of apoptosis was detected, and PLS3 siRNA could not decrease the level of PLS3(T21D)* nor

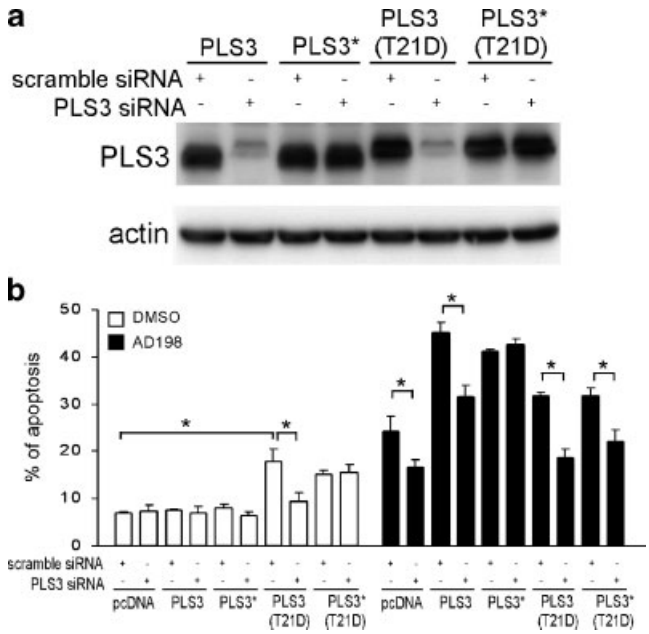

Fig. 5. Rescuing AD198-induced apoptosis by expression of PLS3 transgenes. **a**: Western blotting analysis of PLS3 after rescuing siRNA-mediated downregulation. PLS3* and PLS3(T21D)* represent the PLS mutants containing Gly99 (GGC→GGA), which is located at the center of PLS3 siRNA sequence. HeLa cells were transfected with scrambled control siRNA, PLS3 siRNA, PLS3, PLS3*, PLS3(T21D), or PLS3(T21D)* as indicated for 48 h before harvest. Whole cell lysates were analyzed with Western blotting using PLS3 and β-actin antibodies. **b**: Apoptosis study. HeLa cells were transfected as in (a) and treated with 5 μM AD198 or DMSO only for 16 h. Apoptosis was analyzed with PI staining followed by flow cytometry analysis. The histogram represents the means ± standard deviations from three independent experiments. ''*'' indicates pairs of results that reach statistical significance.

inhibit apoptosis. With AD198 treatment, overexpression of PLS3 enhanced apoptosis compared with cells transfected with the control pcDNA vector. This enhancement could be suppressed by PLS3 siRNA. However, the enhancing effect from overexpression of PLS3* was not inhibited by PLS3 siRNA. Finally, AD198 treatment increased apoptosis in cells expressing PLS3(T21D) or PLS3(T21D)*, which is likely due to the limited efficiency in transfection. This is supported by the observation that co-transfection with PLS3 siRNA decreased the level of apoptosis to the same as cells transfected with PLS3(T21D), indicating that the additional apoptosis after AD198 treatment is due to apoptosis from cells that did not incorporate the PLS3(T21D) expressing vector.

DISCUSSION

Our previous studies demonstrated that PLS3 is phosphorylated by PKC-δ at Thr21 [He et al., 2005] and that PLS3 is a mitochondrial target of PKC-δ-induced apoptosis [Liu et al., 2003a]. Here we investigate whether phosphorylation of PLS3 at Thr21 leads to PLS3 activation and what are the downstream events of PLS3 activation. To study the effect of PLS3 phosphorylation, we constructed a phosphomimetic mutant PLS3(T21D). Even though PLS3 is a pro-apoptotic factor based on our previous studies [Liu et al., 2003b], cells expressing wild-type PLS3 remain viable, which is most likely due to a low baseline PLS3 activity. However, overexpression of PLS3 significantly enhanced apoptosis induced by AD198, a PKC-δ activator [Roaten et al., 2002] that induces PLS3 phosphorylation by PKC-δ at Thr21 [He et al., 2005]. This is in contrast to cells expressing PLS3(T21D), which have a higher degree of apoptosis without AD198 treatment. However, cells expressing PLS3(T21D) have no enhanced apoptosis after AD198 treatment. Downregulation of PKC-δ with siRNA suppressed AD198-induced apoptosis in both control cells and cells expressing wild-type PLS3, but not in cells expressing PLS3(T21D). These findings are consistent with the notion that PLS3 is a downstream effector of PKC-δ-induced apoptosis.

The next question is how phosphorylation of PLS3 by PKC-δ modulates PLS3 activity. In

order to test whether phosphorylation of PLS3 induces its activation, we utilized an enzymatic assay [Epand et al., 2003] based on the phospholipid flip-flop activity of PLS3. We generated recombinant protein of the phosphomimetic mutant PLS3(T21D) to avoid the step of in vitro PKC-δ phosphorylation. This strategy worked and recombinant PLS3(T21D) was shown to be more active than the unphosphorylated wild-type PLS3. We also examined the activity of PLS3(T21A), the phosphorylation-inhibited mutant, and found no difference from that of wild-type PLS3, indicating that inhibition of phosphorylation does not abolish the baseline activity of PLS3. Previously, we found that HeLa cells expressing the PLS3(T21A) could not enhance AD198-induced apoptosis like wild-type PLS3 [He et al., 2005]. These findings prove that PLS3 activity is regulated by PKC-δ-induced phosphorylation.

After we established that PLS3 is phosphorylated and activated by PKC-δ, we examined what is the consequence of PLS3 activation and how that event induces apoptosis. Based on our previous finding in CL distribution in cells expressing wild-type PLS3 or the inactive PLS3(F258V) mutant that abolishes the calcium-binding motif of PLS3 [Liu et al., 2003b], one possibility is that overexpression of PLS3 or activation of PLS3 increases the amount of CL on the mitochondrial surface to facilitate tBid targeting. We thus generated FITC-tBid(G94E) to quantify the tBid-binding capacity on the mitochondrial surface. This probe was developed based on an important assumption that tBid does not insert into the membrane but has a parallel orientation to the membrane surface as described by two independent NMR studies [Gong et al., 2004; Oh et al., 2005]. Based on these two independent observations that tBid does not insert into the membrane, the targets of tBid, mainly CL, must be present on the surface of mitochondria to be accessible by tBid. The FITC-tBid(G94E) probe will not penetrate the outer membrane to interact with CL in the mitochondrial inner membrane and therefore could be used to quantify CL on the mitochondrial surface. The reason to use tBid(G94E) rather than tBid is to avoid the induction of mitochondrial damage [Luo et al., 1998]. Another advantage is that the probe mimics tBid targeting and is thus better than using the antibody against CL as a probe. Other potential related tBid targets, such as monolysocardiolipin (MLCL) [Degli Esposti, 2003; Esposti et al., 2003] and mitochondrial carrier homolog (Mtch2) [Grinberg et al., 2005], could also bind the FITC-tBid(G94E) probe, and were included in the determined tBid-binding capacity. In contrast, Bcl-2 or Bcl-xL are excluded because the BH3 domain of tBid(G94E) is disrupted.

We tested the hypothesis that PLS3 is responsible for translocating CL or monolysocardiolipin to the mitochondrial surface by quantification of tBid-binding capacity on the mitochondrial surface. Mitochondria from cells treated with AD198 had a higher tBid-binding capacity than untreated cells. Since the increase could be due to damage of the mitochondrial outer membrane in AD198-induced apoptotic cells, we examined mitochondria isolated from cells expressing PLS3(T21D) or wild-type PLS3. Mitochondria with expression of PLS3(T21D) had the highest tBid-binding capacity, and those expressing PLS3 exhibited a tBid-binding capacity higher than control mitochondria. Our data support the notion that PLS3 is responsible for increasing tBid-binding capacity in the outer membrane of mitochondria, and phosphorylation of PLS3 at Thr21 will further induce PLS3 activation. Because CL is synthesized in the inner membrane of mitochondria [Schlame et al., 2000], PLS3 is thus a trafficking machinery for moving CL to the outer membrane of mitochondria. CL in the outer membrane is mainly in the contact zone, which is the location where the outer and inner membranes have the closest contact, and would be the natural location when CL is translocated to the outer membrane. The electron microscopic study by Lutter et al. [2001] also reported that tBid(G94E) is localized in the contact zone.

In conclusion, we present evidence to show that phosphorylation of PLS3 by PKC-δ leads to activation of PLS3 using a phospholipid flip-flop assay and analysis of PLS3-induced CL changes. These findings support the importance of PLS3 as a downstream effector in PKC-δ-induced apoptosis. Understanding the function and regulation of PLS3 will greatly facilitate the potential preclinical and clinical development of AD198 or related compounds as novel agents for induction of apoptosis in cancer.

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