

# Protein kinase C $\delta$ amplifies ceramide formation via mitochondrial signaling in prostate cancer cells

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We studied the role of protein kinase C isoform PKC $\delta$  in ceramide (Cer) formation, as well as in the mitochondrial apoptosis pathway induced by anticancer drugs in prostate cancer (PC) cells. Etoposide and paclitaxel induced Cer formation and apoptosis in PKC $\delta$ -positive LNCaP and DU145 cells but not in PKC $\delta$ -negative LN-TPA or PC-3 cells. In contrast, these drugs induced mitotic cell cycle arrest in all PC cell lines. Treatment with Rottlerin, a specific PKC $\delta$  inhibitor, significantly inhibited drug-induced Cer formation and apoptosis in LNCaP cells, as did overexpression of dominant negative-type PKC $\delta$ . Overexpression of wild-type PKC $\delta$  had an opposite effect in PC-3 cells. Notably, etoposide induced biphasic Cer formation in LNCaP cells. The early and transient Cer increase resulted from de novo Cer synthesis, while the late and sustained Cer accumulation was derived from sphingomyelin hydrolysis by neutral sphingomyelinase (nSMase). Cer, in turn, induced mitochondrial translocation of PKC $\delta$  and stimulated the activity of this kinase, promoting cytochrome *c* release and caspase-9 activation. Furthermore, the specific caspase-9 inhibitor LEHD-fmk significantly inhibited etoposide-induced nSMase activation, Cer accumulation, and PKC $\delta$  mitochondrial translocation. These results indicate that PKC $\delta$  plays a crucial role in activating anticancer drug-induced apoptosis signaling by amplifying the Cer-mediated mitochondrial amplification loop.

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## Introduction

In various apoptotic phenomena, an elevation of the intracellular ceramide (Cer) levels occurs in response to a variety of apoptotic stimuli, including anticancer regimens (1). Numerous studies have suggested that lipid ceramide is relevant for apoptosis signaling in response to etoposide (2, 3) and paclitaxel (4). Moreover, a failure to generate ceramide has been associated with apoptosis resistance (5). However, the mechanisms that fail to generate Cer in apoptosis-resistant cells and convey activation signals to the enzyme responsible for Cer formation remain ill-defined.

A novel protein kinase C isoform, PKC $\delta$ , is implicated in apoptotic cell death, and its decreased protein expression is associated with tumor cell promotion (6). We reported recently that PKC $\delta$  protein expression decreased in androgen-independent prostate cancer (PC) cells in which PKC $\delta$  protein degradation is promoted by neuropeptide-mediated Src kinase activation (7). Notably, PKC $\delta$  has been implicated in the mediation of apoptosis in response to various anticancer agents such as etoposide (8) and paclitaxel (9). Recent studies show that a translocation of PKC $\delta$  onto mitochondria induces cytochrome *c* (cyt *c*) release and caspase

activation (9–11). Cer has also been implicated as a primary mediator in signaling to the mitochondrial apoptosis pathway (12, 13). Importantly, PKC $\delta$  and Cer interact with each other. Several investigators have reported that increased Cer can induce PKC $\delta$  translocation in apoptotic cell death (14, 15), thus suggesting the primary role of Cer in the activation of PKC $\delta$ -mediated apoptotic pathway. In contrast, another study showed that a PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which induces apoptosis through PKC $\delta$  activation in PC cells (7, 16), promotes Cer formation in LNCaP PC cells (17), thus leading to the possibility that activated PKC $\delta$  may in turn promote Cer formation in apoptosis. Furthermore, a recent report in which Cer formation was secondary to anticancer drug-induced mitochondrial events (2) argues against Cer playing a primary role in signaling to the mitochondrial apoptosis pathway. As a result, the signaling involving PKC $\delta$  and Cer in mitochondrial apoptosis is complicated, and the precise mechanism remains unclear.

In the present study, we report that PKC $\delta$  is required for the amplification of Cer formation and apoptosis induced by anticancer drugs in PC cells. In addition, we herein present that etoposide induces a biphasic increase

in Cer formation and that Cer formation in each phase is regulated by distinct mechanisms. We demonstrated that PKC $\delta$  mitochondrial translocation induced by an early Cer increase resulted in mitochondrial change followed by caspase-9 activation required for sustained Cer accumulation. These findings suggest that Cer activated by PKC $\delta$  might provide an amplifying signal to mitochondria in a feedback loop of caspase activation.

## Methods

**Cell culture and reagents.** PC cell lines were maintained in either RPMI1640 medium (LNCaP) or MEM (PC-3 and DU145) supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml streptomycin and penicillin, and 10% FCS. LNCaP derivative LN-TPA was maintained in the above media containing 10 nM TPA for more than 3 months as described previously (7, 18). Etoposide and paclitaxel were provided from Bristol-Myers Squibb KK (Tokyo, Japan) Cer analogues and various inhibitors were purchased from Calbiochem-Novabiochem Corp. (La Jolla, California, USA).

**Cell lysate preparation and isolation of mitochondrial and cytosol fractions.** Total cell lysates were prepared with RIPA buffer as described previously (19). For the isolation of mitochondrial and cytosol fractions, cells were collected in PBS and then pelleted by centrifugation. The cell pellets were resuspended in a buffer containing 25 mM Tris (pH 7.4), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT. Protease inhibitors (10  $\mu$ g of leupeptin/ml, 10  $\mu$ g of aprotinin/ml, and 1 mM PMSF) and phosphatase inhibitors (10  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>) were added prior to lysis. Cells were homogenized ten times with a Dounce homogenizer (Wheaton Scientific Products, Millville, New Jersey, USA). Unlysed cells and nuclei were removed by centrifugation at 750 *g* for 10 minutes. The supernatant was centrifuged at 10,000 *g* for 30 minutes, and the resulting pellet was washed once with the same buffer and represents the mitochondrial-enriched fraction. The supernatant was further spun at 100,000 *g* for 1 hour, and the supernatant from this final centrifugation represents the cytosol fraction.

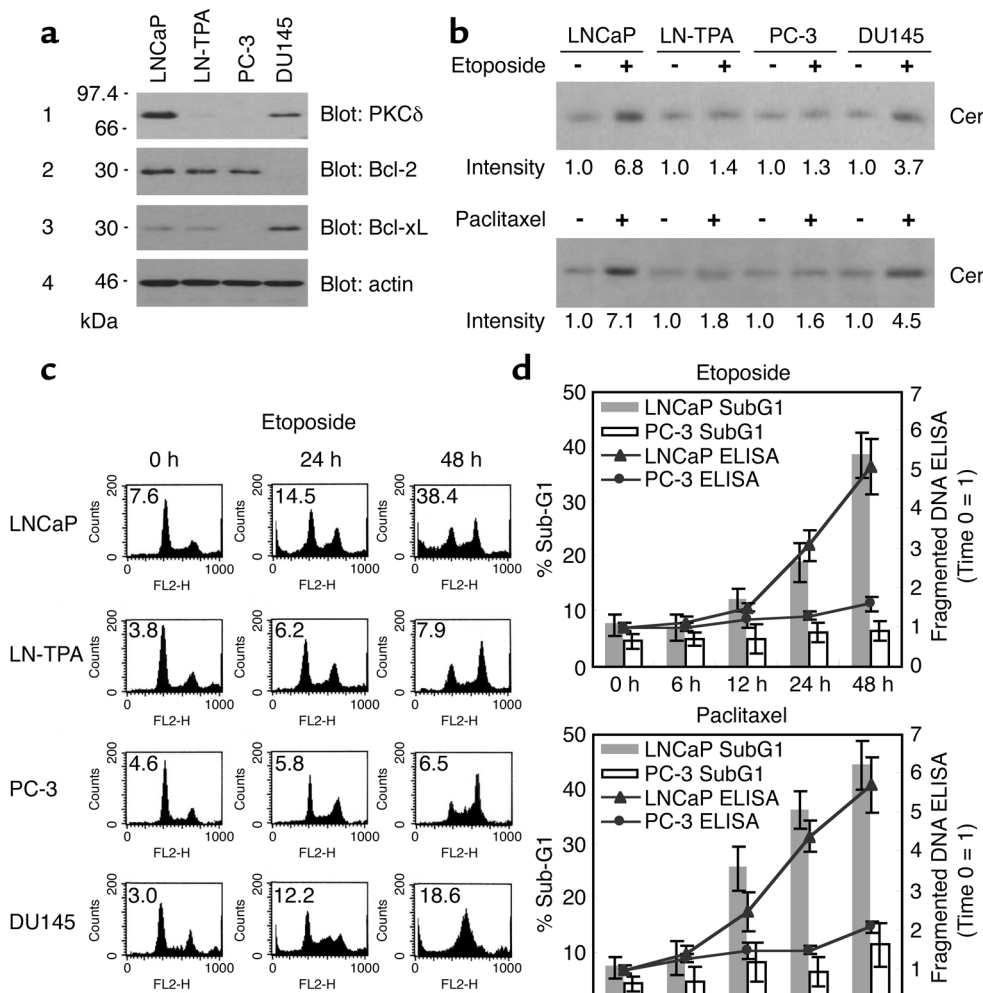
**Immunoblotting.** Immunoblotting procedures were performed as described previously (19) using anti-PKC $\delta$ , anti-Bcl-2 (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), anti-Bcl-xL, and anti-caspase-3 or anti-caspase-9 (1:1,000; New England Biolabs Inc., Beverly, Massachusetts, USA). For the detection of cyt *c* in cytosolic fractions, 20  $\mu$ g of cytosolic fractions were separated on 15% SDS-PAGE. The gels were then transferred, and membranes were immunoblotted with anti-cyt *c* (1:500; Santa Cruz Biotechnology Inc.) and detected. To monitor the loading equality of each lane, membranes were also immunoblotted with anti-actin (1:3,000; Chemicon International, Temecula, California, USA) for total cell lysates and cytosolic fraction or with anti-HSP-60 (1:500; Santa Cruz Biotechnology Inc.) for mitochondrial fraction. Relative intensities of each band were measured by NIH image.

**Overexpression of PKC $\delta$  and dominant negative type of PKC $\delta$  in PC cells.** Replication-deficient adenovirus vectors for PKC $\delta$  (Ax-PKC $\delta$ ), dominant negative type (DN type) (K376A) of PKC $\delta$  (Ax-DNPKC $\delta$ ), and a control vector for LacZ (Ax-LacZ) were constructed and transfected as described previously (20–22). Briefly, subconfluent PC cells were infected with adenovirus vectors at different moi's, and the cells were incubated in the infection media (MEM with 2% L-glutamine and 100  $\mu$ g/ml streptomycin) for 3 hours. After removing the virus, cells were further incubated for specific time periods.

**PKC $\delta$  activity assay.** The equal amounts of protein (500  $\mu$ g per sample) from treated PC cells were immunoprecipitated with anti-PKC $\delta$  Ab (2  $\mu$ g per sample). The washed immunoprecipitates were incubated at room temperature with 40  $\mu$ l of reaction buffer that contained 10  $\mu$ M PKC $\delta$  substrate derived from PKC $\delta$  pseudosubstrate region (23), 20 mM Tris-HCl, pH 7.5, 1 mM CaCl<sub>2</sub>, 10  $\mu$ M magnesium acetate, 1  $\mu$ M TPA, 50  $\mu$ g/ml phosphatidylserine (Sigma-Aldrich, St. Louis, Missouri, USA), 30  $\mu$ M ATP, and 30  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 20 minutes. The reaction tube was centrifuged, and 20  $\mu$ l of the supernatant was spotted on phosphocellulose disk sheets (Invitrogen Corp., Carlsbad, California, USA). The sheets were washed twice with 1% phosphoric acid and twice with distilled water, and samples were analyzed by liquid scintillation. The specific PKC $\delta$  activity was calculated by subtracting the nonspecific catalytic activity from the total catalytic activity and was expressed as fold increase compared with the control.

**Cell cycle analysis and apoptosis assay.** Cell cycle analysis and apoptosis assay were performed by flow cytometry and/or fragmented DNA ELISA as described previously (7, 21). In cell cycle analysis, fragmented apoptotic nuclei are recognized by their subdiploid (sub-G1) DNA content. All experiments were performed at least three times in duplicate.

**Quantitation of Cer and sphingomyelin.** Intracellular Cer and sphingomyelin (SM) levels were determined following metabolic labeling with [<sup>14</sup>C]serine as described (2, 24), with minor modifications. PC cells (10<sup>6</sup>/ml) were incubated for 24 hours with [<sup>14</sup>C]serine (0.2  $\mu$ Ci/ml) in MEM containing 0.3% FCS, washed with PBS, and then treated for specific time periods. After cells were detached with trypsin, total lipids were extracted with 7 ml of chloroform/methanol (1:2 vol/vol), and the phases were separated using 20 mM acetic acid. The extracts were spotted on Silica Gel 60 thin-layer chromatography (TLC) plates (Whatman Japan KK, Tokyo, Japan) and chromatographed with chloroform/methanol/0.22% aqueous CaCl<sub>2</sub> (60:35:5 by volume) or chloroform/methanol/H<sub>2</sub>O/25%NH<sub>4</sub>OH (50:45:2:1 by volume). Radioactive spots were evaluated with the Fujix BAS 2000 PhosphorImager system (Fuji Photo Film Co., Tokyo, Japan). The intensity of each Cer band was measured relative to total radioactivity in phosphatidylethanolamine, which remained unaltered upon stimulation and was expressed as a relative



**Figure 1** Correlation of anticancer drug-induced ceramide formation and apoptosis induction with PKC $\delta$  protein expression in PC cells. (a) Total cell lysates (20  $\mu$ g) from PC cells were analyzed by Western blot analysis as described in Methods using specific Ab's. (b) PC cells were treated with or without 10  $\mu$ M etoposide (upper panel) or 100 nM paclitaxel (lower panel) for 24 hours, and Cer levels were determined as described in Methods. The data are expressed as fold increase relative to each control and are representative of three experiments. (c) DNA flow cytometry histograms of PC cells cultured in media containing 10% FCS treated with or without 10  $\mu$ M etoposide for the indicated periods. The data are representative of three experiments. Percentage of apoptosis (sub-G1 DNA content) is indicated for each sample. (d) LNCaP and PC-3 cells were treated with 10  $\mu$ M etoposide or 100 nM paclitaxel for various periods. Sub-G1 DNA content and fragmented DNA in each sample were measured by cell cycle analysis and ELISA. Bars represent SD. Experiments were repeated three times with similar results.

value to each control set to 1. All experiments were performed at least three times, and the data were substantiated with a diacylglycerol kinase assay.

**Sphingomyelinase assay.** Cell protein extract (100  $\mu$ g) was incubated with 10 nmol of [ $^{14}$ C]SM (the specific radioactivity was adjusted to 10,000 cpm/nmol by the addition of unlabeled SM)/0.4 M Tris/HCl, pH 7.5/0.2% Triton X-100 in a final volume of 100  $\mu$ l at 37°C for 1 hour for neutral sphingomyelinase (nSMase). For acid SMase (aSMase), 0.4 M sodium acetate, pH 4.5, was used instead of Tris/HCl. The reaction was stopped by the addition of 1.5 ml of chloroform/methanol (2:1 vol/vol); then 0.2 ml of doubly distilled water was added to the mixture, vortex-mixed, and centrifuged at 1,000 g for 5 minutes to separate the two phases. The clear aqueous phase was removed to a glass scintillation vial, and the radioactivity was determined by scintillation counting. The experiments were performed at least three times in duplicate.

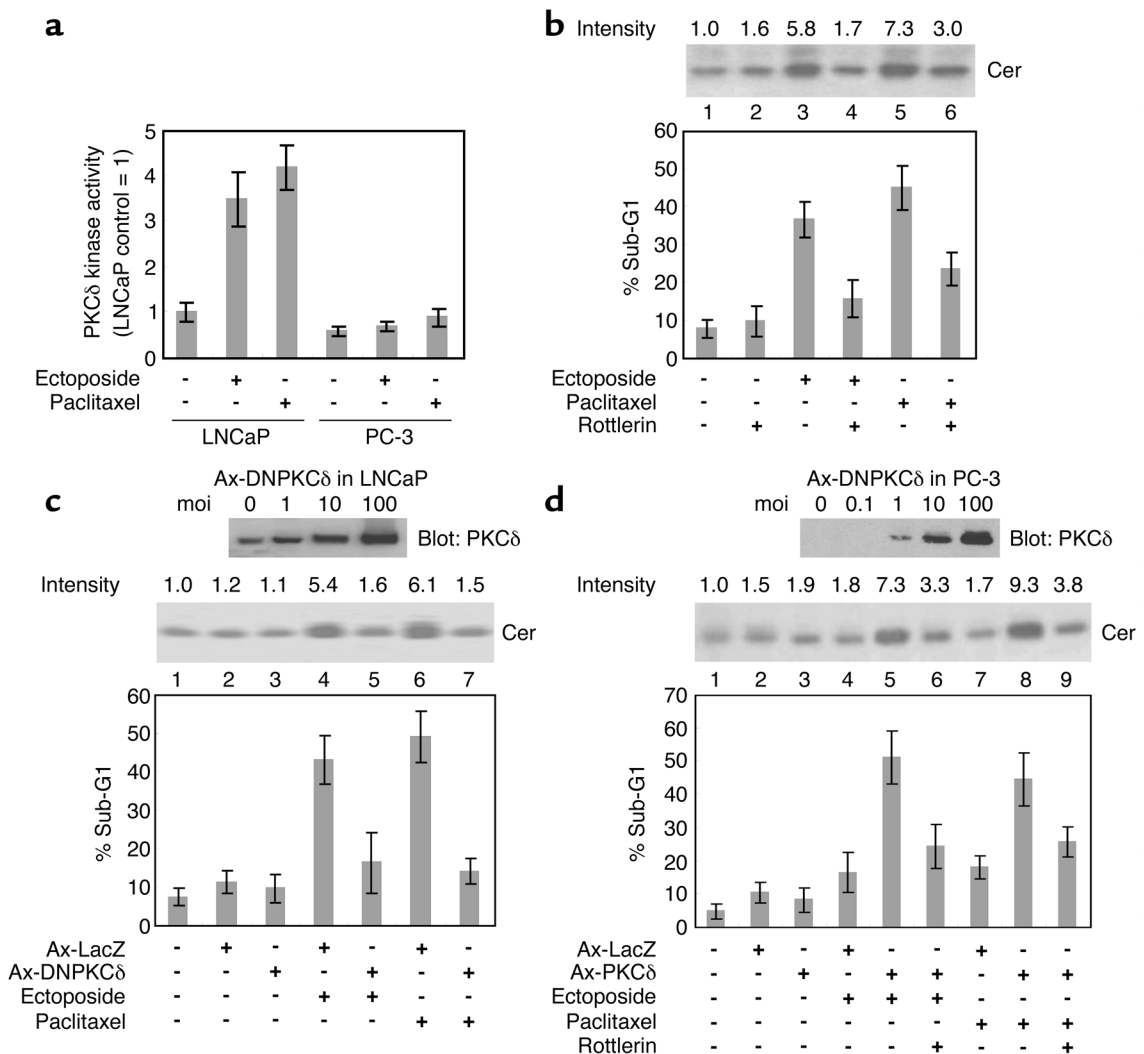
**Measurement of caspase-9 activity.** N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA) cleavage was detected using a kit purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA). Upon cleavage

of the substrate by caspase-9, free pNA light absorbance can be quantified using a microreader plate reader at 405 nm.

**Statistical analysis.** The statistical analysis was performed using an unpaired *t* test. *P* values of less than 0.05 were regarded as statistically significant.

## Results

**Correlation of anticancer drug-induced Cer formation and apoptosis induction with PKC $\delta$  protein expression in PC cells.** Western blot analysis revealed high levels of PKC $\delta$  proteins in total cell lysates derived from LNCaP cells, but low from LN-TPA or PC-3 cells and intermediate from DU145 cells (Figure 1a, panel 1). Bcl-2 families are known to regulate mitochondrial apoptotic process (25). Bcl-2 was expressed in LNCaP, LN-TPA, and PC-3 cells, and BCL-xL was not in PC-3 cells (Figure 1a, panels 2 and 3). Cer quantitation assay by [ $^{14}$ C]serine labeling showed that 10  $\mu$ M etoposide treatment for 24 hours resulted in 6.8- and 3.7-fold increase in Cer formation compared with untreated controls in PKC $\delta$ -positive LNCaP and DU145 cells, respectively, but not in PKC $\delta$ -negative LN-TPA or PC-3 cells (Figure 1b,



**Figure 2**

PKC $\delta$  activity is required for Cer formation and apoptotic cell death in response to anticancer drugs in PC cells. (a) PC cells were treated with or without 10  $\mu$ M etoposide or 100 nM paclitaxel for 6 hours, and PKC $\delta$  activity was measured as described in Methods. Experiments were repeated twice with similar results. (b) LNCaP cells were treated with the complex indicated (10  $\mu$ M etoposide, 100 nM paclitaxel, 10  $\mu$ M Rottlerin), and Cer levels (24-hour treatment, upper panel) and apoptosis (48-hour treatment, graph) were determined. Cer data are expressed as fold increase relative to the control and are representative of three experiments. Experiments were repeated three times with similar results. LNCaP (c) and PC-3 (d) cells, infected with Ax-DNPKC $\delta$  and Ax-PKC $\delta$  at different moi's, are incubated for 24 hours. Cell lysates were analyzed using anti-PKC $\delta$  Ab (upper panels). Note that both endogenous wild-type PKC $\delta$  and DNPKC $\delta$  were detected in LNCaP cells. LNCaP (c) and PC-3 (d) cells, infected with Ax-LacZ, Ax-DNPKC $\delta$ , or Ax-PKC $\delta$  at 10 moi, were treated with the complex indicated (10  $\mu$ M etoposide, 100 nM paclitaxel, 10  $\mu$ M Rottlerin), and Cer levels (middle panels) and apoptosis (graphs) were determined as described in b. For all, bars represent SD. All experiments were repeated three times with similar results.

upper panel). Similar results were obtained by 100 nM paclitaxel treatment (lower panel). Cell cycle analysis showed that 10- $\mu$ M etoposide treatment for 0–48 hours resulted in a marked increase in sub-G1 DNA content in LNCaP and DU145 cells, but not in LN-TPA or PC-3 cells, whereas a marked increase in S or G2 DNA content was induced in all PC cell lines (Figure 1c), thus suggesting that both LN-TPA and PC-3 cells are apoptosis resistant to etoposide even though they sense etoposide-induced DNA damage. Fragmented DNA ELISA showed that treatment with 10  $\mu$ M etoposide or 100 nM paclitaxel resulted in a time-dependent increase in fragmented DNA (apoptotic cells) in

LNCaP cells but not in PC-3 cells, which was well correlated with the results obtained by cell cycle analysis (Figure 1d). These results suggest that anticancer drug-induced Cer formation and apoptosis both correlate well with the protein expression of PKC $\delta$  rather than Bcl-2 or Bcl-xL and that DNA damage-induced mitotic cell cycle arrest in PC cells is not associated with apoptosis induction.

*PKC $\delta$  activity is required for Cer formation and apoptotic cell death in response to anticancer drugs in PC cells.* To assess whether or not PKC $\delta$  activity is needed for anticancer drug-induced Cer formation and apoptosis, we first investigated whether PKC $\delta$  could be activated

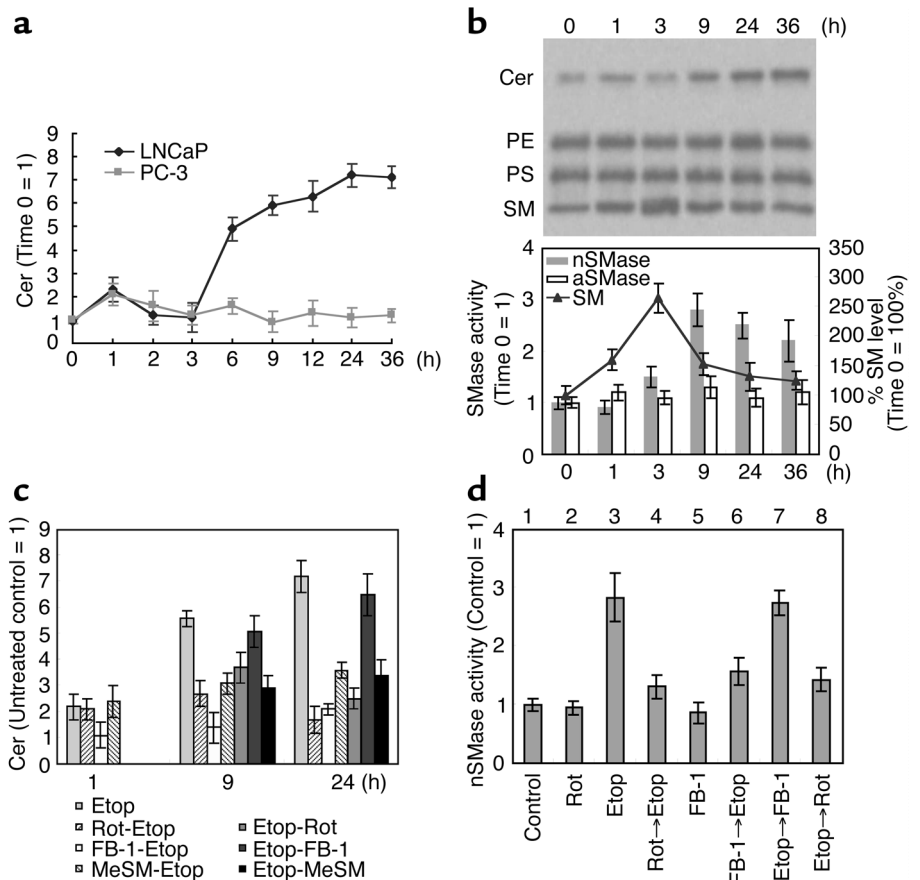
by anticancer drugs. As shown in Figure 2a, incubation with 10  $\mu$ M etoposide or 100 nM paclitaxel for 6 hours resulted in greater than threefold increase in PKC $\delta$  activity in LNCaP cells but not in PC-3 cells. We next cultured LNCaP cells in media containing etoposide or paclitaxel with the specific PKC $\delta$  inhibitor Rottlerin. Incubation of LNCaP cells with 10  $\mu$ M etoposide or 100 nM paclitaxel for 24 hours resulted in 5.8- or 7.3-fold increase in Cer formation, respectively, and incubation for 48 hours resulted in about five- to sixfold increase in sub-G1 DNA content compared with the untreated control (Figure 2b, lanes 3 and 5,  $P < 0.01$  compared with lane 1). Pretreatment with 10  $\mu$ M Rottlerin for 2 hours reversed anticancer drug-induced Cer formation and apoptosis (lanes 4 and 6,  $P < 0.01$ ). Similar results were obtained by overexpression of DNPCK $\delta$  after Ax-DNPCK $\delta$  transfection. We confirmed that DNPCK $\delta$  protein was highly expressed in LNCaP cells at concentrations of 10 moi or more 24 hours after Ax-DNPCK $\delta$  transfection (Figure 2c, upper panel). Culturing of LNCaP cells in media containing 10  $\mu$ M etoposide or 100 nM paclitaxel Ax-DNPCK $\delta$  (10 moi) transfection (Figure 2c, middle panel, 24 hours, and graph, 48 hours, lanes 5 and 7) resulted in a marked decrease in Cer formation and a significant decrease in sub-G1 DNA content ( $P < 0.01$ ) compared with anticancer drug treatment after Ax-LacZ transfection (lanes 4 and 6). The

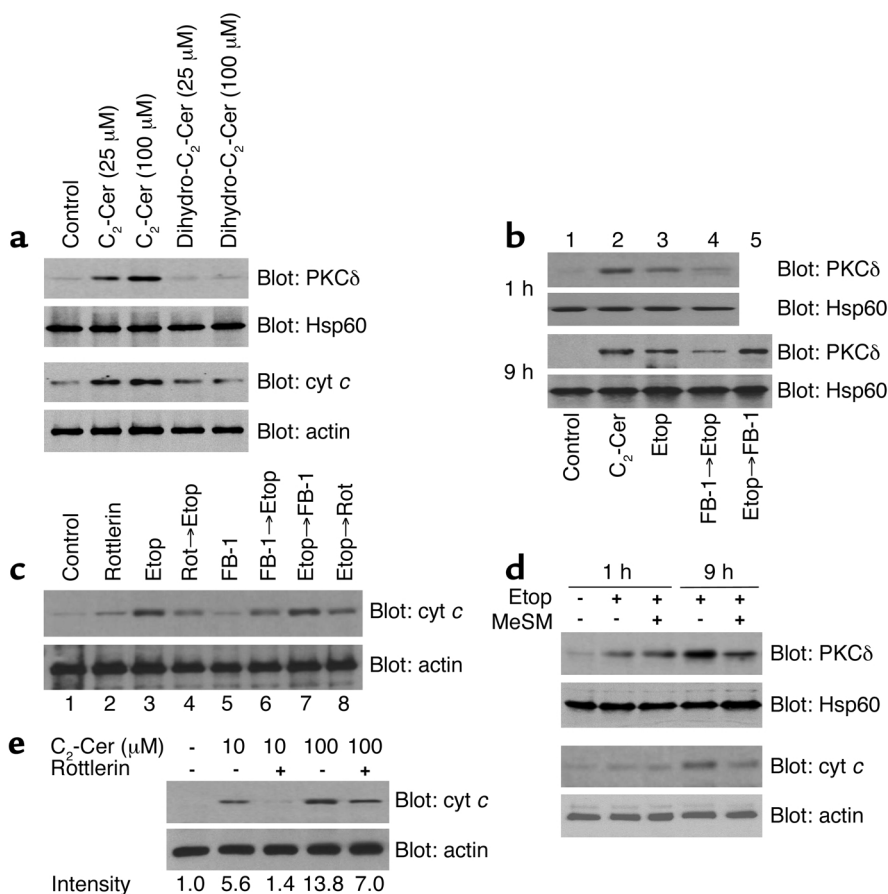
anticancer drug-induced PKC $\delta$  kinase activation in LNCaP cells was significantly inhibited by Ax-DNPCK $\delta$  transfection (data not shown). We further examined whether wild-type PKC $\delta$  expression after Ax-PKC $\delta$  transfection could augment drug-induced Cer formation and apoptosis in PKC $\delta$ -negative PC-3 cells. Western blot analysis confirmed that PKC $\delta$  protein was highly expressed in PC-3 cells at concentrations of 10 moi or more 24 hours after Ax-PKC $\delta$  transfection (Figure 2d, upper panel). Culturing of PC-3 cells in media containing 10  $\mu$ M etoposide or 100 nM paclitaxel following Ax-PKC $\delta$  (10 moi) transfection (Figure 2d, middle panel, 24 hours, and graph, 48 hours, lanes 5 and 8) resulted in greater than fourfold increase in Cer formation and about two- to threefold increase in sub-G1 DNA content ( $P < 0.01$ ) compared with anticancer drug treatment after Ax-LacZ transfection (lanes 4 and 7). Pretreatment of PKC $\delta$ -expressing PC-3 cells with 10  $\mu$ M Rottlerin (lanes 6 and 9) reversed anticancer drug-induced Cer formation and apoptosis ( $P < 0.01$  compared with lanes 5 and 8). These results suggest that PKC $\delta$  protein expression and the kinase activity of PKC $\delta$  are required for anticancer drug-induced Cer formation and apoptosis.

*Etoposide induces a biphasic increase in Cer formation.* It is of interest that an earlier study reported that biphasic Cer accumulation, i.e., an early and transient increase followed by a late and sustained increase in Cer, was

**Figure 3**

Etoposide induces a biphasic increase in Cer formation. (a) LNCaP and PC-3 cells were treated with 10  $\mu$ M etoposide for various periods, and Cer levels were determined. (b) LNCaP cells were treated with 10  $\mu$ M etoposide for various periods, and SM levels are determined by TLC. The nSMase and aSMase were measured as described in Methods. PE, phosphatidylethanolamine; PS, phosphatidylserine. (c) LNCaP cells were treated with the complex (10  $\mu$ M etoposide, 10  $\mu$ M Rottlerin, 50  $\mu$ M FB-1, 100  $\mu$ M MeSM) for the indicated periods, and Cer levels were determined. Etop, etoposide; Rot, Rottlerin. In each treatment, the time at which etoposide was added was set to 0 hours. (d) LNCaP cells were treated with the complex indicated (10  $\mu$ M etoposide, 10  $\mu$ M Rottlerin, 50  $\mu$ M FB-1) for 9 hours, and nSMase activity was determined. In each treatment, the time at which etoposide was added was set to 0 hours. For all, bars represent SD. Experiments were repeated three times with similar results.



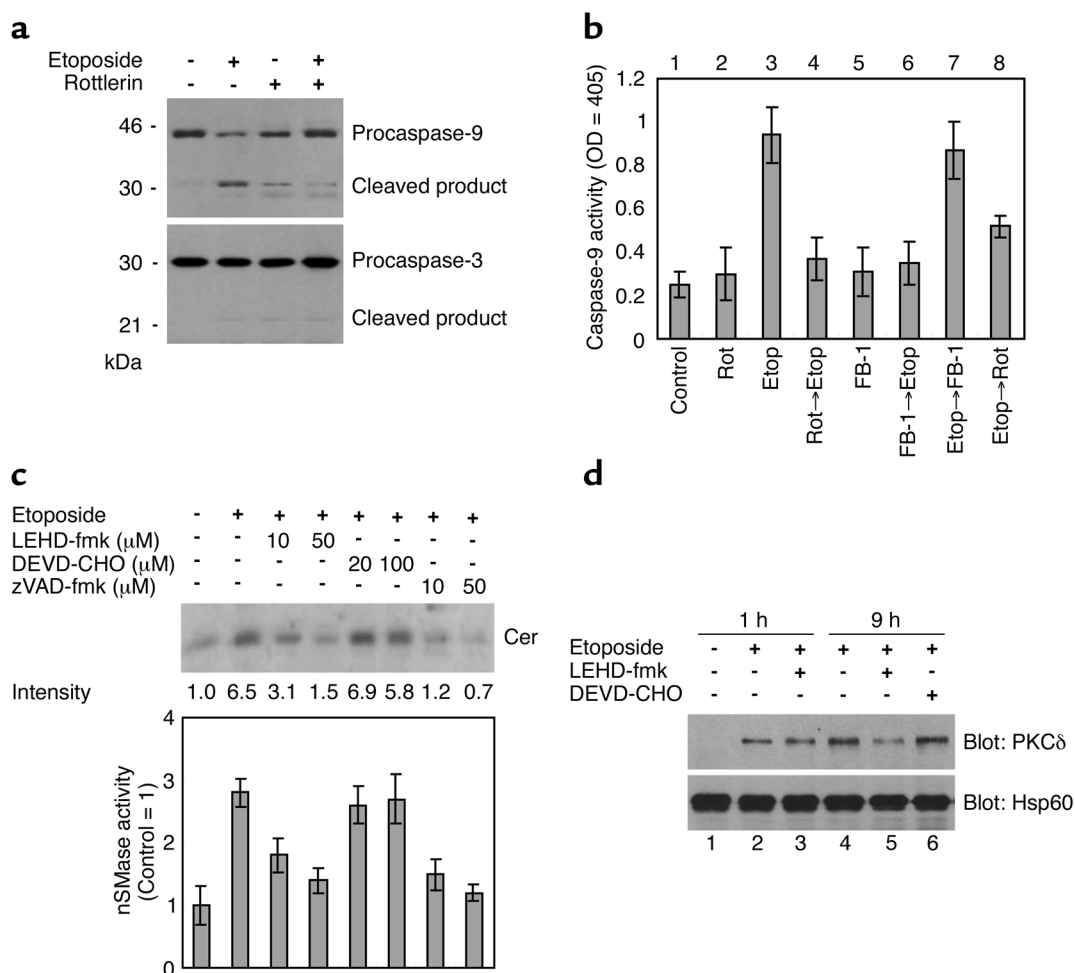


**Figure 4**

Both PKCδ and Cer are required for etoposide-induced mitochondrial events. (a) LNCaP cells were treated with Cer analogues at indicated concentrations for 9 hours, and cells were harvested and separated into mitochondrial and cytosol fractions. Lysates were analyzed using anti-PKCδ Ab and anti-cyt c Ab. (b) LNCaP cells were treated with the complex indicated (25 μM C<sub>2</sub>-Cer, 10 μM etoposide, 50 μM FB-1) for 1 hour or 9 hours, and mitochondrial fractions were analyzed using anti-PKCδ Ab. (c) LNCaP cells were treated with the complex indicated (10 μM etoposide, 10 μM Rottlerin, 50 μM FB-1) for 9 hours, and cytosol fractions were analyzed using anti-cyt c Ab. (d) LNCaP cells were treated with the complex indicated (10 μM etoposide, 100 μM MeSM) for 1 hour or 9 hours, and mitochondrial and cytosol fractions were analyzed using anti-PKCδ Ab and anti-cyt c Ab. (e) LNCaP cells were treated with the complex indicated (C<sub>2</sub>-Cer, 10 μM Rottlerin) for 9 hours, and cytosol fractions were analyzed using anti-cyt c Ab. Intensities of cyt c bands are expressed as fold increase relative to the control.

induced by apoptotic stimuli (26). We investigated the time course of Cer formation induced by etoposide in LNCaP and PC-3 cells. Treatment of LNCaP cells with 10 μM etoposide resulted in about twofold increase in Cer levels within 1 hour, followed by a sustained increase in Cer formation beginning within 6 hours of treatment and increasing by five- to sevenfold within 36 hours (Figure 3a). In PKCδ-negative PC-3 cells, etoposide failed to induce a sustained increase in Cer formation, whereas it induced similar transient Cer increase in LNCaP cells. Cer can result from the SM cycle, and SMase has been implicated in the late Cer formation induced by etoposide as well as CD95 and γ-radiation (2, 24). We therefore investigated the role of the SM cycle and SMase in Cer formation in LNCaP cells. TLC analysis using [<sup>14</sup>C]serine labeling of the total lipids in LNCaP cells showed that etoposide treatment resulted in a marked increase in SM levels at 3 hours, followed by a gradual decrease to the basal level within 36 hours (Figure 3b). SMase assay showed that etoposide treatment for 9 hours and longer resulted in a significant increase in the nSMase activity but not in the aSMase activity ( $P < 0.05$ , Figure 3b, graph). De novo Cer generation pathway is also well-known to be rapidly activated by various anticancer drugs (26, 27). Since we have shown that PKCδ and nSMase have been implicated in etoposide-induced Cer formation in LNCaP cells, we investigated the precise role of PKCδ, nSMase, and de novo Cer synthesis in

etoposide-induced biphasic Cer formation using their inhibitors, Rottlerin, 3-O-methyl-sphingomyelin (MeSM), and FB-1. As shown in Figure 3c, pretreatment of LNCaP cells with 50 μM FB-1 prior to adding 10 μM etoposide resulted in a significant decrease in Cer formation (1, 9, and 24 hours;  $P < 0.01$ ). In contrast, pretreatment with 10 μM Rottlerin or 100 μM MeSM failed to decrease etoposide-induced early Cer formation (1 hour), whereas it was able to significantly inhibit the late Cer formation (9 and 24 hours;  $P < 0.01$ ). These results suggest that the early Cer increase is independent of PKCδ and SM cycle and is thus derived from de novo Cer synthesis, which also has an effect on the late Cer accumulation. We next treated LNCaP cells with these inhibitors later than etoposide treatment. Treatment with Rottlerin or MeSM 3 hours later than the addition of etoposide resulted in a significant decrease in the late Cer formation (9 and 24 hours, respectively,  $P < 0.01$ ), while treatment with FB-1 failed. These results suggest that the late and sustained Cer formation is, in turn, derived from SM cycle and dependent on PKCδ. We investigated whether nSMase activation was regulated by de novo Cer synthesis and PKCδ. Pretreatment with 10 μM Rottlerin or 50 μM FB-1 prior to treatment with 10 μM etoposide for 9 hours (Figure 3d, lanes 4 and 6) or treatment with Rottlerin 3 hours later than etoposide treatment (lane 8) resulted in a significant decrease in the nSMase activity ( $P < 0.01$  compared with lane 3),



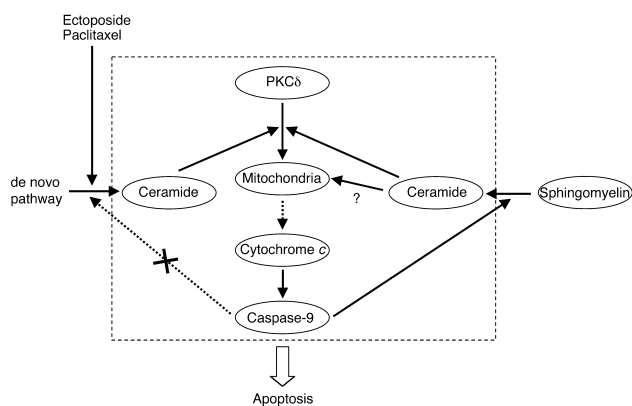
**Figure 5**

Caspase-9 mediates the late Cer accumulation from the SM cycle. (a and b) LNCaP cells were cultured treated with the complex indicated (10 μM etoposide, 10 μM Rottlerin, 50 μM FB-1) for 9 hours, and cell lysates were analyzed by Western blot analysis using anti-caspase-9 Ab and anti-caspase-3 Ab (a), and caspase-9 protease activity was assayed as described in Methods (b). Bars represent SD. Experiments were repeated twice in triplicate with similar results. (c) LNCaP cells were treated with the complex indicated (10 μM etoposide, LEHD-fmk, DEVD-CHO, zVAD-fmk) for 9 hours, and Cer levels (upper panel) and SMase activity (graph) were measured. Cer data are expressed as fold increase relative to the control and are representative of three experiments. Bars represent SD. Experiments were repeated three times with similar results. (d) LNCaP cells were treated with the complex indicated (10 μM etoposide, 50 μM LEHD-fmk, 100 μM DEVD-CHO) for 1 hour or 9 hours, and mitochondrial fractions were analyzed using anti-PKCδ Ab.

while treatment with 50 μM FB-1 3 hours later than etoposide treatment (lane 7) failed. Together, these results suggest that etoposide-induced biphasic Cer increase is mediated by distinct Cer formation pathways and that PKCδ is required for the late and sustained Cer formation derived from the SM cycle.

*Both PKCδ and Cer are required for etoposide-induced mitochondrial events.* PKCδ mitochondrial translocation is important for mitochondrial events (9, 11). We also confirmed that treatment with etoposide resulted in a time-dependent increase in PKCδ mitochondrial translocation followed by cyt *c* release (data not shown). Recent studies suggest that Cer can also induce PKCδ translocation (14, 15), thus leading to the possibility that etoposide-induced Cer formation has an effect on PKCδ mitochondrial translocation followed by cyt *c* release. To clarify this possibility, we first investigated whether one

of the Cer analogues C<sub>2</sub>-Cer can effect PKCδ mitochondrial translocation and cyt *c* release. We confirmed in a preliminary study that C<sub>2</sub>-Cer treatment resulted in a marked increase in the intracellular Cer activity (data not shown, see ref. 28). Incubation of LNCaP with C<sub>2</sub>-Cer (25–100 μM) but not biologically inactive dihydro-C<sub>2</sub>-Cer for 9 hours resulted in marked increase in PKCδ mitochondrial translocation and cyt *c* release (Figure 4a). We next investigated whether de novo Cer synthesis is required for etoposide-induced PKCδ mitochondrial translocation. Incubation of LNCaP with 10 μM etoposide for both 1 hour and 9 hours resulted in an increase in PKCδ mitochondrial translocation (Figure 4b, lanes 1 and 3), which was blocked by pretreatment with 50 μM FB-1 (lane 4). However, treatment with FB-1 3 hours later than the addition of etoposide (lane 5) failed to decrease etoposide-induced Cer formation in



**Figure 6**

The role of PKC $\delta$  in inducing sustained Cer accumulation in mitochondrial apoptosis. Anticancer drugs induce early and transient de novo Cer synthesis. Generated Cer induces PKC $\delta$  mitochondrial translocation, which promotes cyt *c* release and caspase-9 activation dependent on PKC $\delta$  activity. Caspase-9 in turn induces the late and sustained Cer accumulation derived from SM by activating nSMase, which further promotes PKC $\delta$  mitochondrial translocation, thus providing an amplifying signal to mitochondria in a feedback loop of caspase activation.

the late phase. In both phases, 25  $\mu$ M C<sub>2</sub>-Cer markedly induced PKC $\delta$  mitochondrial translocation (lane 2). Similar results were obtained by Western blot analysis for cyt *c* release. Pretreatment with 50  $\mu$ M FB-1 for 2 hours prior to etoposide treatment for 9 hours resulted in a marked decrease in cyt *c* release (Figure 4c, lanes 3 and 6), while treatment with FB-1 3 hours later than etoposide treatment (lane 7) failed. Recent studies suggest that PKC $\delta$  kinase activity is required for such mitochondrial events as cyt *c* release (9, 11). Pretreatment or treatment after administration of etoposide with 10  $\mu$ M Rottlerin resulted in a marked decrease in etoposide-induced cyt *c* release (Figure 4c, lanes 3, 4, and 8). Our results suggest that Cer derived from de novo pathway is required but not sufficient for etoposide-induced PKC $\delta$  mitochondrial translocation followed by cyt *c* release in the late phase. We therefore investigated whether mitochondrial events are also mediated by the SM cycle. As shown in Figure 4d, pretreatment with 100  $\mu$ M MeSM markedly blocked etoposide-induced PKC $\delta$  mitochondrial translocation and cyt *c* release in the late phase (9 hours) but not in the early phase (1 hour). These results suggest that Cer from de novo pathway and the SM cycle mediates etoposide-induced mitochondrial events in different phases. We further investigated whether Cer can induce cyt *c* release independent of PKC $\delta$  since earlier studies suggested that Cer has a direct effect on mitochondria (12, 13). Pretreatment with 10  $\mu$ M Rottlerin for 2 hours prior to 10  $\mu$ M C<sub>2</sub>-Cer for 9 hours resulted in over 90% decrease in cyt *c* release compared with C<sub>2</sub>-Cer alone, while pretreatment with Rottlerin prior to higher concentration (100  $\mu$ M) of C<sub>2</sub>-Cer resulted in about 50% decrease (Figure 4e). Together, these results suggest that etoposide-induced mitochondrial events require both

Cer increase and PKC $\delta$ , although the possibility remains that increased Cer can directly induce cyt *c* release independent of PKC $\delta$ .

*Caspase-9 mediates the late Cer accumulation from SM cycle.* Caspase-9 processing following cyt *c* release is regarded as the apex of the apoptotic cascade (29). We also found that etoposide treatment for 9 hours markedly induced caspase-9 processing in LNCaP cells, which was reversed by pretreatment with 10  $\mu$ M Rottlerin (Figure 5a, upper panel). Of note, treatment with etoposide and/or Rottlerin was found to have little effect on caspase-3 processing (Figure 5a, lower panel). Etoposide-induced caspase-9 activation was substantiated by a caspase-9 activity assay. Treatment with etoposide (Figure 5b, lane 3) resulted in about a four-fold increase in caspase-9 activity compared with the untreated control (lane 1,  $P < 0.01$ ), which was significantly reversed by treatment with Rottlerin or pretreatment with FB-1 (lanes 4, 6, and 8;  $P < 0.01$  compared with lane 3), but not after treatment with FB-1 (lane 7). We next investigated whether etoposide-induced sustained Cer formation resulted from caspase-9 activation, since initiator caspases such as caspase-9 can mediate DNA damage-induced Cer formation (2, 30). Pretreatment of LNCaP cells with the specific caspase-9 inhibitor LEHD-fmk or pan-caspase inhibitor zVAD-fmk resulted in a dose-dependent decrease in Cer formation induced by etoposide treatment for 24 hours, whereas pretreatment with the caspase-3 inhibitor N-acetyl-Asp-Glu-Val-aspartic acid aldehyde (DEVD-CHO) at concentrations of 20–100  $\mu$ M failed to block it (Figure 5c, upper panel). Treatment of LNCaP cells with etoposide plus LEHD-fmk resulted in a significant decrease in Cer formation through the late phase (data not shown). Our findings indicate the possibility that nSMase can be activated by caspase-9. Pretreatment of LNCaP cells with LEHD-fmk or zVAD-fmk resulted in a dose-dependent decrease in nSMase activation induced by etoposide treatment for 9 hours, whereas pretreatment with DEVD-CHO failed (Figure 5c, lower graph). We finally investigated whether PKC $\delta$  mitochondrial translocation dependent on Cer is regulated by caspase-9. As shown in Figure 5d, pretreatment with 50  $\mu$ M LEHD-fmk markedly inhibited 10  $\mu$ M etoposide-induced PKC $\delta$  mitochondrial translocation in the late phase (9 hours, lanes 4 and 5) but not in the early phase (1 hour, lanes 2 and 3). Pretreatment with 100  $\mu$ M DEVD-CHO failed to block etoposide-induced PKC $\delta$  mitochondrial translocation (lane 6). These results suggest that etoposide-induced sustained Cer accumulation and PKC $\delta$  mitochondrial translocation depends predominantly on PKC $\delta$ -mediated caspase-9 activation, which can induce sustained Cer formation via the SM cycle.

## Discussion

The results presented herein help clarify the findings of a previous report on anticancer drug-induced apoptotic cell death in androgen-sensitive LNCaP cells but



not in androgen-independent PC cells such as PC-3 cells (31). We showed that a novel PKC isoform PKC $\delta$  protein, which was highly expressed in LNCaP cells but not in PC-3 cells, was required for apoptosis induced by paclitaxel, which targets microtubule network, as well as etoposide, which inhibits topoisomerase II. Our results are consistent with recent reports in which PKC $\delta$  mitochondrial translocation and its kinase activity have been implicated in the mitochondrial apoptotic pathway (9, 11, 32).

More importantly, our study demonstrated that PKC $\delta$  was required for sustained Cer formation relevant for apoptosis signaling in response to anticancer drugs (2–4). In agreement with a previous study (26), we found that etoposide induced biphasic Cer accumulation in etoposide-sensitive LNCaP cells but not in etoposide-resistant PC-3 cells. While we have shown that de novo Cer generation pathway is required for the early and transient increase in Cer, the importance of this pathway in anticancer drug-induced Cer generation and apoptosis remains controversial (5, 33). We have shown that the early and transient Cer formation is induced even in apoptosis-resistant PC-3 cells and moreover, similar to a recent study (34), we have found that serine palmitoyltransferase rather than Cer synthase is rapidly activated by etoposide treatment in LNCaP cells and even in PC-3 cells (M. Sumitomo et al., unpublished data). Our results highlight the involvement of not only de novo Cer generation pathway but the SM cycle mediated by PKC $\delta$  in anticancer drug-induced Cer accumulation in PC cells. Furthermore, our results suggest that de novo Cer synthesis, activated by PKC $\delta$ -independent mechanisms, is required for transient Cer formation followed by mitochondrial events but it is not sufficient for amplifying these events without PKC $\delta$ . In contrast, the late and sustained Cer formation, dependent on PKC $\delta$  whose mitochondrial translocation and kinase activation is followed by caspase-9 activation, is sufficient for amplifying mitochondrial events. As a result, PKC $\delta$  contributes to sustained Cer formation through caspase-9 activation in mitochondrial events. Once PKC $\delta$  translocates onto mitochondria, it can activate PKC $\delta$ -caspase-9-Cer loop independent of de novo Cer generation pathway, providing an amplifying signal to mitochondria (Figure 6).

It seems obvious that sustained Cer increase plays a crucial role in anticancer drug-induced apoptosis since we found that pretreatment with FB-1 or MeSM blocked etoposide-induced apoptosis in LNCaP and PKC $\delta$ -expressed PC-3 cells (data not shown). However, it remains unclear as to how increased Cer contributes to the effector phase of apoptosis. Interestingly, we found that C<sub>2</sub>-Cer induced marked apoptosis in PKC $\delta$ -negative PC-3 cells and even in LNCaP cells pretreated with LEHD-fmk (M. Sumitomo et al., unpublished data), leading to the possibility that increased Cer that resulted from the mitochondrial amplification loop needs neither PKC $\delta$  nor

the downstream caspase cascade from caspase-9 in order to induce apoptosis. Tepper et al. (35) recently reported that increased Cer could directly alter the cell surface morphology, resulting in apoptosis. In addition, we found that caspase-3 processing was not recognized in etoposide-treated LNCaP cells. Fujii et al. (16) reported similar results in which TPA-induced apoptosis in LNCaP cells was mediated by PKC $\delta$  but was also independent of caspase-3. These reports and the findings of our study may provide new insight supporting the hypothesis that PKC $\delta$ -mediated apoptosis is predominantly mediated by increased Cer via mitochondrial change in an effector caspase-independent manner.

In summary, we clarified a part of the complicated mechanisms that fail to generate Cer in anticancer drug-resistant PC cells. Even in apoptosis-resistant PC cells, DNA damage and transient Cer generation could be induced, but PKC $\delta$  is essential for amplifying Cer accumulation that can further activate mitochondrial apoptosis signaling. A modification of PKC $\delta$  protein expression using neutral endopeptidase, which can block the rapid degradation of PKC $\delta$  protein (7), with anticancer drugs may thus be a potentially effective therapeutic modality for the treatment of PC cells.

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