Tyrosine Phosphorylation of Protein Kinase Cδ Is Essential for Its Apoptotic Effect in Response to Etoposide

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Protein kinase Cδ (PKCδ) is involved in the apoptosis of various cells in response to diverse stimuli. In this study, we characterized the role of PKCδ in the apoptosis of C6 glioma cells in response to etoposide. We found that etoposide induced apoptosis in the C6 cells within 24 to 48 h and arrested the cells in the G1/S phase of the cell cycle. Overexpression of PKCδ increased the apoptotic effect induced by etoposide, whereas the PKCδ selective inhibitor rottlerin and the PKCδ dominant-negative mutant K376R reduced this effect compared to control cells. Etoposide-induced tyrosine phosphorylation of PKCδ and its translocation to the nucleus within 3 h was followed by caspase-dependent cleavage of the enzyme. Using PKC chimera, we found that both the regulatory and catalytic domains of PKCδ were necessary for its apoptotic effect. The role of tyrosine phosphorylation of PKCδ in the effects of etoposide was examined using cells overexpressing a PKCδ mutant in which five tyrosine residues were mutated to phenylalanine (PKCδ5). These cells exhibited decreased apoptosis in response to etoposide compared to cells overexpressing PKCδ. Likewise, activation of caspase 3 and the cleavage of the PKCδ5 mutant were significantly lower in cells overexpressing PKCδ5. Using mutants of PKCδ altered at individual tyrosine residues, we identified tyrosine 64 and tyrosine 187 as important phosphorylation sites in the apoptotic effect induced by etoposide. Our results suggest a role of PKCδ in the apoptosis induced by etoposide and implicate tyrosine phosphorylation of PKCδ as an important regulator of this effect.

Materials and Methods

Materials. An affinity-purified polyclonal anti-PKCε antibody against a polypeptide corresponding to amino acids 726 to 737 of PKCε was purchased from Gibco-BRL Life Technologies (Gaithersburg, Md.). Monoclonal anti-PKCδ antibody directed against the regulatory domain was obtained from Trans-
duction Laboratories (Lexington, Ky.), and polyclonal anti-PKC antibodies were from Transduction Laboratories (Lexington, Ky.). Etoposide was from Alexis Co. (San Diego, Calif.), and an anti-active caspase 3 antibody was obtained from New England Biolabs (Beverly, Mass.). The caspase inhibitors DEVD-FMK, Z-VAD-FMK, and YYVAD were obtained from Calbiochem (La Jolla, Calif.). Leupeptin, apro-
nitin, phenylmethylsulfonyl fluoride (PMSF), and sodium vanadate were ob-
tained from Sigma Chemical Co. (St. Louis, Mo.). The Caspase 3 Cellular Activity Assay Kit PLUS was obtained from BIOMOL (Plymouth Meeting, Pa.), and the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) Kit was from Roche Molecular Biochemicals.

Generation of PKC chimeras. The PKC chimeras were generated by exchang-
ing the regulatory and catalytic domains of PKCα and PKCβ as previously described (1). PKCα and PKCβ were transfected into the chimeras with the PKCα regulatory domain and the PKCβ catalytic domain, and PKCβα refers to the reciprocal chimera. The PKC α dDNAs were subcloned into the metallothionein promoter-driven eukaryotic expression vector (MTH). The vector sequence encodes a C-terminal PKCα-derived 12-amino-acid tag (εMTH) that is added to the expressed proteins (27). The expression of these chimeras and their activities in C6 cells were recently described (5).

Site-directed mutagenesis of PKCβ. Mouse PKCγ was cloned into the pGEM-T vector (Promega, Madison, Wis.) as described previously (5). This plasmid served as our “master” vector for the site-directed mutagenesis, using the Transformer Site-Directed Mutagenesis Kit from Clontech (Palo Alto, Cal-
if.). Conversion of tyrosine residues at sites 52, 64, 155, 187, and 565 into phenylalanine was performed as previously described (5). PKC7 and the PKCβ mutants were subcloned into the metallothionein promoter-driven eukaryotic expression vector (εMTH). A PKCγ K376R dominant mutant was generated as previously described (36).

Construction of PKCβ-GFP fusion protein. cDNAs encoding the murine PKCβ and the PKCβ mutant were fused into the N-terminal enhanced green fluorescent protein (GFP) vector pEGFP-N1 (Clontech Laboratories). The original pEGFP-N1 vector was modified by the insertion of an MmiI site into the plasmid polylinker. The restriction site was created by ligating a phosphorylated linker containing the MmiI site into pEGFP-N1 digested with Snml. The con-
struct was verified by sequencing. The clones containing GFP-PKCβ or GFP-
PKCβS were constructed by the excision of PKCβ or PKCβS from MTH-PKC plasmids by digestion with XhoI and MmiI. The inserts were then ligated into the modified GFP vector by using the same restriction sites. DNA sequencing of the GFPPKC constructs confirmed the intended reading frame.

C6 glial cultures and cell transfection. C6 cells (10^5 cells/ml) were seeded on tissue culture dishes (10-cm diameter) and were grown in medium consisting of Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (0.05 mg/ml). Cells were transfected with the indicated vectors using lipofectamine (Gibco-BRL Life Technolo-
gies) as previously described (6). Experiments were routinely carried out on a clone of the transfected cells, but all the results were confirmed on one pool and two additional individual clones.

For overexpression of the GFP-PKCβ fusion proteins, C6 cells were seeded onto 40-mm round glass coverslips at a density of 5 × 10^4 cells/coverslip. Twenty-four hours later, cells were transfected with the different GFP-PKCβ constructs using Lipofectamine Plus reagent according to the manufacturer’s instructions. All experiments were performed 48 h posttransfection.

Measurements of cell apoptosis. Cell apoptosis was measured using propidium isoside (PI) staining and analysis by flow cytometry and by ELISA (Cell Death Detection ELISA Kit) using anti-histone antibodies. Cells (1 × 10^5/ml) were plated in six-well plates and treated with the indicated treatments for 24 h. Detached cells and tryptophan adherent cells were pooled, fixed in 70% ethanol for 1 h on ice, washed with phosphate-buffered saline (PBS), and treated for 15 min with RNase (50 μg/ml) at room temperature. Cells were then stained with PI (5 μg/ml) and analyzed on a Becton-Dickinson cell sorter.

For anti-histone ELISA (Cell Death Detection ELISA kit), extracts of cells containing histone-associated DNA fragments were incubated in 96-well plates coated with anti-histone antibodies for 2 h. Plates were then washed and incubated with anti-DNA antibodies conjugated to peroxidase for an additional 2 h. Substrate solution was added and absorbance was measured at 405 nm.

Cell viability was also quantitatively assessed by the measurement of lactate dehydrogenase (LDH) in the medium.

Measurement of caspase 3 activity. Caspase 3 activity was measured using the caspase 3 colorimetric assay kit obtained from BIOMOL by using Ac-DEVD-

pNA as a substrate according to the manufacturer’s recommendations.

Nuclear and cytosolic fractionation. Nuclear proteins were prepared according to the method described by Haglund and Rothblum (24). Cells (1 × 10^5 to 5 × 10^6/ml) were washed once with 1 ml of PBS and once with 1 ml of lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2, 0.5 mM PMSF, 10 μg of leupeptin/ml, pH 7.9). Cells were lysed by suspending the cell pellet in 20 μl of lysis buffer containing 0.1% Nonidet P-40 (NP-40) for 10 min on ice. To isolate nuclei, the lysates were microcentrifuged for 5 min at 12,000 g, and the nuclear pellet was washed with lysis buffer without NP-40. Nuclear proteins were obtained by resuspending the nuclear pellet in 20 μl of extraction buffer (420 mM NaCl, 20 mM HEPES, 1.5 mM MgCl_2, 0.2 mM EDTA, and 25% glycerol, pH 7.9) for 10 min at 4°C. The nuclear suspension was microcentrifuged, the pellet was discarded, and the supernatant was diluted in dilution buffer (50 mM KCl, 20 mM HEPES, 0.2 mM EDTA, and 20% glycerol, pH 7.9). Lack of contamination of the nuclear fraction by the plasma membrane was confirmed using the plasma membrane marker NaK ATPase.

Preparation of cell homogenates. Cells were washed and resuspended in se-
rine-free medium. The plates were placed on ice, scraped with a rubber police-
m and centrifuged at 1,400 rpm for 10 min. The supernatants were aspirated, and the cell pellets were resuspended in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% Na deoxycholate, 2% NP-40, 0.2% sodium dodecyl sulfate [SDS], 1 mM PMSF, 50 μg of aprotinin/ml, 50 μM leupeptin, 0.5 mM Na_2VO_3) on ice for 15 min. The cell lysates were centrifuged for 15 min at 14,000
rpm in an Eppendorf microcentrifuge, supernatants were removed, and 2× sample buffer was added.

**Immunoblot analysis.** Lysates (40 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in PBS and subsequently stained with the primary antibody. Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, Calif.), and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham, Arlington Heights, Ill.).

**Immunoprecipitation.** Immunoprecipitation was performed as previously described (5). Briefly, C6 cells overexpressing PKCδ or PKCθ were serum starved overnight and treated for different periods of time with PMA (10 nM) or PDGF (100 ng/ml). The samples were preabsorbed with 25 μl of protein A/G-Sepharose (50%) for 10 min, and immunoprecipitation was performed using 4 μg of antibody/ml for 1 h at 4°C and then incubated with 30 μl of protein A/G-Sepharose

FIG. 2. Effects of rottlerin, PKCδ DN, and PKCθ overexpression on the apoptosis of C6 cells induced by etoposide. C6 cells were treated with etoposide (50 μM) in the absence and presence of rottlerin (5 μM) for 48 h (A) or cells overexpressing control vector (CV), PKCδ DN (B), or PKCθ (C) were treated with etoposide. Cell apoptosis was determined using anti-histone ELISA (A and B) or PI staining and FACS analysis (C). The optical densities of etoposide-treated cells (A) or of etoposide-treated CV cells (B) were designated 100% (total apoptosis), and all other values are presented as percent of this total. (A and B) The results represent the means ± SE of triplicate measurements in each of three experiments. (C) Distributions are from a representative experiment. *, P < 0.001, compared to control cells.
for an additional hour. Following washes, the pellets were resuspended in 25 μl of SDS sample buffer and boiled for 5 min. The entire supernatants were subjected to Western blotting. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were washed and visualized by the enhanced chemiluminescence (ECL) system.

Immuno-fluorescence staining. Cells were grown on glass coverslips. Following etoposide treatment (3 to 24 h), cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) for 10 min. Subsequently, cells were washed in PBS, and after blocking with staining buffer (2% bovine serum albumin [BSA] and 0.1% Triton X-100 in PBS) for 30 min at room temperature, cells were incubated with a mouse (regulatory domain) or a rabbit (catalytic domain) anti-PKC antibody. Following washes in PBS, cells were incubated with an anti-rabbit antibody or with Alexa FluorTM 546 goat anti-mouse IgG for an additional 60 min and were mounted in FluoroGuard antifade reagent. Cells were viewed and photographed using confocal microscopy. The results are from one representative experiment out of five similar experiments.

FIG. 3. Translocation of PKCδ in C6 cells treated with etoposide. C6 cells were treated with etoposide (50 μM) for 0, 3, 6, and 24 h, and the translocation of PKCδ was assessed using immunofluorescence staining. Following fixation with 4% PFA, cells were incubated with a rabbit anti-PKCδ antibody for 1 h and with an anti-rabbit antibody conjugated to fluorescein isothiocyanate. Cells were visualized by confocal microscopy. The results are from one representative experiment out of five similar experiments.

RESULTS

Etoposide induces apoptosis and cell cycle arrest in C6 glioma cells. Treatment of the cells with etoposide arrested the cells in the G1/S phase of the cell cycle and induced cell apoptosis (Fig. 1A). Using PI staining and fluorescence-activated cell sorter (FACS) analysis, we found that approximately 22% ± 3.9% of the cells underwent apoptosis in response to etoposide after 24 h of treatment, whereas approximately 63% ± 7.1% of the cells were apoptotic after 48 h. Similar results were obtained using anti-histone ELISA (Fig. 1B) and using measurements of LDH levels in the cell supernatants (data not shown).

Treatment of the cells with etoposide also resulted in lower cell number and in the appearance of rounded and detached cells, which are characteristic of apoptotic cells (Fig. 1C).

Role of PKCδ in apoptotic effect of etoposide. Recent studies suggested that PKCδ plays a role in the apoptotic effect of etoposide in salivary gland acinar cells (49). To explore the role of PKCδ in the effect of etoposide on C6 cells, we utilized rottlerin, which has been reported to be a PKCδ selective inhibitor (22). Treatment of C6 cells with rottlerin (5 μM) did not affect the basal level of cell apoptosis; however, rottlerin inhibited the apoptotic effect of etoposide (Fig. 2A), reducing cell apoptosis by approximately 50%. Higher concentrations of rottlerin itself induced morphologic changes in the cells and some cell apoptosis and therefore could not be used.

Since the in vitro inhibitory effect of rottlerin on PKCδ activity has recently been subject to controversy (12, 22, 30), we further explored the role of PKCδ in the apoptotic effect of etoposide by employing cells overexpressing a PKCδ dominant-negative mutant (K376R) (36). Cells stably expressing the PKCδ DN mutant were treated with ZnCl2 for 24 h (to induce overexpression through its MTH promoter), followed by a 48-h treatment with etoposide (50 μM). Overexpression of PKCδ DN did not affect the basal level of C6 cell apoptosis; however, rottlerin inhibited the apoptotic effect of etoposide (Fig. 2A), reducing cell apoptosis by approximately 50%. Higher concentrations of rottlerin itself induced morphologic changes in the cells and some cell apoptosis and therefore could not be used.

The role of PKCδ in the apoptotic effect of etoposide was also demonstrated using cells stably overexpressing PKCδ. The
translocation, which are cell and stimulus dependent. To examine the effect of etoposide on the translocation of PKCδ, we treated C6 cells with etoposide for various periods of time and followed the expression of PKCδ using immunofluorescence and confocal microscopy. In control cells, PKCδ was found largely in the cytosol, but with some expression in the nucleus (Fig. 3). Treatment with etoposide induced further translocation of PKCδ to the nucleus. Translocation was observed already after 3 h, and the expression of PKCδ in the nucleus was observed up to 24 h following treatment. We confirmed that etoposide did not induce translocation of PKCδ to the Golgi, endoplasmic reticulum, or the mitochondria, as determined by costaining with these organelle-specific markers (data not shown).

No translocation of PKCα, β, ε, δ, and γ was observed in etoposide-treated cells, whereas some translocation of PKCε to the perinuclear membrane was observed (data not shown).

Cleavage of PKCδ by etoposide. PKCδ undergoes cleavage in response to various apoptotic stimuli (21, 49). To examine the effect of etoposide on the cleavage of PKCδ in our system, we treated C6 cells with etoposide (50 μM) for various periods of time and analyzed cell lysates by using Western blotting. The level of PKCδ decreased following 24 h of etoposide treatment, and a 40-kDa cleavage product of PKCδ appeared and started to accumulate (Fig. 4A). No significant cleavage of PKCα, β, ε, δ, and γ was observed in response to etoposide, and no cleavage products were detected (data not shown).

Since the nuclear translocation of PKCδ preceded its caspase-dependent cleavage, we examined if PKCδ cleavage occurred in the nucleus. We first performed cell fractionation and separated the nuclear and cytosolic fractions. We found that in untreated cells, PKCδ was mainly expressed in the cytosol with some expression in the nucleus. Following etoposide treatment, PKCδ translocated to the nucleus and a cleaved form of PKCδ accumulated only in the nuclear fraction (Fig. 4B). The nuclear marker lamin B was also detected only in the nuclear fraction. Using anti-PKCδ antibodies directed against the regulatory and catalytic domains, we found that the immunostaining of PKCδ using these two antibodies resulted in a similar pattern of nuclear translocation (Fig. 4C). The results of these two experiments suggest that PKCδ translocated to the nucleus, where it underwent cleavage. Since some PKCδ was present in the nucleus of the untreated cells without being cleaved, the cleavage must depend on the etoposide treatment itself as well as on its location.

Caspase 3 is involved in cleavage of PKCδ and apoptosis is induced by etoposide. PKCδ can be cleaved by a caspase-dependent process (21, 34). We therefore examined the effects of the cell-permeable caspase 3 inhibitor DEVD.FMK (20 μM) on the cleavage of PKCδ in the C6 cells. As seen in Fig. 5A, pretreatment of the cells for 1 h with DEVD.FMK significantly reduced the accumulation of the PKCδ cleavage product in response to etoposide. Similar results were obtained with another caspase inhibitor, Z-VAD.FMK (data not shown).

We also examined the ability of the caspase inhibitors to block the apoptosis induced by etoposide in C6 cells. Pretreatment of the cells with either DEVD.FMK or Z-VAD.FMK reduced the apoptotic effect of etoposide by approximately 60% (Fig. 5B), suggesting a role for caspase 3 in the apoptotic process.
response. In contrast, the caspase 1 inhibitor YVAD did not affect etoposide effects in these systems (data not shown).

Since the cleavage of PKCδ occurred in the nucleus, we examined whether active caspase 3 was also expressed in the nucleus in etoposide-treated cells. Using immunofluorescence staining, we found that the active form of caspase 3 was expressed in the nucleus of etoposide-treated cells, whereas a very low expression of active caspase 3 was detected in control cells (Fig. 5C). Similar results were obtained using Western blot analysis of nuclear and cytosolic fractions (Fig. 5D).

**Inhibition of PKCδ blocks cleavage of caspase 3 and PKCδ.** To examine the importance of PKCδ activity in its ability to undergo cleavage, we employed the selective PKCδ inhibitor rottlerin. Pretreatment of the cells with rottlerin (5 μM) reduced the cleavage of caspase 3 in response to etoposide (Fig. 6A). Similarly, cells overexpressing PKCδ DN exhibited a significantly lower caspase 3 activity than control vector cells (Fig. 6B). Finally, the cleavage of PKCδ and the accumulation of the PKCδ catalytic domain were reduced in rottlerin-treated cells (Fig. 6C), suggesting that the cleavage of both caspase 3 and PKCδ is dependent on an active PKCδ.

Both regulatory and catalytic domains of PKCδ are required for apoptotic effect induced by etoposide. In a recent study, we demonstrated that the regulatory domain of PKCδ mediated its inhibitory effect on C6 cell proliferation and on the expression of the astrocytic marker GS (5). The regulatory domain of PKCδ was phosphorylated on tyrosines 155 and 187 in response to PMA and PDGF, respectively (5, 35), and the
To examine the relative contributions of the regulatory and catalytic domains of PKC to its apoptotic effects, we used chimeras between the regulatory and catalytic domains of PKCo and -5 combined at the highly conserved hinge region. The expression and activity of C6 cells overexpressing the different chimeras were already described (5).

Cells were pretreated for 24 h with ZnCl2, followed by etoposide (50 μM) for an additional 48 h. Cells overexpressing PKC5/5 exhibited levels of apoptosis similar to those of control vector-transfected cells. Treatment of the control vector cells with etoposide induced about 60% apoptosis, whereas cells overexpressing PKC5 did not exhibit an increase in tyrosine phosphorylation in response to etoposide (Fig. 8A), suggesting that the tyrosine phosphorylation occurred in the regulatory domain.

Tyrosine phosphorylation of PKC6 is essential for its apoptotic effect. In previous studies, we identified five putative tyrosine phosphorylation sites in PKC6 and generated a PKC6 mutant, in which these five tyrosine phosphorylation sites were mutated to phenylalanine (5). The expression and activity of C6 cells overexpressing this mutant were already described (5). Overexpression of the PKC6 mutant in C6 cells induced an increase in tyrosine phosphorylation in response to etoposide (Fig. 8B), suggesting that the tyrosine phosphorylation occurred in the regulatory domain.

Tyrosine phosphorylation of PKC6 is essential for its apoptotic effect. In previous studies, we identified five putative tyrosine phosphorylation sites in PKC6 and generated a PKC6 mutant, in which these five tyrosine phosphorylation sites were mutated to phenylalanine (5). The expression and activity of C6 cells overexpressing this mutant were already described (5). Overexpression of the PKC6 mutant in C6 cells induced an increase in tyrosine phosphorylation in response to etoposide (Fig. 8B), suggesting that the tyrosine phosphorylation occurred in the regulatory domain.

FIG. 7. Apoptosis of C6 cells overexpressing different PKC chimeras in response to etoposide. C6 cells overexpressing PKCo, PKC5, and the chimeras PKCo/5 and PKC5/α were treated with etoposide for 48 h. Cell apoptosis was determined using anti-histone ELISA. The optical densities of etoposide-treated PKC5-overexpressing cells were designated 100% (total apoptosis), and all other values are presented as a percent of this total. The results represent the means ± SE of triplicate measurements in each of three experiments.
abolished the decrease in the expression of the astrocytic marker GS induced by PMA or PDGF and the decrease in cell proliferation induced by PMA. Expression of the PKC\(\varepsilon\) mutant also resulted in a lower level of tyrosine phosphorylation of PKC\(\varepsilon\) in response to these treatments (5). Using cells expressing PKC\(\varepsilon\), we found that etoposide did not induce a significant increase in tyrosine phosphorylation of PKC\(\varepsilon\) (Fig. 9A). Similarly, treatment of these cells with etoposide resulted in low levels of apoptosis similar to the response observed in the control vector cells when measured after 24 h (Fig. 9B) and in lower levels of apoptosis than control vector cells after 48 h of treatment (Fig. 9C). Thus, the tyrosine phosphorylation of PKC\(\varepsilon\) in the regulatory domain induced by etoposide was essential for the apoptotic effect of PKC\(\varepsilon\) in response to this drug.

Cleavage of caspase 3 and PKC\(\varepsilon\) in cells overexpressing PKC\(\varepsilon\) mutant. To further explore the role of tyrosine phosphorylation of PKC\(\varepsilon\) in the apoptosis induced by etoposide, we compared the activation of caspase 3 in cells overexpressing control vector, PKC\(\varepsilon\), and the PKC\(\varepsilon\) mutant. Using a specific antibody recognizing the cleaved product (17 kDa) of caspase 3, we found that etoposide induced caspase 3 cleavage in the control vector cells. Cells overexpressing PKC\(\varepsilon\) showed a larger amount of the cleaved product, whereas cells overexpressing the PKC\(\varepsilon\) mutant exhibited very low levels of the 17-kDa fragment (Fig. 10A). Similar results were obtained for caspase 3 activity as measured by a caspase 3 colorimetric assay (Fig. 10B). These results suggest that the activation of caspase 3 required a tyrosine phosphorylated form of PKC\(\varepsilon\).

We then examined the cleavage of PKC\(\varepsilon\) and PKC\(\varepsilon\) in response to etoposide. Using the \(\varepsilon\) tag, we were able to detect the cleaved catalytic domain of the exogenous PKC\(\varepsilon\) and PKC\(\varepsilon\). Using the anti-PKC\(\varepsilon\) antibody, we found no detectable cleaved product in the etoposide-treated control vector cells. Accumulation of the catalytic fragment was observed in cells overexpressing PKC\(\varepsilon\), whereas cells overexpressing the PKC\(\varepsilon\) mutant displayed no detectable cleavage (Fig. 10C). Since the kinetics of PKC\(\varepsilon\) phosphorylation is faster than the cleavage and is transient, the phosphorylation presumably plays a role upstream from the actual cleavage.

Translocation of PKC\(\varepsilon\) and the PKC\(\varepsilon\) 5 mutant in response to etoposide. One possible explanation for the differential effects of PKC\(\varepsilon\) and the PKC\(\varepsilon\) mutant on cell apoptosis is their differential translocation following etoposide treatment. We therefore examined the translocation of PKC\(\varepsilon\) and the PKC\(\varepsilon\) mutant in response to etoposide. For these experiments, we used GFP-tagged PKC\(\varepsilon\) wild type or the PKC\(\varepsilon\) mutant. Cells were transiently transfected with the specific construct, and the response of the cells to etoposide was monitored after 6 and 24 h.

We found that etoposide induced a similar pattern of translocation for both PKC\(\varepsilon\) and PKC\(\varepsilon\) (Fig. 11). Thus, stimulation of the cells with etoposide for 6 h induced translocation of PKC\(\varepsilon\)-GFP to the nucleus in about 90 to 95% of the cells,

![Figure 8](http://mcb.asm.org/)

**FIG. 8.** Etoposide induces tyrosine phosphorylation of PKC\(\varepsilon\) in the regulatory domain. Parental C6 cells or cells stably transfected with PKC\(\varepsilon\) or the chimeras PKC\(\varepsilon\)/α and PKC\(\varepsilon\)/δ were treated with etoposide (50 \(\mu\)M) for various periods of time. Cells were then harvested, and immunoblotting (IB) and immunoprecipitation (IP) of PKC\(\varepsilon\) were performed using anti-PKC\(\varepsilon\) (A) or anti-PKC\(\varepsilon\) (B) antibodies as described in Materials and Methods. Following SDS-PAGE, membranes were stained with anti-phosphotyrosine antibody (anti-PY) or with anti-PKC\(\varepsilon\) antibodies. The results are from one representative experiment out of four separate experiments.
similar to the results obtained using immunofluorescent staining of the endogenous PKCδ. Likewise, PKCδ5-GFP translocated to the nucleus in response to etoposide in about 90% of the cells. A similar pattern of nuclear translocation of PKCδ and PKCδ5 was also obtained after 24 h of etoposide treatment (data not shown).

Role of single tyrosine mutants in apoptotic response of etoposide. To identify the specific tyrosines that are involved in the inhibitory effect of PKCδ on cell apoptosis, we examined the role of the single tyrosines 52, 64, 155, 187, and 565. C6 cells were stably transfected with the different PKCδ mutants in which each one of these tyrosines was individually mutated to phenylalanine. The expression and activity of C6 cells overexpressing the different PKC mutants were previously described (35).

The apoptotic effect of etoposide was examined in cells overexpressing the different mutants using PI staining and FACS analysis. We found that cells overexpressing PKCδY52F, PKCδY155F, and PKCδY565F exhibited an enhanced apoptotic response to etoposide similar to that of cells overexpressing PKCδ. In contrast, treatment with etoposide of cells overexpressing PKCδY64F or PKCδY187F resulted in a lower apoptotic response, similar to the response observed with cells overexpressing the PKCδ5 mutant (Fig. 12A). Similar results were obtained with anti-histone ELISA (Fig. 12B). We also found that etoposide did not induce a significant increase in the tyrosine phosphorylation of PKCδY64F and PKCδY187F, similar to the results obtained with PKCδ5 (Fig. 12C). Finally, we found no detectable cleaved product of PKCδ in the etoposide-treated PKCδY64F and PKCδY187F overexpressors (data not shown).

Apoptosis induced by etoposide is not inhibited by Src-kinase inhibitors PP1 and PP2. In a previous study, we showed that Fyn and Lyn can associate with PKCδ and that Fyn asso-

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**FIG. 9.** Tyrosine phosphorylation and cell apoptosis in response to etoposide in cells overexpressing PKCδ and PKCδ5. C6 cells overexpressing control vector (CV), PKCδ, or PKCδ5 were treated with etoposide (50 μM) for 60 min (A), 24 h (B), or 48 h (C). (A) Cells were then harvested, and immunoprecipitation of PKCδ was performed using anti-PKCδ antibody as described in Materials and Methods. Following SDS-PAGE, membranes were stained with anti-phosphotyrosine antibody (anti-PY) or with an anti-PKCδ antibody (rabbit; Santa Cruz). The results represent one of three separate experiments, which yielded similar results. For the measurement of apoptosis, cells were harvested after 24 h (B) or 48 h (C) of treatment and were analyzed using PI staining and FACS analysis (B) or by anti-histone ELISA (C). The optical densities of etoposide-treated PKCδ-overexpressing cells were designated 100% (total apoptosis), and all other values are presented as a percent of this total. The results represent the means ± SE of triplicate measurements in each of three experiments. *, P < 0.001.
ETOPOSIDE INDUCED TYROSINE PHOSPHORYLATION OF PKCδ AND APOPTOSIS

FIG. 10. Activation of caspase 3 and cleavage of PKCδ in cells overexpressing PKCδ and PKCδ5. C6 cells overexpressing control vector (CV), PKCδ, or PKCδ5 were treated with etoposide (50 μM) for 24 h, and the activation of caspase 3 (A and B) and cleavage of PKCδ (C) were determined. Cells were harvested and subjected to SDS-PAGE and Western blot analysis. The membranes were probed with active caspase 3 antibody (A) or with anti-PKCδ that recognizes the ε tag which is located in the catalytic domain of the PKCδ constructs (C). The results represent one of three separate experiments which yielded similar results. The activity of caspase 3 was measured using the Caspase 3 Cellular Activity Assay Kit as described in Materials and Methods (B). Caspase 3 activity was calculated and expressed as picomoles/minute/microgram of protein, and the percent of maximal effect was determined. The results represent the means ± SE of three experiments.

DISCUSSION

In this study, we explored the importance of PKCδ and its tyrosine phosphorylation for the induction of apoptosis in C6 glioma cells upon treatment with etoposide. We found that etoposide induced apoptosis of C6 cells, that this response was partially inhibited by the PKCδ inhibitor rottlerin and PKCδ DN, and that overexpression of PKCδ enhanced the apoptotic effect of etoposide. Etoposide, an inhibitor of topoisomerase II, has been reported to induce apoptosis in a broad range of different cells (29), and PKCδ has previously been implicated in the induction of apoptosis (34, 49). Thus, our results demonstrate that C6 glioma cells exhibit a response to etoposide similar to those of other tumor cells.

Etoposide induced translocation of PKCδ to the nucleus within 3 h of treatment. Various PKC isoforms can be found in the nucleus and in subnuclear compartments (7), and PKC isoforms can undergo nuclear translocation in response to various stimuli, including phorbol esters (55), growth factors (39, 43), and apoptotic stimuli, such as γ-irradiation (58). In addition, increases in the levels of nuclear PKC isoforms were reported in the context of cell proliferation and differentiation (16, 31), suggesting a role of nuclear PKC in these processes. It is presently unclear how PKCδ is transported to the nucleus since PKC isoforms do not contain any known nuclear localization signal, but PKC-binding proteins probably play a role in this process (7). Indeed, in a recent study, it was reported that PKCδ associates with c-Abl via its SH3 domain and undergoes tyrosine phosphorylation and nuclear translocation in complex with c-Abl in response to γ-irradiation (58).

In some systems, the apoptotic effect of PKCδ has been associated with a cleavage of the catalytic domain from the regulatory domain (34, 49). Cleavage of the catalytic domain of PKCδ by caspases has been reported for cells treated with ionizing radiation, tumor necrosis factor alpha, and etoposide (34, 38, 49). We found that etoposide induced cleavage of PKCδ in C6 cells and that this cleavage occurred in the nucleus. The absolute extent of cleavage was somewhat lower than that reported for parotid cells (49), presumably reflecting differences between these cell types, including a lower sensitivity of the C6 cells to etoposide. Both the cleavage of PKCδ by etoposide and its apoptotic effect were partially inhibited by the caspase inhibitor DEVD.FMK, suggesting a role for caspase 3 in these effects, as was reported for other systems (21, 49). Similarly, we found that the inhibition of PKCδ blocked the cleavage of caspase 3 and the cleavage of PKCδ was induced by etoposide. These results suggest the presence of a positive loop between PKCδ and caspase 3, which is dependent on PKCδ activity. Consistent with these results, recent studies demonstrated that caspases, including caspase 3, translocate and act in the nucleus following apoptotic stimuli (15). The partial inhibition of the caspase inhibitors on the apoptotic effect of etoposide was reported for other systems as well (44), and it is probably due to the presence of additional, non-caspase-dependent mechanisms involved in the effects of etoposide (50).

Both the regulatory and catalytic domains of PKCδ were important for the apoptotic effects of PKCδ in response to etoposide. These results are different from those of our previous studies, in which the regulatory domain of PKCδ was responsible for the effects of this isoform on C6 cell proliferation and on G0 expression (5, 35). In various studies, it was suggested that, once cleaved, the catalytic domain of PKCδ mediates the apoptotic effect of this isoform since overexpression of the catalytic fragment alone was able to induce apoptosis (21). Taken together, our results suggest that the regulatory domain contains signals that are important for the cleavage and activation of the catalytic domain.

Etoposide induced tyrosine phosphorylation of PKCδ in the regulatory domain. Tyrosine phosphorylation of PKCδ has been reported in the response to PMA and PDGF in C6 cells (5), in EGF-stimulated keratinocytes (13), in response to acti-
vation of the IgE receptor in RBL-2H3 cells (25, 54), and in response to apoptotic stimuli, such as H9253-irradiation (58) and H2O2 (32). Phosphorylation of tyrosine residues occurs in either the regulatory or catalytic domains of PKC. Thus, PDGF and PMA induced tyrosine phosphorylation on tyrosines 187 and 155, respectively (35). Similarly, activation of the IgE receptor induced tyrosine phosphorylation on tyrosine 52 (54). In contrast, stimulation of the cells with H2O2 and H9253-irradiation induced phosphorylation on tyrosines in the catalytic domain and in the hinge region (32, 33).

The tyrosine phosphorylation of PKC by etoposide appeared to be essential for the apoptosis induced by PKCδ, since the increase in cell apoptosis obtained in cells overexpressing PKCδ was absent in cells overexpressing the PKCδ mutant. These results are similar to our recent findings, which showed a role for tyrosine phosphorylation of PKCδ in the inhibitory effect of this isoform on the expression of the astrocytic marker GS (5) and on C6 cell proliferation (35). Thus, the PKCδ mutant also appears to act in an opposite way to PKCδ in its effect on cell apoptosis.

We found that caspase 3 was cleaved in response to etoposide and that this cleavage was enhanced in cells overexpressing PKCδ but inhibited in cells overexpressing the PKCδ mutant. The mechanisms involved in the enhanced cleavage of caspase 3 in response to overexpression of PKCδ are not known. PKCδ could act directly on caspase 3 to induce its phosphorylation or act indirectly via the phosphorylation of upstream caspases, such as caspase 8 or caspase 9 or other unknown upstream proteins. Indeed, a study by Martins et al. (41) reported that etoposide induced phosphorylation of caspases in HL-60 cells. Similarly, caspase 9, which is upstream of caspase 3, has been shown to be phosphorylated and directly regulated by phosphorylation (8, 20). Whatever the mechanism is for the effects of PKCδ, our data suggest that tyrosine phosphorylation of PKCδ is essential for the ability of this isoform to cause activation of caspase 3. In accordance with our data and with other studies suggesting that the cleavage of PKCδ is mediated by caspase 3 (21, 49), we found that the cleavage of the PKCδ mutant in response to etoposide was also inhibited. The difference in the ability of the PKCδ mutant to markedly inhibit etoposide-induced caspase-dependent cleavage and caspase 3 activity compared to its partial inhibition of etoposide-induced cell apoptosis suggests that tyrosine phosphorylation of PKCδ mediates the caspase-dependent component of the apoptosis induced by etoposide, whereas it is not involved in the caspase-independent pathways.

The effects of tyrosine phosphorylation on the activity of PKCδ or on its function are complex and dependent on the specific system and stimulus. Tyrosine phosphorylation of PKCδ in various systems has been reported to reduce or increase PKCδ activity and may do so in a substrate-specific manner (13, 25). Thus, the phosphorylation of PKCδ in response to etoposide may act by changing the affinity of PKCδ towards specific caspases or upstream proteins.

One of the factors that could provide the basis for the different effects of PKCδ and PKCδ5 is a distinct pattern of

FIG. 11. Translocation of PKCδ and PKCδ5 in etoposide-treated C6 cells. Cells were transiently transfected with GFP-PKCδ or GFP-PKCδ5. After 48 h, cells were treated with etoposide (50 μM) for 6 h and cells were viewed using confocal microscopy. Cells shown are representative of four independent experiments
Translocation. Translocation of PKC to specific cellular compartments could lead to different effects due to the phosphorylation of different substrates and to the association of PKC isoforms with specific proteins present in these locations. One determinant of the localization of PKC following its activation is association with receptors for activated C kinases (RACKs) (42). It is presently not clear to what extent tyrosine kinases can act as RACKs and affect the translocation of PKC isoforms. In a
recent study, we found that mutations in tyrosine residues did not alter the translocation of PKC8 in response to PMA or PDGF (35). In contrast, Ron et al. suggested that Fyn might act as a RACK of PKC8 (51). We found that etoposide induced nuclear translocation of both PKC8 and PKCδ56. Thus, the nuclear translocation of PKC8 does not depend on its phosphorylation, and the differential effects of PKC8 and PKCδ56 on cell apoptosis in response to etoposide do not appear to reflect their different translocation following activation. PKCδ6 is reported to associate with different tyrosine kinases. Thus, p60Src (4, 53, 54, 59), Lyn (54), Fyn (35), and c-Abl (58) can associate with PKCδ6 in either a phosphorylation-dependent or -independent manner. In addition, various reports indicate that PKCδ6 can undergo tyrosine phosphorylation on specific tyrosine residues by Src, Fyn, and c-Abl. The ability of PKCδ6 to be tyrosine phosphorylated on more than one tyrosine suggests that PKCδ6 can associate with different tyrosine kinases. In a recent study, we found that PKCδ6 underwent tyrosine phosphorylation on tyrosines 155 and 187 which was associated with the inhibitory effects of PKCδ6 on cell proliferation and the expression of GS, respectively (35). Our present results indicate that phosphorylation of PKCδ6 on tyrosines 64 and 187 is essential for its apoptotic effect. Our results suggest that Src-related kinases which are involved in the tyrosine phosphorylation of PKCδ6 in response to PDGF are not involved in the apoptosis induced by etoposide. The tyrosine kinase which phosphorylates PKCδ6 in etoposide-treated cells remains to be identified.

In summary, we demonstrated that the apoptosis induced by etoposide involves activation of an as-yet-unidentified tyrosine kinase(s) which phosphorylates PKCδ6 in the regulatory domain. PKCδ6 is then translocated to the nucleus where it directly or indirectly activates caspase 3. Caspase 3 in turn activates the cysteine protease caspase-9 by phosphorylation. Science 285:1318–1321.


