Roles of Tyrosine Phosphorylation and Cleavage of Protein Kinase Cδ in Its Protective Effect Against Tumor Necrosis Factor-related Apoptosis Inducing Ligand-induced Apoptosis*

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Protein kinase Cδ (PKCδ) regulates cell apoptosis in a cell- and stimulus-specific manner. Here, we studied the role of PKCδ in the apoptotic effect of TRAIL in glioma cells. We found that transfection of the cells with a PKCδ kinase-dead mutant (K376R) or with a small interfering RNA targeting the PKCδ mRNA increased the apoptotic effect of tumor necrosis factor-related apoptosis inducing ligand (TRAIL), whereas overexpression of PKCδ decreased it. PKCδ acted downstream of caspase 8 and upstream of cytochrome c release from the mitochondria. TRAIL induced cleavage of PKCδ within 2–3 h of treatment, which was abolished by caspase 3, 8, and 9 inhibitors. The cleavage of PKCδ was essential for its protective effect because overexpression of a caspase-resistant mutant (PKCδD327A) did not protect glioma cells from TRAIL-induced apoptosis but rather increased it. TRAIL induced translocation of PKCδ to the perinuclear region and the endoplasmic reticulum and phosphorylation of PKCδ on tyrosine 155. Using a PKCδY155F mutant, we found that the phosphorylation of PKCδ on tyrosine 155 was essential for the cleavage of PKCδ in response to TRAIL and for its translocation to the endoplasmic reticulum. In addition, phosphorylation of PKCδ on tyrosine 155 was necessary for the activation of AKT in response to TRAIL. Our results indicate that PKCδ protects glioma cells from the apoptosis induced by TRAIL and implicate the phosphorylation of PKCδ on tyrosine 155 and its cleavage as essential factors in the anti-apoptotic effect of PKCδ.

Apoptosis is a genetically controlled process of selective cell deletion involved in normal cell development and turnover (1). Apoptosis is triggered by genotoxic reagents, stress responses, or ligation of membrane death receptors (2). The apoptotic response is characterized by extrinsic and intrinsic pathways (3) that can converge at the mitochondria leading to the release of cytochrome c and activation of caspase 9 and downstream effector caspases (4). Proteins that play a role in this process include the Bcl2 family (5) and the caspases, which are cysteine-dependent aspartate-specific proteases (6, 7). In addition, the apoptotic response is also regulated by various kinases (8), among the best characterized being phosphoinositide 3-kinase/Akt (9), members of the mitogen-activated protein kinase family (10), and various PKC isoforms (11).

PKCδ, a ubiquitously expressed isoform of the novel PKC subfamily (12), has been shown to regulate cell apoptosis in various cellular systems (13). Its function and regulation are clearly complex, however, because PKCδ can be either pro-apoptotic or anti-apoptotic, depending on the specific cellular system and the specific ligand. PKCδ acts in a pro-apoptotic fashion in response to etoposide (14, 15), cytosome arabinoside (16), and in response to UV (17) and γ-radiation (18). The pro-apoptotic function of PKCδ in these systems is typically associated with its cleavage by caspase 3 in the hinge region, which results in a phospholipid-independent activation of the enzyme (19–22). It is noteworthy, that PKCδ can induce apoptosis in a cleavage-independent (23) or a kinase-independent manner (24) in some instances, emphasizing the complex role of PKCδ in the regulation of cell apoptosis.

In other systems, in contrast to the above examples, PKCδ has been clearly shown to function in an anti-apoptotic fashion. Thus, PKCδ played a role in the anti-apoptotic effect of fibroblast-like growth factor in granulosa cells (25) and in serum-deprived PC12 cells (26) and protected macrophages from nitric oxide-induced apoptosis (27). Factors that are associated with the effects of PKCδ on cell apoptosis are its phosphorylation on tyrosine residues and its subcellular localization. Tyrosine phosphorylation of PKCδ occurs in response to many apoptotic stimuli and it has been associated with the apoptotic function of PKCδ (15, 28–31). Similarly, PKCδ translocates to the mitochondria (32), Golgi (31), and nucleus (15, 33) in response to various stimuli and specific apoptosis-related PKCδ substrates have been identified in the mitochondria (34) and nucleus (21, 35).

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL; Apo2 ligand) belongs to the tumor necrosis factor superfamily and induces apoptosis in many transformed cells including some glioma cell lines and primary cultures (36, 37).

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† The abbreviations used are: PKC, protein kinase C; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; NFκB, nuclear factor κB; PMA, phorbol 12-myristate 13-acetate; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; GFP, green fluorescent protein; siRNA, small interfering RNA; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; PI, propidium iodide; FACS, fluorescence activated cell sorter; EGFP, epidermal growth factor protein.
TRAIL acts by formation of the death-inducing signaling complex that is also common to other members of the death receptors (38). Caspase 8 is then activated at the death-inducing signaling complex and leads to two different apoptotic pathways: a mitochondrial-independent pathway via the effector caspases 3 and 7 and a mitochondrial-dependent pathway via activation of caspase 9 (39). The signal transduction pathways that are involved in the TRAIL apoptotic effect are not fully understood. Recent studies reported that TRAIL activates the transcriptional factor NF-κB and c-Jun N-terminal kinase in various cellular systems (40) and that NF-κB (41) and phophatidylinositol 3-kinase/AKT (42) are involved in the resistance of some transformed cells to the apoptotic effect of TRAIL. In addition, PKC signaling, mainly in response to PMA activation, has been implicated in the attenuation of the apoptotic signal induced by TRAIL (43, 44); however, the involvement of the PKCδ isozyme in response to TRAIL has not been described.

In the present study, we examined the role of PKCδ in the apoptotic effect of TRAIL in glioma cells and analyzed the molecular mechanisms underlying its effects. We found that PKCδ protected glioma cells from the apoptosis induced by TRAIL and that this effect was dependent on the phosphorylation of PKCδ on tyrosine 155 and on its caspase-dependent cleavage.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti-PKCδ antibodies (C-20 and C-17) and anti-phospho-PKCδ (Y155F) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRAIL was from PeproTech (Rocky Hill, NJ), anti-calnexin antibody was purchased from BD Biosciences (San Diego, CA), and anti-active caspase 3 antibody, anti-phospho-AKT (Ser473), and anti-AKT were obtained from Cell Signaling (Beverly, MA). The caspase inhibitors DEVD-fmk, Z-VAD-fmk, Z-IETD, and Z-LEHD were obtained from Calbiochem (La Jolla, CA). Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and sodium vanadate were also purchased from Sigma.

Site-directed Mutagenesis of PKCδ—PKCδ cloned into the pEGFP plasmid served as a template vector for the site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Conversion of tyrosine residues at sites 52, 155, and 187 into phenylalanine was performed as previously described (45). A PKCδ K376R mutant was generated using the QuikChange Site-directed Mutagenesis Kit and was confirmed by DNA sequencing. The caspase cleavage site of PKCδ, aspartate 328, was mutated to alanine using the following primers: sense, 5′-GTGACATCTCAGGCAACAGCAG-3′ and antisense, 5′-GGTCCCCGTGTGTTGCTAGGATGTCAC-3′. The mutation was confirmed by DNA sequencing. PKCδ and the PKCδ mutants were subcloned into the pCMV-Tag 2b expression vector (Stratagene) generating PKCδ-GFP and PKCδ-GFP mutants with a N-terminal FLAG tag. The activities and translocations of the PKCδ mutants were already characterized (15, 46).

Generation of PKC Chimeras—The PKCδ chimeras were generated by exchanging the regulatory and catalytic domains of PKCδ and -δ as previously described (15). PKCaδ refers to the chimera with the PKCa regulatory domain and the PKCδ catalytic domain, and PKCδa refers to the reciprocal chimera. The PKCδ cDNAs were subcloned into the pmCV tag 2b (Stratagene) generating PKC chimera with an N-terminal FLAG tag. The expression of these chimeras and their activities were recently described (15, 45).

A172 Glioma Cultures and Cell Transfection—A172 cells were grown in medium consisting of Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (0.05 mg/ml). The cells were transfected by electroporation using the Nucleofector device (Amaxa Inc., Gaithersburg, MD) either with the empty vector or with expression vectors for the PKCδ and PKCδ mutants. Transfection efficiency using nucleofection was about 80–90%.

RNA Transfections—siRNA duplexes (siRNAs) were obtained from Dharmacon (Lafayette, CO) and consisted of a pool of 4 PKCδ siRNA duplexes. Transfection of siRNAs was performed using 50 nm PKCδ or scrambled siRNAs and Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PKCδ protein levels were determined using Western blot analysis.

Adenovirus Preparation and Infection—The AdEasy system was kindly provided by Dr. W. Henkelstein (The Johns Hopkins University School of Medicine, Baltimore, MD) (46). PKCδ and the different PKCδ mutants were first cloned into the pShuttle-CMV vector as previously described (47). The plasmids were then linearized by digestion with Pmel and were transformed into E. coli (Streptomedia) carrying the pAdEasy-1 plasmid that encodes the adenovirus-5 backbone. Recombination was confirmed by restriction analysis. The linearized recombinant plasmids were transfected into HEK293 cells. Viruses were collected after 7–10 days and were further amplified. An adenovirus expressing the LacZ gene was used as a control.

Cells were incubated with 5 multiplicity of infection of the appropriate recombinant adenovirus vectors for 1 h. The medium was then replaced with fresh medium and the cells were used 24–48 h post-infection.

Measurement of Caspase 8 Activity—Caspase 8 activity was measured using the caspase fluorescent substrate/inhibitor QuantiKine kit obtained from R&D Systems (Minneapolis, MN). The activity of caspase 8 was determined using a fluorescent substrate that is cleaved by caspase-8. The fluorescent signal was measured using a fluorescence plate reader.

Measurement of Apoptosis—Apoptosis was measured using a colorimetric apoptosis assay kit obtained from DAKO ( Carpinteria, CA). The assay is based on the detection of the DNA fragmentation that occurs during apoptosis. The DNA fragmentation is detected using a colorimetric assay that is specific for DNA fragmentation.

Preparation of Cell Homogenates—Cell homogenates were prepared using a proteinase K treatment in medium consisting of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin.

Immunoblot Analysis—Immunoblot analysis was performed using the following antibodies: anti-PKCδ (C-20 and C-17), anti-PKCδ (Y155F), and anti-PKCδ (K376R). The membranes were blocked with 5% nonfat dry milk in TBS-T and incubated with the primary antibodies. The blots were then washed with TBS-T and incubated with the secondary antibodies conjugated to horseradish peroxidase. The blots were then washed with TBS-T and incubated with the substrate solution. The bands were visualized using a chemiluminescence detection system.

Preparation of Cell Homogenates—Cell homogenates were prepared using a protease inhibitor cocktail. The cells were homogenized in medium consisting of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin.

Immunoblot Analysis—Immunoblot analysis was performed using the following antibodies: anti-PKCδ (C-20 and C-17), anti-PKCδ (Y155F), and anti-PKCδ (K376R). The membranes were blocked with 5% nonfat dry milk in TBS-T and incubated with the primary antibodies. The blots were then washed with TBS-T and incubated with the secondary antibodies conjugated to horseradish peroxidase. The blots were then washed with TBS-T and incubated with the substrate solution. The bands were visualized using a chemiluminescence detection system.
with 5% dry milk in phosphate-buffered saline and subsequently stained with primary antibody. Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham Biosciences).

Immunofluorescence Staining—Cells were grown on glass coverslips. Following TRAIL treatment, the cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Subsequently, cells were washed in PBS and, after blocking with staining buffer (2% bovine serum albumin and 0.1% Triton X-100 in PBS) for 30 min at room temperature, they were incubated with an anti-PKC antibody. Following washes in PBS, cells were incubated with an anti-rabbit Alexa Fluor 488 antibody for an additional 60 min and mounted in FluoroGuard antifade reagent. Cells were viewed and photographed using confocal microscopy with ×63 magnification at an excitation wavelength of 488 nm. For the visualization of the ER, cells were incubated with mouse anti-calnexin antibody followed by incubation with anti-rabbit Alexa Fluor 546 antibody. For analysis of the translocation of GFP-PKCδ and the GFP-PKCδ kinase-dead (KD) mutant, cells were treated for various time points with TRAIL, fixed in 4% paraformaldehyde (PFA) for 10 min, and mounted in FluoroGuard antifade reagent.

Statistical Analysis—The results are presented as the mean ± S.E. Data were analyzed using analysis of variance and a paired Student’s t test to determine the level of significance between the different groups.

RESULTS

PKCδ Protects Glioma Cells from the Apoptotic Effect of TRAIL—PKCδ regulates cell apoptosis in a cell and stimulus-dependent manner (13). To examine the role of PKCδ in the apoptotic effect of TRAIL we employed the A172 glioma cell line that exhibits high sensitivity to this ligand. Overexpression of a PKCδ kinase-dead (KD) mutant (K376R) using an adenovirus vector (Fig. 1A) did not affect the basal level of A172 cell apoptosis (Fig. 1B). However, it increased the apoptosis induced by TRAIL (Fig. 1, B and C). Similar results were obtained using PI staining and FACS analysis (B and C). In another set of experiments, cells were transfected with control (scrambled) or PKCδ siRNAs using Oligofectamine. PKCδ expression was determined following 72 h (D) and the apoptotic effect of TRAIL (100 ng/ml) was determined after 5 h of treatment (E). Caspase 8 activation was determined in cells treated with TRAIL for 45 min using the fluorescent substrate Ac-IETD-AMC as described under “Experimental Procedures” (F) and cytochrome c release from the mitochondria to the cytosol was determined using Western blot analysis (G).

The results represent the mean ± S.E. of triplicate measurements in each of four experiments (C, E, and F) or are from one representative experiment out of four similar experiments. (A, B, D, and G).
Results.

To examine the role of PKC in TRAIL-induced apoptosis, we found that in addition, we found that in the role of PKC studied. To examine the role of PKC studied. We found that overexpression of PKC did not affect the apoptotic effect of TRAIL, whereas PKC decreased it as already described (Fig. 1C). Cells overexpressing chimeras with the regulatory domain of PKC (PKCα/β) or the catalytic domain of PKC (PKCα/δ) exhibited a similar degree of apoptosis to that of CV cells and none of the chimeras resembled cells overexpressing PKC (Fig. 4A), suggesting that both domains of PKC are required for its protective effect.

To further examine the roles of the regulatory and catalytic domains of PKC in its protective effect we overexpressed these two domains in the A172 cells and examined their effects on cell apoptosis in the absence and presence of TRAIL. We found that overexpression of the PKC catalytic (PKCα-cat) domain induced some cell death in the A172 cells, whereas no apoptosis was observed with the PKC regulatory domain (PKCα-reg, Fig. 4B). The PKCα-reg did not alter the apoptotic response of the cells to TRAIL (Fig. 4B), whereas the PKCα-cat slightly enhanced it. These results further support the results obtained with the PKC chimeras that both domains are required for the protective effect of PKC.

Using the PKCα-cat and PKCα-reg fragments tagged to GFP, we found that the catalytic domain of PKCα accumulated in the cytosol and to a large degree in the nucleus, whereas the regulatory domain of PKCα was expressed throughout the cell (Fig. 4C). The localization of the PKCα-cat and -reg domains was not significantly altered by TRAIL treatment (data not shown).

TRAIL Induces Translocation of PKCα to the Perinuclear Region—One of the important factors in the diverse apoptotic functions of PKCα is its distinct patterns of translocation in response to different stimuli (13). To examine the effect of TRAIL on the translocation of PKCα, we treated A172 cells with TRAIL for various periods of time and followed the localization of the endogenous PKCα using immunofluorescence staining and confocal microscopy. In control cells, PKCα was largely localized to the cytosol with some expression in the nucleus (Fig. 5A). Treatment with TRAIL induced translocation of PKCα to the perinuclear region and in most of the cells this translocation was followed by the exit of PKCα from the nucleus. Translocation was first observed after 15 min, was increased after 30 min, and persisted up to 1 h following TRAIL treatment. After that time PKCα was mainly localized in the cytosol (Fig. 5A) and this localization persisted up to the time of PKCα cleavage (2–3 h post-TRAIL treatment, data not shown).

To further characterize the subcellular localization of PKCα acted as a dominant negative mutant of PKCα by inhibiting its cleavage and protective effect.

Our recent studies (15) and studies from other groups (14, 33) indicate that PKCα plays an essential role in the apoptosis induced by etoposide mainly via its cleaved catalytic fragment. Thus, PKCα plays an opposite role in the apoptosis induced by TRAIL and etoposide. Because PKCα undergoes cleavage in response to both stimuli we also examined the effect of the PKCαD327A mutant in the apoptotic effect of etoposide. The PKCαD327A mutant failed to undergo cleavage in response to etoposide (Fig. 3C), similar to the results obtained with TRAIL. However, in this case the PKCα mutant protected the A172 cells from etoposide-induced apoptosis in contrast to its apoptotic effect in TRAIL-treated cells (Fig. 3D).

Both the Regulatory and Catalytic Domains of PKCα Are Required for Its Protective Effect—The regulatory and catalytic domains of PKC have been associated with the regulation of cell apoptosis (19, 20, 48), however, their role in the protective effect of PKCα has not been explored. To examine the relative contributions of the different domains of PKCα to its protective effect we employed PKC chimeras between the regulatory and catalytic domains of PKCα and -δ combined at the highly conserved hinge region. We found that overexpression of PKCα did not affect the apoptotic effect of TRAIL, whereas PKCα decreased it as already described (Fig. 1C). Cells overexpressing chimeras with the regulatory domain of PKCα (PKCα/β) or the catalytic domain of PKCα (PKCα/δ) exhibited a similar degree of apoptosis to that of CV cells and none of the chimeras resembled cells overexpressing PKCα (Fig. 4A), suggesting that both domains of PKCα are required for its protective effect.

Using the PKCα-cat and PKCα-reg fragments tagged to GFP, we found that the catalytic domain of PKCα accumulated in the cytosol and to a large degree in the nucleus, whereas the regulatory domain of PKCα was expressed throughout the cell (Fig. 4C). The localization of the PKCα-cat and -reg domains was not significantly altered by TRAIL treatment (data not shown).

Translocation of PKCα in TRAIL-treated cells. A172 cells were treated with TRAIL (100 ng/ml) for 0–5 h. The cleavage of PKCα was determined by Western blot using an anti-PKCα antibody that recognizes the catalytic domain (A4) and cell apoptosis was evaluated by poly(ADP-ribose) polymerase cleavage (B). The 40-kDa cleaved form (CF) is marked (A). The role of caspase 3, 8, and 9 in the cleavage of PKCα was determined by pretreatment of the cells with caspase inhibitors DEVD, Z-IETD, and Z-LEHD (10 μM each) for 30 min followed by treatment with TRAIL (100 ng/ml) for 3 h (C). The results represent one of three separate experiments, which gave similar results. PARP, poly(ADP-ribose) polymerase.

The Cleavage of PKCα Is Essential for Its Protective Effect—The cleavage of PKCα generates a constitutively active catalytic fragment that has been associated with the pro-apoptotic function of PKCα in various cellular systems (19–21). However, the role of PKCα cleavage in its protective effect has not been studied. To examine the role of PKCα cleavage in its protective effect against TRAIL-induced apoptosis, we constructed a caspase-resistant PKCα mutant in which the aspartic acid at position 327 was mutated to alanine. We overexpressed the PKCα WT and the PKCαD327A mutant in the A172 cells and examined their cleavage and effects on the apoptosis induced by TRAIL. As presented in Fig. 3A, overexpressed PKCα underwent cleavage in response to TRAIL similar to the endogenous PKCα, whereas the PKCαD327A did not undergo cleavage and no accumulation of a PKCα catalytic fragment was observed in TRAIL-treated cells. In addition, we found that in contrast to the protective effect of the overexpressed PKCα, the PKCα caspase mutant did not protect the A172 cells from TRAIL-induced apoptosis but rather increased it (Fig. 3B). These results suggest that the cleavage of PKCα is essential for the protective effect of PKCα and that the PKCαD327A mutant
Role of Tyrosine Phosphorylation of PKCδ in Its Anti-apoptotic Effect

Following TRAIL treatment we used the ER marker calnexin (49). As shown in Fig. 5B, the ER marker calnexin stained the perinuclear region in a similar pattern to that of PKCδ in the TRAIL-treated cells. Merged images clearly showed co-localization of the green fluorescence of PKCδ and of the red fluorescence of calnexin, suggesting that TRAIL induced translocation of PKCδ to the ER. Similar results were observed using anti-KDEL antibody (Stressgen) as an ER marker (data not shown).

**Fig. 3.** A PKCδ caspase-resistant mutant (D327A) enhances cell apoptosis in response to TRAIL but inhibits cell apoptosis in response to etoposide. A172 cells were infected with adenovirus vectors expressing LacZ (CV), PKCδ, or PKCδD327A. Following 24 h, the cells were treated with TRAIL for 3 h (A) or with etoposide for 18 h (C) and the cleavage of PKCδ was determined using Western blot analysis. Cell apoptosis was determined following a 5-h treatment with TRAIL (B) and 36 h with etoposide (D) using PI staining and FACS analysis. The results are representative of four similar experiments (A and C) or represent the mean ± S.E. of triplicate measurements in each of four independent experiments (B and D).

Phosphorylation of PKCδ on tyrosine 155 is essential for the protective effect of PKCδ and that PKCδY155F acted as a dominant negative of PKCδ. Similarly, we found that overexpression of PKCδ decreased the expression of active caspase 3, whereas overexpression of PKCδY155F slightly increased it as compared with CV cells (Fig. 7B). This apoptotic effect was specific for the PKCδY155F mutant because overexpression of other PKCδ tyrosine mutants, PKCδY52F and PKCδY187F, protected the A172 cells against TRAIL-induced apoptosis, similar to the effect of PKCδ (Fig. 7C).

Phosphorylation of PKCδ on Tyrosine 155 Is Essential for the Translocation and Cleavage of PKCδ—One of the possible mechanisms for the different effects of PKCδ and the PKCδY155F mutant on TRAIL-induced apoptosis is their differential subcellular localization following TRAIL treatment. To examine this point, we followed the translocation of PKCδ and the PKCδY155F mutant tagged with GFP in TRAIL-treated cells. A172 cells were transfected with GFP constructs and the translocation of PKCδ and PKCδY155F was monitored as a function of time following TRAIL treatment. Similar to the endogenous PKCδ, PKCδ-GFP exited the nucleus and translocated to the ER after 15 min of TRAIL treatment and this translocation persisted up to 60 min thereafter (Fig. 8A). In contrast, no significant translocation was observed in the A172 cells transfected with the PKCδY155F-GFP mutant, and in TRAIL-treated cells the PKCδY155F-GFP mutant was localized to the cytoplasm and nucleus similar to the control untreated cells (Fig. 8A). The translocation of the PKCδY155F mutant in response to PMA and etoposide was also examined. We found that PMA induced translocation of the PKCδY155F mutant to the plasma and perinuclear membranes, similar to translocation of the PKCδ-GFP WT (Fig. 8B). Etoposide induces nuclear translocation of PKCδ. We found that the PKCδY155F-GFP translocated to the nucleus in response to etoposide, similar to PKCδ-GFP (Fig. 8B).
The cleavage of PKCδ was essential for its protective effect. We therefore examined whether phosphorylation of PKCδ on tyrosine 155 was associated with the cleavage of PKCδ by TRAIL. Using cells overexpressing PKCδ WT and various PKCδ tyrosine mutants, we found that TRAIL induced the cleavage of PKCδ and that of the PKCδY187F and PKCδY52F mutants but did not induce cleavage of the PKCδY155F (Fig. 8C). Thus, phosphorylation of PKCδ on tyrosine 155 was essential for the cleavage of PKCδ in response to TRAIL. In contrast, phosphorylation of PKCδ on tyrosine 155 was not essential for the cleavage of PKCδ by etoposide (data not shown), suggesting a specific role of this phosphorylation site in the apoptotic pathway activated by TRAIL.

**PKCδ Activates pAKT in TRAIL-treated Cells**—To further delineate the mechanisms underlying the protective effect of PKCδ and the role of tyrosine 155 phosphorylation in its function, we examined the effect of PKCδ on the activation of AKT because this signaling pathway has been implicated in the apoptotic response of various cells to TRAIL (50). We found that, in the A172 cells, TRAIL induced a small and transient increase in the phosphorylation of AKT similar to recent reports (51). Overexpression of PKCδ increased the phosphorylation of AKT in untreated cells; this phosphorylation was further increased in response to TRAIL and persisted up to 60 min thereafter (Fig. 9). This effect of PKCδ was abolished when tyrosine 155 was mutated to phenylalanine, suggesting that phosphorylation of tyrosine 155 was involved in the AKT phosphorylation induced by PKCδ in TRAIL-treated cells.

**DISCUSSION**

In this study we explored the role of PKCδ in the apoptotic effect of TRAIL. Various studies have reported that PKC regulates the apoptotic function of TRAIL using either the PKC activator PMA or various PKC inhibitors (43, 44); however, the role of PKCδ in the effect of TRAIL has not been explored. In an attempt to delineate the molecular mechanisms underlying the anti-apoptotic effect of PKCδ, we focused on three factors that are central for the diverse apoptotic function of PKCδ, its cleavage, localization, and tyrosine phosphorylation (13).

We found that PKCδ protected the A172 cells from the apoptosis induced by TRAIL. Thus, the inhibition of PKCδ activity or expression attenuated the apoptotic effect of TRAIL. PKCδ has been extensively studied for its effects on the regulation of apoptosis in various cellular systems (13, 52). In many of these studies PKCδ acted as a pro-apoptotic kinase and it contributed to the apoptotic effects of a large and diverse apoptotic stimuli, including etoposide (14, 15), γ radiation (18), UV radiation (17), and ceramide (31). However, in other systems PKCδ clearly acted in an anti-apoptotic fashion (25, 26). Thus, PKCδ protected macrophages from NO-induced apoptosis (27) and glioma cells from the apoptosis induced by Sindbis virus infection (28). The basis for the anti- and pro-apoptotic effects of PKCδ is
not understood and, although various downstream targets have been associated with the pro-apoptotic effect of PKC\(\beta\), less is known about the mechanisms involved in its anti-apoptotic effect. We found that PKC\(\beta\) inhibited the release of cytochrome c from the mitochondria and the cleavage of caspase 3, whereas it did not alter the activation of caspase 8 induced by TRAIL. Thus, PKC\(\beta\) acted downstream of caspase 8 activation and upstream of the mitochondrial pathway.

TRAIL-induced cleavage of PKC\(\beta\) within 2–3 h post-treatment and this cleavage was mediated by caspase 3 downstream of caspase 8 and 9 activation. Using a caspase-resistant PKC\(\beta\) mutant (PKC\(\beta\)D327A), we found that, in contrast to PKC\(\beta\) WT, which was cleaved by TRAIL and attenuated its apoptotic effect, the PKC\(\beta\)D327A mutant did not undergo cleavage and increased the apoptotic effect of TRAIL. The enhanced apoptotic effect of this mutant was specific to TRAIL because the same mutant attenuated the apoptotic effect of etoposide. These results suggest that the PKC\(\beta\)D327A acted as a dominant negative of PKC\(\beta\) and that the cleavage of PKC\(\beta\) was essential for the protective effect of PKC\(\beta\) in TRAIL-treated cells. Our results demonstrating a protective effect of the cleaved PKC\(\beta\) against TRAIL-induced apoptosis are the first to show that cleavage of PKC\(\beta\) can provide anti-apoptotic signals. Similar results were recently reported for the cleaved PKC\(\epsilon\) in tumor necrosis factor-\(\alpha\)-treated MCF-7 cells (53); thus also in the case of PKC\(\epsilon\) the cleaved product can exert both pro- and anti-apoptotic effects (53, 54).

The cleavage of PKC\(\beta\) is well documented and it occurs in response to many apoptotic stimuli such as etoposide (15), cisplatin (55), and UV radiation (56). The caspase-dependent cleavage of PKC\(\beta\) leads to the generation of a constitutively active catalytic fragment that is associated with the apoptotic function of PKC\(\beta\) in response to various apoptotic stimuli (15, 19, 20, 55, 56). Moreover, overexpression of the PKC\(\beta\) catalytic fragment has been reported to induce cell apoptosis in various systems by phosphorylating apoptosis related proteins such as p73\(\beta\) and DNA-PK (21, 22). The fate and function of the regulatory domain released following the caspase-dependent cleavage are less understood. However, in a recent study Schultz et al. (48) reported that the regulatory domain of PKC\(\theta\) exerted an apoptotic function when overexpressed, suggesting that both the regulatory and catalytic domains of PKC can regulate cell apoptosis. The cleavage of PKC\(\beta\) and the roles of its catalytic and regulatory domains have not been studied previously in cellular systems where PKC\(\beta\) acted as an anti-apoptotic kinase. In an attempt to determine the roles of the regulatory and catalytic domains of PKC\(\beta\) in its protective effect against TRAIL-induced apoptosis, we employed PKC chimeras between the regulatory and catalytic domains of PKC\(\beta\) and PKC\(\alpha\). We found that both domains were required for the protective effect of PKC\(\beta\), because neither of the chimeras acted like PKC\(\beta\) to protect the A172 cells from TRAIL-induced apoptosis. The involvement of both domains was further confirmed by the experiment of overexpressing the PKC\(\beta\)-reg and PKC\(\beta\)-cat because neither of them protected the A172 cells from the apoptotic effect of TRAIL. These results are similar to our recent results in which the two domains were required for the apoptotic effect of PKC\(\beta\) in response to etoposide (15). Unlike these two systems, the isoform specificity of PKC\(\beta\) for inhibition of glioma cell proliferation depended only on the regulatory domain (45). The mechanisms that are involved in the protective effect of the catalytic domain in this case as compared with its apoptotic effects in other systems are not understood. However, it is noteworthy that the localization of the catalytic domain in TRAIL-treated cells was outside the nucleus as compared with its nuclear localization in response to apoptotic stimuli or when overexpressed (14, 15, 33). Thus, the phosphorylation of different substrates, anti-apoptotic as compared with pro-apoptotic, as a result of this differential localization could account for the different effects of the catalytic domain.

TRAIL-induced translocation of PKC\(\beta\) to the ER within 15 min of treatment. PKC\(\beta\) has been reported to undergo differential translocation to distinct subcellular sites in response to diverse apoptotic stimuli. Thus, PKC\(\beta\) was translocated to the nucleus in response to etoposide (14, 15), to the mitochondria in response to PMA (32) and H\(_2\)O\(_2\) (57), and to the Golgi in response to ceramide (31). Interestingly, we recently found that PKC\(\beta\) translocated to the ER in response to infection of glioma cells with Sindbis virus (28). Similar to its effect in TRAIL-treated cells, PKC\(\beta\) protected glioma cells from the apoptosis induced by SV infection (28). It is currently not clear what is the function of PKC\(\beta\) in the ER and which proteins can be phosphorylated by PKC\(\beta\) at this site. One candidate is Bcl2, which resides in the ER in addition to its mitochondrial local-
Phosphorylation of PKC on tyrosine residues has been implicated as an important mode of regulation of the activity and function of PKCδ and various stimuli phosphorylate PKCδ on distinct tyrosine residues (45, 61, 62). Indeed, phosphorylation of PKCδ on specific tyrosine residues occurs in response to PMA (63), platelet-derived growth factor (39, 64), EGF (65), carbachol, substance P (66), and activation of the IgE receptor (62). In addition, various apoptotic stimuli are associated with the phosphorylation of PKCδ on specific tyrosine residues: H2O2 on tyrosines 311, 332, and 522 (29); etoposide on tyrosines 64 and 187 (15), ceramide on tyrosines 311 and 332 (31); and Sindbis virus infection with phosphorylation of PKCδ on tyrosines 52, 64, and 152 (28). We found that TRAIL induced phosphorylation of PKCδ on tyrosine 155, whereas no phosphorylation was observed on tyrosines 52 or 187. The phosphorylation of tyrosine 155 was essential for the anti-apoptotic effect of PKCδ in TRAIL-treated cells, because a mutation in this tyrosine residue attenuated the protective effect of PKCδ against TRAIL-induced apoptosis. Interestingly, tyrosine 155 has been recently associated with the protective effect of PKCδ against Sindbis virus-induced apoptosis (28).

The phosphorylation of PKCδ on tyrosine 155 occurred prior to the translocation of PKCδ to the ER and was essential for the translocation of PKCδ in response to TRAIL. In contrast, the phosphorylation on tyrosine 155 was not essential for the

FIG. 7. Phosphorylation of tyrosine 155 is essential for the protective effect of PKCδ. A172 cells were transfected with CV, PKCδ, and the PKCδY155F mutant (A and B) or with PKCδ and the PKCδ tyrosine mutants, PKCδY155F, PKCδY52F, and PKCδY187F (C). Cells were then treated with TRAIL (100 ng/ml) for 5 h and apoptosis was determined using PI staining and FACS analysis (A and C). The expression of active caspase 3 (B) was determined using Western blot analysis. The results are representative of five similar experiments (A and B) or represent the mean ± S.E. of triplicate measurements in each of four independent experiments (C).

FIG. 8. Phosphorylation of PKCδ on tyrosine 155 is essential for the translocation and cleavage of PKCδ. A172 cells were transfected with PKCδ and the PKCδY155F mutant tagged to GFP. Cells were then treated with TRAIL for various periods of time and translocation was followed using confocal microscopy (A). The translocation of the PKCδ-GFP and the PKCδY155F-GFP was examined in response to PMA (1 μM, 30 min) and etoposide (50 μg/ml, 3 h) (B). Cells were also transfected with PKCδ or PKCδ tyrosine mutants (Y155F, Y187F, and Y52F), treated with TRAIL for 3 h, and PKCδ cleavage was determined using Western blot analysis (C). The results are representative of four similar experiments. CF, cleaved form.

FIG. 9. PKCδ increases AKT phosphorylation in TRAIL-treated cells. A172 cells overexpressing CV, PKCδ, and PKCδY155F mutants were stimulated with TRAIL for 0–60 min. The phosphorylation of AKT was determined using anti-phospho AKT (Ser473) antibody. The results are representative of three similar experiments.
translocation of PKC\(\delta\) to the plasma and perinuclear membranes in response to PMA or to the nucleus in response to etoposide. Translocation of PKC\(\delta\) is a hallmark of its activation and it occurs via binding of PKC to specific scaffold proteins (RACKS) (67). The role of tyrosine phosphorylation of PKC in its translocation has been explored in various cellular systems. Phosphorylation of atypical PKC at Tyr\(^{256}\) induced exposure of the arginine-rich NLS, which resulted in nuclear translocation of the atypical PKC (68). In contrast, the phosphorylation of PKC\(\delta\) on tyrosines 311 and 322 did not play a role in the translocation of PKC\(\delta\) to the Golgi in response to ceramide (31). Similarly, we recently reported that the phosphorylation of PKC\(\delta\) on tyrosines 187 and 64 was not essential for the nuclear translocation of PKC\(\delta\) in response to etoposide although it was essential for its apoptotic effect (15). The mechanisms by which phosphorylation of PKC\(\delta\) is involved in the translocation of PKC\(\delta\) to the ER is currently not understood, but conformational changes or association of the phosphorylated PKC\(\delta\) with another protein via SH2 or PTB domains are possible mechanisms.

In addition to its translocation to the ER, phosphorylation on tyrosine 155 was also essential for the cleavage of PKC\(\delta\) by TRAIL. The translocation of PKC\(\delta\) to the ER was transient and preceded the cleavage of PKC\(\delta\), suggesting that PKC\(\delta\) was cleaved outside of the ER. Interestingly, the inability of the PKC\(\delta\)Y155F mutant to undergo cleavage by TRAIL was specific to this apoptotic stimulus because this mutation did not interfere with the ability of PKC\(\delta\) to undergo cleavage in response to etoposide. Similarly, mutations in tyrosines 187 and 52 did not attenuate the cleavage of PKC\(\delta\) in response to TRAIL.

TRAIL has been reported to activate both pro- and anti-apoptotic signaling pathways in various cellular systems including ERK (51), c-Jun N-terminal kinase (40), AKT (51), and NF-\(\kappa\)B (40). AKT has been implicated as an important inhibitor of TRAIL-induced apoptosis (50, 69). AKT exerts its anti-apoptotic effect by phosphorylating pro-apoptotic proteins such as BAD, caspase 9, and forkhead transcription factors or by activating anti-apoptotic pathways such as AKT, its affinity toward different substrates, and its association with other proteins via SH2 domains. These results clearly position tyrosine phosphorylation of PKC\(\delta\) as a pivotal factor in the regulation of the diverse functions of PKC\(\delta\) in cell apoptosis.

Although PKC\(\delta\) has been extensively studied as a key kinase in the regulation of cell apoptosis, the mechanisms underlying its anti-apoptotic effects are not well understood. Our results provide new information regarding the factors that play a role in the anti-apoptotic effects of PKC\(\delta\) and implicate tyrosine phosphorylation of PKC\(\delta\) on tyrosine 155 as a key factor in the regulation of AKT phosphorylation and cell survival in glioma cells.

In addition to characterizing the anti-apoptotic role of PKC\(\delta\) in TRAIL-induced apoptosis, our results also have important implications for understanding the factors that regulate the apoptotic function of this key signaling molecule. Table I summarizes the localization, phosphorylation, and cleavage of PKC\(\delta\) in response to TRAIL and etoposide where PKC\(\delta\) acts as an anti- or pro-apoptotic kinase, respectively. Both TRAIL and etoposide induce phosphorylation of PKC\(\delta\) albeit on different residues and in both cases tyrosine phosphorylation is essential for the apoptotic function of PKC\(\delta\). Tyrosine phosphorylation on distinct tyrosine residues can modulate the subcellular localization of PKC\(\delta\), its affinity toward different substrates, and its association with other proteins via SH2 domains. These results confirm that tyrosine phosphorylation of PKC\(\delta\) acts downstream of caspase 8 activation and that the phosphorylation of PKC\(\delta\) on tyrosine 155 is a pivotal factor in the regulation of the diverse functions of PKC\(\delta\) in cell apoptosis.

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