Variable expression of protein kinase Cε in human melanoma cells regulates sensitivity to TRAIL-induced apoptosis

Susan Gillespie, Xu Dong Zhang, and Peter Hersey

Immunology and Oncology Unit, Newcastle Mater Hospital, Newcastle, New South Wales, Australia

Abstract

Protein kinase C (PKC) activation is believed to protect against apoptosis induced by death receptors. We have found however that the effect of activation of PKC on tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis of melanoma differs between cell lines. Pretreatment with phorbol 12-myristate 13-acetate (PMA) led to inhibition of apoptosis in the majority of the melanoma cell lines, but those with relatively low PKCε expression were sensitized to TRAIL-induced apoptosis. Introduction of PKCε into PKCε-low cell lines reversed sensitization of the cells to TRAIL-induced apoptosis by PMA. In contrast, a dominant-negative form of PKCε caused an increase in sensitivity. The changes in sensitivity to TRAIL-induced apoptosis were reflected in similar changes in conformation of Bax and its relocation from the cytosol to mitochondria. Similarly, there were concordant increases or decreases in mitochondrial release of second mitochondria-derived activator of caspase/DIABLO, activation of caspase-3, and processing of its substrates. Activation of PKC seemed to mediate its effects upstream of mitochondria but downstream of caspase-8 and Bid in that pretreatment with PMA did not cause significant changes in the expression levels of TRAIL death receptors, alterations in the levels of caspase-8 activation, or cleavage of Bid. PKC activated the antiapoptotic extracellular signal-regulated kinase 1/2 pathway, but inhibitors of this pathway only partially reversed the protective effect of PKC against TRAIL-induced apoptosis. These results provide further insights into the variable responses of melanoma to TRAIL-induced apoptosis and may help define responsive phenotypes to treatment of melanoma with TRAIL. [Mol Cancer Ther 2005;4(4):668–76]

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptotic signaling is initiated by ligand-induced aggregation of death domains that reside on the cytoplasmic sides of the death receptors. This in turn orchestrates the assembly of adapter components, such as Fas-associated death domain that activate initiator caspases, caspase-8 and caspase-10, leading eventually to activation of effector caspases, such as caspase-3 (1, 2). We have shown in past studies that TRAIL-induced apoptosis of melanoma is largely mediated by the mitochondrial apoptotic pathway and that mitochondrial release of second mitochondria-derived activator of caspase (Smac) plays a critical role by binding to and inhibiting inhibitor of apoptosis protein family members (3, 4). The basis for the variation in Smac release remains largely unknown. It was suggested that the Bcl-2 protein family plays a pivotal role in regulating mitochondrion-mediated apoptosis by interaction between antiapoptotic members, such as Bcl-2, Bcl-x_L, and Mcl-1, and proapoptotic members such as Bax, Bad, and Bid (5). Among the latter, Bax seems essential for TRAIL-induced release of Smac from mitochondria (6, 7).

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases comprising at least 11 isoforms that play fundamental roles in signal transduction pathways that regulate cellular proliferation, differentiation, and apoptosis (8, 9). Activation of PKC by phorbol esters has been shown to have variable effects on apoptosis (10–16). In particular, activation of PKCδ seemed proapoptotic (10–12), whereas activation of PKCε and PKCα was antiapoptotic (13–16). Recent studies have implicated the PKC pathway in the protection of cells from apoptosis induced by death receptor ligation (13, 17–22). Activation of PKC has been reported to abrogate Fas-induced apoptosis through inhibition of death-inducing signaling complex formation by blocking Fas-associated death domain recruitment and thus caspase-8 activation (18–20). A similar mechanism has also been implicated in protection of HeLa cells from TRAIL-induced apoptosis (22). Moreover, inhibition of TRAIL-induced apoptosis by PKC activation was also suggested to occur at the level of proteolytic cleavage of caspase-8 or downstream of caspase-8-mediated Bid cleavage (20, 21).

In the present study, we have examined the potential interaction between PKC-mediated signal transduction

Received 12/10/04; revised 1/21/05; accepted 2/7/05.

Grant support: State Cancer Council, New South Wales, Australia.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Peter Hersey, Immunology and Oncology Unit, Mater Hospital, Room 443, David Maddison Clinical Sciences Building, Corner King & Watt Streets, Newcastle, New South Wales 2300, Australia. Phone: 61-2-49-236828; Fax: 61-2-49-236184. E-mail: Peter.Hersey@newcastle.edu.au

and TRAIL-induced apoptotic signaling pathway in melanoma cell lines. We report that activation of PKC differentially regulates sensitivity of melanoma cells to TRAIL-induced apoptosis by modulating Bax activation, and this seems to be associated with the relative expression levels of PKCε. Deficiency in PKCε expression contributed to sensitization of melanoma to TRAIL-induced apoptosis.

Materials and Methods

Cell Lines

Human melanoma cell lines Me4405, Me1007, IgR3, Mel-FH, Mel-RM, Mel-CV, Mel-AT, and MM200 have been described previously (4). The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia).

Antibodies, Recombinant Proteins, and Other Reagents

Recombinant human TRAIL was supplied by Immunex (Seattle, WA). The preparation was supplied as a leucine zipper fusion protein. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (Castle Hill, New South Wales, Australia). The PKC inhibitor, bisindolylmaleimide I (GF109203X), was from Calbiochem (Kilsyth, Victoria, Australia). The general caspase inhibitor Z-Val-Ala-Asp (OMe)-CH_2F and the caspase-3-specific inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F were from Calbiochem (La Jolla, CA). The rabbit monoclonal antibody (mAb) against active caspase-3 and mouse mAbs against caspase-8 and poly(ADP-ribose) polymerase were from PharMingen (North Ryde, New South Wales, Australia). Mouse mAbs against Bcl-2, Bcl-x_L, Mcl-1, Bax, and phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), rabbit polyclonal antibodies against PKCε, PKCδ, PKCα, and inhibitor of caspase-activated DNase, and phosphorylated PKCε, PKCα, and PKCδ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against Bid was from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody against Smac was a kind gift from Dr. Xiao Dong Wang (Howard Hughes Medical Institute, Dallas, TX). The rabbit polyclonal antibody against ERK1/2 and the mitogen-activated protein kinase (MAPK) kinase inhibitor U0126 were purchased from Promega Corp. (Madison, WI). The rabbit polyclonal antibody against Bax (NT) was purchased from Upstate Biotechnology (Waltham, MA). The mAb against cytochrome *c* oxidase IV was purchased from Molecular Probes (Eugene, UR). Isotype control antibodies used were the ID4.5 (mouse IgG2a) mAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, South Australia, Australia), the 107.3 mouse IgG1 mAb purchased from PharMingen (San Diego, CA), and rabbit IgG from Sigma.

Plasmid Vector and Transfection

The expression construct of pEF Bcl-2 was a kind gift from Dr. David Vaux (Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia), which was transfected into melanoma, cells and the resulting transfectants were maintained as described previously (3, 4).

PKCε Adenovirus Vectors

Recombinant adenoviruses containing cDNA coding for PKCε (Ax-PKCε), a dominant-negative mutant of PKCε (Ax-DN-PKCε), and β-galactosidase (Ax-lacZ) were kind gifts from Dr. H. Shinohara (Tokyo Medical and Dental University, Tokyo, Japan) and were used to infect melanoma cells as described previously (13).

Flow Cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously (4). Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer.

Apoptosis

Melanoma cells were seeded onto 24-well plates (Becton Dickinson, Lane Cove, New South Wales, Australia) overnight. Cells with or without pretreatment with PMA (100 ng/mL) for 30 minutes or GF109203X for 1 hour were then treated with TRAIL (200 ng/mL) for another 24 hours. Apoptotic cells were determined by the propidium iodide (PI) method as described elsewhere (4).

Western Blot Analysis

Methods used were as described previously (4, 23). Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane. The data shown are representative of two individual experiments.

Preparation of Mitochondrial and Cytosolic Fractions

Methods used for subcellular fraction were similar to the methods described elsewhere (4, 23).

Results

PKC Differentially Regulates Sensitivity of Melanoma Cells to TRAIL-Induced Apoptosis

Figure 1A shows that pretreatment with PMA inhibited TRAIL-induced apoptosis to varying degrees in five of eight melanoma cell lines (Mel-RM, Mel-CV, Mel-FH, MM200, and IgR3) ranging from 15% inhibition in IgR3 to ~60% inhibition in Mel-RM and Mel-CV. In contrast, PMA increased TRAIL-induced apoptosis of Me4405 and Mel-AT by ~25% and 13%. Inhibition of PKC with GF109203X resulted in opposing effects to those observed with PMA (Fig. 1B). Me1007, a caspase-8- and Bid-deficient line (2), was resistant to TRAIL-induced apoptosis even in the presence of PMA or GF109203X (Fig. 1A and B). Treatment with PMA or GF109203X alone was not cytotoxic to melanoma cells (Fig. 1A and B).

As shown in Fig. 1C, in the presence of PMA, processing of caspase-3 by TRAIL was reduced in Mel-RM cells but was marginally increased in Me4405 cells. In the presence of GF109203X, it was increased in Mel-RM cells but was reduced in Me4405 cells. Figure 1D shows that pretreatment with PMA resulted in decreased levels of TRAIL-induced processing of poly(ADP-ribose) polymerase and

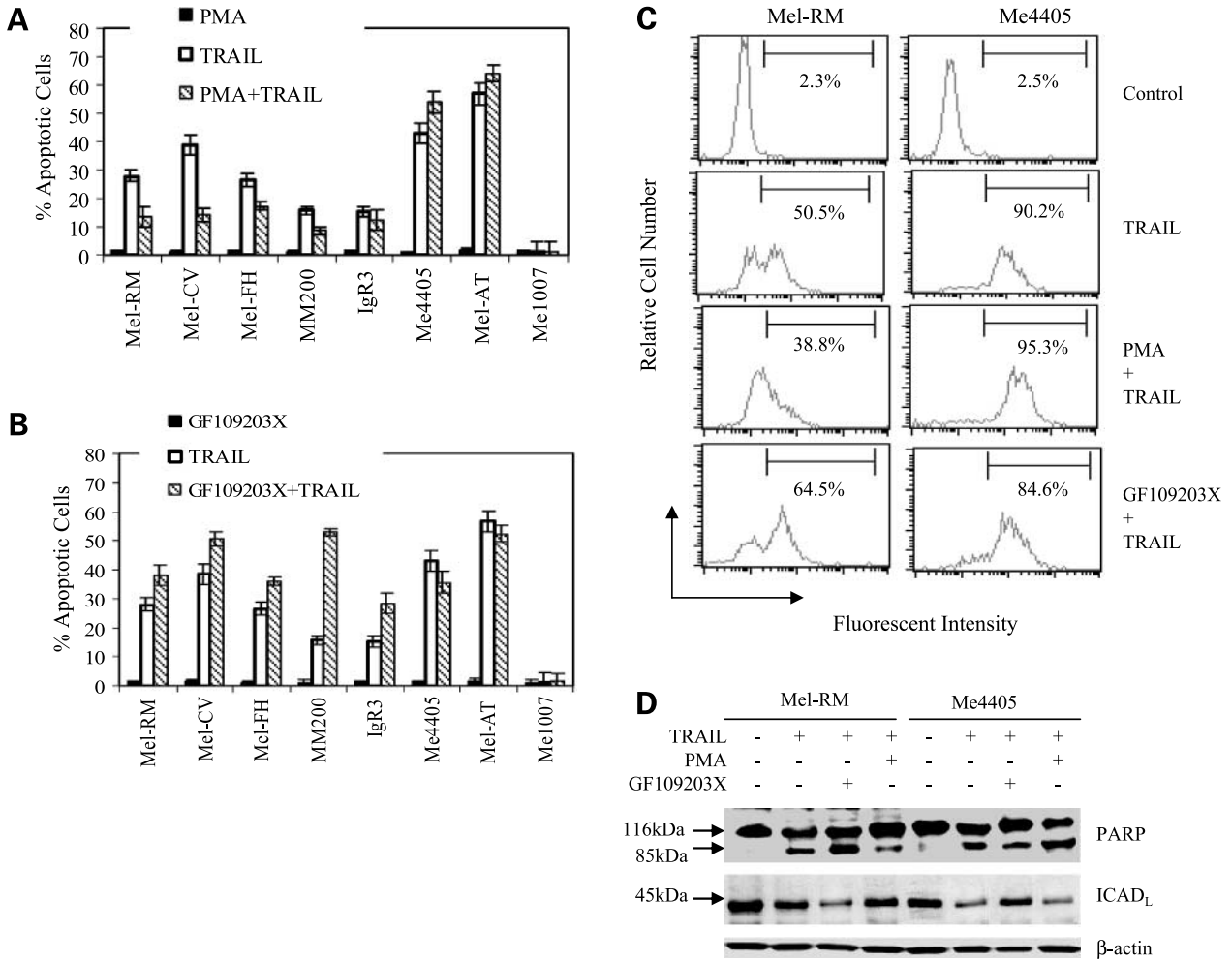

Figure 1. PKC differentially regulates sensitivity of melanoma cells to TRAIL-induced apoptosis. **A** and **B,** melanoma cells with or without pretreatment with PMA (100 ng/mL) for 30 min (**A**) or GF109203X (20 μmol/L) for 1 h (**B**) were treated with TRAIL (200 ng/mL) for another 24 h before measurement of apoptosis by the PI method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **C,** Mel-RM and Me4405 cells with or without pretreatment with PMA (100 ng/mL) for 30 min or GF109203X (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 3 h before the processed form of caspase-3 was measured using a mAb that specifically recognizes the proteolytically processed form of caspase-3 in permeabilized cells using flow cytometry. Representative of three individual experiments. **D,** whole cell lysates from Mel-RM and Me4405 cells treated as in **C** were subjected to Western blot analysis of poly(ADP-ribose) polymerase (*PARP*) and inhibitor of caspase-activated DNase (*ICAD$_L$*).

inhibitor of caspase-activated DNase, two of the key substrates of caspase-3, in Mel-RM cells but elevated levels in Me4405 cells. In contrast, pretreatment with GF109203X led to increased levels in Mel-RM cells but reduced levels in Me4405 cells.

Sensitization of Melanoma Cells to TRAIL-Induced Apoptosis by PMA Is Associated with Deficient PKCε Expression

Among the PKC isoforms that have been implicated in regulation of apoptosis, PKCδ is generally believed to be proapoptotic, whereas PKCε and PKCα are antiapoptotic (10–16). We therefore examined whether the differential effect of PKC on TRAIL-induced apoptosis was due to varying levels of PKCδ, PKCε, and PKCα expression in the cell lines. As shown in Fig. 2A, PKCδ was expressed at high levels in Mel-FH, MM200, IgR3, Mel-RM, and Mel-CV, moderate levels in Mel-AT and Me4405, and low levels in Me1007. PKCα was expressed at moderate to high levels in all but MM200 and Me1007 cells. PKCε was expressed at moderate to high levels in Me1007, MM200, Mel-RM, Mel-CV, Mel-FH, and IgR3 but was at very low levels in Mel-AT and Me4405 cells. It was of note that the latter seemed to be the only two cell lines that were sensitized to TRAIL by PMA (Fig. 1A).

As shown in Fig. 2B, phosphorylated PKCδ could be detected at moderate levels in both Mel-RM and Me4405, whereas low levels of phosphorylated PKCε were seen in Mel-RM cells. Relatively high levels of phosphorylated PKCα were observed in both cell lines. After the addition of TRAIL, an increase in the levels of phosphorylation of

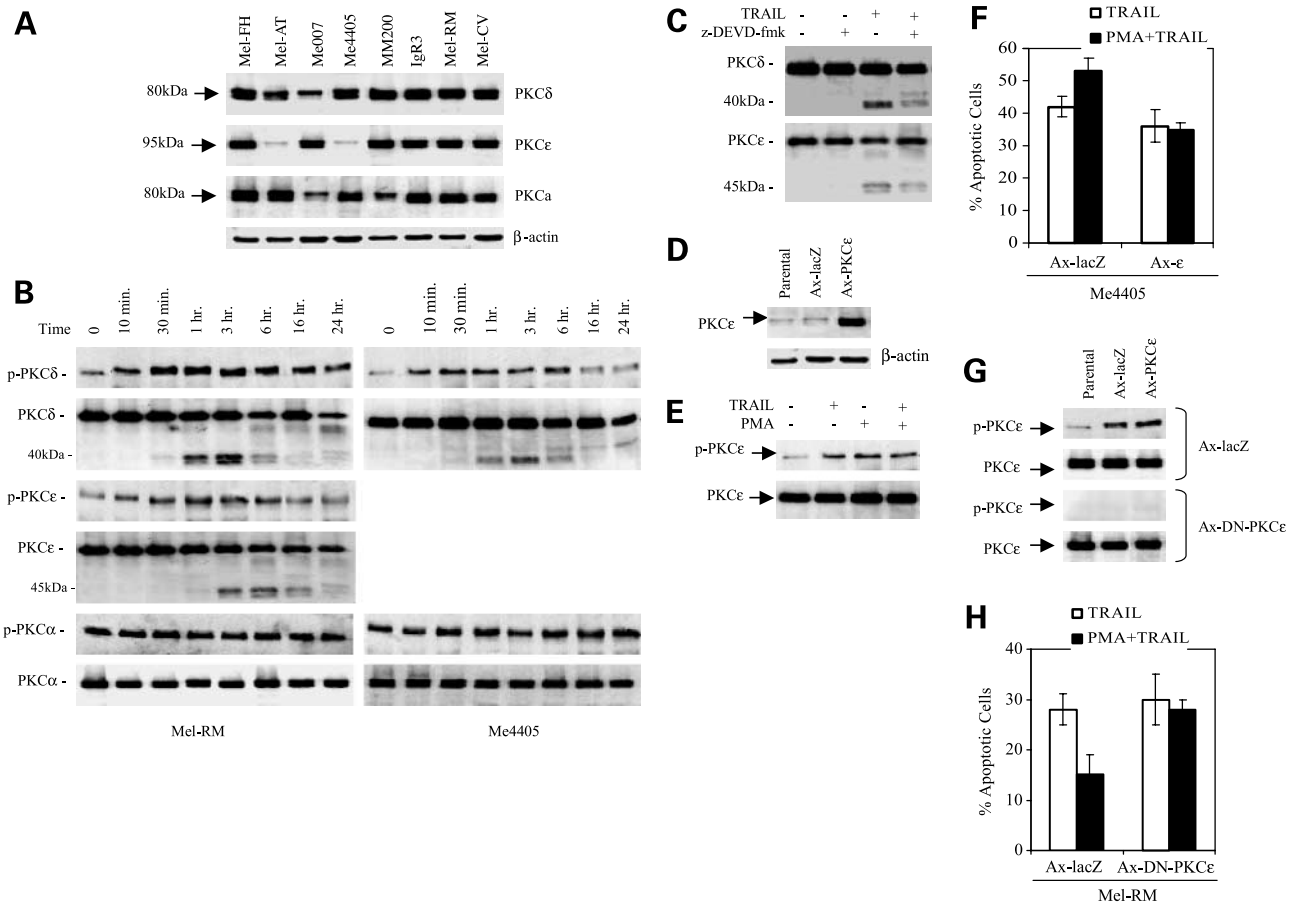

Figure 2. Deficient PKCε expression is associated with sensitivity of melanoma cells to TRAIL-induced apoptosis. **A,** expression of PKCδ, PKCε, and PKCα in melanoma cells. Whole cell lysates from melanoma cells were subjected to Western blot analysis. **B,** TRAIL induced rapid increases in phosphorylation of PKCδ and PKCε. Whole cell lysates from Mel-RM and Me4405 cells with or without treatment with TRAIL (200 ng/mL) for the indicated times were subjected to Western blot analysis. **C,** the caspase-3 specific inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (*z-DEVD-fmk*) inhibited TRAIL-induced proteolytic cleavage of PKCδ and PKCε. Mel-RM cells with or without pretreatment with Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 3 h. Whole cell lysates were subjected to Western blot analysis. **D,** PKCε was expressed in Me4405 cells infected with adenovirus carrying Ax-PKCε but not those infected with adenovirus carrying Ax-lacZ. Whole cell lysates were subjected to Western blot analysis. **E,** Ax-PKCε was phosphorylated by PMA and TRAIL. Whole cell lysates from Mel4405 carrying Ax-PKCε with or without treatment with PMA (100 ng/mL), TRAIL (200 ng/mL), or the combination of both were subjected to Western blot analysis. **F,** Ax-PKCε reverses PMA-mediated sensitization of Me4405 cells to TRAIL-induced apoptosis. Me4405 cells carrying Ax-PKCε or Ax-lacZ with or without pretreatment with PMA (100 ng/mL) for 30 min were treated with TRAIL (200 ng/mL) for another 24 h before measurement of apoptosis by the PI method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **G,** Ax-DN-PKCε inhibits PMA- and TRAIL-mediated phosphorylation of PKCε. Whole cell lysates from Mel-RM carrying Ax-DN-PKCε or Ax-lacZ with or without treatment with PMA (100 ng/mL) or TRAIL (200 ng/mL) for 30 min were subjected to Western blot analysis. **H,** Ax-DN-PKCε reverses PMA-mediated protection of Mel-RM cells from TRAIL-induced apoptosis. Mel-RM cells carrying Ax-DN-PKCε or Ax-lacZ with or without pretreatment with PMA (100 ng/mL) for 30 min were treated with TRAIL (200 ng/mL) for another 24 h before measurement of apoptosis by the PI method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE.

PKCδ was detected as soon as 10 minutes, with a peak at 30 to 60 minutes after treatment. An increase in phosphorylation of PKCε was also observed in Mel-RM cells with the same kinetics. In contrast, TRAIL did not induce any change in phosphorylation status of PKCα.

Figure 2B also shows that a 40-kDa band was detected in both Mel-RM and Me4405 in Western blot analyses of PKCδ ~1 hour after exposure to TRAIL. This band persists until 16 hours after TRAIL treatment. Similarly, a weak 45-kDa band was detected in Western blot analyses of PKCε in Mel-RM with a corresponding decrease in the expression levels of the native form of PKCε. These are consistent with proteolytic cleavage of PKCδ and PKCε by activated caspase-3 as described before (24–26). TRAIL-induced caspase-3 activation in melanoma cells is detectable as soon as 30 minutes after exposure to TRAIL and peaks at 3 hours (4). We therefore treated Mel-RM cells with the caspase-3 specific inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F for 1 hour before adding TRAIL for another 3 hours. Figure 2C shows that inhibition of caspase-3 markedly blocked the appearance of TRAIL-induced smaller forms of PKCδ and PKCε. No indication of proteolytic cleavage of PKCα was found after treatment with TRAIL.

To confirm a role of PKCε in regulation of sensitivity of melanoma cells to TRAIL-induced apoptosis, we introduced PKCε into PKCε-deficient Me4405 cells by using an adenovirus expression system (13). Figure 2D and E shows that PKCε was expressed at high levels in cells infected with adenovirus carrying the cDNA for PKCε (Ax-PKCε) and was readily phosphorylated by treatment with PMA or TRAIL. Pretreatment with PMA did not cause any increase in TRAIL-induced apoptosis in Me4405 cells expressing Ax-PKCε (Fig. 3F).

We next infected PKCε-expressing Mel-RM cells with adenovirus carrying cDNA for a dominant-negative form of PKCε (Ax-DN-PKCε). Figure 2G and H shows that the Ax-DN-PKCε markedly blocked phosphorylation of PKCε induced by either PMA or TRAIL and reversed protective effects of PMA on TRAIL-induced apoptosis in Mel-RM cells.

PKC Regulates Sensitivity of Melanoma Cells to TRAIL-Induced Apoptosis Downstream of Bid Cleavage but Upstream of Mitochondrial Apoptotic Events

Figure 3A shows that pretreatment with either PMA or GF109203X did not cause any significant alteration in the processing of pro-caspase-8 or Bid. Consistent with this is that treatment with PMA or GF109203X for 3, 6, or 16 hours did not cause any change in the expression levels of TRAIL-R1 or TRAIL-R2 on the cell surface (data not shown).

As shown in Fig. 3B, TRAIL induced rapid release of Smac from mitochondria into the cytosol, which was markedly blocked by PMA but was enhanced by GF109203X pretreatment in Mel-RM cells. In contrast, TRAIL-induced release of Smac in Me4405 was potentiated by pretreatment with PMA but was reduced by pretreatment with GF109203X.

The role of mitochondria in PKC-mediated regulation of sensitivity to TRAIL-induced apoptosis was further confirmed by transfecting cDNA encoding Bcl-2 into Mel-RM cells (Fig. 3C). Figure 3D shows that TRAIL-induced apoptosis was completely inhibited in Bcl-2 transfectants irrespective of the presence of PMA or GF109203X.

PKC Modulates TRAIL-Induced Conformational Changes of Bax and Its Translocation from the Cytosol to Mitochondria

Translocation of Bax from the cytosol to mitochondria plays a key role in TRAIL-induced mitochondrial changes (27, 28). This involves a conformational change in Bax that exposes its NH_2 terminus that is otherwise not accessible for binding by Bax-NH_2-terminal epitope-specific antibodies in intact cells (28, 29). We studied if PKC affects TRAIL-induced conformational change of Bax by using an antibody directed against the NH_2-terminal region of Bax in flow cytometry (28, 29). As shown in Fig. 4A, pretreatment with PMA resulted in a marked decrease in the conformational change of Bax induced by TRAIL in Mel-RM cells but led to a moderate increase in Me4405 cells. In contrast, pretreatment with GF109203X promoted the conformational change of Bax in Mel-RM cells but slightly inhibited it in Me4405 cells.

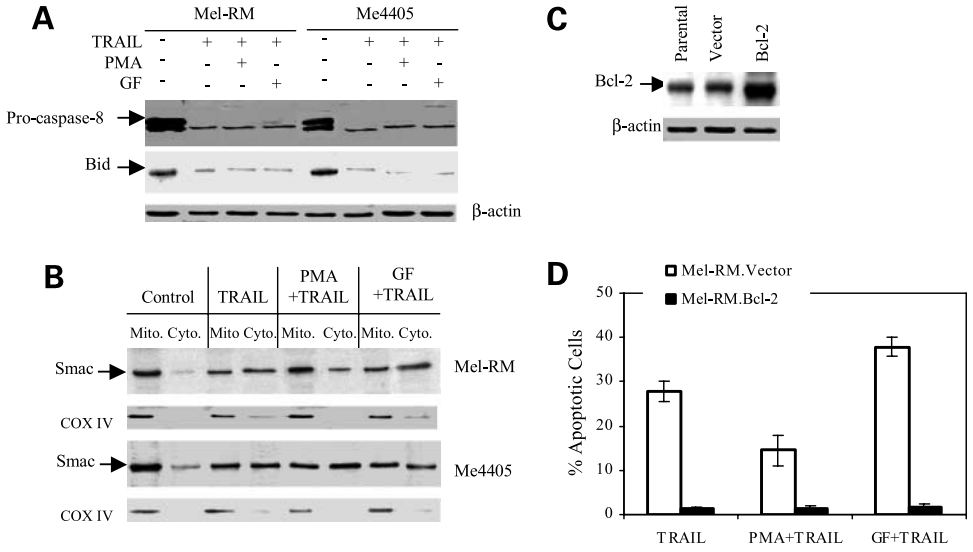

Figure 3. PKC regulates sensitivity of melanoma cells to TRAIL-induced apoptosis downstream of Bid cleavage but upstream of mitochondrial apoptotic events. **A,** whole cell lysates from Mel-RM and Me4405 cells with or without treatment with TRAIL (200 ng/mL) in the presence or absence of PMA (100 ng/mL) or GF109203 (*GF*; 20 μmol/L) were subjected to Western blot analysis. **B,** regulation of mitochondrial release of Smac/DIABLO by PKC activation. Mel-RM and Me4405 with or without pretreatment with PMA (100 ng/mL) for 30 min or GF109203X (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 3 h. Mitochondrial (*Mito.*) and cytosolic (*Cyto.*) fractions were subjected to Western blot analysis. Western blot analysis of cytochrome *c* oxidase IV (*COX IV*) levels was included to show relative purity of the mitochondrial fractions. **C,** Bcl-2 was overexpressed in Mel-RM cells transfected with the cDNA encoding Bcl-2. Whole cell lysates were subjected to Western blot analysis. **D,** overexpression of Bcl-2 inhibited apoptosis induced by TRAIL regardless of the activation status of PKC. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone with or without pretreatment with PMA (100 ng/mL) for 30 min or GF109203X (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 24 h. The percentage of apoptotic cells was quantitated by the PI method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE.

Figure 4B shows that 3 hours after TRAIL treatment a considerable amount of Bax was observed in the mitochondrial fractions with a corresponding decrease in the expression in the cytosol. Pretreatment with PMA caused a decrease, whereas pretreatment with GF109203X caused an increase in the amount of Bax that underwent translocation in Mel-RM cells. In contrast, Bax translocation in Me4405 was potentiated by pretreatment with PMA but was inhibited by pretreatment with GF109203X.

Role of PKC-Mediated Activation of ERK1/2 in Protection of Melanoma from TRAIL-Induced Apoptosis

We have reported previously that Mel-RM and, to a lesser extent, Me4405 were sensitized to TRAIL-induced apoptosis by pretreatment with the MAPK kinase–specific inhibitor U0126 (23). We examined whether this MAPK pathway may be involved in PKC-mediated inhibition of TRAIL-induced apoptosis. In the presence of U0126, TRAIL-induced apoptosis was increased in both cell lines (Fig. 5A). Pretreatment with U0126 before adding PMA and TRAIL partially reversed the protective effect of PMA on TRAIL-induced apoptosis in Mel-RM cells but had no influence on the sensitizing effect of PMA on Me4405. The percentage of inhibition afforded by PMA decreased from 41.5% to 30.9% in the presence of U0126. Figure 5B shows that pretreatment with GF109203X markedly blocked both TRAIL- and PMA-induced phosphorylation of ERK1/2 in both Mel-RM and Me4405 cells.

Discussion

Several recent studies have suggested that activation of PKC plays an important role in protecting cells from TRAIL-induced apoptosis (13, 19, 21). We show in this study, however, that activation of PKC differentially regulates sensitivity of melanoma to TRAIL-induced apoptosis in a cell line–dependent manner. This was shown by PMA-induced decrease or increase in the levels of caspase-3 activation, cleavage of its substrates, and percentage of apoptotic cells induced by TRAIL in the different cell lines. It is likely that the discrepancies between these results and those reported previously are due to the differences in the types of cell lines used in the different studies (13, 19, 21). Nevertheless, the limited numbers of cell lines examined in previous studies might have prevented them from observing a complete spectrum of responses to the effects of activation of PKC on TRAIL-induced apoptosis.

Among the PKC isoforms, PKCδ is generally believed to be proapoptotic (10–12), whereas PKCε and PKCα are antiapoptotic (13–16). In view of this, we studied the relationship of the expression of these isoforms in melanoma cell lines to their sensitivity to TRAIL-induced apoptosis. The results indicate that the expression levels of PKCε especially may play an important role in determining sensitivity of melanoma to apoptosis induced by TRAIL. This was supported by studies using adenovirus vector expression systems to express PKCε in

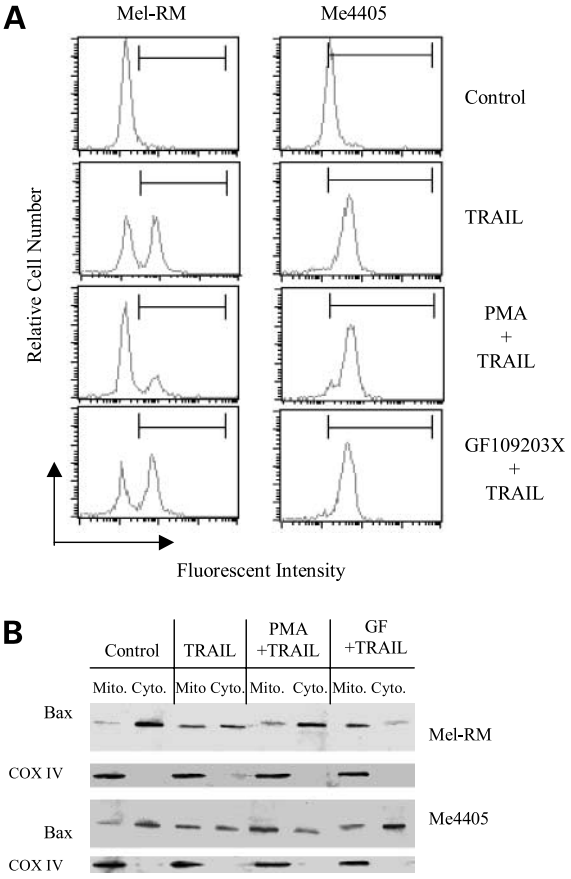

Figure 4. Regulation of TRAIL-induced conformational changes of Bax and its translocation to mitochondria by PKC. **A,** pretreatment with PMA or GF109203X resulted in an increase or decrease in the conformational changes of Bax. Mel-RM and Me4405 cells with or without pretreatment with PMA (100 ng/mL) for 30 min or GF109203X (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 3 h. Conformational changes of Bax were measured in flow cytometry analyses using a Bax-NH_2-terminal epitope-specific antibody in permeabilized cells. Representative of three individual experiments. **B,** pretreatment with PMA or GF109203X resulted in an increase or decrease in translocation of Bax from the cytosol to mitochondria. Mel-RM and Me4405 with or without pretreatment with PMA (100 ng/mL) for 30 min or GF109203X (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 3 h. Mitochondrial and cytosolic fractions were subjected to Western blot analysis. Western blot analysis of cytochrome *c* oxidase IV levels was included to show relative purity of the mitochondrial fractions.

the PKCε-deficient Me4405 cells, which reversed the potentiating effect of PMA on TRAIL-induced apoptosis. In contrast, expression of a dominant-negative PKCε in PKCε-expressing Mel-RM cells reversed the protective effect of PMA on TRAIL-induced apoptosis. These results are similar to those suggesting that PKCε may protect against TRAIL-induced apoptosis of glioma cells (13). Our present results also support a proapoptotic role of PKCδ in melanoma in that cells with lower levels of PKCε that were sensitized to TRAIL-induced apoptosis by PMA (Me4405 and Mel-AT) had relatively high levels of PKCδ. PKCα was reported to be involved in protection against apoptosis in several types of cells (15, 16), but we

did not find any relationship between the expression levels of PKCα and sensitivity of melanoma cells to TRAIL-induced apoptosis. Others have shown that manipulation of PKCα levels had little effect on apoptosis in response to the chemotherapeutic drug etoposide (30). The role that other isoforms of PKC may have in regulation of the sensitivity of melanoma cells to TRAIL is not known. Although the present results do not exclude a possible role for other PKC isoforms, such as PKCλ and PKCζ (31–33), they do however suggest that the relative contents of PKCε may play an important part in determining sensitivity of melanoma cells to apoptosis induced by TRAIL.

We showed that both PKCδ and PKCε are constitutively phosphorylated in melanoma cells and that TRAIL induces a rapid increase in their phosphorylation. These studies suggest that activation of PKC by TRAIL may provide positive or negative regulation of sensitivity of cells to TRAIL-induced apoptosis. This was supported by studies showing that a PKC inhibitor had opposing effects to PMA on sensitivity to TRAIL-induced apoptosis. Activation of PKC by TRAIL was reported to be protective in pancreatic adenocarcinoma cells (34). The present results, however, suggest that TRAIL-mediated activation of PKC protects melanoma cells from TRAIL-induced apoptosis in cells that express relatively high levels of PKCε but promotes apoptosis in those that express relatively low levels of PKCε.

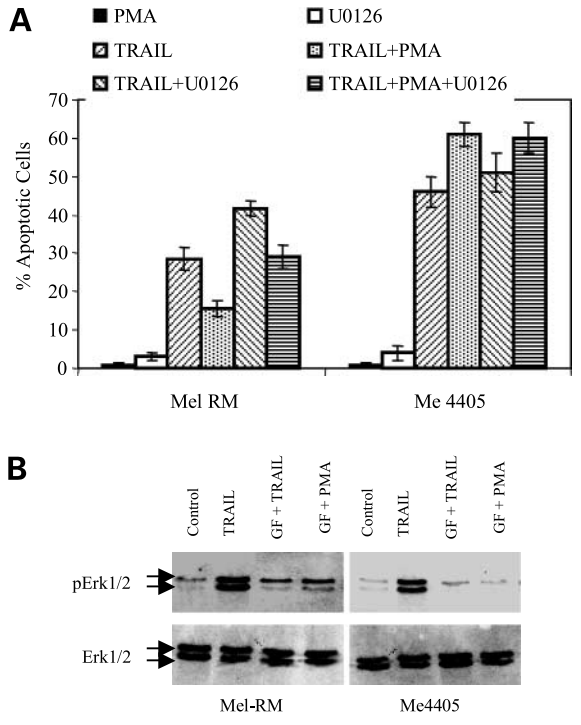

Figure 5. Activation of ERK1/2 is partially responsible for protection of melanoma cells by PKC from TRAIL-induced apoptosis. **A,** inhibition of ERK1/2 reverses the protective effect of PKC on Mel-RM cells. Mel-RM and Me4405 cells with or without pretreatment with U0126 (20 μmol/L) were sequentially treated with PMA (100 ng/mL) and TRAIL (200 ng/mL). The percentage of apoptotic cells was then quantitated by the PI method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **B,** inhibition of PKC blocked TRAIL-induced ERK1/2 activation. Mel-RM and Me4405 cells with or without pretreatment with PMA (100 ng/mL) for 30 min or GF109203X (20 μmol/L) for 1 h were treated with TRAIL (200 ng/mL) for another 1 h. Whole cell lysates were subjected to Western blot analysis.

The site in the apoptotic pathway at which PKC acts to affect TRAIL-induced apoptosis remains disputed (19–22). Whereas some studies showed that inhibition of TRAIL-induced apoptosis by activation of PKC occurred at the level of proteolytic cleavage of pro-caspase-8 (24), others showed that activation of PKC inhibited TRAIL-induced mitochondrial apoptotic events downstream of Bid cleavage (19, 21). More recently, it was shown that activation of PKC interfered with Fas-associated death domain recruitment to the death-inducing signaling complex (22). Our present results showed that the activation of PKC regulates TRAIL-induced apoptosis of melanoma by modulating Bax activation: (*a*) pretreatment with PMA or GF109203X did not cause significant changes in the expression levels of TRAIL death receptors, alterations in activation of caspase-8, and cleavage of Bid; (*b*) pretreatment with PMA resulted in either increased or decreased mitochondrial release of Smac/DIABLO, activation of caspase-3, and processing of its substrates; (*c*) overexpression of Bcl-2 markedly inhibited TRAIL-induced apoptosis in the presence of PMA or GF109203X; and (*d*) pretreatment with PMA or GF109203X led to increased or decreased levels in the conformational changes of Bax and its translocation from the cytosol to mitochondria.

A possible explanation for modulation of Bax activation by PKC is that PKC may regulate the expression of Bcl-2 family members. However, there was no change in the expression levels of Bcl-2, Bcl-x_L, Mcl-1, Bax, or Bak up to 12 hours after treatment with PMA or GF109203X when activation of Bax had already occurred (data not shown). It was reported recently that PKCε interacts with Bax and promotes survival of human prostate cancer cells (35). This was tested in the present studies in melanoma cells by coimmunoprecipitation with a mAb against Bax or an antibody against PKCε with or without exposure of cells to TRAIL, but no such association could be identified (data not shown). Given the complexity of multiple PKC isoforms that may act on Bax by different mechanisms, further studies using PKC isoform ''knockouts'' may assist in elucidating the role they may play in modulating Bax activation induced by TRAIL.

Our data also revealed that TRAIL induced proteolytic cleavage of PKCδ and PKCε, which seemed to be caspase-3 dependent in that a caspase-3-specific inhibitor markedly blocked the appearance of the smaller forms of PKCδ and PKCε. Treatment with either PMA or GF109203X did not induce appreciable levels of caspase-3 activation (data not shown) but was able to regulate TRAIL-induced caspase-3 activation by modulating mitochondrial apoptotic events. The latter is inhibitable by overexpression of Bcl-2 in melanoma cells (23, 36). We

therefore believe that, even in the presence of PMA or GF109203X, Bcl-2 is able to inhibit cleavage of PKCδ and PKCε and exerts its inhibitory effect on TRAIL-induced apoptosis by blocking TRAIL-induced release of mitochondrial apoptotic factors and subsequent activation of caspase-3.

PKC activates the MAPK ERK1/2 pathway by stimulating the Raf-Ras-MAPK kinase pathway in different cell types (26, 27). In this study, we showed that the protective effect of activation of PKC is at least in part associated with activation of ERK1/2 induced by TRAIL (23). This is because inhibition of ERK1/2 by the MAPK kinase–specific inhibitor partially reversed the protective effect on melanoma cells against TRAIL afforded by PMA. In addition, activation of ERK1/2 seems to be downstream of PKC in that inhibition of PKC markedly blocked TRAIL-induced activation of ERK1/2. However, the current studies also clearly show that an ERK1/2-independent mechanism(s) exists in PKC-mediated protection of melanoma against TRAIL-induced apoptosis. Previous studies have also reported that activation of PKC inhibited apoptosis by both MAPK-dependent and MAPK-independent pathways (19, 21). PKC-mediated activation of nuclear factor-κB is known to play a role in protection of many types of cells from death receptor-induced apoptosis (34).

We believe that these results provide further important insights into the variable responses of melanoma cells to TRAIL-induced apoptosis. Measurement of relative contents of PKC isoforms may help define sensitive and resistant melanoma phenotypes to treatment based on TRAIL-induced apoptosis, and studies on this aspect are continuing. Should specific inhibitors of PKCε become available, these in combination with TRAIL would be a promising regimen in treatment of melanoma and worthy of further exploration.

Acknowledgments

We thank Drs. N. Oyaizu and H. Shinohara for the adenovirus constructs.

References

1. Griffith TS, Lynch DH. A molecule with multiple receptors and control mechanisms. Curr Opin Immunol 1998;10:559 – 63.

2. Hersey P, Zhang XD. How melanoma cells evade trail-induced apoptosis. Nat Rev Cancer 2001;1:142 – 50.

3. Thomas W, Zhang XD, Franco A, Nguyen T, Hersey P. TRAIL induced apoptosis of melanoma is associated with perinuclear clustering of mitochondria and changes in mitochondrial membrane potential. J Immunol 2000;165:5612 – 20.

4. Zhang XD, Zhang XY, Nguyen DT, Gray C, Hersey P. Regulation of TRAIL-induced apoptosis of melanoma by release of Smac/DIABLO from mitochondria. Cancer Res 2001;61:7339 – 48.

5. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322 – 6.

6. Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev 2002; 16:33 – 45.

7. Ravi R, Bedi A. Requirement of BAX for TRAIL/Apo2L-induced apoptosis of colorectal cancers: synergism with sulindac-mediated inhibition of Bcl-x(L). Cancer Res 2002;62:1583 – 7.

8. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992;258:607 – 14.

9. Newton AC. Protein kinase C: structure, function, and regulation. J Biol Chem 1995;270:28495 – 8.

10. Majumder PK, Pandey P, Sun X, et al. Mitochondrial translocation of protein kinase Cδ in phorbol ester-induced cytochrome *c* release and apoptosis. J Biol Chem 2000;275:21793 – 6.

11. Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH. Protein kinase Cδ targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol Cell Biol 1999;19:8547 – 58.

12. Fujii T, Garcia-Bermejo ML, Bernabo JL, et al. Involvement of protein kinase Cδ (PKCδ) in phorbol ester-induced apoptosis in LNCaP prostate cancer cells. Lack of proteolytic cleavage of PKCδ. J Biol Chem 2000;275:7574 – 82.

13. Shinohara H, Kayagaki N, Yagita H, et al. protective role of PKCε against TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in glioma cells. Biochem Biophys Res Commun 2001;284:1162 – 7.

14. Basu A, Lu D, Sun B, Moor AN, Akkaraju GR, Huang J. Proteolytic activation of protein kinase C-ε by caspase-mediated processing and transduction of antiapoptotic signals. J Biol Chem 2002;277:41850 – 6.

15. Whelan RD, Parker PJ. Loss of protein kinase C function induces an apoptotic response. Oncogene 1998;16:1939 – 44.

16. Matassa AA, Kalkofen RL, Carpenter L, Biden TJ, Reyland ME. Inhibition of PKCα induces a PKCδ-dependent apoptotic program in salivary epithelial cells. Cell Death Differ 2003;10:269 – 77.

17. Gomez-Angelats M, Bortner CD, Cidlowski JA. Protein kinase C (PKC) inhibits Fas receptor-induced apoptosis through modulation of the loss of K^+ and cell shrinkage. A role for PKC upstream of caspases. J Biol Chem 2000;275:19609 – 19.

18. Gomez-Angelats M, Cidlowski JA. Protein kinase C regulates FADD recruitment and death-inducing signaling complex formation in Fas/CD95-induced apoptosis. J Biol Chem 2001;276:44944 – 52.

19. Sarker S, Ruiz-Ruiz C, Lopez-Rivas A. Activation of protein kinase C inhibits TRAIL-induced caspases activation, mitochondrial events and apoptosis in a human leukemic T cell lines. Cell Death Differ 2001;8: 172 – 81.

20. Meng XW, Heldebrant MP, Kaufmann SH. Phorbol 12-myristate 13-acetate inhibits death receptor-mediated apoptosis in Jurkat cells by disrupting recruitment of Fas-associated polypeptide with death domain. J Biol Chem 2002;277:3776 – 83.

21. Sarker M, Ruiz-Ruiz C, Robledo G, Lopez-Rivas A. Stimulation of the mitogen-activated protein kinase pathway antagonizes TRAIL-induced apoptosis downstream of BID cleavage in human breast cancer MCF-7 cells. Oncogene 2002;21:4323 – 7.

22. Harper N, Hughes MA, Farrow SN, Cohen GM, MacFarlane M. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. J Biol Chem 2003;278:44338 – 47.

23. Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. Oncogene 2003;22: 2869 – 81.

24. Emoto Y, Manome Y, Meinhardt G, et al. Proteolytic activation of protein kinase Cδ by an ICE-like protease in apoptotic cells. EMBO J 1995; 14:6148 – 56.

25. Datta R, Kojima H, Yoshida K, Kufe D. Caspase-3-mediated cleavage of protein kinase Cθ in induction of apoptosis. J Biol Chem 1997;272: 20317 – 20.

26. Goerke A, Sakai N, Gutjahr E, et al. Induction of apoptosis by protein kinase Cδ is independent of its kinase activity. J Biol Chem 2002;277: 32054 – 62.

27. Tafani M, Cohn JA, Karpinich NO, Rothman RJ, Russo MA, Farber JL. Regulation of intracellular pH mediates Bax activation in HeLa cells treated with staurosporine or tumor necrosis factor-α. J Biol Chem 2002;277:49569 – 76.

28. Desagher S, Osen-Sand A, Nichols A, et al. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. J Cell Biol 1999;144:891 – 901.

29. Bellosillo B, Villamor N, Lopez-Guillermo A. Spontaneous and drug-induced apoptosis is mediated by conformational changes of Bax and Bak in B-cell chronic lymphocytic leukemia. Blood 2002;100:1810 – 6.

30. Mandil R, Ashkenazi E, Blass M, et al. Protein kinase Cα and protein kinase Cδ play opposite roles in the proliferation and apoptosis of glioma cells. Cancer Res 2001;61:4612 – 9.

31. Smith L, Chen L, Reyland ME, et al. Activation of atypical protein kinase Cζ by caspase processing and degradation by the ubiquitin-proteasome system. J Biol Chem 2000;275:40620 – 7.

32. Berra E, Municio MM, Sanz L, Frutos S, Diaz-Meco MT, Moscat J. Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. Mol Cell Biol 1997;17:4346 – 54.

33. Pongracz J, Tuffley W, Johnson GD, et al. Changes in protein kinase C isoenzyme expression associated with apoptosis in U937 myelomonocytic cells. Exp Cell Res 1995;218:430 – 8.

34. Trauzold A, Wermann H, Arlt A, et al. CD95 and TRAIL receptor-mediated activation of protein kinase C and NF-κB contributes to apoptosis resistance in ductal pancreatic adenocarcinoma cells. Oncogene 2001;20:4258 – 69.

35. McJilton MA, Van Sikes C, Wescott GG. Protein kinase Cε interacts with Bax and promotes survival of human prostate cancer cells. Oncogene 2003;22:7958 – 68.

36. Zhang XD, Gillespie SK, Borrow J, Hersey P. The histone deacetylase inhibitor suberic bishydroxamate regulates the expression of multiple apoptotic mediators and induces apoptosis by triggering changes in mitochondrial membrane permeability of melanoma cells. Mol Cancer Ther 2004;3:425 – 35.