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Downregulation of Bid is associated with PKCε-mediated TRAIL resistance

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent as it selectively kills tumor cells but spares normal cells. Resistance to TRAIL by tumor cells limits its therapeutic use. We have previously shown that protein kinase C-ε (PKCε) acts as an antiapoptotic protein in MCF-7 breast cancer cells. In the present study, we have investigated the mechanism(s) by which PKCε contributes to TRAIL resistance. Overexpression of PKCε inhibited caspase-8 and -9 activation, release of mitochondrial cytochrome c and cell death induced by TRAIL, but did not interfere with the recruitment of caspase-8 to the death-inducing signaling complex. Knockdown/inhibition of PKCε resulted in enhanced sensitivity to TRAIL. The level of Bcl-2 was increased and Bid was decreased by PKCε at both the protein and mRNA level but PKCε had no effect on Bax. Knockdown of Bcl-2 by siRNA reversed TRAIL resistance in PKCε-overexpressing cells, whereas depletion of Bid contributed to TRAIL resistance in MCF-7 cells. A decrease in Bid content was also associated with inhibition of TRAIL-induced caspase-8 activation. Furthermore, PKCε depletion or overexpression of DN-PKCε was associated with a decrease in Bcl-2 protein level. Thus, our results suggest that PKCε acts upstream of mitochondria and mediates TRAIL resistance via both Bcl-2 and Bid in MCF-7 cells.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor-α (TNF) superfamily, induces apoptosis in a variety of tumor cells with minimal toxicity to normal cells.[1] Thus, TRAIL is particularly valuable for cancer therapy. Resistance to TRAIL-mediated apoptosis by tumor cells, however, limits its therapeutic use.[2,3] An understanding of the mechanism(s) of TRAIL resistance is essential to improve its application in cancer therapy.

TRAIL-mediated apoptosis involves ligand-dependent tri-merization of TRAIL receptors DR4 or DR5, and the recruitment and activation of the death-inducing signaling complex (DISC) composed of Fas-associated death domain (FADD) and initiator procaspase-8.[4–6] Active caspase-8 subsequently cleaves and activates the effector caspase-3 and -7, ultimately resulting in apoptosis.[4,5] Depending on the cell type, TRAIL can also amplify cell death through the mitochondrial pathway.[7] Cross-talk between the receptor-mediated pathway and mitochondria occurs when active caspase-8 cleaves Bid and the truncated Bid translocates to the mitochondria causing activation of Bax/Bak, release of cytochrome c and activation of caspases.[8–11] Downregula-tion/mutations in caspase-8 or increased expression of FLIP can contribute to TRAIL resistance.[3,12–15] The presence of antiapoptotic proteins, such as Akt or Bcl-2 family members can also contribute to TRAIL resistance by affecting either the extrinsic or the intrinsic cell death pathway.[2,10,15–18]

Protein kinase C (PKC), a family of phospholipid-dependent serine/threonine kinases, is emerging as a critical regulator of cellular responses to TRAIL.[12,14,18–24] The PKC family consists of 11 isoforms that are classified as conventional (α, β1, β2 and γ), novel (δ, ε, η and θ) or atypical (ζ and ι/λ).[25] PKCs are involved in the regulation of cell proliferation, differentiation as well as cell death.[26] PKCε is the only PKC isoform that has been reported to have oncogenic poten-tial.[27,28] There is also considerable evidence that PKCε acts as an antiapoptotic protein.[23,29,30]

Activation of PKC was shown to protect against TRAIL-induced apoptosis in several cell lines, including cervical cancer, multiple myeloma, glioma and breast can-cer.[19,20,22,23] There are conflicting reports as to the mechan-isms by which PKC exerts its protective effect. In cervical cancer HeLa cells, PKC activation hindered apical events in the TRAIL receptor-mediated pathway by interfering with FADD recruitment to the DISC.[20] PKC inhibitors inhibited FLIP and cIAP-2 expression in multiple myeloma cells.[12,14] In melanoma and breast cancer MCF-7 cells, PKC acted upstream of mitochondria but downstream of caspase-8 and Bid.[19,22] In the latter study, the effect of phorbol myristic acetate (PMA) was examined but the contribution of a specific PKC isoform was not tested. The mechanism of

TRAIL resistance may vary depending on the PKC isozyme.[14,18,19,24,31]

PKCε has been implicated in inhibiting TRAIL-induced apoptosis in glioma and melanoma cells.[18,19,23] Increased levels and activity of PKCε were associated with TRAIL resistance,[19] and overexpression of PKCε inhibited TRAIL-induced apoptosis in these cells.[18,23] It has been reported that cellular susceptibility to TRAIL cannot be explained by the status of TRAIL receptor expression but does correlate with PKCε level.[23] Furthermore, PKCε overexpression did not modify the cell surface expression of TRAIL receptors in erythromyeloid cell lines.[21] It has been reported that mitochondria constitute an important target in overcoming inherent resistance to TRAIL.[2,3,10,16,19,22,32] However, PKCε did not affect Bcl-2 and Bax in glioma cells.[18] Thus, the mechanisms underlying the protective effect of PKCε and its regulation of components of the mitochondrial pathway in TRAIL-induced cell death are incompletely understood.

We have previously reported that PKCε protects MCF-7 breast cancer cells against receptor-initiated apoptosis.[30] In this study, we have examined the mechanism(s) of the antiapoptotic effect of PKCε in TRAIL-induced cell death. We report for the first time that PKCε inhibited TRAIL-mediated apoptosis by simultaneously increasing Bcl-2 and decreasing Bid expression in MCF-7 cells. Additionally, a decrease in Bid was associated with inhibition of TRAIL-induced caspase-8 activation. These results explain how PKCε could inhibit TRAIL-induced apoptosis by acting upstream of mitochondria at the level of Bid and Bcl-2.

Results

Overexpression of PKCε delays TRAIL-induced cell death. We have previously shown that PKCε protects cells against TNF-induced cell death in MCF-7 cells.[30] We determined whether PKCε overexpression would exert a protective effect against TRAIL-induced apoptosis. MCF-7 cells overexpressing PKCε (MCF-7/PKCε) or vector-transfected control cells (MCF-7/Neo) were treated with different concentrations of TRAIL, and cell viability was determined by the 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) assay. Figure 1a revealed that the IC_{50} of TRAIL for MCF-7/Neo cells (0.027 nM) was almost eightfold less than MCF-7/PKCε cells (0.16 nM). The IC_{50} of TRAIL for MCF-7/Neo cells was equivalent to untransfected MCF-7 cells (0.021 nM, data not shown). As MCF-7 cells transfected with the empty vector can control for any non-specific effect associated with transfection and G418 selection, we primarily used MCF-7/Neo cells instead of MCF-7 cells for comparison with MCF-7/PKCε cells.

MCF-7 cells lack caspase-3 and therefore they do not undergo DNA fragmentation or exhibit characteristic morphological features of apoptosis, such as chromatin condensation.[33] Poly(ADP-ribose) polymerase (PARP) is a substrate for both caspase-3 and -7, and the cleavage of full-length 116-kDa PARP to the 85-kDa cleaved form is a convenient way to monitor cell death. Figure 1b shows that TRAIL induced a concentration-dependent increase in PARP cleavage; the 85-kDa cleavage product of PARP was evident when MCF-7/Neo

cells were treated with 0.03 nM TRAIL. We have tested two different stable clones of MCF-7 cells expressing PKCε and both clones exhibited resistance to TRAIL-induced cell death. Unless otherwise mentioned, we have used clone 1 in all subsequent experiments and these cells are designated as MCF-7/PKCε. Overexpression of PKCε decreased TRAIL-induced PARP cleavage and a 10-fold higher concentration of TRAIL was necessary to cause equivalent PARP cleavage in MCF-7/PKCε cells. In contrast, 0.01 nM TRAIL was sufficient to induce PARP cleavage in MCF-7 cells expressing dominant-negative PKCε.

We also examined the effect of PKC overexpression on the time course of PARP cleavage. As shown in Figure 1c, PARP cleavage in MCF-7/Neo cells was evident at 6 h of treatment with TRAIL, whereas MCF-7/PKCε cells exhibited a delayed response to TRAIL, with PARP cleavage evident at 18 h of treatment. As prolonged treatment with TRAIL may cause further cleavage of PARP, it is sometime difficult to demonstrate a time-dependent increase in cleaved PARP. The levels of full-length PARP in TRAIL-treated MCF-7/PKCε cells were greater compared to MCF-7/Neo cells. Thus, overexpression of PKCε in MCF-7 cells delayed TRAIL-induced cell death.

We also examined the effect of PKCε overexpression on the clonogenic survival. As shown in Figure 1d, treatment of vector-transfected cells with 0.1 nM TRAIL caused a substantial decrease in colony formation, such that 37 and 10% colonies survived when MCF-7/Neo cells were treated with TRAIL for 10 and 20 h, respectively. In contrast, similar treatment of MCF-7/PKCε with TRAIL decreased clonogenic survival only to 76 and 67%. These results suggest that PKCε protected MCF-7 cells from loss of clonogenic survival induced by TRAIL.

Overexpression of PKCε does not affect TRAIL receptor expression and caspase-8 recruitment to the DISC. As binding of TRAIL to its receptors causes recruitment of FADD and caspase-8 to form DISC resulting in activation of caspase-8, we examined if overexpression of PKCε interferes with the recruitment of caspase-8 to the DISC. Figure 2 shows that PKCε overexpression had no effect on the levels of DR4 and DR5. In addition, the abundance of FADD and caspase-8 associated with the DISC did not decrease by PKCε overexpression. A slight decrease in procaspase-8 in the DISC of MCF-7/Neo cells compared to MCF-7/PKCε could be due to processing of procaspase-8 during 30 min incubation with 500 ng/ml TRAIL to generate the cleaved fragment. These results suggest that a decrease in caspase-8 recruitment to the DISC was not responsible for TRAIL resistance of MCF-7/PKCε cells.

Overexpression of PKCε inhibits TRAIL-induced caspase activation. Although TRAIL primarily acts through the receptor-initiated pathway, it can also amplify cell death through the mitochondrial pathway.[7] We therefore examined the effect of PKCε on TRAIL-induced activation of apical caspase-8 and -9 of the extrinsic and intrinsic pathways, respectively (Figure 3). Treatment of MCF-7/Neo cells with TRAIL caused a concentration-dependent increase in processing of both procaspase-8 and -9. MCF-7 cells do
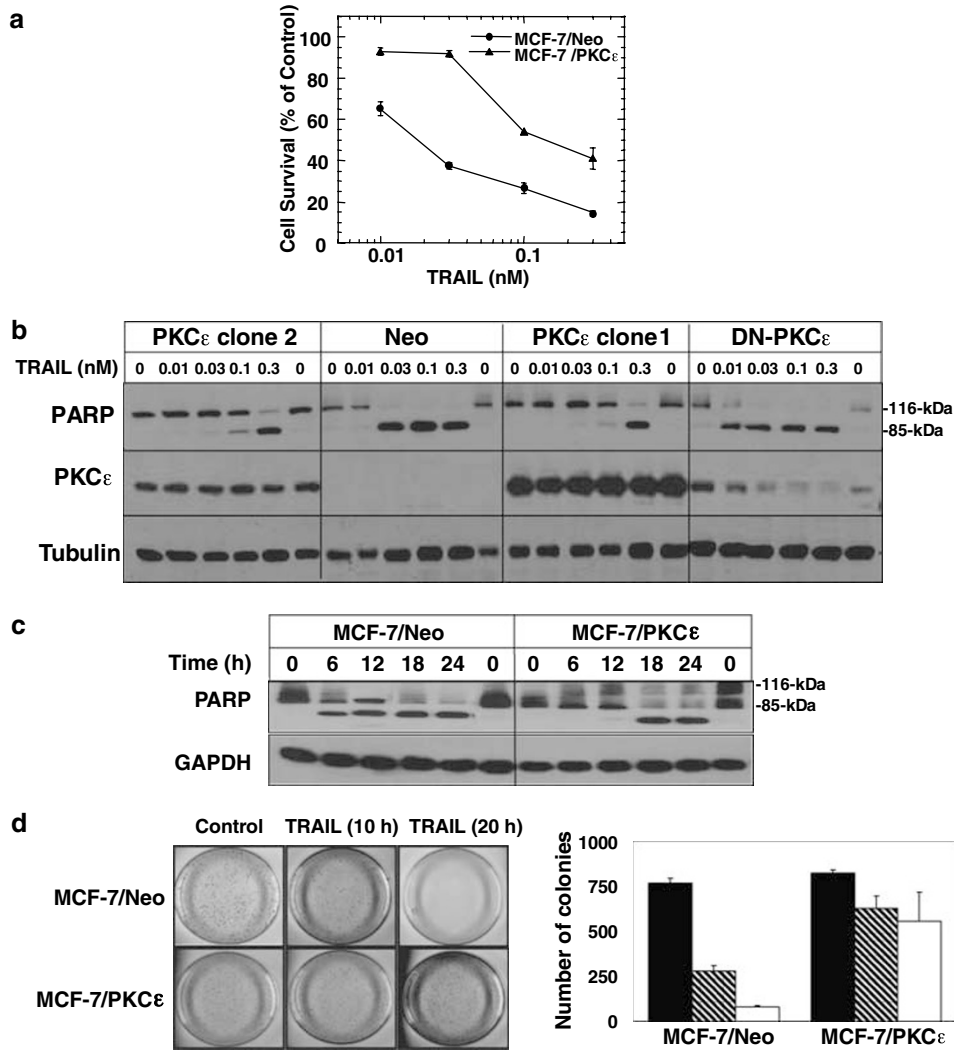
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Figure 1 Time-course and concentration–response of TRAIL-induced cell death in MCF-7/Neo and MCF-7/PKCε cells. (a) Cells were treated with the indicated concentrations of TRAIL for 48 h. Cell viability was determined by the MTT assay as described in Materials and Methods. Data are the mean ± S.E.M. of three separate experiments. (b) Cells were treated with varying concentrations of TRAIL for 10 h, and Western blot analysis was performed with total cell lysates using the indicated antibodies. Tubulin was used as the loading control. (c) Cells were treated with 0.1 nM TRAIL for the indicated periods of time, and Western blot analysis was performed with total cell lysates using the indicated antibodies. GAPDH was used as the loading control. (d) Cells were treated without (solid bar) or with 0.1 nM TRAIL for 10 h (hatched bar) or 20 h (open bar) and clonogenic assay was performed as described in Materials and Methods. The results are representative of two independent experiments. The bar graph represents average colonies ± S.D. of duplicate plates
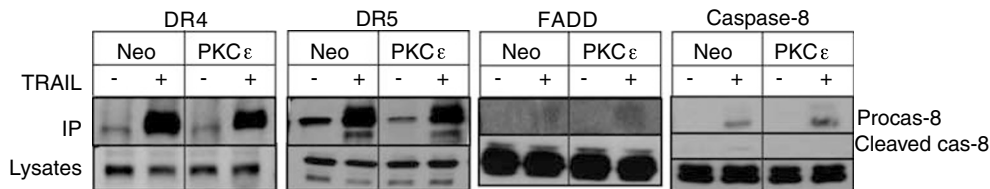

Figure 2 Effect of PKCε overexpression on the recruitment of caspase-8 and FADD to TRAIL receptors. MCF-7/Neo and MCF-7/PKCε cells were treated with or without FLAG-TRAIL for 30 min and DISCs were immunoprecipitated as described in Materials and Methods. Western blot analyses were performed with the indicated antibodies. The results are representative of two independent experiments

not express functional caspase-3, but the activation of effector caspase-7 and -6 was increased by TRAIL treatment. PKCε overexpression attenuated TRAIL-induced activation of both apical caspase-8 and -9, as well as downstream effector caspase-6 and -7 (Figure 3). Thus, PKCε not only affects the extrinsic cell death pathway but also suppresses signaling through the intrinsic or mitochondrial cell death pathway.
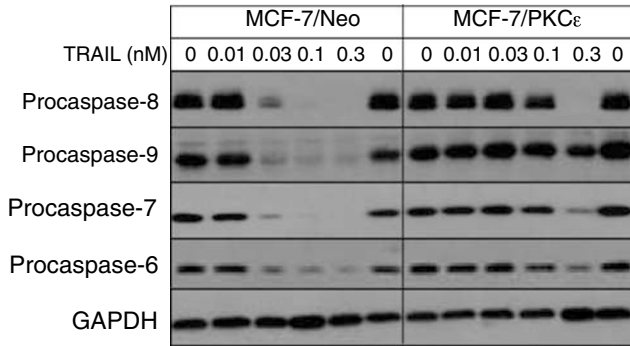
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Figure 3 Overexpression of PKCε inhibits TRAIL-induced caspase activation. MCF-7/Neo and MCF-7/PKCε cells were treated with varying concentrations of TRAIL for 10 h and Western blot analysis was performed with total cell lysates using the indicated antibodies. GAPDH was used as the loading control
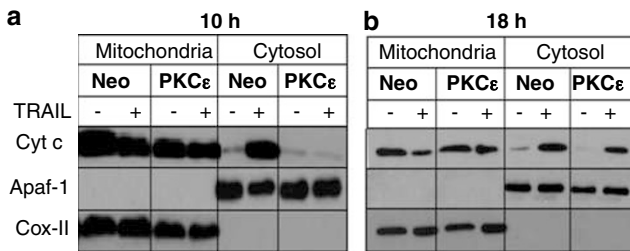

Figure 4 Overexpression of PKCε inhibits TRAIL-induced cytochrome c release. MCF-7/Neo and MCF-7/PKCε cells were treated with 0.3 nM TRAIL for **(a)** 10 h and **(b)** 18 h. Cells were fractionated into cytosol and heavy membrane fraction enriched in mitochondria and Western blot analysis was performed with the indicated antibodies. Cytochrome oxidase subunit 2 and Apaf-1 were used as mitochondrial and cytosolic markers, respectively

Overexpression of PKCε inhibits TRAIL-induced cytochrome c release. Cytochrome c release is a critical event in the mitochondrial cell death pathway. We therefore examined the effect of PKCε overexpression on TRAIL-induced cytochrome c release from the mitochondria. Heavy membrane enriched in mitochondria and cytosolic fractions were isolated from MCF-7/Neo and MCF-7/PKCε cells after treatment with 0.3 nM TRAIL for 10 (Figure 4a) or 18 h (Figure 4b). Treatment of MCF-7/Neo cells with TRAIL caused an increase in cytochrome c in the cytosol. Overexpression of PKCε in MCF-7 cells prevented TRAIL-induced cytochrome c release when treated with TRAIL for 10 h (Figure 4a). However, when cells were treated with 0.3 nM TRAIL for 18 h, we could detect cytochrome c release in MCF-7/PKCε cells although the extent of cytochrome c release was less compared to MCF-7/Neo cells (Figure 4b). The level of Apaf-1, a cytosolic protein, was not affected by TRAIL treatment. Cytochrome oxidase subunit 2, a mitochondrial marker, was detected only in the mitochondrial fraction. These results suggest that PKCε overexpression inhibits the mitochondrial cell death pathway by inhibiting release of cytochrome c from the mitochondria.

Overexpression of PKCε upregulates Bcl-2 and downregulates Bid expression. As Bcl-2 family proteins regulate the mitochondrial cell death pathway, we compared

levels of the Bcl-2 family proteins in MCF-7/Neo and MCF-7/PKCε cells. As shown in Figure 5a, the level of Bcl-2 was increased whereas the level of Bid was decreased in two stable clones of MCF-7 cells overexpressing PKCε compared to MCF-7/Neo cells. Based on densitometric scanning of several independent experiments, there was a 2.5-fold increase in Bcl-2 ($P < 0.05$) and a 3.3-fold decrease in Bid ($P < 0.05$) in MCF-7/PKCε cells compared to MCF-7/Neo cells (Figure 5b). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of RNA isolated from these cell lines also revealed an increase in Bcl-2 and a decrease in Bid mRNA in MCF-7/PKCε cells (Figure 5c). Similar results were obtained when two different primers were used during RT-PCR. The proteasome inhibitor MG132 had no effect on the decrease in Bid in MCF-7/PKCε cells (data not shown). These results suggest that PKCε regulates Bid and Bcl-2 at the level of transcription. However, levels of Bax mRNA and protein were equivalent in both cell lines. Thus, an increase in the ratio of antiapoptotic Bcl-2 to proapoptotic Bid may contribute to TRAIL resistance in MCF-7/PKCε cells. PKCε overexpression did not decrease the levels of other antiapoptotic proteins, such as X-linked inhibitor of apoptosis (XIAP) or c-FLIP (Figure 5d).

Depletion of Bid inhibits TRAIL-induced apoptosis in MCF-7 cells. To determine the functional significance of Bid downregulation in MCF-7/PKCε cells, we overexpressed Bid in MCF-7/PKCε cells. The transfection efficiency of Bid plasmid in MCF-7/PKCε was low (7–8%) and overexpression of Bid in MCF-7/PKCε cells by itself caused cell death (Figure 6). Therefore, we were unable to isolate stable clones. Nevertheless, these results suggest that overexpression of Bid can reverse antiapoptotic effect of PKCε. We then depleted Bid using siRNA and determined cell death in response to TRAIL. Figure 7a shows that knockdown of Bid by siRNA selectively decreased Bid but not Bcl-2 or Bax. Depletion of Bid resulted in protection from TRAIL-induced cleavage of PARP in MCF-7 (Figure 7b) as well as MCF-7/Neo cells (Figure 7c). The effect of untransfected cells was similar to control siRNA-transfected cells (data not shown). We also stained cells with Annexin V-Alexa 488 conjugate and propidium iodide (PI) to monitor apoptosis. As shown in Figure 8, 0.1 nM TRAIL increased cell death to 72% in both untransfected and control siRNA-transfected MCF-7 cells. Knockdown of Bid inhibited TRAIL-induced apoptosis in MCF-7 cells to 12%. These results suggest that a decrease in proapoptotic Bid in MCF-7/PKCε cells may contribute to TRAIL resistance.

Treatment with TRAIL also resulted in the cleavage of Bid and the truncated Bid (t-Bid) was visible in TRAIL-treated MCF-7 (Figure 7b) or MCF-7/Neo cells (Figure 7c). Interestingly, depletion of Bid by siRNA was associated with a decrease in the cleavage of full-length Bid (Figure 7b and c). As Bid is cleaved by caspase-8, we examined the effect of Bid knockdown on caspase-8 processing. Figure 7b shows that depletion of Bid inhibited processing of not only procaspase-9 but also of procaspase-8.

Depletion of Bcl-2 potentiates TRAIL-induced apoptosis in MCF-7 cells. We also examined the consequence of
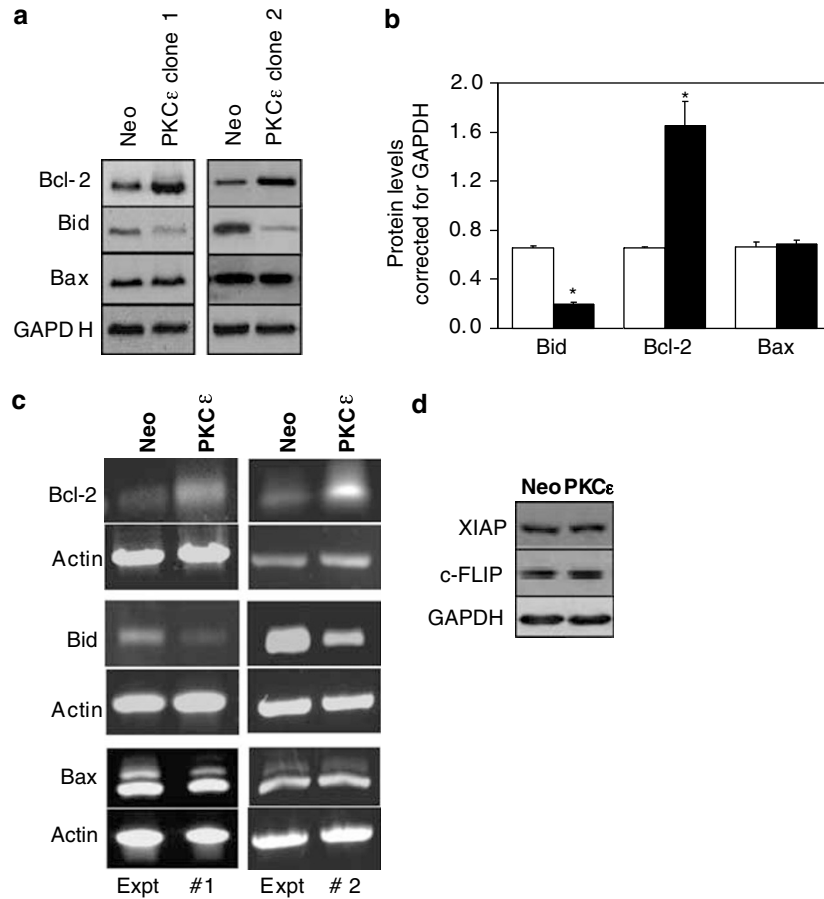
Figure 5 Overexpression of PKCε upregulates Bcl-2 and downregulates Bid. (**a**) Total cell extracts from MCF-7/Neo and MCF-7/PKCε cells were analyzed by Western blot analysis with the indicated antibodies. GAPDH was used to control for loading differences. (**b**) Densitometric quantification of three separate experiments corrected for loading. Data are the mean ± S.E.M. Asterisks indicate a significant difference from MCF-7/Neo cells ($P < 0.05$) using paired Student's t-test. Open bar, MCF-7/Neo; closed bar, MCF-7/PKCε. (**c**) RT-PCR analysis was performed for detecting Bid, Bcl-*2*, Bax and actin expression. The results are representative of three independent experiments. Two experiments with two different primers are shown. (**d**) Western blot analysis of total cell lysates was performed with the indicated antibodies. GAPDH was used to control for loading differences
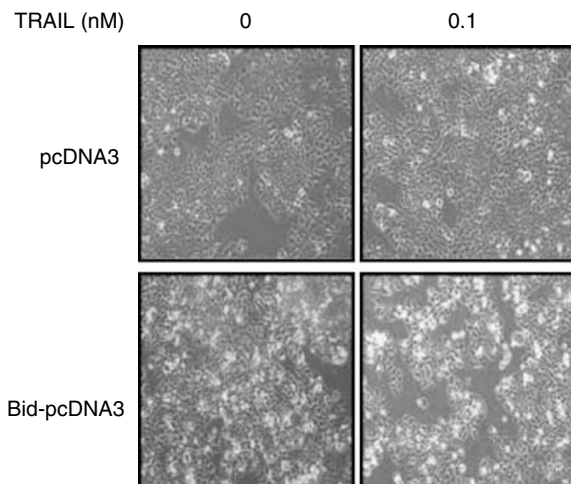

Figure 6 Effect of Bid overexpression on cell death. MCF-7/PKCε cells were transfected with empty vector or vector containing Bid as described under Materials and Methods. After 24 h of transfection, cells were treated with 0.1 nM TRAIL for 8 h and cellular morphology was examined under a microscope. The results are representative of two independent experiments

Bcl-2 depletion on TRAIL-induced cell death in MCF-7 cells. Knockdown of Bcl-2 decreased the level of Bcl-2 but not of Bid (Figure 9a) or Bax (data not shown). Depletion of Bcl-2 in MCF-7 cells enhanced TRAIL-induced cell death as evident by the cleavage of PARP (Figure 9a) and by Annexin V/PI staining (Figure 8). Although 0.01 nM TRAIL had little effect on cell death in untransfected or control siRNA-transfected MCF-7 cells, it caused 35% cell death in Bcl-2-depleted cells (Figure 8). However, Bcl-2 knockdown had no additional effect on cell death in MCF-7 and MCF-7/Neo cells treated with 0.1 or 1.0 nM TRAIL (Figures 8, 9a and b). Knockdown of Bcl-2 also enhanced TRAIL-induced activation of caspase-8 and -9 as well as cleavage of Bid in MCF-7 cells (Figure 9a). As MCF-7/PKCε cells overexpressed Bcl-2, we also examined if depletion of Bcl-2 would reverse TRAIL resistance in these cells. Knockdown of Bcl-2 restored the sensitivity of MCF-7/PKCε cells to TRAIL as judged by PARP cleavage (Figure 9b) and Annexin V dye binding assay (Figure 9c). Thus, upregulation of Bcl-2 may contribute to TRAIL resistance in PKCε-overexpressing cells.
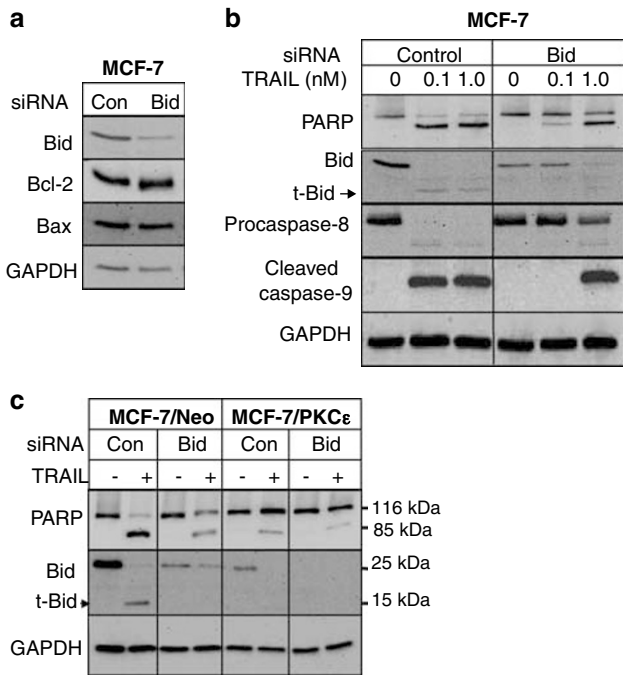
Figure 7 Depletion of Bid attenuates TRAIL-mediated PARP cleavage, caspase activation and cell death. (**a**) Western blot analysis was performed with total cell lysates from MCF-7 cells treated with control or Bid siRNA. (**b**) Bid was depleted from MCF-7 cells using siRNA. Cells were then treated with or without 0.1 nM or 1.0 nM TRAIL for 10 h. Western blot analysis was performed with the indicated antibodies. (**c**) MCF-7/Neo and MCF-7/PKCε cells transfected with control or Bid siRNA were treated with or without 1.0 nM TRAIL for 10 h. Western blot analysis was performed with the indicated antibodies. GAPDH was used to control for loading differences. The results are representative of three independent experiments

Knockdown of PKCε increases TRAIL sensitivity. To further examine the link between Bcl-2, Bid and the antiapoptotic function of PKCε, we depleted PKCε by siRNA. Figure 10 shows that knockdown of PKCε by siRNA enhanced cellular sensitivity to TRAIL as determined by activation of caspase-8 and -9, PARP cleavage (Figure 10a) and Annexin V dye binding assay (Figure 10b). Although TRAIL (0.01 nM) had little effect on cell death in untransfected and control siRNA-transfected MCF-7 cells, it caused approximately 70% cell death in PKCε-depleted cells (Figure 10b). Knockdown of PKCε decreased Bcl-2 level (Figure 10c). Based on densitometric scanning of three independent experiments, PKCε depletion was associated with a 2.2-fold decrease in Bcl-2. PKCε knockdown caused only a modest increase in Bid (Figure 10c). Expression of dominant-negative PKCε also caused a similar decrease in Bcl-2 and an increase in Bid (Figure 10d). Thus, although the levels of both Bcl-2 and Bid are important determinants of PKCε-mediated TRAIL resistance, decrease in Bcl-2 may be primarily responsible for TRAIL sensitization in PKCε-depleted cells.

Discussion

The ability of TRAIL to kill cancer cells is counteracted by the presence of antiapoptotic proteins, contributing to TRAIL resistance. In the present study, we have investigated the mechanism(s) by which PKCε contributes to TRAIL resistance. Our results show that an increase in antiapoptotic Bcl-2 and a decrease in proapoptotic Bid were associated with PKCε-mediated TRAIL resistance in MCF-7 cells.

There have been several reports that suggest that PKC acts upstream of the mitochondrial pathway to regulate cell death by TRAIL, although the site of action of PKCε in the cell death pathway is not well established.[18,19,22,23] The mitochondrial cell death pathway is regulated by the interaction between pro- and antiapoptotic members of the Bcl-2 family. Several studies have implicated Bax as an important mediator of cellular responsiveness to TRAIL.[16,34,35] Bax-deficient colon cancer cells were resistant to TRAIL-induced apoptosis.[2] Bax, but not Bak, sensitized leukemic and prostate cancer cells to TRAIL.[35,36] PKCε-deficient LNCaP cells were sensitive to PMA-induced apoptosis and forced expression of PKCε in these cells conferred resistance to PMA-mediated apoptosis via interaction with Bax.[34] We have shown that the levels of antiapoptotic Bcl-2 as well as proapoptotic Bid were regulated by PKCε in MCF-7 breast cancer cells, whereas PKCε had no effect on the level of Bax. It has been reported that EPO-driven upregulation of PKCε level renders differentiating erythroid cells resistant to TRAIL likely via Bcl-2 upregulation.[21] We have shown that overexpression of PKCε increased Bcl-2 not only at the protein level but also at the mRNA level. In addition, knockdown of PKCε by siRNA decreased Bcl-2 level. Furthermore, depletion of Bcl-2 by siRNA reversed the antiapoptotic effect of PKCε. Taken together, these results provide direct evidence that Bcl-2 is involved in the antiapoptotic function of PKCε in MCF-7 breast carcinoma cells. Depletion of Bcl-2 not only enhanced TRAIL-induced activation of caspase-9 but also of caspase-8. As caspase-8 can also be cleaved by effector caspases, such as caspase-6, activation of caspase-8 could be a consequence of TRAIL-induced activation of caspase-9. Although MCF-7 cells do not express caspase-3, which is believed to cleave caspase-6,[37] TRAIL did induce processing of caspase-6 in MCF-7 cells and PKCε inhibited cleavage of procaspase-6.

It is generally believed that the antiapoptotic effect of PKCε occurs downstream of caspase-8 activation and Bid cleavage.[18,19,21,22,38] We also found that overexpression of PKCε did not alter the levels of TRAIL receptors or interfere with the recruitment of caspase-8 to the DISC. Overexpression of PKCε caused a decrease in Bid protein and mRNA in MCF-7 cells. Consequently, the level of truncated Bid in response to TRAIL treatment was also lower in PKCε-overexpressing cells. However, depletion of PKCε caused only a slight increase in Bid level. It is conceivable that although overexpression of PKCε represses Bid transcription, a decrease in constitutive level of PKCε had no effect on Bid expression. Alternatively, as Bid is cleaved when cells undergo apoptosis and depletion of PKCε alone induces cell death, it may be difficult to demonstrate increase in Bid following depletion of PKCε. Consistent with this notion, we have found that overexpression of Bid not only induced cell death in PKCε-overexpressing cells (Figure 6), but also resulted in cleavage of Bid (data not shown). We do not know if forced expression of Bid results in mitochondrial localization causing release of cytochrome c and activation of caspases resulting in cleavage
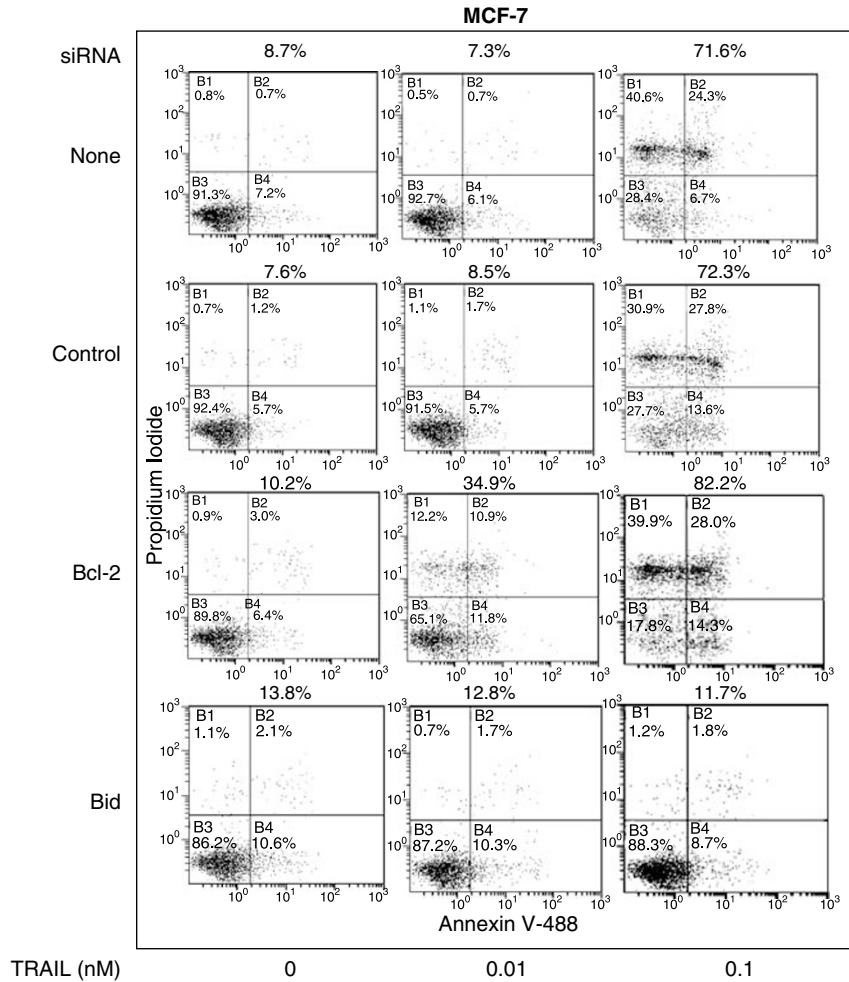
Figure 8 Depletion of Bcl-2 or Bid affects TRAIL resistance in MCF-7 cells. MCF-7 cells were transfected with or without control, Bcl-2 or Bid siRNA and then treated with the indicated concentrations of TRAIL for 10 h. Cells were stained with Annexin V-Alexa 488 conjugate and PI and analyzed by flow cytometry. The results are representative of two independent experiments

of Bid. In addition, knockdown of Bid by siRNA decreased TRAIL sensitivity in MCF-7 cells, suggesting the importance of Bid in regulating TRAIL sensitivity in these cells.

Overexpression of PKCε in MCF-7 cells caused inhibition of caspase-8 and -9 processing in response to TRAIL. The effect of PKCε on caspase-8 activation may be a consequence of the decrease in Bid expression. We have shown that knockdown of Bid in fact inhibited TRAIL-induced cleavage of Bid and activation of caspase-8. Thus, Bid is not only a substrate for caspase-8 but it can also regulate its activation. We propose that in response to TRAIL, activation of caspase-8 results in cleavage of Bid and translocation of t-Bid to mitochondria, resulting in release of cytochrome *c*, activation of caspase-9 and cell death. This cross-talk drives more caspase-8 cleavage by a feed-forward mechanism. As the level of Bid is decreased by PKCε overexpression, the level of t-Bid generated by TRAIL-induced activation of casapse-8 is less in PKCε-overexpressing cells. As PKCε also induces antiapoptotic Bcl-2, the ratio of antiapoptotic and proapoptotic Bcl-2 family members is increased further. Inhibition of the mitochondrial cell death pathway results in a decrease in Bid

cleavage and inhibition of TRAIL-induced caspase-8 activation.

TRAIL is a highly promising candidate for the treatment of cancer because it exhibits selective cytotoxicity to cancer cells. Some tumor cells, however, evade TRAIL-induced apoptosis owing to the presence of antiapoptotic proteins. It has been reported that tumor cells that overexpress PKCε are resistant to TRAIL.[19,23] The elucidation of the mechanism of TRAIL resistance is highly significant for its effective application in the clinic. We provide direct evidence that both Bcl-2 and Bid are important for PKCε-mediated TRAIL resistance. Thus, Bcl-2 family proteins could be targeted to reverse TRAIL resistance in breast cancer cells.
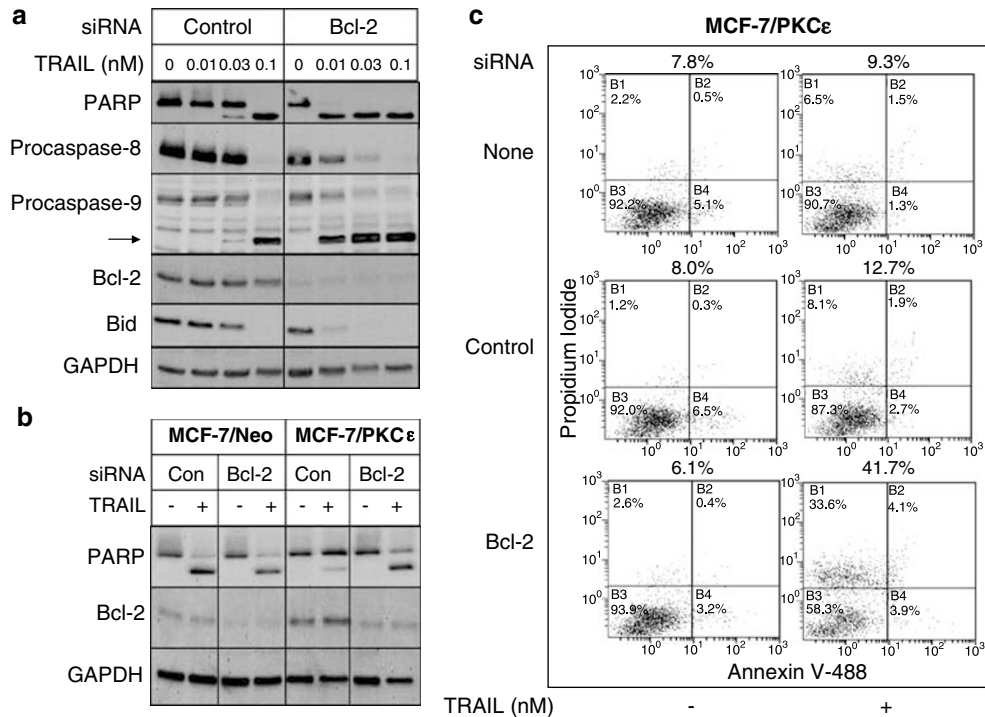
Figure 9 Depletion of Bcl-2 abrogates TRAIL resistance in MCF-7/PKCε cells. (**a**) MCF-7 cells were transfected with control siRNA or siRNA targeted against Bcl-2. Cells were then treated with the indicated concentrations of TRAIL for 10 h. Western blot analysis was performed with the indicated antibodies. GAPDH was used to control for loading differences. (**b**) MCF-7/Neo and MCF-7/PKCε cells were transfected with control or Bcl-2 siRNA and then treated with 1.0 nM TRAIL for 10 h. Western blot analysis was performed with the indicated antibodies. GAPDH was used to control for loading differences. (**c**) MCF-7/PKCε cells were transfected with or without control or Bcl-2 siRNA and then treated with 0.1 nM TRAIL for 10 h. Cells were then stained with Annexin V-Alexa 488 conjugate and PI, and analyzed by flow cytometry. The results are representative of two independent experiments

Inc. (Santa Cruz, CA, USA). Polyclonal antibody to Bid and monoclonal antibody to caspase-8 were purchased from BioSource (Camarillo, CA, USA). Monoclonal antibody to PARP, XIAP, FADD, caspase-7 and polyclonal antibody to caspase-9 were purchased from Pharmingen (San Diego, CA, USA). Polyclonal antibody to caspase-6 was obtained from Cell Signaling Technologies (Beverly, MA, USA). MTT and crystal violet were purchased from Sigma (St Louis, MO, USA). siRNA SMARTpool against PKCε, Bcl-2, Bid and nontargeting SMARTpool siRNA were obtained from Dharmacon (Lafayette, CO, USA). Annexin V-Alexa 488 and PI were obtained from Molecular Probes (Eugene, OR, USA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL, USA).

Cell culture and transfection. MCF-7 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine and kept in a humidified incubator at 37°C with 95% air and 5% CO_2. MCF-7/Neo, MCF-7/PKCε and MCF-7/DN-PKCε cells were generated as described previously.[30] MCF-7/PKCε cells were co-transfected with either 2 μg of empty vector or Bid-pcDNA3.1 together with 2 μg SV40-β-gal plasmids using Fugene HD reagent (Roche Biomedicals) according to the manufacturer's instructions. Transfection efficiency was determined by β-galactosidase expression.

Knockdown of PKCε and Bcl-2 family members. siRNAs against PKCε, Bcl-2, Bid or nontargeting SMARTpool were introduced into MCF-7 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, cells were seeded one day before transfection. Lipofectamine 2000 and siRNA diluted in Opti-minimal essential medium (MEM) were mixed gently at a ratio of 300 ng lipofectamine 2000 : 133 ng siRNA and incubated at room temperature for 15–20 min. Culture medium was replaced with Opti-MEM and 100 μl of siRNA:lipofectamine 2000 complexes were added to cells. After 4–6 h, fresh culture medium was added to cells. After 48 h, following siRNA transfection, cells were treated with TRAIL.

Assessment of cell viability by MTT assay. Exponentially growing MCF-7, MCF-7/Neo and MCF-7/PKCε cells were plated in microtiter plates and incubated in a humidified incubator at 37°C with 5% CO_2. The following day, cells were treated with or without different concentrations of TRAIL as described in the text and incubated for 48 h. The number of viable cells was determined using the dye MTT.

Clonogenic assay. MCF-7/Neo or MCF-7/PKCε cells were seeded in 10-cm tissue culture dish and allowed to attach overnight. Cells were treated with or without 0.1 nM TRAIL for the indicated periods. Following incubation, the media were replaced with fresh media. Cells were then incubated in a humidified incubator at 37°C and 5% CO_2 until there were at least 50 cells per colony in untreated cells. At the end of the incubation, cells were washed with phosphate-buffered saline (PBS) and incubated with a 0.25% crystal violet solution for 15 min. Colonies were counted using the colony-counting tool in the BioChemi System (UVP).

RT-PCR analysis. Total RNA was isolated using RNA STAT-60 from Tel-Test Inc, (Friendswood, TX, USA), as per the manufacturer's protocol and subjected to RT reaction using Promega (Madison, WI, USA) reverse transcriptase enzyme (ImProm-II™). PCR amplifications were performed using the following primers: Bax, forward, 5′-GGC CCA CCA GCT CTG AGC AGA TCA T-3′; reverse, 5′-CCT GGT CTT GGA TCC AGC CCA ACA G-3′. Bcl-2, forward, 5′-TAT AAG CTG TCG CAG AGG GGC TA-3′; reverse, 5′-GTA CTC AGT CAT CCA CAG GGC GAT-3′ and forward, 5′ GAC AGT CCC ATC AAA ACT CC 3′; reverse, 5′ ATG ATC AGG TCC TTT TTC CA 3′. Bid, forward, 5′-ATG GAC TGT GAG GTC AAC GGC GC-3′; reverse, 5′-CCC GGC CAG AAA TGG GAT GGA CT-3′ and forward, 5′ GCA TGT CAA CAG CGT TCC TA 3′; reverse: 5′ GGA ACC TGC ACA GTG GAA AT 3′. Actin, forward, 5′-TAC AAT GAG CTG CGT GTG GCT-3′; reverse, 5′-ATC CAC ATC TGC TGG AAG GTG GA-3′. Cycle conditions for all PCRs were set up for 40 cycles as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The products were resolved on a 1% agarose gel containing ethidium bromide.

a

MCF-7

siRNA | Control | PKCε
TRAIL | − + | − +

PKCε

PARP — 116 kDa
— 85 kDa

Caspase-8

Procaspase-9

GAPDH

b

MCF-7

c

MCF-7

siRNA | Control | PKCε

Bcl-2

GAPDH

MCF-7

siRNA | Control | PKCε

Bid

GAPDH

d

MCF-7/Neo | MCF-7/PKCε | MCF-7/DN-PKCε
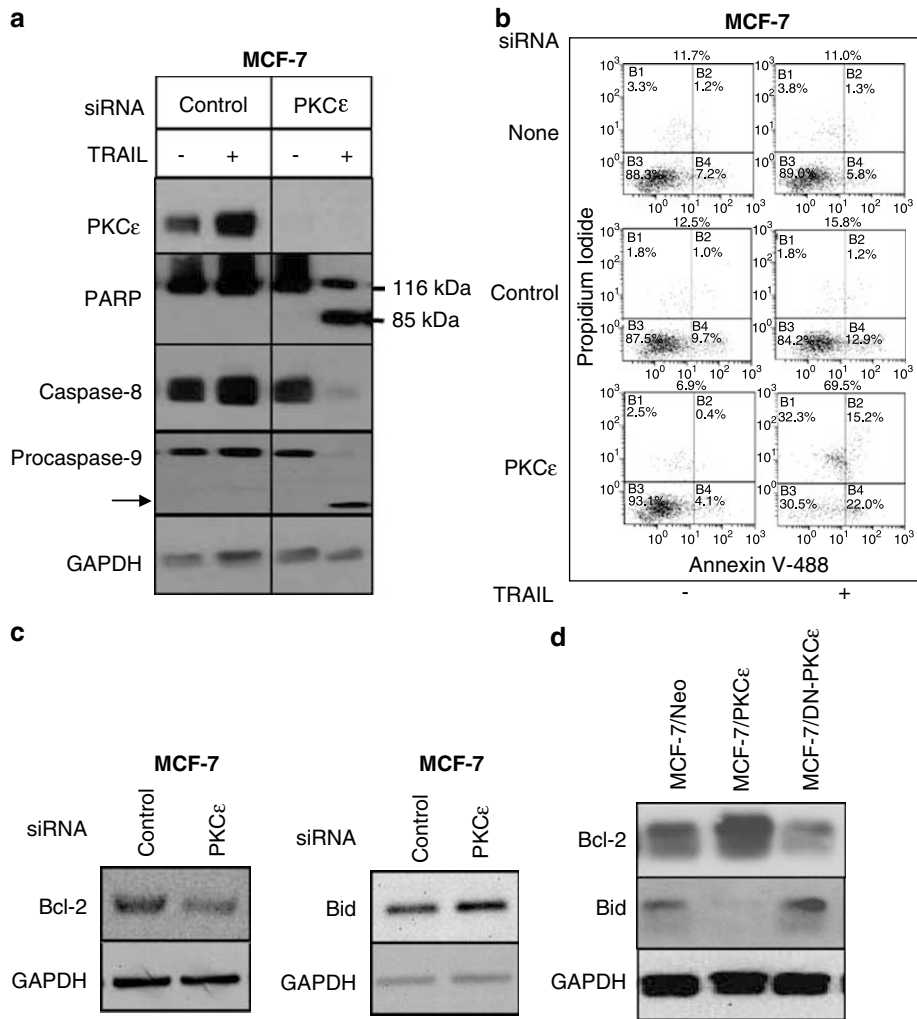
Bcl-2

Bid

GAPDH

Figure 10 Depletion of PKCε increases TRAIL sensitivity. (**a**) MCF-7 cells transfected with control siRNA or siRNA targeted against PKCε were treated with 0.01 nM TRAIL for 10 h. Western blot analysis was performed with the indicated antibodies and GAPDH was used as the loading control. (**b**) MCF-7 cells transfected with or without control siRNA or siRNA targeted against PKCε were treated with 0.01 nM TRAIL for 10 h. Cells were stained with Annexin V-Alexa 488 conjugate and PI, and analyzed by flow cytometry. The results are representative of two independent experiments. (**c**) MCF-7 cells were transfected with control siRNA or siRNA targeted against PKCε. Cell lysates were analyzed by Western blotting with Bcl-2 or Bid antibody. GAPDH was used to control for loading differences. Results are representative of three independent experiments. (**d**) Cell lysates from MCF-7/Neo, MCF-7/PKCε and MCF-7/DN-PKCε were analyzed by Western blotting with the indicated antibodies. Results are representative of two independent experiments

Preparation of cytosolic and mitochondrial extracts. Following treatment with or without TRAIL, cells were collected, washed twice with PBS and subcellular fractionation was performed as described previously.[39]

Immunoblot analysis. Equivalent amounts of protein from total cellular extracts were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene difluoride membrane. Immunoblot analyses were performed as described previously.[39]

DISC analysis. Analysis of DISC formation was performed as described previously[40] with some modifications. Briefly, cells were treated with 500 ng/ml human recombinant soluble FLAG-tagged TRAIL and 1.5 μg/ml anti-FLAG monoclonal antibody (enhancer) for 30 min. Cells were lysed in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% TX-100, 0.5% NP-40, 25 mM β-glycerophosphate, 10 mM NaF, 1.0 mM Na₃VO₄ and protease inhibitor cocktail, and protein complexes were precipitated by incubation with protein A/G agarose. Beads were washed four times with immunoprecipitation buffer and denatured by

boiling in Laemmli buffer containing β-mercaptoethanol. Proteins were separated by SDS-PAGE and Western blot analyses were performed.

Annexin V/PI-binding assay. Cells were treated with or without TRAIL. At the end of the incubation, both detached cells and attached cells were collected and washed with PBS. Cells were then stained with Annexin V-Alexa 488 conjugate and PI according to the manufacturer's protocol and analyzed using a flow cytometer (Coulter Epics).

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