PKCε induces Bcl-2 by activating CREB

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Abstract. Protein kinase C epsilon (PKCε) is a transforming oncogene and an important anti-apoptotic protein. We previously demonstrated that overexpression of PKCε in MCF-7 breast cancer cells caused an increase in anti-apoptotic Bcl-2 and a decrease in pro-apoptotic Bid, attenuating tumor necrosis factor-α (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. The objective of our present study was to determine the mode of induction of Bcl-2 by PKCε in breast cancer cells. siRNA silencing of either PKCε or Akt in MCF-7 cells, which overexpress Akt, decreased Bcl-2 protein and mRNA levels. However, knockdown of PKCε, but not Akt, led to the decrease in Bcl-2 at both protein and mRNA levels in MDA-MB-231 breast cancer cells, which overexpress PKCε but contain little constitutively-active Akt. Knockdown of PKCε decreased phosphorylation of cAMP response element-binding protein (CREB) at Ser133 in MDA-MB-231 cells, and depletion of CREB by siRNA decreased Bcl-2 at both the protein and mRNA levels. In addition, knockdown of CREB sensitized MDA-MB-231 cells to TRAIL-mediated cell death. These results suggest that PKCε regulates Bcl-2 induction through activation of the transcription factor CREB.

Introduction

The protein kinase C (PKC) family of proteins have emerged as key regulators of signal transduction and cellular responses. They represent a family of phospholipid-dependent serine/threonine kinases consisting of 10 isozymes that are classified into classic (α, ß1, ß2 and γ), novel (δ, ε, η and θ), and atypical PKCs (ζ and ι/λ). These isozymes differ in their structure, function and biochemical properties (1). PKCε has been reported to function as a transforming oncogene (2,3) and is overexpressed in several types of cancers (4,5). It also functions as an anti-apoptotic protein in several systems (6-8).

There are two major pathways of cell death: the receptor-mediated or extrinsic pathway and the mitochondrial or intrinsic pathway (9). Binding of ligands to the tumor necrosis factor-α (TNF) superfamily triggers recruitment of initiator caspase-8 via adaptor proteins to form a death-inducing signaling complex (DISC), resulting in the activation of this initiator caspase. Once activated, caspase-8 can activate caspase-3 to trigger cell death (10-12). The mitochondrial or intrinsic cell death pathway is triggered by cellular stress, such as DNA damage, which induces the release of mitochondrial cytochrome c. This results in the Apaf-1-mediated recruitment and activation of initiator caspase-9 followed by activation of caspase-3 or -7 (13). Bcl-2 family proteins regulate the mitochondrial integrity (14). The receptor-mediated pathway may be amplified by the cleavage of pro-apoptotic Bcl-2 family protein Bid, which translocates to the mitochondria resulting in the release of cytochrome c and activation of caspase-9 and its downstream caspases (14). PKCε has been shown to inhibit both extrinsic and intrinsic cell death pathways (15,16).

Our laboratory recently demonstrated that PKCε exerts its anti-apoptotic effect during TNF- and TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibiting the mitochondrial cell death pathway (17). Ectopic expression of PKCε in MCF-7 cells was associated with an increase in anti-apoptotic Bcl-2 and a decrease in pro-apoptotic Bid at both the mRNA and protein levels (17). PKCε has also been demonstrated to protect against cell death through the induction of Bcl-2 in various other cellular models (8,18,19). We have shown that in MCF-7 breast cancer cells, PKCε functions upstream of Akt (20) and that Akt-mediated phosphorylation and stabilization of Hdm2 causes downregulation of p53, resulting in decrease in pro-apoptotic Bid (21). Recent reports also suggest that PKCε forms a signaling complex with Akt in mediating the induction of Bcl-2 in vascular endothelial cells (18). The mechanism by which PKCε induces Bcl-2 expression in breast cancer cells, however, is not clear.

cAMP response element-binding protein (CREB) has been implicated in the transcriptional regulation of *Bcl-2* gene expression (22). The Bcl-2 promoter region contains a CREB-binding site at its upstream promoter region (23,24). Phosphorylation of CREB at Ser133 is required for its transcriptional activation (25-27). Akt has been shown to activate CREB by phosphorylating at Ser133 residue (25,26).

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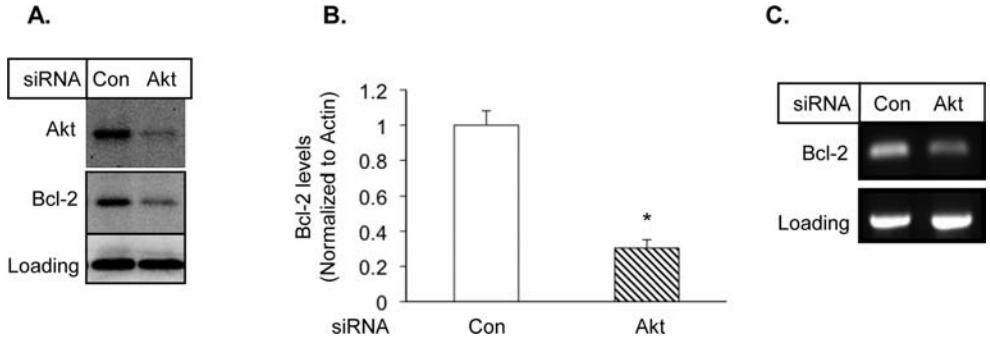

Figure 1. Depletion of Akt in MCF-7 cells decreased the levels of Bcl-2. MCF-7 cells were transfected with control or Akt siRNA. (A) Forty-eight hours after transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used as a control for loading differences. (B) Densitometric quantification of Bcl-2 from at least three separate experiments corrected for loading. Data are the mean ± S.E.M. The asterisk (*) indicates significant difference from control siRNA-transfected cells ($P<0.05$) using paired Student's t-test. (C) Twenty-four hours after transfection, reverse transcription (RT)-PCR analysis was performed for detecting *BCL2* mRNA as described in Materials and methods.

Furthermore, it has been reported that Akt induces Bcl-2 expression through the transcription factor CREB (28,29). Activation of PKC by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has also been reported to phosphorylate and thereby activate CREB in murine cardiac cells (30). In the present study, we examined if PKCε induces Bcl-2 via CREB during TRAIL-induced apoptosis in breast cancer cells, and if Akt is involved in PKCε-mediated induction of Bcl-2. Our results show that PKCε regulates Bcl-2 induction through activation of the transcription factor CREB. Furthermore, PKCε can activate CREB via Akt-independent mechanism.

Materials and methods

Materials. Human recombinant TRAIL was purchased from R&D systems (Minneapolis, MN). Monoclonal antibody to Bcl-2 and polyclonal antibody to PKCε were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to Akt, CREB and phospho-CREB (Ser133) were purchased from Cell Signaling Technologies (Beverly, MA). Small interfering RNA (siRNA) Smartpool against Akt, CREB, PKCε and non-targeting Smartpool siRNA were obtained from Dharmacon (Lafayette, CO). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Propidium iodide was obtained from Molecular Probes (Eugene, OR). The enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). YO-PRO-1 staining was obtained from Invitrogen (Carlsbad, CA).

Cell culture. MCF-7 and MDA-MB-231 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.

siRNA transfection. siRNA Smartpool against *AKT*, *CREB* and *PRKCE* (PKCε) or non-targeting Smartpool were introduced into MCF-7 and MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen) as described previously (17). Briefly, cells were seeded one day before transfection. RNA was isolated following 24 h after siRNA transfection for RT-PCR analysis. For Western blot analysis, the cells were treated with or without TRAIL 48 h after siRNA transfection.

Reverse transcriptase (RT)-PCR analysis. Total RNA was isolated using RNA STAT-60 from Tel-Test Inc (Friendswood, TX) as per the manufacturer's protocol, and was subjected to reverse transcription reaction using Promega (Madison, WI) reverse transcriptase enzyme (ImProm-II™). Polymerase chain reaction (PCR) amplifications were performed using the following primers: *BCL2*, forward, 5' GAC AGT CCC ATC AAA ACT CC 3'; reverse, 5' ATG ATC AGG TCC TTT TTC CA 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.

Immunoblot analysis. Equivalent amounts of protein from total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane. Immunoblot analyses were performed as described previously (31).

Cell death analysis by YO-PRO-1/PI assay. Following TRAIL treatment for 12 h, the control and siRNA-transfected cells were assessed for apoptotic and necrotic cells using YO-PRO-1 and propidium iodide (PI) staining. Cells were labeled with 0.5 μM YO-PRO-1 and 2 μM PI by incubating at 37˚C for 15 min. Labeling of the cells was visualized using a Zeiss Axiovert 40 inverted microscope with the AxioVision Rel 4.6 software (Zeiss, Göttingen, Germany). Cells staining positively for YO-PRO-1 only (green fluorescent membranes) were scored as apoptotic. Cells staining positively with propidium iodide (red fluorescent nuclei) were scored as necrotic. Merged green and red fluorescent images indicated cell death by both apoptosis and necrosis, respectively (32).

Results

PKCε can induce Bcl-2 via the Akt-independent pathway. We have previously shown that PKCε regulates the Bcl-2 level in

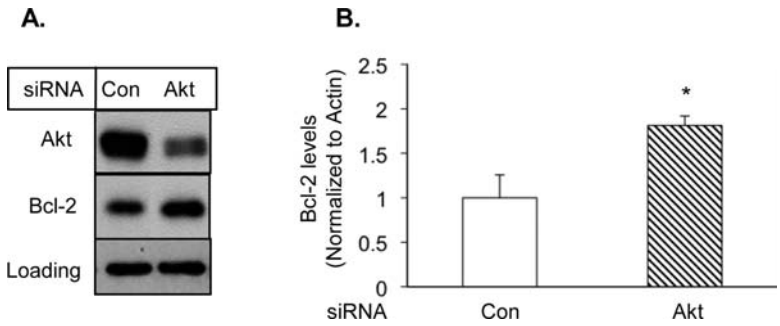

Figure 2. Depletion of Akt in MDA-MB-231 cells failed to decrease the Bcl-2 level. MDA-MB-231 cells were transfected with control or Akt siRNA. (A) Forty-eight hours after transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used to control for loading differences. (B) Densitometric quantification of Bcl-2 from at least three separate experiments corrected for loading. Data are the mean ± S.E.M. The asterisk (*) indicates significant difference from control siRNA-transfected cells (P<0.05) using paired Student's t-test.

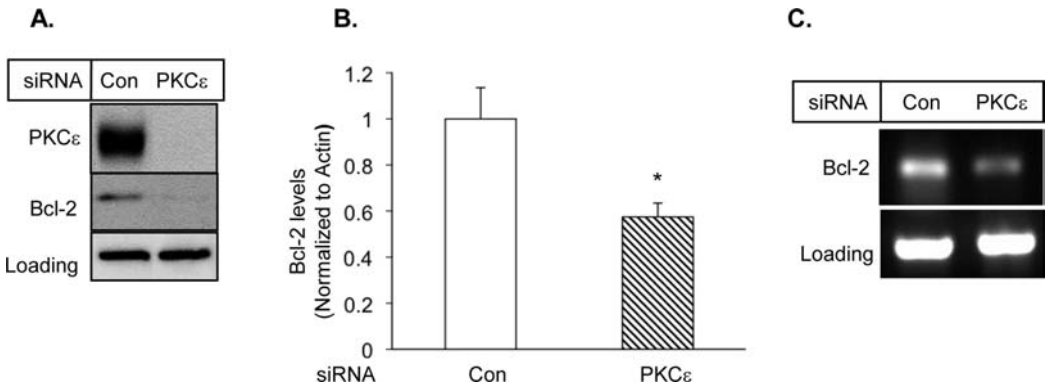

Figure 3. Knockdown of PKCε in MDA-MB-231 cells decreased the levels of Bcl-2. Cells were transfected with control or PKCε siRNA. (A) Forty-eight hours after transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used to control for loading differences. (B) Densitometric quantification of Bcl-2 from at least three separate experiments corrected for loading. Data are the mean ± s.e.m. The asterisk (*) indicates significant difference from control siRNA-transfected cells (P<0.05) using paired Student's t-test. (C) Twenty-four hours after transfection, RT-PCR analysis was performed for detecting *BCL2* mRNA as described in Materials and methods.

MCF-7 cells. Since Akt has been shown to upregulate Bcl-2 in pheochromocytoma PC12 cells (28) and we have shown that PKCε acts upstream of Akt in MCF-7 cells (20), one possibility is that PKCε induces Bcl-2 levels via Akt. We therefore examined whether Akt also regulates Bcl-2 expression in MCF-7 breast cancer cells. As shown in Fig. 1, silencing of Akt in MCF-7 cells by siRNA decreased Bcl-2 at the protein (Fig. 1A and B) and mRNA (Fig. 1C) levels. Densitometric scanning of three independent experiments revealed a 3.0-fold decrease in the Bcl-2 protein levels as a result of Akt depletion (Fig. 1B).

While MCF-7 cells overexpress Akt, MDA-MB-231 breast cancer cells express high levels of PKCε but little constitutively-active Akt (33). We therefore investigated if Akt also regulates Bcl-2 expression in these cells. Fig. 2 shows that knockdown of Akt failed to decrease the level of Bcl-2 in MDA-MB-231 cells and rather increased Bcl-2 level. However, depletion of PKCε in MDA-MB-231 cells by siRNA decreased Bcl-2 at both the protein (Fig. 3A) and mRNA (Fig. 3C) level. Densitometric scanning of three individual experiments demonstrated approximately a 2-fold decrease in Bcl-2 protein levels following PKCε knockdown (Fig. 3B). Thus, PKCε may regulate Bcl-2 levels independent of Akt.

Depletion of CREB downregulates Bcl-2 expression. Since CREB is reported to be a positive regulator of Bcl-2 expression (22) and both Akt and PKC have been implicated in phosphorylating CREB at Ser133 (25), we examined if CREB regulates Bcl-2 in MCF-7 and MDA-MB-231 cells. Knockdown of CREB in MDA-MB-231 cells was associated with a decrease in Bcl-2 at the protein (Fig. 4A and B) and mRNA (Fig. 4C and D) levels. However, the levels of other anti-apoptotic Bcl-2 family proteins, such as Bcl-X_L or Mcl-1, were not decreased by CREB knockdown. In fact, depletion of CREB was associated with an increase in Bcl-X_L. Knockdown of CREB in MCF-7 cells also caused a decrease in Bcl-2 (Fig. 5). Thus, CREB may regulate Bcl-2 levels regardless of the status of Akt.

To determine whether PKCε acts upstream of CREB, we examined the consequence of PKCε depletion on CREB phosphorylation at Ser133 residue. The knockdown of PKCε was associated with a decrease in CREB phosphorylation at Ser133 site in both MDA-MB-231 (Fig. 6A) and MCF-7 (Fig. 6B) cells. Thus, PKCε may regulate CREB phosphorylation and activation.

Knockdown of CREB enhances TRAIL-mediated cell death. We then examined the effect of CREB knockdown on

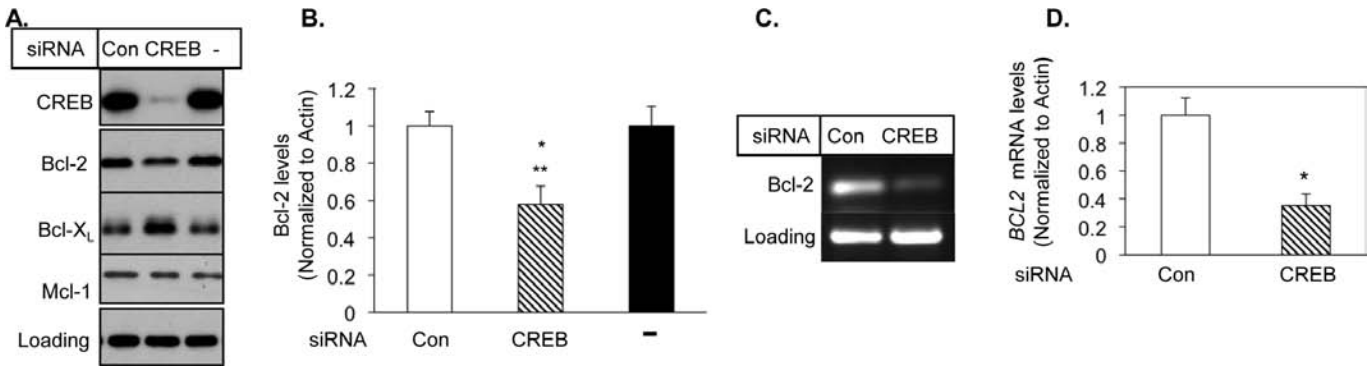

Figure 4. Knockdown of CREB in MDA-MB-231 cells decreased the levels of Bcl-2. Cells were transfected with control or CREB siRNA. (A) Forty eight hours after transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used to control for loading differences. (B) Densitometric quantification of Bcl-2 from at least three separate experiments corrected for loading. Data are the mean ± S.E.M. The asterisks (**) indicate significant difference from control siRNA-transfected cells (P<0.01) and the asterisk (*) indicates significant difference from non-transfected cells (P<0.05) using paired Student's t-test. (C) Twenty-four hours after transfection, RT-PCR analysis was performed for detecting *BCL2* mRNA as described in Materials and methods. (D) Densitometric quantification of *BCL2* mRNA from at least three separate experiments corrected for loading. Data are the mean ± S.E.M. The asterisk (*) indicates significant difference from control siRNA-transfected cells (P<0.05) using paired Student's t-test.

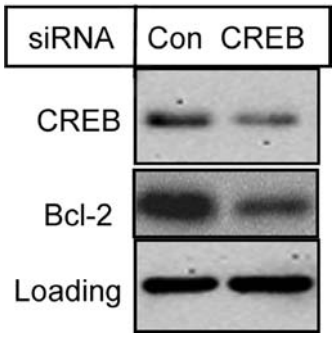

Figure 5. Knockdown of CREB in MCF-7 cells decreased the levels of Bcl-2. Cells were transfected with control or CREB siRNA. (A) Forty-eight hours after transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used to control for loading differences.

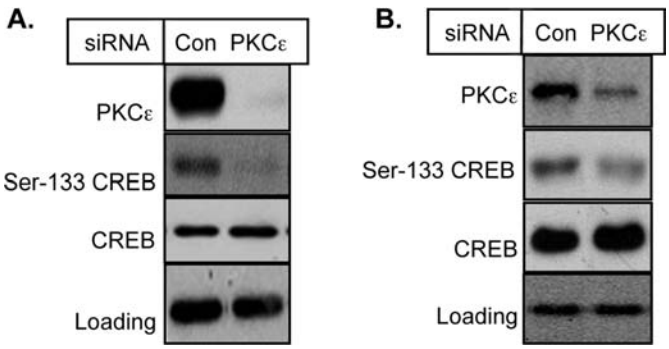

Figure 6. Silencing PKCε in MDA-MB-231 and MCF-7 cells decreased the levels of CREB phosphorylation at Ser133 site. MDA-MB-231 and MCF-7 cells were transfected with control or PKCε siRNA. (A and B) Forty-eight hours after transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. Actin was used to control for loading differences.

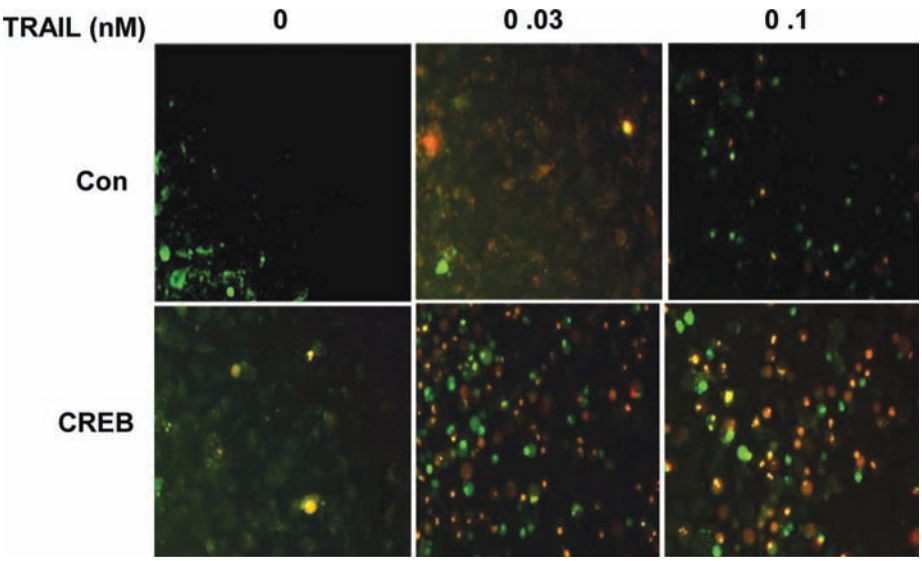

Figure 7. Knockdown of CREB in MDA-MB-231 cells increased TRAIL-mediated cell death. Cells were transfected with control or CREB siRNA. Forty-eight hours after transfection, the cells were treated with the indicated concentrations of TRAIL for 12 h. After TRAIL treatment the cells were treated with YO-PRO-1 and PI as indicated in the Methods and materials. They were incubated for 15 min and visualized for fluorescent staining using Zeiss Axiovert 40 inverted microscope. The dead cells were detected as described in the Materials and methods.

TRAIL-mediated cell death in MDA-MB-231 cells. We monitored cell death by staining cells with the cell-impermeable dye YO-PRO-1 which stains apoptotic cells but not live cells (32), and with the cell-permeable dye propidium iodide (PI). In the control siRNA-tranfected cells, TRAIL had little effect on cell death as evident by the staining of YO-PRO-1 and PI whereas CREB-depleted cells were sensitized to TRAIL-induced cell death (Fig. 7). Thus, CREB may be important in contributing to TRAIL resistance in MDA-MB-231 cells.

Discussion

The involvement of PKCε in cell survival is well-documented (4,34). We previously demonstrated that PKCε exerts its anti-apoptotic function during receptor-mediated cell death by increasing the level of anti-apoptotic Bcl-2 and decreasing the level of pro-apoptotic Bid (17). We have also shown that PKCε regulates the expression of Bid via the Akt/Hdm2/p53 pathway (21). The results of our present study demonstrate that PKCε regulates the expression of anti-apoptotic Bcl-2 through the transcriptional factor CREB.

Bcl-2 promoter was shown to contain a cyclic AMP-responsive element in its upstream promoter region (P1 region). This site appeared to play a major role in the induction of Bcl-2 expression to rescue apoptosis during B-cell activation (23). It was reported that the induction of Bcl-2 was dependent on PKC, but not, protein kinase A (PKA) because TPA led to an increase in Bcl-2 in these cells. In NIH 3T3 cells, synergistic activation of CREB by forskolin and TPA was abolished by the PKC inhibitor GF109203X (27). However, the identity of the PKC isozyme responsible for CREB activation was not known. Later, it was shown that TPA phosphorylates CREB at Ser133 (30). PKCε was implicated in phosphorylating CREB at Ser133 site, since TPA induced translocation of PKCε to the nucleus. While it is well established that PKC phosphorylates CREB, the isozyme responsible for phosphorylating CREB may vary with cell type and stimulus. Several PKC isozymes, including PKCα, -δ and -ε have been implicated in phosphorylating CREB and enhancing its transcriptional activity.

It has also been demonstrated that CREB is a target for Akt, as overexpression of Akt stimulated target gene expression via phosphorylation of CREB at Ser133 site (35). Subsequent studies showed that insulin-like growth factor-1, as well as Akt, caused upregulation of Bcl-2 through CREB in PC12 cells (24,28). Akt/CREB axis was also implicated in transcriptional activation of Bcl-2 by complement factor 5a in HL-60-derived neutrophils (22), or t-Darpp (dopamine and cyclic AMP-regulated phosphoprotein) in gastric cancer AGS cells (29).

We have previously shown that overexpression of PKCε in MCF-7 breast cancer cells enhanced TNF-induced activation of Akt and that knockdown of PKCε attenuated activation of Akt by TNF (20). Furthermore, PKCε exerted its anti-apoptotic effect at least in part via Akt (20). Inhibition/depletion of both Akt and PKCε sensitized these cells to TNF and TRAIL (20,33). We have previously shown that PKCε upregulates Bcl-2 mRNA and protein in response to TRAIL (17) and the present study shows that Akt also regulates Bcl-2 at the transcriptional and protein levels. Moreover, in human vascular endothelial cells, PKCε caused the induction of Bcl-2 by forming a signaling complex with Akt (18). Thus, it is conceivable that PKCε induces Bcl-2 levels via Akt.

The highly-aggressive and metastatic MDA-MB-231 breast cancer cells overexpress PKCε but contain little phosphorylated Akt (33,34). The PKC-specific inhibitor bisindolylmalemide sensitized these cells to TNF-induced apoptosis whereas the PI3K/Akt inhibitor Ly294002 had little effect (33), suggesting that PKCε exerts its anti-apoptotic effect independent of Akt. We therefore exploited these cells to determine if PKCε regulates the Bcl-2 level directly or via Akt. We found that knockdown of PKCε decreased Bcl-2 mRNA and protein levels and this was associated with a decrease in CREB phosphorylation. Furthermore, knockdown of CREB decreased Bcl-2 levels and sensitized cells to TRAIL, suggesting that PKCε may exert its anti-apoptotic effect through CREB-mediated induction of Bcl-2. However, knockdown of Akt not only failed to decrease Bcl-2 levels but rather enhanced it. It is not clear why depletion of Akt caused upregulation of Bcl-2. Nevertheless, these results demonstrate that PKCε does not require Akt to induce Bcl-2 levels in MDA-MB-231 cells.

TRAIL is an attractive target for cancer therapy because it exhibits selective cytotoxicity to cancer cells but spares normal cells. However, some cancer cells escape TRAIL-induced cell death due to the presence of anti-apoptotic proteins, and this resistance to TRAIL limits its therapeutic use. Bcl-2 family proteins play important roles in TRAIL resistance. Our observations reveal that in breast cancer cells which lack active Akt, PKCε may induce Bcl-2 levels by activating CREB. Therefore, CREB may also be important in contributing to TRAIL resistance.

Acknowledgements

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