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Protein Kinase C-θ Mediates a Selective T Cell Survival Signal Via Phosphorylation of BAD

Martin Villalba, 2 Paul Bushway, and Amnon Altman 2

Protein kinase C (PKC)-activating phorbol esters protect T cells from Fas-induced apoptosis. However, the mechanism of this protective effect and the identity of the relevant PKC isoform(s) are poorly understood. Here, we show that PKCθ plays a selective and important role in this protection. Fas triggering led to a selective caspase-3-dependent cleavage of the enzyme and proteasome-mediated degradation and inactivation of its catalytic fragment. These events preceded the onset of apoptosis. Pharmacological inhibition of PKCθ promoted Fas-mediated apoptosis in three different types of T cells. Conversely, constitutively active PKCθ (and, to a lesser degree, PKCε) selectively protected T cells from Fas-induced apoptosis. We provide evidence that the distant Bcl-2 family member, BAD, is a PKCθ substrate, is phosphorylated by TCR stimulation, and can mediate at least in part the anti-apoptotic effect of PKCθ. The Journal of Immunology, 2001, 166: 5955–5963.

T riggering of the Ag-specific TCR initiates signaling cascades that induce T cell activation and proliferation. Once the Ag has been cleared, distinct mechanisms terminate the response and eliminate or inactivate the excess of Ag-specific T cells. One such mechanism is the process known as activation-induced cell death (AICD). 3 It involves the TCR-induced expression of the Fas ligand (FasL) on the surface of T cells. Once FasL is expressed, it induces T cell apoptosis through activation of its receptor, Fas. Aberrations in this pathway may also play a role in the survival and expansion of malignant lymphocytes. In addition, non-T cells expressing FasL on their surface can eliminate Fas-expressing T cells. This process is important for the maintenance of the so-called immune-privileged sites and for the development of certain tumors that escape an immune response.

TCR engagement induces activation of protein kinase C (PKC), a family of Ser/Thr kinases that consists of three subfamilies: cPKC (or conventional PKCs: α, β1, β2, and γ), aPKC (or atypical PKC: ζ and λ), and nPKC (or novel PKCs: δ, ε, θ, and μ). Phorbol ester stimulation, which activates PKC, protects various cells, including T cells, from apoptosis (1–5). However, the PKC isoform(s) involved in this process has not been definitively identified in T cells.

Among T cell-expressed PKC enzymes, PKCθ plays a particularly important role in TCR/CD28 signaling pathways (6). PKCθ was isolated as an nPKC isoform expressed selectively in T cells (7). It displays several unique functions in T cells, including its ability to activate the c-Jun N-terminal kinase (JNK)/AP-1 pathway and induce the IL-2 gene in synergy with calcineurin (8–10), and its translocation to the site of cell contact between Ag-specific T cells and APCs, where it colocalizes with the TCR to form the central core of the T cell supramolecular activation cluster (11). PKCθ translocation is mediated by a Vav-regulated process that also involves Rac and the cytoskeleton (12). In addition, we recently found that CD28 costimulation promotes TCR-induced PKCθ translocation and activation. PKCθ then activates NF-κB and the CD28 response element in the IL-2 gene promoter (13, 14), providing evidence that PKCθ is functionally coupled to TCR/CD28 costimulation. The importance of PKCθ in T cell activation is indicated by the findings that PKCθ-deficient mature T cells display a severe activation defect in response to TCR/CD28 ligands (15).

In this work, we analyzed the role of PKCθ function in TCR- and/or PMA-induced survival signals that protect T cells from Fas-induced apoptosis. We show that activated PKCθ provides a protective survival signal to T cells, which can be accounted for, in part, by its ability to phosphorylate BAD.

Materials and Methods

Abs and reagents

Protein A/G Plus-agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs generated against human PKCε (aa270–427), PKCθ (aa114–289), PKCε (aa1–175), PKCθ (aa21–217), and PKCγ (aa94–590) were obtained from Transduction Laboratories (Lexington, KY). The anti-actin mAb was obtained from ICN Pharmaceuticals (Aurora, OH), and anti-human or anti-mouse Fas mAbs were obtained from Upstate Biotechnology (Lake Placid, NY) or BD PharMingen (San Diego, CA), respectively. PD98059 and polyclonal Abs against native BAD or phospho-BAD (Ser112 or Ser116) were obtained from New England Biolabs (Beverly, MA). The anti-human CD28 and CD3 mAbs and PE- or FITC-conjugated annexin were purchased from BD PharMingen. Donkey anti-rabbit or sheep anti-mouse IgG Abs and GST-Sepharose 4B were obtained from Amersham (Piscataway, NJ), and the anti-Xpress tag mAb was purchased from Invitrogen (San Diego, CA). An anti-14-3-3-3 Ab that recognizes 14-3-3-3r and 14-3-3-3r isoforms has been described previously (16). Z-VaL-Ala-Asp-CH₂-F (Z-VAD) was obtained from Kamiya Biomedical (Seattle, WA). SB203580 was a gift from SmithKline Beecham Pharmaceuticals (Philadelphia, PA). LY294002, wortmannin, MG132, rottlerin, and G06976 were purchased from Calbiochem (San Diego, CA). The Isoler
Phosphorylation inhibition with BAY 11-7082 was purchased from Biomol (Plymouth Meeting, PA). All other reagents were obtained from Sigma (St. Louis, MO).

**Plasmids and NF-κB reporter assay**

The cDNAs encoding Xpress epitope-tagged human wild-type PKCα and PKCε, the corresponding constitutively active (A/E) mutants, and the dominant negative (kinase-inactive; K/R) mutants of these PKCs as well as rat PKCε and mouse PKCζ in the eukaryotic expression vector pEBNeo have been described previously (12, 13, 17). A green fluorescent protein (GFP) expression plasmid in the pEF vector was obtained from Dr. G. Baier (University of Innsbruck, Innsbruck, Austria). The cDNAs encoding wild-type or dominant negative (SS32/36AA) IkBa in the eukaryotic expression vector pCMV4 were obtained from Dr. W. C. Greene (University of California, San Francisco, CA). The mammalian expression plasmid encoding murine BAD (pEBG-mBAD) was acquired from New England Biolabs. The BAD-coding region was excised by double digest with BamHl and NotI and ligated into the corresponding sites in the bacterial expression vector pGEX-5X-1 (Pharmacia, Kalamazoo, MI). The resulting expression vector pCMV4 were obtained from Dr. W. C. Greene (University of California, San Francisco, CA). The mammalian expression plasmid encoding murine BAD (pEBG-mBAD) was acquired from New England Biolabs. The BAD-coding region was excised by double digest with BamHI and NotI and ligated into the corresponding sites in the bacterial expression vector pGEX-5X-1 (Pharmacia, Kalamazoo, MI). The cultures were grown to log phase, induced with 0.5 mM isopropyl β-D-thiogalactoside, and GST-BAD was isolated on GST-Sepharose 4B. The NF-κB-luciferase reporter plasmid and the method for assaying its activity have been described previously (13).

**Cell culture and transfection**

Jurkat E6.1 T cells, SV40 large T Ag-transfected human leukemic Jurkat T (Jurkat-TAg), and A1.1 T hybridoma cells were grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1 × MEM nonessential amino acid solution (Life Technologies), and 100 U/ml each of penicillin G and streptomycin. Cells in a logarithmic growth phase were transfected with the indicated amounts of plasmid DNAs by electroporation as described previously (12, 17). Human PBMC were prepared from healthy volunteers by standard Ficoll-Hypaque centrifugation and cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1 × MEM nonessential amino acid solution (Life Technologies), and 100 U/ml each of penicillin G and streptomycin. Cells in a logarithmic growth phase were transfected with the indicated amounts of plasmid DNAs by electroporation as described previously (12, 17). Human PBMC were prepared from healthy volunteers by standard Ficoll-Hypaque centrifugation and cultured in the presence of an activating anti-CD3 mAb (OKT3; 1 μg/ml) and notl and ligated into the corresponding sites in the bacterial expression vector pGEX-5X-1 (Pharmacia, Kalamazoo, MI). The resulting expression vector pCMV4 were obtained from Dr. W. C. Greene (University of California, San Francisco, CA). The mammalian expression plasmid encoding murine BAD (pEBG-mBAD) was acquired from New England Biolabs. The BAD-coding region was excised by double digest with BamHI and NotI and ligated into the corresponding sites in the bacterial expression vector pGEX-5X-1 (Pharmacia, Kalamazoo, MI). The cultures were grown to log phase, induced with 0.5 mM isopropyl β-D-thiogalactoside, and GST-BAD was isolated on GST-Sepharose 4B. The NF-κB-luciferase reporter plasmid and the method for assaying its activity have been described previously (13).

**Immunoprecipitation and immunoblotting**

Cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM sodium pyrophosphate, 1 mM Na3VO4 plus 1% Nonidet P-40, and 10 μg/ml each of aprotinin and leupeptin) for 10 min on ice. After centrifugation (16,000 × g, 10 min, 4°C), the supernatants were incubated with optimal concentrations of Abs for 1 h at 4°C, followed by 30 μl of protein A/G Plus-agarose and overnight incubation at 4°C; for GST-BAD precipitation, they were incubated with GST-Sepharose 4B for 1 h at 4°C. Samples were washed four times in lysis buffer, and precipitates were dissolved in Laemmli buffer and resolved by SDS-PAGE. Electrophoresed samples were processed for immunoblot analysis as previously described (12, 17).

**Induction and assessment of apoptosis**

Cells were induced to undergo apoptosis by treatment with an anti-human or anti-mouse Fas mAb (50 ng/ml) or by serum deprivation. Apoptosis was assessed by staining the cells using annexin V-FITC or annexin V-PE and analyzing them on a FACScan analyzer following the manufacturer’s instructions. In experiments involving pretreatment with PKC inhibitors or Z-VAD, apoptosis was determined by propidium iodide (PI) staining (17). In experiments involving PBLs, the cellular shrinkage associated with apoptosis was also assessed by the decrease in light scatter.

**Results**

Fas ligation induces selective activation and subsequent cleavage and degradation of PKCθ

DNA-damaging agents induce selective cleavage of PKCθ in U937 cells, and this caspase-3-induced event has been linked to activation of PKCθ and inhibition of apoptosis (18). We determined whether Fas ligation also causes PKCθ cleavage in T cells. Treatment of Jurkat T cells with an anti-Fas mAb induced cleavage of PKCθ, as indicated by the time-dependent decrease in the expression of intact PKCθ and the corresponding increase in the level of its regulatory fragment (Fig. 1a, top panel). This effect was selective, because PKCo remained intact during this process (Fig. 1a, middle panel; see also Fig. 3b), even at times when 80% of the cells were annexin positive (data not shown; Fig. 4). As a control, actin also remained intact up to 24 h after anti-Fas treatment (Fig. 1a and b, bottom panels). PKCθ cleavage (Fig. 1b, top panel) and cell death (Fig. 1c) were blocked in parallel by the caspase-3 inhibitor Z-VAD, suggesting that Fas-induced PKCθ cleavage is mediated by caspase-3. The caspase-3 substrate poly(ADP-ribose) polymerase displayed a kinetically similar cleavage to that of PKCθ under the same conditions (data not shown).
The caspase-dependent cleavage of PKC\(\theta\) correlated with loss of its catalytic fragment, as indicated by immunoblotting with a C-terminal-specific Ab (Fig. 1d). This loss suggested that Fas ligation induces degradation and subsequent inactivation of the catalytic fragment of PKC\(\theta\). This possibility was analyzed by assessing the effect of a selective proteasome inhibitor, MG132, on the Fas-mediated change in PKC\(\theta\) expression. MG132 pretreatment inhibited the degradation of PKC\(\theta\), resulting in the appearance of an intact catalytic fragment, which was detectable even 24 h after Fas ligation (Fig. 1d). Thus, Fas triggering appears to induce selective, proteasome-mediated degradation of the catalytic fragment of PKC\(\theta\), subsequent to the caspase-mediated cleavage of the holoenzyme.

**PKC\(\theta\) induces a selective anti-apoptotic effect in T cells**

The Fas-induced degradation and inactivation of PKC\(\theta\) raised the possibility that PKC\(\theta\) mediates a survival signal that is ablated following its degradation, thereby allowing the Fas-associated apoptotic machinery to become fully functional. We initially used a pharmacological approach to address this question. We previously demonstrated that rottlerin blocked PKC\(\theta\) activity, but not the activity of Ca\(^{2+}\)-dependent PKC\(\varepsilon\) (17). We took advantage of this property to demonstrate the role of PKC\(\theta\) in regulation of FasL expression (17) and activation of NFAT (12) or NF-\(\kappa B\) (13). Thus, we evaluated the effects of rottlerin pretreatment on Fas-mediated apoptosis.

In untreated cells, anti-Fas induced a relatively weak apoptosis after short periods, which was first observed after about 3 h and reached a level of 20–25% above background at 6 h (Fig. 2a). Rottlerin pretreatment alone induced a slight apoptotic response. However, the combination of rottlerin pretreatment and Fas ligation induced massive apoptosis that reached a level of 50–60% after 4 h. The concentration of rottlerin used in this experiment was previously found to inhibit the anti-CD3/CD28-induced activation of PKC\(\theta\) in T cells (13). For comparison, we tested in a similar experiment the effects of G06976, a PKC inhibitor selective for Ca\(^{2+}\)-dependent PKCs (19), which did not inhibit PKC\(\theta\). In contrast to rottlerin, G06976 did not have any detectable effect on the basal or anti-Fas-induced apoptosis (Fig. 2b), even when used at concentrations that completely blocked the activity of PKC\(\varepsilon\).

To demonstrate that these effects are not unique to Jurkat T cells, we performed similar experiments with two other types of T cells, i.e., A1.1 T hybridoma cells (Fig. 2c), which have often been used as a model of AICD, and anti-CD3-activated human peripheral blood T cells (Fig. 2d). We observed an effect similar to that obtained in Jurkat cells, i.e., a synergy between anti-Fas and rottlerin in the induction of apoptosis. The relatively low level of apoptosis displayed by activated normal T cells (Fig. 2d) is consistent with other studies (20). Because rottlerin was originally found to inhibit PKC\(\varepsilon\) (21), we examined the expression of this isoenzyme in the different T cells. The results (Fig. 2e, top panel) demonstrate that Jurkat cells of three different origins and 8-day
anti-CD3-activated (i.e., T cell-enriched) human PBLs expressed barely detectable levels of PKC\(\delta\). In contrast, fresh PBLs, a B human lymphoma line (Dakiki), or a cerebellum extract readily expressed detectable PKC\(\delta\). PKC\(\theta\) displayed a reciprocal expression pattern (Fig. 2e, middle panel). We also confirmed earlier findings (1–5) by showing that pretreatment of Jurkat T cells with PMA (which activates cPKC and nPKC enzymes) protected the cells from Fas-induced apoptosis, as indicated by the ~70% reduction in the level of apoptotic cells (Fig. 2f). In contrast, a calcium ionophore, ionomycin, did not

**FIGURE 3.** PKC\(\theta\) selectively induces a T cell survival signal through its catalytic activity. a, Jurkat-TAg cells (1 \(\times\) 10\(^3\)) were transfected with the indicated constitutively active (A/E) PKC plasmids or empty vector (5 \(\mu\)g each) together with a GFP expression vector (2.5 \(\mu\)g). Two days later, the cells were treated with an anti-Fas mAb for the indicated times. The number of GFP-positive apoptotic cells was determined by annexin V-PE staining and FACS analysis. The data are presented as the mean ± SE of three experiments. b, Jurkat E6.1 cells (1 \(\times\) 10\(^6\)) were treated with an anti-Fas mAb for the indicated times. Cell lysates were fractionated by SDS-PAGE and immunoblotted with PKC-specific mAbs to visualize the full-length PKC isoforms. c, Jurkat-TAg cells were cotransfected with empty vector (5 \(\mu\)g), constitutively active (A/E; 5 \(\mu\)g), wild-type (wt; 10 \(\mu\)g), or dominant negative (K/R; 10 \(\mu\)g) PKC\(\theta\) plus GFP. Cells were treated and analyzed as described in a. The data represent the mean ± SE of two experiments. The similar expression levels of the different PKC constructs were verified by immunoblotting with an anti-Xpress Tag mAb (data not shown).

**FIGURE 4.** NF-\(\kappa\)B does not have a major role in the PKC\(\theta\)-mediated survival signal. a, Jurkat-TAg cells (1 \(\times\) 10\(^3\)) were cotransfected with wild-type (wt) or dominant negative (S/S) IkB plasmids (10 \(\mu\)g each), PKC\(\theta\)-A/E (5 \(\mu\)g), and a GFP reporter plasmid (2.5 \(\mu\)g). After 2 days, the cells were stimulated with an anti-Fas mAb for the indicated times, and the proportion of apoptotic cells in the GFP-positive population was determined by annexin V-PE staining. b, Inhibition of PKC\(\theta\)-induced NF-\(\kappa\)B activation by dominant negative IkB was assessed as a positive control for the effectiveness of IkB transfection. c, Jurkat-TAg cells were transfected with PKC\(\theta\)-A/E plus GFP plasmids. Two days later, the cells were stimulated for 6 h with anti-Fas in the presence of different concentrations of MG132. Apoptosis was determined as described in a. Immunoblotting with the corresponding Abs confirmed similar expression levels of the PKC\(\theta\) and IkB plasmids, respectively (data not shown).
modulate the apoptotic response in the absence or the presence of PMA. In parallel experiments, we found that costimulation with PMA plus ionomycin was required to induce IL-2 production by the same cells (data not shown). Because the αPKC subfamily does not respond to PMA, and only the cPKC subfamily requires Ca$^{2+}$ for full activation, these findings suggest that the PKC isoform involved in T cell survival most likely belongs to the nPKC subfamily.

To more directly assess the role of PKCθ in mediating a T cell survival signal, we transiently transfected Jurkat cells with constitutively active mutants of different PKC isoforms and determined their effects on Fas-mediated apoptosis. We previously showed that these mutants are functional in T cells, and furthermore, they are expressed at similar levels (12, 13, 17). The cells were cotransfected with wild-type PKCθ (i.e., θ and ε) protected cells from undergoing apoptosis following Fas ligation (Fig. 3a), and PKCθ was more active than PKCε. PKCα and PKCζ were essentially inactive. Moreover, in agreement with Fig. 1, Fas engagement induced a selective loss of PKCθ (Fig. 3b). PKCα and PKCζ remained intact during the whole period of treatment, and the level of PKCε started to decrease after a long period of incubation (20 h) and, to a small extent, by comparison with PKCθ. These data show that PKCθ protects T cells from Fas-induced apoptosis, and that PKCθ is selectively cleaved after Fas engagement.

To study the role of the catalytic activity of PKCθ in this protective effect, we used different forms of PKCθ plasmids (Fig. 3c). Only the constitutively active mutant (A/E) conferred effective protection. The kinase-inactive (K/R) mutant was completely ineffective, and in fact, it increased the level of cell death. Minimal protection was observed with wild-type PKCθ. However, PMA treatment of cells transfected with wild-type PKCθ induced greater protection compared with empty vector-transfected cells (data not shown). These results suggest that PKCθ must be activated to protect T cells from apoptosis. The next series of experiments was undertaken to elucidate the protective pathway activated by PKCθ.

Role of NF-κB in the PKCθ-mediated survival signal

We recently found that PKCθ activates the NF-κB cascade in T cells (13). This transcription factor protects different cell types from apoptosis (22). Therefore, we studied whether NF-κB is involved in the PKCθ-mediated survival signal. Jurkat cells were cotransfected with constitutively active PKCθ (A/E) together with wild-type IκBα or a dominant negative, unphosphorylatable form.
PKCθ induces activation of both extracellular-signal-regulated kinase and JNK in T cells (8–10). To study the roles of these two pathways in PKCθ-mediated survival, we pretreated Jurkat cells with different inhibitors. The mitogen-activated protein/extracellular signal-regulated kinase 1 inhibitor PD98059 and the p38/JNK inhibitor SB203580, which was found to inhibit only p38 at 4 μM and both p38 and JNK at 40 μM (23), were both ineffective in reversing the PKCθ-mediated protective signal (data not shown). Similarly, the phosphoinositide 3-kinase inhibitors, LY294002 and wortmannin, as well as combinations of these drugs did not have a significant effect on the ability of PKCθ to protect the cells from Fas-mediated apoptosis (data not shown). These negative results suggest an alternative pathway through which PKCθ mediates its protective effect.

BAD is a substrate, and mediates the survival signal, of PKCθ

The finding that rottlerin synergizes with an anti-Fas Ab to induce rapid and massive cell death suggested that this inhibitor affects a post-transcriptional event mediated by PKCθ. The Bcl-2 family member, BAD, is phosphorylated by different kinases on three Ser residues, i.e., Ser112, Ser136, and Ser155. This phosphorylation inhibits apoptosis by preventing BAD from translocating to the mitochondria, where it binds to Bcl-2 or Bcl-xL, and inhibits their anti-apoptotic activity (24). Therefore, we studied whether PKCθ could play a role in the phosphorylation of BAD. These experiments were performed using serum-starved cells to reduce the basal phosphorylation of BAD. In unstimulated cells, basal BAD phosphorylation was observed at both Ser112 and Ser136 (Fig. 5a). Costimulation with anti-CD3 plus anti-CD28 Abs increased phosphorylation at both residues, and this effect was essentially abolished by pretreatment with the PKCθ inhibitor, rottlerin (Fig. 5a).

That this phosphorylation occurs at sites relevant for the anti-apoptotic effect of phospho-BAD (24) is also supported by the finding that BAD immunoprecipitated from anti-CD3-stimulated cells was associated with increased levels of endogenous 14-3-3 (Fig. 6b).

Because rottlerin pretreatment consistently sensitized cells to certain apoptotic stimuli (e.g., Fig. 2), we determined whether rottlerin affects the basal phosphorylation of BAD in unstimulated cells. Fig. 5b shows that rottlerin pretreatment caused dephosphorylation of endogenous BAD, mainly at Ser136. This dephosphorylation was first detected at 1 h and preceded cell death, consistent with the idea that this event predisposes T cells to apoptosis. These results suggest that basal PKCθ activity promotes cell survival by maintaining basal BAD phosphorylation.

Next, we determined the effect of transfected constitutively active PKCθ on the phosphorylation of BAD in serum-starved Jurkat leukemia or A1.1 hybridoma cells. PKCθ-A/E induced BAD phosphorylation in both Jurkat and A1.1 (Fig. 5c) cells. This phosphorylation occurred on both Ser112 and Ser136, and it was blocked by rottlerin.

We also tested the abilities of different PKC isoforms, i.e., α and θ, to phosphorylate BAD in vitro. For comparison, we also examined the phosphorylation of myelin basic protein (MBP), a common PKC substrate. PKCα was ~3-fold more effective than PKCθ in inducing MBP phosphorylation (Fig. 5d, top panel). Conversely, PKCθ was about 8-fold more effective than PKCα in phosphorylating recombinant GST-BAD (second panel from top). The two kinases did not phosphorylate GST (data not shown). Thus, by comparison with PKCα, PKCθ appears to have considerable selectivity for BAD. PKCθ phosphorylated BAD mainly at Ser136, and, to a lesser degree, at Ser112. In contrast, PKCα did not

of IkBα (SS32/36AA or IkB-S/S). However, under the same conditions, both wild-type and IkB-S/S should block the NF-κB pathway by retaining NF-κB in the cytoplasm and preventing its nuclear translocation. Neither of these IkBα plasmids had a significant effect on PKCθ-mediated protection following Fas engagement (Fig. 4a). However, under the same conditions, IkB-S/S completely blocked the activation of a NF-κB reporter plasmid induced by PKCθ-A/E (Fig. 4b). We confirmed these results by using MG132, which blocks NF-κB activation by preventing IkBα degradation in the proteosome. We have previously shown that MG132 blocks PKCθ-induced NF-κB activation in T cells (13). MG132 had a small effect on the protective signal provided by PKCθ, reducing it by 27 and 43% at concentrations of 1 and 10 μM, respectively (Fig. 4c). However, at these concentrations MG132 had a toxic effect, suggesting that the increase in cell death in PKCθ-transfected cells may be due to its toxic effect rather than inhibition of a PKCθ-mediated survival signal (Fig. 4c). Similar results were obtained using an IkB phosphorylation inhibitor, BAY 11-7082 (data not shown), indicating that IkB degradation and the resulting activation of NF-κB do not play a major role in mediating the protective effect of PKCθ.
induce detectable phosphorylation of these serine residues. Therefore, it can be concluded that PKC\(\theta\) preferentially phosphorylates BAD at sites that are relevant to the anti-apoptotic function of phospho-BAD. The bottom panel shows that equal amounts of BAD were present in all kinase reactions. We further studied this kinase selectivity in vivo by comparing the effect of rottlerin with that of \(\text{G60}\text{976}\). Consistent with the results shown in Fig. 5, a and c, rottlerin blocked the phosphorylation of Ser\(^{136}\) and Ser\(^{112}\) in BAD induced by anti-CD3/CD28 Abs, but it had no effect on serum-induced phosphorylation (Fig. 5e). \(\text{G60}\text{976}\), in contrast, was incapable of blocking the same phosphorylation event. In agreement with these results, Fig. 6a shows that a dominant negative PKC\(\theta\) mutant (PKC\(\theta\)-K/R) blocked BAD phosphorylation induced by anti-CD3.

Phosphorylated BAD is sequestered in the cytosol by binding to 14-3-3 proteins, so we next decided to study whether CD3 stimulation increases the amount of BAD-associated endogenous 14-3-3 protein in T cells. Under resting conditions, a small amount of 14-3-3 coimmunoprecipitated with BAD. This amount was increased 8-fold by \(\alpha\)-CD3 (Fig. 6b). Similar amounts of BAD were present in all immunoprecipitates (bottom panels).

The ability of PKC\(\theta\) to phosphorylate BAD at Ser\(^{136}\) and Ser\(^{112}\) and the reported anti-apoptotic effect of phosphorylation at these sites by other kinases (24–30) prompted us to examine whether transient PKC\(\theta\) overexpression can inhibit the ability of overexpressed BAD to promote apoptosis. Compared with vector-transfected cells, transfection with BAD induced a slight increase in the basal or anti-Fas-induced apoptosis of the cells (Fig. 6c). Although this increase was small, it was consistently observed in three of three experiments. This increase was reversed by coexpressed PKC\(\theta\)-A/E; in addition, PKC\(\theta\) inhibited by \(~70\%\) the anti-Fas-induced apoptosis in cells that were not transfected with BAD. We further analyzed the effect of PKC\(\theta\) on death induced by serum deprivation (Fig. 6d). Under these conditions, the promoting effect of BAD on cell apoptosis was more prominent. Thus, BAD increased the basal or serum starvation-induced apoptosis of Jurkat cells by 160 and 97\%, respectively. The BAD-induced increase in cell death was still reversed by PKC\(\theta\).

Discussion

Understanding the mechanisms that control the growth and death of immune cells is a key step for developing new therapeutic strategies for eliminating cancer or treating autoimmune diseases. In this study we analyzed the roles of distinct PKC isoforms in Fas-mediated T cell apoptosis. This analysis was prompted by findings that stimulation with PKC-activating phorbol esters protects various cell types from apoptosis (1–5) and, conversely, that pharmacological PKC inhibition facilitates apoptosis, including that in T cells (31, 32). However, the mechanism(s) through which PKC mediates this protective effect as well as the identity of the relevant PKC isoform(s) have not been established.

Here, we demonstrate that, in T cells, PKC\(\theta\) provides a survival signal that protects the cells from Fas-mediated apoptosis. We also show that Fas ligation causes caspase-mediated PKC\(\theta\) cleavage and proteasome-mediated degradation of the catalytic fragment. Finally, our results show that the proapoptotic protein, BAD, is phosphorylated under stimulation conditions that also activate PKC\(\theta\), i.e., combined CD3/CD28 costimulation and, moreover, that BAD is a substrate for PKC\(\theta\). These results are consistent with a very recent report showing that PKC\(\theta\) induces BAD phosphorylation through activation of Rsk (33). It is possible that two alternative pathways, which lead to BAD phosphorylation, are activated by PKC\(\theta\), but, depending on BAD, Rsk, and PKC\(\theta\) localization, one pathway would be predominant over the other. This is consistent with the recent finding that BAD is a PKC target in nonhemopoietic cells (29), although the identity of the relevant PKC isoform(s) has not been established in this study. Our results show that PKC\(\theta\) transduces signals from TCR/CD28 to BAD. Various studies demonstrated that CD28 costimulation provides a survival signal that protects T cells from AICD (34–40). This survival signal can be mediated by several mechanisms, e.g., up-regulation of the anti-apoptotic protein Bcl-x \(_L\) and increased production of IL-2 (34, 35). The protective role of PKC\(\theta\) revealed by the current study implies an additional protective pathway associated with CD28 costimulation and is consistent with our earlier findings that PKC\(\theta\) integrates signals from the TCR/CD3 complex and CD28 (13).

Several findings support the conclusion that PKC\(\theta\) is the major PKC isoform that protects T cells from Fas-mediated apoptosis and most likely accounts for the anti-apoptotic effect of phorbol ester in T cells. First, the protective effect of phorbol ester was not modulated by ionomycin (Fig. 2f), suggesting that a member of the novel Ca\(^{2+}\)–independent PKC subfamily is involved. Second, rottlerin, which selectively inhibits PKC\(\theta\) activity in vitro and in intact T cells (12, 13, 17), synergized with anti-Fas to induce cell death (Fig. 2, a, c, and d), whereas the cPKC inhibitor, \(\text{G60}\text{976}\), did not have any effect under similar conditions (Fig. 2b). Third, PKC\(\theta\) was more effective than other PKC isoforms in protecting cells from Fas-mediated apoptosis (Fig. 3a); although PKC\(\epsilon\) also displayed a significant protective effect. Fourth, a dominant negative PKC\(\theta\) construct blocked CD3-mediated BAD phosphorylation (Fig. 6a). Nevertheless, we cannot rule out the possibility that other Ca\(^{2+}\)–independent PKCs, e.g., PKC\(\epsilon\), which is relatively abundant in T cells, also play a protective role. This idea is consistent with our observation, and in agreement with others (33), that constitutively active PKC\(\epsilon\) also protected T cells from apoptosis, albeit less potently than PKC\(\theta\) (Fig. 3a). However, PKC\(\epsilon\), which is also inhibited by rottlerin (21), most likely has a minor, if any, role in this process, because it was barely expressed in the cells used (Fig. 2e) (17).

Activated NF-\kappaB can protect cells from apoptosis (22). Our experiments revealed that blocking of I\(\kappa\)B\(\alpha\) function by the corresponding dominant negative mutant (Fig. 4a) or inhibition of the proteasome machinery (Fig. 4c) did not greatly interfere with the protective effect of PKC\(\theta\) on Fas-mediated apoptosis, suggesting that NF-\kappaB is not the major pathway involved in this effect.

The function of BAD was mostly studied in systems in which apoptosis is induced by growth factor deprivation of hemopoietic or neuronal cells (25, 26, 41). The role of BAD in protecting lymphoid cells from apoptosis induced by the interaction of death receptors with their ligands (e.g., Fas/FasL) is less clear, particularly because these cells express low levels of BAD (42). In addition, BAD appears to regulate apoptotic events associated with mitochondria, and it has been proposed that Fas engagement induces a cascade of events that is independent of mitochondria (43). Thus, the idea that phosphorylation of BAD is a potentially important mechanism for suppression of apoptosis by Fas is antithetical to data suggesting that Fas can induce apoptosis independent of the mitochondria-dependent pathway governed by BAD and other Bcl-2 family proteins. However, it is becoming increasingly clear that cross-talk exists between mitochondria-dependent and death receptor-mediated apoptosis pathways (43). Furthermore, BAD can cooperate with a Fas-mediated signal to promote T cell death. For example, T cells derived from BAD-transgenic mice are highly susceptible to apoptotic stimuli, including Fas ligation (44). Moreover, the same study demonstrated that Fas ligation induces marked up-regulation of BAD expression in thymocytes. In addition, BID, a BH3 domain-containing proapoptotic Bcl-2 family member, is a specific proximal substrate of caspase8 in the Fas and...
TNF-α apoptotic signaling pathways. Although full-length BID is localized in cytosol, truncated BID (tBID) translocates to mitochondria and thus transduces apoptotic signals from cytoplasmic membrane to mitochondria. tBID induces first the clustering of mitochondrial damage induced by caspase-8 (45–48). The finding that BAD acts as a key regulator of T cell apoptosis and T cell development (44) coupled with the selective expression of PKCζ in T cells and its unique role in TCR signaling (6, 15) suggest that our findings and those of others (33) are biologically relevant in the context of the balance between TCR/CD28-mediated survival signals and Fas-mediated apoptosis.

The outcome of PKC activation vis-à-vis cellular apoptosis, as well as the contributions of distinct PKC isoforms to protection from apoptosis, are likely to be stimulus and cell type specific, as indicated by several examples. Thus, pharmacological PKC inhibition was found to rescue neuronal cells from apoptosis induced by growth factor deprivation (49), and this effect probably reflects the promoting role of PKCζ in neuronal apoptosis (50). Furthermore, another PKC isozyme, i.e., PKCuα, was found to protect COS cells from apoptosis (51). Lastly, a nPKC isoform (probably PKCζ) was reported to promote, rather than inhibit, the glucocorticoid-induced apoptosis of thymocytes (52). These examples strongly suggest that the nature of survival pathways is dependent on the cellular context and/or the particular triggering stimulus. In this regard, leukemic or activated T cells are more susceptible than resting cells to Fas-mediated apoptosis (53, 54). Based on our findings (Fig. 2), it would be interesting to determine whether strategies designed to selectively inhibit the function of PKCζ can synergize with death receptor agonists to facilitate the elimination of autoreactive T cells in vivo, consistent with a recent study (55).

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References