Protein Kinase C θ and ε Promote T-cell Survival by a Rsk-dependent Phosphorylation and Inactivation of BAD*

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Both MAPK and protein kinase C (PKC) signaling pathways promote cell survival and protect against cell death. Here, we show that 12-O-tetradecanoylphorbol-13-acetate (TPA) prevents Fas-induced apoptosis in T lymphocytes. The effect of TPA was specifically abolished by the PKC inhibitor GF109203X and by dominant negative PKCθ, PKCe, and PKCα, suggesting that novel and conventional PKC isoforms mediate phorbol ester action. Moreover, TPA stimulated phosphorylation of BAD at serine 112, an effect abrogated by GF109203X but not by the MEK inhibitor PD98059. Expression of constitutively active PKC increased the phosphorylation of BAD at serine 112 but not at serine 136. Additionally, Fas-mediated cell death was enhanced by overexpression of a catalytically inactive form of p90Rsk (Rsk2-KN). Finally, Rsk2-KN abolished the protective effect of constitutively active PKC and totally blocked phosphorylation of BAD on serine 112. Thus, novel PKCθ and PKCe rescue T lymphocytes from Fas-mediated apoptosis via a p90Rsk-dependent phosphorylation and inactivation of BAD.

In several cell lines, apoptosis is antagonized by growth factors and hormones and, more generally, by stimuli that promote cell survival. Interleukin 3 and insulin-like growth factor 1 exert their antiapoptotic effect through activation of phosphatidylinositol 3-kinase, which, in turn, leads to activation of the serine/threonine protein kinase B (PKB/Akt), which promotes cell survival by phosphorylating BAD at Ser112 (1–6). Interestingly, interleukin 3, through activation of a mitochondrial membrane-based protein kinase A, also stimulates phosphorylation of BAD at Ser112 (6, 7). When phosphorylated at Ser112 or Ser136, BAD is complexed to the cytosolic 14.3.3 protein. Association of BAD with 14.3.3 prevents its dimerization with the antiapoptotic Bcl-XL protein, thus favoring cell survival (6). Furthermore, brain-derived neurotrophic factor and agonists such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)¹ have been shown to phosphorylate BAD at Ser112 (8, 9). Brain-derived neurotrophic factor exerts its antiapoptotic effect in a mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase pathway (8). The mechanism of action of TPA remains unclear, although MAPK-dependent (10–13) and -independent pathways have been described (9). TPA is a tumor promoter that binds and activates members of a family of serine/threonine protein kinases termed protein kinase C (PKC). PKC comprised at least 12 isotypes that have been classified into three groups according to their structure and cofactor requirement: (a) conventional PKCs (PKCα, PKCβI, and PKCγ) are diacylglycerol- and calcium-dependent, (b) novel PKCs (PKCδ, PKCe, PKCγ, PKCθ, and PKCμ) are diacylglycerol-dependent but calcium-independent, and (c) atypical PKCs (PKCe, PKCε, and PKCδ) are not activated by phorbol esters but can bind diacylglycerol (14, 15). Overexpression of PKCe, PKCα, or PKCe increases the resistance of cells to apoptosis, and PKC inhibitors are known to sensitize cells to apoptosis (16–20). Additionally, Fas ligation-induced apoptosis in Jurkat T cells resulted in a blockade of cellular PKC activity, suggesting a link between the two events (21). Although involvement of PKC in the suppression of apoptosis has been demonstrated recently, the mechanisms by which PKC promotes cell survival remain to be elucidated.

EXPERIMENTAL PROCEDURES

Reportor Plasmids, Transfections, and Luciferase Assays—Transfections of Jurkat T cells were done by electroporation with simple electric shock (320 V, 960 microfarads) using the gene pulsar system (Bio-Rad). Cells were transfected with 5 μg of the c-fos SRE luciferase vector with or without 14 μg of the different PKC mutants or transfected with 5 μg of a SRE luciferase reporter plasmid with 14 μg of the constitutively active PKC isoforms (14) in presence or absence of 14 μg of Rsk2-KN (8). Cells were exposed to the different effectors as indicated in the figure legends. Two days after transfection, soluble extracts were harvested in lysis buffer (Promega) and assayed for luciferase activity. Luciferase activity was normalized by protein amount. Transfections of HEK 293 cells were performed with a calcium phosphate transfection method (Stratagene). HEK 293 cells were transfected with 2.5 μg of glutathione S-transferase-BAD (New England Biolabs) with or without 2.5 μg of empty vector or vector encoding the PKC mutant constructs in presence or absence of Rsk2-KN. Two days after transfection, cells were exposed to different effectors for the indicated time as described in figure legends and then lysed to perform Western blotting experiments.

Western Blot Assays—Jurkat T cells or HEK 293 cells were incubated with different effectors for the times indicated in the figure legends, and

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† The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; SRE, serum response element; HEK, human embryonic kidney.
then the cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 20 mM EDTA, 100 mM NaF, 10 mM Na3VO4, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 20 μg/ml aprotinin, and 1% Nonidet P-40. Proteins were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membrane (Immobilon, Millipore), and then exposed to the appropriate antibodies. BAD was detected with polyclonal phospho-specific BAD Ser112 or Ser136 antibodies (New England Biolabs; dilution, 1:1000) or with an antibody that recognizes BAD regardless of its phosphorylation state (New England Biolabs; dilution, 1:1000) and with a secondary peroxidase-conjugated anti-rabbit antibody at a 1:10000 dilution. Caspase 3, PKCε, PKCu, and PKCa were detected with monoclonal antibodies (Transduction Laboratory) at 1:4000 and 1:1000 dilution, respectively, for PKC in saturation buffer and with a secondary anti-mouse antibody at a 1:5000 dilution. Proteins were visualized with the Amersham ECL system.

Fig. 1. TPA promotes cell survival in a PKC-dependent pathway. A, Jurkat T cells were left untreated or incubated with GF109203X (Calbiochem; 2 μM) or PD98059 (Calbiochem; 20 μM) for 30 min before treatment with TPA (Sigma Chemical Co.; 100 ng/ml) and CH11 (Euromedex; 80 ng/ml) for 4 h. The cells were then lysed and analyzed for fragmented DNA. B, Jurkat T cells were exposed to the different effectors as described in A. Immunoblotting was done with a mouse monoclonal antibody that recognizes human caspase 3. C, Jurkat T cells were transiently transfected with a SRE luciferase vector. Thirty-six h later, cells were exposed as described in A and then assessed for their luciferase activity. Results are expressed as a percentage of the luciferase activity from unstimulated cells. Data are the means ± S.E. of three independent experiments.

Fig. 2. PKCe, PKCθ, and PKCa block Fas-induced apoptosis. A, Jurkat T cells were transfected with a c-fos SRE luciferase vector together with either an empty vector or an expression vector encoding the constitutively active (CA) or dominant negative (DN) PKC mutants. Two days after transfection, cells were assayed for their luciferase activity. Lysates from mock-transfected cells or cells transfected with PKC constructs were immunoblotted with mouse monoclonal antibodies that recognize either PKCe, PKCθ, or PKCa, and the results are representative of several experiments. The fold stimulation over the basal c-fos SRE luciferase evoked by PKC transfection is shown at the top of the column. B, Jurkat T cells were transfected as described in A. Two days after transfection, cells were preincubated for 30 min with GF109203X (2 μM) or PD98059 (20 μM) before exposure to CH11 (80 ng/ml) for 4 h. Cells were then assayed for their luciferase activity. The fold stimulation over the basal c-fos SRE luciferase evoked by PKC transfection when cells were treated with CH11 is shown at the top of the column. C, Jurkat T cells were transfected with DN-PKC mutants as described in A. Two days after transfection, cells were exposed to TPA (100 ng/ml) for 4 h and then assayed for their luciferase activity. Results of transfection in A–C are expressed as a percentage of the luciferase activity from unstimulated cells. Data are the means ± S.E. of three independent experiments.

RESULTS AND DISCUSSION

Apoptosis is characterized by cytoplasmic shrinkage, chromatin condensation, and nuclear DNA fragmentation and culminates in cellular death (22). In Jurkat T cells, induction of apoptosis by CH11, an anti-Fas monoclonal antibody that mimics the proapoptotic effect of Fas ligand, results in the disappearance of intact DNA and in internucleosomal DNA fragmentation (Fig. 1A). In the presence of TPA no DNA ladder,
indicative of fragmentation, was observed, demonstrating that this phorbol ester protects cells from apoptosis and may promote cell survival (Fig. 1A) (12). The caspase family of proteins consists of more than a dozen proteins, among which caspase 3 is crucial for the final step of the apoptotic program. CH11-induced activation of caspase 3, as followed by the disappearance of its 32-kDa zymogen form in Western blotting, was also blocked by treatment with TPA (Fig. 1B). The effect of TPA on both DNA fragmentation and caspase 3 activation was completely abrogated by the PKC inhibitor GF109203X (GF109203X) but was weakly sensitive to the MEK inhibitor PD98059 (PD98059, Fig. 1, A and B). These results indicate that the extracellular signal-regulated kinase pathway plays only a minor role in the protective effect of TPA. Execution of the apoptotic program is achieved through cleavage by caspases of numerous cellular proteins that are essential for cell proliferation and survival (23). Among these substrates, we previously identified the serum response factor as a target for caspase 3 during CH11-mediated apoptosis (24). In Jurkat T cells, cleavage of serum response factor results in a drastic inhibition of the activity of a c-fos SRE luciferase reporter plasmid (Fig. 1C) (24). Interestingly, TPA was found to counteract the inhibitory effect of CH11 on SRE activity and to concomitantly abrogate serum response factor cleavage (Fig. 1C) (24). Consistent with DNA fragmentation and caspase 3 immunoblotting analysis, the protective effect of TPA on SRE activity was completely abolished by GF109203X but only weakly affected by PD98059. These results indicate that SRE luciferase activity can be used as a reporter to monitor the apoptotic process. To define which PKC isoforms were involved in the protective effect of TPA on cell death, we verified the activity of several PKC mutant constructs on CH11-mediated apoptosis. We first assessed the ability of PKC mutant constructs to mediate SRE activation. Jurkat cells were transiently cotransfected with the SRE luciferase vector and different PKC mutant constructs. As observed in HEK 293 cells, constitutively active mutants of PKCε, PKCθ, and PKCα stimulated SRE activity (with PKCθ > PKCε > PKCe), whereas dominant negative mutants of these PKCs were found to weakly inhibit the basal promoter activity (Fig. 2A) (25). Immunoblotting of lysates from mock-transfected cells or cells transfected with either PKCε, PKCθ, or PKCα revealed...
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that the level of expression of the corresponding proteins was comparable (Fig. 2A). We then studied the effect of PKC constructs on CH11-induced apoptosis. Introduction of the constitutively active form of PKCe and PKCθ totally prevented the CH11-induced inhibition of SRE activity, whereas the effect of PKCa was less pronounced (Fig. 2B). On the other hand, constitutively active PKCa failed to induce the death of Jurkat T cells (data not shown). The protective effect evoked by constitutively active PKCe, PKCθ, and to a lesser extent, PKCa was abolished by GF109203X (Fig. 2B). Furthermore, dominant negative mutants of PKC had no protective effect but rather increased the inhibitory effect of CH11 on SRE activity, suggesting that they behave as proapoptotic stimuli (Fig. 2B). Additionally, dominant negative PKCe, PKCθ, and PKCa impaired the stimulatory effect of TPA on SRE activity, indicating that each of these PKC isoforms may mediate the effect of TPA, although PKCa again appears to be the most potent (Fig. 2C). These results strongly suggest that TPA, through the activation of novel and probably conventional PKC isoforms, protects cells from apoptosis. To better understand how these PKC isoforms exert their antiapoptotic function, we investigated the possible participation of an important regulator of the cell death machinery, BAD. First, immunoblotting of protein lysates prepared from untreated, TPA-treated, or CH11-treated Jurkat T cells with phospho-specific BAD antibodies revealed that TPA increased BAD phosphorylation at Ser112, whereas CH11 dramatically reduced the phosphorylation of BAD at the same site (Fig. 3, A and B). This result indicates that CH11, which stimulates apoptosis through the activation of death receptors, not only leads to caspase activation but also plays a role in the regulation of the phosphorylation state of BAD. Additionally, TPA promotes cell survival and BAD phosphorylation at Ser112 in Jurkat T cells. In HEK 293 cells, TPA also stimulated phosphorylation of BAD at Ser112 but not at Ser136, in agreement with the results of Tan et al. (9) (Fig. 3C). In Jurkat T cells and in HEK 293 cells, phosphorylation of BAD at Ser112 was drastically inhibited by GF109203X, whereas PD98059 had no significant ability to inhibit Ser112 phosphorylation (Fig. 3, B and C). Thus, although PKC has been previously shown to activate the MAPK pathway, MAPK activation is unlikely to be the major signaling pathway by which TPA abrogates apoptosis (26–28). Furthermore, we observed that Ly294002 had no effect on the TPA-induced rise in BAD Ser112 phosphorylation, ruling out the involvement of Akt in the protective effect of TPA (data not shown). Interestingly, introduction of constitutively active PKCe, PKCθ, and PKCa in HEK 293 cells also led to phosphorylation of BAD at Ser112 (Fig. 4A) but not at Ser136 (Fig. 4B). Moreover, in cells overexpressing dominant negative PKC, phosphorylation of BAD at Ser112 was significantly reduced (Fig. 4A), indicating that under basal conditions, BAD is already phosphorylated in a PKC-dependent fashion at Ser112. Finally, dominant negative PKC and, more particularly, PKCθ blocked TPA-induced stimulation of BAD at Ser112, demonstrating that TPA-induced cell survival is mediated by these PKC isoforms (Fig. 4C). Until now, it has not been possible to show a direct phosphorylation of BAD by PKC (9). On the other hand, it has recently been demonstrated that the MAPK-activated p90 ribosomal S6 kinase family (Rsk), a downstream target of extracellular signal-regulated kinase, phosphorylates BAD at Ser112 both in vitro and in vivo, and Rsk has been reported to protect cells from BAD-induced apoptosis (8, 9, 29, 30). Using the SRE luciferase assay (24), we found that introduction, in Jurkat T cells, of a catalytically inactive form of Rsk2, Rsk2-KN, not only markedly decreased the protective effect of constitutively active PKCe, PKCθ, and PKCa but also potentiated the inhibitory effect of CH11 (Fig. 5A). Immunoblotting of HEK 293 cell lysates with the phospho-specific BAD Ser112 antibody revealed that expression of Rsk2-KN abrogated constitutively active PKC-induced phosphorylation of BAD at Ser112 (Fig. 5B). Taken together, these results demonstrate that Rsk is involved in the protective effect of TPA.

In this report, we showed that PKCθ, PKCe, and, to a lesser extent, PKCθ trigger BAD phosphorylation at Ser112, thus preventing Fas-induced cell death and promoting cell survival. In conclusion, we demonstrate that phorbol esters promote cell survival essentially through a PKC-Rsk-dependent, MAPK-independent pathway that leads to phosphorylation and inactivation of BAD.

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