p90RSK Blocks Bad-mediated Cell Death via a Protein Kinase C-dependent Pathway

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Yi Tan‡, Hong Ruan, Matthew R. Demeter, and Michael J. Comb
From the Cell Signaling Laboratory, New England Biolabs, Beverly, Massachusetts 01915

Although activation of protein kinase C (PKC) is known to promote cell survival and protect against cell death, PKC targets and pathways that serve this function have remained elusive. Here we demonstrate that two potent activators of PKC, 12-O-tetradecanoylphorbol-13-acetate and bryostatin, both stimulate phosphorylation of Bad at Ser112, a site known to regulate apoptotic cell death by interleukin-3. PKC inhibitors but not PI 3-kinase/Akt inhibitors block 12-O-tetradecanoylphorbol-13-acetate-stimulated Bad phosphorylation. PKC isoforms tested in vitro were unable to phosphorylate Bad at Ser112, suggesting that PKC acts indirectly to activate a downstream Bad kinase. p90RSK and family members RSK-2 and RSK-3 are activated by phorbol ester and phosphorylate Bad at Ser112 both in vitro and in vivo. p90RSK stimulates binding of Bad to 14-3-3 and blocks Bad-mediated cell death in a Ser112-dependent manner. These findings suggest that p90RSK can function in a PKC-dependent pathway to promote cell survival via phosphorylation and inactivation of Bad-mediated cell death.

Survival factors prevent cells from undergoing cell death or apoptosis by inhibiting the execution of the cell death program. Recently, the convergence of signaling pathways activated by IL-3 and other survival factors with the cell death machinery has been traced to their ability to stimulate phosphatidylidylinositol (PI) 3-kinase (1–3) and inactivate the apoptotic factor Bad (4, 5). IL-3 treatment of immune cells stimulates the phosphorylation of Bad at two sites, Ser112 and Ser136 (5). Phosphorylation of Bad at these sites inhibits binding of Bad to Bcl-xL and blocks Bad-mediated cell death in a Ser112-dependent manner. These findings suggest that p90RSK can function in a PKC-dependent pathway to promote cell survival via phosphorylation and inactivation of Bad-mediated cell death.

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‡ To whom correspondence should be addressed: Cell Signaling Laboratory, New England Biolabs, 32 Tzer Rd., Beverly, MA 01915. Tel.: 978-927-5054 (ext. 393); Fax: 978-922-7069; E-mail: tan@neb.com.

1 The abbreviations used are: IL, interleukin; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; CREB, cAMP-response element-binding protein; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; GST, glutathione S-transferase; MBP, maltose-binding protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; TUNEL, terminal deoxynucleotidyl transferase-mediated x-dUTP nick end labeling.
in a PKC-dependent fashion and that in vitro p90<sub>RSK</sub> can directly phosphorylate Bad at Ser<sup>112</sup>. Overexpression of p90<sub>RSK</sub> in HEK293 cells stimulates Bad phosphorylation at Ser<sup>112</sup>. Furthermore, treatment of HEK293 cells with TPA or overexpression of p90<sub>RSK</sub> suppresses Bad-mediated cell death. These results suggest that the effects of phorbol ester and PKC on cell survival act via a pathway involving a p90<sub>RSK</sub>-dependent phosphorylation of Bad at Ser<sup>112</sup>. Our results suggest that the PKC/p90<sub>RSK</sub> pathway may play an important role in regulating cell death decisions at the Bcl-xL checkpoint via phosphorylation and inactivation of Bad.

**EXPERIMENTAL PROCEDURES**

**Materials**—Forskolin, 3-isobutyl-1-methyl-xanthine, TPA, fetal calf serum, and mammalian cell culture media were from Sigma. Phosphorylation state-specific Bad antibodies (Ser<sup>112</sup>) and (Ser<sup>136</sup>) and control Bad antibodies were from New England Biolabs. p90<sub>RSK</sub>-specific monoclonal antibody was from Transduction Laboratories. RSK-2- and RSK-3-specific antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MEK protein kinase inhibitor PD98059 and protein kinase A were from New England Biolabs, and SB203580 was kindly provided by John Lee and Peter Young. PKC inhibitors G06983, G06976, and bisindolylmaleimide, wortmannin, LY294002, and rapamycin were from Calbiochem, bryostatin was from Alexis; β-galactosidase stain kit was from Invitrogen; and in situ cell death detection kit for the TUNEL assay was from Roche Molecular Biochemicals. The Phototope-HRP Chemiluminescent Western Detection Kit was from New England Biolabs.

**Cell Culture**—HEK293 cells were maintained in modified Eagle’s medium supplemented with 10% (v/v) horse serum. COS1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum.

**Plasmid and DNA Constructions**—The GST-Bad mammalian expression vector was constructed by fusion of the complete coding sequence of Bad amplified from a mouse brain cDNA library (CLONTECH) and cloned into the BamHI/NotI sites of eukaryotic expression vector pEG. pEGB expresses an amino-terminal GST fusion of the cloned gene under the control of the strong, constitutively active human EF-1 α promoter (63). GST-Bad S112A, S136A, and S112A/S136A mammalian expression vectors were constructed by transferring BglII/AfeI fragments from pCMVBadS112A, pCMVBadS136A, and pCMVBadS112A/S136A (prepared from Stanley Korsmeyer) into a GST-Bad mammalian expression vector. Maltose-binding protein (MBP)-p90<sub>RSK</sub> was kindly provided by Michael Yaffe and Lewis Cantley. pMT2-p90<sub>RSK</sub> was provided by Christian Bjorbaek.

**Phosphoantibody Production**—The anti-phospho-Bad (Ser<sup>112</sup>) and anti-phospho-Bad (Ser<sup>136</sup>) antibodies were generated by immunizing rabbits with synthetic phosphopeptides covalently coupled to keyhole limpet hemocyanin. The presence of phosphospecific immunoreactivity was detected by enzyme-linked immunosorbent assays using both the phosphorylated and the nonphosphorylated peptides. After purification of IgG using protein A-agarose, the phosphopeptide-specific antibodies were purified by first passing IgG over immobilized, nonphosphorylated peptide, to remove antibodies reactive with the nonphosphorylated epitopes. The nonabsorbed fraction was then passed over a column of immobilized phosphopeptide. After extensive washing, the retained immunoglobulins were eluted at low pH, rapidly neutralized, dialyzed, and concentratet.

**Transient Transfections**—Transient transfection of HEK293 cells was performed as described previously (35) with various amounts of expression plasmid as described in the figure legends. The total amount of DNA transfected was maintained at 20 μg with pCMV3. Following calcium phosphate transfections, cells were glycerol-shocked and incubated for 24 h in media containing 10% fetal calf serum and for 18 h without serum. Cells were treated with 200 μM TPA for the indicated times.

**14-3-3 Fusion Protein Pull Down**—MBP-14-3-3 proteins bound to maltose beads were mixed with extracts prepared from HEK293 cells transfected with Bad expression vectors and treated with or without TPA and lysed in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cell extracts were incubated overnight at 4°C; beads were washed twice with cell lysis buffer and twice with phosphate-buffered saline; and proteins were eluted with SDS sample buffer for Western analysis.

**Cell Transfection and Western Blot Analysis**—HEK293 cells were transiently transfected with Bad expression vectors and treated with or without TPA for the indicated times. Cells were harvested, cell lysates subjected to SDS-PAGE, and Western blots were probed using the appropriate phosphospecific antibodies or antibodies directed against Bad Ser<sup>112</sup> (S112-p), Ser<sup>136</sup> (S136-p), and total Bad (tBad) as indicated.

**Immunoblotting**—Cell extracts were prepared by lysing cells in 100 μl of SDS sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 0.15% 2-mercaptoethanol, 0.02% bromphenol blue). Extracts from 10<sup>5</sup> cells (20 μl) were fractionated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature; incubated with primary antibody overnight at 4°C; incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature; and finally visualized using the Phototope-HRP Chemiluminescent Western Detection Kit.

**Protein Kinase Assay**—p90<sub>RSK</sub> activity was detected by immunoprecipitation of p90<sub>RSK</sub> from HEK293 cell extracts. HEK293 cells were pretreated with different inhibitors for 1 h and then treated with 200 μM TPA for 30 min. Cells were lysed in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 μM/ml leupeptin). Extracts from 5 × 10<sup>5</sup> cells were incubated with p90<sub>RSK</sub> antibody overnight and with protein A beads for 3 h at 4°C by gentle rocking. Immunocomplexed beads were washed twice with cell lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.4, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>). Immunocomplexes were resuspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg of GST-Bad protein or MBP-Bad and Bad mutation and incubated for 30 min at 30°C. Kinase reactions were terminated with SDS sample buffer, and Bad phosphorylation was detected by immunoblotting.

**In situ-mediated Protein-Peptide Ligation**—Wild-type and mutant peptides spanning Bad amino acid sequences from 106 to 141 were chemically synthesized with a cysteine added to the N terminus of each peptide. Phosphopeptides were synthesized by incorporating phosphoacceptor serine at position 112 or 136. Mutant peptides were synthesized with the following changes: Ser<sup>112</sup> changed to Ala, Ser<sup>136</sup> changed to Ala, or the double mutant where both Ser<sup>112</sup> and Ser<sup>136</sup> were changed to Ala. Fusion proteins were prepared by incubating each peptide with 34860
bacterially expressed MBP-paramyosin isolated and purified using the IMPACT protein expression system (New England Biolabs) using the Mxe GyrA intein as described by Evans et al. (36). Following the peptide ligation reaction, MBP fusion proteins were purified away from free peptides by binding to maltose beads.

**TUNEL Assay**—HEK293 cells were transfected with pCMVBad, plus or minus p90RSK, 24 h after transfection, cells were serum-starved for 18 h and then fixed with 4% paraformaldehyde solution for 30 min, blocked with 0.3% H2O2 in methanol for 30 min, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After extensive washing, cells were incubated with terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotide mixture for 1 h at 37 °C, washed three times with phosphate-buffered saline, incubated with anti-fluorescein antibody conjugated with horseradish peroxidase for 30 min, washed, and then developed with 3,3′-diaminobenzidine tetrahydrochloride solution.

**X-Gal Staining**—After transfection and treatment, cells were fixed in 4% paraformaldehyde solution for 10 min at room temperature. After washing three times with phosphate-buffered saline, cells were incubated with 1 mg/ml X-Gal in N,N-dimethylformamide, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM MgCl2 in phosphate-buffered saline buffer for 24 h. Blue color-containing cells were examined under a microscope.

**RESULTS**

**Protein Kinase C Activators Stimulate Bad Phosphorylation at Ser112**—Phosphorylation state-specific antibodies (37) directed against Ser112 and Ser136 of Bad were generated as described under “Experimental Procedures.” The antibodies were shown to be highly specific for the phosphorylated epitope by enzyme-linked immunosorbent assays and by Western blotting using phosphorylated and nonphosphorylated GST-Bad protein (data not shown). To further characterize antibody site specificity, a series of MBP-Bad fusion proteins was constructed by ligating to the C terminus of MBP chemically synthesized Bad peptides or Bad peptides phosphorylated at Ser112, Ser136, or both sites (see “Experimental Procedures” and Ref. 36). As shown in Fig. 1A, phospho-Bad Ser112 antibody only recognizes MBP-Bad fusion protein when phosphorylated at Ser112 or Ser136 and Ser136, whereas phospho-Bad Ser136 antibody only recognizes MBP-Bad fusion protein when phosphorylated at Ser136 or Ser112 and Ser136. These results demonstrate a high degree of specificity for both antibodies to their specific phosphoserine residues. We next examined antibody specificity in vivo. HEK293 cells were transfected with plasmids encoding wild-type GST-Bad or GST-Bad S112A, S136A, or S112A/S136A mutants. Cell extracts were prepared and immunoblotted using phosphospecific antibodies directed against Bad Ser112 (Fig. 1B, top) or Bad antibody (Fig. 1B, bottom). Treatment of HEK293 cells with TPA increased the phosphorylation of wild-type GST-Bad at Ser112. Mutation of Ser112 to Ala blocked the TPA-induced immunoreactivity. Mutation of Ser136 to Ala did not block TPA-induced Ser112 phosphorylation, demonstrating the site specificity of the phospho-Bad Ser112 antibodies. Wild-type and mutant GST-Bad proteins were expressed at similar levels as determined using Bad antibody (Fig. 1B, bottom).

We next explored other agents that might also stimulate phosphorylation of Bad at Ser112 using the phosphospecific Bad Ser112 antibody. Treatment of GST-Bad transfected HEK293 cells with TPA or epidermal growth factor produced the largest induction of GST-Bad phosphorylation at Ser112. Forskolin, UV irradiation, and platelet-derived growth factor also reproducibly stimulated phosphorylation at Ser112 (Fig. 2A). TPA rapidly induced a sustained phosphorylation of Bad, evident within 5 min of treatment, that remained elevated 8 h after treatment (Fig. 2B). Bryostatin, another activator of PKC, as well as TPA induced endogenous Bad Ser112 phosphorylation in COS1 cells (Fig. 2C).

**Protein Kinase C Inhibitors Block Bad Phosphorylation**—Since phorbol esters are known to activate classical and novel PKC isoforms as well as several other growth-associated pathways including the MAP kinase cascade, we next used selective inhibitors to determine the role of several different intracellular signal transduction pathways on Bad phosphorylation at
and β1, but to have no effect on the Ca2+-independent isoforms ε and δ (38). The potent inhibition by bisindolylmaleimide I and the inability of GO6976 to block TPA-mediated signaling to Bad suggest a role for the Ca2+-independent, novel PKC isoforms.

**p90^RSK Phosphorylates Bad at Ser112 in Vitro**—We next sought to identify the TPA-activated protein kinase phosphorylating Bad at Ser112. Since both TPA and bryostatin induce Bad phosphorylation and PKC inhibitors block Bad phosphorylation, we first tested the ability of various PKC isoforms to directly phosphorylate Bad at Ser112. Several different conventional (α, β1, β2, γ), novel (ε, δ), and atypical (ζ) PKC isoforms were tested for their ability to phosphorylate Bad in vitro. Surprisingly, although the PKC isoforms were able to phosphorylate various control proteins and peptides, all of the isoforms tested were unable to phosphorylate Bad at Ser112 (data not shown). This result suggested that PKC may activate a downstream protein kinase that in turn phosphorylates Bad at Ser112.

The role of p90^RSK was next investigated, since this protein kinase is activated by TPA downstream of PKC (39). The substrate specificity of p90^RSK in vitro is reported to be consistent with the RXX motif in which Bad Ser112 is found (28). To test whether p90^RSK could phosphorylate Bad at Ser112, p90^RSK was immunoprecipitated from mock- or TPA-treated HEK293 cells using a p90^RSK-specific monoclonal antibody. Immunocomplex kinase assays were performed using wild-type and mutant MBP-Bad fusion proteins as substrates, and Bad phosphorylation was detected by immunoblotting using a phospho-Bad Ser112 antibody (Fig. 4A). TPA stimulated p90^RSK kinase activity that phosphorylated wild-type Bad and the Bad S136A mutant but not the S112A and S112A/S136A mutants. Immunocomplexes were also incubated in a kinase assay using recombinant GST-Bad fusion protein as substrate, and Bad phosphorylation was detected by immunoblotting using phospho-Bad Ser112 antibody (Fig. 4A). TPA stimulated p90^RSK kinase activity that phosphorylated wild-type Bad and the Bad S136A mutant but not at Ser112. These results suggest that in vitro p90^RSK phosphorylates Bad at Ser112 but not significantly at Ser136.

We next reasoned that if p90^RSK was downstream of PKC on a pathway leading to Bad phosphorylation at Ser112, then p90^RSK activity should show a profile of inhibitor sensitivity similar to that observed for Bad Ser112 phosphorylation. To test this, we examined the sensitivity of p90^RSK kinase activity to 20 μM PD98059, 100 nM GO6983, 100 nM GO6976, and 100 nM bisindolylmaleimide I pretreating cells for 1 h with each inhibitor. Following a 30-min TPA treatment, cell extracts were prepared, p90^RSK was immunoprecipitated, and kinase activity was determined as described above. As observed for Bad Ser112 phosphorylation (Fig. 3A), pretreatment with either GO6983 or bisindolylmaleimide I blocked p90^RSK activity (Fig. 4B). Pretreatment with PD98059 and GO6976 resulted in little or no effect on p90^RSK activity, in good agreement with their effects on Bad Ser112 phosphorylation (compare Fig. 3A with Fig. 4B). To exclude the possibility that these inhibitors block p90^RSK activity directly, p90^RSK was immunoprecipitated from TPA-treated HEK293 cell extracts, and immunocomplexes were preincubated in the presence of 20 μM PD98059 and 1 μM of each of the three different PKC inhibitors (GO6983, GO6976, or bisindolylmaleimide I) in vitro for 1 h at 37 °C. Kinase assays were then carried out using GST-Bad as substrate. As shown in Fig. 4C, even a 10-fold increase over the concentrations of GO6983 and bisindolylmaleimide I required to block p90^RSK activity in vitro had no effect on p90^RSK activity in vitro (Fig. 4B).

[Fig. 2. Inhibitors of protein kinase C but not inhibitors of MAPK, Akt, and p70S6 kinase block TPA-stimulated Bad phosphorylation at Ser112. A, HEK293 cells were transfected with plasmids encoding GST-Bad and serum-starved as described in the legend to Fig. 2. Cells were treated with 100 nM PKC inhibitors GO6983, GO6976, and bisindolylmaleimide; 40 μM p38 MAPK inhibitor SB203580; 20 μM MEK1 inhibitor PD98059; 2 μM PI 3-kinase inhibitors wortmannin and 10 μM LY294002; and 10 nM p70 S6 kinase inhibitor rapamycin. After a 60-min pretreatment with the inhibitors, cells were stimulated with 200 nM TPA for 30 min prior to harvest. B, dose response of PKC inhibitors upon TPA-stimulated Bad phosphorylation at Ser112. HEK293 cells were transfected without or with plasmids encoding GST-Bad as indicated and treated with different amounts of inhibitors and TPA as described above. In both A and B, immunoblotting was carried out using phospho-Bad Ser112 antibody (top panels) or control Bad antibody (bottom panels).]

Ser112. Treatment of HEK293 cells with rapamycin (a selective mTOR/FRAP inhibitor) or two different PI 3-kinase inhibitors, wortmannin and LY294002, had no effect on TPA-stimulated Bad phosphorylation. Inhibitors of MEK and the MAPK cascade (PD98059), or p38 MAPK (SB203580) had very small effects on TPA-stimulated Ser112 phosphorylation. Inhibitors of MEK and the MAPK cascade (PD98059) or GO6983, a potent inhibitor of PKCα, -β1, -γ, and -δ isoforms, also blocked TPA-stimulated Bad phosphorylation. Significantly, treatment with GO6976 had little or no effect on TPA-stimulated Ser112 phosphorylation until concentrations of 1 μM were reached. This inhibitor has been reported to selectively inhibit Ca2+-dependent PKC isoforms α,
were performed using GST-Bad as a substrate. Phosphorylation of Bad was detected by immunoblotting using phospho-Bad Ser112 antibody as described above and incubated with 20 μM PD98059, 100 nM GO6976, 100 nM GO6983, and 100 nM bisindolylmaleimide, respectively for 1 h, and then treated with TPA for 30 min. Cells were harvested, and p90RSK was immunoprecipitated as described above. Immunocomplex kinase assays were performed using GST-Bad S112A, S136A, or S112A/S136A mutants; and pMT2 p90RSK as indicated. After 24 h, cells were deprived of serum, and 18 h later they were treated with medium or with medium plus 200 nM TPA for 30 min prior to harvest. Immunoblotting was carried out using phospho-Bad Ser112 antibody (top panel), or Bad antibodies (bottom panel). 

**Comparison of p90RSK and TPA**—in vivo and in vitro stimulation of Bad phosphorylation. A, TPA-induced p90RSK activity phosphorylates Bad at Ser112. HEK293 cells were grown in normal medium for 24 h and deprived of serum for 18 h followed by treatment with or without 200 nM TPA for 30 min prior to harvesting. Cell extracts were prepared, and p90RSK was immunoprecipitated using p90RSK-specific antibody. Immunocomplexes were incubated in a kinase reaction using wild-type and mutant MBP-Bad fusion proteins as substrates, and Bad phosphorylation was detected by immunoblotting using phospho-Bad Ser112 antibody. B, inhibitors of PKC but not extracellular signal-regulated kinase 1/2 block p90RSK activity in HEK293 cells. HEK293 cells were grown as described above and incubated with 20 μM PD98059, 100 nM GO6976, 100 nM GO6983, and 100 nM bisindolylmaleimide, respectively for 1 h, and then treated with TPA for 30 min. Cells were harvested, and p90RSK was immunoprecipitated as described above. Immunocomplex kinase assays were performed using GST-Bad S112A as a substrate. Phosphorylation of Bad was detected by immunoblotting using phospho-Bad Ser112, (top) and phospho-Bad Ser136- (bottom) specific antibodies. C, inhibitors of PKC do not inhibit p90RSK activity in vitro. Immunoprecipitated p90RSK from TPA-treated 293 cells was preincubated with 20 μM PD98059, 1 μM GO6976, 1 μM GO6983, and 1 μM bisindolylmaleimide for 1 h in vitro at 37 °C. Kinase assays were then performed using GST-Bad as a substrate. Phosphorylation of Bad was detected by Western blotting using phospho-Bad Ser112 antibody. As shown in Fig. 5A, phosphorylation at Ser112 in the absence of TPA treatment, but not Ser136 to Ala, blocks p90RSK-induced phosphorylation. RSK-2 and RSK-3 (data not shown). To test the ability of RSK-2 and RSK-3 to stimulate Bad phosphorylation in vivo, we transfected HEK293 cells with pMT2-RSK2, pMT2-RSK3, and GST-Bad. As for p90RSK, overexpression of either RSK-2 or RSK-3 stimulated Bad phosphorylation that was further enhanced after TPA treatment (Fig. 5B). 

**p90RSK and TPA Suppress Bad-mediated Cell Death via 14-3-3 Isoforms**—Phosphorylation of Bad at Ser112 and Ser136 has been reported to mediate 14-3-3 binding, thereby sequestering Bad to cellular compartments that block its cytotoxicity (5). We next asked whether treatment of HEK293 cells with TPA or overexpression of p90RSK would stimulate binding of Bad to 14-3-3 proteins. Cell extracts were prepared from control cells or cells transfected with GST-Bad and pMT2-p90RSK or treated with TPA for 30 min. The cell extracts were incubated with MBP-14-3-3 or δ fusion proteins immobilized on amyllose beads. The interaction of GST-Bad with 14-3-3 proteins was monitored by Western blotting using phospho-Bad Ser112 antibody. As shown in Fig. 6, both TPA and p90RSK stimulate binding of GST-Bad to 14-3-3 or δ isoforms.
positive cells exhibiting condensed nuclei from five different fields in each experiment. S.D. values were determined by counting comparable fields from three separate experiments. As shown in Fig. 7A, transfection of COS1 cells with wild-type Bad followed by 18-h serum starvation resulted in the death of a large majority of transfected cells (70 ± 10%). Bad-mediated cell death was substantially suppressed by treatment with TPA for 6 h (34 ± 5%).

To determine whether p90RSK could also block Bad-mediated cell death, we cotransfected p90RSK together with Bad into HEK293 cells. Transfection of HEK293 cells with wild-type Bad (Fig. 7B) or GST-Bad (Fig. 7C) followed by 18-h serum starvation resulted in dramatic cell death, as determined by the TUNEL assay (Fig. 7B) or by morphological analysis of β-galactosidase-positive cells (Fig. 7C) as described above for COS1 cells. In HEK293 cells, Bad-mediated cell death was completely suppressed by cotransfection with p90RSK (Fig. 7B). Similarly, cotransfection with p90RSK completely suppressed cell death mediated by GST-Bad (Fig. 7C). To examine the Bad phosphorylation sites required for p90RSK suppression, we cotransfected GST-Bad S112A, S136A, and S112A/S136A mutants together with p90RSK. Like wild-type GST-Bad, all GST-Bad mutants induce the death of a majority of transfected cells, and cell death induced by Bad S136A mutants can be suppressed by cotransfection with p90RSK. However, cell death induced by Bad S112A or S112A/S136A mutants was not rescued by p90RSK, suggesting that phosphorylation of Bad at Ser112 is necessary for p90RSK to function as a death suppressor.

**DISCUSSION**

Although a wide variety of growth and survival factors activate PKC, and PKC activation is known to promote cell survival, the PKC effectors mediating cell survival are largely unknown. Experiments in this study identify a mechanism whereby the activation of PKC promotes cell survival. PKC activation by TPA is shown to result in the rapid phosphorylation of Bad at Ser112, and this is correlated with inhibition of Bad-mediated cell death. While PKC does not appear to directly phosphorylate Bad at Ser112 in vitro, we show by several different criteria that the protein kinase, p90RSK, acts downstream of PKC and in turn can directly phosphorylate Bad at Ser112. First, TPA-activated p90RSK phosphorylates Bad at Ser112 in vitro; second, the effects of PKC inhibitors on Bad phosphorylation are tightly correlated with their effects on p90RSK activity and phosphorylation in vivo. Finally, overexpression of p90RSK in HEK293 cells stimulates Bad Ser112 phosphorylation and effectively blocks Bad-induced cell death. Taken together, these findings identify a pathway involving the sequential activation of PKC and p90RSK and phosphorylation of Bad at Ser112 that may function generally to suppress Bad-mediated cell death and promote cell survival.

Studies by Zha et al. (5) first identified Bad Ser112 and Ser136 as two sites that when phosphorylated by IL-3 were able to block the cytotoxic effects of Bad. Several recent studies have identified a survival pathway leading to Bad phosphorylation that involves PI 3-kinase-dependent activation of Akt, followed by phosphorylation of Bad at Ser112 (12, 13, 40, 41). Datta et al. demonstrated that Akt activation results in the selective phosphorylation of Bad at Ser136 but not Ser112. The pathway identified by Datta et al. is blocked by the PI 3-kinase inhibitors wortmannin and LY294002 and involves the Akt-dependent phosphorylation of Bad Ser136 (12). The PKC/p90RSK pathway we have identified is not blocked by PI 3-kinase inhibitors, leads primarily to Bad phosphorylation at Ser112, and does not involve Akt, since TPA does not appreciably stimulate Akt activity in the cells we have examined (data not shown). The
FIG. 7. TPA or transfection with p90RSK blocks Bad-mediated cell death in a Ser112-dependent manner. A, COS1 cells were transfected with plasmids encoding Rous sarcoma virus-β-Galactosidase, alone or together with wild-type Bad as indicated. 24 h after transfection, cells were grown in the medium without serum for 18 h and then treated with 200 nM TPA for 6 h as indicated. Cells were fixed, and X-gal staining was performed as described under “Experimental Procedures.” Apoptotic cells were quantitated by scoring the X-gal-stained cells showing condensed nuclei and cytoplasmic blebs from five different comparable fields. The S.D. value was calculated based on three independent experiments.

B, HEK293 cells were transfected with empty vectors or plasmids encoding wild-type Bad alone or together with pMT2-p90RSK as indicated. 24 h after transfection, cells were serum-starved for 18 h, fixed, and analyzed by the TUNEL assay as described under “Experimental Procedures.” The percentage of apoptotic cells was quantitated by counting the amount of TUNEL positive cells from five different fields. The S.D. was calculated based on three independent experiments.

C, HEK293 cells were transfected with the indicated combination of plasmids encoding Rous sarcoma virus-β-galactosidase; GST-Bad; GST-Bad S112A, S136A, or S112A/S136A mutants; and/or pMT2-p90RSK. 24 h after transfection, the cells were serum-starved for 18 h and then fixed and analyzed by X-gal staining.
PKC/p90RSK pathway therefore represents a route distinct from the PI 3-kinase/Akt pathway, leading to Bad phosphorylation and cell survival. Accumulating evidence suggests that multiple pathways converge on Bad to determine cell survival. Survival factors such as IL-3 also increases cAMP levels, resulting in activation of protein kinase A. Membrane-based protein kinase A has recently been identified as a Bad kinase phosphorylating Bad at Ser112 (14). Calcineurin, a calcium-dependent phosphatase, can dephosphorylate Bad at both Ser112 and Ser136 and promote apoptosis in a calcium-dependent fashion (15). In addition, several recent studies have identified a Bad-independent pathway mediating cell survival in response to IL-4 and insulin-like growth factor-1 (7, 8), suggesting the existence of Bad-independent survival mechanisms.

The precise pathway leading from PKC to the activation of p90RSK is still unclear. Since PKC has been reported to activate the MAPK kinase cascade at several levels including Ras and Raf (42, 43), and MAPK can directly phosphorylate p90RSK at Thr562 in vitro and in vivo (34, 39), it seems plausible to assume that PKC regulates p90RSK via the MAPK cascade. However, blockade of MAPK with PD98059, a selective MEK inhibitor (44), has only a very small inhibitory effect on TPA-stimulated Bad phosphorylation. Because the MEK inhibitor PD98059 completely blocks TPA-stimulated MAPK phosphorylation (data not shown), it is unlikely that the inability of PD98059 to block p90RSK activity and Bad phosphorylation arises from incomplete blockade of MAPK. These studies suggest that PKC activates p90RSK in a fashion that does not depend upon the MAPK cascade. One such mechanism might be the recruitment of a PDK1-like enzyme to p90RSK, since these kinases bind and phosphorylate PKC within its activation loop (45). Another might be direct phosphorylation of p90RSK by PKC at activation sites such as Ser391. Ser391 is located within the spacer region between p90RSK NTD and CTD kinase domains and is a major target of autophosphorylation by the CTD (34, 46). This site conforms to the consensus Phe-X-Phe-Ser-Phe located within the C-terminal sections of most PKC isozymes as well as Akt and p70 S6 kinase and is a critical site regulating the activity of Akt as well as p70 S6 kinase. Since PKC autophosphorylates at this site (47–49), it is possible that it may also phosphorylate similar sites on other kinases such as p90RSK.

Activation of PKC by IL-3 and other survival factors has been observed in many different cell types, although the physiological role of PKC activation and the PKC isozymes mediating survival has been difficult to define. The observation that both IL-3 and granulocyte-macrophage colony-stimulating factor induce diacylglycerol without mobilizing Ca2+ suggested a role for the diacylglycerol-dependent but Ca2+-independent novel PKC isozymes (50). Recently, transfection experiments overexpressing PKCε demonstrated that this novel PKC isoform extends cell survival in the absence of IL-3 (51). PKCε is involved in the protection of cardiac myocytes from hypoxia-induced cell death (52) as well as protection against tumor necrosis factor-α-induced apoptosis (53). PKCε is also known to increase the RNA and protein expression of Bcl-2 (51). Experiments in our study also suggest a role for the Ca2+-independent novel PKC isozymes based upon the potent inhibition seen with bisindolylmaleimide I and GO6983 and the lack of inhibition using GO6976. The conventional PKC isoform PKCε has recently been implicated in an antiapoptotic response in COS1 cells (54) and in phosphorylating Bcl-2 at Ser70, a site critical for Bcl-2 antiapoptotic activity (25). The atypical PKC isoforms δ and λ also play a role in regulating cellular susceptibility to drug-induced (55) and UV-induced apoptosis (56). However, since activation of these isoforms is sensitive to wortmannin and is not activated by TPA, it seems unlikely that they contribute to TPA-induced Bad phosphorylation.

p90RSK has not been previously implicated in cell survival; however, the activity of this enzyme has long been known to be induced by many different growth and survival factors (13). Although this enzyme is phosphorylated and activated by MAPK and is considered a major output of the MAPK cascade, its biological significance has remained obscure, since few physiological substrates have been identified. The finding that NGF induces transcription of c-Fos via p90RSK-dependent phosphorylation of CREB at Ser133 (33) suggests that p90RSK or its closely related isofrom RSK-2 may play an important role in nerve growth factor-mediated regulation of gene expression via CREB and its DNA binding site, the cAMP-responsive element. TPA and PKC are reported to induce Bel-2 expression and rescue immature B cells from apoptosis via a cAMP-responsive element site located within the Bel-2 promoter (57). Furthermore, IL-3 stimulates phosphorylation of CREB at Ser133 (58). These observations suggest that the PKC/p90RSK pathway outlined in this study may function more generally as part of a signal transduction cascade transmitting survival signals to the Bcl-2 checkpoint, regulating the phosphorylation status and cellular expression of Bad, Bel-2, and perhaps other as yet unidentified targets.

Induction of apoptosis appears to be the major mechanism of action of most, if not all, effective chemotherapeutic and radiation-based cancer treatments (59). Hence, the success of chemo- and radiation-based therapy may largely be dependent upon their ability to override existing anti-apoptotic survival pathways. There is considerable evidence that the PKC survival pathway may limit apoptosis induced by radiation and chemotherapy. PKC decreases c-Myc-induced apoptosis in small lung cancer cells (60) and protects endothelial cells against radiation-induced apoptosis (61). PKC inhibitors stimulate apoptosis in human malignant glioma cells (21) and lymphoma cells (24), while TPA prevents apoptosis in chronic lymphocytic leukemia cells (62). In addition, tumor cell sensitivity to araC is greatly enhanced by disruption of the PKC pathway (23). Our findings suggest that disruption of the PKC/p90RSK pathway may also serve to augment chemotherapeutic-induced apoptosis and suggest p90RSK as a novel target for intervention.

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