BAD Ser-155 Phosphorylation Regulates BAD/Bcl-XL Interaction and Cell Survival*

The BH3 domain of BAD mediates its death-promoting activities via heterodimerization to the Bcl-XL family of death regulators. Growth and survival factors inhibit the death-promoting activity of BAD by stimulating phosphorylation at multiple sites including Ser-112 and Ser-136. Phosphorylation at these sites promotes binding of BAD to 14-3-3 proteins, sequestering BAD away from the mitochondrial membrane where it dimerizes with Bcl-XL to exert its killing effects. We report here that the phosphorylation of BAD at Ser-155 within the BH3 domain is a second phosphorylation-dependent mechanism that inhibits the death-promoting activity of BAD. Protein kinase A, RSK1, and survival factor signaling stimulate phosphorylation of BAD at Ser-155, blocking the binding of BAD to Bcl-XL. RSK1 phosphorylates BAD at both Ser-112 and Ser-155 and rescues BAD-mediated cell death in a manner dependent upon phosphorylation at both sites.

BAD is one member of a group of “BH3 domain only” proteins that appears to transduce death signals from the cytoplasm to the mitochondrial membrane and induce apoptosis (1–3). Intracellular survival pathways can inhibit cell death by phosphorylating BAD on several different sites blocking death-promoting activity. Studies by Zha et al. (2) first identified BAD Ser-112 and Ser-136 as two sites that when phosphorylated in response to interleukin-3 blocked the cytotoxic effects of BAD. Survival factors such as interleukin-3 activate a phosphatidylinositol 3-kinase-dependent pathway involving the activation of Akt followed by the phosphorylation of BAD at Ser-136 (4–6). Interleukin-3 signaling can also activate protein kinase C-, MAPK, and RSK1-dependent pathways that stimulate the phosphorylation of BAD at Ser-112 (7–11). Interleukin-3 can also increase cAMP levels resulting in activation of PKA. Membrane-based PKA has been identified as a BAD kinase that phosphorylates BAD at Ser-112 (12). Calcineurin, a calcium-dependent phosphatase, promotes apoptosis by dephosphorylating BAD at both Ser-112 and Ser-136 (13). Phosphorylation at Ser-112 and Ser-136 creates binding sites for the phospho-specific binding of 14-3-3 proteins, which retain BAD in the cytoplasm and prevent cytotoxic interactions with Bcl-XL at the mitochondrial membrane (2, 5, 7).

Although the precise mechanism these molecules use to induce cell death is still unknown, deletion analysis of BAD has demonstrated the importance of the BH3 domain in mediating both its heterodimerization with Bcl-XL and its death-promoting activity (14). Site-specific mutagenesis of the closely related BH3 domain of BAX has revealed the importance of the hydrophobic surface of the BH3 α-helix for dimerization and cell death activity (15). BAX is thought to interact with Bcl-XL at the mitochondrial membrane by fitting into an α-helical groove. The binding of Bcl-XL by the BH3 domain may cause Bcl-XL to release Apaf1 or regulate other Bcl-XL activities resulting in a caspase 9-initiated cascade of proteolysis and induction of apoptosis (16, 17). In the present study, we demonstrate that BAD can be phosphorylated at Ser-155, which is located within the middle of the hydrophobic amphipathic face of the BH3 α helix. Phosphorylation at Ser-155, similar to phosphorylation at Ser-112 and Ser-136, inhibits BAD-induced death-promoting activity but does so in a different manner. Phosphorylation at Ser-155 does not induce 14-3-3 binding and cytotoxic sequestration but instead directly blocks BH3-dependent dimerization with Bcl-XL.

**EXPERIMENTAL PROCEDURES**

Materials—Forskolin, 3-isobutyl-1-methylxanthine (IBMX), TPA, isoproterenol, fetal calf serum, and mammalian cell culture medium were obtained from Sigma. Epidermal growth factor and insulin-like growth factor I were from Life Technologies, Inc. Phosphorylation state-specific anti-BAD/Ser-155 antibody, control anti-BAD antibodies, and the chemiluminescent Western detection kit were obtained from Cell Signaling Technology. Bcl-XL-specific antibodies were purchased from Santa Cruz. RSK1-specific monoclonal antibody was from Transduction Laboratories. Protein kinase A, calmodulin-dependent protein kinase, casein kinase I, casein kinase II, and ERR2 were purchased from New England Biolabs. Protein kinase C was purchased from Calbiochem, and the X-gal staining kit was from Invitrogen. The QuickChange site-directed mutagenesis kit was obtained from Stratagene, and the FuGENE 6 transfection reagent was from Roche Molecular Biochemicals.

Plasmid and DNA Constructions—The GST-BAD mammalian expression vector was constructed by fusion of the complete coding sequence of BAD that has been amplified from a mouse brain cDNA library (CLONTECH) and cloned into the BamHI/NolI sites of the eukaryotic expression vector pEBG. pEBG expresses an amino-terminal GST fusion of the cloned gene under the control of the strong constitutively active human EF-1α promoter (18). GST-BAD S112A/S136A mammalian expression vectors were constructed as described previously (7). GST-BAD S155A and S155E mammalian expression vectors were constructed by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit. pMT2-RSK2, was provided by Joseph Avruch and pMT2-RSK1 and RSK3 were provided by Christian Bjorbaek. The pEEAK 10-Bcl-XL vector was constructed by fusion of the complete coding sequence of Bcl-XL amplified from pCMV-Bcl-XL (provided by Stanley Koremeyer) and cloned into the EcoRI/NolI site of the pEEAK10 vector. MBP-Bcl-XL vector was constructed by fusion of the complete coding sequence of Bcl-XL into the EcoRI/NolI of pMalC2 vector.

Anti-Phospho-BAD Antibody Production—The anti-phospho-BAD-
(Ser-155) antibodies were generated by immunizing rabbits with synthetic phosphopeptides covalently coupled to keyhole limpet hemocyanin. The presence of phospho-specific immunoreactivity was detected by the enzyme-linked immunosorbent assay using both phosphorylated and nonphosphorylated peptides. After purification of IgG using protein A-agarose, phosphopeptide-specific antibodies were purified by first passing the antibodies over immobilized nonphosphorylated peptide to remove antibodies that are reactive to 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, dialoged, and concentrated.

**Protein Phosphorylation in Vitro—**2 μg of GST-BAD protein were incubated with different kinases in 50 μl of kinase buffer (25 mM Tris, pH 7.4, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2) and 200 μM ATP for 30 min at 30 °C. Kinase reactions were terminated with SDS sample buffer, and BAD phosphorylation was detected by immunoblotting.

**Cell Culture and Transfections—**HEK293 cells were maintained in modified minimum Eagle’s medium supplemented with 10% (v/v) horse serum. Transient transfection of HEK293 cells was performed as described previously (19) with 2 μg of GST-BAD, GST-BAD S112A/S136A, or GST-BAD S155A and 10 μg of the catalytic subunit of PKA or 10 μg of pMT-RSK1, RSK2, or RSK3 in 10-cm² plates. The total amount of transfected DNA was maintained at 20 μg with pEGB. Following calcium phosphate transfections, cells were glycero-shocked and incubated for 24 h in medium containing 10% horse serum and for 18 h without serum. Cells were treated with regulators for the indicated times. For the survival assay, HEK293 cells were transfected with 0.5 μg of Rous sarcoma virus β-galactosidase, 1 μg of GST-BAD or BAD mutants, and 0.5 μg of pMT-RSK1, using FuGENE 6 transfection reagent. 24 h after transfection, cells were grown in medium without serum for 18 h and then fixed for X-gal staining.

**Immunoblotting—**Cell extracts were prepared by lysing 5 × 10⁶ cells in 100 μl of SDS sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 0.15 mM 2-mercaptoethanol, 0.02% bromophenol blue). Extracts from 10⁵ cells (20 μl) were fractionated by 12% SDS-polyacrylamide gel electrophoresis and 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 visualized using the chemiluminescent Western detection kit.

**Immunoprecipitation—**HEK293 cells were transfected with plasmids encoding pEAK10-Bcl- XL, wild-type GST-BAD, or GST-BAD containing S155A or S155E mutants as indicated. After 24 h, the cells were deprived of serum and 18 h later were harvested in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cell extracts were incubated with Bcl- XL polyclonal antibody overnight at 4 °C and protein A-conjugated beads for 2 h. Beads were washed twice with cell lysis buffer and once with phosphate-buffered saline, and proteins were eluted with SDS sample buffer for Western analysis.

**MPP-14-3-3 and MPP-Bcl-XL Fusion Protein Pull Down Assays—**MPP-14-3-3 or MPP-Bcl-XL proteins bound to maltose beads were mixed with extracts prepared from HEK293 cells transfected with plasmids encoding wild-type BAD, BAD mutants, or pMT-RSK1, treated with or without TPA, and lysed in cell lysis buffer. 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.

**RESULTS**

As shown in Fig. 1, Ser-155 lies within the BH3 region of BAD (asterisk). Ser-155 is embedded in a canonical protein kinase A motif (RRXS) suggesting that this residue may be phosphorylated by β-casein kinase I in certain kinases. To test the idea that phosphorylation at this residue might alter the pro-apoptotic function of the BAD BH3 domain, we generated a phosphorylation state-specific antibody (20) directed against Ser-155 of BAD as described under “Experimental Procedures.” The antibody was shown to be highly specific for the phosphorylated epitope by enzyme-linked immunosorbent assay (data not shown) and by Western blotting using phosphorylated and nonphosphorylated GST-BAD proteins (Fig. 2A). We used this antibody to examine the ability of several different protein kinases to phosphorylate BAD at Ser-155 in vitro with a GST-BAD fusion protein as substrate. As demonstrated in Fig. 2A, of the protein kinases tested, only protein kinase A was able to phosphorylate BAD at Ser-155. This observation led us to test whether in vivo activation of protein kinase A using forskolin would stimulate phosphorylation of BAD at Ser-155. HEK293 cells were transfected with plasmids encoding wild-type GST-BAD or GST-BAD S112A/S136A or S155A mutants. Cell extracts were prepared and immunoblotted using phospho-specific antibodies directed against BAD Ser-155 and BAD Ser-12 as well as phosphorylation state-independent anti-BAD antibodies (Fig. 2B). Forskolin increased the phosphorylation of GST-BAD at both Ser-155 and Ser-112. The site specificity of the anti-phospho-BAD(Ser-155) and -BAD(Ser-112) antibodies was analyzed using the mutant BAD proteins. As expected, mutation of either Ser-155 or Ser-112 to alanine eliminated immunoreactivity with the corresponding phospho-specific antibody. Although the S136A/S112A double mutant is unable to bind 14-3-3 (2), it was phosphorylated at Ser-155 in response to forskolin, indicating that Ser-155 can be phosphorylated in the absence of phosphorylation at Ser-112 and Ser-136. As shown in Fig. 2C, forskolin induced a rapid and sustained phosphorylation of BAD at Ser-155. Transfection of HEK293 cells with the catalytic subunit of protein kinase A also increased the phosphorylation of BAD at Ser-155 (Fig. 2D).

We also explored other agents that might stimulate phosphorylation of BAD at Ser-155. Treatment with TPA, isoprotorex- nol, forskolin, or insulin-like growth factor produced the largest increase in phosphorylation at Ser-155 (Fig. 3A). TPA rapidly induced a sustained phosphorylation of BAD that was evident within 5 min of treatment and remained elevated 8 h after treatment (Fig. 3B). Because TPA activates RSK1 in these cells (7), next we tested whether RSK1 phosphorylates BAD at Ser-155. RSK1 was immunoprecipitated from mock or TPA-treated HEK293 cells using a RSK1-specific monoclonal antibody. Immunocomplex kinase assays were performed using wild-type and mutant GST-BAD fusion proteins as substrates, and BAD phosphorylation was detected by immunoblotting using anti-phospho-BAD(Ser-155) antibody (Fig. 4A). Immunoprecipitated RSK1 phosphorylated BAD at Ser-155, and this activity was increased after treatment with TPA. To examine whether RSK1 might also phosphorylate BAD at Ser-155 in vivo, HEK293 cells were cotransfected with plasmids encoding RSK1 and GST-BAD. Overexpression of RSK1 induced BAD phosphorylation at Ser-155 in the absence of TPA treatment, and Ser-155 phosphorylation was further stimulated by TPA in vivo.

![Fig. 1. Alignment of proapoptotic BH3 proteins with Bcl-XL. Asterisk, alignment of Ser-155 of mouse BAD with corresponding residues of other BH3 proteins (human BIM, mouse BID, human BAK, mouse BAX, human Bcl-XL, and human BIK) with the PKA/RSK1 consensu sequence.](image-url)
and treated with 30 μM forskolin at Ser-155. HEK293 cells were transfected as described above for PKA, dependent protein kinase II. GST phosphatase was expressed from bacteria and phosphorylated in vitro with the catalytic subunit of PKA, and its ability to bind to Bcl-XL was measured by retention on immobilized MBP-Bcl-XL maltose beads. As shown in Fig. 5A, PKA treatment strongly inhibited binding of wild-type BAD to Bcl-XL but had no effect on the binding of BAD S155A to Bcl-XL. Consistent with the inhibitory effect of phosphorylation at Ser-155, we also observed that BAD S155E blocked binding to Bcl-XL both in vitro (Fig. 5A) and in vivo (Fig. 5B). As shown in Fig. 5B, significant levels of wt BAD and BAD S155A were coimmunoprecipitated with Bcl-XL whereas BAD S155E was not. We also examined BAD-Bcl-XL complex formation using immobilized Bcl-XL to “pull down” BAD from extracts prepared from HEK293 cells transfected with BAD wt, S155A, or S155E. As shown in Fig. 5C, BAD wt and S155A were retained on the Bcl-XL beads whereas BAD S155E was not. Together, these results suggest that phosphorylation of Ser-155 or mutation of Ser-155 to glutamic acid blocks binding of BAD to Bcl-XL.

As shown in Fig. 6, BAD wt and BAD S155A were potent cell death stimulators. In HEK293 cells 24 h after transfection with BAD or BAD S155A, there was approximately 25% survival of transfected cells. Cotransfection of BAD together with RSK1 can largely reverse BAD-mediated cell death. BAD S155A or BAD S112A/S136A/S155E increased cytotoxicity and impaired RSK-mediated survival whereas BAD S155E significantly reduced cell death when compared with wt or S155A-mutated BAD. Mutation of all three phosphorylation sites renders the triple mutant S112A/S136A/S155A even more cytotoxic with no significant rescue afforded by RSK1.

**DISCUSSION**

Our results indicate that survival signals act in at least two ways to inhibit the cell-killing activity of BAD, first by phosphorylating BAD at Ser-112 and Ser-136 to stimulate binding of 14-3-3 proteins and sequestering BAD from the mitochondrial membrane (2) and second by phosphorylation of BAD at Ser-155, which directly inhibits BH3 function and interaction with Bcl-XL. Phosphorylation of BAD at Ser-155 is predicted to introduce a charged phosphate directly between the BH3 hydrophobic face and the Bcl-XL hydrophobic pocket (14, 21). This
is consistent with the effects of BAD Ser-155 mutations as well as previous studies involving mutagenesis of BAD and BAX BH3 domains (14, 15). The BH3 motif surrounding Ser-155 is most similar to BAX where detailed mutagenesis has shown the importance of Gly-67 (the BAD Ser-155 corresponding residue) in mediating BAX heterodimerization (15). As observed here for BAD S155E, BAX G67E blocked heterodimerization and profoundly reduced BAX death-promoting activity whereas G67A had little effect (15). Analysis of the three-dimensional structure of another BH3 domain only protein, BID (22–24), has established that the BH3 hydrophobic face is buried within the molecule and is unmasked by caspase 8-mediated cleavage, activating the BID-induced death-promoting activity (21, 23, 25). The above observations suggest that regulation of BH3 domain accessibility by covalent modification may be a general feature of the death-promoting BH3 domain only proteins.

The two currently understood survival pathways involve activation of either the Ras-MAPK pathway (8, 9, 26, 27) and/or the phosphatidylinositol 3-kinase-Akt pathway (4–6, 28–33). These pathways signal independently to two different sites on BAD; the Akt pathway signals to Ser-136 on BAD (4, 5) whereas the MAPK pathway signals to Ser-112 (7–11) and Ser-155. Because phosphorylation at both Ser-136 and Ser-112 is required for cooperative high affinity 14-3-3 binding (2, 34), complete cytosolic sequestration may require activation of both pathways. Our work demonstrates that RSK phosphorylates BAD at both Ser-112 and Ser-155 and rescues BAD-mediated cell death in a manner dependent upon phosphorylation at both sites. Additional work is needed to determine whether Ser-155 phosphorylation serves a fail-safe function to reduce inappropriate
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FIG. 6. RSK1 blocks BAD-mediated cell death in a Ser-155 and Ser-112-dependent manner. HEK293 cells were transfected with the indicated combination of plasmids encoding Rous sarcoma virus β-galactosidase; GST-BAD (WT); or S155A, S155E, S112A/S136A, S112A/S136A/S156A mutants with or without RSK1. 24 h after transfection, cells were serum-starved for 18 h, fixed, and analyzed by X-gal staining. Quantitation of cell survival was performed by counting X-gal positive apoptotic cells with highly condensed nuclei were excluded.) The standard deviation (bars) was calculated based on four independent experiments. X-gal staining was carried out as described previously (7).

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