Growth Factors Inactivate the Cell Death Promoter BAD by Phosphorylation of Its BH3 Domain on Ser<sup>155</sup>*

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The Bcl-2 family protein BAD promotes apoptosis by binding through its BH3 domain to Bcl-x<sub>L</sub> and related cell death suppressors. When BAD is phosphorylated on either Ser<sup>112</sup> or Ser<sup>136</sup>, it forms a complex with 14-3-3 in the cytosol and no longer interacts with Bcl-x<sub>L</sub> at the mitochondria. Here we show that phosphorylation of a distinct site Ser<sup>155</sup>, which is at the center of the BAD BH3 domain, directly suppressed the pro-apoptotic function of BAD by eliminating its affinity for Bcl-x<sub>L</sub>. Protein kinase A functioned as a BAD Ser<sup>155</sup> kinase both in vitro and in cells. BAD Ser<sup>155</sup> was found to be a major site of phosphorylation induced following stimulation by growth factors and prevented by protein kinase A inhibitors but not by inhibitors of the phosphatidylinositol 3-kinase/Akt pathway. Growth factors inhibited BAD-induced apoptosis in both a Ser<sup>112</sup>/Ser<sup>136</sup> and a Ser<sup>155</sup>-dependent fashion. Thus, growth factors engage an anti-apoptotic signaling pathway that inactivates BAD by direct modification of its BH3 cell death effector domain.

Bcl-2 family proteins are important regulators of apoptosis that function during development and other physiological processes but also contribute to pathological conditions associated with inappropriate cell survival such as cancer (1, 2). This still expanding family contains both pro- and anti-apoptotic members characterized by the presence of one or more Bcl-2 homology (BH) domains. Of these domains, BH3 has proven to be a key element in pro-apoptotic Bcl-2 homologs that mediate both protein binding and cell death functions (1, 3). In Caenorhabditis elegans, the BH3-containing protein EGL-1 functions at the most proximal point in a pathway required for all programmed cell death, and in mammalian cells BH3 proteins transduce signals from cell surface receptors to a central cell death pathway regulated by Bcl-2 (4–6). BAD is a “BH3-only” pro-apoptotic Bcl-2 family member whose function is regulated by phosphorylation in response to survival factors such as nerve growth factor, insulin-like growth factor-1, and interleukin-3 (7–9). In its unphosphorylated state, BAD forms heterodimers with anti-apoptotic Bcl-2 homologs and promotes cell death. These activities are inhibited by phosphorylation of BAD on either of two serine residues, Ser<sup>112</sup> or Ser<sup>136</sup> (8). The serine/threonine kinase Akt that is activated by growth factors through a PI 3-kinase-dependent mechanism phosphorylates BAD on Ser<sup>136</sup> (9, 10). The ribosomal S6 kinases and a mitochondria-localized cAMP-dependent protein kinase (PKA) have been reported to phosphorylate BAD on Ser<sup>112</sup> (11, 12) following stimulation by growth factors and interleukin-3, respectively. When BAD is phosphorylated on these sites, it is sequestered in the cytosol in a complex with 14-3-3 and fails to interact with Bcl-x<sub>L</sub> at mitochondrial membranes (8). The present study provides genetic, biochemical, and biological evidence that growth factor-induced phosphorylation on the novel site Ser<sup>155</sup> is within the functionally critical BH3 domain, directly blocks BAD binding to Bcl-x<sub>L</sub> and may represent another major regulatory mechanism of BAD inactivation.

Experimental Procedures

Constructs—A cDNA for murine BAD was obtained by reverse transcription-PCR using mRNA isolated from FL5.12 cells and the following PCR primers: 5′-GCCCTCCAGATCCAAGATGGAACC-3′ and 5′-GGACCCGGGTAGAATCCCGGATG-3′. When this cDNA, which encodes the 204-amino acid BAD protein (7), was cloned into pcDNA3 (Invitrogen) and was expressed in cells, the protein product was significantly larger than the endogenous BAD protein (approximately 30 kDa versus 23 kDa, respectively) detected with an anti-BAD antibody (C-20, Santa Cruz Biotechnology, Inc.). A methionine residue at position 43 of the mouse sequence corresponds to the first methionine residue of the human BAD. We generated a cDNA for the shorter form of murine BAD by using an upstream PCR primer surrounding the second methionine residue (5′-TGGAGACCACTCAGTCCAGAT-3′) and the same downstream primer as above. The expressed construct co-migrated with the endogenous BAD of FL5.12 cells, suggesting that the shorter form of BAD (162 amino acids) is the major translation product of BAD in these cells.

Serine residues 112, 136, 134, and 155 were substituted with alanine, and serine 155 was also substituted with aspartic acid using PCR-mediated mutagenesis in the context of the shorter form of BAD. (Amino acid numbering was not changed to be consistent with previous conventions; serine positions are actually 70, 94, 92, and 113, respectively.) The nucleotide sequence of BAD and its mutants was confirmed by DNA sequencing. The cDNA of BAD and its mutants were cloned into a pcDNA3-HA vector, which introduces the HA epitope at the amino terminus of the human BAD. We generated a cDNA for the shorter form of murine BAD by using an upstream PCR primer surrounding the second methionine residue (5′-TGGAGACCACTCAGTCCAGAT-3′) and the same downstream primer as above. The expressed construct co-migrated with the endogenous BAD of FL5.12 cells, suggesting that the shorter form of BAD (162 amino acids) is the major translation product of BAD in these cells.

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amide gels and transferred to nitrocellulose membranes. Blots were blocked in Western wash buffer (40 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Nonidet P-40) with 5% bovine serum albumin for 1 h at room temperature. Blots were incubated with primary antibody diluted in Western wash buffer with 3% bovine serum albumin at room temperature for 1–2 h. Blots were washed and incubated with secondary horseradish peroxidase-coupled antibody diluted in Western wash buffer with 1.5% bovine serum albumin and washed extensively. Proteins were detected by the ECL method according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Anti-HA antibody (3F10) was from Roche Molecular Biochemicals. Anti-Akt (sc1618) was from Santa Cruz Biotechnology, Inc. Anti-Bad (9671S) was from New England BioLabs, and anti-activated EGF receptor (E12120) was from Transduction Laboratories.

*In Vitro Kinase Assay*—DNA encoding BAD and various mutants was excised from pcDNA3 as a BamHI-EcoRI fragment and inserted into the BamHI-EcoRI cloning sites of pGEX-2T (Amersham Pharmacia Biotech). GST fusion proteins were expressed in *Escherichia coli* strain DH5α. Cells (500 ml) were grown to an *A*_{595} of 0.7–0.9 at 37 °C and induced with 2 mM isopropyl-β-D-thiogalactopyranoside at 30 °C overnight. Cells were collected by centrifugation and suspended in 20 ml of HBSS (10 mM Hepes, pH 7.5, 3.4 mM EDTA, 150 mM NaCl) plus 1% (v/v) Triton X-100 and 10 mM β-mercaptoethanol. Cells were lysed by two passages through a Microfluidizer M110S (Microfluidics), and cell debris was removed by centrifugation for 30 min at 20,000 × g. A 20% ammonium sulfate precipitation was performed on the cell lysate to remove aggregated protein, and GST-BAD was purified from the supernatant by glutathione-agarose chromatography. The fractions containing GST-BAD were pooled and concentrated using a Centriprep 3 (Amicon), and the protein concentration was determined by the Bradford assay (Bio-Rad). *In vitro* kinase assays were carried out in 30-μl volumes containing 10 mM Hepes, pH 7.5, 100 mM NaCl, 12 mM MgCl₂, 1 mM dithiothreitol, 15 μCi of [γ-32P]ATP (6000 Ci/mmol, NEN Life Science Products), 11 units of protein kinase A (catalytic subunit from bovine heart, Calbiochem), and 1 μg of purified GST-BAD. Reactions were incubated for 30 min at 30 °C and terminated by the addition of SDS-polyacrylamide gel electrophoresis sample buffer.

*Antibody Development*—The anti-phospho-Ser_{155} BAD antibody was produced at Bio-Synthesis Inc. (Lewisville, TX). A polypeptide of the sequence NH₂-GCQRYGRELRRMpSDESVDSF-COOH where pS is phosphoserine was synthesized at >70% purity, conjugated to keyhole limpet hemocyanin, and injected into rabbits. Immune serum (10 ml) was collected, and the fraction containing the anti-phospho-Ser_{155} antibody was purified using protein A-Sepharose and reacted with the lower unphosphorylated band (see Fig. 2C), and failed to recognize either Ser_{112} or Ser_{136}-phosphorylated BAD (data not shown).

**Apoptosis Assay**—HeLa cells were plated in 12-well plates at 5 × 10⁴ cells/well 1 day prior to transfection. Cells were transfected with BAD (or Bcl-xL mutants) and β-galactosidase expression plasmids in a ratio of 4:1 (0.6 μg:0.15 μg) using the Superfect transfection reagent (Qiagen). Twenty-four hours after transfection, cells were cultured in serum-free medium or in serum-free medium plus EGF for an additional 12 h. β-galactosidase activity was measured in extracts using a fluorogenic substrate (MUG, Bio-Rad FluorAce) β-galactosidase reporter assay. 170–3150. The loss of β-galactosidase activity in these assays reflects apoptosis and elimination of the transfected cells, and the β-galactosidase reductions were reversed by the addition of a broad spectrum caspase inhibitor, Z-VAD-fluoromethylketone (where Z is benzoxycarbonyl, data not shown).

**Competition Binding Assay**—Immunol 2 (Dynatech) microtiter plates were coated with 5 μg/ml neutravidin (50 μg/well, Pierce) in sodium bicarbonate buffer, pH 9.0, overnight at 4 °C. All remaining steps were conducted at room temperature. Plates were washed two times with phosphate-buffered saline containing 0.1% Tween 20 (wash buffer) and blocked for 1 h with 0.2 ml/well 1% normal goat serum in phosphate-buffered saline. Following two additional washes, 1.25 μg/ml Bak BH3 19-mer peptide (residues 71–89) that was biotinylated at the amino terminus, was added to the wells in a ratio of 10 μg of 10 mM Hepes buffer, pH 7.2, containing 150 mM KCl, 5 mM MgCl₂, 1 mM EGTA, and 0.2% Nonidet P-40 (Nonidet P-40 buffer). After 30 min, the wells were washed twice with wash buffer, and GST-Bcl-xL (0.25 μM in 50 μl of Nonidet P-40 buffer) was added in the absence or presence of BAD or Bak BH3 peptides. Following a 1-h incubation, the plates were washed twice with wash buffer, and the amount of bound GST-Bcl-xL was determined by enzyme-linked immunosorbent assay using an anti-GST primary antibody and a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Jackson) with ABTS (Zymed Laboratories Inc.) as substrate. Five washes were conducted following each 1-h antibody incubation. GST-Bcl-xL fusion protein was produced in *E. coli* by a similar procedure that was described for the production of GST-BAD (see above).

**RESULTS**

**Ser**_{155} Is a Third Phosphorylation Site of BAD—Immunoblot analysis of a BAD mutant that was unable to be phosphorylated on Ser_{112} and Ser_{136} revealed that BAD was phosphorylated on a third site. BAD S112A/S136A expressed in COS-7 cells transiently transfected with HA-BAD S112A/S136A was electrophoresed on a large dimension 16% SDS-polyacrylamide gel and were followed by Western blot analysis with an anti-HA antibody. Samples of cell lysate were treated with a phosphatase (+PP) or were left untreated (−PP). Untreated lysates were also incubated with either GST-Bcl-xL (Xl) or GST (at 10 μg/ml) for 2 h at 4 °C. GST fusion protein complexes were captured by incubation with glutathione beads and washed with radioimmune precipitation buffer (Pull-down). B, the BAD S112A/S136A double mutant and the HA-tagged triple serine substitution mutants of BAD, S112A/S134A/S136A and S112A/S136A/S155A, were transiently expressed in COS-7 cells. For the control, cells were transfected without plasmid DNA (mock). Cell lysates were prepared (treated either with a phosphatase (PP) or left untreated) and HA-BAD proteins were detected by Western blot analysis using anti-HA antibody. C, HA-tagged wild-type BAD (WT) and BAD S155A were transiently expressed in HeLa cells. After serum starvation for 12 h, the cells were treated with EGF (50 ng/ml for 15 min) or fetal calf serum (FCS, 20%, 15 min). HA-BAD and its phosphorylated forms were detected in cell lysates by anti-HA Western blot analysis.

![Image](Image 363x473 to 499x729)

**FIG. 1.** Ser_{155} is a third phosphorylation site of BAD. A, lysates of COS-7 cells transiently transfected with HA-BAD S112A/S136A were electrophoresed on a large dimension 16% SDS-polyacrylamide gel and were followed by Western blot analysis with an anti-HA antibody. Samples of cell lysate were treated with a phosphatase (+PP) or were left untreated (−PP). Untreated lysates were also incubated with either GST-Bcl-xL (Xl) or GST (at 10 μg/ml) for 2 h at 4 °C. GST fusion protein complexes were captured by incubation with glutathione beads and washed with radioimmune precipitation buffer (Pull-down). B, the BAD S112A/S136A double mutant and the HA-tagged triple serine substitution mutants of BAD, S112A/S134A/S136A and S112A/S136A/S155A, were transiently expressed in COS-7 cells. For the control, cells were transfected without plasmid DNA (mock). Cell lysates were prepared (treated either with a phosphatase (PP) or left untreated) and HA-BAD proteins were detected by Western blot analysis using anti-HA antibody. C, HA-tagged wild-type BAD (WT) and BAD S155A were transiently expressed in HeLa cells. After serum starvation for 12 h, the cells were treated with EGF (50 ng/ml for 15 min) or fetal calf serum (FCS, 20%, 15 min). HA-BAD and its phosphorylated forms were detected in cell lysates by anti-HA Western blot analysis.
Inactivation of BAD by Phosphorylation of its BH3 Domain

Protein Kinase A Is a BAD Ser<sup>155</sup> Kinase in vitro and in vivo—The sequence surrounding Ser<sup>155</sup> of mammalian PKA, XRXXXX (16), to alanine mutation significantly reduced the overall extent of BAD phosphorylation in transfected HeLa cells and eliminated the slowest migrating phosphorylated form of BAD observed following stimulation with serum or EGF (Fig. 1C). Phosphorylation on Ser<sup>155</sup> was confirmed directly by demonstrating reactivity of BAD to a phospho-Ser<sup>155</sup>-specific antibody (see below). Interestingly, Ser<sup>155</sup> is located at the center of the BH3 domain of BAD, the structural element that mediates BAD/Bcl-x<sub>L</sub> dimerization (13–15).

Serine 134 eliminated the slower migrating band of the doublet when expressed in COS-7 cells (BAD S112A/S136A/S155A; Fig. 1B) suggesting that Ser<sup>155</sup> could be a third site of phosphorylation on BAD. A single Ser<sup>136</sup> to alanine mutation significantly reduced (S155A single mutant) or completely abolished (S112A/S136A/S155A triple mutant) phosphorylation (Fig. 2A). In contrast, mutation of Ser<sup>155</sup> dramatically reduced (S155A single mutant) or completely abolished (S112A/S136A/S155A triple mutant) phosphorylation (Fig. 2A) indicating that Ser<sup>155</sup> is the major phosphorylation site of PKA in vitro.

To test whether PKA may function as a Ser<sup>155</sup> kinase of BAD in vivo, we examined whether known activators of PKA would stimulate BAD Ser<sup>155</sup> phosphorylation in cells. Forskolin, which stimulates PKA through the activation of adenylyl cyclase, rapidly induced a sustained gel mobility shift of BAD S112A/S136A in transfected HeLa cells that was sensitive to phosphatase treatment (Fig. 2B). In contrast, forskolin had no effect on the BAD S112A/S136A/S155A mutant, indicating that Ser<sup>155</sup> is the likely site of phosphorylation by a cAMP-dependent kinase in vivo (Fig. 2B). An anti-phospho-Ser<sup>155</sup>-specific BAD antibody (see "Experimental Procedures") reacted with the phosphorylated bands of wild-type BAD and BAD S112A/ S136A induced by forskolin in transfected HeLa cells (Fig. 2C, upper panels). This antibody is specific for phospho-Ser<sup>155</sup>-BAD; it failed to react with BAD S155A or the BAD triple mutant although the expression levels of BAD and the BAD mutants were at similar levels (Fig. 2C, lower panels). These results provide direct evidence for BAD phosphorylation on Ser<sup>155</sup>. Because adenylyl cyclase is a target of activated G protein-coupled receptors (18), we also examined whether BAD Ser<sup>155</sup> phosphorylation would respond to ligands of G protein-coupled receptors. Of the ligands tested in HeLa cells, L-epinephrine rapidly induced a gel mobility shift of BAD S112A/

![Fig. 2. Protein kinase A is a BAD Ser<sup>156</sup> kinase in vitro and in vivo.](image)

![Fig. 3. Growth factors induce phosphorylation on BAD Ser<sup>155</sup> by a PKA-dependent mechanism, independent of PI 3-kinase/Akt.](image)
S136A (Fig. 2D) that was blocked by mutation of BAD Ser\(^{155}\) to alanine or by the co-expression of PKI (17) (data not shown). Together, these results demonstrate that BAD Ser\(^{155}\) can be phosphorylated in cells either by PKA directly or through a PKA-dependent pathway.

**Growth Factors Induce Phosphorylation of BAD Ser\(^{155}\) by a PKA-dependent Mechanism That Is Independent of PI 3-Kinase/Akt**—Growth factors suppress the pro-apoptotic function of BAD in part through the activation of Akt, which functions as a BAD Ser\(^{136}\) kinase (9, 10). We examined whether growth factor stimulation would also induce Ser\(^{155}\) phosphorylation of BAD. EGF stimulated the phosphorylation of BAD on Ser\(^{155}\) in transfected HeLa cells, which was blocked by co-expression of PKI (Fig. 3A). To test whether phosphorylation of Ser\(^{155}\) is likewise dependent on the PI 3-kinase/Akt pathway, we treated transfected HeLa cells with wortmannin, an inhibitor of PI 3-kinase, and analyzed BAD Ser\(^{155}\) phosphorylation following EGF stimulation. Wortmannin treatment prevented the activation of endogenous Akt by PI 3-kinase following EGF stimulation but did not impair EGF-induced phosphorylation of BAD on Ser\(^{155}\) (Fig. 3B). In parallel assays, BAD Ser\(^{155}\) phosphorylation was prevented by the addition of the PKA inhibitor H89 without affecting the phosphorylation of Akt. The EGF receptor kinase inhibitor AG1478 blocked the phosphorylation of both Akt and BAD Ser\(^{155}\). These results demonstrate that EGF stimulates the phosphorylation of BAD Ser\(^{155}\) through a PKA-dependent mechanism that is distinct from the PI 3-kinase/Akt pathway.

To examine whether endogenous BAD is phosphorylated on Ser\(^{155}\) in response to growth factors, serum-starved Rat-1 fibroblasts were stimulated with PDGF, endogenous BAD was immunoprecipitated, and Ser\(^{155}\) phosphorylation was analyzed by Western blot with the anti-phospho-Ser\(^{155}\) antibody. PDGF induced the phosphorylation of endogenous BAD on Ser\(^{155}\), which was significantly reduced by pretreatment of cells with the PKA inhibitor H89 but not the PI 3-kinase inhibitor wortmannin (Fig. 3, C and D).

**Phosphorylation of BAD on Ser\(^{155}\) Correlates with Cell Survival**—The pro-apoptotic function of BAD requires its ability to heterodimerize with Bcl\(_x\)-L and related proteins (13–15). Thus, the phosphorylation of Ser\(^{155}\) in response to growth/survival factors (Fig. 3) and its apparent inhibitory effect on binding to Bcl\(_x\)-L (Fig. 1A) suggest that it may suppress the pro-apoptotic activity of BAD. In keeping with this possibility, mutation of BAD Ser\(^{155}\) to a residue that cannot be phosphorylated enhanced the pro-apoptotic function of BAD in transient transfection assays. BAD S155A showed a modest but highly reproducible enhancement of toxicity compared with wild-type BAD, and the S112A/S136A/S155A triple mutant consistently exhibited greater toxicity than the S112A/S136A double mutant (Fig. 4A, open bars). Western blot analysis demonstrated that the enhanced toxicity of these mutants could not be attributed to elevated levels of protein expression in these assays (Fig. 4A, inset).

EGF stimulation, which induces BAD Ser\(^{155}\) phosphoryla-
Phosphorylation of the BAD BH3 domain on Ser\textsuperscript{155} directly blocks BAD/Bcl-x\textsubscript{L} heterodimerization. A, the structure of murine BAD is shown schematically with BH3, and the sites of serine phosphorylation are indicated (top). The BH3 domain sequences from BAD orthologs are shown in alignment with BH3 domains from other known pro-apoptotic Bcl-2 family proteins. The sequence of zebrafish BAD was deduced from an expressed sequence tag clone (GenBank\textsuperscript{\textregistered} accession no. AI332108). B, synthetic BAD BH3 peptides were incubated at the indicated concentrations with GST-Bcl-x\textsubscript{L}. The binding of BH3 peptides to Bcl-x\textsubscript{L} was measured by the ability of the peptides to block the subsequent binding of GST-Bcl-x\textsubscript{L} to a Bak BH3 peptide immobilized on a microtiter plate as detected by enzyme-linked immunosorbent assay (triplicate samples). Bars indicate mean ± S.D. BAD peptides corresponded to residues 143–168 that were either unphosphorylated (BADBH3) or phosphorylated on Ser\textsuperscript{155} (BADBH3-P). A Bak BH3 peptide (residues 71–89) was used as a positive control for competition binding. C, [\textsuperscript{35}S]methionine-labeled wild-type BAD (WT) or BAD S155A was produced by in vitro translation (IVT), and incubated with PKA as described in the legend to Fig. 2 except that unlabeled ATP (200 \mu M) was used. Following the kinase reaction, aliquots were incubated for 1 h with either GST or GST-Bcl-x\textsubscript{L} (1 \mu M), and protein complexes were captured on glutathione-agarose beads. Proteins bound to beads (Bound) and samples of reactions prior to incubation with beads (Total) were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**DISCUSSION**

Mutational studies have provided strong evidence that BAD promotes apoptosis by dimerizing with Bcl-x\textsubscript{L} and related proteins (13), emphasizing the importance of understanding how...
phosphorylation regulates this interaction. Our findings suggest that phosphorylation on different sites within BAD have mechanistically distinct consequences; Ser\textsuperscript{155} phosphorylation directly prevents heterodimerization by abolishing the affinity of BAD BH3 for Bcl-x\textsubscript{L} whereas phosphorylation on Ser\textsuperscript{112} and Ser\textsuperscript{136} that are located outside the BH3 domain may inhibit dimerization with Bcl-x\textsubscript{L} indirectly. In particular, phosphorylation on Ser\textsuperscript{112} or Ser\textsuperscript{136}, but not Ser\textsuperscript{155}, generates a consensus binding site for the cytosolic protein 14-3-3 that may bind and alter the subcellular distribution of BAD to prevent interaction with Bcl-x\textsubscript{L} at mitochondrial membranes (8).

Phosphorylation of BAD provides an important link between extracellular survival factors and the intrinsic cell death pathway regulated by Bcl-2. The prevailing model for anti-apoptotic signaling by growth/survival factors emphasizes a pathway involving the PI 3-kinase-dependent activation of Akt, phosphorylation of BAD on Ser\textsuperscript{136}, and subsequent dissociation from Bcl-x\textsubscript{L}. Our findings identify a distinct anti-apoptotic pathway that leads to the biochemical inactivation of the BH3 domain through a PKA-dependent but PI 3-kinase/Akt-independent mechanism. PKA-mediated phosphorylation of BAD Ser\textsuperscript{155} may explain, at least in part, the anti-apoptotic effects of PKA activation that have been observed in some settings (20–22) and PKI 3-kinase-independent survival signals triggered by growth factors (23, 24). Precisely how growth factors activate PKA in cells is not clear although there have been reports showing that PDGF activates PKA by stimulating its release from cell membrane (25) and that EGF activates PKA via Grb-2-mediated recruitment of PKA to the EGF receptor (26). Mitochondrial membrane-localized PKA was identified as an interleukin-3-induced kinase of BAD Ser\textsuperscript{112} (12). This is a paradox because our results indicate that Ser\textsuperscript{112} is a poor substrate for PKA relative to Ser\textsuperscript{155} both in vitro and in cells. It is possible that Ser\textsuperscript{155} may prove to be an important target for the mitochondria-localized PKA because the BAD substrate used in that study was a mutant lacking Ser\textsuperscript{155} (12).

The convergence of multiple growth factor-stimulated pathways on BAD underscores the important role of this BH3 protein in anti-apoptotic signaling by cell surface receptors. It has been generally proposed that BAD kinases modify the unphosphorylated form of BAD bound to Bcl-x\textsubscript{L} at mitochondrial membranes, thereby releasing free active Bcl-x\textsubscript{L}. Both PKA and Akt, however, are unable to phosphorylate BAD in vitro on residues Ser\textsuperscript{155} and Ser\textsuperscript{136}, respectively, when BAD is in a pre-existing complex with Bcl-x\textsubscript{L}.\textsuperscript{2} Although substrate accessibility may be different in cells, this finding suggests that PKA and Akt may inactivate BAD not by dissociating existing BAD-Bcl-x\textsubscript{L} complexes but rather by preventing its accumulation in a dephosphorylated "active" state. The relative significance of PKA- and Akt-mediated pathways to the inactivation of BAD in different settings remains to be determined. In tumor cell lines, our preliminary results indicate that basal BAD Ser\textsuperscript{155} phosphorylation is higher than in non-transformed cells and is more refractory to dephosphorylation upon growth factor deprivation.\textsuperscript{3} This raises the possibility that the inactivation of BAD BH3 through Ser\textsuperscript{155} phosphorylation makes an important contribution to the suppression of apoptosis in cancer cells.

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\textsuperscript{2} X.-M. Zhou and R. J. Lutz, unpublished observations.

\textsuperscript{3} X.-M. Zhou and Y. Liu, unpublished observations.