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14-3-3 Proteins and Survival Kinases Cooperate to Inactivate BAD by BH3 Domain Phosphorylation

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Summary

The Bcl-2 homology 3 (BH3) domain of prodeath Bcl-2 family members mediates their interaction with prosurvival Bcl-2 family members and promotes apoptosis. We report that survival factors trigger the phosphorylation of the proapoptotic Bcl-2 family member BAD at a site (Ser-155) within the BAD BH3 domain. When BAD is bound to prosurvival Bcl-2 family members, BAD Ser-155 phosphorylation requires the prior phosphorylation of Ser-136, which recruits 14-3-3 proteins that then function to increase the accessibility of Ser-155 to survival-promoting kinases. Ser-155 phosphorylation disrupts the binding of BAD to prosurvival Bcl-2 proteins and thereby promotes cell survival. These findings define a mechanism by which survival signals inactivate a proapoptotic Bcl-2 family member, and suggest a role for 14-3-3 proteins as cofactors that regulate sequential protein phosphorylation events.

Introduction

Survival factors, acting through their cognate cell surface receptors, activate intracellular signaling cascades that promote survival by suppressing an intrinsic cellular death program (Vaux and Korsmeyer, 1999). Proteins of the Bcl-2 family are components of a survival factor-regulated checkpoint in the cellular death machinery (Reed, 1998). Bcl-2 family members promote either survival (e.g., BCL-2, BCL-X_L, and the *C. elegans* Bcl-2 homolog CED-9) or apoptosis (e.g., BAK, BAD, and the *C. elegans* BCL-2 homolog EGL-1). Although the precise mechanism by which the Bcl-2 proteins function remains unclear, their ability to homo- and heterodimerize has given rise to the hypothesis that the balance between prosurvival and prodeath Bcl-2 family members determines whether a cell lives or dies (Oltvai and Korsmeyer, 1994). In living cells, the tilt toward survival is maintained, in part, by mechanisms that prevent proapoptotic Bcl-2 family members from interacting with and inactivating prosurvival Bcl-2 family members (Zha et al., 1996; Goping et al., 1998; Li et al., 1998; Luo et al., 1998; Puthalakath et al., 1999). When cells are challenged with apoptotic stimuli, death-promoting Bcl-2 family members become activated, and the equilibrium within the Bcl-2 family protein network shifts toward apoptosis. Apoptosis then occurs through a cascade of events that culminates in the release of cytochrome c from the mitochondria and subsequent death protease activation (Reed, 1998).

A subset of the proapoptotic Bcl-2 family proteins are localized to the cytoplasm under conditions of cellular survival and translocate to the mitochondria under apoptotic conditions, making these Bcl-2 family members attractive candidates for molecular switches that regulate cell death in response to changes in the extracellular environment (Zha et al., 1996; Li et al., 1998; Luo et al., 1998). One proapoptotic molecule that belongs to this subset of the Bcl-2 family is BAD (Yang et al., 1995; reviewed in Datta et al., 1999). Structural and functional analysis indicates that a key role of survival factors is to inhibit the activity of BAD. In the absence of survival stimuli, endogenous BAD is dephosphorylated and localized to the outer mitochondrial membrane where BAD binds to prosurvival Bcl-2 family members via its Bcl-2 homology 3 (BH3) domain, an amphipathic α helix that is conserved in all proapoptotic Bcl-2 family members and is critical to the death-promoting function of these proteins. Survival factors, acting through kinases such as Akt and PKA, induce endogenous BAD phosphorylation at two evolutionarily conserved sites, Ser-112 and Ser-136, which leads to the translocation of BAD from the mitochondria to the cytoplasm and the inhibition of BAD-dependent death (reviewed in Datta et al., 1999).

It has been proposed that the phosphorylation of BAD at Ser-112 and/or Ser-136 induces a conformational change in BAD that blocks its ability to interact with prosurvival Bcl-2 family members such as BCL-X_L (Wang et al., 1999). Secondary structural prediction models suggest that BAD Ser-112 lies within an N-terminal α helix, while Ser-136 lies within an adjacent unstructured loop domain. However, deletion analysis and mutagenesis have revealed that the ability of BAD to regulate apoptosis is conferred exclusively by its C-terminal BH3 domain (Kelekar et al., 1997; Ottilie et al., 1997; Zha et al., 1997). Although phosphorylation of Ser-112 and Ser-136 is critical for inactivating BAD, it is not clear how phosphorylation of the BAD N terminus effects a change in the activity of the distal BAD BH3 domain and leads to the disruption of the BAD/BCL-X_L complex.

Phosphorylation of BAD at Ser-112 and Ser-136 promotes the interaction of BAD with 14-3-3 proteins, a large family of proteins that bind to phosphoserine and phosphothreonine-containing ligands and are thought to act as molecular chaperones (Muslin et al., 1996; Yaffe et al., 1997). The binding of BAD to 14-3-3 correlates with

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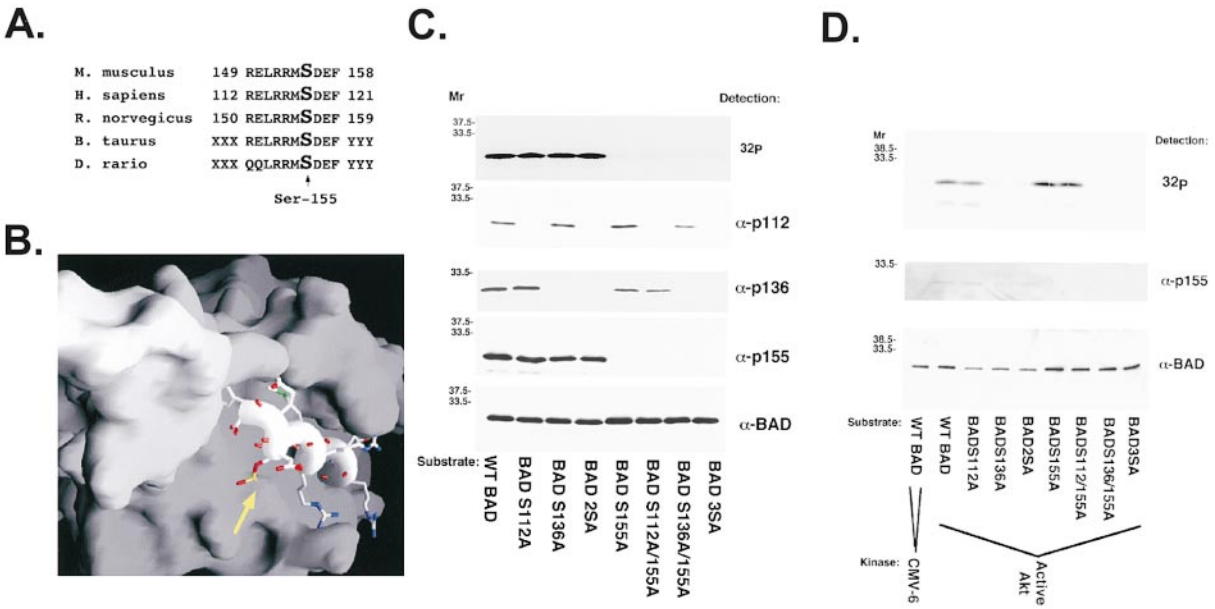

Figure 1. PKA Directly Phosphorylates BAD Ser-155 In Vitro

(A) Multiple sequence alignment of the C-terminal segment of BAD BH3 domains from multiple species. Bovine and zebrafish sequences are derived from expressed sequence tags (dbEST accession numbers AW425056 and AI332008) that encode BAD homologs.

(B) The modeled structure of the BCL-X_L:phospho-Ser155 BAD peptide complex based on the BCL-X_L:BAK peptide structure shown in GRASP surface representation (see Experimental Procedures). Side chains of the BAD BH3 domain (white helix) are depicted in stick representation. The hydrophilic residues of the BH3 domain that form the solvent-accessible hydrophilic face of the BAD BH3 amphipathic helix are shown in blue; the hydrophobic side chains of the BAD BH3 domain that interact with BCL-X_L are largely obscured in this view. Phosphorylated Ser-155 is just above the BCL-X_L surface indicated by an arrow (yellow); as shown, the BCL-X_L/BAD interaction cannot accommodate phosphorylated BAD Ser-155, as the phosphate group collides with the molecular surface of BCL-X_L (yellow arrow).

(C) BAD Ser-155 is a major PKA phosphorylation site. Kinase assay using .25 U purified PKA (HMK, Sigma) to phosphorylate 100 ng recombinant WT BAD or various BAD mutants. Samples generated in parallel were separated by SDS-PAGE and subjected to phosphorimager scanning (upper panel) or Western blot (lower panels) with anti-BAD antisera, or phosphospecific antibodies as indicated.

(D) Akt primarily phosphoryates BAD Ser-136 but can phosphorylate Ser-155 in vitro. Immunocomplex kinase assay using anti-HA immunoprecipitates from stable balb/c 3T3 cell lines expressing either constitutively active Akt (clone 4-23 mΔPH) or CMV-6 vector using 100 ng/lane recombinant BAD as a substrate. Parallel samples were separated by SDS-PAGE, and subjected to phosphorimager scanning (upper panel), or Western blotting with anti-BAD Abs or anti-BAD155 pAbs (lower panels).

BAD inactivation, and 14-3-3 proteins promote cellular survival, consistent with a causative role for 14-3-3 proteins in blocking the ability of BAD to induce cell death (Hsu et al., 1997; Xing et al., 2000). However, it is not known whether 14-3-3 binding to phosphorylated BAD is sufficient to displace BAD from BCL-X_L or is sufficient to inactivate the proapoptotic activity of BAD.

Here we show that survival signals block apoptosis by phosphorylating the proapoptotic Bcl-2 family member BAD at Ser-155, a site within its BH3 domain. In the absence of survival stimuli, BAD is dephosphorylated and bound to BCL-X_L. Upon exposure to survival factors, Ser-136 phosphorylation is induced, leading to the recruitment of 14-3-3 proteins to BAD. We find that 14-3-3 only weakly binds to the BAD/BCL-X_L complex but effectively increases the accessibility of Ser-155 to survival kinases. Phosphorylation of BAD Ser-155 disrupts the interaction between the hydrophobic face of the BAD BH3 domain and the hydrophobic groove in BCL-X_L, causing BAD to dissociate from BCL-X_L. BAD then translocates from the outer mitochondrial membrane to the cytoplasm, where it forms a stable complex with 14-3-3 proteins. These observations identify Ser-155 as a site whose phosphorylation is critical for cellular survival, and raise the possibility that BH3 domain phosphorylation may be a general mechanism by which survival factors inactivate proapoptotic Bcl-2 family members. In addition, these findings suggest a new role for 14-3-3 proteins as cofactors that cooperate with kinases to promote sequential phosphorylation events.

Results

By interrogating the BAD primary sequence for consensus phosphorylation motifs that are potential targets for protein kinases that promote cell survival, we identified a phosphorylation site for PKA (RRXS) within the BAD BH3 domain (Figure 1A) (Songyang et al., 1994). Sequence alignment of BAD from species ranging from human to zebrafish reveals that this site (Serine-155 in mouse) is evolutionarily conserved, suggesting that Ser-155 phosphorylation may regulate BAD function. Modeling of the BAD/BCL-X_L interaction based upon a previously derived NMR structure of the BAK BH3 domain complexed to BCL-X_L (Sattler et al., 1997) indicated that Ser-155 of BAD points toward the hydrophobic cleft of BCL-X_L (Figure 1B). Phosphorylation of BAD Ser-155 is therefore predicted to interfere with the ability of BAD to bind to BCL-X_L due to the addition of a bulky, negatively charged phosphate group to the sterically constrained hydrophobic interaction interface between BAD and BCL-X_L. A recently generated solution structure of the BAD BH3 domain complexed to BCL-X_L verifies this

prediction (S. W. F., unpublished data). We hypothesized that because Ser-155 is embedded within an evolutionarily conserved PKA phosphorylation site and is well positioned within the BH3 domain to regulate the BAD/BCL-X_L interaction, BAD Ser-155 may serve as a critical site of phosphorylation by protein kinases that promote cell survival.

Phosphorylation of BAD Ser-155 In Vitro and In Vivo

To test whether PKA functions as a BAD Ser-155 kinase, we assessed the ability of purified PKA to phosphorylate recombinant BAD in vitro. We generated mutant forms of recombinant BAD in which serines 112, 136, and 155, either singly or in combination, were converted to alanine so that BAD could no longer be phosphorylated at these sites. Wild-type and mutant forms of BAD were incubated with PKA and [$\gamma-^{32}$P]ATP, and the reaction products were analyzed by SDS-PAGE followed by autoradiography. As shown in Figure 1C, PKA phosphorylates wild-type BAD protein, as well as BAD protein with Ser-112 mutated to alanine (S112A), Ser-136 mutated to alanine (S136A), or both sites mutated to alanine (2SA). In contrast, the ability of PKA to phosphorylate BAD in which Ser-155 was mutated to alanine (S155A) was substantially diminished, suggesting that Ser-155 is a major PKA phosphorylation site on BAD. To verify that BAD Ser-155 is directly phosphorylated by PKA, we generated phosphospecific antibodies that recognize BAD only when it is phosphorylated at Ser-155 (anti-BAD155 pAbs). Western blotting with the anti-BAD155 pAbs revealed that these antibodies recognize recombinant wild-type BAD phosphorylated in vitro with PKA but fail to recognize BADS155A. Using phosphospecific antibodies that specifically recognize BAD phosphorylated at Ser-112 or Ser-136, we also found that PKA phosphorylates BAD at both Ser-112 and Ser-136. Taken together, these results suggest that Ser-155 is the major phosphoacceptor site for PKA on BAD, but that PKA also phosphorylates Ser-112 and Ser-136.

The serine/threonine kinase Akt phosphorylates BAD at Ser-136 in vitro and in vivo (Datta et al., 1999). To address whether Akt phosphorylates BAD at Ser-155, we immunoprecipitated active Akt from cells and subjected this immunoprecipitate to an in vitro kinase assay using recombinant BAD as a substrate. While Akt phosphorylates wild-type BAD, BADS155A, and BADS112/S155A, the ability of Akt to phosphorylate BADS136A was substantially diminished, suggesting that Ser-136 is the major phosphoacceptor site for Akt (Figure 1D). Nevertheless, Western blotting of Akt-phosphorylated BAD with anti-BAD155 pAbs reveals that Akt can weakly phosphorylate Ser-155. These findings suggest that although Akt is principally a Ser-136 kinase, it is also capable of phosphorylating BAD at Ser-155 in vitro.

We next tested whether PKA and Akt could induce the phosphorylation of BAD at Ser-155 in cells. CCL39 fibroblasts were cotransfected with BAD expression vectors and plasmid constructs encoding the catalytic subunit of PKA or constitutively active Akt. Western blotting revealed that active PKA potently induced the phosphorylation of BAD at Ser-155 and led to a shift in the migration of BAD during SDS-PAGE, which has previously been reported to reflect dual phosphorylation of

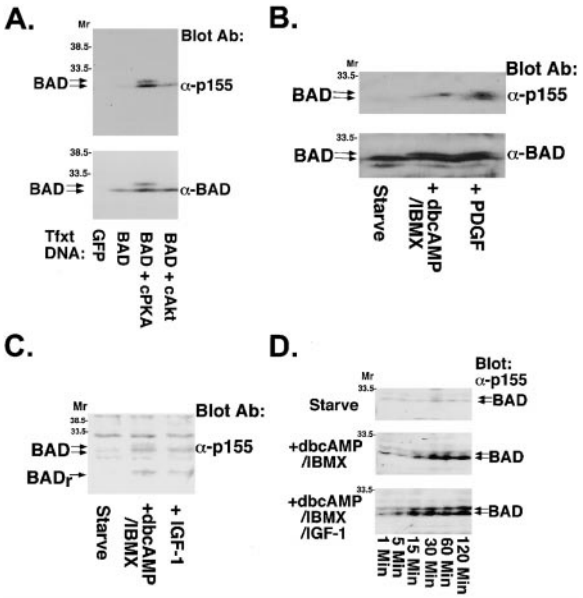

Figure 2. Survival Factors and Kinases Induce Ser-155 Phosphorylation In Vivo

(A) The catalytic subunit of PKA and constitutively active Akt induce Ser-155 phosphorylation in vivo. CCL39 fibroblasts were transfected with control vector (GFP), WT BAD vector, a vector encoding the catalytic subunit of PKA (cPKA), or a vector encoding a constitutively active mutant of Akt (cAkt) as indicated. Cells were left overnight, starved for 6 hr, and lysed in 1% NP40 lysis buffer. Lysates were subjected to SDS-PAGE and Western blotting with anti-BAD Abs or anti-BAD155 pAbs.

(B) PKA agonists and PDGF induce endogenous Ser-155 phosphorylation in fibroblasts. Balb/c 3T3 cells were starved overnight and either treated with vehicle or with dbcAMP (100 μM)/IBMX (50 μM) or platelet-derived growth factor (20 ng/ml) for 15 min before lysis in 2x LSB. Lysates were subjected to SDS-PAGE, and analyzed by Western blotting with anti-BAD Abs or anti-BAD155 pAbs.

(C) PKA agonists and IGF-1 induce endogenous Ser-155 phosphorylation in primary neurons. Cerebellar granule neurons were cultured in 10%FBS/25 mM KCl/basal medium eagle (BME) for 6 days and then stimulated with either vehicle, dbcAMP(100 μM)/IBMX(50 μM), or IGF-1 (50 ng/ml) before lysis in 1%NP40 buffer, immunoprecipitation with anti-BAD antibodies, SDS-PAGE, and Western blotting with anti-BAD155 pAbs. BAD_r refers to a BAD-related band that likely represents an N-terminal truncation of endogenous BAD.

(D) IGF-1 enhances PKA-mediated endogenous BAD Ser-155 phosphorylation. Cerebellar granule neurons were cultured and treated as in (C) for the times indicated before lysis in 2x LSB, SDS-PAGE, and analysis by Western blotting.

BAD at Ser-112 and Ser-136 (Figure 2A) (Zha et al., 1996). Active Akt also induced the phosphorylation of BAD at Ser-155. In contrast to PKA, Akt did not induce a phosphorylation-dependent mobility shift in BAD, consistent with previous data demonstrating that Akt is principally a Ser-136 kinase. The anti-BAD155 pAbs recognize both the shifted and unshifted forms of BAD, suggesting that phosphorylation of BAD at both Ser-112 and Ser-136 is not required for Ser-155 phosphorylation.

To address further the regulation of BAD by PKA and Akt in vivo, cells were stimulated with activators of PKA and Akt, and the level of endogenous BAD phosphorylation at Ser-155 was assessed. Balb/c 3T3 fibroblasts were stimulated with either the cAMP mimic dibutyryl-cyclic-AMP (dbcAMP) and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) or platelet-derived

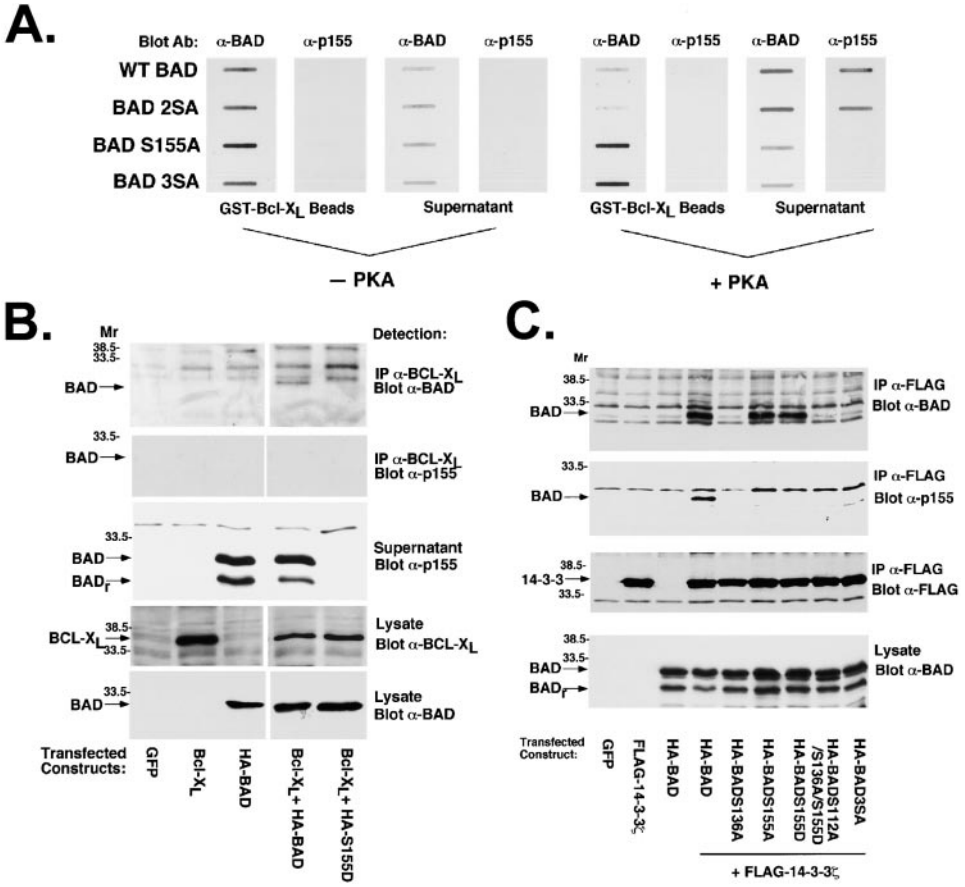

Figure 3. Phosphorylation of BAD at Ser-155 Disrupts the Ability of BAD to Bind BCL-X_L

(A) In vitro phosphorylation of BAD at Ser-155 disrupts BCL-X_L binding. Two hundred nanograms of recombinant WT or mutant BAD was either prephosphorylated with .25 U PKA or mock incubated at 30° for 30 min. GST-BCL-X_L beads were then incubated for 1 hr in 1% NP40 lysis buffer with the BAD proteins. After incubation, the supernatants and pellets were separately isolated and slot blotted with anti-BAD or anti-BAD155 pAbs.

(B) Mutation of BAD Ser-155 to aspartic acid disrupts the ability of BAD to bind to BCL-X_L in cells. HEK 293 cells were transfected with expression vectors encoding BCL-X_L and either WT BAD or the BAD S155D mutant. Thirty-six hours after transfection, cells were lysed in .2% NP40 lysis buffer, and the whole-cell lysates, anti-BAD immunoprecipitates, and immunoprecipitation supernatants were separately isolated and subjected to SDS-PAGE and Western blotting with the antibodies indicated.

(C) The 14-3-3/BAD interaction depends on Ser-136 phosphorylation, and not Ser-155 phosphorylation in cells. HEK 293 cells were transfected with expression constructs encoding 14-3-3ζ and either WT BAD, the BAD alanine phosphorylation site mutants, or BADS155D. Cells were lysed and immunoprecipitated as in (B), except that 1% NP40 lysis buffer was used for lysis and washing, and anti-BAD was used as the immunoprecipitating antibody. Lysates, supernatants, and immunoprecipitates were analyzed by Western blotting with the antibodies indicated.

growth factor (PDGF). Western blotting of lysates derived from these cells revealed that both PKA agonists and PDGF induced the phosphorylation of BAD at Ser-155 (Figure 2B). Both dbcAMP/IBMX and PDGF induced a shift in the migration of BAD on SDS-PAGE, suggesting that these stimuli induced the phosphorylation of BAD at both Ser-112 and Ser-136. As shown in Figure 2C, PKA agonists and IGF-1 also induced the phosphorylation of BAD at Ser-155 in cerebellar granule neurons, and treatment of these cells simultaneously with PKA agonists and IGF-1 promoted enhanced BAD Ser-155 phosphorylation (Figure 2D). Taken together, these findings demonstrate that endogenous BAD is inducibly phosphorylated at Ser-155 in multiple cell types in response to survival stimuli, including PKA agonists and growth factors.

BAD Ser-155 Phosphorylation Disrupts BCL-X_L Binding

Given the location of Ser-155 within the BAD BH3 domain, we investigated whether Ser-155 phosphorylation disrupts the ability of BAD to bind BCL-X_L. GST-BCL-X_L protein coupled to glutathione beads was used as an affinity reagent to precipitate recombinant BAD, and the ability of BAD phosphorylation to influence the BCL-X_L/BAD interaction was assessed. Wild-type BAD and the various BAD phosphorylation site mutants (BAD2SA, BADS155A, and BAD3SA, a derivative in which all three phosphorylation sites were mutated to alanine) interacted with BCL-X_L in the absence of PKA (Figure 3A). However, when BAD and the BAD derivatives were preincubated with PKA, those BAD variants that had an intact Ser-155 phosphorylation site no longer interacted

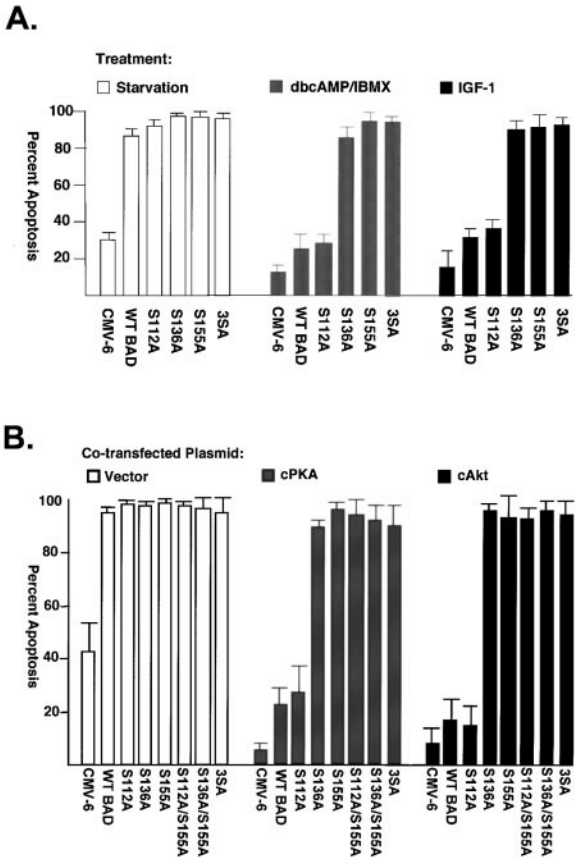

Figure 4. Survival Factors and Survival Kinases Require Phosphorylation of Ser-136 and Ser-155 to Block BAD-Mediated Apoptosis

(A) IGF-1 and PKA agonists block BAD-mediated death in a Ser-136 and Ser-155-dependent manner. Quantitation of percent apoptosis based on nuclear morphology of transfected cells as identified by positive β galactosidase staining. After transfection with indicated BAD expression plasmids, cells were allowed to recover for 24 hr and then switched into media containing either no stimuli, the PKA agonists dbcAMP (100 μM) and IBMX (50 μM), or IGF-1 (50 ng/ml). Cells were fixed after 10 hr, immunostained, and scored in a blinded manner. Bars indicate the standard error of the mean, and all of the bars have P values $<.05$; each bar represents 300-800 transfected neurons.

(B) PKA and Akt suppress BAD-mediated death in a manner dependent on Ser-136 and Ser-155 phosphorylation. Quantitation similar to (A), of neurons cotransfected with either a constitutively active Akt allele (cAkt), or the catalytic subunit of PKA (cPKA) and BAD derivatives as indicated. Cells were allowed to recover 24 hr after transfection in culture media, and then switched into basal medium eagle for 24 hr before fixation, staining, and blinded scoring.

with BCL-X_L, whereas the BAD mutants in which Ser-155 was converted to an alanine still interacted with BCL-X_L. In addition, blotting the GST-BCL-X_L beads and the supernatants with anti-BAD155 pAbs revealed that none of the BAD that precipitated with GST-BCL-X_L was phosphorylated at Ser-155. Instead, BAD phosphorylated at Ser-155 was detected exclusively in the supernatant and therefore dissociated from BCL-X_L. These data indicate that phosphorylation of BAD at Ser-155 blocks binding of BAD to BCL-X_L.

To assess the degree to which BAD phosphorylation interferes with BCL-X_L binding, we performed fluorescence anisotropy experiments using recombinant BCL-X_L and 25-mer peptides that represent the BAD BH3 domain. Wild-type and S155A peptides bound to BCL-X_L with extremely high affinity, with dissociation constants of .4 nmol and .1 nmol, respectively (see Experimental Procedures). In contrast, a BAD BH3 peptide that was synthesized with a phosphoserine in the place of Ser-155 bound to BCL-X_L with a dissociation constant of 8100 nmol, indicating that phosphorylation of BAD at Ser-155 effectively disrupts the binding of BAD to BCL-X_L in vitro.

We next asked if Ser-155 phosphorylation disrupted the BAD/BCL-X_L interaction in vivo. As it is difficult to obtain stoichiometric phosphorylation of a single site in cells, we generated a derivative of BAD in which Ser-155 was mutated to aspartic acid (S155D), an acidic amino acid whose negative charge may mimic the effects of phosphorylation. Fluorescence anisotropy experiments verified that this mutation effectively abrogated the binding of the BAD BH3 domain to BCL-X_L. WT BAD and BADS155D were expressed in 293 cells, and their ability to interact with coexpressed BCL-X_L was assessed by Western blotting. As shown in Figure 3B, WT BAD coimmunoprecipitated with BCL-X_L. In addition, BAD with any or all of the phosphorylation sites mutated to alanine coimmunoprecipitated with BCL-X_L (data not shown). However, mutation of Ser-155 to aspartic acid completely blocked the ability of BAD to coimmunoprecipitate with BCL-X_L, suggesting that phosphorylation at Ser-155 disrupts the BCL-X_L/BAD interaction within cells (Figure 3B). Because endogenous kinases phosphorylate a fraction of expressed BAD in 293 cells at multiple sites including Ser-155, we asked whether the Ser-155-phosphorylated form of BAD interacts with BCL-X_L in cells. BAD that coimmunoprecipitated with BCL-X_L was not phosphorylated at Ser-155, while BAD that failed to interact with BCL-X_L and was therefore in the immunoprecipitation supernatant was phosphorylated at Ser-155. These findings indicate that the phosphorylation of BAD at Ser-155 blocks the ability of BAD to interact with BCL-X_L in vivo.

We next investigated if Ser-155 phosphorylation affects the ability of BAD to interact with 14-3-3 proteins. Wild-type BAD effectively coimmunoprecipitated with 14-3-3ζ when expressed in 293 cells (Figure 3C). However, mutation of Ser-136 to alanine abrogated the BAD/14-3-3 interaction, suggesting that Ser-136 phosphorylation is required for BAD to bind 14-3-3. Mutation of Ser-112 to alanine, or mutation of Ser-155 to either alanine or aspartate, did not substantially influence the ability of BAD to coimmunoprecipitate with 14-3-3 (data not shown, Figure 3C), suggesting that Ser-112 or Ser-155 phosphorylation is not required for the BAD/14-3-3 interaction. In addition, Western blotting the 14-3-3 immunoprecipitates with anti-BAD155 pAbs revealed that 14-3-3 interacts with BAD that is phosphorylated at Ser-155 in vivo. These results suggest that Ser-155 phosphorylation disrupts the interaction of BAD with BCL-X_L but does not significantly affect the ability of BAD to interact with 14-3-3 proteins.

Ser-136 and Ser-155 Phosphorylation Are Both Necessary to Block BAD-Mediated Death in Primary Neurons

The observation that BAD phosphorylation at Ser-155 interferes with the ability of BAD to interact with prosurvival Bcl-2 family members such as BCL-X_L raised the

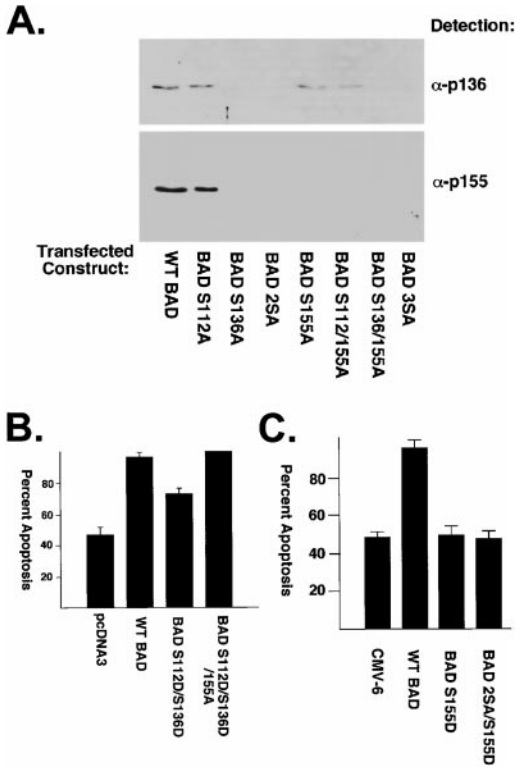

Figure 5. BAD Ser-136 Phosphorylation Leads to Ser-155 Phosphorylation

(A) BAD Ser-136 phosphorylation is required for BAD Ser-155 phosphorylation. HEK 293 cells were transfected with BAD expression vectors as indicated and lysed in 1% NP40 lysis buffer after 36 hr. Lysates were subjected to SDS-PAGE and Western blotted with anti-BAD136 pAbs or anti-BAD155 pAbs.

(B and C) Ser-155 disrupts the ability of BAD to promote programmed cell death at a step after Ser-136 phosphorylation. Transfection, treatment, and quantitation identical to Figure 4 except that the cells were transfected with the plasmids indicated. Note that the 50% death of vector-transfected cells is typical of cultured cerebellar neurons deprived of survival factors for 24 hr. All of the bars have P values <.05.

possibility that phosphorylation of Ser-155 disrupts the ability of BAD to promote apoptosis. To investigate this hypothesis, we tested the ability of BAD and various phosphorylation site mutants to induce apoptosis in cultured cerebellar granule neurons. For this analysis, we cotransfected various BAD alleles into the neurons together with a plasmid expressing the reporter β galactosidase to facilitate the identification of transfected cells, and scored transfected cells in a blinded manner based on nuclear morphology.

Neurons transfected with BAD exhibited a classic apoptotic phenotype, with shrunken, pyknotic nuclei and fragmented neuritic arbors. The vast majority of BAD-transfected neurons underwent apoptosis (Figure 4A). Treatment of BAD-transfected neurons with IGF-1 or dbcAMP/IBMX, which activates PKA, blocked the ability of wild-type BAD or BADS112A to induce cell death. However, BADS155A or BADS136A-transfected neurons were not capable of being rescued by PKA agonists or by IGF-1. PKA and Akt, the survival kinases activated downstream of dbcAMP/IBMX and IGF-1, respectively, also suppressed BAD-mediated death in a manner dependent on Ser-136 and Ser-155 phosphorylation (Figure 4B). Taken together, these findings demonstrate that survival factors and kinases require the phosphorylation of both Ser-136 and Ser-155 to block BAD-mediated apoptosis.

BAD Ser-136 Phosphorylation Leads to Subsequent Ser-155 Phosphorylation

The requirement for phosphorylation of both Ser-136 and Ser-155 to inactivate BAD is consistent with a model of BAD inactivation in which phosphorylation at one site (i.e., Ser-136) is required for the phosphorylation of a second site (i.e., Ser-155), whose phosphorylation then disrupts the ability of BAD to bind to prosurvival Bcl-2 family members and to cause cell death. To test whether phosphorylation of BAD at Ser-136 leads to subsequent phosphorylation of Ser-155, we asked whether Ser-136 phosphorylation was required for the phosphorylation of Ser-155 in cells. BAD and various BAD phosphorylation site mutants were expressed in 293 cells, and the phosphorylation state of Ser-136 and Ser-155 was analyzed by Western blotting with phosphospecific antibodies. As shown in Figure 5A, BAD is phosphorylated by endogenous kinases in 293 cells at both Ser-136 and Ser-155. Mutation of either Ser-112 or Ser-155 to alanine had no effect on the ability of Ser-136 to be phosphorylated. However, mutation of Ser-136 to alanine abolished the phosphorylation of BAD at Ser-155 within cells, suggesting that Ser-136 phosphorylation is required for Ser-155 phosphorylation.

Given the in vivo data demonstrating that BAD Ser-136 phosphorylation is required for Ser-155 phosphorylation, we tested whether the ability of phosphorylation at Ser-136 to block BAD-mediated apoptosis depended on subsequent phosphorylation of Ser-155. To address this question, we took advantage of the previous finding that acidic substitution of Ser-112 and Ser-136 can mimic the effect of phosphorylation at these sites (Wang et al., 1999). Although we tested all of the possible combinations of acidic residue substitutions at Ser-112 and Ser-136 (data not shown), only mutation of both Ser-112 and Ser-136 to aspartic acid (BADS112D/S136D) partially blocked the ability of BAD to promote cell death in neurons (Figure 5B). We asked whether the defect in cell death induced by acidic substitution of BAD Ser-112 and Ser-136 required the phosphorylation of BAD at Ser-155 by transfecting cells with a BADS112D/S136D derivative in which Ser-155 was mutated to alanine. In contrast to BADS112D/S136D, the BADS112D/S136D/S155A mutant was not impaired in its ability to promote cell death and induced apoptosis to a level comparable to that observed in cells transfected with WT BAD. This finding suggests that in order for Ser-136 and/or Ser-112 phosphorylation to inactivate the apoptotic function of BAD, Ser-155 must also be phosphorylated.

Based upon the hypothesis that BAD phosphorylation at Ser-136 blocks BAD-mediated death by inducing the phosphorylation of BAD at Ser-155, we predicted that mutation of Ser-155 to aspartic acid, which mimics phosphorylation and disrupts the binding of BAD to BCL-X_L, would block the ability of BAD to promote cell

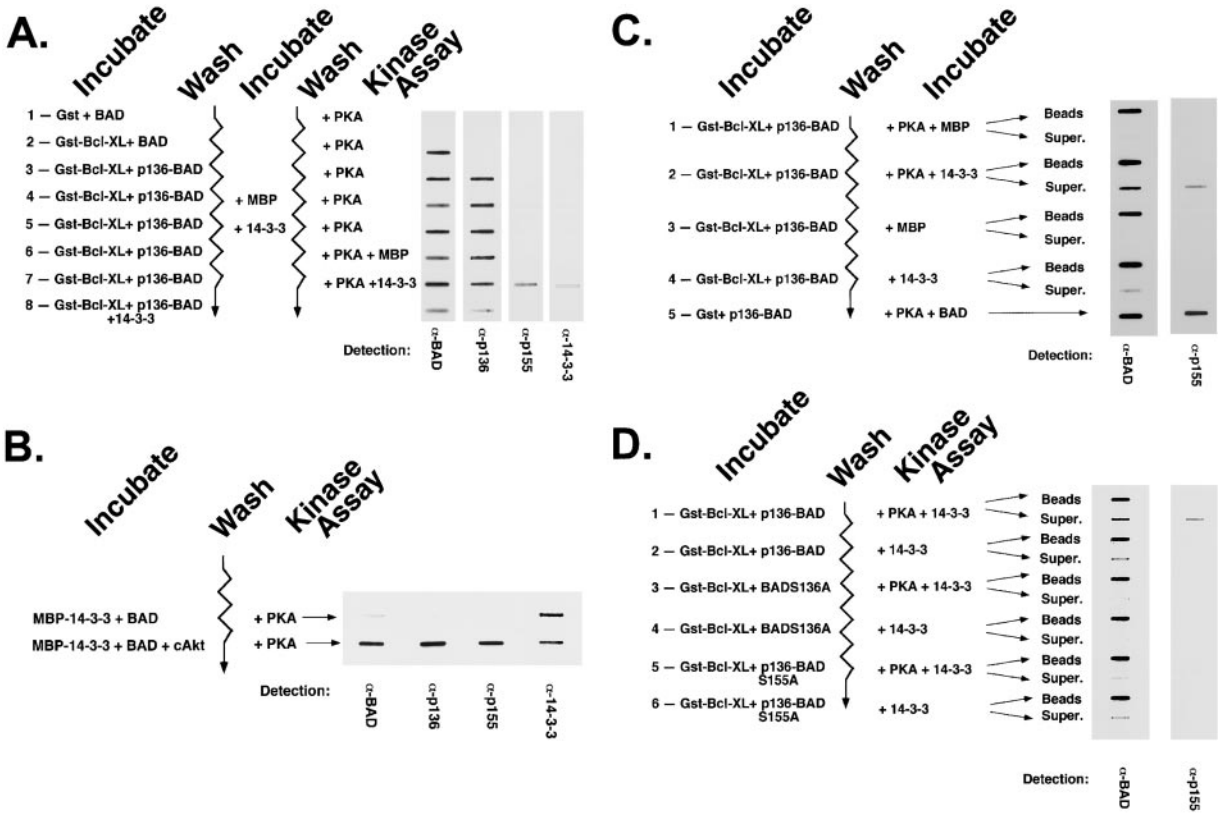

Figure 6. BAD Ser-136 Phosphorylation Recruits 14-3-3 Proteins that Increase Access of Ser-155 to Ser-155 Kinases, Thereby Disrupting the BAD/BCL-X_L Complex

(A) PKA and 14-3-3 cooperate to induce phosphorylation of BAD Ser-155 when BAD is bound to BCL-X_L. Wild-type recombinant BAD was phosphorylated at Ser-136 in vitro by active Akt purified from baculovirus. BAD proteins were then added to glutathione beads coupled to either GST protein alone or GST-BCL-X_L (as indicated) in the presence of .5% NP40 lysis buffer. Beads were washed and incubated with either MBP or MBP-14-3-3ϵ protein (as indicated) for 1 hr, then washed again, and in vitro kinase assays performed on the beads with purified PKA or PKA plus MBP or MBP-14-3-3ϵ protein (as indicated). Assays were stopped by the addition of 2x LSB and slot blotted with the indicated antibodies.

(B) Ser-136-phosphorylated BAD bound to 14-3-3 is effectively phosphorylated by PKA at Ser-155. Recombinant BAD was phosphorylated in vitro by active Akt purified from baculovirus and then incubated with MBP-14-3-3ϵ and amylose beads for 1 hr in 1% NP40 lysis buffer. Precipitates were washed and subjected to a PKA kinase assay for 15 min. Assays were terminated with the addition of 2x LSB and slot blotted with the antibodies indicated.

(C and D) PKA phosphorylation of BAD Ser-155 releases BAD from BCL-X_L. The assay was carried out as in (A), but the reactions were terminated with the addition of EDTA. Supernatants and beads were separately isolated, buffered in 2x LSB, and slot blotted with anti-BAD Abs or anti-BAD155 pAbs.

death even when Ser-136 and/or Ser-112 cannot be phosphorylated. Consistent with phosphorylation at Ser-155 inactivating the ability of BAD to promote apoptosis, the BADS155D construct did not promote apoptosis above the level observed in vector-transfected cells that were deprived of trophic support for 24 hr (Figure 5C). To address whether phosphorylation of BAD Ser-155 inactivates BAD even when BAD Ser-136 is not phosphorylated, we generated a mutant BAD allele in which Ser-112 and Ser-136 were converted to alanine, but Ser-155 was converted to aspartic acid. As shown in Figure 5C, mutation of BAD Ser-155 to aspartate completely abrogated the ability of BAD to promote cell death even when Ser-112 and Ser-136 were converted to alanine. Taken together, these data suggest that Ser-136 phosphorylation inhibits BAD-mediated death by inducing Ser-155 phosphorylation, which disrupts the binding of BAD to prosurvival Bcl-2 proteins and thereby suppresses the ability of BAD to promote apoptosis.

Ser-136 Phosphorylation Recruits 14-3-3 Proteins, which Increase Ser-155 Accessibility to Kinases

The mechanism by which BAD Ser-136 phosphorylation leads to Ser-155 phosphorylation was next investigated. We hypothesized, based upon transfection experiments in a number of cell lines (data not shown), that Ser-155 phosphorylation may require Ser-136 phosphorylation only when BAD is bound to BCL-X_L. The necessity for additional events, such as Ser-136 phosphorylation, to induce Ser-155 phosphorylation was suggested by the molecular modeling of the BCL-X_L/BAD complex (Figure 1A), which revealed that when BAD is bound to BCL-X_L, Ser-155 is buried in the BCL-X_L/BAD interface and is likely inaccessible to kinases. To verify this conclusion experimentally, glutathione-coupled BCL-X_L/BAD complexes were isolated and subsequently incubated with PKA and ATP. As shown in Figure 6A (lane 2), BAD interacted with BCL-X_L in this assay as assessed by slot blotting with anti-BAD antibodies. However, incubation

of the BAD/BCL-X_L complexes with PKA did not lead to Ser-155 phosphorylation under conditions where PKA did phosphorylate free BAD at Ser-155 (lane 2 of Figure 6A, and Figure 1A). These results support the structural data indicating that the hydroxyl group of BAD Ser-155 faces toward the hydrophobic face of BCL-X_L and is therefore inaccessible to kinases when BAD is bound to BCL-X_L.

Based upon the finding that Ser-136 phosphorylation is necessary for Ser-155 phosphorylation in cells, we tested the hypothesis that BAD Ser-136 phosphorylation induces a conformational change in the BAD/BCL-X_L complex that renders BAD Ser-155 accessible to kinases. BAD prephosphorylated at Ser-136 (p136BAD) by Akt interacted as effectively as unphosphorylated BAD with BCL-X_L, suggesting that phosphorylation of Ser-136 alone does not induce a conformational change in BAD that causes BAD to dissociate from BCL-X_L (Figure 6A, lane 3). However, we found that phosphorylation of BAD Ser-136 also did not alter the BAD/BCL-X_L complex in a manner that allows PKA to phosphorylate BAD at Ser-155 (Figure 6A, lane 3). This result indicates that phosphorylation of BAD at Ser-136 alone does not render BAD Ser-155 accessible to kinases when BAD is bound to BCL-X_L.

We next tested the possibility that 14-3-3 binds to the BCL-X_L/p136BAD complexes and induces a change in the complex that allows BAD to be phosphorylated at Ser-155. Glutathione beads coupled to preassembled GST-BCL-X_L/p136BAD complexes were incubated with recombinant MBP-14-3-3ϵ protein, and then washed and subjected to a PKA kinase assay. As shown in Figure 6A (lane 5), incubation of GST-BCL-X_L/p136BAD with 100-fold molar excess of MBP-14-3-3 did not enhance the ability of PKA to phosphorylate BAD at Ser-155. However, we found that although 14-3-3 effectively bound to free p136BAD (Figure 6B), 14-3-3 did not stably bind to the p136BAD/BCL-X_L complexes (Figure 6A, lane 5). We therefore considered the possibility that 14-3-3 weakly interacts with p136BAD/BCL-X_L complexes, forming a transient ternary complex that increases the accessibility of kinases to BAD Ser-155. To test this hypothesis, an in vitro kinase reaction was performed using PKA and the p136BAD/BCL-X_L complexes, both in the presence and the absence of MBP-14-3-3 proteins. We found that in the presence of 14-3-3 proteins, PKA was now capable of phosphorylating BAD at Ser-155 (Figure 6A, lane 7). The ability of 14-3-3 to enhance the accessibility of Ser-155 when BAD was in the BAD/BCL-X_L complex required Ser-136 phosphorylation, as 14-3-3 proteins did not induce Ser-155 phosphorylation when BAD Ser-136 was mutated to alanine (Figure 6D, lane 3). These results suggest that 14-3-3 proteins, through an unstable interaction with the BCL-X_L/p136BAD complex, promote the ability of Ser-155 kinases to phosphorylate BAD Ser-155 when BAD is bound to BCL-X_L.

We next investigated whether the 14-3-3 and PKA-mediated phosphorylation of BAD at Ser-155 induced the release of BAD from the BAD/BCL-X_L complexes. GST-BCL-X_L/p136BAD complexes were coincubated with PKA and/or 14-3-3, and the reaction supernatants were then isolated and analyzed for the presence of released BAD by slot blotting. Incubation of 14-3-3 protein alone with the GST-BCL-X_L/p136BAD complexes displaced only a small amount of BAD from BCL-X_L (Figures 6C, lane 4). In contrast, when the BCL-X_L/p136BAD complexes were coincubated with PKA and 14-3-3, the reaction supernatants contained substantially more BAD immunoreactivity (Figure 6C, lane 2), indicating that PKA enhances the release of BAD from GST-BCL-X_L when incubated in the presence of 14-3-3. The ability of PKA to promote the release of BAD from BCL-X_L depends on Ser-155 phosphorylation, as mutation of Ser-155 to alanine abrogated the ability of PKA to induce enhanced BAD release into the supernatant (Figure 6D, lanes 5 and 6). Taken together, these findings suggest that phosphorylation of Ser-155 promotes the dissociation of BAD from BCL-X_L in a 14-3-3-dependent manner.

Discussion

A Model for BAD Inactivation

This study identifies a mechanism by which survival signals inactivate the proapoptotic Bcl-2 family member BAD. In the absence of survival factors, BAD is dephosphorylated and tightly bound to prosurvival Bcl-2 family members such as BCL-X_L at the outer mitochondrial membrane, where BAD initiates apoptosis by inhibiting BCL-X_L function (Figure 7, stage 1). BAD and BCL-X_L interact through an interface formed by the hydrophobic cleft of BCL-X_L and the hydrophobic face of the amphipathic BH3 domain of BAD. Upon treatment of cells with survival stimuli, BAD becomes phosphorylated at Ser-136 (Figure 7, stage 2). This phosphorylation site lies outside of the BAD/BCL-X_L interaction interface, and phosphorylation of BAD at Ser-136 does not influence the ability of BAD to bind to BCL-X_L, suggesting that this phosphorylation event alone is not likely to inactivate BAD. Survival factor-mediated phosphorylation of BAD at Ser-136 subsequently recruits 14-3-3 proteins to the BAD/BCL-X_L complex. However, 14-3-3 interacts with phosphorylated ligands (such as phosphoBAD) with dissociation constants ranging from 100 to 300 nmol (Yaffe et al., 1997), whereas BAD binds to BCL-X_L with a dissociation constant of 400 pmol, suggesting that 14-3-3 binding likely does not substantially disrupt the BAD/BCL-X_L complexes. Nevertheless, 14-3-3 proteins effectively increase the accessibility of BAD to Ser-155 kinases, which then phosphorylate BAD within the BAD BH3 domain. Phosphorylation of the BAD BH3 domain permanently blocks the ability of BAD to bind to BCL-X_L due to electrostatic and steric constraints, thus disrupting the interaction between BAD and BCL-X_L (Figure 7, stage 3). Once detached from BCL-X_L, BAD stably binds to 14-3-3 proteins and is localized to the cytoplasm (Figure 7, stage 4).

Survival stimuli may induce the inactivation of proapoptotic BAD through multiple pathways. Neurotransmitters activate PKA, which likely inactivates BAD by directly or indirectly inducing the phosphorylation of BAD Ser-136 and by directly phosphorylating BAD at Ser-155. Growth factors, which activate Akt and in some cases PKA, may induce BAD inactivation by a similar mechanism in which Akt and/or PKA directly phosphorylates BAD at both Ser-136 and Ser-155. A fraction of PKA, which is targeted to the mitochondria by AKAP

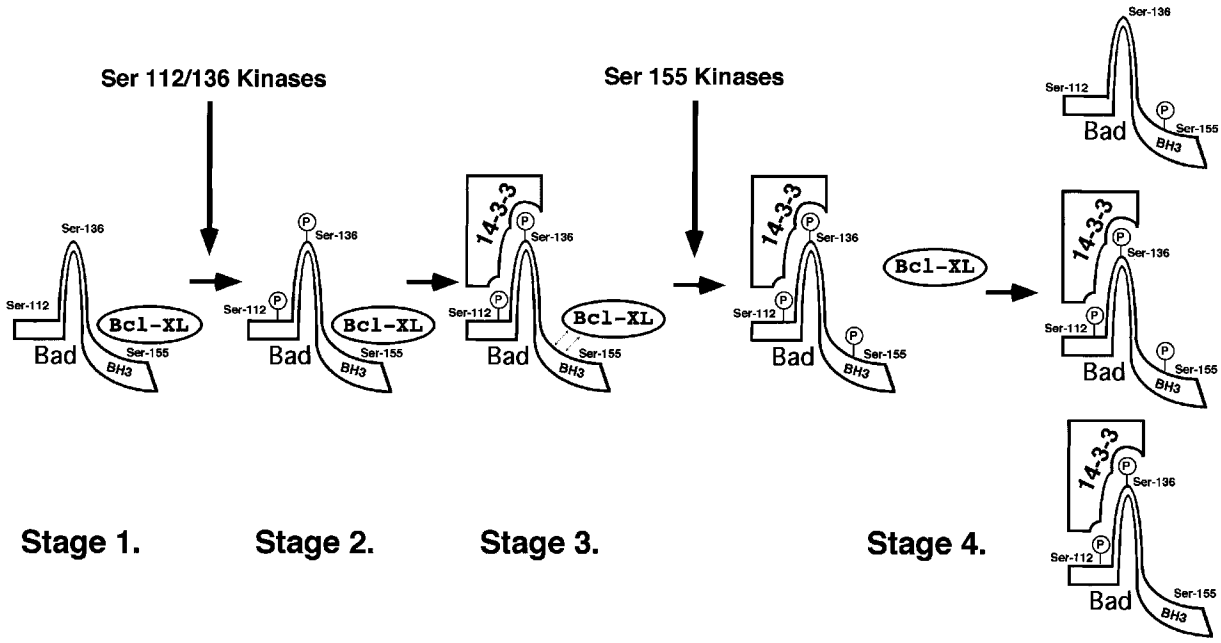

Figure 7. A Model for BAD Inactivation

Survival signals activate kinases that induce the phosphorylation of BAD at Ser-136 and Ser-112 (Stage 1 and 2). 14-3-3 proteins then bind to the BAD/BCL-X_L complexes in a Ser-136-dependent manner (Stage 3). Ser-112 phosphorylation may enhance the binding of 14-3-3 to BAD at this stage. 14-3-3 proteins weaken the effective interaction of BAD and BCL-X_L and increase the access of Ser-155 kinases to BAD Ser-155. Phosphorylation of Ser-155 permanently disrupts the BCL-X_L/BAD complex and inactivates BAD (Stage 4).

proteins and has been shown to be important for BAD phosphorylation, may be crucial for Ser-155 phosphorylation in this context (Harada et al., 1999). Alternatively, growth factors may principally regulate Ser-136 phosphorylation, which through 14-3-3 binding increases the accessibility of Ser-155 to basally active kinases. The mechanism and kinases by which growth factors induce Ser-155 phosphorylation remain to be fully characterized.

Implications for Bcl-2 Family Regulation and for 14-3-3 Protein Function

The observation that BAD is inactivated by phosphorylation at Ser-155 has important implications for the understanding of the regulation of Bcl-2 family members. Phosphorylation of BH3 domains may be a general mechanism by which trophic factors block the apoptotic activity of Bcl-2 family members. The proapoptotic Bcl-2 family member bNIP3 contains a consensus PKA phosphorylation site (KKNS) at the residue homologous to BAD Ser-155 within the bNIP BH3 domain. The Bcl-2 family member BAX also contains an evolutionarily conserved sequence within its BH3 domain that corresponds to a PKA phosphorylation site. It is possible that bNIP3 and BAX, like BAD, may be inactivated by survival signals through phosphorylation within their BH3 domains. Consistent with this possibility, Akt and PKA block BAX-mediated apoptosis (Kennedy et al., 1999; S. R. D. and M. E. G., unpublished data).

The findings presented in this paper also suggest an unappreciated role for 14-3-3 proteins in BAD inactivation. 14-3-3 proteins have been previously proposed to inactivate BAD by displacing BAD from BCL-X_L. Consistent with this possibility, incubating BCL-X_L/BAD complexes with 100-fold molar excess of 14-3-3 protein induced the dissociation of a small fraction of the BAD bound to BCL-X_L. However, this effect depends on high concentrations of 14-3-3 protein, as addition of equimolar amounts of 14-3-3 to BCL-X_L/p136BAD complexes did not detectably induce the release of BAD from BCL-X_L (data not shown). This failure of 14-3-3 to substantially disrupt the BAD/BCL-X_L complexes is consistent with the measured affinity of the BAD BH3 domain for BCL-X_L being 100- to 1000-fold higher than the affinity of phosphorylated BAD for 14-3-3. The ability of 14-3-3 proteins to displace BAD from BCL-X_L at the outer mitochondrial membrane may therefore not be sufficient to account for the growth factor-mediated inactivation of BAD. Consistent with this possibility, genetic data indicate than an additional event, Ser-155 phosphorylation, is required for growth factors to block BAD-mediated death. The data presented in this study support a role for 14-3-3 proteins in which they act to block BAD-mediated death by promoting the accumulation of BAD that is phosphorylated at Ser-155 and therefore incapable of binding to BCL-X_L. Although there are many possible means by which 14-3-3 may facilitate BAD Ser-155 phosphorylation, one potential mechanism is that 14-3-3 may compete with BCL-X_L for binding to BAD, thus liberating a small quantity of BAD from BCL-X_L and rendering this population of free BAD accessible to Ser-155 kinases. In order for 14-3-3 to promote BAD Ser-155 phosphorylation, the binding of 14-3-3 to BAD would have to induce the simultaneous dissociation of

BAD from BCL-X_L, whose hydrophobic cleft would otherwise obstruct kinase access to BAD Ser-155. We were unable to isolate a ternary complex containing 14-3-3, BAD, and BCL-X_L either in vivo (data not shown) or in vitro (Figure 6A), suggesting that stable 14-3-3 binding to BAD and stable BCL-X_L binding to BAD are indeed mutually exclusive. Given that BAD has a substantially higher affinity for BCL-X_L than it has for 14-3-3, BAD that is dissociated from BCL-X_L by forming a BAD/14-3-3 complex is likely rapidly recaptured by BCL-X_L. Consistent with this possibility, BCL-X_L is capable of significantly displacing p136BAD from p136BAD/14-3-3 complexes in vitro (data not shown). However, survival-promoting Ser-155 kinases such as PKA may prevent BCL-X_L from rebinding to BAD by phosphorylating BAD at Ser-155 while BAD is bound to 14-3-3. In such a model, BAD Ser-155 kinases, cooperating with 14-3-3 proteins, would steadily increase the amount of BAD dissociated from BCL-X_L by mass action. The detailed mechanism by which 14-3-3 proteins induce Ser-155 phosphorylation remains to be clarified, and the data presented in this manuscript do not exclude either additional roles for 14-3-3 in BAD inactivation, or the existence of additional constraints on 14-3-3/BAD complexes that may influence the binding of BAD to BCL-X_L. However, the genetic and biochemical data suggest that 14-3-3 blocks BAD activity, at least in part, by promoting Ser-155 phosphorylation, which induces the dissociation of BAD and BCL-X_L.

The finding that 14-3-3 facilitates the access of kinases to Ser-155 of Ser-136-phosphorylated BAD indicates that 14-3-3, in addition to its defined role as a molecular chaperone, may also act as a cofactor that participates in sequential protein phosphorylation events. Akt-mediated phosphorylation of the forkhead-related (FKHR) transcription factor at Ser-253, which leads to 14-3-3 binding, has been identified as a gatekeeper event that facilitates the phosphorylation of FKHR at other sites (Brunet et al., 1999; Nakae et al., 2000). As with BAD, 14-3-3 binding to FKHR may facilitate the access of kinases to other phosphorylation sites on FKHR. Interestingly, 14-3-3 proteins function as dimers, and many of the proteins that bind 14-3-3, including Raf and PKC, are enzymes involved in signal transduction (Van Der Hoeven et al., 2000). Thus, 14-3-3 may both increase the accessibility of substrates (e.g., BAD, FKHR) to kinases and serve to localize kinases to their substrates. It will be important in the future to examine whether 14-3-3 proteins regulate a variety of sequential phosphorylation events, and to clarify the biological importance of 14-3-3-regulated phosphorylation.

Experimental Procedures

Antibodies

To generate anti-BADp155, a phosphopeptide of the sequence NLWAAQRYGRELRRMpSDEFVDSFKK was synthesized, coupled to keyhole limpet hemocyanin (Pierce), and injected into New Zealand white rabbits. Anti-BADp112 Abs and anti-BADp136 Abs were described previously (Datta et al., 1997). Anti-BAD C-terminal antibodies (sc-943) were obtained from Santa Cruz Biotechnology, anti-HA 12CA5 antibodies were obtained from Boehringer Mannheim, anti-β-gal antibodies were obtained from 5 Prime-3 Prime, and anti-FLAG monoclonal antibodies were obtained from Sigma, and used according to manufacturers' protocols. Anti-BAD112 pAbs, anti-BAD136 pAbs, and anti-BAD155 pAbs were diluted 1:1000 in 5% milk/1% BSA for use in Western blots.

Plasmids and Proteins

Bacterial and mammalian BAD expression vectors, protein purification methods, and kinase assay methods were described previously (Datta et al., 1997). To introduce additional mutations (including S155A, S112D, S112E, S136D, S136E, and S155D), the Quickchange mutagenesis kit was used (Stratagene). The bacterial expression vector for GST- BCL-X_L was obtained from J. Yuan (Harvard Medical School). GST-BCL-X_L was inducibly expressed in E. coli strain BL21 HMS 175 (DE5)pLysS and was purified over glutathione-Sepharose resin (Sigma) under native conditions. MBP-14-3-3 was purified as described previously (Yaffe et al., 1997).

Cell Culture, Transfections, and Stimulations

Balb/c, 293, PC12, and cerebellar granule cells were cultured, transfected, stimulated, and immunostained as described previously (Datta et al., 1997). Both primary anti-β-gal antibodies and Cy3-coupled secondary antibodies (Molecular Probes) were used at a dilution of 1:750 in 5% BSA/phosphate-buffered saline/.05% Tween-20 for immunostaining.

Molecular Modeling

The BCL-X_L:BAD BH3 peptide complex was modeled using the NMR structure of the BCL-X_L:BAK BH3 peptide complex as a template (Sattler et al., 1997). Threading of the BAD BH3 sequence (GRELRR MSDEF) on the BAK BH3 sequence (GRQLAIIGDDI) was optimized using SwissPdbViewer 3.1 (Guex and Peitsch, 1997) and QUANTA, then modeled using ProModII (Peitsch 1996; Guex and Peitsch, 1997) followed by energy minimization using Gromos96 with 200 cycles of steepest descent followed by 300 cycles of conjugate gradient minimization. Figures were generated using Molscript and GRASP (Kraulis 1991; Nicholls et al., 1991).

Fluorescence Anisotropy

The relative affinity of peptides encompassing the WT BAD BH3 domain (KGGQRYGRELRRMSDEFEG) and similar peptides with modifications at Ser-155 (BADS155A BH3, BADS155D BH3, and BADS155PO4 BH3) for BCL-X_L were determined by using a fluorescence polarization based competitive assay with a fluorescein-labeled BAD peptide complexed to recombinant BCL-X_L. Peptide dilutions were buffered in 120 mM sodium phosphate (pH 7.55) with .01% bovine gamma globulin and .1% sodium azide. To each dilution, BCL-X_L protein complexed to fluorescein-labeled BAD peptide was added to 114 nM, and the tubes were incubated for 5 min at 35°C before total intensity and polarization were read. To extract the binding affinities of the unlabeled peptides, an analytical approach for equilibrium competition was applied (Dandliker et al., 1981), using the model development program MINSQ (V. 4.03, Micromath Scientific Software).

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