

Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery

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Summary

Growth factors can promote cell survival by activating the phosphatidylinositol-3'-OH kinase and its downstream target, the serine-threonine kinase Akt. However, the mechanism by which Akt functions to promote survival is not understood. We show that growth factor activation of the PI3'K/Akt signaling pathway culminates in the phosphorylation of the BCL-2 family member BAD, thereby suppressing apoptosis and promoting cell survival. Akt phosphorylates BAD in vitro and in vivo, and blocks the BAD-induced death of primary neurons in a site-specific manner. These findings define a mechanism by which growth factors directly inactivate a critical component of the cell-intrinsic death machinery.

Introduction

Regulation of cell survival is crucial to the normal physiology of multicellular organisms. The appropriate suppression of cell death is important in a number of contexts, ranging from the sculpting of the nervous system during development to adaptive responses during adulthood. In addition, perturbation of normal survival mechanisms—leading to either excessive cell death or survival—may play a role in a large number of disease processes.

A number of well-characterized peptide factors promote cell survival, including the neurotrophins and growth factors such as insulin-like growth factor I (IGF-1) and platelet-derived growth factor (PDGF) (Segal and Greenberg, 1996; Stewart and Rotwein, 1996; reviewed in Attaliotis and Mercola, 1997). The characterization of signal transduction pathways activated by these factors has led to the identification of proteins that are critical mediators of cell survival. For example, the phosphatidylinositol-3'-OH kinase (PI3'K) has been recently shown to be involved in the survival of a number of different cell types. Pharmacologic or genetic blockade of PI3'K activity can

suppress the ability of trophic factors to promote survival; conversely, in many cell types PI3'K activity is sufficient to promote survival (Scheid et al., 1995; Vemuri and McMorris, 1996; Yao and Cooper, 1995, 1996; D'Mello et al., 1997; Dudek et al., 1997). There are a number of PI3'K isoforms, the most well-characterized of which is a heterodimer composed of an 85 kDa regulatory subunit and a catalytic 110 kDa subunit. The best elucidated mechanism by which growth factors activate PI3'K involves the association of the p85 subunit with specific phosphotyrosines on either the cytoplasmic domain of growth factor receptors or on receptor-associated adapter proteins. PI3'K may also directly interact with and be activated by the small G protein Ras (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994, 1996). Thus, through several possible protein-protein interactions, growth factor receptor activation recruits PI3'K to the membrane where the PI3'K p110 subunit phosphorylates phosphoinositides at the D-3 position. PI3'K-generated phospholipids then elicit a diverse set of cellular responses (reviewed in Carpenter and Cantley, 1996).

One target of PI3'K is the serine-threonine kinase c-Akt, also known as PKB (protein kinase B) and RAC-PK (related to A and C protein kinase). c-Akt (or RAC α) is the prototypical member of a family of mammalian Akt isoforms that includes RAC β and RAC γ (Bellacosa et al., 1991; Jones et al., 1991; Coffey and Woodgett, 1992; Konishi et al., 1995). Akt may be regulated by both phosphorylation and by the direct binding of PI3'K lipid products to the Akt pleckstrin homology (PH) domain. Other mechanisms of Akt activation may also exist, as PI3'K-independent Akt stimuli have been identified (Burgering and Coffey, 1995; Franke et al., 1995, 1997; Alessi et al., 1996, 1997; Datta et al., 1996; Kohn et al., 1996; Didichenko et al., 1996; Klippel et al., 1997).

Akt is a general mediator of growth factor-induced survival and has been shown to suppress the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage (Ahmed et al., 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997). Thus, a signaling pathway has been defined in which growth factor receptor activation leads to the sequential activation of PI3'K and Akt, which then, through as-yet undescribed mechanisms, promotes cell survival and blocks apoptosis.

An unanswered question is how activation of the PI3'K/Akt signaling pathway promotes cell survival and suppresses apoptosis. Known *in vivo* substrates for Akt include the glycogen synthase kinase-3, and possibly the p70 ribosomal S6 kinase, although neither of these proteins has yet been shown to play a role in cell survival (Burgering and Coffey, 1995; Cross et al., 1995; Franke et al., 1995; Alessi et al., 1996; Kohn et al., 1996). One mechanism by which Akt may promote survival is through the inhibition of a component of the cell death machinery. Among molecules central to the regulation of cell death in eukaryotes are members of the BCL-2 family of proteins. The *C. elegans* protein CED-9 and its

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mammalian proto-oncogene homolog BCL-2 have been shown by genetic means to promote cell survival. Several members of the BCL-2 family (including BCL-2, BCL-X_L, MCL-1, A1, and BAG-1) promote survival while other members (including BCL-X_S, BAD, BAX, and BAK) promote cell death. BCL-2 family proteins homo- and heterodimerize, and the balance between particular homo- and heterodimers is thought to be critical to the maintenance of cell survival or the induction of death. The mechanisms by which BCL-2 family members function are not well understood, but may include the formation of ion channels and the regulation of proteases involved in programmed cell death (Hengartner and Horvitz, 1994; reviewed in Boise et al., 1995; Steller, 1995; Chinnaiyan and Dixit, 1996; Merry and Korsmeyer, 1997; Reed, 1997).

It is not known how the activity of BCL-2 family members is regulated by specific signal transduction molecules that are activated by survival signals. One possibility is that growth factor-regulated protein kinases phosphorylate a BCL-2 family member and regulate its function. One likely target of phosphorylation by growth factor-regulated kinases is the BCL-2 family member BAD (Yang et al., 1995). BAD function is modulated by phosphorylation at two sites, serine 112 (Ser-112) and serine 136 (Ser-136) (Gajewski and Thompson, 1996; Wang et al., 1996; Zha et al., 1996). In hematopoietic cells the cytokine Il-3, under conditions where it promotes cell survival, induces the phosphorylation of BAD at Ser-112 and Ser-136. In the absence of phosphorylation of these sites, BAD is thought to induce cell death, possibly via the formation of heterodimers with BCL-X_L and the concomitant generation of BAX homodimers. In contrast, the Il-3-mediated phosphorylation of either the Ser-112 or the Ser-136 site may promote the survival of hematopoietic cells. Phosphorylation has been correlated with binding of BAD to the 14-3-3t protein, which may sequester BAD from BCL-X_L, thus promoting cell survival. However, the kinases that catalyze BAD phosphorylation and thereby promote cell survival have not been characterized.

We hypothesized that the PI3'K/Akt pathway may lead to BAD phosphorylation and may thereby suppress cell death and promote cell survival. In this report, we show that Akt phosphorylates BAD at Ser-136 in vitro and in vivo. In addition, IGF-1 and Akt function to block BAD-mediated death by phosphorylating BAD at Ser-136. These findings reveal a mechanism by which a growth factor-regulated kinase cascade that mediates cell survival inhibits the death-promoting activity of a component of the intrinsic cell death machinery.

Results

By using peptides derived from the site on GSK-3 known to be phosphorylated by Akt, it was recently established that Akt preferentially phosphorylates substrates that conform to the sequence RXXXS (Alessi et al., 1996). Both the Ser-112 (RSRHSS) and Ser-136 (RGRSRS) phosphorylation sites on BAD conform to this general consensus sequence (Zha et al., 1996). Based upon this observation and the finding that the effects of growth

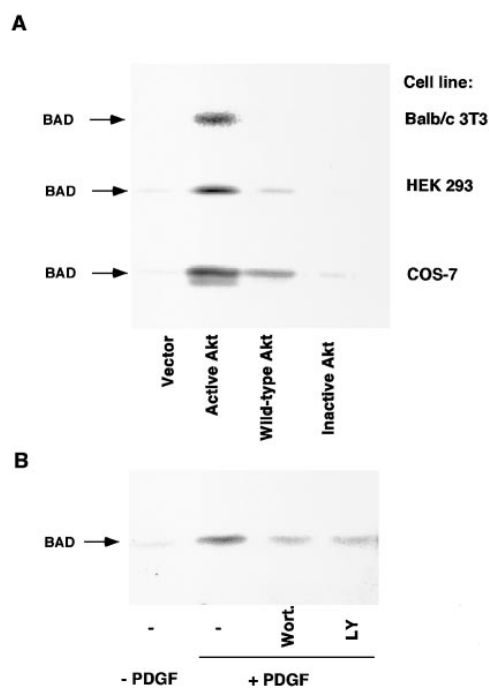


Figure 1. Akt Phosphorylates BAD In Vitro

(A) Expressed Akt phosphorylates recombinant BAD in an immunocomplex kinase assay. Immunocomplex kinase assay using anti-HA immunoprecipitates (12CA5 MAb) of hemagglutinin-tagged Akt constructs expressing CMV-6 control (Vector), constitutively active HA-m Δ 4-129Akt (Active Akt), HA-Akt (Wild-type Akt), or kinase-inactive HA-AktK179M (Inactive Akt) expressed in Balb/c 3T3 cells, HEK 293 cells, or COS-7 cells using recombinant wild-type BAD (100 ng/rxn) as a substrate. Immunoblotting revealed that cells expressed Akt constructs equally.

(B) Activated endogenous Akt phosphorylates BAD in a PI3'K-dependent manner. Immunocomplex kinase assay using endogenous Akt immunoprecipitated (Santa Cruz anti-Akt1) from equal amounts of protein extracted from starved and PDGF-stimulated Balb/c 3T3 cells using recombinant BAD (100 ng/rxn) as a substrate. Cells were pretreated for 45 min with either vehicle, wortmannin, (20 nM), or LY 294002 (10 μ M).

factors on cell survival are often mediated by the PI3'K/Akt signaling pathway, we asked whether Akt might promote survival by phosphorylating BAD.

The PI3'K-Dependent Kinase Akt Phosphorylates BAD at Ser-136 In Vitro

To test whether Akt functions as a BAD kinase in vitro, wild-type or mutant forms of hemagglutinin-tagged Akt were expressed in a variety of cell lines, immunoprecipitated with an anti-HA antibody, and assayed in an immunocomplex kinase assay for their ability to phosphorylate recombinant BAD. Akt constructs that were expressed in cells included hemagglutinin-tagged wild-type Akt (HA-Akt), an Akt derivative rendered constitutively active by targeting it to the plasma membrane with a myristoyl tag (HA-m Δ 4-129Akt), and an Akt derivative rendered kinase-inactive by point mutation within the Akt catalytic domain (HA-AktK179M) (Franke et al., 1995; Kohn et al., 1996; Dudek et al., 1997). As shown in Figure 1A, anti-HA immunoprecipitates prepared from cells transfected with constitutively active Akt or, to a lesser

extent, wild-type Akt were found to induce BAD phosphorylation in immunocomplex kinase assays. Anti-HA immunoprecipitates from cells transfected with vector or the kinase-inactive Akt failed to phosphorylate BAD significantly. In addition, endogenous Akt immunoprecipitated from 3T3 cells stimulated with PDGF, but not Akt immunoprecipitated from starved cells, phosphorylated BAD (Figure 1B). Consistent with a role for the PI3'K/Akt pathway in BAD phosphorylation in vitro, pretreatment of cells with the wortmannin, an irreversible inhibitor of PI3'K, or LY 294002, a structurally unrelated PI3'K inhibitor, before PDGF stimulation blocked Akt phosphorylation of BAD in an immunocomplex kinase assay (Figure 1B).

Previous experiments established that there are two phosphorylation sites that regulate BAD function, the Ser-112 site (RSRHSpYP) and the Ser-136 site (RQRSTpSTP) (Zha et al., 1996). These are the only sites within BAD that correspond to the defined RXRXXS Akt consensus phosphorylation sequence. To facilitate the identification of the Akt phosphorylation site on BAD, recombinant BAD mutant proteins were generated in which Ser-112 (BADS112A), Ser-136 (BADS136A), or both (BAD2SA) were converted to alanine so that BAD could no longer be phosphorylated at these sites. Mutation of these sites had no effect on the levels of expression of these proteins in either *E. coli* or eukaryotic cells, and the mutation of individual sites did not disrupt the ability of BAD to bind to 14-3-3 β in vitro (data not shown).

To test the importance of BAD Ser-112 and Ser-136 for Akt-mediated BAD phosphorylation in vitro, cells were transfected with various Akt expression constructs, and the ability of immunoprecipitated Akt derivatives to phosphorylate wild-type BAD and various BAD phosphorylation site mutants was assessed. As shown in Figure 2A, Akt activation resulted in the phosphorylation of wild-type BAD and the phosphorylation of BAD containing a Ser-112 mutation. In contrast, ³²P incorporation was not evident in BADS136A or in BAD2SA, indicating that the BAD Ser-136 phosphoacceptor site is critical to Akt-mediated BAD phosphorylation. In addition, Akt isolated from PDGF-stimulated 3T3 cells or NGF-stimulated PC12 cells significantly induced the phosphorylation of a peptide substrate composed of the BAD sequence encompassing Ser-136, but only slightly induced the phosphorylation of a peptide composed of the BAD sequence surrounding Ser-112 (data not shown). To verify that mutation of the BAD Ser-136 site did not disrupt the conformation of the BADS136A protein and render it refractory to all phosphorylation, the BAD proteins were coincubated with purified PKA and (γ -³²P)ATP in vitro. PKA phosphorylated wild-type BAD and BADS136A to the same extent in vitro but poorly phosphorylated BADS112A and BAD2SA, consistent with the previous identification of BAD Ser-112 as a major PKA phosphorylation site (Zha et al., 1996) (Figure 2B).

To further characterize the sites in BAD phosphorylated by Akt, tryptic fragments of wild-type and mutant BAD proteins that had been phosphorylated by activated Akt in vitro were subjected to two-dimensional mapping analysis. Two-dimensional maps of ³²P-labeled tryptic fragments of wild-type BAD revealed one major phosphopeptide spot. This tryptic peptide migrated

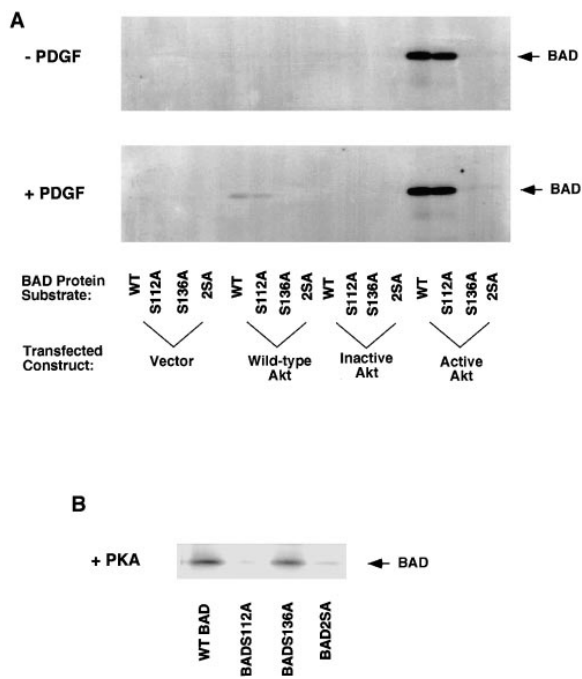


Figure 2. Akt Phosphorylates BAD at Serine 136 In Vitro

(A) Expressed Akt phosphorylates recombinant BAD and BADS112A, and not BADS136A or BAD2SA. Immunocomplex kinase assay using anti-HA immunoprecipitates (12CA5 MAb) from Balb/c 3T3 cells transfected with hemagglutinin-tagged Akt constructs expressing CMV-6 vector control (Vector), HA-Akt (Wild-type Akt), kinase-inactive HA-AktK179M (Inactive Akt), and constitutively active Akt HA-m Δ 4-129Akt (Active Akt) using recombinant wild-type BAD (WT BAD), BADS112A, BADS136A, or BAD2SA as substrates (100 ng/rxn). Experiments were performed after 6 hr of serum starvation followed by injection of either control PBS (- PDGF) or 20 ng/ml PDGF (+ PDGF).

(B) Protein kinase A phosphorylates mainly Ser-112. Kinase assay using recombinant wild-type BAD, BADS112A, BADS136A, and BAD2SA (100 ng/rxn) as substrates and purified PKA (Boehringer Mannheim, 4 mU) as an in vitro kinase.

identically to a ninhydrin-stained synthetic phosphopeptide corresponding to the predicted BAD tryptic fragment that includes BAD Ser-136 (Figure 3A, left panels). Tryptic maps of Akt-phosphorylated BADS112A protein appeared identical to maps of wild-type BAD (data not shown); in contrast, mutation of the BAD Ser-136 site, either in the context of BADS136A protein or BAD2SA protein, resulted in maps devoid of any detectable ³²P, indicating that the BAD Ser-136 site is required for Akt to phosphorylate BAD (Figure 3A, right panels). As a control, BAD phosphorylated with PKA was also subjected to tryptic 2-D mapping, and tryptic fragment migration was compared to the 2-D migration of a synthetic phosphopeptide containing the putative BAD Ser-112 tryptic fragment. The BAD Ser-112 tryptic peptide and the BAD Ser-112 synthetic peptide migrated similarly, and the BAD Ser-112 peptide migrated quite differently from the tryptic fragment containing BAD Ser-136 (Figure 3A, middle panels). These experiments suggest, therefore, that BAD Ser-136 is the major site for BAD phosphorylation by Akt.

To unequivocally identify BAD Ser-136 as a site involved in Akt-mediated BAD phosphorylation in vitro, a

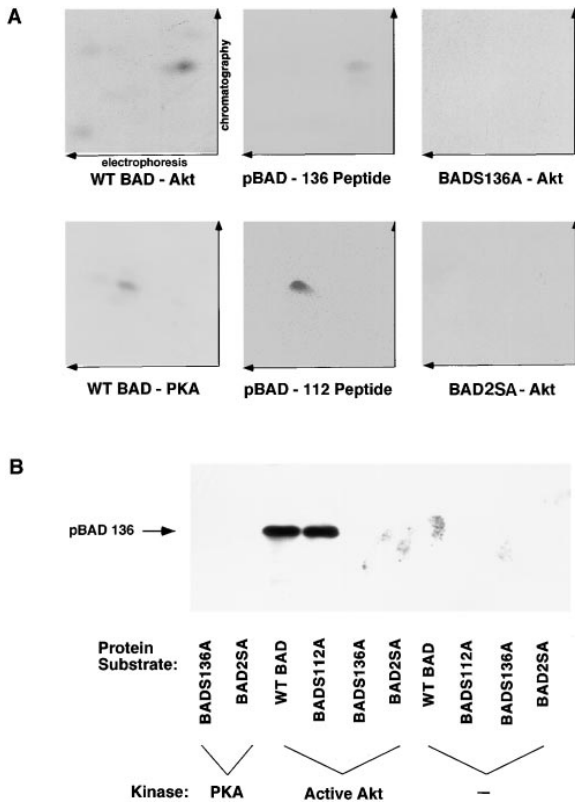


Figure 3. Phosphotryptic and Phosphopeptide Analyses Demonstrate That Akt Phosphorylates BAD at Ser-136

(A) Akt phosphorylates one major tryptic peptide. 2-D maps of tryptic fragments of recombinant BAD phosphorylated by Akt were generated by immunoprecipitating a constitutively active Akt expressed in transfected Balb/c 3T3 cells, phosphorylating wild-type BAD (WT BAD-Akt, upper left panel), BADS136A (BADS136A-Akt, upper right panel), or BAD2SA (BAD2SA-Akt, lower right panel), and trypsinizing the proteins. Fragments were separated using electrophoresis in the horizontal dimension (pH 8.9) and chromatography in the vertical dimension. TLC plates were then subjected to autoradiography. An identical protocol was used to generate a map for BAD phosphorylated by purified PKA (WT BAD-PKA, lower left panel). Mobility of synthetic phosphopeptides containing the predicted BAD Ser-136 tryptic fragment (pSAPPNLWAARQ; pSer-136 Peptide, upper middle panel) and the predicted BAD Ser-112 tryptic fragment (HSPSY-PAGTEEDGMEELSPFR; pSer-112 Peptide, lower middle panel) were subjected to similar TLC separation and detected by ninhydrin staining.

(B) A phosphospecific antibody detects Akt phosphorylation of BAD at Ser-136. Western blot using anti-BAD136 pAbs of recombinant BADS136A or BAD2SA phosphorylated by purified PKA (Boehringer Mannheim, 4 mU) *in vitro* BAD, BADS112A, BADS136A, and BAD2SA phosphorylated *in vitro* by constitutively active HA-m Δ 4-129Akt (Active Akt) and unphosphorylated BAD, BADS112A, BADS136A, and BAD2SA expressed in and isolated from Balb/c 3T3 cells.

polyclonal antibody was raised that specifically recognizes BAD phosphorylated at Ser-136 and fails to recognize BAD that is not phosphorylated at Ser-136. This antibody was generated by injecting rabbits with an immunogen composed of a synthetic phosphopeptide containing phosphorylated BAD Ser-136. As shown in Figure 3B, upon Western blotting the antibodies to phosphorylated Bad Ser-136 (anti-BAD136 pAbs) recognized BAD and BADS112A that had been phosphorylated by Akt *in vitro*, but failed to recognize mutant BAD

proteins in which Ser-136 was changed to an alanine. In parallel control experiments, the anti-BAD136 pAb failed to recognize unphosphorylated forms of BAD, BADS112A, BADS136A, and BAD2SA, and failed to recognize BAD Ser-136 mutants that had been phosphorylated at Ser-112 by PKA. These control experiments demonstrate that the anti-BAD136 pAbs recognize only BAD phosphorylated at Ser-136, and do not recognize BAD phosphorylated at Ser-112. Therefore through the use of phosphospecific antisera we have demonstrated that Akt phosphorylates BAD Ser-136 *in vitro*.

The PI3'K-Akt Pathway Leads to BAD Ser-136 Phosphorylation *In Vivo*

We next examined the importance of the PI3'K-Akt pathway for the phosphorylation of BAD *in vivo*. In these experiments, we took advantage of the previous finding that BAD phosphorylated on both Ser-112 and Ser-136 undergoes a shift in mobility on SDS-PAGE gels, due to the appearance of a more slowly migrating form of BAD (Wang et al., 1996; Zha et al., 1996). For example, BAD protein isolated from Il-3-treated hematopoietic cells that had been stably transfected with BAD expression constructs ran as a doublet on SDS-PAGE gels; this doublet resolved to a monomer after potato acid phosphatase treatment, strongly suggesting that this shift was the result of a phosphorylation event. Mutational analysis revealed that both Ser-112 and Ser-136 were required for this phosphorylation shift (Wang et al., 1996; Zha et al., 1996). We assayed the *in vivo* phosphorylation status of endogenous BAD using this shift assay. Brief exposure of Balb/c 3T3 cells to platelet-derived growth factor (PDGF), which is a potent survival factor for a number of cell types, induced a shift in BAD migration on SDS-PAGE (Figure 4A). A similar shift in migration was observed in BAD isolated from PC12 cells treated with the survival factor NGF (data not shown). Thus, in the absence of survival factors, endogenous BAD is less phosphorylated, and when survival factors such as Il-3, PDGF or NGF are added to cells, BAD becomes newly phosphorylated within minutes, as indicated by its slower migration on SDS-PAGE.

We next tested the possibility that growth factors trigger BAD phosphorylation in a PI3'K/Akt pathway-dependent manner. Consistent with this possibility, treatment of cells with wortmannin or LY 294002 blocked the PDGF-induced BAD phosphorylation (Figure 4A). In contrast, treatment with PD 98059, an inhibitor of MEK (a component of the Ras/MAPK pathway), or rapamycin, a specific inhibitor of the p70S6K pathway, did not significantly block BAD phosphorylation. PDGF-induced MAPK activity was inhibited by PD 98059, p70^{S6K} activity was inhibited by rapamycin, and Akt activity was inhibited by wortmannin, demonstrating that these drugs were effective in these cells (data not shown). Therefore, growth factor-induced BAD phosphorylation on at least one of the two phosphorylation sites requires PI3'K pathway activity, but not the activity of MAPK or activity of p70^{S6K}. These findings suggest that although Akt and p70^{S6K} are both dependent on PI3'K activity and share a similar consensus phosphorylation sequence (RXRXXS), Akt but not p70^{S6K} is likely to mediate BAD phosphorylation *in vivo*. These data are also consistent

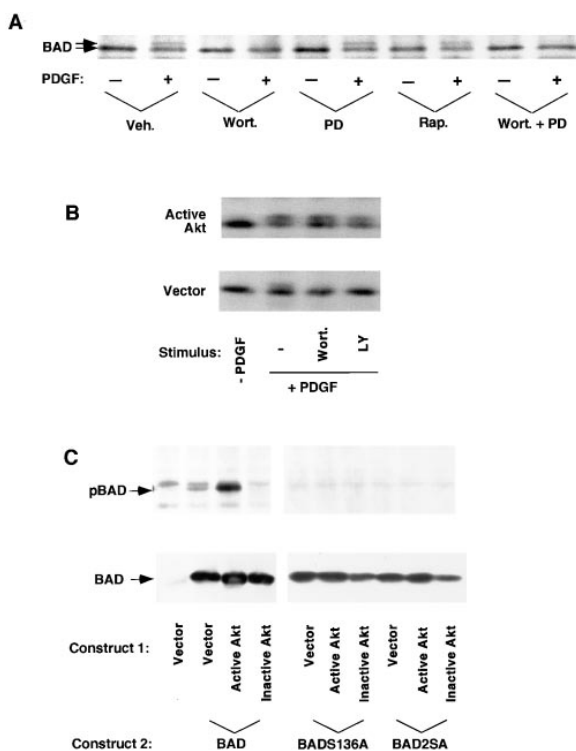


Figure 4. PI3'K/Akt Pathway Mediates BAD Phosphorylation In Vivo
(A) PDGF treatment of Balb/c 3T3 cells results in PI3'K-dependent BAD hyperphosphorylation. Anti-BAD Western blot (Santa Cruz anti-BAD C-terminal Ab) of anti-BAD immunoprecipitates (Santa Cruz anti-BAD N-terminal Ab) from cells starved for 6 hr, and then either treated with control PBS (- PDGF) or with 20 ng/ml PDGF (+ PDGF). Cells were also treated with control DMSO (Veh.), PI3'K inhibitor wortmannin (Wort.), the MEK inhibitor PD98059 (PD), the p70^{S6K} inhibitor rapamycin (Rap.), or a combination of wortmannin and PD98059 (Wort. + PD). Use of the PI3'K-inhibitor LY294002 also blocked BAD phosphorylation in this experiment (data not shown).
(B) Akt activity induces the phosphorylation of endogenous BAD in vivo. Experiment similar to (A), except the cells used are Balb/c 3T3 cells stably transfected with either CMV-6 (Vector) or HA-m Δ 4-129Akt (Active Akt).
(C) Constitutively active Akt increases BAD Ser-136 phosphorylation, and kinase-inactive Akt blocks BAD Ser-136 phosphorylation in vivo. HEK 293 cells were cotransfected with CMV-6 (vector), hemagglutinin-tagged constructs encoding BAD or BAD mutants (BADS136 and BAD2SA), and constructs encoding CMV-6 control (Vector), constitutively active HA-m Δ 4-129Akt (Active Akt), or a kinase-inactive HA-Akt-K179M (Inactive Akt). Transfected cells were either immunoprecipitated with an antibody directed against the BAD N terminus (Santa Cruz N-term. Ab) or the BAD136 pAb and immunoprecipitates were subjected to anti-HA (12CA5 Mab) Western blotting.

with previous findings establishing a role for Akt but not p70^{S6K} in mediating cell survival.

We next investigated whether PI3'K-regulated BAD phosphorylation is triggered by Akt in vivo. To facilitate this analysis, stable Balb/c 3T3 cell lines were generated that express a constitutively active Akt construct or carry a control vector construct. We were unable to obtain stably transfected cell lines that express the kinase-inactive Akt construct, perhaps because expression of this kinase-inactive Akt induced apoptosis, as demonstrated in previous studies (Dudek et al., 1997; Kauffmann-Zeh et al., 1997). Consistent with the findings in

Figure 4A, BAD isolated from cells stably transfected with control vector underwent a PDGF-induced shift in mobility that reflects an increase in the BAD phosphorylation at two sites previously shown to be Ser-112 and Ser-136 (Figure 4B). This mobility shift was blocked by pretreatment of the cells with either wortmannin or LY 294002, suggesting that PI3'K activity is required for phosphorylation of at least one of the sites on BAD. BAD isolated from cells that had been stably transfected with the construct encoding constitutively active Akt also underwent a PDGF-induced shift in mobility. However, unlike control cells, the BAD mobility shift was not blocked by treatment of cells with wortmannin or LY 294002. Thus, when Akt is constitutively active, BAD phosphorylation becomes independent of PI3'K activity. This result suggests that Akt activity is sufficient to phosphorylate endogenous BAD in vivo, and that Akt may mediate PI3'K-dependent BAD phosphorylation. In addition, the fact that constitutive Akt activity in the absence of PDGF stimulation was incapable of inducing a BAD mobility shift, which requires phosphorylation simultaneously at both Ser-112 and Ser-136, suggests that Akt phosphorylates only one site on BAD in vivo. A second PDGF-inducible kinase or kinase cascade that remains to be identified is most likely responsible for the phosphorylation of BAD at the second site in these cells. Results similar to those described were also obtained in experiments using independently isolated stable cell lines transfected with either vector or constitutively active Akt constructs (data not shown).

We next attempted to identify the site on BAD phosphorylated by Akt in vivo. This analysis focused on the ability of Akt to phosphorylate BAD at Ser-136 in vivo, because BAD Ser-136 is the preferred Akt phosphorylation site in vitro. Transfection of HEK 293 cells with wild-type BAD and immunoprecipitation with the anti-BAD136 pAb revealed that BAD Ser-136 was basally phosphorylated in these cells (Figure 4C, lane 2). This basal phosphorylation was greatly enhanced when BAD was cotransfected with a construct that encodes a constitutively active Akt construct, demonstrating that Akt activity induces BAD Ser-136 phosphorylation in vivo (Figure 4C, lane 3). Importantly, phosphorylation of BAD at Ser-136 was completely blocked by the expression of a kinase-inactive Akt (Figure 4C, lane 4). The finding that expression of a constitutively active Akt promotes the phosphorylation of BAD Ser-136, and that expression of kinase-inactive Akt blocks the phosphorylation of BAD at Ser-136 by endogenous kinases suggests that Akt may be a major in vivo mediator of BAD Ser-136 phosphorylation.

Additional biochemical evidence for the hypothesis that Akt is a relevant BAD kinase is derived from the observation that the major inducible BAD kinase activity in PDGF-stimulated 3T3 cells was found to cofractionate with both Akt immunoreactivity and Akt kinase activity after Mono-Q fractionation (data not shown). In addition, preliminary evidence from cotransfection experiments suggests that Akt and BAD associate upon overexpression in vivo. Western blotting of BAD immunoprecipitates from cells cotransfected with HA-tagged Akt constructs and wild-type BAD demonstrated that Akt coimmunoprecipitated with BAD, suggesting that Akt

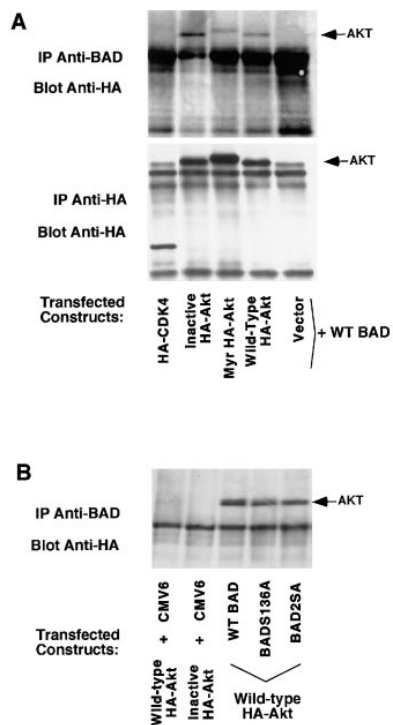


Figure 5. Akt and BAD Coprecipitate

(A) Overexpression of Akt and BAD results in interaction independently of Akt kinase activity. Anti-HA (12CA5 MAb) Western blot (upper panel) of anti-BAD immunoprecipitates (Santa Cruz N-term Ab) prepared from HEK 293 cells cotransfected with a wild-type BAD expression construct and either constitutive Akt (Myr HA-Akt), kinase-inactive HA-AktK179M (Inactive HA-Akt), HA-Akt (Wild-type HA-Akt), or a hemagglutinin-tagged CDK4 construct (HA-CDK4) as a negative control. Mobility of proteins on SDS-PAGE was determined by anti-HA Western blotting (lower panel) of anti-HA immunoprecipitates isolated from split lysates.

(B) Overexpression of Akt and BAD results in interaction independently of an intact Ser-136. Anti-HA Western blot (12CA5 MAb) of anti-BAD immunoprecipitates (Santa Cruz N-term Ab) of HEK 293 cells cotransfected with constructs expressing either HA-Akt (Wild-type HA-Akt) or kinase-inactive HA-AktK179M (Inactive HA-Akt) and constructs expressing either CMV-6 control (Vector), wild-type BAD (WT BAD), BADS136A, or BAD2SA.

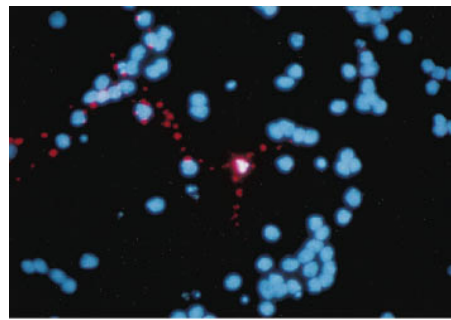
and BAD interact in vivo (Figure 5A). Interestingly, formation of this potential Akt/BAD complex did not depend on the kinase activity of Akt, as a kinase-inactive mutant form of Akt associated with BAD. In addition, phosphorylation of BAD at Ser-136 is not required for the observed Akt/BAD interaction, as both the BADS136A and the BAD2SA derivatives were capable of associating with Akt (Figure 5B). It remains to be demonstrated that endogenous Akt and BAD can interact, and under what conditions such a Akt/BAD complex is generated. However, taken together, these data raise the possibility that Akt and BAD proteins may interact, and provide support for the hypothesis that Akt is an in vivo BAD kinase.

IGF-1 and Akt Suppress BAD-Mediated Cell Death in Primary Neurons in a Ser-136-Dependent Manner

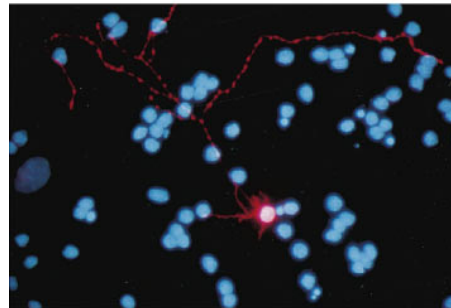
The finding that Akt activation triggers BAD Ser-136 phosphorylation in vitro and in vivo suggests that growth

factors that utilize the PI3'K-Akt pathway to promote survival may suppress BAD-mediated cell death by phosphorylating BAD at Ser-136. To investigate this possibility, we took advantage of a well-established neuronal culture system that has recently been used effectively to characterize the importance of the PI3'K/Akt pathway in IGF-1-mediated survival (D'Mello et al., 1993; Galli et al., 1995; Miller and Johnson, 1996; Dudek et al., 1997). Survival of postmitotic cerebellar granule neurons is promoted by defined survival factors, including IGF-1; withdrawal of trophic support from these neurons, which express BAD protein, induces synchronous apoptotic cell death (data not shown; Dudek et al., 1997). We previously demonstrated in these cells that IGF-1 specifically activates the PI3'K-Akt pathway, and not alternate survival pathways such as the Ras-MAPK-p90^{RSK} pathway, and that IGF-1-mediated induction of the PI3'K-Akt pathway is both necessary and sufficient for IGF-1 to promote the survival of these cells (Dudek et al., 1997).

To test the possibility that the IGF-1-PI3'K-Akt survival pathway suppresses BAD-mediated death, we cotransfected granule cells with expression constructs encoding various BAD derivatives and a construct encoding the marker gene β -galactosidase to allow the identification of transfected neurons. As previously established, transfected cells effectively expressed both β -gal and the cotransfected plasmid, and the stability of β -gal during apoptosis allowed effective identification of dying neurons (Dudek et al., 1997). Transfection of neurons with constructs encoding BAD resulted in dramatic induction of nuclear pyknosis and neurite fragmentation, consistent with apoptotic death (Figure 6). This death appeared phenotypically identical to the death induced by trophic factor withdrawal. To quantitate the levels of death induced by BAD, in a blinded manner we scored death based upon nuclear morphology, which we have previously found corresponds to TUNEL-positive apoptosis in these cells (Dudek et al., 1997). As shown in Figure 6, transfection of granule cells with wild-type BAD followed by 8 hr of starvation resulted in the death of the large majority of transfectants ($78.5\% \pm 2.5\%$ apoptosis, see also Figures 7A and 7B, pcDNA3 versus wild-type BAD). We then asked whether IGF-1 inhibits BAD-induced death in transfected cells. BAD-mediated death was substantially suppressed by treatment of transfectants with the survival factor IGF-1 ($37.5\% \pm 10\%$ apoptosis, Figure 7A). To determine if IGF-1 suppresses BAD-mediated death by inducing BAD phosphorylation at either BAD Ser-112 or BAD Ser-136, expression constructs encoding BADS112A, BADS136A, and BAD2SA were transfected into cells that were either starved or treated with IGF-1. IGF-1 suppression of BAD-mediated death required Ser-136, as IGF-1 blocked the death induced by both BAD and BADS112A, but not the death induced by BADS136A and BAD2SA. Thus, IGF-1, which promotes survival via PI3'K and Akt, inhibits BAD-mediated death via site-specific phosphorylation of BAD at Ser-136. This IGF-1 survival effect does not require the presence of the Ser-112 site on BAD, suggesting that the major kinases mediating the IGF-1 survival effect require the presence of only BAD Ser-136 to block BAD induction of death.



WT BAD



WT BAD + Active Akt

Figure 6. Transfection of Granule Cells with Active Akt Suppresses BAD-Mediated Death

Anti- β -gal immunostaining (red) of cerebellar granule cells cotransfected with expression plasmids encoding the marker β -galactosidase and wild-type BAD excluding (upper panel) or including (lower panel) a construct encoding constitutively active HA-m Δ 4-129Akt (Active Akt). Nuclear morphology was visualized by bisbenzamide costaining (blue). White arrows indicate nuclei of transfected cells.

Given the established role for PI3'K and Akt in mediating IGF-1 survival in cerebellar granule cells, and the demonstration that Akt can phosphorylate BAD in vitro and in vivo, we examined if Akt activity is sufficient to suppress BAD-induced cell death in the absence of IGF-1 in a Ser-136-dependent manner. To test whether a constitutively active Akt could substitute for IGF-1 in preventing BAD-mediated death, we transfected constructs encoding BAD and an active form of Akt into granule cells. Nearly all of the neurons ($97.7\% \pm 1.3\%$) that were transfected with BAD and deprived of survival factors for 16 hr, as quantitated in Figure 7B, underwent apoptosis (versus $41.0\% \pm 5.5\%$ for pcDNA3 vector alone). BAD-mediated death was almost wholly suppressed by cotransfection with a constitutive Akt derivative ($8.3\% \pm 1.2\%$, ANOVA with Bonferroni correction, $p < 0.0001$) (Figure 7B). Neurons cotransfected with Akt and BAD appeared healthy and robust, with elaborate processes, well-defined nuclei, and large soma (Figure 6). To demonstrate that Akt could suppress BAD-mediated death that was due entirely to BAD's death-promoting activity, the ability of Akt to suppress BAD-mediated

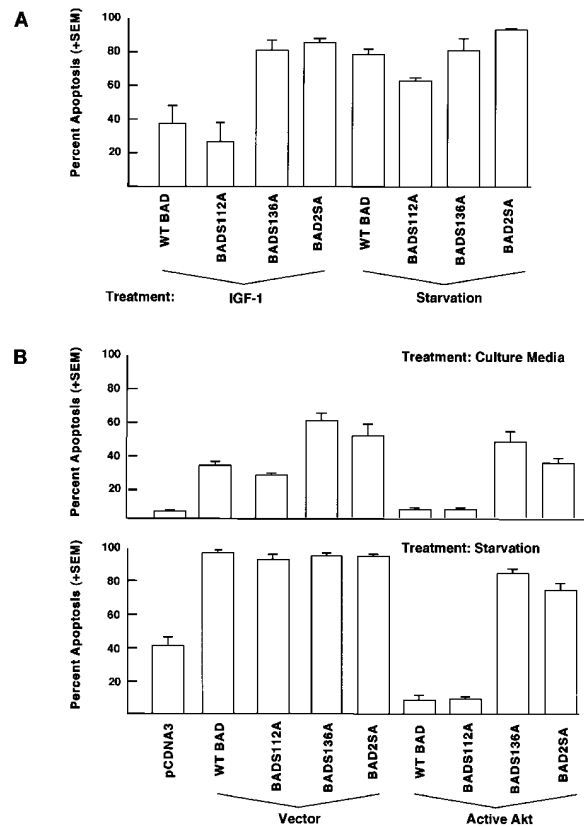


Figure 7. IGF-1 and Akt Suppress BAD-Mediated Death in Primary Neurons in a BAD Ser-136-Dependent Manner

(A) IGF-1 suppresses BAD-mediated death of transfected neurons in a BAD Ser-136-dependent manner. Quantitation of percent apoptosis based upon nuclear morphology of positively transfected neurons (as identified by β -gal staining) expressing constructs encoding either wild-type BAD (WT BAD), BADS112A, BADS136A, or BAD2SA. After transfection, neurons were allowed to recover overnight, and then placed in either starvation media or IGF-1-containing media (50 ng/ml) for 8 hr, fixed, and scored in a blinded manner. At this time point (8 hr), cells that were not transfected with BAD showed no increase in cell death in the presence of IGF-1, compared to the basal level of apoptosis seen in full survival medium (data not shown). Bars indicate the standard error of the mean; each bar represents at least 400 scored transfected cells.

(B) Akt suppresses BAD-mediated death of transfected neurons in a BAD Ser-136-dependent manner. Quantitation similar to (A) of neurons cotransfected with expression constructs encoding either vector (pECEC) or a constitutively active HA-m Δ 4-129Akt (Active Akt) and constructs expressing either CMV-6 control (Vector), wild-type BAD (WT BAD), BADS112A, BADS136A, or BAD2SA. Neurons were treated and stained as in (A), but treatment time in either starvation media or full survival media (10% bovine calf serum + 25 mM KCl) was 16 hr. Bars indicate the standard error of the mean; each bar represents at least 500 scored transfected cells from three independent experiments.

death was also tested in the presence of survival media containing serum and depolarizing concentrations of KCl, conditions that likely activate multiple survival pathways. Transfected BAD was capable of killing neurons cultured in full survival media, although the activation of multiple survival pathways—one or more of which may act independently of BAD—caused partial suppression of BAD-mediated death ($32.0\% \pm 2.3\%$, Figure 7B).

Under these conditions, the death that was detected was almost totally blocked by cotransfection of a constitutive Akt derivative ($5.0\% \pm 1\%$, ANOVA with Bonferroni correction $p < 0.0001$). Thus, Akt activity is sufficient to dramatically suppress the BAD-mediated death of transfected neurons.

The phosphorylation sites involved in the Akt-mediated inhibition of BAD-induced death were examined by transfecting neurons with expression vectors encoding BADS112A, BADS136A, and BAD2SA. Each of these constructs induced the death of essentially all transfected neurons when the cells were deprived of survival factors (for example, BADS112A induced apoptosis in $93.7\% \pm 3.4\%$ of transfectants, Figure 7B). However, constitutive Akt was capable of suppressing the death induced by BAD only when the BAD Ser-136 site was intact. The death induced by BAD and BADS112A was effectively suppressed by the cotransfection of a constitutively active Akt construct; in contrast, death induced by BAD constructs in which Ser-136 was converted to alanine (BADS136A and BAD2SA) was not rescued by cotransfection with an active Akt. Consistent with the findings in the absence of survival factors, expression of constitutive Akt also suppressed BAD-induced apoptosis in full survival media in a Ser-136-dependent manner (Figure 7B). These findings support the conclusion that IGF-1 and its target kinase Akt potentially block BAD-induced apoptosis and promote survival by triggering phosphorylation of BAD at Ser-136.

Discussion

Experiments in this study reveal that Akt phosphorylates BAD both *in vitro* and *in vivo* and that Akt-mediated phosphorylation of BAD effectively blocks BAD-induced cell death. Taken together, these findings demonstrate that activation of growth factor receptors can suppress apoptosis induced by BAD through a pathway involving the sequential induction of phosphoinositide-3'-OH kinase activity, Akt activity, and finally BAD phosphorylation. Because both PI3'K and Akt have been shown to promote survival in a variety of cellular contexts, the PI3'K-Akt-BAD pathway may represent a general mechanism by which growth factors promote cell survival.

In addition, these findings implicate a particular phosphorylation site on BAD, serine 136, in the suppression of BAD-mediated death by Akt. We show by a number of criteria that Akt is a potent BAD Ser-136 kinase *in vitro*. Since constitutively active Akt phosphorylates both endogenous and transfected BAD, and because disruption of Akt kinase activity results in the loss of BAD Ser-136 phosphorylation, Akt is also likely a major BAD Ser-136 kinase *in vivo*. This conclusion is supported by the observation that in cerebellar granule cells, an intact BAD Ser-136 phosphorylation site is required for both IGF-1 and its downstream kinase Akt to prevent BAD-mediated cell death. In contrast, Akt failed to significantly phosphorylate BAD at Ser-112, and the mutation of BAD Ser-112 to an alanine had no effect on the ability of IGF-1 or Akt to suppress BAD-mediated cell death. These observations suggest that Akt primarily triggers BAD phosphorylation at Ser-136 and that phosphorylation at this site is sufficient to promote survival.

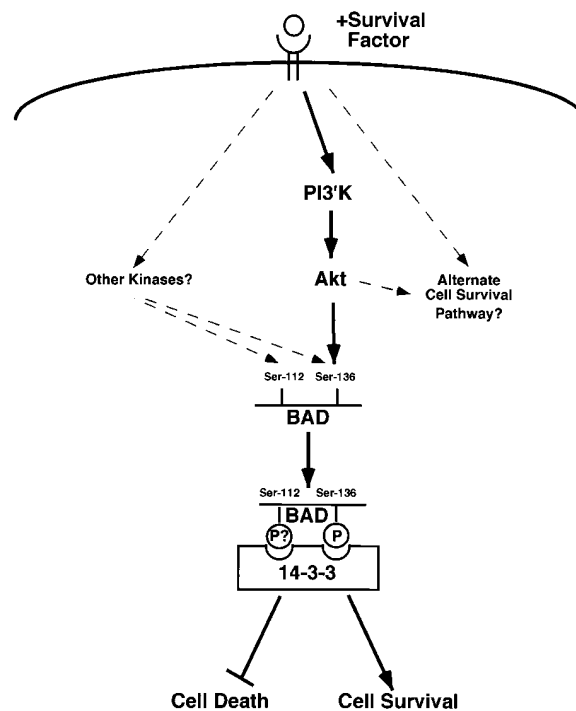


Figure 8. Model of Survival Signaling to BAD

The delineated signaling pathway mediates survival by the sequential activation of growth factor receptors, PI3'K, and Akt, which phosphorylates BAD Ser-136. Both PI3'K- and BAD-independent survival mechanisms likely also exist.

In other cell types, such as Il-3-dependent hematopoietic cells, prevention of cell death may involve both BAD Ser-112 and BAD Ser-136 (Zha et al., 1996). It may be that in these cells Akt is involved in BAD Ser-112 phosphorylation. Alternatively, Il-3 and other stimuli such as PDGF may promote survival by activating other kinase cascades to phosphorylate BAD at either Ser-112 or Ser-136. A number of signaling molecules in addition to Akt have been identified as potential mediators of survival stimuli, including PKA, components of the Ras-MAPK-p90^{RSK} pathway, and the calcium/calmodulin-dependent kinases (Rukenstein et al., 1991; Hack et al., 1993; Xia et al., 1995). Preliminary evidence suggests that p90^{RSK} or purified CaMKII can phosphorylate BAD at either Ser-112 or Ser-136 *in vitro* (data not shown). Thus, BAD may be a general target of kinases induced by a diverse set of survival stimuli including growth factors and cytokines, agents that cause increases in intracellular calcium, and neurotransmitters whose effects are mediated by adenylate cyclases.

The presence of two phosphorylation sites on BAD also suggests that the simultaneous activation of different survival pathways may result in the concomitant phosphorylation of BAD Ser-112 and Ser-136 by different kinase cascades (Figure 8). The relevance of such a model is supported by the observation that optimal cell survival *in vitro* is often best supported by a combination of survival factors, rather than a single factor (Barres et al., 1993; Meyer-Franke et al., 1995).

The phosphorylation of BAD may lead to the prevention of cell death via a mechanism that involves the

selective association of the phosphorylated forms of BAD with 14-3-3 protein isoforms. This BAD/14-3-3 interaction can occur when BAD becomes phosphorylated at Ser-112 and/or Ser-136, and the induced association of BAD with 14-3-3 appears to prevent BAD association with BCL-X_L or BCL-2. Preliminary evidence suggests that constitutively active Akt can induce the association of BAD and 14-3-3 ζ , and that kinase-inactive Akt does not induce this association event. In addition, Akt-induced BAD/14-3-3 ζ association depends on the presence of BAD Ser-136, as mutation of Ser-136 to alanine abolishes the BAD/14-3-3 ζ interaction (data not shown). It has been proposed that in the absence of BAD phosphorylation, BAD may bind to BCL-X_L or BCL-2 and suppress survival by inducing BAX homodimer formation. The prodeath function of BAD could also be a direct consequence of BAD's heterodimerization with BCL-X_L or BCL-2, and may not involve BAX heterodimerization (Yang et al., 1995; Gajewski and Thompson, 1996; Zha et al., 1996). The role for BAD in regulating death may not be ubiquitous, as there are cell types in which BCL-X_L and BCL-2 do not play significant roles in survival, and in these cell types overexpression of BAD does not induce death (Yang et al., 1995). However, genetic and cell biological evidence suggests that BCL-X_L, BCL-2, and BAX likely have broad and important roles in organismal physiology, and thus, the prevention of BAD-related death by growth factor regulation of BAD phosphorylation may be a major mechanism by which growth factors induce cell survival.

Phosphorylated BAD may also play an active role in promoting survival. Although there is as yet no direct evidence supporting this hypothesis, an active survival function for BAD is suggested by the finding that the interaction between BAD and 14-3-3 proteins is induced by phosphorylation at BAD Ser-136. 14-3-3 isoforms have been found to associate with a number of cellular signaling molecules, including KSR, cdc25, Raf-1, and PI3'K (reviewed in Morrison, 1994; Burbelo and Hall, 1995). Given our preliminary evidence that overexpression of BAD and Akt can result in the formation of a BAD/Akt complex, it is possible that BAD brings Akt to the 14-3-3 complex, where Akt may phosphorylate additional signaling molecules to promote survival. Further dissection of 14-3-3 protein complexes that assemble after growth-factor treatment may reveal novel roles for phosphorylated BAD and Akt in promoting survival.

The identification of BAD as an Akt substrate expands the list of *in vivo* Akt targets. Because Akt mediates multiple physiological responses in addition to survival, including GLUT4 translocation and changes in glycogen metabolism, Akt likely has additional substrates *in vivo* (Cross et al., 1995; Kohn et al., 1996). These novel targets could also play a role in promoting survival and preventing cell death. The possibility that Akt phosphorylates multiple substrates is interesting in light of the observation that neuronal survival promoted by blocking the terminal stages of apoptosis frequently results in neurons that survive but are shrunken and nonfunctional (Deckwerth et al., 1996; Deshmukh et al., 1996). In contrast, neurons whose survival is promoted by Akt activity are healthy and appear functional, with arborized processes and large nuclei (this study; Dudek et al., 1997).

Therefore, Akt may promote both survival and the functional integrity of neurons and other cells by phosphorylating a number of targets, including BAD and other substrates that remain to be identified.

Experimental Procedures

Materials

Throughout this paper, all Akt constructs are based on the wild-type Akt sequence. Unless otherwise noted, wortmannin was used at a concentration of 20 nM, rapamycin at a concentration of 20 nM, PD 98059 at a concentration of 20 μ M, and LY 294002 at a concentration of 10 μ M.

Antibodies

To generate anti-BAD136 pAg, a phosphopeptide of the sequence CSPFRGRSRpSAPPN (Tufts Synthesis Facility, Tufts Medical School, Boston, MA), was synthesized and coupled to keyhole limpet hemocyanin (Pierce). To generate anti-BAD136 pAbs, this antigen was injected into New Zealand White rabbits (Covance Research Products, Denver, PA), from which serum was collected approximately every three weeks. Serum was affinity-purified by passing it over a protein A-Sepharose column (Pharmacia), eluting the bound pAbs with 100 mM glycine (pH 2.5), passing this eluate over an agarose-iodoacetyl column (Pierce) to which was coupled a synthetic peptide of the sequence CSPFRGRSRpSAPPN (Biopolymers Laboratory, Harvard Medical School, Boston, MA), and collecting the flow-through.

Other antibodies used in this study include polyclonal anti N-terminal BAD antibody sc-941 (Santa Cruz), polyclonal anti C-terminal BAD antibody sc-943 (Santa Cruz), monoclonal anti-BAD antibody B31420 (Transduction Labs), polyclonal anti-Akt1 sc-1618 (Santa Cruz), polyclonal anti-Akt1 antibody 31 (generous gift of Dr. Morris Birnbaum), and monoclonal anti-hemagglutinin antibody 12CA5 (Boehringer Mannheim).

BAD Plasmid Cloning, Mutagenesis, and Protein Purification

The full-length murine BAD cDNA was cloned by RT-PCR of RNA isolated from adult mouse whole brain using a forward primer of sequence TCC-AGG-ATC-CAA-ATG-GGA-ACC and a reverse primer of sequence CGT-CGA-ATT-CGC-CCA-CAC-CCA-ATA-TGG-CG. The cloned cDNA was digested with BamHI and EcoRI and subcloned into the polylinker of the vector pCDNA3. The HA tag was added to this cDNA by PCR amplifying the full-length BAD cDNA with the forward primer 5'-GCC-GGT-ACC-ATG-TAC-CCA-TAC-GAT-GTT-CCA-GAT-TAC-G CT-GGA-ACC-CCA-AAG-C AG-CCC-3', which contains the nucleotide sequence for the HA tag linked to the second amino acid of BAD. This cDNA was digested with KpnI and EcoRI and cloned into pCDNA3. Point mutation of Ser-112 and Ser-136 was accomplished by PCR primer mutagenesis. To generate wild-type and mutant polyhistidine-tagged BAD bacterial expression constructs, a NdeI site was generated near the BAD start codon. The gene was then cloned into the NdeI and BamHI sites of a pET-16b expression vector. The mutant poly His-tagged BAD constructs were generated by swapping the mutant region between AvrII and BamHI sites with the corresponding region of wild-type BAD. The sequences of all wild-type and mutant BAD constructs were verified by automated sequencing. Wild-type and mutant BAD proteins were inducibly expressed in *E. coli* strain BL21 HMS175 (DE5)pLysS and purified over ProBond resin (Invitrogen). All PCR primers were synthesized at the Harvard Biopolymers Facility (Harvard Medical School, Boston, MA), and all restriction enzymes and polymerases were obtained from New England Biolabs.

Cell Lines, Transfection, and Stimulation Protocols

Balb/c 3T3 and 293 cells were transfected by treating the cells with calcium phosphate precipitates for 12 hr, then changing to fresh media for 12 hr, and lysing the cells. COS cells were transfected by the DNA/DEAE-dextran (Pharmacia)/chloroquine (Sigma) method. PDGF stimulation of 3T3 cells was accomplished by starving the cells in DMEM containing 0% FBS for 6 hr, and injecting control PBS or PDGF-BB (Upstate Biotechnology Incorporated) into the

dish at a final concentration of 20 ng/ml. Balb/c 3T3 stable transfectants were generated by cotransfecting either CMV-6 or HA-m Δ 4-129Akt with the plasmid pBABE-PURO (1:10 ratio) by the calcium phosphate method in standard media supplemented with 1.25 μ g/ml puromycin (Sigma). Individual clones were picked and expression of the Akt plasmid was detected by anti-HA immunoblotting.

Protein Methods

Immunoprecipitations, kinase assays, and Western blotting were carried out as described previously (Dudek et al., 1997). Two-dimensional tryptic mapping was carried out by isolating, treating the pellet with performic acid, trypsinizing overnight with TPCK-trypsin (Worthington Enzymes), washing extensively in water, and spotting onto cellulose TLC plates (VWR). Electrophoresis was carried out in pH 8.9 for 30 min at 1000 V using a Hunter TLC apparatus, and chromatography was carried out in a phosphochromatography buffer containing 37.5% n-butanol, 25% pyridine, and 0.75% glacial acetic acid. Control Ser-112 (HSPYPAGTEEDEGMEEELSPFR) and Ser-136 (pSAPPNLWAAQR) tryptic phosphopeptides were synthesized and run as controls (Dana Farber Molecular Biology Core Facility, Boston, MA).

Granule Cell Cultures, Immunostaining, Transfection, and Survival Assays

Neuronal cultures and scoring were carried out as described previously (Dudek et al., 1997). Cells were transfected at DIV 4-5 essentially as in Dudek et al., 1997, except that transfections were done in Hank's salt-buffered MEM (at pH 7.85) in ambient air. One day after transfection, cells were incubated an additional 8 or 16 hr in treatment solutions, then fixed with paraformaldehyde, blocked in 3% BSA solution, and immunostained.

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