Developmental Cell, Vol. 3, 631–643, November, 2002, Copyright ©2002 by Cell Press

Survival Factor-Mediated BAD Phosphorylation Raises the Mitochondrial Threshold for Apoptosis

Sandeep Robert Datta,[1] Ann M. Ranger,[2] Michael Z. Lin,[1] James Fitzhugh Sturgill,[1] Yong-Chao Ma,[1] Chris W. Cowan,[1] Pieter Dikkes,[1] Stanley J. Korsmeyer,[2] and Michael E. Greenberg[1,3]
[1]Division of Neuroscience
Children's Hospital and
Department of Neurobiology
Harvard Medical School
Boston, Massachusetts 02115
[2]Departments of Pathology and Medicine
Harvard Medical School
Howard Hughes Medical Institute
Dana-Farber Cancer Institute
Boston, Massachusetts 02115

Summary

Growth factor suppression of apoptosis correlates with the phosphorylation and inactivation of multiple proapoptotic proteins, including the BCL-2 family member BAD. However, the physiological events required for growth factors to block cell death are not well characterized. To assess the contribution of BAD inactivation to cell survival, we generated mice with point mutations in the BAD gene that abolish BAD phosphorylation at specific sites. We show that BAD phosphorylation protects cells from the deleterious effects of apoptotic stimuli and attenuates death pathway signaling by raising the threshold at which mitochondria release cytochrome c to induce cell death. These findings establish a function for endogenous BAD phosphorylation, and elucidate a mechanism by which survival kinases block apoptosis in vivo.

Introduction

Growth factors play critical roles in promoting the survival of many different cell populations during metazoan development (Raff, 1992). As the nervous system develops, neurons compete for limiting quantities of neurotrophic factors that promote the survival of those neurons that successfully innervate their targets (Segal and Greenberg, 1996). Likewise, cells of the immune system secrete cytokines whose activity induces the survival of populations of lymphocytes that are essential to normal immune function (Plas et al., 2002). Growth factors also function in mature organisms to promote the survival of cells challenged with apoptotic stimuli such as tumor necrosis factor α (TNFα) or ionizing radiation (Rosen et al., 1999; Venters et al., 2000).

The importance of growth factors in attenuating cell death has focused recent efforts on identifying the intracellular signaling pathways that transduce survival signals. A number of growth factor-regulated protein kinase cascades have been implicated in survival signaling, including the PI3K/Akt, Ras/MAPK/Rsk, and PKA pathways (Datta et al., 1999; Talapatra and Thompson, 2001). These survival kinases are thought to prevent apoptosis by inactivating proapoptotic proteins whose unrestrained activity would otherwise consign cells to death. The potential targets of survival kinases include regulators of mitochondrial function such as the BCL-2 family member BAD, regulators of metabolism such as GSK-3, and transcription factors of the Forkhead family such as FOXO3a (reviewed in Brunet et al., 2001; Datta et al., 1999). Although these survival kinase substrates have been suggested to play a role in growth factor-dependent cell survival, a gap in the understanding of survival factor function arises from the absence of evidence that phosphorylation and inactivation of these survival kinase substrates is important for cell survival under physiological conditions. As such, it is not clear whether inactivation of BAD, FOXO3a, or other potential survival kinase substrates is required for cell survival.

The proapoptotic BCL-2 family member BAD has been suggested to link survival signals to the mitochondrial cell death machinery (Zha et al., 1996). BAD belongs to a subfamily of BCL-2 proteins that are similar to BCL-2 in that they share a BCL-2 homology 3 (BH3) domain. These "BH3-only" proteins serve as molecular sentinels whose activity or expression is regulated in response to specific death stimuli (Puthalakath and Strasser, 2002). BAD is a BH3-only protein whose activity is regulated by extracellular survival signals. Growth factors induce the phosphorylation of BAD at three sites, Ser-112, Ser-136, and Ser-155, which inactivates the proapoptotic activity of BAD (Datta et al., 2000; Lizcano et al., 2000; Virdee et al., 2000; Zha et al., 1996). Several kinases that have been implicated in survival signaling have been proposed to mediate BAD phosphorylation, including Akt, Rsk, PAK, p70^{S6K}, and PKA (Bonni et al., 1999; Datta et al., 1999; Harada et al., 2001; Schurmann et al., 2000; Tan et al., 1999). Upon growth factor withdrawal, BAD is dephosphorylated at Ser-112, Ser-136, and Ser-155, and this active form of BAD binds to prosurvival BCL-2 family members at the mitochondria. The binding of BAD to prosurvival BCL-2 proteins is followed by the oligomerization of the proapoptotic BCL-2 proteins BAX and BAK, which results in mitochondrial dysfunction, cytochrome c release, caspase activation, and apoptotic death (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001).

The phosphorylation of endogenous BAD at Ser-112, Ser-136, and Ser-155 occurs under many conditions in which growth factors promote cell survival in cell culture (reviewed in Datta et al., 1999). This correlation has led to the suggestion that BAD phosphorylation is essential for survival factors to block apoptosis. Consistent with a role for BAD inactivation in survival signaling, overexpressed BAD induces cell death that can be blocked by survival kinases. However, it remains to be determined whether growth factors require the phosphorylation and inactivation of BAD to promote cell survival when BAD is expressed in its normal physiological contexts.

To address the physiological importance of BAD

[3]Correspondence: michael.greenberg@tch.harvard.edu

phosphorylation in trophic factor-mediated cell survival, we generated mutant mice (*BAD*$^{3SA/3SA}$) in which the three regulatory serines in BAD (Ser-112, Ser-136, and Ser-155) were converted to alanines, rendering endogenous BAD refractory to phosphorylation. We reasoned that any specific defects observed in *BAD*$^{3SA/3SA}$ mutant mice would reveal the circumstances in which BAD inactivation by phosphorylation is required for normal cell function. We show here that in select cell types, survival factors require BAD phosphorylation to prevent developmental apoptosis, as growth factor-dependent survival is attenuated in *BAD*$^{3SA/3SA}$ cells from the immune and nervous systems. These findings demonstrate that BAD dephosphorylation alone, even when survival factors are present, is sufficient to promote cell death in some circumstances.

The analysis of *BAD*$^{3SA/3SA}$ mice also reveals that a primary function of BAD phosphorylation is to allow growth factors to block cell death induced by a variety of apoptotic stimuli. Growth factors can block cell death induced by either the cell-extrinsic apoptotic pathway (which is activated by death cytokines such as TNFα), or by the cell-intrinsic apoptotic pathway (which is activated after cellular damage). Activation of either pathway results in increased activity of proapoptotic BCL-2 family members, which induce mitochondrial dysfunction to cause cell death. We find that growth factors require endogenous BAD phosphorylation to protect cells from death induced by activation of cell-intrinsic or cell-extrinsic death pathways because BAD phosphorylation raises the threshold at which mitochondria release cytochrome c. Taken together, these findings identify a biological function for growth factor-mediated BAD phosphorylation in regulating cellular responses to death signals.

Results

Generation of BAD Knockin Mice

To generate BAD knockin mice (*BAD*$^{3SA/3SA}$) in which each of the regulatory serines (Ser-112, Ser-136, and Ser-155) was converted to alanine, we constructed a targeting vector in which mutations in these three serines were introduced into the genomic BAD sequence and linked to silent restriction fragment length polymorphisms (RFLPs) to facilitate genotyping (Figure 1A). This targeting vector, which contained a positive selection marker flanked by recognition sites for the FlpE recombinase (FRT-Neo-FRT) successfully recombined with the endogenous BAD locus of embryonic stem (ES) cells, as revealed by Southern blotting (Figure 1B). After germline transmission of the mutant BAD allele in mice was established, the FRT-Neo-FRT cassette was excised from the genomic BAD sequence by crossing *BAD*$^{3SA(NEO)/+}$ animals with transgenic mice that express the FlpE recombinase (Farley et al., 2000). Excision of the neomycin gene was confirmed by Southern blotting to detect the presence or absence of the FRT-Neo-FRT cassette, and a PCR-based assay was performed to detect the RFLPs linked to the BAD serine-to-alanine mutations (Figure 1C).

Western blot analysis of protein extracts of whole brain (Figure 1D) and other organs (data not shown) revealed that the amounts of BAD protein expressed in *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ tissues were similar. In contrast, Western blotting of whole-brain lysate with an antibody that specifically recognizes BAD when it is phosphorylated at Ser-155 detected Ser-155-phosphorylated BAD in wild-type tissue, but failed to detect phosphorylated BAD in tissue obtained from *BAD*$^{3SA/3SA}$ mice (Figure 1D). Similar results were obtained using antibodies that specifically recognize BAD when it is phosphorylated at Ser-112 or Ser-136 (data not shown), indicating that we had generated animals in which endogenous BAD is no longer capable of being phosphorylated at the three critical regulatory sites (Ser-112, Ser-136, and Ser-155), but in which BAD levels and patterns of expression are normal.

To establish that mutation of the regulatory serines in BAD did not create a BAD molecule that functions in a dominant manner to interfere with survival-promoting kinases, we assessed the expression levels and phosphorylation status of Akt, PAK, and p90^{Rsk}, three kinases proposed to mediate BAD phosphorylation. As shown in Figure 1E, the expression levels and phosphorylation status of these kinases was similar in *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ brain lysates, as were the levels and phosphorylation status of other survival signaling substrates, including FOXO3a and CREB (Figure 1F; Datta et al., 1999). In addition, the levels of expression of BCL-2 family members were also not significantly altered in the *BAD*$^{3SA/3SA}$ animals (Figure 1G). These data demonstrate that the serine-to-alanine mutations in endogenous BAD render BAD unresponsive to survival signals, but do not apparently alter the signaling pathways that regulate cell survival upstream of BAD or the expression levels of other BCL-2 family members.

A Requirement for BAD Phosphorylation during Immune System Development

Homozygous *BAD*$^{3SA/3SA}$ mice were obtained at the normal Mendelian frequency, and histological analysis of most tissues from *BAD*$^{3SA/3SA}$ animals revealed no gross abnormalities. However, examination of immune tissues revealed that mutant BAD animals exhibit a defect in the growth factor-dependent survival of lymphocytes. Both the weight of the thymus (37.5 $\pm$ 1.3 mg versus 22 $\pm$ 1.0 mg, $p < 0.0007$) and the number of cells in the thymus ($6.4 \times 10^7 \pm 0.7$ cells versus $3.5 \times 10^7 \pm 0.1$ cells, $p < 0.01$) were significantly lower in *BAD*$^{3SA/3SA}$ than in *BAD*$^{+/+}$ adult mice (Figure 2A). Therefore, we sought to determine whether BAD phosphorylation was required for the appropriate development of T cells. In the thymus, T cell development progresses in a stepwise manner, each stage of which is characterized by the expression of a unique complement of cell surface markers (Shortman and Wu, 1996). An early T cell progenitor in the thymus, the pre-pro-T cell, expresses the CD44 marker but does not express either the CD25 marker or the T cell receptor. As pre-pro-T cells differentiate into pro-T cells, they express CD25 and undergo T cell receptor rearrangement. As pro-T cells differentiate into pre-T cells, they express the T cell receptor, and as maturation continues, they express the CD4 and/or CD8 coreceptor molecules. To analyze thymic development in *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ mice, the numbers of thymocytes at each

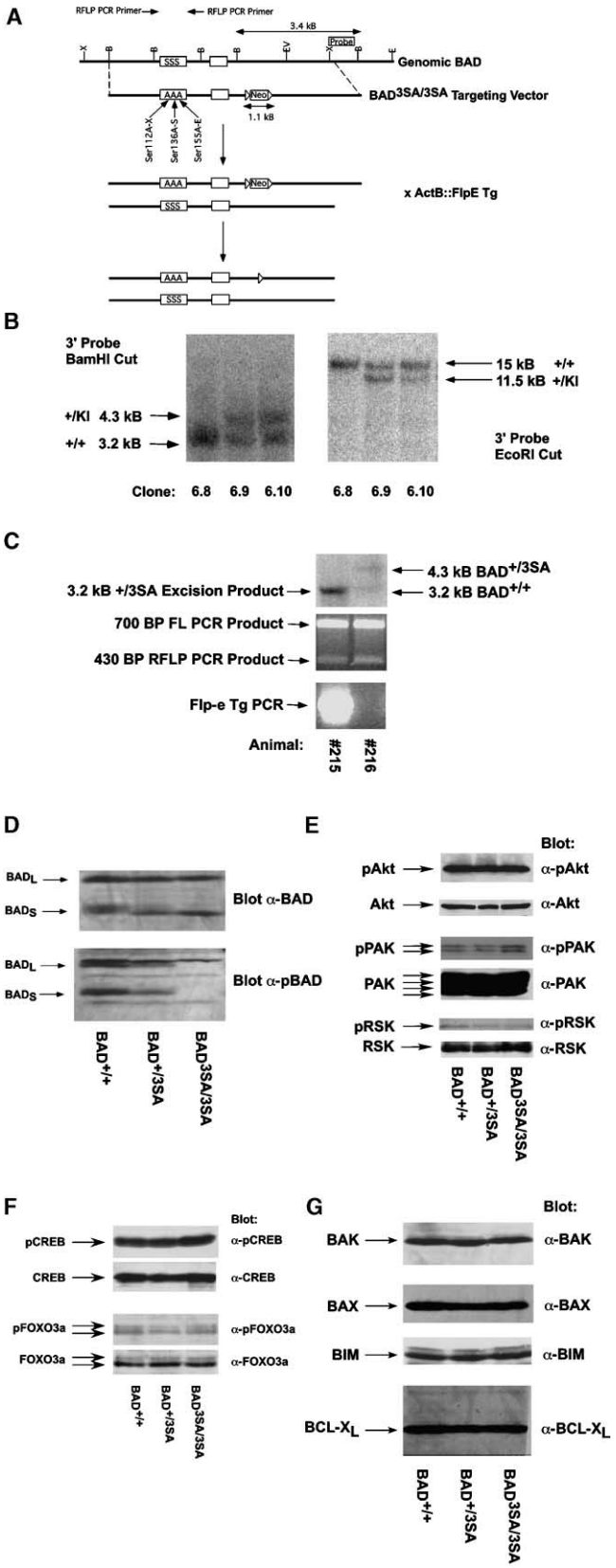

Figure 1. Generation and Characterization of *BAD*$^{3SA/3SA}$ Mice

(A) Targeting strategy to generate *BAD*$^{3SA/3SA}$ mice. Map of the BAD locus indicates restriction markers (X, XbaI; B, BamHI; EV, EcoRV; S, SmaI; E, EcoRI) and exons (open boxes). Serine-to-alanine mutations were linked to RFLPs as indicated, and a PGK-NEO cassette with flanked FRT sites (triangles) was inserted into the targeting vector downstream of the BAD 3′ UTR. After generation of *BAD*$^{3SA(NEO)/+}$ animals (see below), animals were crossed to FlpE transgenic animals to excise the Neo cassette.

(B) Southern blots indicating appropriate gene targeting as assessed using the probe indicated in (A). The left panel shows increased fragment length due to FRT-Neo-FRT cassette insertion into the BamHI fragment, whereas the right panel shows decreased length of the EcoRI fragment due to successful introduction of an EcoRI RFLP into BAD exon 3.

(C) Analysis of excision of the FRT-Neo-FRT cassette. Animals depicted are progeny of a *BAD*$^{3SA(NEO)/+}$ male crossed to a FlpE transgenic female. The lower panel is an EtBr-stained agarose gel of PCR for the FlpE transgene. The middle panel is a similar gel of a PCR fragment for the RFLP mutation marker after digestion with SmaI, demonstrating that both animals have a single copy of the *BAD*$^{3SA/3SA}$ mutant allele. The top panel is a Southern blot with the probe indicated in (A), demonstrating that the FRT-Neo-FRT cassette has successfully been excised in animal #215.

(D) Western blots of SDS lysates of whole brain, probed with either C-20 anti-BAD (α-BAD), or anti-p155BAD (α-pBAD). BAD_L is full-length mouse BAD, and BAD_S is an isoform of BAD missing the BAD N terminus. BAD_S is the predominant form of BAD in most tissues (S.R.D. and M.E.G., unpublished data). The band above the BAD_L band is nonspecific. The shift in BAD_S mobility in the upper panel observed in the *BAD*$^{+/+}$ and *BAD*$^{+/3SA}$ lanes is indicative of phosphorylation, and the increased apparent intensity of the BAD_S band in the mutant animals reflects the collapse of this shift (Zha et al., 1996).

(E–G) Western blots of SDS lysates of whole brain, probed with the antibodies indicated. Two-dimensional gel electrophoresis of similar lysates from *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ brains followed by Western blotting with a phospho-Akt substrate antibody (CST) were also performed, and no differences between the wild-type and mutant animals was detected (data not shown).

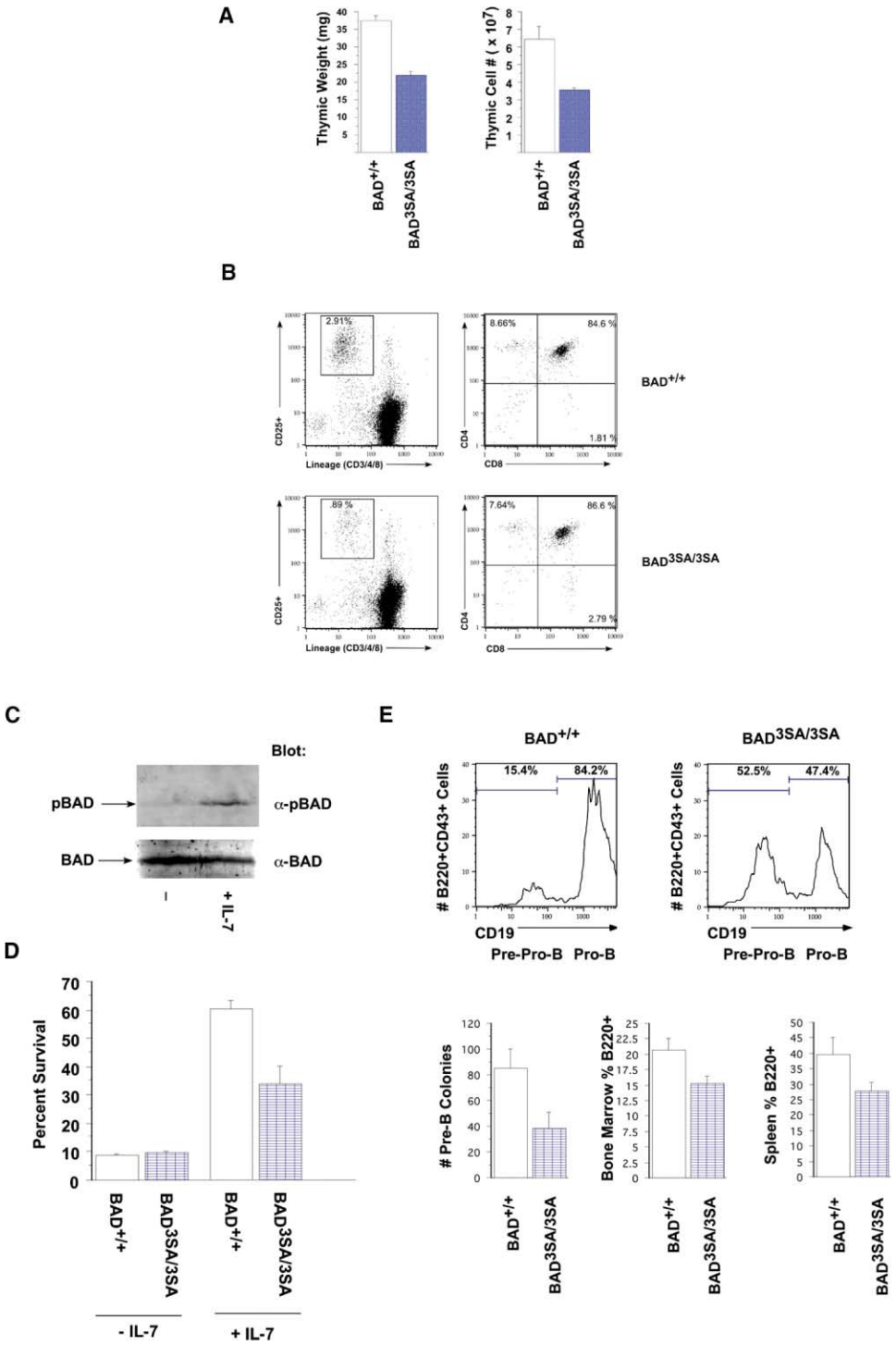

Figure 2. Pro-T and -B Cell Survival Defects in the *BAD*$^{3SA/3SA}$ Mouse

(A) Weight (left) and cell number (right) of thymuses isolated from 3-month-old animals (n = 6).

(B) Representative FACS analysis of CD25+/CD3−CD4−CD8− pro-T cells (left), and CD4/CD8 ratios (right) of 3-month-old littermate animals. No significant differences in the CD4/CD8 ratios were observed when *BAD*$^{3SA/3SA}$ and *BAD*$^{+/+}$ mice were compared.

(C) Western blot of thymocytes isolated from *RAG2*$^{-/-}$ animals. Thymuses from 3-week-old *RAG2*$^{-/-}$ animals were dissociated and the resultant thymocytes were cultured in either no survival factors or in 10 ng/ml IL-7 for 6 hr. More than 95% of viable cells from *RAG2*$^{-/-}$ thymuses were CD25+CD3−CD4−CD8− as assessed by FACS analysis (data not shown). Whole-cell lysates were resolved by SDS-PAGE and subjected to Western blot analysis with the antibodies indicated.

(D) Survival of sorted CD25+CD3−CD4−CD8− pro-T cells from *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ mice after 24 hr culture in either no survival factors or 10 ng/ml IL-7. Survival was assessed by trypan blue exclusion.

(E) FACS sorting for B220+CD43+CD19− pre-pro-B cells and B220+CD43+CD19+ pro-B cells (upper panel). Data shown are representative of differences observed in four pairs of 3-month-old wild-type and mutant animals. The lower left panel depicts the number of IL-7-dependent methylcellulose pre-B colonies recovered from bone marrow of indicated mice after 1 week of culture in a methylcellulose matrix (n = 3). The lower right panels depict numbers of B220+ B cells in bone marrow and spleen as assessed by FACS sorting (n = 5).

stage of development were analyzed by fluorescence-activated cell sorting (FACS). These experiments revealed that *BAD*$^{3SA/3SA}$ mice, when compared to wild-type animals, have significantly fewer CD25+ pro-T cells (50.6 ± 9.1 percent of wild-type; Figure 2B). Consistent with this finding, the proportion of CD44+CD25− pre-pro-T cells was increased in *BAD*$^{3SA/3SA}$ thymuses (180.9 ± 27.5 percent of *BAD*$^{+/+}$). However, no significant difference in the absolute number of pre-pro-T cells in *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ thymuses was detected, indicating that *BAD*$^{3SA/3SA}$ thymuses contain normal numbers of pre-pro-T cells, but decreased numbers of pro-T cells. BAD phosphorylation apparently does not regulate the development of cells generated after the pro-T stage, as the relative proportions of CD4+ and CD8+ cells, and the expression of the T cell receptor and markers of T cell activation, were normal in *BAD*$^{3SA/3SA}$ mice. Because pro-T cells give rise to the vast majority of the cells in the thymus, these results suggest that the low thymic weight and cellularity observed in the *BAD*$^{3SA/3SA}$ animals is due to a requirement for endogenous BAD phosphorylation in the development or survival of pro-T cells.

Since the cytokine interleukin-7 (IL-7) has been shown to be required for the survival of prothymocytes in vivo (Peschon et al., 1994; von Freeden-Jeffry et al., 1995), we tested the possibility that the observed loss of pro-T cells in *BAD*$^{3SA/3SA}$ mice was due to the inability of IL-7 to promote the survival of these cells. To obtain an adequate number of cells to perform biochemical analysis, we isolated pro-T cells from 3-week-old *RAG2*$^{-/-}$ animals, as thymocytes from these mice are developmentally arrested at the pro-T stage (Shinkai et al., 1992). Consistent with the possibility that in pro-T cells IL-7 normally promotes survival by phosphorylating and inactivating BAD, we found by Western blot analysis that BAD was expressed in pro-T cells from *RAG2*$^{-/-}$ mice, and became newly phosphorylated after treatment with IL-7 (Figure 2C). These findings led us to test whether the loss of pro-T cells observed in the *BAD*$^{3SA/3SA}$ mutant mice reflects a failure of IL-7 to promote the survival of *BAD*$^{3SA/3SA}$ pro-T cells. We isolated pro-T cells from pooled *BAD*$^{+/+}$ or *BAD*$^{3SA/3SA}$ thymuses by cell sorting, and assessed the ability of IL-7 to promote the survival of these cells. As shown in Figure 2D, IL-7 potentiated the survival of cultured wild-type pro-T cells, but was less effective at promoting the survival of *BAD*$^{3SA/3SA}$ pro-T cells. These findings demonstrate that in wild-type pro-T cells, IL-7 promotes survival in part by phosphorylating and inactivating BAD.

Pro-B cells also require IL-7 for their survival in vivo (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Analysis of cells of the B lineage revealed a specific defect in pro-B cells in *BAD*$^{3SA/3SA}$ mice. FACS analysis of B cell precursors revealed that the bone marrow of *BAD*$^{3SA/3SA}$ mice contained fewer pro-B cells than did the bone marrow of wild-type mice (Figure 2E). Additional defects were also detected at later stages of B cell development in *BAD*$^{3SA/3SA}$ mice, which may reflect the lower numbers of pro-B cells in mutant animals. For example, *BAD*$^{3SA/3SA}$ bone marrow produced fewer pre-B cell colonies than did *BAD*$^{+/+}$ bone marrow after in vitro culture in methylcellulose-containing medium. In addition, the total number of B cells in the bone marrow and the total number of mature splenic B cells were lower in *BAD*$^{3SA/3SA}$ animals relative to their wild-type counterparts (Figure 2E). These findings demonstrate that BAD phosphorylation is important for the normal development of pro-B and pro-T cells, and are consistent with a model in which IL-7-mediated BAD phosphorylation is important for the ability of IL-7 to promote cell survival.

Growth Factor-Mediated BAD Phosphorylation Is Required for Protection of Cells from Multiple Apoptotic Stimuli

The observation that BAD mutant mice exhibit defects in the growth factor-dependent survival of immune cells led us to investigate whether BAD phosphorylation plays a more general role in cell survival. We assessed the ability of specific survival factors to block cell death in cultured cerebellar granule neurons (D'Mello et al., 1993; Dudek et al., 1997). In the absence of survival factors, cultured cerebellar granule neurons from *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ mice underwent apoptosis to a similar extent (Figure 3A). This finding is not surprising, given that endogenous BAD is dephosphorylated in cerebellar granule neurons upon removal of survival stimuli (Datta et al., 2000; Gleichmann et al., 2000). As growth factors, including the insulin-like growth factor 1 (IGF-1), induce endogenous BAD phosphorylation in wild-type neurons but cannot stimulate BAD phosphorylation in *BAD*$^{3SA/3SA}$ neurons, we compared the ability of IGF-1 to promote the cell survival of *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ cerebellar granule cells. We found that after 48 hr of treatment with IGF-1, significantly more wild-type neurons survived than *BAD*$^{3SA/3SA}$ neurons, indicating that BAD phosphorylation contributes to the IGF-1-mediated survival of cerebellar granule neurons (Figure 3A).

Despite the defects observed in IGF-1-mediated survival in *BAD*$^{3SA/3SA}$ neurons, IGF-1 was still capable of partially blocking the apoptosis of *BAD*$^{3SA/3SA}$ cells, suggesting that IGF-1 and other growth factors may also inhibit other proapoptotic proteins to block cell death. One such target may be FOXO3a, a transcription factor of the Forkhead family, whose activity is regulated by growth factor-dependent phosphorylation (Brunet et al., 2001). In the presence of survival factors, FOXO3a is phosphorylated and localized to the cytoplasm, whereas in the absence of survival factors, FOXO3a is dephosphorylated and localized to the nucleus, where it induces many genes including several that promote cell death. Although implicated in the induction of cell death, FOXO3a, when rendered constitutively active and overexpressed, only weakly induces apoptosis in neurons (Brunet et al., 1999). This finding raises the possibility that under physiological conditions, FOXO3a might collaborate with other proapoptotic proteins to promote cell death. If this were found to be true, survival factors might be expected to coordinately inactivate FOXO3a and other proapoptotic proteins, such as BAD, to effectively block apoptosis. To test whether BAD dephosphorylation sensitizes cells to the proapoptotic activity of FOXO3a, cultured *BAD*$^{+/+}$ or *BAD*$^{3SA/3SA}$ cerebellar granule cells were transfected with constructs expressing a constitutively active mutant of FOXO3a, in which the phosphorylation sites normally induced by growth factors are mutated to alanines (FOXO3aTM; Figure 3B).

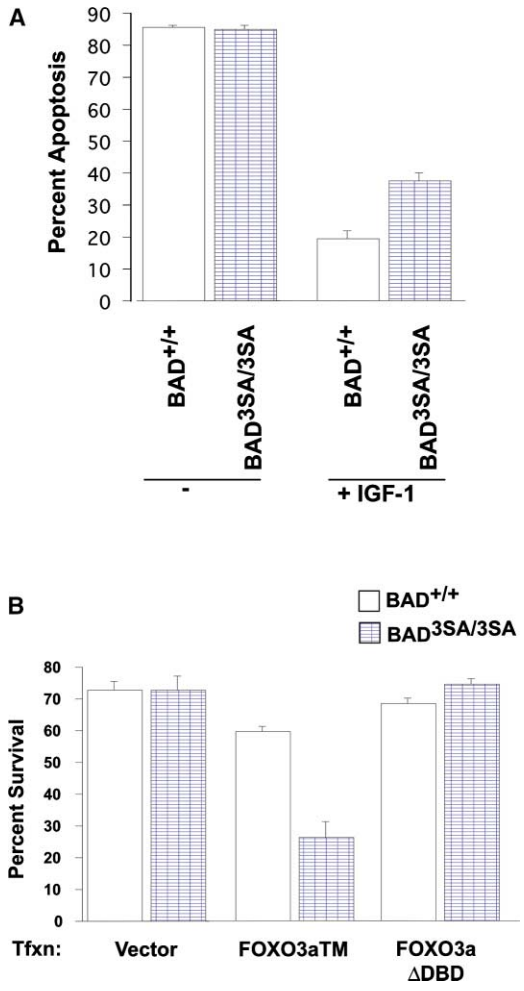

Figure 3. Analysis of Cerebellar Granule Neurons from *BAD*$^{3SA/3SA}$ Mice

(A) Cerebellar granule cells were isolated from P5 mouse pups, cultured for 5 days, and then either starved or treated with 50 ng/ml IGF-1 for 48 hr. Cell survival was assessed in a blinded manner by neurite fragmentation and nuclear morphology after Hoechst 33342 staining (n = 3).

(B) Cerebellar granule neurons were isolated as in (A), cotransfected with the indicated constructs and a β-galactosidase transfection marker, and after 24 hr were switched into IGF-1. After a culture of cells in IGF-1 for 12 hr, cells were fixed and stained with an anti-β-gal antibody, and positive transfectants were scored as in (A) (n = 3).

As previously demonstrated, FOXO3aTM only weakly induced death in wild-type cerebellar granule neurons cultured in the presence of IGF-1, suggesting that IGF-1 suppresses cell death by inducing the phosphorylation of additional target molecules. One such target molecule is likely BAD, as transfection of *BAD*$^{3SA/3SA}$ mutant neurons with FOXO3aTM resulted in the robust induction of cell death in the presence of IGF-1 (Figure 3B). This effect required the ability of FOXO3aTM to bind to DNA, as mutation of the DNA binding domain of FOXO3aTM abrogated the ability of FOXO3aTM to promote the death of either wild-type or *BAD*$^{3SA/3SA}$ neurons. These results indicate that growth factors promote survival by inducing the phosphorylation and inactivation of multiple proapoptotic molecules, such as BAD and FOXO3a. These results also demonstrate that activation of a second apoptotic pathway more effectively reveals the proapoptotic function of dephosphorylated BAD.

The observation that BAD dephosphorylation sensitizes cells to apoptosis induced by active FOXO3a led us to investigate whether the functional synergy between BAD and FOXO3a is specific, or whether it reflects a more general ability of dephosphorylated BAD to sensitize cells to the activation of other apoptotic signaling pathways. If this were found to be true, then endogenous BAD phosphorylation would be essential for the normal ability of growth factors to attenuate cell death caused by proapoptotic signaling. To address this possibility, we generated mouse embryo fibroblasts (MEF) from E13.5 *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ embryos. MEFs are well suited for studying whether BAD phosphorylation blocks death induced by death receptor activation or genotoxic stress, since the phosphorylation state of BAD in MEFs can be regulated by the addition or withdrawal of growth factors (Figure 4A). In addition, *BAD*$^{3SA/3SA}$ MEFs appear healthy and do not exhibit an increased rate of spontaneous apoptosis relative to *BAD*$^{+/+}$ MEFs in the presence or absence of growth factors (data not shown).

To test the possibility that endogenous BAD phosphorylation in MEFs protects cells from the potentially lethal effects of apoptotic stimuli, we challenged MEFs with cell-extrinsic activators of death receptor signaling (such as TNFα or anti-Fas antibodies that ligate and activate the Fas receptor) or DNA-damaging agents (such as γ irradiation or the chemotherapeutic agent etoposide). We cultured both *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ MEFs in 15% fetal calf serum, IGF-1, or platelet-derived growth factor (PDGF), conditions that induce BAD phosphorylation, treated the cells with either death receptor ligands or DNA-damaging agents, and assessed the relative levels of cell death. As shown in Figures 4B and 4C, BAD phosphorylation effectively countered cell death induced by anti-Fas antibodies, TNFα, γ irradiation, and etoposide in wild-type MEFs. Notably, growth factors that induce BAD phosphorylation and cell survival in *BAD*$^{+/+}$ MEFs failed to block apoptosis induced by either death receptor activation or DNA-damaging agents in *BAD*$^{3SA/3SA}$ MEFs (Figures 4B and 4C). Analysis of the responses of *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ MEFs cultured in survival factors to a range of doses of anti-Fas antibodies and TNFα revealed that constitutive BAD dephosphorylation lowered the threshold at which activation of the cell-extrinsic pathway of cell death induces apoptosis (Figure 4D). In contrast, as shown in Figures 4B and 4C, when cultured in the absence of survival factors, *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ MEFs underwent equivalent levels of cell death after treatment with anti-Fas antibodies, TNFα, γ irradiation, or etoposide, as might be expected, as BAD is dephosphorylated in the absence of survival factors in both wild-type and *BAD*$^{3SA/3SA}$ cells. These results demonstrate that endogenous BAD phosphorylation is required for survival factors to block death induced by apoptotic signaling.

Survival factors were able to block cell death induced by cell-extrinsic death stimuli in the absence of new protein synthesis (Figures 4B and 4D), but were unable to block cell death in the absence of PI3K activity, as treatment of MEFs with LY294002, a chemical inhibitor of PI3K, blocked the antiapoptotic activity of survival

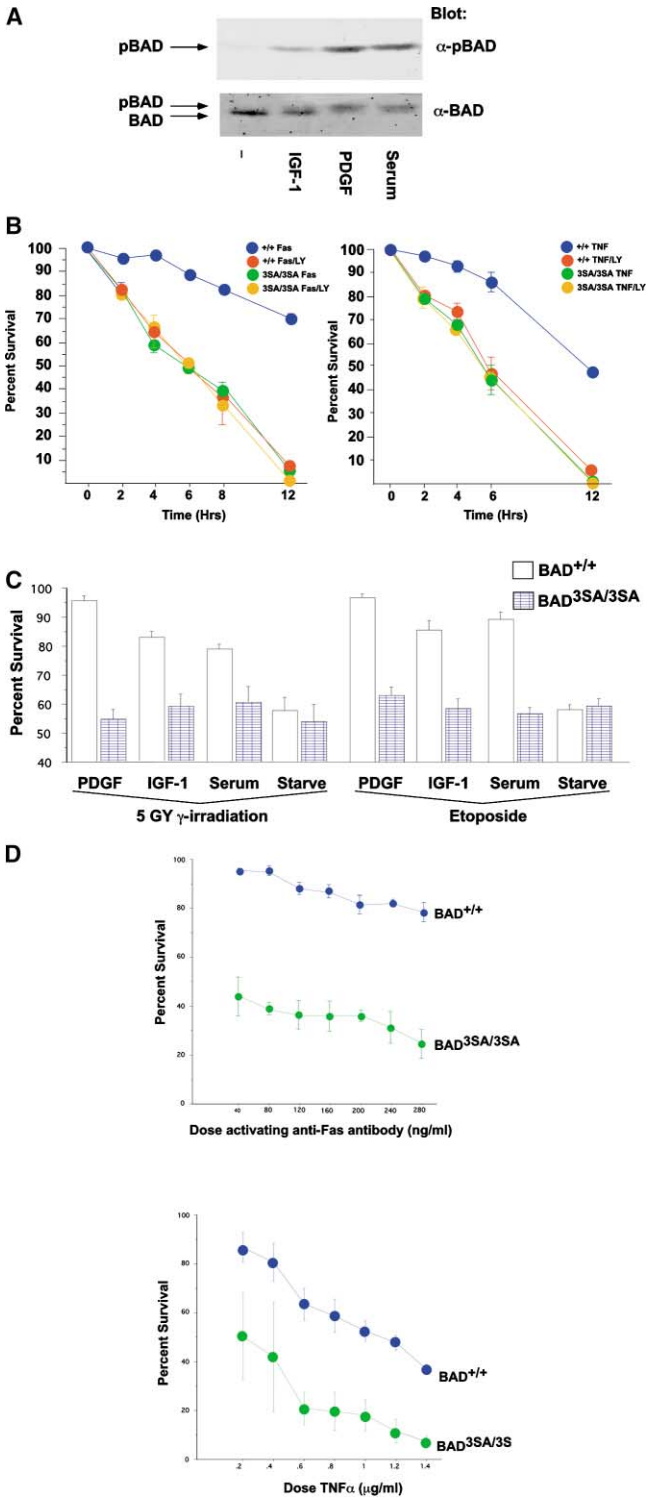

Figure 4. Survival Factors Require BAD Phosphorylation to Block Apoptosis Induced by Cell-Extrinsic and Cell-Intrinsic Death Pathways

(A) Western blots of wild-type MEF cell lysates assessing BAD phosphorylation levels. MEFs were plated, allowed to reach confluence, and deprived of survival factors. MEFs were then stimulated as indicated, lysed in RIPA buffer, and subjected to Western blot analysis after SDS-PAGE with either the anti-BAD c-20 antibody (α-BAD) or the anti-p112BAD antibody (α-pBAD) as indicated.

(B) Time course of death induced in primary MEFs cultured in serum after treatment with either 200 ng/ml Jo2 anti-Fas antibody or 1 ng/ml TNFα and 200 μg/ml cyclohexamide ($n = 3$). Some cells were also cotreated with 30 μM LY294002 as indicated. Note that treatment of cells with cyclohexamide alone did not induce cell death, and that similar results were obtained when cells were cultured in IGF-1 or PDGF (data not shown). Similar data have been obtained utilizing an independently isolated pair of MEF lines.

(C) MEFs were cultured in the media indicated and were treated with either 5 Gray γ irradiation or 400 μM etoposide for 24 hr ($n = 3$).

(D) Conditions in (D) are similar to (A) and (B), except that MEFs were treated with doses of anti-Fas antibodies or TNFα and 200 μg/ml CHX as indicated, and death was assessed at 8 hr posttreatment ($n = 3$).

factors (Figure 4B). These findings suggest that growth factors suppress the effectiveness of apoptotic signaling at least in part through the PI3K-dependent post-translational modification of proteins such as BAD. Taken together, these results demonstrate that a function of growth factor-mediated BAD phosphorylation is to promote the resistance of cells to proapoptotic stimuli.

BAD$^{3SA/3SA}$ Mice Show Increased Sensitivity to Apoptotic Stimuli

We next considered whether growth factor-mediated BAD phosphorylation also protects whole animals from death signals. We initially focused on the responses of *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ animals to systemic activation of death receptor signaling, which has been implicated in a wide variety of infectious and noninfectious pathologies,

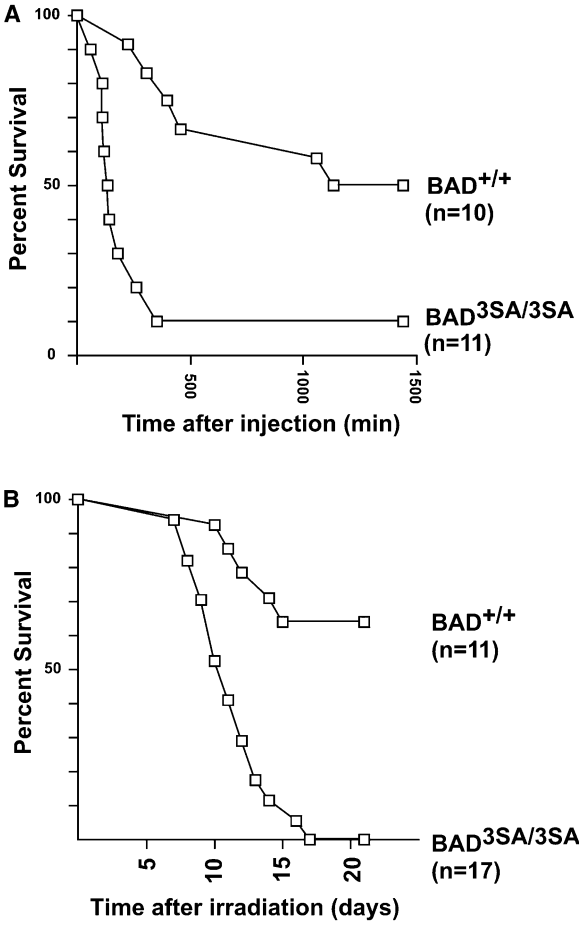

Figure 5. BAD Phosphorylation Is Required for Normal In Vivo Responses to Death Receptor Signaling and to γ Irradiation

(A) Five- to seven-week-old mice were injected into the retroorbital plexus with 0.2 μg/g anti-Fas antibody brought up to 50 μl total volume in PBS, and time of death was assessed after injection. Retroorbital injection with PBS alone did not induce any mortality. (B) Adult female mice were subjected to 8.5 Gy of γ irradiation, and mortality was assessed over the time period indicated. Similar results were obtained with male mice, although the kinetics of death were accelerated (data not shown).

including fulminant hepatitis, vascular leak syndrome, and encephalomyelitis (Licon Luna et al., 2002; Rafi et al., 1998; Yin et al., 1999). Injection of mice with anti-Fas antibodies that activate Fas signaling induces multiorgan damage and causes rapid death due to hepatocellular apoptosis (Yin et al., 1999). Western blot analysis revealed that BAD was both expressed and phosphorylated in liver hepatocytes, suggesting that BAD phosphorylation plays a role in normal tissue homeostasis (data not shown). To test the responses of *BAD*$^{3SA/3SA}$ mice to in vivo activation of Fas signaling, we first defined an intravenous dose of anti-Fas antibodies (0.2 μg/g) at which 50% of 5- to 7-week-old wild-type mice died within 24 hr. We then compared the kinetics and extent of animal death after anti-Fas antibody injection in the *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ mice. As shown in Figure 5A, *BAD*$^{3SA/3SA}$ mice were hypersensitive to injection with activating anti-Fas antibodies, as 90% of injected *BAD*$^{3SA/3SA}$ animals died, with an average time to death of 121 $\pm$ 54 min (n = 11). In contrast, only 54% of *BAD*$^{+/+}$ mice died after anti-Fas antibody injection, with an average time to death of 495 $\pm$ 23 min (Figure 5A; n = 10). These findings reveal that dephosphorylation of BAD sensitizes animals to the potentially lethal effects of systemic activation of the Fas receptor pathway, and suggest that BAD phosphorylation plays a role in the whole organism by attenuating the apoptotic effects of death receptor signaling.

We next asked whether BAD phosphorylation also regulates the response of animals to DNA damage, with which mammalian organisms must contend throughout the course of a normal lifespan. Exposure of animals to γ irradiation induces organismal death, and growth factors have been shown to block γ irradiation-induced death, although the mechanisms by which survival factors attenuate DNA damage-mediated apoptosis were not clear (Neta et al., 1993; Rosen et al., 1999). To determine whether BAD phosphorylation regulates organismal responses to genotoxic damage, we subjected both *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ mice to 8.5 Gray of whole-body γ irradiation, and assessed mouse survival over a 3-week period. As shown in Figure 5B, *BAD*$^{3SA/3SA}$ mice are hypersensitive to the effects of γ irradiation compared to *BAD*$^{+/+}$ mice. All of the *BAD*$^{3SA/3SA}$ mice (n = 17) subjected to γ irradiation died within the experimental period, as opposed to approximately 35% of control mice (n = 11). We conclude that the maintenance of BAD in a phosphorylated and inactivated state renders mice less sensitive to the potentially deleterious effects of DNA damage.

BAD Phosphorylation Alters the Threshold for Mitochondrial Cytochrome C Release

The observation that BAD phosphorylation attenuates organismal and cellular responses to death cues led us to investigate the mechanism by which dephosphorylation of BAD sensitizes cells to proapoptotic signaling. As the molecular basis for Fas receptor-dependent apoptosis is well characterized in many cell types (Li and Yuan, 1999), we investigated the mechanism by which BAD dephosphorylation sensitizes cells to the effects of Fas signaling. In both MEFs and mature thymocytes, ligation of the Fas receptor induces the nucleation of a receptor-proximal multiprotein complex that includes the death protease caspase-8. In thymocytes and other type I cells, cell death is induced by a caspase-8-dependent caspase cascade that does not require mitochondrial cytochrome c release (Scaffidi et al., 1998). In MEFs and other type II cells, cell death is a consequence of caspase-8-mediated cleavage of the BH3-only protein BID. BID cleavage generates active tBID, which translocates to the mitochondria, induces cytochrome c release, and causes apoptosis (Li et al., 1998; Luo et al., 1998). As shown in Figure 6A, cultured *BAD*$^{+/+}$ and *BAD*$^{3SA/3SA}$ thymocytes did not exhibit any differences in the level of death induced by treatment of these cells with anti-Fas antibodies. This result suggests that BAD dephosphorylation does not alter the activation of death receptors, or death receptor-induced caspase activity. Consistent with this interpretation, Western blot analysis demonstrated that death receptor activation induced

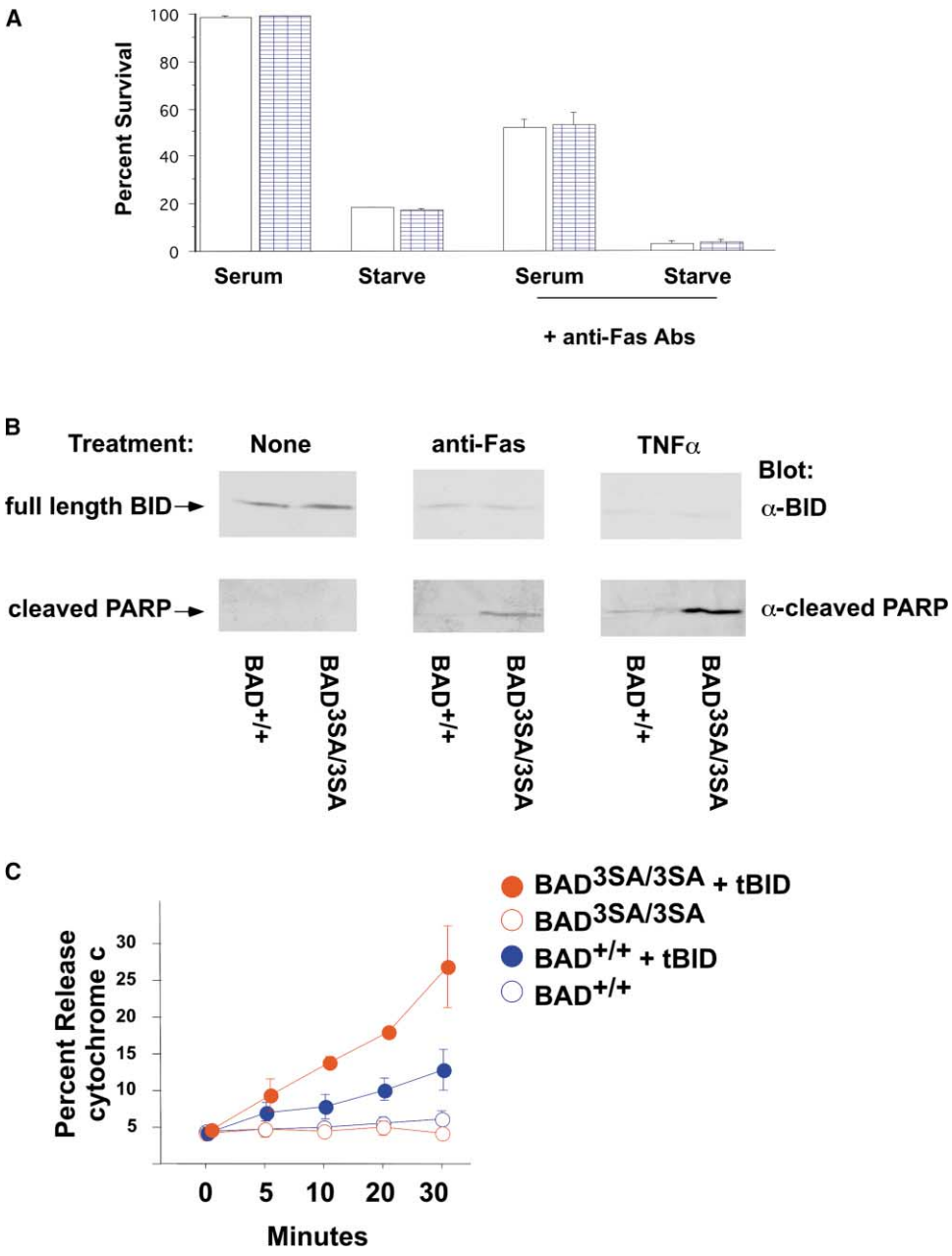

Figure 6. Survival Factor-Dependent BAD Phosphorylation Raises the Mitochondrial Threshold for Cytochrome C Release

(A) Thymocytes were isolated, cultured in the indicated media, treated with either no stimulus or 200 ng/ml anti-Fas antibody and 1 μg/ml cyclohexamide, and the levels of apoptosis assessed by trypan blue exclusion after 6 hr of culture (n = 3). Treatment of thymocytes with either 5 Gy of γ irradiation or etoposide induced increased cell death in *BAD*$^{3SA/3SA}$ thymocytes (hatched bars) relative to *BAD*$^{+/+}$ thymocytes (white bars) when cultured in serum (data not shown)

(B) Western blot of RIPA lysates of MEFs with either α-BID antibody (top panel), or α-cleaved PARP antibody (bottom panel) after the indicated treatment. In the top panel only, cells were preincubated with the caspase-9 inhibitor LEHD-CHO to prevent caspase-3-mediated activation of caspase-8. In both panels, protein levels were normalized before loading by BCA assay. Loss of immunoreactivity with the full-length BID antibody reflects BID cleavage.

(C) Cytochrome c release from isolated liver mitochondria. Mitochondria were isolated from liver by differential centrifugation, purified by Percoll gradient, and then incubated for the time indicated with either vehicle or 150 μM recombinant tBID (see Experimental Procedures).

the cleavage of BID in *BAD*$^{+/+}$ MEFs, but that BID was cleaved to an equal extent in *BAD*$^{3SA/3SA}$ MEFs in the absence or presence of death receptor activation (Figure 6B).

The finding that BAD dephosphorylation does not alter apoptotic responses to Fas signaling in type I cells suggests that BAD promotes apoptosis by acting at the mitochondria, consistent with previous data indicating that BAD interacts with antiapoptotic BCL-2 family members localized at the mitochondria. In addition,

DNA-damaging agents and death receptor signaling in type II cells induce apoptosis in a manner that is dependent upon an increase in the activity of proapoptotic BCL-2 family members that act at the mitochondria (Puthalakath and Strasser, 2002). These observations led us to hypothesize that dephosphorylated BAD might sensitize cells to apoptotic stimuli by lowering the mitochondrial threshold at which proapoptotic BCL-2 family members trigger cytochrome c release. To test this hypothesis, we assessed the level of tBID-induced cytochrome c release from isolated mitochondria from $BAD^{+/+}$ and $BAD^{3SA/3SA}$ livers. Purified $BAD^{3SA/3SA}$ mitochondria exhibited increased cytochrome c release relative to $BAD^{+/+}$ mitochondria (27.7 $\pm$ 5.6 percent release versus 13.4 $\pm$ 2.7 percent release at 30 min), indicating that BAD dephosphorylation sensitizes mitochondria to the activity of tBID, a downstream effector of Fas signaling (Figure 6C). As assessed by Western blot, $BAD^{3SA/3SA}$ MEFs also exhibited biochemical characteristics consistent with excessive release of cytochrome c after death receptor signaling, such as increased caspase-3-dependent cleavage of the poly-ADP-ribose polymerase relative to that observed in $BAD^{+/+}$ MEFs (Figure 6B). These findings indicate that a function of growth factor-dependent BAD phosphorylation in both animals and cultured cells is to raise the threshold at which mitochondria release cytochrome c in response to apoptotic stimuli.

Discussion

The findings in this manuscript define a role for growth factor-dependent BAD phosphorylation in increasing the resistance of cells to apoptotic stimuli. We find that growth factors are unable to block effectively apoptosis induced by either the cell-intrinsic or cell-extrinsic death pathways in $BAD^{3SA/3SA}$ cells, consistent with a role for endogenous BAD phosphorylation in mediating survival signals. This defect is also reflected at the organismal level, as $BAD^{3SA/3SA}$ animals are more susceptible than $BAD^{+/+}$ animals to the effects of Fas receptor ligation and ionizing radiation. Finally, growth factor-dependent BAD phosphorylation increases the ability of mitochondria to withstand proapoptotic signals, as mitochondria in which BAD is constitutively dephosphorylated release more cytochrome c in response to tBID than do wild-type mitochondria. As growth factors induce endogenous BAD phosphorylation in a large number of cell types, these results suggest that BAD phosphorylation plays a general role in raising the threshold at which mitochondria release cytochrome c and induce apoptosis in response to death signals. We conclude that BAD phosphorylation serves as a mechanism by which cells assess whether growth factors are present or absent during a proapoptotic stimulus, to allow cells to respond appropriately when faced with conflicting extracellular survival and death cues.

Several lines of evidence suggest that BAD dephosphorylation specifically lowers the mitochondrial threshold for cell death, but does not nonspecifically cause cells to be generally unhealthy. $BAD^{3SA/3SA}$ mice appeared as healthy as wild-type counterparts when housed in sterile conditions, $BAD^{3SA/3SA}$ mice survived to adulthood, and histological analysis of tissues in the $BAD^{3SA/3SA}$ mouse revealed little evidence of excessive cell death. $BAD^{3SA/3SA}$ MEFs did not exhibit defects in proliferation, and did not undergo spontaneous apoptosis at a higher rate than $BAD^{+/+}$ MEFs (Figure 4 and data not shown). The cell volume of $BAD^{3SA/3SA}$ thymocytes was not altered compared to wild-type cells, suggesting that the ability of $BAD^{3SA/3SA}$ cells to maintain cell size, an indirect measure of cellular health, is not impaired (data not shown). In addition, BAD dephosphorylation did not sensitize cells to apoptosis that occurs independently of mitochondrial dysfunction, as mature thymocytes cultured from $BAD^{3SA/3SA}$ mice did not exhibit enhanced sensitivity to death receptor signaling when compared to wild-type thymocytes. In contrast, BAD dephosphorylation sensitized MEFs and animals to apoptosis induced by death receptor signaling and DNA-damaging agents, which cause mitochondrial damage through the activation of proapoptotic BCL-2 family members (Figure 4). Taken together, these findings suggest that BAD phosphorylation and dephosphorylation specifically regulate the responses of cells to apoptotic stimuli that induce mitochondrial dysfunction.

The mechanism by which dephosphorylated BAD cooperates with other proapoptotic BCL-2 family members such as tBID to stimulate mitochondrial dysequilibrium and cytochrome c release is not yet clear. The ability of dephosphorylated BAD to sensitize mitochondria to the proapoptotic effects of tBID may reflect the ability of dephosphorylated BAD to lower the effective concentration of prosurvival BCL-2 family members such that less tBID is needed to induce BAK and BAX activation and cell death (Wei et al., 2001). If such a mechanism operates, one would predict that when prosurvival BCL-2 family members are less abundant, cells would be more sensitive to the effects of apoptotic stimuli. This prediction is supported by the finding that mice in which prosurvival BCL-2 family members have been deleted are hypersensitive to the effects of both cell-intrinsic and cell-extrinsic apoptotic signaling (Ma et al., 1995; Veis et al., 1993).

The low number of prolymphocytes observed in $BAD^{3SA/3SA}$ mice may be caused by the inactivation of BCL-2 and related proteins by dephosphorylated BAD. The loss of pro-T and pro-B cells in the $BAD^{3SA/3SA}$ mice may also be a consequence of the DNA rearrangements that occur during the pro-T and pro-B stages of lymphocyte development. Evidence suggests that physiological DNA rearrangements can induce the DNA damage response (Danska and Guidos, 1997). Prolymphocytes that are undergoing DNA rearrangement may therefore be particularly vulnerable to death when BAD is dephosphorylated, as active BAD sensitizes cells to the proapoptotic effects of the DNA damage response. Such a mechanism may explain the finding that $BAD^{3SA/3SA}$ mice exhibit a specific defect in prolymphocytes, rather than in most other cells, in which DNA rearrangements are believed not to occur.

Additional defects whose cellular basis is not yet understood were also noted in the $BAD^{3SA/3SA}$ mice, including low body weight, uterine atrophy, and ovarian dysfunction (data not shown). However, the largely normal development of $BAD^{3SA/3SA}$ mice was somewhat surprising and suggests that survival factors block cell death

during development through mechanisms in addition to BAD phosphorylation. One potential target that may mediate the ability of growth factors to block apoptosis is the proapoptotic transcription factor FOXO3a. While BAD dephosphorylation alone is not generally sufficient to induce cell death during development, our findings support a model in which BAD cooperates with FOXO3a to regulate developmental apoptosis. The mechanism that underlies this cooperativity has not yet been characterized, but the ability of FOXO3a to activate genes such as Fas ligand and BIM, which activate the cell-extrinsic and cell-intrinsic death pathways, suggests that death signals from FOXO3a and BAD are functionally integrated at the mitochondria. The synergy between BAD and FOXO3a also suggests that growth factors do not block cell death by the phosphorylation and inactivation of a single molecule in mammals, but rather through the coordinated inactivation of multiple apoptotic regulators that interact at the mitochondria to promote cell death.

Both FOXO3a and BAD have been proposed to be regulated by PI3K and downstream kinases such as Akt that have been implicated in promoting cell survival. There is evidence that in some cell types, Akt directly phosphorylates and inactivates BAD (reviewed in Datta et al., 1999). However, it remains to be determined whether Akt activity and BAD phosphorylation are functionally linked in vivo. *BAD*$^{3SA/3SA}$ mice phenocopy many of the defects observed in mice with homozygous mutations in the Akt1 gene, including hypersensitivity to Fas and TNFα signaling in MEFs, low body weight, increased apoptosis of thymocytes, and hypersensitivity to γ irradiation (Figures 4 and 5, and data not shown; Chen et al., 2001; Cho et al., 2001). Given the similarities between the *Akt1*$^{-/-}$ and the *BAD*$^{3SA/3SA}$ phenotypes, we speculate that under some circumstances, Akt1 may inhibit mitochondria-mediated death by inactivating BAD.

In addition to its proposed role in cell survival during development, the PI3K/Akt pathway has been implicated in tumorigenesis. Increased activity of the PI3K/Akt cascade has been implicated in the ability of tumors to tolerate genomic damage, and in the resistance of tumors to radio- or chemotherapy (Nicholson and Anderson, 2002). The findings we report here and the recent analysis of *BAD*$^{-/-}$ mice, which both exhibit aberrant responses to DNA damage and are cancer prone (A.M.R. and S.J.K., submitted), are consistent with a role for BAD phosphorylation in oncogenesis. Since BAD dephosphorylation renders cells sensitive to DNA-damaging agents but does not lead to the death of most cells under normal conditions, pharmacological agents that induce targeted BAD dephosphorylation have the potential to resensitize tumors to death induced by localized radio- or chemotherapy without causing excessive damage to normal tissues.

A large number of proapoptotic substrates for survival-promoting kinases have been proposed, but the physiological importance of these targets in mediating the effects of survival signals in vivo has been unclear. The identification of the biological function of growth factor-dependent inactivation of BAD fills a critical gap in our understanding of survival signaling by demonstrating the importance of the phosphorylation-mediated inactivation of a survival kinase target.

Experimental Procedures

Materials

All reagents unless otherwise noted were obtained from Sigma chemicals, and the various BAD, CREB, PAK, and FOXO3a antibodies were generated and utilized as described previously (Brunet et al., 1999; Datta et al., 1997, 2000; Shamah et al., 2001). The pAkt, p90^{RSK}, pp90^{RSK}, and cleaved PARP antibodies (CST), the BID, BIM, and BAK antibodies (R&D Systems), the β-galactosidase antibody (5′, 3′), the Akt and BCL-X$_L$ antibodies (UBI), CD8-FITC, CD4-PE, CD25-PE, CD3-FITC, CD4-FITC, CD19-biotin, B220-PE, and CD43-FITC (BD Pharmingen), and the RED670 secondary reagent (GIBCO) were all used according to manufacturers' protocols. IGF-1 (Roche) was used at 50 ng/ml, PDGF (UBI) was used at 20 ng/ml, and IL-7 (R&D Systems) was used at 10 ng/ml. Jo2 α-Fas antibodies (BD Pharmingen) were used at 200 ng/ml and TNFα (UBI) was used at 1 μg/ml unless otherwise noted.

Cloning and Targeting of BAD Genomic Locus

The BAD cDNA was used to probe a λ phage 129/SvJ mouse genomic DNA library, and a 12 kB XbaI fragment from a positive clone was subcloned into pBR322 to generate pBR322gBAD. Details of the isolation and characterization of this fragment will be published elsewhere (A.M.R. and S.J.K., submitted). Serine-to-alanine mutations of Ser-112, Ser-136, and Ser-155 with silent RFLPs (encoding an XbaI site linked to Ser-112A, an SmaI site linked to Ser-136A, and an EcoRI site linked to Ser-155A) were introduced into BAD exon 3 by QuickChange using pBR322gBAD as a template. A 900 bp SexA1/RsrII fragment containing mutations only at the three sites was swapped into a new pBR322gBAD construct. An FRT-PGK-NEO-FRT cassette was cloned into an NsiI site 1.4 kB distal to the BAD 3′ UTR, and a diphtheria toxin-negative selection cassette was introduced into the genomic BAD/pBR322 junction at the 5′ end of the BAD locus. This vector was electroporated into 129 J1 ES cells, and standard characterization, injection (into C57B6 blastocysts), and molecular biology techniques were used to identify homologous recombinants. The SexA1/RsrII fragment was reisolated from mice positive for the marker RFLPs, cloned into a TOPO vector, and sequenced to verify that *BAD*$^{3SA/+}$ mice indeed carried the appropriate mutations.

Cell Culture and Biochemistry

Thymocytes were isolated by mechanical dissociation, and dead cells were removed with the MACS dead cell removal kit (Miltenyi Biotechnology) and cultured in RPMI 1640-supplemented penicillin/streptomycin, glutamine, 10 mM HEPES (pH 7.2), and 50 μM β-mercaptoethanol. When thymocytes were cultured in full media, this mixture was supplemented with 10% FBS (Hyclone). MEFs were isolated from E13.5 embryos from heterozygote crosses, and subjected to posthoc genotyping as described previously (Brown et al., 1998). Low-passage number MEFs (passages 2–4) were cultured in DMEM supplemented with 15% FBS, nonessential amino acids, and penicillin/streptomycin. In all experiments, MEFs were allowed to reach confluence, and in experiments comparing starvation conditions to those with serum or growth factors, cells were deprived of serum for 36 hr before stimulation. MEF results were verified using two independently isolated pairs of both wild-type and mutant MEF lines. All death receptor experiments were carried out in the presence of 200 ng/ml cyclohexamide. MEFs were lysed in RIPA buffer (10 mM Tris [pH 7.2], 150 mM NaCl, 1% NaDOC, 1% TX-100, 0.1% SDS, 1 mM sodium vanadate), and normalized for protein content by BCA assay (Pierce). In indicated experiments, cells were pretreated with 50 μM LEHD-CHO (Calbiochem) for 2 hr before stimulation with death receptor agonists and lysis. Cerebellar granule cell culture and transfection were performed as previously described (Datta et al., 1997). Apoptosis in thymocytes was determined by trypan blue inclusion, and apoptosis in MEFs and cerebellar granule neurons was detected by nuclear morphology after Hoechst 33342 staining. Pre-B colonies were cultured in Methocult pre-B methylcellulose media (Stem Cell Technologies) according to the manufacturer's protocol.

Cytochrome C Release Assay

Mitochondrial isolation and the cytochrome c release assay were performed using the Quantikine cytochrome c assay kit (R&D Systems) according to the manufacturer's protocol.

Acknowledgments

This work was supported by National Institutes of Health PO1 grant HD 24926 (M.E.G.) and the Mental Retardation Research Center grant NIHP30-HD18655. S.J.K. was supported in part by NIH award R37CA50239. S.R.D. was supported by a Medical Scientist Training Program grant, and M.Z.L. was supported by a National Defense Science and Engineering Graduate fellowship. M.E.G. acknowledges the generous support of the F.M. Kirby Foundation to the Division of Neuroscience. A.M.R. is the Edmond J. Safra Irvington Institute Fellow. The authors would like to thank the gene targeting facility of the Children's Hospital MRRC, including Tam Thompson, Yiping Zhao, and Hong Ye for their assistance with generation of the BAD knockin mice. We thank Susan Dymecki and Frank Farley for generously providing FlpE transgenic mice, and Emer Clarke for assistance with the pre-B colony assay. The authors would also like to thank Linda Hu for assistance with antibodies, and Alex Katsov, Pei Ho, Anne Brunet, Janine Zieg, and members of the Greenberg laboratory for support and critical reading of the manuscript.

Received: July 8, 2002
Revised: October 11, 2002

References

Bonni, A., Brunet, A., West, A.E., Datta, S.R., Takasu, M.A., and Greenberg, M.E. (1999). Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science *286*, 1358–1362.

Brown, J.R., Nigh, E., Lee, R.J., Ye, H., Thompson, M.A., Saudou, F., Pestell, R.G., and Greenberg, M.E. (1998). Fos family members induce cell cycle entry by activating cyclin D1. Mol. Cell. Biol. *18*, 5609–5619.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Brunet, A., Datta, S.R., and Greenberg, M.E. (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr. Opin. Neurobiol. *11*, 297–305.

Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., et al. (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. *15*, 2203–2208.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell *8*, 705–711.

Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F., and Birnbaum, M.J. (2001). Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J. Biol. Chem. *276*, 38349–38352.

Danska, J.S., and Guidos, C.J. (1997). Essential and perilous: V(D)J recombination and DNA damage checkpoints in lymphocyte precursors. Semin. Immunol. *9*, 199–206.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231–241.

Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. Genes Dev. *13*, 2905–2927.

Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B., and Greenberg, M.E. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol. Cell *6*, 41–51.

D'Mello, S.R., Galli, C., Ciotti, T., and Calissano, P. (1993). Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc. Natl. Acad. Sci. USA *90*, 10989–10993.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., and Greenberg, M.E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science *275*, 661–665.

Farley, F.W., Soriano, P., Steffen, L.S., and Dymecki, S.M. (2000). Widespread recombinase expression using FLPeR (flipper) mice. Genesis *28*, 106–110.

Gleichmann, M., Weller, M., and Schulz, J.B. (2000). Insulin-like growth factor-1-mediated protection from neuronal apoptosis is linked to phosphorylation of the pro-apoptotic protein BAD but not to inhibition of cytochrome c translocation in rat cerebellar neurons. Neurosci. Lett. *282*, 69–72.

Harada, H., Andersen, J.S., Mann, M., Terada, N., and Korsmeyer, S.J. (2001). p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc. Natl. Acad. Sci. USA *98*, 9666–9670.

Li, H., and Yuan, J. (1999). Deciphering the pathways of life and death. Curr. Opin. Cell Biol. *11*, 261–266.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491–501.

Licon Luna, R.M., Lee, E., Mullbacher, A., Blanden, R.V., Langman, R., and Lobigs, M. (2002). Lack of both Fas ligand and perforin protects from flavivirus-mediated encephalitis in mice. J. Virol. *76*, 3202–3211.

Lizcano, J.M., Morrice, N., and Cohen, P. (2000). Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. Biochem. J. *349*, 547–557.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell *94*, 481–490.

Ma, A., Pena, J.C., Chang, B., Margosian, E., Davidson, L., Alt, F.W., and Thompson, C.B. (1995). Bclx regulates the survival of double-positive thymocytes. Proc. Natl. Acad. Sci. USA *92*, 4763–4767.

Neta, R., Williams, D., Selzer, F., and Abrams, J. (1993). Inhibition of c-kit ligand/steel factor by antibodies reduces survival of lethally irradiated mice. Blood *81*, 324–327.

Nicholson, K.M., and Anderson, N.G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. Cell. Signal. *14*, 381–395.

Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C.B., et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J. Exp. Med. *180*, 1955–1960.

Plas, D.R., Rathmell, J.C., and Thompson, C.B. (2002). Homeostatic control of lymphocyte survival: potential origins and implications. Nat. Immunol. *3*, 515–521.

Puthalakath, H., and Strasser, A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. *9*, 505–512.

Raff, M.C. (1992). Social controls on cell survival and cell death. Nature *356*, 397–400.

Rafi, A.Q., Zeytun, A., Bradley, M.J., Sponenberg, D.P., Grayson, R.L., Nagarkatti, M., and Nagarkatti, P.S. (1998). Evidence for the involvement of Fas ligand and perforin in the induction of vascular leak syndrome. J. Immunol. *161*, 3077–3086.

Rosen, E.M., Fan, S., Rockwell, S., and Goldberg, I.D. (1999). The molecular and cellular basis of radiosensitivity: implications for understanding how normal tissues and tumors respond to therapeutic radiation. Cancer Invest. *17*, 56–72.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J. *17*, 1675–1687.

Schurmann, A., Mooney, A.F., Sanders, L.C., Sells, M.A., Wang,

H.G., Reed, J.C., and Bokoch, G.M. (2000). p21-activated kinase 1 phosphorylates the death agonist Bad and protects cells from apoptosis. Mol. Cell. Biol. *20*, 453–461.

Segal, R.A., and Greenberg, M.E. (1996). Intracellular signaling pathways activated by neurotrophic factors. Annu. Rev. Neurosci. *19*, 463–489.

Shamah, S.M., Lin, M.Z., Goldberg, J.L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R.L., Corfas, G., Debant, A., and Greenberg, M.E. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. Cell *105*, 233–244.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Shortman, K., and Wu, L. (1996). Early T lymphocyte progenitors. Annu. Rev. Immunol. *14*, 29–47.

Talapatra, S., and Thompson, C.B. (2001). Growth factor signaling in cell survival: implications for cancer treatment. J. Pharmacol. Exp. Ther. *298*, 873–878.

Tan, Y., Ruan, H., Demeter, M.R., and Comb, M.J. (1999). p90(RSK) blocks Bad-mediated cell death via a protein kinase C-dependent pathway. J. Biol. Chem. *274*, 34859–34867.

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229–240.

Venters, H.D., Dantzer, R., and Kelley, K.W. (2000). A new concept in neurodegeneration: TNFα is a silencer of survival signals. Trends Neurosci. *23*, 175–180.

Virdee, K., Parone, P.A., and Tolkovsky, A.M. (2000). Phosphorylation of the pro-apoptotic protein BAD on serine 155, a novel site, contributes to cell survival. Curr. Biol. *10*, R883.

von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J. Exp. Med. *181*, 1519–1526.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727–730.

Yin, X.M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K.A., and Korsmeyer, S.J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature *400*, 886–891.

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell *87*, 619–628.

Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R., and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev. *15*, 1481–1486.