BAD is a pro-survival factor prior to activation of its pro-apoptotic function

So Young Seo\textsuperscript{1}, Ying-bei Chen\textsuperscript{2}, Iva Ivanovska\textsuperscript{2}, Ann M. Ranger\textsuperscript{4}, Suk J. Hong\textsuperscript{3}, Valina L. Dawson\textsuperscript{3}, Stanley J. Korsmeyer\textsuperscript{4}, David S. Bellows\textsuperscript{2}, Yihru Fannjiang\textsuperscript{2} and J. Marie Hardwick\textsuperscript{1,2,3,}\* 

\textsuperscript{1}W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA  
\textsuperscript{2}Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, USA  
\textsuperscript{3}Department of Neurology, Johns Hopkins School of Public Health, Baltimore, Maryland 21205, USA  
\textsuperscript{4}Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 USA  

Running title: Anti-death function of BAD

*Corresponding author:  
J. Marie Hardwick  
Johns Hopkins, E5140 BSPH  
615 N Wolfe St.  
Baltimore, Maryland 21205 USA  
Phone 410-955-2716  
Office 410-614-3110  
Fax 410-955-0105  
hardwick@jhu.edu
Abstract
The mammalian BAD protein belongs to the BH3-only subgroup of the BCL-2 family. In contrast to its known pro-apoptotic function, we found that endogenous and overexpressed BADL can inhibit cell death in neurons and other cell types. Several mechanisms regulate the conversion of BAD from an anti-death to a pro-death factor, including alternative splicing that produces the N-terminally truncated BADS. In addition, caspases convert BADL into a pro-death fragment that resembles the short splice variant. The caspase site that is cleaved during cell death following growth factor (IL-3) withdrawal is conserved between human and murine BAD. A second cleavage site that is required for murine BAD to promote death following Sindbis virus infection, gamma irradiation and staurosporine treatment is not conserved in human BAD, consistent with the inability of human BAD to promote death with these stimuli. However, loss of the BAD N-terminus by any mechanism is not always sufficient to activate its pro-death activity, indicating that the N-terminus is a regulatory domain rather than an anti-death domain. These findings suggest that BAD is more than an inert death factor in healthy cells, but is also a pro-survival factor prior to its role in promoting cell death.

Keywords: apoptosis/BAD/BCL-2/caspase/neuron/phosphorylation
Introduction
Healthy mammalian cells constitutively express pro-apoptotic BCL-2 family members such as the BH3-only protein BAD and the multi-domain proteins BAX and BAK. These proteins are generally regarded as latent death factors that are normally held in check and must be activated to exhibit their death-inducing functions. However, it is possible that pro-death BCL-2 family proteins are not only latent death factors, but also carry out important functions in healthy cells. Consistent with this idea, BAX and BAK can function as potent anti-death factors in cultured neurons and in mouse models (1-4). This dual function of BAK may be explained by its ability to decrease neuronal excitability in healthy neurons, as this function is consistent with both its ability to promote death during development (based on the phenotype of double Bax/Bak knockout mice) and its ability to inhibit death induced by excitotoxic stimuli in postnatal animals (4,5). Consistent with this dual role for BAX and BAK, the anti-apoptotic family members human BCL-2, BCL-xL, and C. elegans CED-9 can function as pro-death factors (6-8). Several mechanisms appear to contribute to activation of Bcl-2 family proteins to exhibit their pro-death function. BAX undergoes conformational changes, relocates to mitochondria, may oligomerize with other BAX molecules in the mitochondrial membrane, and can be cleaved by calpain to enhance its pro-death activity (9-12). Cleavage of BCL-2 and BCL-xL by caspases produces C-terminal fragments that behave similar to pro-apoptotic BAX in that they localize to mitochondria, induce cytochrome c release and can form pores in synthetic membranes that are sufficient in size to pass cytochrome c and larger molecules (7,13,14). However, non-proteolytic events can also explain the pro-death effects of BCL-2 (15).

Several mechanisms have been proposed to explain how BAD and other BH3-only proteins facilitate cell death by inhibiting anti-apoptotic BCL-2 proteins, activating pro-death BAX and BAK, or otherwise regulating mitochondrial structure (16-18). BAD plays an important role in connecting the growth factor signaling pathway with the cell death pathway. Recent genetic evidence confirm that when a variety of cell types are deprived of their extracellular survival signals, BAD is dephosphorylated to assume its pro-death function (19,20). Though it is logical that the pro-death function of BAD must be inactivated or suppressed in healthy cells, this does not preclude the possibility that BAD and other pro-death BCL-2 family proteins have anti-death activity or alternative biochemical functions that benefit healthy cells.
prior to a death stimulus. Analogously, cytochrome c has a key function in the mitochondrial respiratory chain of healthy cells but promotes caspase activation in the cytosol following a death stimulus (21).

BAD-deficient mice are developmentally normal by most criteria, including normal B and T cell development (20). However, isolated thymocytes, fibroblasts and epithelial cells from BAD-deficient mice were found to be variably resistant to cell death induced by growth factor withdrawal, consistent with a pro-death function of BAD under these conditions (20). Furthermore, BAD-deficient mice develop B cell lymphomas and other malignancies, and this propensity is dramatically increased by exposure to limited gamma irradiation (20). Again, this tumor-suppressor phenotype is consistent with a pro-death function for BAD. But seemingly inconsistent with a pro-death function, BAD was reported to promote glycolysis, a pathway induced by the same growth factors that “inactivate” the pro-death function of BAD (22). That is, BAD-deficient mice and derived hepatocytes have a reduced capacity to utilize glucose, apparently due to the requirement for BAD to assemble active glucokinase (liver hexokinase) complexes (22). Thus, it is paradoxical that a pro-death factor facilitates utilization of glucose in the first step of the glycolytic pathway. This paradox could be resolved if prior to its conversion into a pro-death factor, BAD has a normal cellular function that promotes maintenance/survival of the cell. However, BAD and other BH3-only proteins have not previously been found to play a physiological role in promoting cell survival.

Here we show that BAD can function as a potent inhibitor of cell death and is converted into a pro-death factor by multiple mechanisms including splicing, dephosphorylation and caspase cleavage at death stimulus-specific sites. The N-terminus of BAD is important but not essential for anti-death function in all paradigms, suggesting that the N-terminus is a guard against activation of pro-death function rather than a direct anti-death domain. These findings are consistent with the possibility that other “pro-death” Bcl-2 family proteins may have normal cell functions that promote survival.

Materials and Methods

Plasmids and transfections. Murine BADL (Stratagene) and human BADs (Science Reagents) cDNAs were inserted into pSG5 (Stratagene) or derivative pDB59 (containing an N-terminal HA
tag) for expression in mammalian cells or *in vitro* transcription/translation. Point mutants of BAD were generated by two-step PCR and those clones with a perfect sequence were selected for further study. Cell lines were transfected with Fugene 6 (Roche). Primary cortical neurons were transfected with calcium phosphate as previously described (23,24). Briefly, the media of cortical neurons were replaced with DMEM and saved. DNA/calcium phosphate precipitate was prepared by mixing 1 ug GFP plasmid and 5 ug of the expression vector for wt or D61A mBAD in 250 mM CaCl₂ with 2x HBS (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 15 mM D-glucose, 42 mM Hepes, pH 7.05). After 25-30 min, 120 ul of the DNA/calcium phosphate precipitate were added drop-wise to each 60-mm-diameter dish and incubated in 5% CO₂ incubator. Twenty-five min after the first precipitate formed, cells were washed three times with DMEM and returned to saved medium.

**Viruses and animals.** Coding sequences for wild type and mutant BAD were subcloned from pSG5/pDB59 into the Sindbis virus vectors dsTE12Q (for infection of newborn mice) and dsNSV (for infection of 5-week old mice) (3,25), or into the retrovirus construct pLXSN (26) for generation of recombinant viruses. Control Sindbis viruses contain the reverse (non-coding) orientations of mBAD₁, viral KSBcl-2 or other irrelevant cDNA of similar size to ensure equivalent replication rates between viruses by maintaining the same genome size in experimental and control viruses. The data obtained with various control viruses were indistinguishable (thereby eliminating concerns about anti-sense effects of mBAD). Data presented here were generated with Sindbis viruses prepared from at least two independently derived constructs. Mice were inoculated by intracranial injection with 5000 plaque forming units (pfu) of Sindbis virus or buffer diluent alone. Cultured cell lines and dissociated rat/mouse primary cortical neurons were infected with 5 pfu per cell. Organotypic cultures were infected with 5x10⁶ pfu per tissue slice. BAD knockout mice and genotyping strategy were described previously (20). Upon receiving the BAD knockout, it was backcrossed once to a C57BL/6 mouse and the colony was interbreed over a 2-year period. All experiments were performed on littermates from heterozygous crosses and subsequently correlated to the genotype.
Cell lines, viability assays and immunoblots. COS-1 (African green monkey kidney), BHK (baby hamster kidney) and HEK 293 (human embryonic kidney) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 ug/ml streptomycin. Ba/F3 (murine hematopoietic) were grown in RPMI 1640 supplemented with IL-3 (26). Stable BHK cell lines were established by cotransfection with a neomycin-resistance plasmid (at a 1:10 ratio with BAD-expressing plasmids) and selected in 0.5 ug/ml G418. Individual clones were separated, maintained in G418 and analyzed only at low passage numbers. Cell lysates were harvested in RIPA buffer containing protease inhibitors (aprotinin, benzamidine, chymostatin, leupeptin, pepstatinA and phenylmethyl sulfonyl fluoride) and separated by SDS-PAGE for immunoblot analysis. Viability of various cell lines was determined by trypan blue dye exclusion, by propidium iodide staining and flow cytometry or fluorescence microscopy, or by co-transfection/[β]-GAL assay as described previously (27). IL-3 withdrawal-induced cell death was performed as previously described (7).

Primary neurons. Dissociated cultures of (postmitotic) cortical neurons were prepared from E15-16 Sprague-Dawley rats or E13-14 mice as previously described (23) and plated in 24 well dishes (1x10^5 per well) with 5-fluoro-2-deoxyuridine from days 3-6 after plating to inhibit expansion of non-neuronal cells that constitute <10% of the cultures (data not shown). Death of infected cortical neurons was assessed by determining the percent of neurons that were stained with propidium iodide (PI) relative to the total number of Hoechst stained nuclei. Randomly selected fields were imaged and printed for counting. NMDA and staurosporine treatments were performed (4). Transfected cortical neurons were scored for viability /death based on morphology of GFP positive cells. Those cells with no processes or with two or more fragmented processes were scored as non-viable. Hippocampal and spinal cord tissues were dissected from newborn mice and four 200 um slices were cultured on one Millipore membrane per well with Basal Medium Eagle (BME, GIBCO/BRL) plus 25% horse serum in 6 well dishes (28). Viability of organotypic hippocampal or spinal cord cultures was assessed by incubating cultures for 16 hours in 0.1 mg/ml PI. Relative PI intensity was determined by computer-based image analysis (4).
In vitro caspase cleavage. $^{35}$S-labeled, in vitro transcribed/translated proteins were digested with recombinant purified caspase-3 (2,000 U), or caspase-7 (750 U) as previously described (29).

Results

BAD switches from anti-death to pro-death function during neuron maturation. Sindbis virus can induce classic morphological and biochemical characteristics of apoptotic cell death in a variety of cell lines and in neurons (30,31). The use of Sindbis virus as a vector to express both anti- and pro-death BCL-2 family proteins in cell cultures and in animals has faithfully revealed the endogenous function of these proteins (1,4,32). Therefore, the function of BAD was quantified by inserting a cDNA encoding the widely studied long form of murine BAD into the Sindbis virus genome and measuring the ability of mBAD$_L$ to modulate the outcome of a Sindbis virus infection. Infection of COS-1 cells with recombinant Sindbis virus encoding murine BAD$_L$ (SV-mBAD$_L$) confirmed the expected pro-death function of transiently overexpressed mBAD$_L$. That is, BAD accelerated Sindbis virus-induced cell death compared to control viruses (SV-control) (Fig. 1A and 4B). In contrast, BCL-xL inhibited Sindbis virus-induced cell death as previously reported (33).

Because Sindbis virus is neuronotropic, the same recombinant viruses were used to determine the function of overexpressed BAD in neurons of organotypic hippocampal slice cultures prepared from 3 day-old mice (4,34). Contrary to expectation, mBAD$_L$ significantly reduced Sindbis virus-induced neuronal death compared to control virus-infected cultures, suggesting that mBAD$_L$ protects hippocampal neurons from virus-induced death (Fig. 1B and 7B). In contrast, another pro-death BH3-only family member, BIM$_S$ was a potent pro-death factor in this model (Fig. 1B). This finding with BIM strongly argues against the possibility that the anti-death function of BAD results from the indirect effects of its pro-death function.

To determine if endogenous BAD is an anti-death factor in neurons, dissociated embryonic cortical neurons were prepared from bad-deficient mice and infected with Sindbis virus as a death stimulus. Consistent with the overexpression model, more cell death occurred in bad$^{-/-}$ cortical neurons and spinal cord slices compared to cultures prepared from bad$^{+/+}$ or
bad+/− control littermates, indicating that endogenous BAD is protective in these immature neurons (Fig. 1C). Spinal cord slice cultures prepared from newborn bad+/+ and bad−/− mice yielded similar results when exposed to Sindbis virus or NMDA to induce an excitotoxic death. Thus, endogenous BAD also protects neurons against a non-viral death stimulus. Confidence in these findings is enhanced because they were generated in primary cells derived from several different animals per genotype. Genotypes of cultures and mice were confirmed by immunoblot analysis (Fig. 1D).

The tropism of Sindbis virus for central nervous system neurons in mice results in neuronal death that strongly correlates with mouse mortality (4,31). Furthermore, endogenous and overexpressed BCL-2 (via the Sindbis virus vector) protects newborn mice from neuronal apoptosis (32,34). Therefore, BAD was evaluated by a similar approach. Infection of newborn bad−-deficient mice with Sindbis virus (SV-control) caused increased mouse mortality compared to control bad+/+ and bad−/- littermates, consistent with a protective function of BAD (data not shown). However, the interpretation of this result is complicated by the fact that these mice are defective for production of antigen-stimulated antibody (20), which is critical for clearing Sindbis virus from infected mouse brains (35). Therefore, to confirm that BAD protects in animals, 3-day-old wild type mice were infected with recombinant Sindbis virus expressing mBADL. These mice had increased survival compared to those infected with control virus, consistent with the protective function of mBADL (Fig. 1E). In contrast, the pro-death BH3-only protein BIMs significantly accelerated mouse mortality, arguing that the protective effect is specific to mBADL (Fig. 1E).

In contrast to newborn mice, mBADL failed to significantly alter the rate or extent of mortality in mature (5-week old) mice (Fig. 1E). To confirm this age-dependent shift in BAD function using an alternate model, BAD was evaluated in dissociated embryonic rat cortical neurons that were allowed to mature in culture 2-14 days prior to infection. Overexpression of mBADL (SV-mBADL) protected 2 day-old cultures, similar to the function of endogenous BAD (compare Fig. 1F and 1C). Unlike BAD, BAX was modestly pro-death in these cells as expected. In contrast to the younger cultures, mBADL exhibited consistent pro-death activity in 8-14 day-old cultures (Fig. 1F). Viability of cortical neurons was determined by dual staining with Hoechst to detect nuclei and propidium iodide (PI) to detect dead cells as shown for
representative fields (Fig. 1G). These findings indicate that BAD is converted from a pro-

survival to a pro-death or neutral factor as postmitotic cortical neurons mature in culture and \textit{in vivo} (Fig. 1H).

\textbf{Caspase cleavage of mBAD promotes age-dependent neuronal death.} To pursue the mechanisms responsible for converting BAD into a pro-death factor as neurons mature, a role for proteolysis was examined based on the observation that other anti-apoptotic BCL-2 family members can be converted into killer proteins when cleaved by caspases (7). In addition, the pro-
death function of murine BAD was reported to be enhanced when BAD is cleaved by caspases at Asp56 and Asp61 (36), positions roughly analogous to the caspase cleavage sites in BCL-2, BCL-xL and the BH3-only protein BID. Using \textit{in vitro} translated $^{35}$S-labeled mBAD, we failed to detect cleavage at Asp56, as the only cleavage site detected with recombinant purified caspase-3 and caspase-7 was at Asp61 (Fig. 2A). Mutation of the only other potential caspase cleavage site in this region, Asp71, also did not appear to alter cleavage patterns (Fig. 2B).

To determine if caspase cleavage contributes to the pro-death function of BAD in mature neurons, 14-day rat cortical cultures and 5 week-old mice were infected with Sindbis virus expressing a point mutant of mBAD in which the aspartate 61 cleavage site was changed to alanine (D61A). Compared to wild type BAD and controls, this mutant significantly inhibited death of mature cortical neurons (Fig. 2C), reduced mortality in mature mice (Fig. 2D) and decreased the extent of hind limb paralysis (reflecting spinal cord motor neuron function, data not shown). Thus, the neutral/pro-death function of wild type mBAD in mature neurons was converted to anti-death activity by a point mutation in the Asp61 cleavage site. Uncleavable BAD also protected against a non-viral death stimulus, as cortical neurons transfected with mBAD(D61A) were protected from cell death induced by staurosporine (Fig. 2E). These results suggest that caspase cleavage of BAD at position 61 is required to activate its pro-death function in these models (Fig. 2F).

\textbf{Caspases convert mBAD from an anti- to a pro-death factor in cell lines.} To determine if caspase cleavage serves to regulate mBAD function in non-neuronal cells, stable CCL10 BHK cell lines were generated that overexpress wild type mBAD or uncleavable
mutants in which Asp56, Asp61 and Asp71 were changed to Ala (Fig. 3A). Consistent with the established pro-death function of BAD, cells expressing wild type mBADL were more susceptible to cell death induced by gamma-irradiation in a dose-dependent manner compared to control cells stably transfected with the Neo vector alone. However, double or triple point mutations in potential caspase cleavage sites caused mBADL to exhibit a gain of anti-death activity (Fig. 3B). Similar results were obtained with the same cell lines when cell death was induced by Sindbis virus infection or staurosporine (STR) treatment (data not shown). Mutation of the caspase cleavage site impaired cleavage of BAD in these cells, as immunoblot analysis of staurosporine-treated cells revealed the predicted 21kD cleavage product of wild type mBADL while the full-length D61A/71A mutant was stabilized and cleavage-resistant (Fig. 3C). Another series of stably transfected cell clones were generated in the related BHK-21 cell line, this time including the single point mutant D61A (Fig. 3D). Single, double or triple cleavage site mutants of mBADL significantly inhibited cell death induced by virus infection (Fig. 3E) and staurosporine treatment (Fig. 3F). Like cortical neurons, the Asp61 site appears to be primarily responsible for activating the pro-death function of mBADL, consistent with the cleavage site specificity of recombinant caspases in vitro.

Results obtained with these stable cell lines could be potentially misleading because only those cells that are resistant to the pro-death functions of overexpressed BAD will survive the selection process to produce clonal cell lines. Although this is unlikely to explain the gain of anti-death function of uncleavable mBADL, we returned to transient assays using either transfected plasmids or Sindbis virus vectors. Consistent with the observations of many labs, transfection of mBADL into HEK293 cells induced cell death in the absence of an additional death stimulus, albeit less efficiently than BAX (Fig. 4A). Also consistent with the findings of others (36,37), the pro-death function of BAD increased when the N-terminus (amino acids 2-61) was deleted to mimic caspase-cleaved mBADL (ΔN61). However, pro-death function of mBADL was completely dependent on the Asp61 cleavage site because the D61A and triple D56/61/71A mutants had no killing activity, and even reduced the slight toxic effects of transfection compared to vector control (Fig. 4A). Similar results were obtained in transiently transfected COS-1 cells (data not shown). To verify that uncleavable BAD inhibits death in cell lines, an additional death stimulus was applied. Transiently overexpressed caspase-resistant BAD (via the
Sindbis virus vector) protected COS-1 cells (Fig. 4B) and BHK cells (data not shown) from Sindbis virus-induced death as efficiently as human BCL-xL. The generally enhanced cell death by truncated △N61 over wild type mBADL in the same assays (Fig. 4A, B) supports the hypothesis that mutation of Asp61 serves to inhibit proteolytic cleavage of BAD rather than ablating some other intrinsic pro-death function of BAD. Immunoblot analysis of infected COS-1 and BHK cells confirmed that wild type mBADL was cleaved in apoptotic cells to produce a fragment that comigrates with an engineered △N61BAD, while uncleavable D61/71A mBADL remained stable (Fig. 4C and data not shown).

**Splicing regulates activation of hBAD pro-death activity.** There are two prominent forms of murine BAD proteins expressed in cell lines and tissues, the widely studied mBADL and the shorter mBADs that is produced by alternative splicing and lacks exon 1-encoded N-terminal sequence (20) (Fig. 5A). In contrast, only one form of human BAD, corresponding to mBADs, has been identified, and current annotation of the human genome indicates only three exons that correspond to exons 2-4 in the mouse genome. Another striking difference between mouse and human BAD is that human BAD lacks the critical Asp61 caspase cleavage site of mBADL. This aspartate is conserved between mouse and other mammalian BAD proteins, but human BAD has a Glu at this position. Although caspases from other species are known to cleave following Glu (e.g. *C. elegans* CED-3) (38), the absence of Asp61 in hBAD casts doubt on the significance of this cleavage event at least in humans (Fig. 5B).

To determine if hBADs is susceptible to caspase cleavage at Asp14 and Asp29 (corresponding to Asp56 and Asp71 of mBADL), *in vitro* translated 35S-labeled human BADs was incubated with purified caspases. In contrast to murine BAD, recombinant caspase-3 cleaved hBADs at both Asp14 and Asp29, because both sites must be mutated to block *in vitro* cleavage of human BADs (Fig. 5C). Unlike murine BAD, no caspase-7 cleavage products of human BADs were observed (lane 3). To determine if caspases also regulate the function of hBADs, stable CCL10-BHK cell lines were generated. However, hBADs was found to be toxic based on the observation that no cell clones expressing hBADs survived selection in several experiments where numerous cell colonies were produced with transfected uncleavable hBADs mutant or control vector. Nevertheless, cells expressing uncleavable human BADs (D14/29A) were not
protected from gamma irradiation, but instead had 10-30% increased cell death (Fig. 5D). Thus, while caspase cleavage enhanced the pro-death activity of hBAD\textsubscript{s}, cleavage of this short form of hBAD was not required for pro-death function. In this way, the shorter splice variant of human BAD resembles the caspase cleavage product of mBAD\textsubscript{L} (Fig. 5B).

These observations suggested that humans have only the pro-death version of BAD and lack the longer anti-death form of BAD found in mice. However, some commercially available antibodies to human BAD were advertised to detect a larger ~29kD BAD\textsubscript{L} species in human cells that corresponds in size to mBAD\textsubscript{L} (Santa Cruz and Upstate Biotechnology). Similarly, immunoblot analysis for endogenous BAD in cell lines derived from human and other species revealed two endogenous BAD proteins corresponding in size to transfected murine BAD\textsubscript{L} and transfected human BAD\textsubscript{s} (Fig. 5E, and data not shown). The short form of endogenous BAD is unlikely to be a cleavage product because it is recognized by a BAD antibody that fails to detect the engineered caspase cleavage product \textsuperscript{DN61-mBAD}.

To explore the possibility that humans have an anti-apoptotic form of BAD with additional N-terminal sequences, the amino acid sequence from exon 1 of murine BAD was used in a blast search of the human genome. A single hit was found on chromosome 11 starting approximately 500 nucleotides upstream of the known human \textit{BAD} gene. By joining the putative human exon 1 sequences at predicted splice sites, the predicted human BAD protein has a 43 amino acid N-terminal extension (Fig. 5B). However, concerted effort failed to produce a cDNA for hBAD\textsubscript{L}. This may be due in part to the predicted extensive secondary structure in the 5’ region of hBAD (using Mfold).

**Death stimulus-specific cleavage site specificity is conserved in mouse and human BAD.** To determine if the predicted human BAD\textsubscript{L} protein has anti-death activity, an engineered cDNA (with alternate codon usage to avoid potential hairpins) was tested in stably transfected CCL10-BHK cell clones (Fig. 6A). Both the wild type and the double cleavage site mutant (corresponding to amino acids Asp57 and Asp72 in hBAD\textsubscript{L}) failed to alter cell death triggered by staurosporine treatment (Fig. 6B). Similarly, neither protein exhibited pro- or anti-death activity in a transient assay of Sindbis virus-induced death (Fig. 6C). Immunoblot analysis also failed to detect a stable cleavage product of hBAD\textsubscript{L} in virus-infected cells, while mBAD\textsubscript{L} was processed.
under the same conditions (Fig. 6D). Thus, human BAD<sub>L</sub> may fail to kill cells because it lacks the critical Asp61 cleavage site required to convert murine BAD<sub>L</sub> into a pro-death factor by these death stimuli.

We sought a death paradigm involving the Asp56 or 71 cleavage sites that are conserved between humans and mice. In stably transfected Ba/F3 cell lines that are dependent on IL-3, human BAD<sub>L</sub> lacking both cleavage sites (D57/D72A), but not the single mutant (D72A) or the wild type, suppressed cell death induced by IL-3 withdrawal (Fig. 6E). Consistent with this observation, wild type hBAD<sub>L</sub> protein levels were reduced ~70% following IL-3 withdrawal, while the uncleavable mutant was stabilized (Fig. 6F). The hBAD<sub>L</sub> cleavage fragment was either unstable or not efficiently recognized by the antibody. Thus, cleavage at hBAD<sub>L</sub> Asp57 (alone or in combination with Asp71) appears to abolish the anti-death activity of human BAD during growth factor withdrawal. Analogous results were obtained with murine BAD<sub>L</sub> in a series of stably transfected Ba/F3 cell clones (Fig. 6H), where mutation of Asp61 (or Asp61 and 71) in mBAD<sub>L</sub> provided minimal or no protection compared to vector controls. However, mutation of all three potential cleavage sites (Asp56/Asp61/Asp71) provided significant protection (Fig. 6G). The somewhat variable expression levels of various BAD proteins in different clonally derived cell lines (Fig. 6H; also see Figs. 3A, 3D, and 6A) prior to induction of cell death were apparently inconsequential and did not alter the conclusions. Further analysis of one of these cell lines indicates that there are no obvious compensatory changes in the endogenous expression levels of other anti- and pro-death BCL-2 proteins that could explain the protective effect of BAD (Fig. 6I). Thus, Asp56 of mBAD<sub>L</sub> and Asp57 of hBAD<sub>L</sub> make a critical contribution to the conversion of BAD function in growth factor withdrawal in Ba/F3 cells. This is in sharp contrast to the cell death paradigms described above (irradiation, virus infection and staurosporine treatment) that were tested in one or more cell types (COS-1, HEK293, BHK, primary neurons and/or mice), where Asp61 was apparently the only cleavage site required to convert murine BAD into a pro-death factor (Fig. 6J).

Caspase cleavage of mBAD<sub>L</sub> is not sufficient to kill immature neurons. Of all the experiments described above, the only example where mutation of the caspase cleavage site was not required for BAD to exhibit death was in immature neurons, either in culture or in newborn
mice. This suggests that in immature neurons mBAD\textsubscript{L} is either not cleaved or does not require its N-terminus for anti-death activity. To address this issue, an engineered cleavage fragment of mBAD\textsubscript{L} ([N61] was expressed via the Sindbis virus vector. In 2-day-old dissociated neuron cultures, [N61]-BAD increased cell death compared to the control (Fig. 7A). Therefore, removal of the BAD N-terminus was sufficient to activate its pro-death function in immature cortical cultures. In contrast, [N61]-BAD failed to enhance neuronal death in brain slices, although this deletion largely abolished its protective function (Fig. 7B). Furthermore, [N61]-BAD protected newborn mice almost as efficiently as wild type mBAD\textsubscript{L} (Fig. 7C). Therefore, other mechanisms are apparently required to activate the pro-death function of BAD when neuronal connections are maintained in the immature cultured tissues and animals.

Dephosphorylation of BAD in response to growth factor deprivation promotes cell death in thymocytes, cerebellar granule neurons and fibroblasts (19). Therefore, to determine if dephosphorylation could impair the anti-death function of BAD, Ser residues at known phosphorylation sites (S112, S136 and S155) were changed to Ala to mimic the unphosphorylated protein, or to Glu to mimic phosphorylated BAD. In 2-day cortical neurons, the triple S/A mutant (Fig. 7A) and the single S136A mutant (not shown) failed to protect against Sindbis virus-induced death, while the glutamate mutant was indistinguishable from wild type mBAD\textsubscript{L} (Fig. 7A). Consistent with this observation, mutation of S136A largely abolished the anti-death function of mBAD\textsubscript{L} in the whole animal model (Fig. 7C). In contrast, the Ser mutations had no significant effect on BAD function in 8-day cultures, where cleavage of mBAD\textsubscript{L} appears to be the primary regulator of its function (Fig. 7A). Thus, it appears that dephosphorylation of mBAD\textsubscript{L} at S136 and perhaps other sites is also required to abolish anti-death function in immature neurons. As one compares dissociated neurons to neurons in tissue slices and finally to neurons in the whole animals, the pro-death function of BAD becomes progressively more difficult to activate as the model approaches normal physiological conditions (Fig. 7D).

**Discussion**

Here we demonstrate that the BH3-only protein BAD can inhibit cell death induced by different death stimuli in a variety of cell types. Depending on the model system, the anti-death
function of BAD can be abolished or converted into pro-death function by multiple mechanisms including proteolysis, splicing and dephosphorylation. The general assumption has been that BAD is a latent death factor in healthy cells and is activated to kill cells by dephosphorylation (39) or by caspase cleavage during IL-3 withdrawal (36), herpesvirus infections (40), exposure to TGFβ (37) and raloxifene treatment (41). A revised viewpoint consistent with these earlier findings is that BADL is not simply an inert factor in healthy cells, but has an active pro-survival function prior to initiation of programmed cell death. The question remains as to whether the anti-death function of BAD is biochemically related or unrelated to its pro-death function. Along this line, the interactions between anti- and pro-death BCL-2 family members, which are widely thought to modulate cell death/survival, may be distinct from their normal cellular pro-survival mechanisms. The available evidence does not definitively distinguish between these possibilities, and both mechanisms could co-exist. Nevertheless, the hypothesis that BAD has a normal cellular function that is not necessarily related to its pro-death activity is supported by the recent report that BAD is a regulator of cellular metabolism by facilitating utilization of glucose in the glycolytic pathway (22). While the function of BAD in glycolysis could conceivably contribute to cell death through increased exposure of cells to damaging byproducts of respiration, the role of BAD in glycolysis appears to be beneficial to cells and their host. This is based on the observation that glucose induces phosphorylation of BAD and that BAD-deficient mice exhibit characteristics of diabetes (22). Furthermore, dephosphorylated (pro-death) BAD also decreases glucokinase activity and suppresses glycolysis in hepatocytes. Similarly, BAK and BCL-xL may have alternative biochemical functions in healthy cells where they regulate neuronal excitability (4,42).

It is inherently challenging to distinguish between the lack of a pro-death function and the presence of a pro-survival function. Results derived from a knockin mouse expressing S112/136/155A mutant BAD indicate that endogenous unphosphorylated BAD causes decreased cell viability, consistent with the long-standing interpretation that phosphorylated BAD is an inactivate pro-death protein (19). However, these data are simultaneously compatible with a second conclusion that phosphorylated BAD actively inhibits programmed cell death prior to a death stimulus. This conclusion is supported by the observation that knockin unphosphorylated BAD causes decreased cell viability with or without a death stimulus (19). Our observation that
BAD inhibits cell death could appear to contradict the observation that BAD-deficient primary hepatocytes are resistant to cell death induced by glucose deprivation or other stimuli (20,22). To the contrary, knockout mice lack both the anti- and pro-death functions of BAD by definition. Therefore, decreased death in the absence of BAD would be expected under conditions where BAD is normally converted into a killer protein, particularly if this occurs by an irreversible event such as proteolysis.

Analogous to BAD, the anti-apoptotic family members BCL-2 and BCL-xL can be converted into pro-apoptotic factors when cleaved by caspases or other proteases. Cell lines overexpressing uncleavable BCL-2 and BCL-xL proteins have increased resistance to cell death induced by growth factor withdrawal and virus infections compared to wild-type proteins (7,8). Cleavage of the *C. elegans* BCL-2 homologue CED-9 by the pro-death *C. elegans* caspase CED-3 testifies to the conservation of this proteolytic event, though the function of this event in *C. elegans* is not understood (38). The striking reversal of pro-death to anti-death function by mutation of the caspase cleavage sites in BAD is likely accomplished by one or both consequences of a two-pronged mechanism, preserving full-length BADL to carry out its pro-survival function, and blocking the release of a pro-death fragment. It is also possible that the cleavage site itself is involved in the inhibitory process as suggested for CED-9 and BCL-xL (8,38). Caspase cleavage of the unrelated proteins c-IAP1 (cellular inhibitor of apoptosis) and retinoblastoma protein (Rb) can also promote cell death (43-45). A knockin mouse in which the caspase cleavage site near the C-terminus of Rb was abolished confirms the importance of proteolysis in regulating Rb function (45). Thus, a single caspase cleavage site can have profound biological consequences. However, we found that mechanisms other than caspase cleavage can convert BAD from an anti-death to a pro-death protein. Similarly, BCL-2 can promote cell death without being cleaved when it binds to Nur77 (15).

Different caspases appear to cleave BAD at different sites in response to different death stimuli. The Asp56/57 site in murine/human BADL plays a significant role in cell death induced by growth factor deprivation in Ba/F3 cells, while Asp61 is the critical site for a variety of other cell types and death stimuli. This idea is further supported by our observation that recombinant caspase-3 cleaves mBADL at Asp61 but not at Asp56 (Fig. 2), and caspase-7 cleaves murine but not human BAD (Fig. 5). Furthermore, recombinant caspase-2, -4, -6 and -9 failed to cleave
murine/human BAD (data not shown). However, if BAD is a sentinel that monitors glycolysis or other function and serves to activate the death pathway upon loss of growth factor stimulation, then one would expect BAD to be cleaved in cells by initiator caspases (e.g. caspase-2, -8, -9) rather than effector caspases (e.g. caspase-3, -7). Indeed, the specificities of recombinant caspases (especially initiator caspases that are normally bound to regulatory subunits) are not necessarily expected to faithfully mimic apoptotic cells. Consistent with this idea, BCL-xL is readily cleaved by caspase-3 in vitro to produce a pro-death fragment, yet in COS-1 cells where BAD is converted to a pro-death factor by caspase cleavage, BCL-xL remains anti-apoptotic (see Fig. 1A) and is not detectably cleaved (data not shown).

Posttranslational modifications that occur in dying cells could also determine which cleavage sites are utilized in BAD, as demonstrated for BID (46). However, we observed that the triple Ser/Glu mutations at 112, 136 and 155 in murine BAD unexpectedly increased susceptibility of in vitro translated BAD to recombinant caspase-3 (data not shown). This might be inconsequential in immature neurons where “cleaved” N61BAD still protects (Fig. 7C). However, this enhanced susceptibility to caspase-3 was reversed and further suppressed by addition of the BAD binding partner 14-3-3 in the same experiments. Although we did not detect age-dependent changes in 14-3-3 expression to explain the age-dependent conversion of BAD function in neurons (data not shown), a switch in protein function could easily be overlooked by simply measuring expression levels. Similarly, caution should be used when interpreting the significance of altered levels of anti- and pro-death BCL-2 family members in patient samples and elsewhere.

Proteolytic cleavage and dephosphorylation of BAD are not the only mechanisms that govern the activation of pro-death function, because the short splice variant of BAD lacking exon 1 structurally and functionally resembles its proteolytic cleavage product (Fig. 5). Furthermore, the mouse and human genomes have yet another conserved initiation codon and reading frame further upstream of exon 1, suggesting the existence of an extra-long version of BAD, perhaps analogous to the three N-terminal splice variants of another BH3-only protein BIM (47). In addition to splicing, the presence of extensive predicted secondary structure in exon 1 and upstream regions raises the possibility that an IRES-like element is involved in selection of alternative translation initiation sites, providing yet another caspase-independent mechanism.
Selective use of cap-dependent and IRES-dependent translation can be differentially regulated by physiological stimuli including apoptosis (48). Thus, there are potentially multiple mechanisms for regulating the pro-survival and pro-death functions BAD.

Because removal of the BAD N-terminus enhances cell death in most (but not all) circumstances, we might be led to think that the N-terminus encodes an essential anti-death function. However, neither BAD nor BAK requires its N-terminus for anti-death activity in intact tissue (hippocampal tissues slices and whole animals) (Fig. 7) (4). Also, murine BADs, which lacks its N-terminus, was the predominant form of BAD identified in glucokinase complexes (22). Similarly, CED-9 lacking its N-terminal CED-3 cleavage fragment partially retains its anti-death activity in C. elegans (38). These observations suggest that the N-termini of BCL-2 family proteins may not contribute directly to their anti-death or alternate biochemical functions, but instead are required under some conditions to block the activation of their own pro-death functions. Thus, the N-terminus may serve as a safety cap.

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REFERENCES


Figure legends

Fig. 1. Cell type and age-dependent regulation of cell death by mBAD. (A) Cell death of COS-1 cells was determined by trypan blue staining at 24 h post infection with the indicated recombinant Sindbis viruses (SV) encoding HA-tagged murine BADL or HA-tagged human BCL-xL proteins (mean +/-SE of three independent experiments). (B) Neuronal death in tissue slices cultured for 3 days prior to infection was determined by fluorescence-based computer imaging of propidium iodide (PI) staining at 68 hours post infection with recombinant viruses expressing mBADL or mBIMs (mean +/-SE of three independent experiments). (C) Cell death of 2-day-old cultures of dissociated cortical neurons prepared from individual bad+/+, bad+/- and bad-/- embryos was determined by counting >3000 dual-stained (PI + Hoechst) cells in 15 randomly selected fields per sample at 0h and 24h post infection with Sindbis virus (SV-control) and subsequently correlated to genotypes. Neuronal death in 2-day-old cultures of spinal cord slices prepared from genotyped newborn littermates was determined as described for panel B (bad+/- cultures were not tested). Data shown are the mean +/-SE for three independent experiments (* p<0.05 compared to bad-/-). (D) Immunoblot of lysates from neuronal cultures described in panel C or brain tissue from bad+/+ (Wt) and bad-/- (KO) mice using anti-BAD antibody C-20 (Santa Cruz). (E) Percent mortality following intracranial injection of 3-day-old CD-1 mice (n >65 per group) or 5-week-old C57BL/6 mice (n >30 per group) with the indicated recombinant Sindbis viruses. (F) Cell death of rat embryonic cortical neurons was determined at 48 hours post infection with the indicated recombinant viruses as described for panel C (counting >1000 cells per sample in each of three independent experiments). An immunoblot for BAD in SV-control (SV-C) and SV-mBADL (mB)-infected cortical neuron cultures is shown. (G) Representative fields of PI/Hoechst-stained cortical neurons from panel F are shown. (H) Diagram summarizing the age-dependent function of BAD. The precise neuron population responsible for virus-induced mortality is not known, but is likely to involve the brain stem that regulates vital functions.

Fig. 2. Caspase cleavage of mBAD promotes death in mature neurons. (A,B) In vitro translated, 35S-labeled wild type murine BAD (mBADL) and derived point mutants were treated with buffer only, caspase-3 or –7, and analyzed by SDS-PAGE and autoradiography. (A
potential unidentified caspase-7 cleavage product is marked with a dash.) (C) Cell death of infected rat cortical neurons was determined as described for Fig. 1F-G. (D) Mortality of 5-week-old C57BL/6 mice (n>20 per group from three independent experiments) was determined as described for Fig. 1E. (E) Viability was determined by morphology of GFP-positive cells at 48 hours after cotransfection of 6-7 day-old rat cortical neuron cultures with the indicated plasmids (5 ug) plus GFP (1 ug) in the absence or presence of 150 nM staurosporine during the final 24 hours. (F) Diagram summarizing the role of caspases in converting BAD into a pro-death factor in mature neurons.

**Fig. 3.** Uncleavable mBAD protects stable cell lines from apoptotic stimuli. (A) Immunoblot analysis of the stably transfected CCL10-BHK cells expressing the indicated HA-tagged constructs (individual cell clones are numbered), loading 20-25 ug/lane and blotting with anti-BAD antibody C20 (Santa Cruz). (B) Death of the cells shown in panel A was determined by trypan blue staining at 48 hours after exposure to the indicated doses of gamma irradiation (mean +/-SE for three independent experiments counting >150 cells per sample). (C) Immunoblot analysis of the indicated stable CCL10-BHK cells with or without treatment with 1 uM staurosporine (STR) for 3 hours was performed as described for panel A. Arrows mark full-length and cleaved BAD. (D) Immunoblot analysis (anti-BAD C20) of individual BHK-21 cell clones (numbered) stably transfected with the indicated constructs. (E,F) Cell death of BHK-21 cells lines from panel D was determined by PI staining and flow cytometry and/or manual counting at 24 h post infection with control Sindbis virus or 3 h after treatment with 1 uM staurosporine. Data presented are the mean +/-SE from duplicate determinations in each of three independent experiments.

**Fig. 4.** Murine BAD enhances cell death while uncleavable BAD suppresses cell death in transient assays. (A) Percent death of HEK293 cells at 48 hours after transfection of the indicated plasmids was determined by morphology. (B) Percent cell death of COS-1 cells was determined at 48h after infection with the indicated recombinant Sindbis viruses. Data were compiled from three independent experiments analyzed by PI staining and flow cytometry, or by
trypan blue staining counting \( >200 \) cells per sample. (C) Immunoblot of BHK cell lysates at 24 hours post infection using anti-BAD antibody C-20 (Santa Cruz).

**Fig. 5. Regulation of human BAD function by splicing.**

(A) Diagram of the long and short mouse and human BAD proteins produced by alternative splicing. Caspase cleavage site positions are numbered. (B) Alignment of predicted N-terminal amino acid sequences of BAD from mouse, rat, *Bos taurus* (cow, BF043486), *Sus scrofa* (pig, BF441436) and human chromosome 11 (h chr 11). ESTs for human slice variants 2 (NM_032989) and 1 (NM_004322) also lack the long N-terminus. The predicted N-termini of long and short splice variants (arrow heads) and potential caspase cleavage sites (thick vertical lines with amino acid numbers) are indicated. (C) Autoradiograph of *in vitro* translated (untagged) human BAD\(_5\) (hBAD\(_5\)) and derived point mutants as described for Fig. 2A. (D) Cell death of the indicated stably transfected CCL10-BHK cell clones (numbered) following exposure to the indicated doses of gamma irradiation was determined as described for Fig. 3B. (No cells transfected with wt human BAD\(_5\) survived selection.) An immunoblot of untreated cell extracts is shown. (E) Immunoblot of endogenous or transfected BAD. Cell lysates were prepared from the indicated cell lines (left) or from 293 cells transfected with plasmids (2 ug) encoding the indicated untagged BAD constructs (right) and detected by immunoblot with anti-BAD antibodies H-168 (N-terminus) or C-20 (C-terminus).

**Fig. 6. A death stimulus-specific cleavage site is conserved in human and mouse BAD.** (A) Immunoblot analysis (anti-BAD C-20) of human BAD in untreated CCL10-BHK cell clones (numbered) stably transfected with the indicated constructs. (B) Cell death in the cell lines shown in panel A was determined by counting fluorescence microscopy images of PI stained samples following treatment with 1 \( \mu \)M staurosporine (STR). Data presented are the mean +/- SE from duplicate determinations in each of three independent experiments. (C) Percent death of CCL10-BHK cells at 24 h post infection with the indicated recombinant viruses was determined by PI staining and flow cytometry in three independent experiments (mean +/- SE). (D) Immunoblot analysis of the indicated stable cell lines from panel C and Fig. 3D-E (anti-BAD C20). (E) Death of individual stably transfected Ba/F3 cell clones (numbered) expressing the
indicated human BAD$_L$ proteins was determined by PI staining and flow cytometry at the indicated time points following IL-3 withdrawal. (F) Immunoblot analysis of the cells shown in panel E using anti-BAD antibody (R&D Systems). Relative densitometry values of BAD normalized against actin blots are indicated. (G) The function of murine BAD$_L$ and derived mutants was assessed as described in panel E. Data presented are the average +/- range of two independent experiments for each of 4 clonally derived cell lines of mBAD$_L$(D56/61/71A), and the mean +/-SE of three independent experiments for all other constructs in panels E and G. (H) Immunoblot analysis of untreated Ba/F3 cell lines used in panel G (anti-BAD R&D System). (I) Immunoblot analysis of a Ba/F3 cell line (+IL-3) expressing mBAD(D56/61/71) using antibodies against BCL-xL (BioCarta), BCL-2 (Pharmingen), BAX (Upstate), BAK (Upstate) and actin (ICN/MP Biomedicals). (J) Diagram summarizing the role of conserved and unconserved caspase cleavage sites in human and mouse BAD$_L$.

**Fig. 7. Caspases and phosphorylation regulate mBAD function in immature neurons. (A)** Cell viability of 2- and 8-day-old embryonic rat cortical neuron cultures following infection with the indicated recombinant viruses was determined by PI/Hoechst double staining as described for Fig. 1F-G. 

**(B)** Cell death of infected hippocampal slice cultures was determined as described for Fig. 1B. Data presented are the mean+/−SE for 3 independent experiments. Representative images of PI-stained hippocampal slice cultures show a pattern of cell death consistent with the neurotropism of Sindbis virus. 

**(C)** Percent mortality of 3 day-old CD-1 mice (n>100 per group) infected with the indicated viruses was determined as described for Fig. 1E. 

**(D)** Diagram summarizing the role of caspases and phosphorylation in regulating BAD function in immature neurons.
**Figure 1**

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A. COS-1 cell death (% trypan)

B. 3-day hippocampal slice

C. 2-day cortical neurons

D. Cortical culture, Whole brain, Spinal cord slice

E. 3-day mice, 5-week mice

F. Cortical neurons

G. SV-Control, SV-mBAD

H. Cell death/mortality

- Cell lines
- ≥ 6-day dissociated cortical neurons
- ≤ 4-day CNS tissue slices
- Newborn mice
**Figure 2**

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Figure 4
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Figure 5
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Figure 7
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A

Cortical neurons

Cell death (% PI+)

Culture age

2-day

8-day

SV-Control

SV-wt mBAD_L

SV-D61A

SV-ΔN61mBAD

SV-S112/136/155A

SV-S112/136/155E

B

3-day hippocampal slice

Cell death (% PI intensity)

Mock

SV-control

SV-mBAD

SV-D61A

SV-ΔN61mBAD

C

3-day mice

Percent mortality

Mock

SV-Control

SV-mBAD

SV-ΔN61mBAD

SV-S112/136/155A

SV-S112/136/155E

D

ΔN61-mBAD_L

Tissue slice death

Mouse mortality

2-day cortical neuron death

Newborn mouse mortality

Caspase

mBAD_L

Caspase

P

P
BAD is a pro-survival factor prior to activation of its pro-apoptotic function
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