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Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice

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Trophic factor deprivation (TFD)-induced apoptosis in sympathetic neurons requires macromolecular synthesis-dependent BAX translocation, cytochrome *c* (cyt *c*) release, and caspase activation. Here, we report the contributions of other intrinsic and extrinsic pathway signals to these processes. Sympathetic neurons expressed all antiapoptotic BCL-2 proteins examined, yet expressed only certain BH3-only and multidomain proapoptotic BCL-2 family members. All coexpressed proapoptotic proteins did not, however, exhibit functional redundancy or compensatory expression, at least in the *Bax*^{-/-}, *Bak*^{-/-}, *Bim*^{-/-}, *Bid*^{-/-}, and *Bad*^{-/-} neurons examined. Although the subcellular distribution or posttranslational modification of certain BCL-2 proteins changed with TFD, neither transcriptional

nor posttranslational mechanisms regulated the expression or subcellular localization of BID, BAD, or BAK in this paradigm. Despite modest induction of *Fas* and *FasL* expression, Fas-mediated signaling did not contribute to TFD-induced apoptosis in sympathetic neurons. Similar findings were obtained with K⁺ withdrawal-induced apoptosis in cerebellar granule neurons, a model for activity-dependent neuronal survival in the CNS. Thus, expression alone does not guarantee functional redundancy (or compensation) among BCL-2 family members, and, at least in some cells, extrinsic pathway signaling and certain BH3-only proteins (i.e., BID and BAD) do not contribute to BAX-dependent cyt *c* release or apoptosis caused by TFD.

Introduction

In mammals, signaling cascades culminating in apoptotic cell death can be divided into two broad categories: the “intrinsic” (i.e., apoptosome) and the “extrinsic” (i.e., death receptor and perforin/granzyme) pathways. Current evidence suggests the following model for activation of the intrinsic pathway of apoptosis. A death signal induces the release of mitochondrial proteins, such as cytochrome *c* (cyt *c*)* (Liu et

al., 1996) and Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), through an unknown mechanism that may involve a pore formed by proapoptotic BCL-2 family proteins and/or the permeability transition pore. Once released, Smac/Diablo releases inhibitor-of-apoptosis protein-mediated inhibition of procaspases, whereas cyt *c* forms a complex with Apaf-1 and procaspase-9, which in the presence of ATP or dATP, becomes activated, resulting in further caspase activation, cleavage of cellular substrates, and cell death (Liu et al., 1996; Li et al., 1997; Zou et al., 1997).

In contrast, so-called “extrinsic” pathway signals, such as those mediated by death receptors of the TNF receptor superfamily, activate the caspase cascade more directly. For example, interaction of Fas with its ligand (FasL) triggers formation of a death-inducing signaling complex (DISC) that includes the critical adaptor molecule FADD, which in turn recruits procaspase-8. According to the induced-proximity model (Salvesen and Dixit, 1999), procaspase-8 undergoes

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*Abbreviations used in this paper: CG, cerebellar granule; CGN, CG neuron; cyt *c*, cytochrome *c*; P-Jun, phospho-c-Jun S63; PCD, programmed cell death; SCG, superior cervical ganglion; TFD, trophic factor deprivation.

Key words: Bax; Bad; Bid; caspase; Fas

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autoproteolytic cleavage, forming active caspase-8, which in turn can activate other procaspases, culminating in cleavage of cellular substrates, and apoptosis.

Crosstalk between the intrinsic and extrinsic pathways can occur. For example, activation of caspase-8 through Fas induces cleavage of full-length (p22) BID. The COOH-terminal p15 tBID fragment then translocates to mitochondria, where it causes (either directly or indirectly) cyt *c* release, resulting in apoptosome formation, caspase activation, and cell death (Li et al., 1998; Luo et al., 1998).

Regulation of the activities of BCL-2 proteins is complex and includes both transcriptional (e.g., EGL-1, BIM, and HRK) and posttranslational (e.g., BAD, BID, BAX) mechanisms. In the case of the multidomain proapoptotic protein BAX, a critical regulatory mechanism is subcellular compartmentalization. Apoptotic stimuli result in the translocation of BAX from the cytosol to mitochondria, leading to multimerization, integration, and cyt *c* release, culminating in caspase activation and apoptosis. However, the mechanisms responsible for triggering these events remain poorly defined.

The regulation of BAX function has been studied extensively in the context of trophic factor deprivation (TFD)-induced apoptosis in neonatal sympathetic neurons, an *in vitro* paradigm that recapitulates the physiological cell death that these cells undergo *in vivo* during development. Apoptosis in this model requires *de novo* protein synthesis (Martin et al., 1988) and caspase activation (Deshmukh et al., 1996; Troy et al., 1996; McCarthy et al., 1997). Most important, in contrast to other paradigms in which the regulation of BAX has been examined, sympathetic neurons absolutely require endogenous BAX expression (Deckwerth et al., 1996; Deshmukh and Johnson, 1998) and translocation (Putcha et al., 1999, 2000) for cyt *c* release, caspase activation, and apoptosis.

Multidomain proapoptotic BCL-2 family members such as BAX and BAK may serve redundant functions in the regulation of cell death (Lindsten et al., 2000; Wei et al., 2001). Such redundancy may be true not only for multidomain proapoptotic BCL-2 proteins, but also for members of the BH3-only subfamily. For example, targeted deletion of BIM, a BH3-only protein induced during TFD in sympathetic neurons, confers partial protection against cyt *c* release and apoptosis (Putcha et al., 2001), consistent with functional compensation by another BH3-only protein, HRK, which is also induced with similar kinetics in this paradigm (Imaizumi et al., 1997). Therefore, reproduction of the phenotype seen in *Bax*^{-/-} mice, in which cyt *c* release and apoptotic cell death are completely prevented in many neurons, may require inactivation of at least both BIM and HRK. Because both proapoptotic and antiapoptotic BCL-2 proteins often exhibit overlapping spatial and temporal expression patterns, these findings suggest that functional redundancy among BCL-2 family members may be a common theme in the regulation of cell death.

Here, we report that neonatal sympathetic neurons expressed all antiapoptotic BCL-2 proteins examined and several multidomain and BH3-only proapoptotic BCL-2 family members. However, targeted deletion of only certain proteins (i.e., BAX or BIM) inhibited TFD-induced cyt *c* re-

lease and cell death. Moreover, neither transcriptional nor posttranslational mechanisms appeared to regulate the expression and subcellular distribution of several pro- and antiapoptotic BCL-2 proteins in this paradigm. Finally, although NGF deprivation induced *Fas* and *FasL* expression, analysis of *lpr* and *gld* mice indicates that *Fas*/*FasL* signaling did not contribute to TFD-induced apoptosis in these neurons. Similar results were obtained with K⁺ withdrawal in cerebellar granule neurons (CGNs), a model of activity-dependent neuronal survival in the CNS.

Taken together, these observations indicate the following. First, expression alone did not guarantee functional redundancy or compensation among proapoptotic BCL-2 family members. Second, neither BID nor BAD contributed to BAX-dependent cyt *c* release and its downstream sequelae in these paradigms. And finally, extrinsic pathway signaling had little, if any, role in TFD-induced neuronal apoptosis.

Results

Expression of BCL-2 family members during neuronal apoptosis

BCL-2 proteins regulate programmed cell death (PCD) in two well-characterized models of neuronal apoptosis: NGF deprivation in superior cervical ganglion (SCG) neurons (a PNS population) and K⁺ withdrawal in CGNs (a CNS population).

To study the regulation of TFD-induced apoptosis by other pro- and antiapoptotic BCL-2 family members, we examined their expression during NGF deprivation in SCG neurons (Fig. 1 A) and K⁺ withdrawal in CGNs (Fig. 1 B). For SCG neurons, these experiments were conducted in the presence of the broad-spectrum caspase inhibitor, BAF; for CGNs, the studies were done with neurons from *Bax*^{-/-} mice. In both cases, these experimental conditions were chosen to allow “early” signaling events genetically upstream of the BAX/BCL-2 and caspase checkpoints to occur, while preventing cell death. In contrast to BIM, which was significantly induced during cell death in both paradigms, expression of the multidomain proteins BAK, BAX, BCL-2, BCL-X_L, and BCL-w and the BH3-only proteins BID and BAD did not change significantly during the first 24 h after deprivation. At later time points (e.g., 72 h), expression of these proteins, like that of all proteins, declined.

With respect to the aforementioned findings, several points warrant mention. First, we observed neither loss of full-length (p22) BID nor appearance of truncated (p15) BID (tBID), suggesting that activation of the extrinsic pathway leading to caspase-8-dependent cleavage of BID is not critical to cell death in either model. Second, RT-PCR analysis indicated that both SCG and CG neurons express several other known antiapoptotic members (e.g., *Mcl-1* and *Bfl-1/A1*), but only express a select repertoire of proapoptotic proteins, both multidomain and BH3-only. For example, neither neuronal population showed detectable expression of either *Bok* or *Bik* mRNA (unpublished data). Third, although we observed changes in the migration of BAD consistent with trophic factor-dependent phosphorylation of BAD (Figs. 1 and 2; and unpublished data), our efforts to

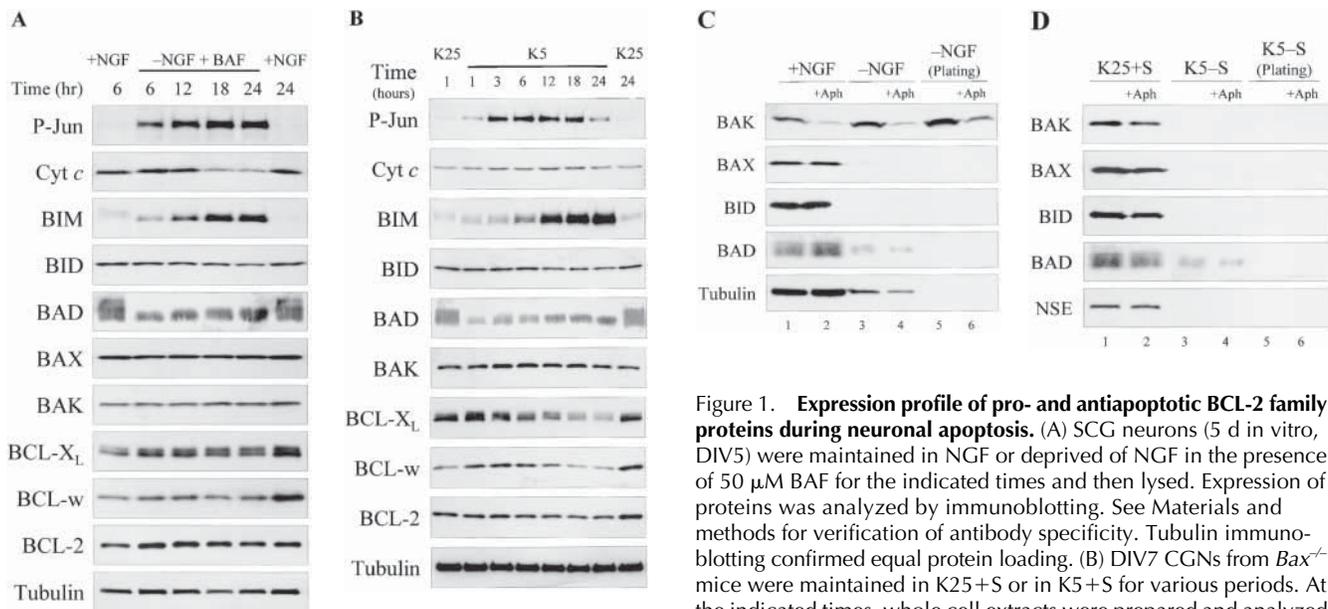


Figure 1. Expression profile of pro- and antiapoptotic BCL-2 family proteins during neuronal apoptosis. (A) SCG neurons (5 d in vitro, DIV5) were maintained in NGF or deprived of NGF in the presence of 50 μ M BAF for the indicated times and then lysed. Expression of proteins was analyzed by immunoblotting. See Materials and methods for verification of antibody specificity. Tubulin immunoblotting confirmed equal protein loading. (B) DIV7 CGNs from *Bax*^{-/-} mice were maintained in K25+S or in K5+S for various periods. At the indicated times, whole cell extracts were prepared and analyzed by Western blotting. (C) Neonatal rat SCG neurons were plated in NGF (lanes 1–4) or in α NGF (lanes 5 and 6) in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of aphidicolin (Aph). At DIV5, the neurons were maintained in NGF (lanes 1 and 2) or deprived of NGF (lanes 3–6) in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of aphidicolin (Aph). All cells were lysed 96 h later (DIV9), and expression of proteins was analyzed by immunoblotting. Tubulin immunoblotting tracked neuronal survival. Fluorodeoxyuridine was present in all cultures. (D) P7 rat CGNs were plated in K25+S (lanes 1–4) or in K5–S (lanes 5 and 6). Aph was added to some cultures (lanes 2, 4, and 6) 24–36 h later. At DIV7, the neurons were switched to K25+S (lanes 1 and 2) or K5–S (lanes 3–6) in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of Aph. All cells were lysed 96 h later (DIV11), and expression of proteins was analyzed by immunoblotting. Neuron-specific enolase (NSE) immunoblotting tracked neuronal survival.

examine changes in the phosphorylation status of BAD associated with cell death in both SCG and CG neurons were hindered by the poor reactivity and specificity of commercially available phosphospecific antibodies (see Materials and methods). Finally, neither *Bax* deletion nor caspase inhibition inhibited c-Jun phosphorylation, an indicator of JNK activation, in these paradigms (Fig. 1; Miller et al., 1997; Deckwerth et al., 1998). However, *Bax* deletion, but not caspase inhibition, prevented the loss of mitochondrial *cyt c*. Therefore, like SCG neurons (Deshmukh and Johnson, 1998; Putcha et al., 2000), CGNs require BAX for *cyt c* release (Fig. 1) and cell death (Miller et al., 1997).

Finally, Sun et al. (2001) recently reported that SCG neurons express *N-Bak*, a BH3-only splice variant of *Bak*, but not full-length *Bak*, which they suggest is expressed only by the nonneuronal cells in SCG cultures, which represent $\leq 5\%$ of the cells present in these cultures. In light of this report and the phenotype of *Bak*^{-/-} neurons, we evaluated whether the BAK expression observed in cultures of SCG and CG neurons derived solely from nonneuronal cells by examining expression in the presence and absence of antimetabolic agents (Fig. 1, C and D, respectively). We chose immunoblotting over immunocytochemistry because no BAK antibodies we tested were specific for the latter application, whereas at least one was specific for the former (see Materials and methods). As shown in Fig. 1, C and D, CG, but not SCG, neurons express BAK; neither appears to express N-BAK (unpublished data). Both neuronal populations express BAD, BID, and BAX (Fig. 1, C and D). Therefore, for the remainder of the paper, “SCG

or CG neurons” will designate SCG or CG neuronal cultures (containing no more than 5% nonneuronal cells).

In sum, both SCG and CG neurons selectively express multiple members of all three subfamilies of BCL-2 proteins. However, like EGL-1 in nematodes (Conradt and Horvitz, 1998), only BH3-only proteins, such as BIM and HRK, are induced during TFD in these neurons. Intriguingly, the BCL-2 family expression patterns of SCG and CG neurons, as well as their dependence on particular family members (e.g., BAX and BIM) for TFD-induced apoptosis, are remarkably concordant.

Subcellular distribution of multidomain and BH3-only BCL-2 family members during neuronal apoptosis

The sequestration of cell death effectors, such as BAX and *cyt c*, within distinct subcellular compartments has recently emerged as a common theme in the regulation of apoptosis. Therefore, we examined the localization of endogenous BCL-2, BCL-X_L, BCL-w, BAD, BID, and BAK by using subcellular fractionation. As shown in Fig. 2, BCL-2 and BAK were found exclusively in the heavy membrane fraction enriched in mitochondria; BCL-X_L and BCL-w localized to both the cytosolic and mitochondrial fractions; BAD and BID were found exclusively in the cytosolic fraction. With NGF deprivation, we observed loss of the cytosolic pools of BCL-X_L and BCL-w, raising the possibility that selective degradation of these proteins may contribute to the translocation of BAX required for TFD-induced neuronal apoptosis (see Discussion). Furthermore, consistent with the findings shown in Fig. 1 A, we did not observe loss of p22 BID

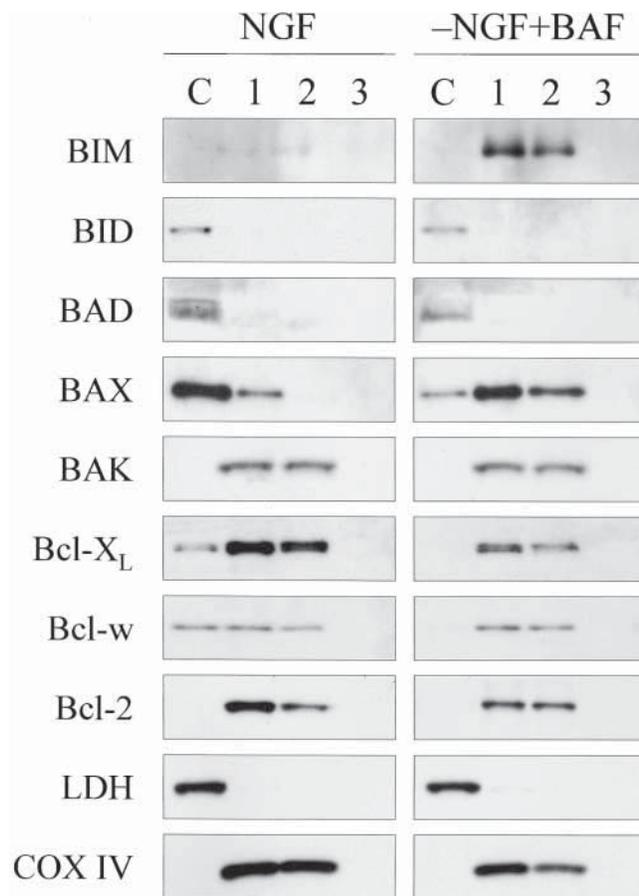


Figure 2. Subcellular distribution of pro- and antiapoptotic BCL-2 family proteins during neuronal apoptosis. DIV5 SCG neurons were maintained in NGF or deprived of NGF in the presence of 50 μ M BAF for 30 h. The subcellular localization of BCL-2 proteins was examined by fractionation with alkali extraction by using 0.2 M Na_2CO_3 (pH 11.5). SDS (1% wt/vol) served as a positive control for the extraction of integral membrane proteins. C, cytosol; HM, heavy membrane; 1, isotonic buffer; 2, 0.2 M Na_2CO_3 ; 3, 1% SDS. Lactate dehydrogenase and cytochrome oxidase subunit IV (COX IV) served as markers for the purity of cytosolic (C) and heavy membrane (HM) fractions, respectively, and as markers for equal protein loading. Markers for the endoplasmic reticulum and Golgi body labeled only the microsomal fraction (not depicted here). Please note that the BAX, BIM, and cyt *c* blots have been reported previously in Putcha et al. (2000, 2001) and are included only for comparison.

or appearance of p15 tBID in either the cytosolic or mitochondrial fractions, suggesting that BID was not cleaved, nor did BID or tBID translocate to mitochondria, during NGF deprivation in SCG neurons. Finally, the cytosolic localization of BAD did not change during TFD (unpublished data).

To determine whether BCL-2 proteins associated with mitochondria were integrated into mitochondrial membranes, we performed alkali extraction of the mitochondrial fractions from NGF-maintained and -deprived SCG neurons. In this assay, mitochondrial integral membrane proteins are resistant to extraction by 0.2 M Na_2CO_3 , yet sensitive to extraction by 1% SDS, whereas peripheral membrane proteins are extracted by both treatments. Using this assay,

we previously demonstrated that, after induction or translocation from the cytosol to mitochondria, BIM and BAX, respectively, integrate into the mitochondrial outer membrane (Putcha et al., 2000, 2001). Similar analyses indicated that mitochondrial BAK, BCL-2, BCL-w, and BCL-X_L were integral membrane proteins in mitochondria (Fig. 2).

Fas-mediated extrinsic pathway signaling does not contribute to TFD-induced neuronal apoptosis

Extrinsic pathway signaling through death receptors, such as Fas, is critical for cellular homeostasis in the immune system in response to both physiological and pathological cues. Recently, several groups suggested that Fas/FasL signaling contributes to cell death during development and/or in response to apoptotic stimuli, such as TFD (Brunet et al., 1999; Le-Niculescu et al., 1999; Raoul et al., 1999), chemotherapeutic agents (Friesen et al., 1996), or ischemia (Martin-Villalba et al., 1999). Since these stimuli generally require macromolecular synthesis for complete and efficient neuronal cell death (Martin et al., 1988; D'Mello et al., 1993; Miller and Johnson, 1996), the groups proposed that cell death in these models requires transactivation of Fas and FasL, presumably followed by cell autonomous and noncell autonomous Fas-dependent apoptosis. However, other reports cast doubt on this hypothesis (Eischen et al., 1997; Gerhardt et al., 2001).

To study the contribution of Fas–FasL interactions to neuronal apoptosis in SCG neurons, we first examined mRNA expression of *Fas* and *FasL* during TFD. As shown in Fig. 3 A, NGF withdrawal induced a modest (\sim 2-fold) elevation in *Fas* and *FasL* during TFD. Similarly, induction of *Fas* and *FasL* occurs during cell death caused by cytosine arabinoside (AraC) in sympathetic neurons (unpublished data) or by K^+ deprivation in CGNs (Brunet et al., 1999; Le-Niculescu et al., 1999), two macromolecular synthesis-dependent models of neuronal PCD. Taken together, these findings suggest that Fas–FasL interactions may contribute to TFD-induced neuronal apoptosis in SCG and CG neurons.

To evaluate the requirement for Fas signaling during TFD-induced neuronal apoptosis, we examined survival after K^+ or NGF withdrawal in CG and SCG neurons, respectively, from *lpr* and *gld* mice, which harbor loss-of-function mutations in Fas and FasL, respectively. As shown in Fig. 3 B, the *lpr* and *gld* mutations altered neither the extent nor the kinetics of K^+ deprivation-induced apoptosis in CGNs, consistent with the findings of Gerhardt et al. (2001). Similarly, the mutations did not prevent TFD-induced apoptosis in sympathetic neurons (survival at 72 h relative to NGF-maintained sister cultures: *wt/wt* = $2.74 \pm 0.57\%$; *lpr/lpr* = $2.31 \pm 0.33\%$; *gld/gld* = $1.85 \pm 2.05\%$; $n = 3$). Furthermore, activation of extrinsic pathway signaling by $\text{TNF}\alpha$, soluble FasL, or agonistic Fas antibody Jo-2 did not induce cell death in NGF-maintained sympathetic (unpublished data) or K^+ -maintained CGNs (Gerhardt et al., 2001). Finally, targeted deletion of *Bid*, a critical target for death receptor-mediated apoptosis, did not alter the time-course or extent of cyt *c* release or cell death in either SCG or CG neurons (see Fig. 5). Thus, although withdrawal of survival factors induced expression of both *Fas* and *FasL* in

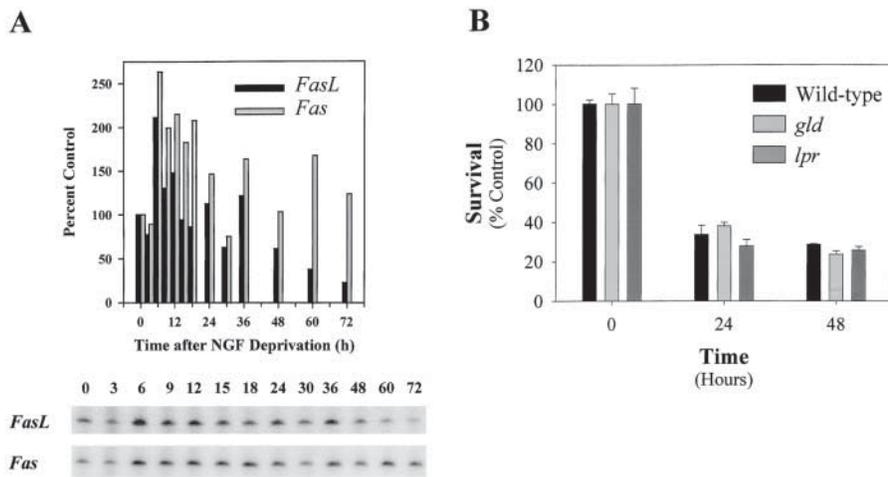
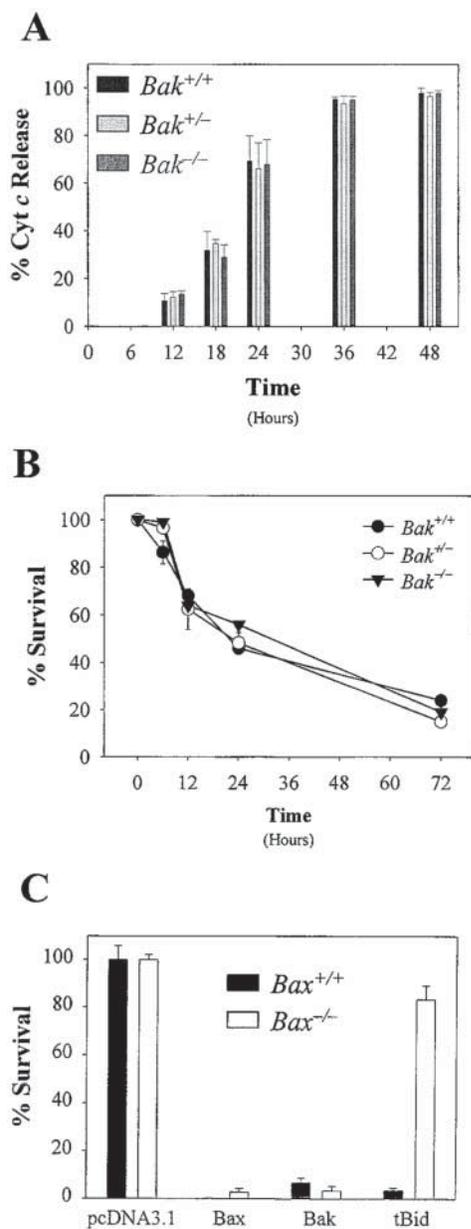


Figure 3. TFD induces *Fas* and *FasL* expression in neurons, but inactivating mutations in *Fas* and *FasL* do not alter the kinetics of neuronal apoptosis.

(A) DIV5 SCG neurons were deprived of NGF for the indicated times. RT-PCR analysis with primers specific for *Fas* and *FasL* was performed. Quantitation (relative to $t = 0$ h) was performed by using ImageQuant (Molecular Dynamics). Similar results were obtained in an independent timecourse. (B) DIV7 CGNs from wild-type (C57BL/6), *lpr*, or *gld* mice were maintained in K25+S or K5+S for various periods. At the indicated times, cells were stained with the vital dye calcein AM and photographed. A naïve observer then counted the number of calcein AM-stained neurons in the photomicrographs. Mean \pm SEM, $n = 3$.



SCG and CG neurons, extrinsic pathway signaling mediated by Fas (and presumably by other death receptors) is not required for—and may not contribute to—neuronal apoptosis in these particular paradigms.

Deletion of *Bak*, *Bid*, or *Bad* does not attenuate TFD-induced neuronal apoptosis

Hepatocytes, embryonic fibroblasts, and some lymphocytes—and perhaps other mitotic nonneuronal cells—may require both BAX and BAK for cyt *c* release and cell death in response to certain intrinsic (e.g., TFD) or extrinsic (e.g., TNF/ActD) pathway stimuli (Lindsten et al., 2000; Wei et al., 2001), suggesting that BAX and BAK are functionally redundant, at least in a cell type- and stimulus-specific manner. We examined the generality of such functional redundancy by examining TFD-induced neuronal apoptosis in *Bax*^{-/-} and *Bak*^{-/-} mice. As reported previously, *Bax* deletion completely prevents cyt *c* release and PCD in NGF-deprived SCG and K⁺-deprived CG neurons (Fig. 1; Deckwerth et al., 1996; Miller et al., 1997; Deshmukh and Johnson, 1998; Putcha et al., 2000); in contrast, *Bak* deletion did not alter the extent or kinetics of cyt *c* release or apoptosis in either cell type (Fig. 4, A and B). Therefore, despite coexpression (Fig. 1) and appropriate subcellular local-

Figure 4. *Bak* deletion does not alter the kinetics or extent of cyt *c* release or apoptosis in SCG or CG neurons. (A) DIV5 SCG neurons from *Bak*^{+/+}, *Bak*^{+/-}, or *Bak*^{-/-} mice were maintained in NGF or deprived of NGF in the presence of 50 μ M BAF for various periods. At the indicated times, cells were fixed and immunostained for cyt *c*. Then, the number of cells exhibiting diffuse cytosolic cyt *c* was determined by a naïve observer. At all time points, NGF-maintained neurons showed punctate, mitochondrial cyt *c*. Mean \pm SEM, $n = 3$ –4. (B) DIV7 CGNs from *Bak*^{+/+}, *Bak*^{+/-}, or *Bak*^{-/-} mice were maintained in K25+S or K5+S for various periods. At the indicated times, cells were stained with the vital dye calcein AM and lysed. The fluorescence of an aliquot of the lysate was then measured on a TiterTek fluorescence plate reader. Mean \pm SEM, $n = 3$ –4. (C) DIV5 CGNs from *Bax*^{+/+} or *Bax*^{-/-} mice were cotransfected with EGFP and the indicated vector. The number of GFP-positive cells was counted 48 h later and expressed as a percentage of vector (pcDNA3.1)-transfected cells. Mean \pm SD, $n = 2$ in duplicate.

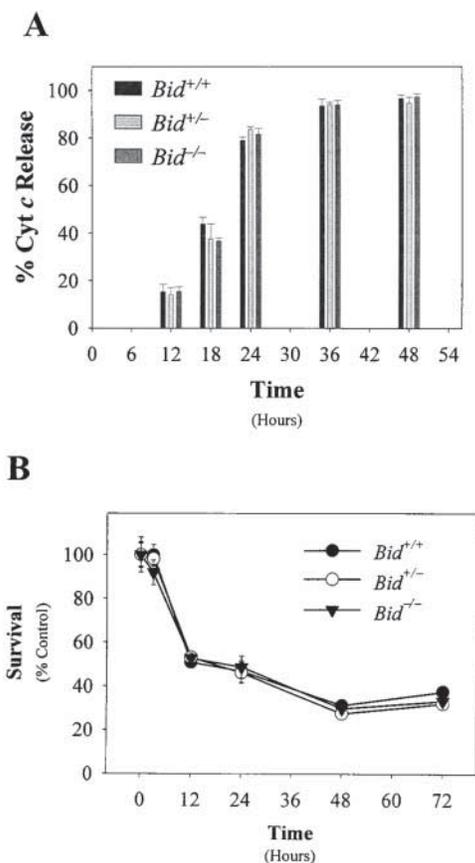


Figure 5. *Bid* deficiency does not alter the timecourse or extent of cyt *c* release or apoptosis in SCG or CG neurons. (A) DIV5 SCG neurons from *Bid*^{+/+}, *Bid*^{+/-}, or *Bid*^{-/-} mice were maintained in NGF or deprived of NGF in the presence of 50 μ M BAF for various periods. At the indicated times, cells were fixed and immunostained for cyt *c*. Then, the number of cells exhibiting diffuse cytosolic cyt *c* was determined by a naive observer. At all time points, NGF-maintained neurons showed punctate, mitochondrial cyt *c*. Mean \pm SEM, $n = 3-6$. (B) DIV7 CGNs from *Bid*^{+/+}, *Bid*^{+/-}, or *Bid*^{-/-} mice were maintained in K25+S or K5+S for various periods. At the indicated times, cells were stained with the vital dye calcein AM and lysed. The fluorescence was then measured on a TiterTek fluorescence plate reader. Mean \pm SEM, $n = 3-4$.

ization (Fig. 2; and unpublished data), BAX and BAK were not functionally redundant in CG neurons.

To examine this issue of functional redundancy further, we examined whether reintroduction of either *Bax* or *Bak* by transient transfection could overcome the cell death defective phenotype conferred by *Bax* deletion in CGNs. As shown in Fig. 4 C, overexpression of either *Bax* or *Bak*, but not *tBid*, killed *Bax*^{-/-} CGNs. Thus, at least in CGNs, BAX and BAK were not functionally redundant at physiological levels of expression, but can be functionally equivalent when overexpressed.

Although redundancy did not exist among multidomain proapoptotic BCL-2 proteins in CGNs, such redundancy may exist between BH3-only proteins in these same cells. Specifically, reproduction of the *Bax*^{-/-} phenotype, in which cyt *c* release and apoptosis are completely prevented, may require inactivation of at least both *Bim* and

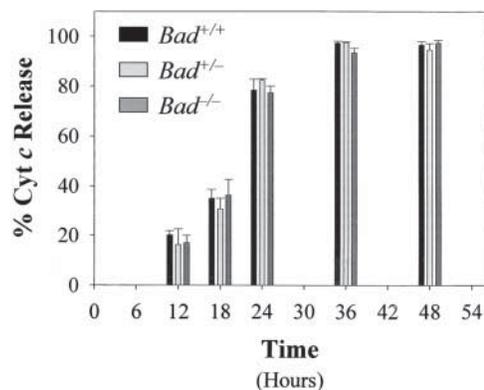


Figure 6. *Bad* deletion does not alter the kinetics or extent of cyt *c* release or apoptosis in SCG neurons. DIV5 SCG neurons from *Bad*^{+/+}, *Bad*^{+/-}, or *Bad*^{-/-} mice were maintained in NGF or deprived of NGF in the presence of 50 μ M BAF for various periods. At the indicated times, cells were fixed and immunostained for cyt *c*. Then, the number of cells exhibiting diffuse cytosolic cyt *c* was determined by a naive observer. At all time points, NGF-maintained neurons showed punctate, mitochondrial cyt *c*. Mean \pm SEM, $n = 3-4$.

Hrk in these neurons (Putchá et al., 2001). However, SCG and CG neurons also express at least two other BH3-only proteins, namely BID and BAD, which have been widely implicated in processes (e.g., BAX translocation and integration and BAX/BAK-dependent cyt *c* release) required for apoptosis in these particular cells. Therefore, we examined whether targeted deletion of *Bid* or *Bad* inhibited neuronal apoptosis in SCG and CG neurons. As shown in Figs. 5 and 6, respectively, *Bid* or *Bad* deficiency did not affect either the extent or kinetics of cyt *c* release or apoptosis after NGF deprivation in SCG neurons (Figs. 5 A and 6 A) or K⁺ withdrawal in CG neurons (Fig. 5 B; and unpublished data). Thus, despite coexpression of multiple BH3-only proteins (i.e., BAD, BID, BIM, and HRK), only BIM—and we predict HRK—regulated BAX-dependent cyt *c* release and neuronal apoptosis in SCG and CG neurons.

Inactivation of critical components of the intrinsic and extrinsic pathways does not inhibit posttranslational regulation of apoptosis by NGF

SCG neurons from *Bak*^{-/-}, *Bid*^{-/-}, or *Bad*^{-/-} mice and from their wild-type and heterozygote littermates were deprived of NGF in the presence of BAF for 18 h; the pattern of cyt *c* staining was assessed. In parallel cultures, NGF was readded, and the pattern of cyt *c* staining was determined 18 h later. As shown in Fig. 7, the gene dosage of *Bak*, *Bid*, or *Bad* did not affect NGF-mediated acute arrest of cyt *c* release, a measure of the ability of NGF to prevent apoptosis posttranslationally. Taken together with the results shown in Figs. 4-6, these findings suggest that, in sympathetic neurons, BAK, BID, and BAD were not required for TFD-induced apoptosis and did not contribute to the regulation of neuronal PCD by either transcriptional or posttranslational mechanisms. Furthermore, these proteins were not required for K⁺ withdrawal-induced apoptosis in CGNs.

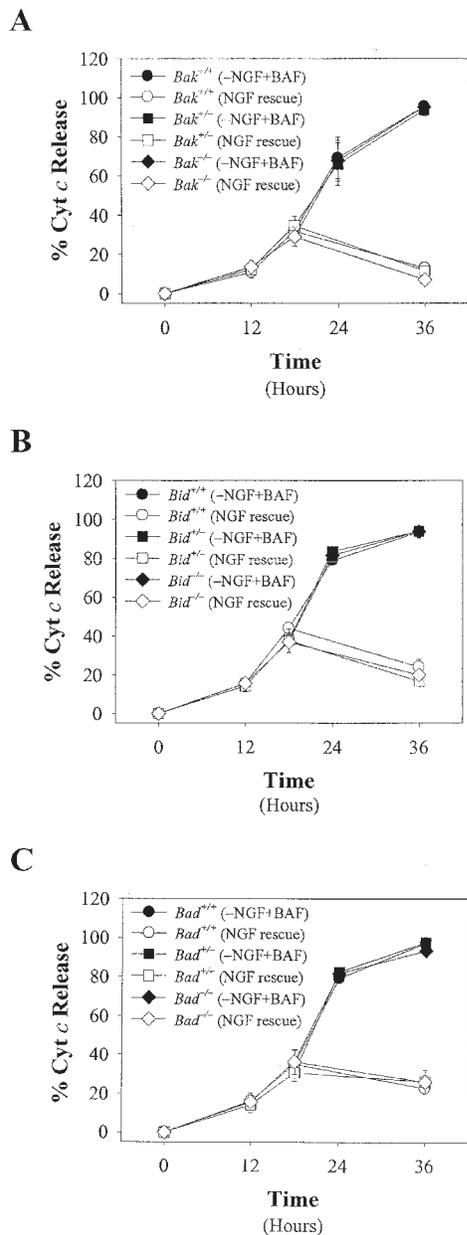


Figure 7. Inactivation of *Bak*, *Bid*, or *Bad* does not affect post-translational regulation of neuronal apoptosis by NGF. DIV5 SCG neurons from *Bak*^{+/+}, *Bak*^{+/-}, or *Bak*^{-/-} (A); *Bid*^{+/+}, *Bid*^{+/-}, or *Bid*^{-/-} (B); or *Bad*^{+/+}, *Bad*^{+/-}, or *Bad*^{-/-} (C) mice deprived of NGF in the presence of 50 μ M BAF for various periods. At the indicated times, cells were fixed and immunostained for cyt c. In parallel, neurons that were deprived of NGF in the presence of 50 μ M BAF for 18 h were washed and rescued with 300 ng/ml NGF and 50 μ M BAF. After an additional 18 h, these cells were fixed and immunostained for cyt c. Then, the number of cells exhibiting diffuse cytosolic cyt c was determined by a naïve observer. Mean \pm SEM; $n = 3-4$ (A), $n = 3-6$ (B), and $n = 3-4$ (C).

Targeted deletion of *Bax*, *Bak*, *Bim*, *Bid*, or *Bad* does not cause compensatory changes in the expression of other BCL-2 family proteins

The findings described above indicate that at least some multidomain and BH3-only BCL-2 proteins were not functionally redundant, at least in SCG and CG neurons, despite

coexpression. To determine whether functional compensation by changes in the expression of other family members might account for the absence of neuronal PCD defects in NGF-deprived sympathetic neurons from *Bak*^{-/-}, *Bid*^{-/-}, and *Bad*^{-/-} mice, we examined expression of other BCL-2 proteins for which we had reliable reagents, in SCG neurons from these mice and from their wild-type littermates. As shown in Fig. 8, we did not observe any such compensatory changes in the expression of pro- or antiapoptotic BCL-2 proteins. Furthermore, we did not observe such changes in several other tissues (e.g., cortex, cerebellum, liver, and spleen; unpublished data). Furthermore, TFD induced BIM expression in *Bax*^{-/-}, *Bak*^{-/-}, *Bid*^{-/-}, and *Bad*^{-/-} sympathetic neurons (Fig. 8; and unpublished data). Finally, as previously reported for SCG neurons from mice overexpressing *Bcl-2* (Putcha et al., 1999) or lacking *Bax* (Deckwerth et al., 1998) or *Bim* (Putcha et al., 2001), activation of the JNK signaling pathway (as indicated by phosphorylation of c-Jun), which is critical for apoptosis in this paradigm, occurred normally in *Bak*^{-/-}, *Bid*^{-/-}, and *Bad*^{-/-} sympathetic neurons (Fig. 8; and unpublished data).

Discussion

SCG and CG neurons expressed all antiapoptotic BCL-2 proteins examined, yet only expressed certain BH3-only and multidomain proapoptotic BCL-2 family members. All coexpressed proapoptotic proteins did not, however, exhibit functional redundancy or compensatory expression, at least in the *Bax*^{-/-}, *Bak*^{-/-}, *Bim*^{-/-}, *Bid*^{-/-}, and *Bad*^{-/-} neurons examined here. For example, despite expression and appropriate subcellular localization, BAK was not functionally redundant with BAX, at least in CG neurons. Similarly, targeted deletion of *Bim*, but not *Bid* or *Bad*, attenuated these processes, consistent with BIM sharing functional redundancy with another BH3-only protein, presumably HRK, but not BID or BAD. Furthermore, neither transcriptional nor posttranslational mechanisms appeared to regulate the expression or subcellular localization of BID, BAD, or BAK in these paradigms. Finally, despite modest induction of *Fas* and *FasL* expression, Fas-mediated signaling in particular—and presumably extrinsic pathway signaling more generally—did not contribute to TFD-induced PCD in these neurons.

Antiapoptotic BCL-2 proteins in neuronal apoptosis: an abundance of riches

Over a decade after the cloning of its founding member, the BCL-2 family is still an enigma whose precise biochemical mechanisms remain unclear. In the case of antiapoptotic members, many questions remain: do they promote cell survival in vertebrates by regulating apoptosome activation through direct protein-protein interactions as in *C. elegans*, by regulating mitochondrial permeability or bioenergetics, by other mechanisms, or some combination of all of the above? If mitochondria are the focus of their actions, do antiapoptotic BCL-2 proteins act solely by inactivating their proapoptotic counterparts or do they possess intrinsic, survival-promoting properties? If so, does the hypothesized “rheostat” (Oltvai and Korsmeyer, 1994) involve direct pro-

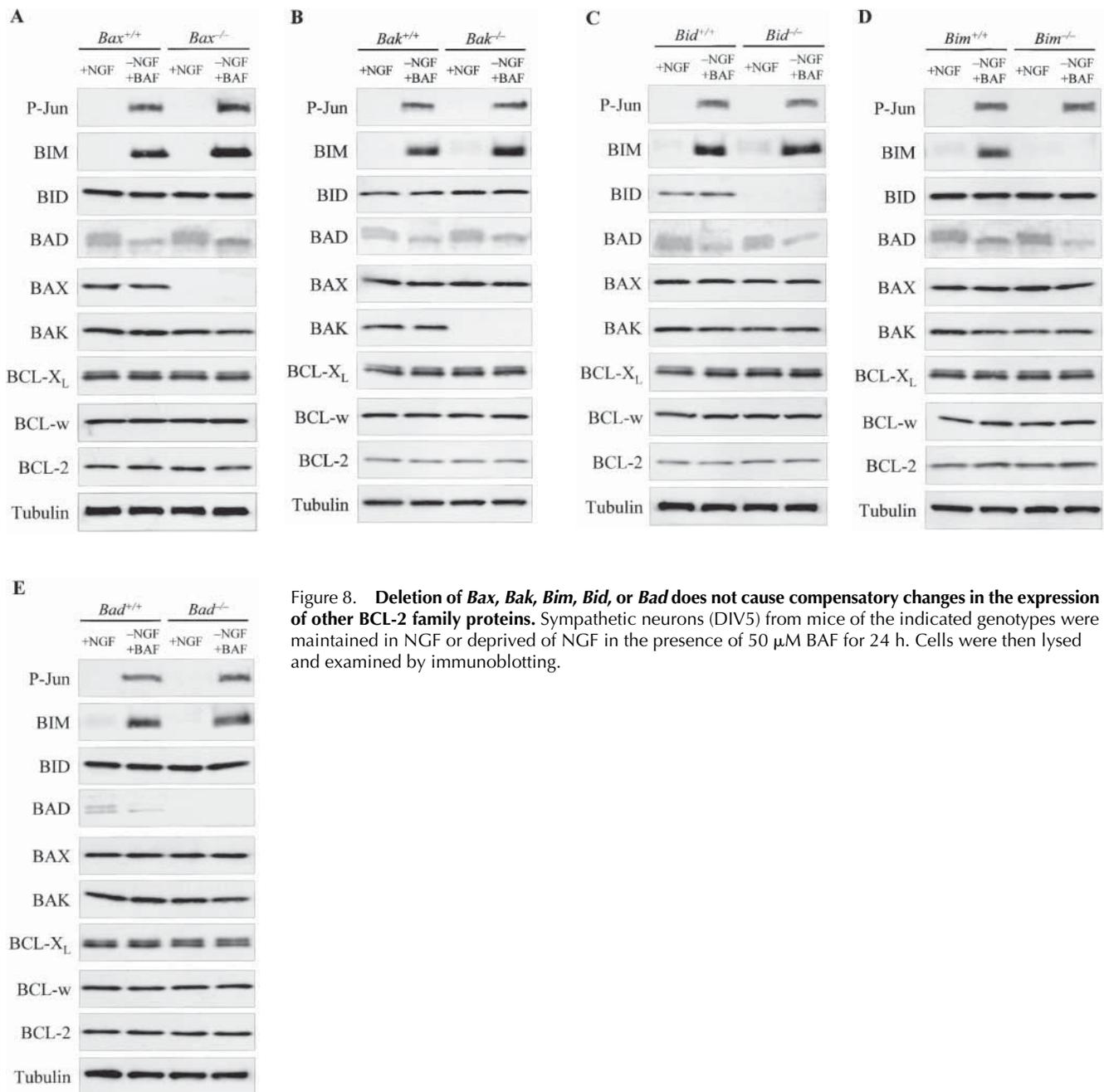


Figure 8. Deletion of *Bax*, *Bak*, *Bim*, *Bid*, or *Bad* does not cause compensatory changes in the expression of other BCL-2 family proteins. Sympathetic neurons (DIV5) from mice of the indicated genotypes were maintained in NGF or deprived of NGF in the presence of 50 μ M BAF for 24 h. Cells were then lysed and examined by immunoblotting.

tein-protein interactions and what are the critical partners *in vivo*? And finally, what are the functional forms of BAX, BCL-2, and so forth—monomers, homodimers, homomultimers, heterodimers, heteromultimers, or some combination of all of these? Disparate answers to all these questions exist in the literature.

TFD in SCG and CG neurons sheds light on some of these questions. Both cell types express all antiapoptotic BCL-2 proteins examined (Figs. 1 and 2; and unpublished data). Like most proteins, total levels of BCL-2, BCL-X_L, and BCL-w remained essentially unchanged until relatively late in the cell death process, after BAX translocation and cytochrome *c* release (Fig. 1). However, subtle changes in the subcellular distributions of these proteins were intriguing. For example,

BCL-2 was found as an integral membrane protein in the heavy membrane and microsomal fractions, which include mitochondria and ER, respectively, in both NGF-maintained and -deprived sympathetic neurons (Fig. 2; unpublished data). In contrast, BCL-X_L and especially BCL-w were distributed more evenly between the cytosolic and mitochondrial compartments in NGF-maintained SCG neurons, and TFD caused loss of the cytosolic pools of these proteins, with relative sparing of the mitochondrial pools. Such selective degradation of cytosolic BCL-w and BCL-X_L might facilitate the translocation of BAX to mitochondria. However, a recent report on the tertiary structure of BAX suggests that its BH3 domain, which is required for heterodimerization with BCL-X_L and BCL-w, may not be exposed for such in-

teractions (Suzuki et al., 2000). Taken together with other observations that interactions between BAX and antiapoptotic BCL-2 proteins expressed at endogenous levels may not occur in the absence of conformation-altering detergents (Hsu and Youle, 1997; Antonsson et al., 2001), these findings cast doubt on the existence a physical BAX/BCL-w/BCL-X_L rheostat in the cytosol of these neurons. We do not favor the hypotheses that cytosolic BCL-w and/or BCL-X_L functions as the anchor that prevents cytosolic BAX from translocating to the mitochondria in NGF-maintained SCG neurons, or that selective loss of these pools (e.g., by ubiquitination and proteasomal degradation) is the trigger for BAX translocation during apoptosis. Instead, as described elsewhere (Harris and Johnson, 2001), we propose that BAX exists in dynamic equilibrium in at least two subcellular compartments (i.e., cytosol and mitochondria) and that BH3-only proteins disrupt this equilibrium by inactivating antiapoptotic BCL-2 proteins, thereby indirectly promoting BAX translocation and its downstream sequelae (see Fig. 9).

Multidomain proapoptotic BCL-2 proteins in neuronal apoptosis: similar but not equal

A limitation of current gene targeting technologies (in mice) is the difficulty interpreting the absence of a phenotype when ablating the function of a single or even several members of vertebrate multigene families as large as the BCL-2 (~21 members), caspase (~14 members), and inhibitor-of-apoptosis protein (~6 members) families. Accordingly, functional redundancy (Lindsten et al., 2000; Zheng et al., 2000; Wei et al., 2001) or compensation (Harlin et al., 2001) among members may explain why targeted deletion of individual genes often produces little or no cell death phenotype, at least with certain paradigms. The mechanisms underlying compensatory changes in expression, whereby cells from knock-out mice cannot only sense diminished expression of one family member but can also up-regulate, sometimes dramatically (Harlin et al., 2001), expression of another functionally redundant member, are unclear. However, those mechanisms responsible for functional redundancy among family members seem self-evident, namely that similar proteins can function similarly. In the case of the multidomain proapoptotic subfamily of BCL-2 proteins consisting of BAX, BAK, and BOK, the hypothesis—substantiated by two recent reports (Lindsten et al., 2000; Wei et al., 2001)—is that BAX and BAK are essentially interchangeable. Findings reported in this paper demonstrate that this is not true in all cell types.

CG neurons express both BAX and BAK, but not BOK (Figs. 1 and 2, and unpublished data). However, *Bax* deletion alone confers complete, long-term protection (Miller et al., 1997), whereas *Bak* deficiency did not change either the kinetics or extent of apoptosis (Fig. 4). Moreover, inhibition of cell death by *Bax* deletion can be overcome by reintroduction of either *Bax* or *Bak* (but not *tBid*) by transient transfection (Fig. 4 C). Finally, targeted deletion of either did not cause compensatory changes in the expression (Fig. 8) or subcellular localization (unpublished data) of the other. Thus, at physiological levels of expression, BAX and BAK may not always be functionally redundant; however, when overexpressed, they can be.

BH3-only proapoptotic BCL-2 proteins in neuronal apoptosis: functional redundancy and implications for BAX-dependent cyt *c* release

Emerging within the BCL-2 family of proteins is a subfamily of proapoptotic molecules that share homology only within the BH3 domain. When overexpressed, many of these “BH3-only” proteins (e.g., BIM and HRK), like their multidomain cousins, localize to mitochondria, cause cyt *c* release, and induce cell death that can be attenuated by caspase inhibition or cooverexpression of antiapoptotic BCL-2 proteins (Imaizumi et al., 1997; Inohara et al., 1997; O'Connor et al., 1998).

Recently, we reported that TFD significantly induces expression of the BH3-only protein BIM in multiple neuronal populations (in vivo and in vitro), including SCG, DRG, and CG neurons (Putcha et al., 2001). In both sympathetic and CGNs, induction of BIM (Putcha et al., 2001) and HRK (Harris and Johnson, 2001) occurs upstream of (or in parallel with) the BAX/BCL-2 and caspase checkpoints. Moreover, in CG neurons, cell death caused by ectopic overexpression of BH3-only proteins (e.g., BID, BIM, or HRK) requires BAX (Fig. 4 C; Harris and Johnson, 2001). Finally, *Bim* deletion delayed, but did not prevent, cyt *c* release and apoptosis in SCG and CG neurons, suggesting that BIM shared functional redundancy with other BH3-only proteins (e.g., HRK).

Both SCG and CG neurons expressed a limited repertoire of BH3-only proteins, including BAD, BID, BIM, and HRK (Figs. 1 and 2; and unpublished data). Since *Hrk*^{-/-} mice are not yet available, we examined whether BAD or BID was responsible for this redundancy. Analysis of cells from *Bid*^{-/-} and *Bad*^{-/-} mice indicated that neither BID nor BAD contributed to neuronal apoptosis in NGF-deprived SCG or K⁺-deprived CG neurons. Furthermore, neither transcriptional nor posttranslational mechanisms appeared to regulate BID or BAD in these paradigms (Figs. 1 and 2).

To our knowledge, TFD in SCG and CG neurons is the only primary cell culture paradigm entirely dependent on the expression and translocation of BAX alone for cyt *c* release, caspase activation, and apoptosis (Deckwerth et al., 1996; Miller et al., 1997; Deshmukh and Johnson, 1998; McGinnis et al., 1999; Putcha et al., 1999, 2000), although this may also be true for other neuronal populations (Deckwerth et al., 1996; Li et al., 2001). Therefore, these models are excellent for studying the physiological mechanisms responsible for endogenous BAX translocation and BAX-dependent cyt *c* release. Moreover, macromolecular synthesis is required for BAX translocation, cyt *c* release, and apoptosis during TFD in SCG neurons (Martin et al., 1988; Deckwerth et al., 1996; Deshmukh and Johnson, 1998; Neame et al., 1998; Putcha et al., 1999), and the BH3-only proteins BIM and HRK are induced in cells destined to die and require BAX to mediate cell death (Imaizumi et al., 1997; Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001). Taken together, these findings clearly suggest that BH3-only proteins may mediate BAX translocation and its downstream sequelae. However, our results are consistent with several possibilities, which need not be mutually exclusive, for how this may occur (Fig. 9 A).

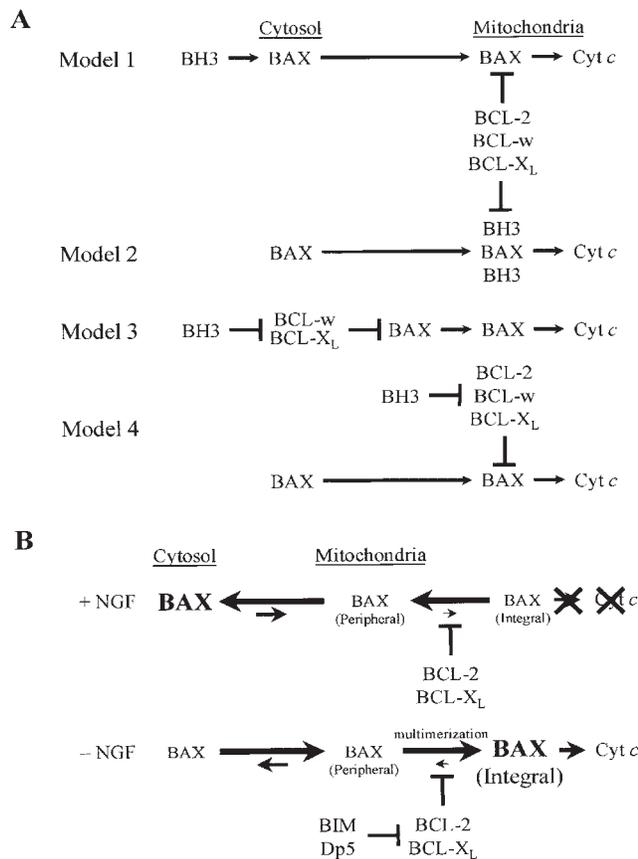


Figure 9. BH3-only proteins indirectly trigger BAX translocation, cytochrome *c* release, caspase activation, and apoptosis during TFD-induced neuronal cell death. (A) Shown are four proposed models depicting how BH3-only proteins, such as BIM and HRK, may promote BAX translocation and its downstream sequelae during TFD in SCG and CG neurons. Models 1 and 2 invoke direct interaction of BH3 donors with BAX, whereas models 3 and 4 involve BH3-only proteins indirectly triggering BAX translocation via inactivating interactions with antiapoptotic BCL-2 proteins. We favor model 4 for reasons discussed in the text. (B) A dynamic equilibrium model for BAX translocation. During TFD in SCG and CG neurons, BIM and HRK are induced and inactivate antiapoptotic BCL-2 proteins, thereby altering the equilibrium between cytosolic and mitochondrial pools of BAX, culminating in apparent BAX translocation, multimerization, and integration, followed by cytochrome *c* release, caspase activation, and apoptotic cell death. See text for more details.

First (model 1), BH3-only proteins may interact directly with BAX in the cytosol to trigger its translocation to mitochondria, where it integrates and mediates the release of apoptogenic intermembrane-space proteins, such as cytochrome *c*. Alternatively (model 2), BH3-only proteins may interact directly with BAX at mitochondria to form a multiprotein complex that mediates cytochrome *c* release. We do not favor these models for several reasons. First, the tertiary structure of soluble, cytosolic BAX seems inconsistent with BAX interacting with BH3-only proteins in the cytosol, arguing against model 1. Specifically, the BH1 and BH2 domains, which presumably would mediate interactions with a “BH3 donor,” are not accessible for such interactions (Suzuki et al., 2000). (Whether the “BH3-binding pocket” formed by BH1 and BH2 becomes accessible upon integration into the

outer mitochondrial membrane by BAX or BAK is unclear.) Second, to date, only three known or putative BH3-only proteins may be cytosolic: BID, BAD, and MAP-1. Although the physiological role of MAP-1 is unknown, deletion of neither BID nor BAD altered the timecourse or extent of TFD-induced cytochrome *c* release or apoptosis in SCG or CG neurons, arguing against model 1 for these proteins (Figs. 5 and 6; and unpublished data). Therefore, neither tBID, BID, nor BAD appear to be the cytosolic triggers for BAX translocation and its downstream sequelae in SCG and CG neurons. Finally, deletion of *Bim* (and presumably *Hrk*) attenuates BAX-dependent cytochrome *c* release (Putcha et al., 2001). However, neither protein appears to be cytosolic (Inohara et al., 1997; Putcha et al., 2001), which argues against model 1, or can interact directly with BAX (Inohara et al., 1997; O’Connor et al., 1998), which argues against models 1 and 2.

Next, BH3-only proteins may indirectly promote BAX-dependent cytochrome *c* release by interacting with and inactivating antiapoptotic BCL-2 proteins, either in the cytosol (model 3) or at mitochondria (model 4), thereby freeing BAX to multimerize and integrate, forming structures that release cytochrome *c*. Both models are generally consistent with the genetics and biochemistry of PCD in *C. elegans* in which a BH3-only protein (i.e., EGL-1 in worms, BIM and HRK in vertebrate neurons) is induced in cells destined to die and interacts with and inactivates an antiapoptotic BCL-2 protein, leading to apoptosome formation, caspase activation, and apoptosis. (A caveat, of course, concerns the requirement for cytochrome *c* release in vertebrates, but not in worms.) However, we favor model 4 for several reasons. First, antiapoptotic BCL-2 proteins may not interact directly with BAX in the cytosol, except in the presence of conformation-altering nonionic detergents (Hsu and Youle, 1997; Antonsson et al., 2001), which may mimic a membrane environment, arguing against model 3, but for model 4. Second, the tertiary structure of BAX may be inconsistent with interactions with antiapoptotic BCL-2 proteins in the cytosol (Suzuki et al., 2000), which argues against model 3, but not model 4. Third, BIM and HRK, two BH3-only proteins that we propose are critical for TFD-induced apoptosis in neurons, do not interact directly with BAX and appear to localize to mitochondria. Moreover, the proapoptotic activity of these proteins generally correlates with their ability to interact with, and inactivate, their antiapoptotic cousins (Wang et al., 1996; Inohara et al., 1997; O’Connor et al., 1998). Finally, this hypothesis may help explain a “threshold effect” observed with both BIM and HRK. Specifically, neurons can tolerate a certain level of BIM and HRK induction without releasing cytochrome *c* or undergoing cell death (Harris and Johnson, 2001; Putcha et al., 2001). For example, during TFD in *Bcl-2*-overexpressing SCG and CG neurons, BIM levels increase with time (to a point greater than that seen in wild-type littermates) and then decline precipitously as cells begin to die; in contrast, in *Bax*^{-/-} neurons, BIM levels continue to increase (to levels exceeding even those seen in *Bcl-2*-overexpressing cells) and only begin to decline days later when significant decreases in global macromolecular synthesis begin to dismantle the synthetic machinery needed to sustain the induction. Accordingly, overexpression of *Bcl-2* only attenuates TFD-induced cytochrome *c* release and apoptosis in SCG and CG

neurons (Tanabe et al., 1998; Putcha et al., 1999), whereas *Bax* deletion completely prevents both (Figs. 1 and 4; Deckwerth et al., 1996; Miller et al., 1997; Deshmukh and Johnson, 1998; Putcha et al., 2000). The same is true for CGN death caused by ectopic overexpression of BH3-only proteins (Fig. 4 C; Harris and Johnson, 2001).

Therefore, we propose that cellular BAX exists in equilibrium between two states (Fig. 9 B), soluble in the cytosol or peripherally associated with mitochondria, in NGF-maintained sympathetic neurons. With TFD, BH3-only proteins are induced and localize to the outer mitochondrial membrane, where they interact with and inactivate antiapoptotic BCL-2 proteins, thereby allowing BAX peripherally associated with mitochondria or translocated from the cytosol to multimerize and integrate. This creates a thermodynamic "sink" that shifts the BAX equilibrium strongly (and perhaps irreversibly) in favor of mitochondrial localization, resulting in further translocation and integration into the mitochondrial outer membrane. In this scenario, inhibitors of de novo protein synthesis should prevent BAX translocation (consistent with our earlier findings [Putcha et al., 1999]) by preventing induction of BH3-only proteins (Putcha et al., 2001). However, the macromolecular synthesis dependence of BAX translocation in this paradigm also has two possible interpretations that may prove indistinguishable by approaches such as immunocytochemistry and subcellular fractionation. First, BH3-only proteins may directly cause BAX translocation, a possibility we do not favor for reasons alluded to above. Second, BH3-only proteins may indirectly promote BAX translocation by inhibiting the inhibitors (i.e., antiapoptotic BCL-2 proteins). If true, this second hypothesis predicts that ectopic overexpression of BH3-only proteins (e.g., BIM, HRK, BID, or tBID) or multidomain proapoptotic proteins (e.g., BAX Δ TM or BAK Δ TM) should induce BAX translocation and BAX-dependent *cyt c* release, caspase activation, and apoptosis with appropriate kinetics in cells that require BAX for these processes. Although incomplete, the available data is consistent with this prediction (Harris and Johnson, 2001; Wei et al., 2001; Zong et al., 2001). Therefore, we suggest that BH3-only proteins are triggers, albeit indirect, for BAX translocation and its downstream sequelae during TFD-induced neuronal apoptosis.

Materials and methods

Reagents

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Other reagents and their sources were collagenase and trypsin (Worthington Biochemical); caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF) (Enzyme Systems Products). Medium lacking NGF (AM0) consisted of Eagle's MEM with Earle's salts (Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 20 μ M fluorodeoxyuridine, 20 μ M uridine, and 3.3 μ g/ml aphidicolin. AM50 medium consisted of AM0 medium plus 50 ng/ml mouse 2.5S NGF (Harlan Bioproducts). For CGNs, two different media were used: K5+S (basal medium Eagle's [Life Technologies] containing 10% dialyzed fetal bovine serum, 5 mM KCl, 100 U/ml penicillin, and 100 μ g/ml streptomycin) and K25+S (K5+S plus 20 mM KCl). The breeding and genotyping of *Bax*^{-/-}, *Bak*^{-/-}, *Bad*^{-/-}, *Bid*^{-/-}, and *Bim*^{-/-} mice have been described previously (Knudson et al., 1995; Bouillet et al., 1999; Yin et al., 1999; Lindsten et al., 2000).

Sympathetic neuronal cultures

Primary cultures of sympathetic neurons were established from the SCG of neonatal mice or rats by using previously described methods (Johnson and

Argiro, 1983; Deckwerth et al., 1996). Neurons were grown in AM50 for ~5 d in vitro (DIV) and then either maintained in AM50 or treated as follows: For NGF deprivation, cultures were rinsed with AM0, followed by the addition of AM0 containing goat anti-mouse 2.5S NGF neutralizing antiserum (anti-NGF) (Ruit et al., 1990). For NGF deprivation in the presence of BAF, 50 μ M BAF was added to AM0 containing anti-NGF.

CGN cultures

Primary cultures of CGNs were obtained as described previously (Miller and Johnson, 1996; Miller et al., 1997). Briefly, postnatal day 7 (P7) cerebella were dissected, trypsinized, triturated, and plated into K25+S medium at a density of 2.3×10^5 cells/cm² in four-well dishes (Nunc) coated with 0.1 mg/ml poly-L-lysine. To reduce the number of nonneuronal cells, 3.3 μ g/ml aphidicolin was added to the medium 36 h after plating. At DIV7, CGNs were either maintained in K25+S or switched to K5+S after washing once with the respective medium. Cell viability was then assessed at the indicated times by taking photomicrographs of representative fields of cells labeled with calcein AM (Molecular Probes) as described previously (Miller and Johnson, 1996). Alternatively, viability was assessed by lysing the calcein AM-stained cells and measuring the fluorescence on a TiterTek fluorescence plate reader (emission = 485/excitation = 538).

RT-PCR analysis, CGN transfection, subcellular fractionation, alkali extraction, and immunoblotting

RT-PCR analysis, CGN transfection, fractionation, extraction, and immunoblotting of sympathetic neurons were performed as described previously (Estus et al., 1994; Putcha et al., 2000). Primary antibodies included α -tubulin (1:100000; Sigma-Aldrich); Bad (C-20; 1:200; Santa Cruz Biotechnology, Inc.); Bak (1:1000; Upstate Biotechnology); Bax (1:1000; Upstate Biotechnology); Bid (1:1000; Zymed or 1:1000; R&D Systems); Bim/Bod (1:1000; StressGen); Bcl-2 (10C4; 1:500; NeoMarkers); Bcl-X_L (H-9; 1:1000; Santa Cruz Biotechnology, Inc.); *cyt c* (1:1000; BD PharMingen); cytochrome oxidase subunit IV (COX4) (Molecular Probes); lactate dehydrogenase (1:1000; Rockland Immunochemicals); neuron-specific enolase (1:1000; Chemicon); phospho-c-Jun S63 (P-Jun) (1:1000; New England Biolabs, Inc.). Bcl-w antibodies were provided by L.A. O'Reilly and S. Cory (Walter and Eliza Hall Institute, Melbourne, Australia). Appropriate HRP-conjugated secondary antibodies (NEB and Jackson ImmunoResearch Laboratories) were diluted 1:5000–1:10000.

All antibodies (except those for Bcl-2, Bcl-w, and Bcl-X_L that had been tested in other contexts) were tested for specificity by immunoblotting with tissues (e.g., spleen, thymus, liver, kidney, cortex, and cerebellum) from knockout mice. In our experience, many antibodies to Bcl-2 family proteins are not specific by this criterion. Even fewer are specific by immunocytochemistry. Although we have found several examples of antibodies that are specific by Western but are not specific by immunocytochemistry, the converse has never been true. A listing of the antibodies for which we have results is available online at <http://www.jcb.org/cgi/content/full/jcb.200110108/DC1>.

Cell counts: commitment point experiments

Sympathetic neurons from mice of the indicated genotypes were deprived of NGF as described above. At various times after deprivation, cultures were rinsed with AM0 and then incubated in AM50 for 5–7 d. Neurons were then counted after washing with PBS, fixing with 4% paraformaldehyde for 30 min at 4°C, washing again with PBS, and staining with toluidine blue as described previously (Deshmukh et al., 1996).

Cell counts: P-Jun and Cyt c immunocytochemistry

Neuronal cultures were immunostained as described previously (Putcha et al., 1999). Briefly, sympathetic neurons that had been maintained in NGF for ~5 DIV in AM50 were deprived of NGF in the presence of the caspase inhibitor BAF (as described above). At various times after deprivation, cultures were fixed and immunostained with anti-P-Jun (1:200; NEB) or anti-*cyt c* (1:1000; BD PharMingen) antibodies. The respective secondary antibodies were Cy3-conjugated donkey anti-rabbit and anti-mouse IgG (1.5 mg/ml; Jackson ImmunoResearch Laboratories), respectively, diluted 1:400. For each time point, the number of cells that had acquired a nuclear staining pattern for P-Jun or had lost the punctate staining pattern for *cyt c* was determined by a naïve observer from a random sampling of 100–200 cells. All experiments were conducted in the presence of BAF to prevent any cell loss that would otherwise affect the counts.

For acute rescue experiments in which NGF was readded to cultures after 18 h of NGF deprivation, cultures were rinsed with AM0 and then incubated in AM0 medium containing 300 ng/ml NGF in the presence of 50 μ M BAF.

Online supplemental material

Online supplemental Figs. S1–S4 show representative immunoblots of various commercially available antibodies to BAX (Fig. S1), BAK (Fig. S2), BIM (Fig. S3), and BID (Fig. S4) tested in tissues from the respective knock-out mice and their wild-type littermates. Similar experiments were performed for antibodies to other cell death–related proteins (e.g., Apaf-1, caspases, and other BCL-2 family proteins). In addition, immunocytochemistry experiments were conducted in SCG and/or CG neurons with certain antibodies. We occasionally did not evaluate antibodies that proved nonspecific by immunoblotting for immunocytochemistry because, in our experience, although some antibodies are specific by immunoblotting but not by immunocytochemistry, the converse has never been true. (That is, every antibody that is nonspecific by immunoblotting is also nonspecific by immunocytochemistry.) All results are tabulated in Table S1.

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