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BCL-2, BCL-X_L Sequester BH3 Domain-Only Molecules Preventing BAX- and BAK-Mediated Mitochondrial Apoptosis

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Summary

Critical issues in apoptosis include the importance of caspases versus organelle dysfunction, dominance of anti- versus proapoptotic BCL-2 members, and whether commitment occurs upstream or downstream of mitochondria. Here, we show cells deficient for the downstream effectors Apaf-1, Caspase-9, or Caspase-3 display only transient protection from "BH3 domain-only" molecules and die a caspase-independent death by mitochondrial dysfunction. Cells with an upstream defect, lacking "multidomain" BAX, BAK demonstrate long-term resistance to all BH3 domain-only members, including BAD, BIM, and NOXA. Comparison of wild-type versus mutant BCL-2, BCL-X_L indicates these antiapoptotics sequester BH3 domain-only molecules in stable mitochondrial complexes, preventing the activation of BAX, BAK. Thus, in mammals, BH3 domain-only molecules activate multidomain proapoptotic members to trigger a mitochondrial pathway, which both releases cytochrome c to activate caspases and initiates caspase-independent mitochondrial dysfunction.

Introduction

Core components of the cell death pathway have been identified by genetic and biochemical approaches and have proven conserved from nematodes to mammals. Downstream of death signals, execution programs, which include a cascade of caspase enzymes and mitochondrial organelle dysfunction, manifest irreversible damage to cellular constituents (Green and Reed, 1998). The BCL-2 family of proteins is a critical death regulator that resides immediately upstream of the mitochondria (Adams and Cory, 1998). Genetic analysis of the developmental cell deaths in the nematode *Caenorhabditis elegans* identified four components of the core death pathway: a "BH3 domain-only" molecule EGL-1, an antiapoptotic BCL-2 homolog CED-9, a caspase CED-3, and its adaptor CED-4 (Horvitz, 1999). In one example of a mammalian cascade, the initiator Caspase-9 is activated upon binding with a CED-4 homolog, Apaf-1 (Zou et al., 1997), in a complex with cytochrome c, which subsequently activates the effector Caspase-3 (Li et al., 1997).

Multiple apoptotic signals release cytochrome c from the mitochondrial intermembrane space to activate Apaf-1, coupling this organelle to caspase activation. BCL-2 family members are major regulators of mitochondrial integrity and mitochondria-initiated caspase activation. The BCL-2 family possesses both antiapoptotic and proapoptotic members. In general, the antiapoptotic members display sequence conservation throughout all four BCL-2 homology domains (BH1-4). Proapoptotic BCL-2 members can be further subdivided into more fully conserved, "multidomain" members possessing homology in BH1-3 domains and "BH3 domain-only" members that display sequence homology only within this amphipathic α-helical segment which serves as the critical death domain (Chittenden et al., 1995; Wang et al., 1996). The ratio between the antiapoptotic and the multidomain proapoptotic BCL-2 members helps determine the susceptibility of cells to a death signal (Oltvai et al., 1993). Evolving evidence indicates the multidomain members demonstrate active and inactive conformations. In viable cells, the multidomain proapoptotic members appear to be inactive monomers residing at the mitochondria (BAK) or in the cytosol (BAX) (Wolter et al., 1997). Following multiple death stimuli, BAX translocates to and is found homooligomerized at mitochondria, while BAK is also homooligomerized into higher order multimers apparently representing an active conformation (Gross et al., 1998; Wei et al., 2000).

BH3 domain-only molecules display differential interactions with selected multidomain BCL-2 members as assessed by multiple binding assays (O'Connor et al., 1998; Oda et al., 2000; Wang et al., 1996; Yang et al., 1995). This prompted a model in which the BH3 domain-only molecules could be subdivided into two groups: molecules typified by BID, which can bind and appear to activate proapoptotic BAX, BAK versus molecules such as BIM, BAD, and NOXA, which preferentially bind to and thus are presumed to function by inhibiting antiapoptotic BCL-2, BCL-X_L (Huang and Strasser, 2000; Kelekar and Thompson, 1998). The proapoptotic activity of BH3 domain-only molecules is kept in check by either transcriptional control or posttranslational modifications. Caspase-8 cleavage of cytosolic BID is followed by its translocation to mitochondria (Luo et al., 1998), resulting in an allosteric conformational activation of BAK and BAX, including their intramembranous oligomerization (Eskes et al., 2000; Wei et al., 2000). Studies of recombinant BAX in liposomes indicate it can form a multimeric pore capable of the initial efflux of cyto-

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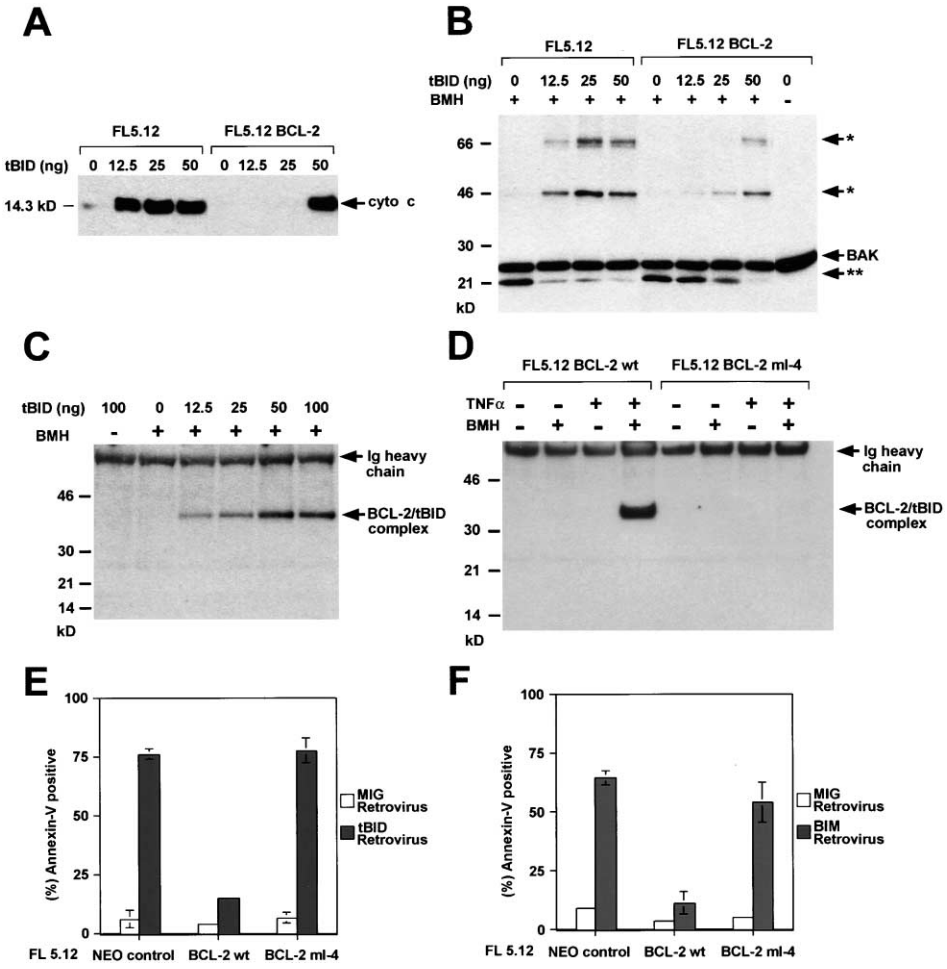

Figure 1. BCL-2 Inhibits Apoptosis by Sequestering the BH3 Domain-Only Molecules

(A) Mitochondria isolated from FL5.12 parental cells and cells expressing BCL-2 were incubated with increasing concentrations of recombinant tBID for 30 min at 30°C, after which supernatants were analyzed for cytochrome c by immunoblotting.

(B) Mitochondria isolated from FL5.12 parental cells or BCL-2 expressing cells were incubated with recombinant tBID for 30 min at 30°C and then treated with 10 mM BMH crosslinker. The conformation of BAK was determined by immunoblot. The single asterisk denotes BAK complexes consistent with dimers or trimers. The double asterisk denotes the inactive BAK conformer.

(C) Mitochondria isolated from BCL-2 expressing FL5.12 cells were incubated with the indicated amounts of recombinant tBID for 30 min at 30°C and then treated with 10 mM BMH crosslinker. The mitochondrial pellets were lysed in RIPA buffer, followed by immunoprecipitation with the 6C8 anti-BCL-2 monoclonal Ab. The BCL-2/tBID complex was detected by an anti-BID Ab immunoblot.

(D) FL5.12 cells expressing either wild-type (wt) or mutant (G145E) BCL-2 were subjected to TNFα plus CHX for 5 hr and then mitochondria were isolated and incubated with BMH crosslinker. Immunoprecipitation was performed as in (C). The BCL-2/tBID complex was detected by an anti-BID Ab immunoblot.

(E) FL5.12 parental (Neo) cells or cells expressing wt BCL-2 or BCL-2 ml-4 (G145E) were infected with tBID expressing or control (MIG) retroviruses. Death of GFP expressing transduced cells is presented as mean ± 1 SD of Annexin-V positive cells at 24 hr post infection from three experiments.

(F) Cells as in (E) were infected with BIM expressing or control (MIG) retroviruses, and death was quantitated.

chrome c (Saito et al., 2000). Additional events transpire in vivo which result in the release of even larger molecules (Kluck et al., 1999; Marzo et al., 1998; Shimizu et al., 1999).

Unresolved issues include whether anti- or proapoptotic BCL-2 members will prove dominant. A major uncertainty is whether antiapoptotic BCL-2 members sequester another "CED-4 like" molecule at the mitochondria, just as Apaf-1 localizes to cytosol and does not bind substantially to BCL-2 members (Hausmann et al., 2000). Finally, it is critical to determine whether the definitive commitment to apoptosis occurs proximal to mitochondria or whether it can be distal to mitochondria.

Results

BCL-2 and BCL-X_L Sequester BH3 Domain-Only Molecules, Preventing the Allosteric Activation of BAK and the Release of Cytochrome C

To assess the role of antiapoptotic BCL-2 and BCL-X_L, we turned to purified mitochondria. Mitochondria isolated from FL5.12 parental cells that are susceptible

to apoptotic stimuli released cytochrome c after addition of recombinant tBID. Mitochondria from BCL-2-expressing cells displayed a dose-dependent resistance to tBID-induced cytochrome c release (Figure 1A). While 12.5 ng of recombinant tBID was sufficient to release all the intramitochondrial cytochrome c from FL5.12 mitochondria, 50 ng was required for BCL-2-overexpressing mitochondria. Addition of tBID eliminated the "inactive" BAK conformer and shifted BAK into higher order crosslinked complexes while the presence of BCL-2 blocked this dose-dependent tBID effect (Figure 1B).

Irreversible, homobifunctional maleimide crosslinkers demonstrated a stable BCL-2/tBID complex (Figures 1C and 1D). A ~40 kDa tBID/BCL-2 complex consistent with a heterodimer of one BCL-2 molecule (25 kDa) plus one tBID molecule (15 kDa) reached a plateau at ≥50 ng recombinant tBID added to mitochondria (100 μg of mitochondrial total protein) (Figure 1C). The binding capacity of BCL-2 at mitochondria is saturable and correlates with availability of free tBID to activate BAK and release cytochrome c (Figures 1A–1C).

In intact cells expressing BCL-2, an abundant ~40 kDa tBID/BCL-2 complex was present after TNFα treatment (Figure 1D). We next assessed BCL-2 loss-of-function mutant (BCL-2 ml-4) bearing a G145E mutation within BH1 (Yin et al., 1994) which failed to form complexes with tBID (Figure 1D). Cells expressing the BCL-2 G145E mutant but not wild-type (wt) BCL-2 underwent apoptosis following infection with a tBID expressing retrovirus (Figure 1E). Moreover, BIM also killed cells with mutant BCL-2 ml-4 but not wt BCL-2 (Figure 1F). Consistent with this, stable complexes of BIM and tBID were detected with wt but not mutant BCL-2 (data not shown).

Antiapoptotic BCL-2, BCL-X_L could function by heterodimerizing with multidomain BAX, BAK effectors or the BH3 domain-only ligands. Extensive mutagenesis of BCL-X_L suggested that BCL-X_L can inhibit apoptosis independent of its heterodimerization with BAX or BAK (Cheng et al., 1996). Such a mutant BCL-X_L bearing F131V and D133A substitutions (designated mt1) that does not bind BAX or BAK could still be coimmunoprecipitated with tBID, BIM, or BAD (Figures 2A and 2B). In contrast, a loss-of-function mutant of BCL-X_L with G138E, R139L, and I140N substitutions (designated mt8), which failed to protect cells from apoptotic signals, failed to bind tBID, BIM, or BAD (Figures 2A and 2B). When MEFs were sequentially infected with retroviruses expressing BCL-X_L and tBID, BIM, or BAD, we found the ability of BCL-X_L proteins to inhibit apoptosis mirrored their ability to bind tBID, BIM, or BAD (Figure 2C).

Absence of Downstream Effectors Provides Transient Protection, while Absence of BAX, BAK Confers Long-Term Resistance

We compared cells deficient for *Apaf-1* (Yoshida et al., 1998), *Caspase-9* (Kuida et al., 1998), or *Caspase-3* (Kuida et al., 1996) bearing a defect downstream of mitochondria with *Bax, Bak* DKO cells inhibited upstream of mitochondria. *Apaf-1*-, *Caspase-9*-, or *Caspase-3*-deficient MEFs remained viable for 24 hr following retroviral expression of GFP and tBID, exhibiting normal morphology and remaining Annexin-V negative. However, by 48 hr and beyond, these tBID-expressing MEFs

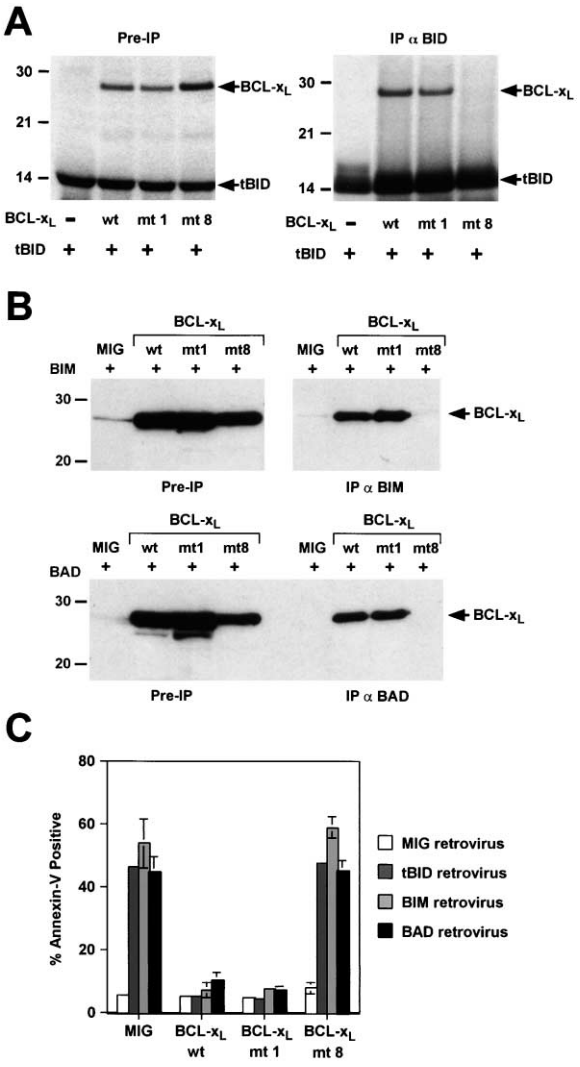

Figure 2. The Antiapoptotic Activity of BCL-X_L Correlates with Its Ability to Bind to BH3 Domain-Only Members, Not BAX, BAK

(A) BCL-X_L wt or BCL-X_L mutants (mt1, mt8) and tBID were cotranslated in vitro in the presence of [^{35}S]methionine. An autoradiogram revealed each translation reaction contained approximately equivalent amounts of BCL-X_L and tBID (Pre-IP, left panel). Reticulocyte lysates in 0.2% NP-40 lysis buffer were immunoprecipitated with anti-BID Ab and analyzed on 10% Nu-PAGE gels followed by autoradiography (IPα BID, right panel). The same pattern of tBID heterodimerization was confirmed in MEFs following retroviral transduction.

(B) Coimmunoprecipitation of BIM or BAD with wt or mutant BCL-X_L. MEFs were serially infected with retrovirus expressing BCL-X_L wt or BCL-X_L mt1, mt8, or control retrovirus (MIG) followed by BIM, BAD, or control (MIG) virus. Cells lysed in 0.2% NP-40 buffer were immunoprecipitated with anti-BIM or anti-BAD Ab and complexes detected by anti-BCL-X Ab. Equal amounts of BIM or BAD were immunoprecipitated in each lane (data not shown).

(C) SV40-transformed MEFs were infected with retrovirus expressing BCL-X_L wt or mt1, mt8 or control virus (MIG). After 24 hr, the cells were transduced with tBID, BIM, or BAD expressing or control (MIG) retrovirus. Cell death was quantitated after another 24 hr for tBID and BIM or 36 hr for BAD. Values shown are mean ± 1 SD of three experiments.

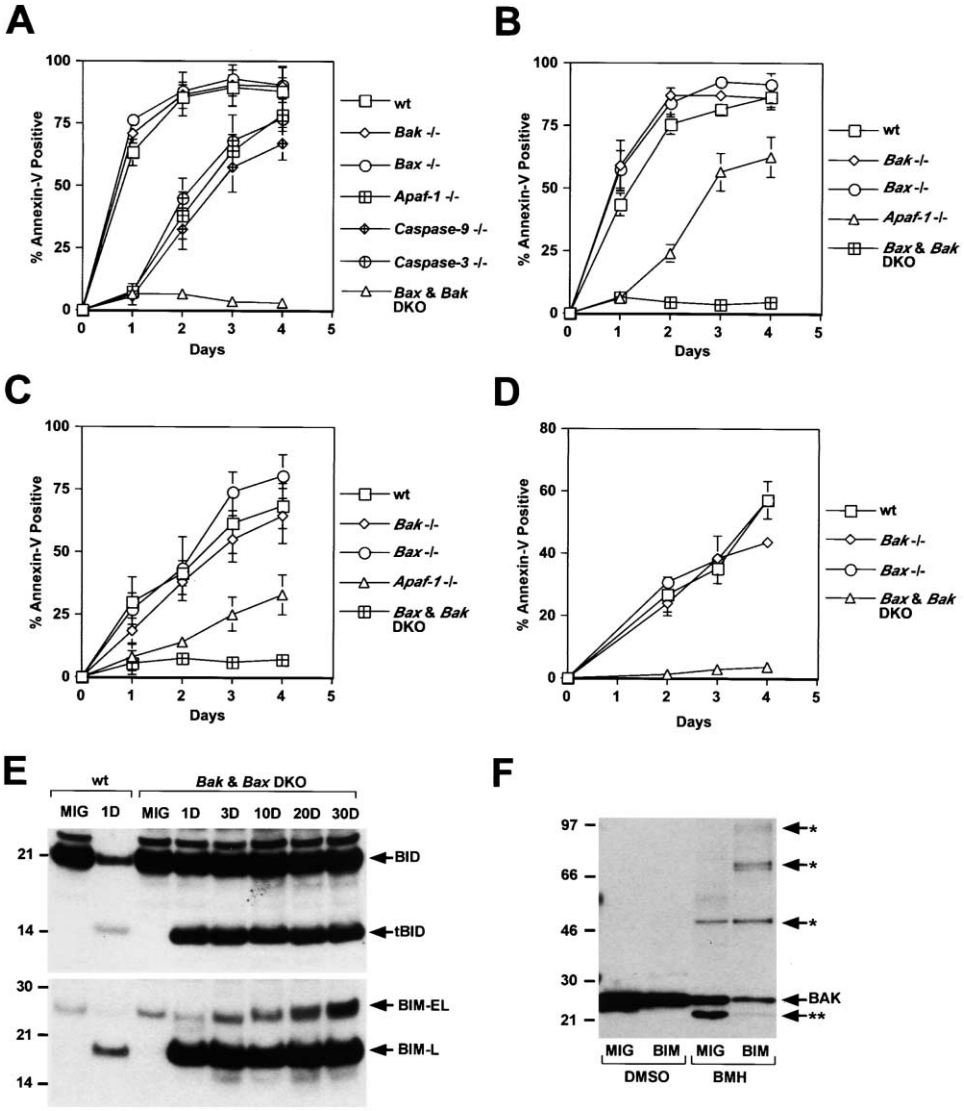

Figure 3. Deficiency of Proapoptotic Molecules Upstream of Mitochondria (BAX, BAK) but Not Downstream of Mitochondria (Apaf-1, Caspase-9, Caspase-3) Provides Long-Term Protection from tBID, BIM, BAD, and NOXA

(A) Time course of tBID-induced apoptosis in selected transformed MEFs. Cell death was quantitated as described above.

(B) BIM-induced apoptosis in selected MEFs.

(C) BAD-induced apoptosis in selected MEFs.

(D) NOXA-induced apoptosis in selected MEFs.

(E) BID and BIM immunoblots of whole cell lysates (50 μg protein) from MEFs infected with tBID or BIM-L expressing or control (MIG) retrovirus at indicated time points. BID denotes endogenous expression in MEFs, whereas tBID represents retroviral vector mediated expression. BIM-EL denotes endogenous expression in MEFs, whereas BIM-L is of retroviral vector origin.

(F) Mitochondria isolated from wt MEFs infected with BIM expressing or control retrovirus (MIG) were treated with the crosslinker BMH or with DMSO control buffer. BAK species were assessed by immunoblot where single asterisk denotes crosslinked BAK complexes consistent with dimers, trimers, or tetramers. The double asterisk denotes the inactive BAK conformer reflecting an intramolecular crosslink.

began to die, displaying progressive Annexin-V staining as well as uptake of the vital dyes trypan blue and propidium iodide. The survival curves of *Apaf-1*-, *Caspase-9*-, or *Caspase-3*-deficient cells were similar and intermediate between wt and *Bax, Bak* doubly deficient cells. In contrast, *Bax, Bak* doubly deficient cells proved completely resistant (Figure 3A), as they continued to express GFP in 90%–95% of cells and remained negative for Annexin-V (<10%) over a period of 35 days. An immunoblot substantiated high levels of tBID in long-term viable DKO cells in contrast to low levels in wt cells, which were killed by tBID (Figure 3E).

The *Bax, Bak* DKO cells also proved completely resistant to all tested BH3 domain-only molecules, including BIM, BAD, and NOXA, which demonstrate selective interaction with antiapoptotic BCL-2 members (Figures 3B–3D). Long-term survival assessed over 30 days occurred despite high level expression of these BH3 domain-only molecules, as evidenced by BIM immunoblot (Figure 3E). MEFs singly deficient for *BAX* or *BAK* were

as sensitive as wt cells to BIM, BAD, or NOXA (Figures 3B–3D). Reexpression of BAX or BAK alone was insufficient to kill, but did restore the capacity of BIM or BAD to kill DKO MEFs (data not shown). In contrast, MEFs deficient in downstream *Apaf-1* once again displayed only early protection from BIM- or BAD-induced death (Figures 3B and 3C). This constellation of findings supports a model in which BIM, BAD, or NOXA require and work upstream to induce the activation of multidomain BAX, BAK. Consistent with this, BIM eliminated the faster-migrating inactive BAK conformer and increased the amount of BAK in higher order oligomers consistent in size with dimers, trimers, and tetramers (Figure 3F). Similar findings were observed in MEFs expressing the other BH3 domain-only proteins.

Apaf-1-Deficient Cells Demonstrate a Caspase-Independent Death by Mitochondrial Dysfunction

Overexpression of tBID, BIM, or BAD resulted in activation of Caspase-3 in wild-type, *Bax*, or *Bak* singly deficient cells, but not in either DKO or *Apaf-1*-deficient cells. Importantly, no activated Caspase-3 was observed in *Apaf-1*-deficient cells up to 4 days post expression of the BH3 domain-only molecules, a time when cell death was already evident. Fluorogenic substrate assays using either DEVD-AFC for Caspase-3 and -7, VDVAD-AFC for Caspase-2, or VEID-AFC for Caspase-6 also revealed no measurable activity in *Apaf-1*-deficient cells expressing tBID, BIM, or BAD (data not shown). A broad-spectrum caspase-inhibitor zVAD-fmk did not prolong the survival of *Apaf-1*-deficient cells either.

tBID expressing *Apaf-1*$^{-/-}$ cells released cytochrome c, but did not exhibit marked chromatin condensation or nuclear fragmentation as determined by Hoechst staining. By 40–48 hr post retroviral infection, the tBID expressing *Apaf-1*-deficient cells exhibited substantial mitochondrial depolarization as evidenced by loss of TMRM staining, a mitochondria-specific fluorophore (Figure 4A). In contrast, tBID expressing *Bax, Bak* DKO cells displayed strong mitochondrial TMRM staining (Figure 4A).

Discussion

We demonstrate that all tested BH3 domain-only molecules kill cells deficient for the downstream effectors *Apaf-1*, *Caspase-9*, or *Caspase-3* by a caspase-independent process of mitochondrial dysfunction. The absence of detectable caspase activity even in the presence of BCL-2, BCL-X_L argues against the participation of an unidentified upstream CED-4-like molecule in mammals (Figure 4B). In contrast, all BH3 domain-only members absolutely require multidomain BAX, BAK to induce apoptosis, regardless of their selective binding affinity. Thus, the definitive commitment to cell death occurs proximal to mitochondria at the step of BAX, BAK activation. Finally, instructive BCL-2, BCL-X_L mutant molecules strongly support a model in which a principal role of antiapoptotic BCL-2, BCL-X_L is to sequester arriving BH3 domain-only molecules in stable mitochondrial complexes, thus preventing the allosteric activation of BAX, BAK and a subsequent mitochondrial program of apoptosis (Figure 4B).

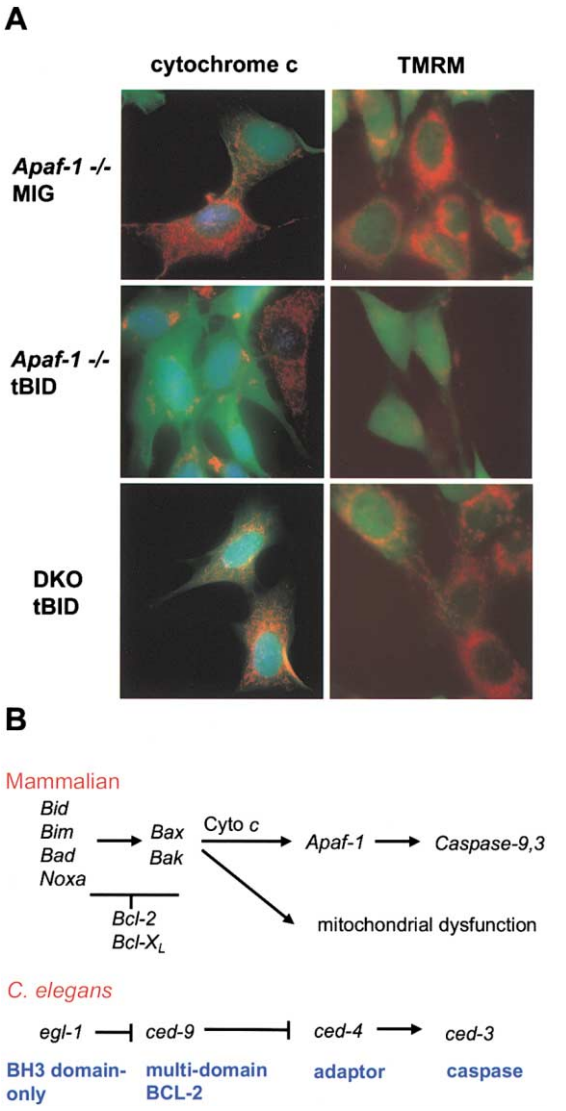

Figure 4. Mitochondrial Dysfunction and the Core Apoptotic Pathway

(A) tBID triggers cytochrome c release and mitochondrial depolarization in *Apaf-1*-deficient, but not *Bax*, *Bak* doubly deficient (DKO), MEFs. MEFs were infected with tBID expressing or control retrovirus. Cells were fixed after 40 hr and assessed for transduction by GFP expression (green), nuclear morphology by Hoechst dye (blue), and immunostained with anti-cytochrome c antibody (red). An overlap of GFP and cytochrome c results in an orange yellow hybrid color (left panel). To monitor mitochondrial depolarization, MEFs were loaded with TMRM, which is retained proportional to mitochondrial transmembrane potential (right panel). Red fluorescence (TMRM) and green fluorescence (GFP) were monitored simultaneously in living cells (right panel).

(B) Model of Anti- and Proapoptotic BCL-2 Member Regulation of Apoptosis

Schematic representation of the mammalian core apoptotic pathway based on data in this paper compared to the pathway in *C. elegans*. Both display a pathway in which a BH3 domain-only interacts with a multidomain BCL-2 member upstream of an adaptor and a caspase. All mammalian pathway BH3 domain-only molecules require multidomain proapoptotic BAX, BAK to initiate a cytochrome c, Apaf-1-driven caspase activation and a caspase-independent mitochondrial dysfunction. Antiapoptotic BCL-2, BCL-X_L inhibit the activated BH3 domain-only molecules.

A collection of seemingly disparate prior observations now fit a common construct. A simple "displacement model" in which BH3 domain-only molecules occupy BCL-2, BCL-X_L and thereby prevent their binding to and inhibition of BAX, BAK (Yang et al., 1995) does not appear to fully account for the findings. One possibility is that BH3 domain-only molecules result in the allosteric conformational activation of BAX, BAK through direct or indirect mechanisms. In viable, normal cells the majority of proapoptotic BAX, BAK versus antiapoptotic BCL-2, BCL-X_L molecules are in different compartments. Most of the BCL-2, BCL-X_L is not complexed with BAX, BAK prior to the addition of detergent (Gross et al., 1998; Hsu and Youle, 1997). The three-dimensional structure of BAX and molecular modeling of BAK indicate that heterodimerization with BCL-2, BCL-X_L would require a conformational alteration (Sattler et al., 1997; Suzuki et al., 2000). Consistent with this, we have noted an increase in BCL-2/BAK heterodimers following death stimuli, suggesting that activation of BAK enables its binding to BCL-2 (Figure 4B). A genetic approach to this question which crossed gain- and loss-of-function models of *Bcl-2* and *Bax* indicated that BCL-2 and BAX could function independent of one another (Knudson and Korsmeyer, 1997). BAX, which is downstream, does not require BCL-2 to promote apoptosis, and the capacity of BCL-2 to repress apoptosis in the absence of BAX can be attributed to sequestering BH3 domain-only molecules. The competition for BH3 domain-only molecules by BCL-2, BCL-X_L versus BAX, BAK demonstrated here helps explain how the ratio between anti- and proapoptotic subfamilies serves as a rheostat that determines susceptibility (Oltvai et al., 1993). Activation of BAX, BAK by BH3 domain-only molecules leads to cytochrome c efflux and, later, to mitochondrial depolarization (Figure 4A). Depletion of cytochrome c will lead to respiratory chain defects which at early time points can be rescued by exogenous cytochrome c (Mootha et al., 2001). However, at later time points, irreversible damage occurs to mitochondria.

The basic tenets of a core apoptotic pathway appear common to all metazoans (Figure 4B). Mammals evolved additional complexity as BCL-2 members expanded in number to accommodate a panoply of death signals in multiple cell types. The added multidomain proapoptotic BAX, BAK molecules are essential core components required for the function of all upstream BH3 domain-only members, whereas antiapoptotic multidomain BCL-2, BCL-X_L principally sequester BH3 domain-only rather than CED-4 like molecules. Of note, in *Drosophila* the multidomain BCL-2 homologs to date have all proven proapoptotic (Vernooy et al., 2000). Moreover, *C. elegans* CED-9 has a death-promoting as well as a death-protecting activity, since the presence of CED-9 in certain mutant backgrounds increases cell deaths (Hengartner and Horvitz, 1994). Finally, comparison of cells with defects upstream and downstream of mitochondria indicates a lethal program of mitochondrial dysfunction can still kills cells in the absence of detectable caspase activation (Figure 4B).

Experimental Procedures

Plasmid Construction and Retrovirus Production

Expression plasmids including pcDNA3-t*Bid*, and SG5-*Bcl-*x_L wild-type, mutant 1, or mutant 8 were described previously (Cheng et al., 1996). Murine t*Bid*, *Bim-L*, *Bad*, *Bax*, and *Bak* as well as human *Bcl-*x_L wild-type, mt 1, and mt 8 were cloned into the retroviral expression vector MSCV-IRES-GFP (pMIG).

Amphotropic retroviruses were generated by transfecting 293 GPG packaging cell line with pMIG vector containing indicated Bcl-2 members. Using this approach, we can routinely transduce more than 90% (95% on average) of SV40 transformed MEFs, as determined by GFP expression. The level of infection obtained with primary MEFs was typically less (~50%–80%). Spin inoculation of retrovirus was utilized to infect FL5.12 cells. Routinely, ~60% of FL5.12 cells were infected as determined by flow cytometric detection of GFP.

Cell Viability Assay

Cell death was quantitated by Annexin-V-Cy3 (BioVision) staining according to manufacturer's protocols, followed by flow cytometric analysis using a FACScalibur (Becton Dickinson). For retroviral infection of primary MEFs and FL5.12 cells, the uninfected population (GFP negative cells) was excluded for the determination of % Annexin-V positivity due to a 50%–80% infection rate.

Immunoprecipitation

Immunoprecipitation of tBID/BCL-X_L, BIM/BCL-X_L, and BAD/BCL-X_L complexes was performed as previously described (Cheng et al., 1996). For immunoprecipitation of the BCL-2/tBID complex, mitochondrial lysates (in RIPA buffer) were immunoprecipitated with 6C8 anti-BCL-2 antibody, separated on NuPAGE (Novex) gels, and analyzed by anti-BID as well as anti-BCL-2 immunoblotting.

Immunoblot Analysis

Antibodies used for immunoblot included anti-cytochrome c (75981A, Pharmingen), anti-BAK (Upstate Biotechnology), anti-BID (Wang et al. 1996), anti-BCL-2 (6C8 and /100, Pharmingen), anti-BIM (Calbiochem), anti-BAD (C20, Santa Cruz), and anti-BCL-X (7B2). Antibody detection was accomplished using an enhanced chemiluminescence method (New England Nuclear).

Indirect Immunofluorescence Microscopy and TMRM labeling

Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were sequentially incubated with anti-cytochrome c antibody (clone 6H2.B4, Pharmingen), Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (Molecular Probes), and Hoechst 33258 (Molecular Probes). Images were acquired using a SPOT camera (Diagnostics, Inc.) mounted on Nikon Eclipse E600.

For monitoring mitochondrial membrane potential, cells following retroviral infection were loaded with 20 nM tetramethylrhodamine methylester (TMRM) (Molecular Probes) in PBS at 37°C for 20 min. Images were collected using an inverted microscope (Olympus IX50).

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