PROGRAMMED CELL DEATH AND THE REGULATION OF HOMEOSTASIS

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I. INTRODUCTION

Programmed cell death plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms. Genetic and molecular analysis from nematodes to humans has indicated that the pathway of cellular suicide is highly conserved. Although the capacity to carry out apoptosis seems to be inherent in all cells, the susceptibility to apoptosis varies markedly and is influenced by external and cell-autonomous events. Considerable progress has been made in identifying the molecules that regulate the apoptotic pathway at each level. Of note, both positive and negative regulators, often encoded within the same family of proteins, characterize the extracellular, cell surface, and intracellular steps (Fig. 1).

A variety of physiologic death signals as well as pathological cellular insults trigger the genetically programmed pathway of apoptosis (Vaux and Korsmeyer, 1999). Apoptosis manifests in two major downstream execution programs: the caspase pathway and organelle dysfunction, of which mitochondrial dysfunction is best characterized (Green and Reed, 1998; Thornberry and Lazebnik, 1998) (Fig. 1). The BCL-2 family members play a pivotal role in deciding whether a cell will live or die as they reside upstream to irreversible cellular damage and focus much of their efforts at the level of mitochondria (Fig. 1).

The founder of this family, the BCL-2 proto-oncogene, was discovered at the chromosomal breakpoint of t(14;18) bearing human B-cell lymphomas. The BCL-2 family of proteins has markedly expanded and includes both pro- as well as antiapoptotic molecules (Fig. 2). Indeed, the ratio between

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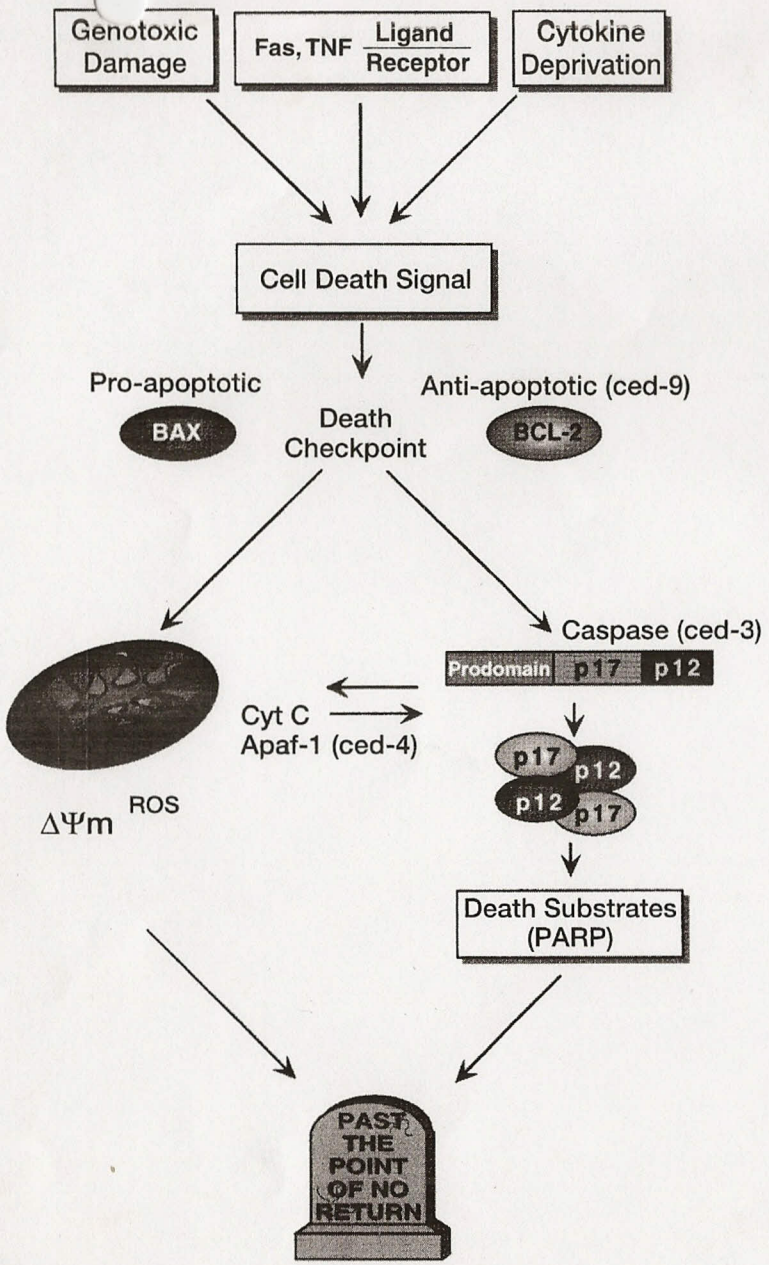

these two subsets helps determine, in part, the susceptibility of cells to a death signal (Oltvai et al., 1993) (Fig. 1). An additional characteristic of members of this family is their frequent ability to form homo- as well as heterodimers, suggesting neutralizing competition between these proteins. A further characteristic of probably functional significance is their ability to become integral membrane proteins. The BCL-2 family members possess up to four conserved BCL-2 homology (BH) domains designated BH1, BH2, BH3, and BH4, which correspond to α helical segments (Adams and Cory, 1998; Kelekar and Thompson, 1998; Yin et al., 1994) (Fig. 2). Many of the antiapoptotic members display sequence conservation in all four domains. The proapoptotic molecules frequently display less sequence conservation of the first α helical segment, BH4. Deletion and mutagenesis studies argue that the amphipathic α helical BH3 domain serves as a critical death domain in the proapoptotic members. This concept is supported by an emerging subset of "BH3-domain only" members that display sequence homology only within the BH3 domain and to date are all proapoptotic. However, the three-dimensional structure of at least one "BH3-domain only" molecule, BID, demonstrates a very similar overall α helical content to the antiapoptotic molecule BCL-X_L (Chou et al., 1999; McDonnell et al., 1999). Many BCL-2 family members also carry a C-terminal hydrophobic domain, which, in the case of BCL-2, is essential for its targeting to membranes such as the mitochondrial outer membrane (Nguyen et al., 1993).

Substantial amounts of the pro- versus the antiapoptotic BCL-2 members localize to separate subcellular compartments before a death signal. Antiapoptotic members are initially integral membrane proteins found in the mitochondria, endoplasmic reticulum, or nuclear membrane (de Jong et al., 1994; Hockenbery et al., 1990; Krajewski et al., 1993; Zhu et al., 1996). In contrast, a substantial fraction of the proapoptotic members localize to cytosol or cytoskeleton before a death signal (Gross et al., 1998; Hsu et al., 1997; Puthalakath et al., 1999). After a death signal, the proapoptotic members that have been examined to date undergo a conformational change that enables them to target and integrate into membranes, especially the mitochondrial outer membrane.

Fig. 1. Schematic model of mammalian cell death pathway. A major checkpoint in the common portion of this pathway is the ratio of proapoptotic (BAX) to antiapoptotic (BCL-2) members. Downstream of this checkpoint are two major execution programs: the caspase pathway and mitochondrial dysfunction.

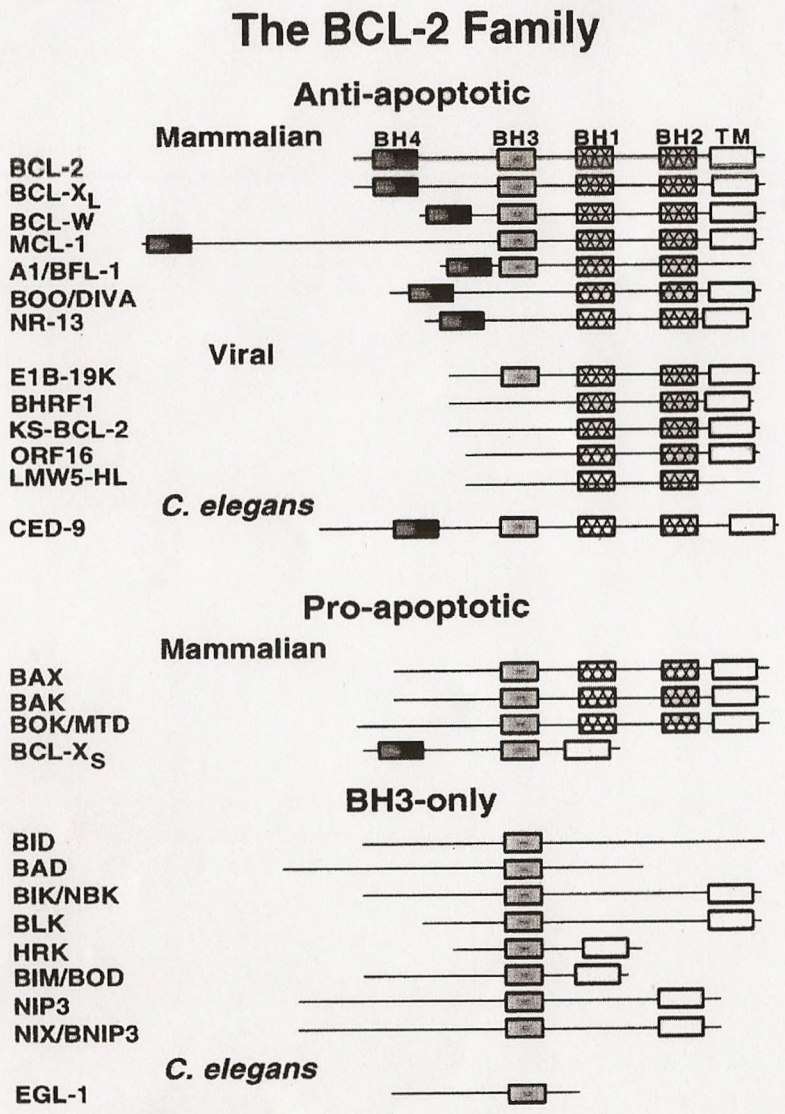

Fig. 2. Summary of antiapoptotic and proapoptotic BCL-2 family members. BCL-2 homology regions are indicated with similar shaded boxes *(BH1–BH4)*. The carboxy-terminal hydrophobic domain is indicated with an open box *(TM)*.

II. Dimerization and Translocation of BAX

The first proapoptotic homolog, BAX, was identified as a partner protein that co-immunoprecipitated with BCL-2 (Oltvai et al., 1993). Activation of the proapoptotic molecule BAX seems to involve subcellular translocation and dimerization. In viable cells, a substantial portion of BAX is monomeric and found either in the cytosol or loosely attached to membranes. After a death stimulus, cytosolic/monomeric BAX translocates to the mitochondria where it is an integral membrane and cross-linkable as homodimers and higher-order multimers (Gross et al., 1998; Walter et al., 1997) (Fig. 3). An FKBP-BAX molecule indicated that enforced dimerization by the bivalent ligand FK1012 results in its translocation to mitochondria, where it was capable of killing cells despite the presence of survival factor and BCL-X_L.

After death signals, membrane-associated BAX becomes a more integral membrane protein and alters the exposure of its N-terminal domain

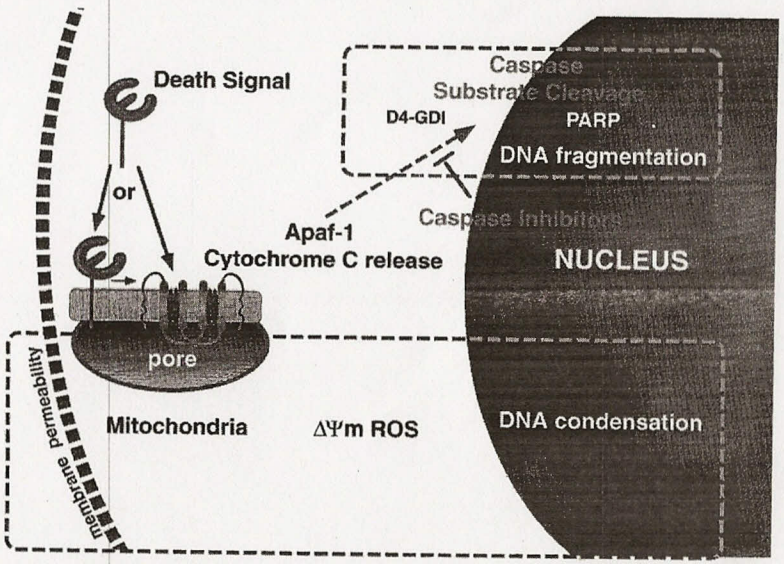

Fig. 3. Schematic model of BAX activation cell death. A death signal activates BAX, resulting in the translocation of a BAX monomer in the cytosol to homodimerized, integral mitochondrial membrane BAX. Downstream effects include the activation of caspases and a program of mitochondrial dysfunction.

(Desagher et al., 1999; Goping et al., 1998). Full-length BAX only targets mitochondria invitro when in the presence of a cytosol signaled for apoptosis, whereas removal of the NH2-terminal 20 amino acids of BAX enabled its targeting in the absence of an activated cytosol (Goping et al., 1998). The presence of an antiapoptotic molecule such as BCL-2 or BCL-X_L can inhibit the activation of BAX after a death signal (Gross et al., 1998) (Fig. 3). Inactive BAX is monomeric and in the cytosol or loosely associated with membranes, whereas BCL-2 is an integral membrane protein heavily localized to mitochondria. Taken together, one model is that the N-terminus of BAX or BAK is concealed to keep the molecule in a closed configuration until an activation stimulus results in a conformational change that manifests in its release.

III. BAX Activation Induces Mitochondrial Dysfunction and Apoptosis

Additional evidence that BAX and BAK could themselves initiate death was provided by transient transfection and inducible expression systems, where each proved sufficient to induce apoptosis without an additional stimulus (McCarthy et al., 1997; Xiang et al., 1996). BAX-induced death-activated caspases, which cleaved endogenous substrates in the nucleus (PARP) and cytosol (D4-GDI) (Fig. 4). However, although caspase inhibitors successfully blocked protease activity and could prevent a FAS-induced death, it did not block BAX-induced death. The localization of BCL-2 (Hockenbery et al., 1990) and subsequently other family members to the mitochondria prompted an assessment of mitochondrial function. Although the cleavage of substrates and the final degradation of DNA was prevented, mitochondrial dysfunction still occurred. BAX-induced alterations in mitochondrial membrane potential, production of reactive oxygen species, and plasma membrane permeability apparently occurred despite the inhibition of measurable caspase activity (Xiang et al., 1996).

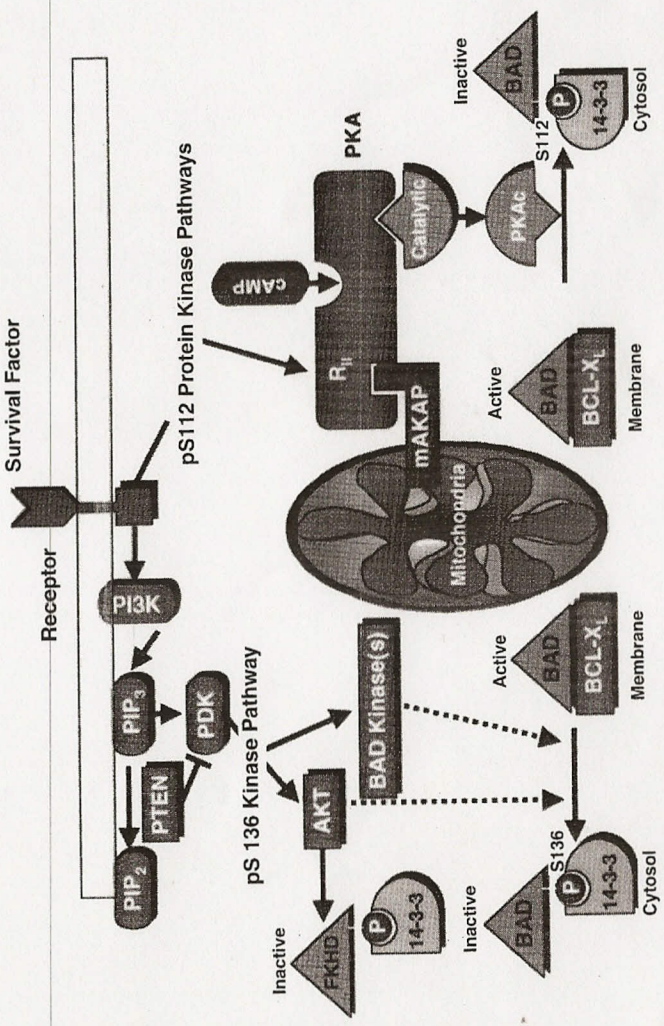

Fig. 4. Dual impact of IL-3-induced phosphorylation of BAD on two serine sites by distinct kinase pathways. The phosphorylated BAD is sequestered by 14-3-3 in the cytosol as the inactive form, which releases BCL-X_L to promote survival. Only nonphosphorylated BAD can heterodimerize with membrane-bound BCL-X_L, which seems to be the active form that inhibits BCL-X_L. Two distinct BAD kinase pathways include mitochondrial-tethered PKA via a mitochondrial AKAP and RII holoenzyme complex for S112. The S136 kinase resides downstream of PI-3K.

The ability of BAX multimers to kill cells is consistent with genetic evidence that BAX can function independently of BCL-2 (Knudson and Korsmeyer, 1997). How might integral membrane BAX kill? One possibility may relate to the capacity of BAX to form distinct ion-conductive pores (Antonsson et al., 1997; Schlesinger et al., 1997). This may include regulating an electrochemical gradient, altering mitochondrial volume homeostasis, or, as our recent experiments indicate, releasing critical products residing in the intermembrane space such as cytochrome c. Overall, these data favor a model in which a death signal results in the activation of BAX. This conformational change in BAX manifests in its translocation, mitochondrial membrane insertion, oligomerization, and a program of mitochondrial dysfunction that results in cell death (Fig. 3).

IV. Phosphorylation Inactivates BAD

In the presence of a survival factor, the "BH3-domain only" molecule BAD is phorphorylated on two serine sites (S112 and S136) and sequestered in the cytosol by the 14-3-3 molecule (Zha et al., 1996) (Fig. 4). After a death signal (IL-3 deprivation), BAD is dephosphorylated and found in association with BCL-X_L/BCL-2. The BH3 domain of BAD appears essential for its interaction with BCL-X_L/BCL-2 (Kelekar et al., 1997; Ottilie et al., 1997; Zha et al., 1997). This has the dual impact of dictating BAD's location as well as its protein partners. Thus, it appears that phosphorylation regulates the exposure of the BH3 domain of BAD. This hypothesis would be consistent with the prediction that the nonphosphorylated BAD is constitutively active due to the exposure of the hydrophobic face of the BH3 domain (McDonnell et al., 1999). Functionally, the rapid phosphorylation of BAD that follows IL-3 connects proximal survival signaling with the BCL-2 family, resetting this checkpoint for apoptosis. Thus, identifying the signaling components between the surface receptor and BAD, including the identification of BAD kinase(s), is a critical objective to interconnect signal transduction and distal death effector mechanisms.

To date, several kinases have been shown to phosphorylate and inactivate BAD. AKT/PKB/RAC, a Ser/Thr kinase downstream of phosphatidylinositol 3-kinase, is site specific for Ser136 of BAD (del Peso et al., 1997; Blume-Jensen et al., 1998; Datta et al., 1997). However, immunodepletion studies argue AKT is not the major kinase responsible for phosphorylation of BAD following IL-3 receptor engagement (Harada et al., 1999). In addition to BAD, AKT has been demonstrated to promote survival by phosphorylating and inactivating caspase-9 (Cardone et al., 1998) and the transcription factor FKHRL1 (Brunet et al., 1999).

Mitochondrial membrane-based cAMP-dependent protein kinase (PKA) was identified by column chromatography purification as a BAD Ser 112 site-specific kinase (Harada et al., 1999). The differential phosphorylation of highly selected protein substrates is a key element in the surprising specificity that follows engagement of surface receptors. Signaling cascades downstream of receptors often use rather ubiquitous intermediates, raising important questions as to how activated kinases and phosphatases preferentially phosphorylate target substrates. An attractive "targeting hypothesis" holds that phosphorylation is controlled, at least in part, by localization of individual kinases and phosphatases to distinct subcellular compartments (Hubbard and Cohen, 1993; Pawson and Scott, 1997). A striking example of subcellular targeting of kinase activity is the type II cAMP-dependent kinase (PKA) holoenzyme (Dell'Acqua and Scott, 1997). The PKA holoenzyme complex is comprised of an inhibitory regulatory R subunit dimer that holds two catalytic C subunits in a dormant state. Another property of the RII dimer is its direction of the subcellular location of PKA holoenzyme through its association with a series of tethering proteins known as A-kinase-anchoring proteins (AKAPs). AKAPs represent a functionally related family of signaling proteins that contain a conserved anchoring domain that associates with RII and unique targeting sequences that direct the PKA holoenzyme complex to specific subcellular addresses including the mitochondria (Carr et al., 1992; Huang et al., 1997; Lin et al., 1995). Thus, PKA represents an attractive subcellular-focused kinase/substrate interaction in which an outer mitochondrial membrane, AKAP (D-AKAP-1/S-AKAP84), tethers the PKA holoenzyme to the organelle where active BAD does its damage. Upon exposure to a survival factor, the localized catalytic subunit of PKA phosphorylates mitochondrial-based BAD, resulting in its inactivation and movement to the cytosol (Fig. 4).

V. BCL-2 Is Inactivated by Phosphorylation in its Unstructured Loop at G_2/M

BCL-2 is also phosphorylated in vivo, and this modification has been demonstrated to affect its antiapoptotic activity (Chang et al., 1997; Haldar et al., 1995; Ito et al., 1997). Phosphorylation within the flexible loop of

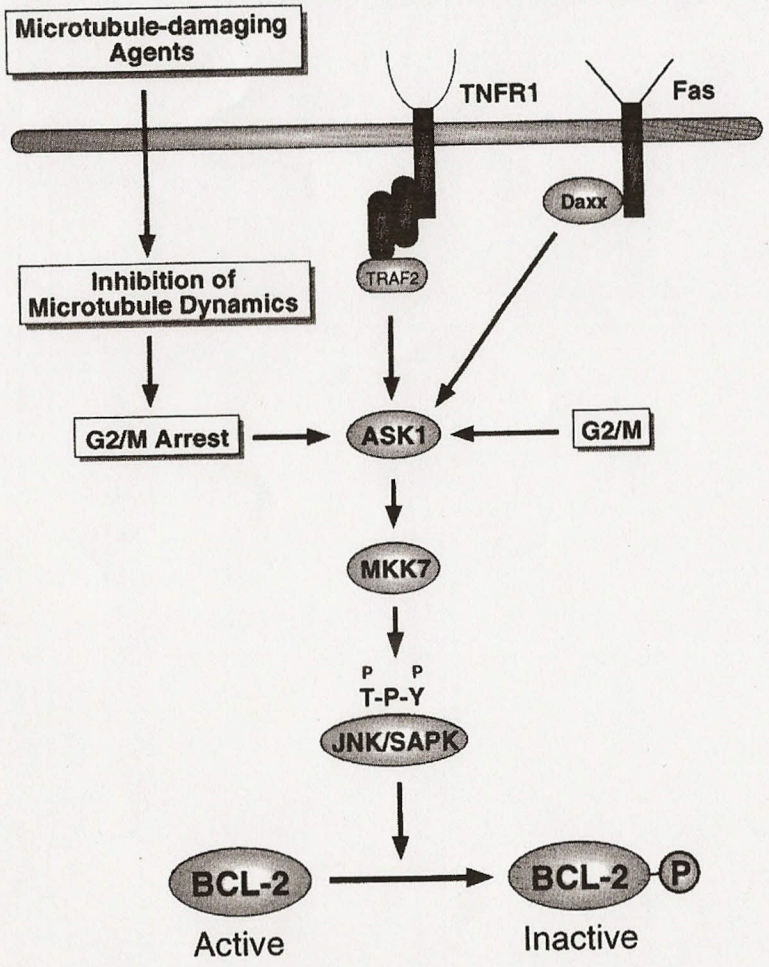

Fig. 5. Schematic representation of the ASK1/MKK7/JNK1 pathway in BCL-2 phosphorylation. ASK1, a MAP3K, is activated by extracellular and intracellular stimuli to induce JNK pathway activation. JNK phosphorylates BCL-2, inactivating its antiapoptotic function. TNFR1, tumor necrosis factor receptor 1.

BCL-2 appears to induce a conformational change that dictates its function. Multiple signal transduction pathways are capable of modifying BCL-2 family members to reset susceptibility to apoptosis. We used two-dimensional peptide mapping and sequencing to identify three residues (Ser70, Ser87, and Thr69) within the unstructured loop of BCL-2 that were phosphorylated in response to microtubule-damaging agents, which also arrest cells at G_2/M. Changing these sites to alanine conferred more antiapoptotic activity on BCL-2 after physiologic death signals as well as paclitaxel, indicating that phosphorylation is inactivating. An examination of cycling cells enriched by elutriation for distinct phases of the cell cycle revealed that BCL-2 was phosphorylated at the G_2/M phase of the cell cycle. G_2/M-phase cells proved more susceptible to death signals, and phosphorylation of BCL-2 appeared to be responsible, as a Ser70Ala substitution restored resistance to apoptosis. We noted that ASK1 and JNK1 were normally activated at G_2/M phase, and JNK was capable of phosphorylating BCL-2. Expression of a series of wild-type and dominant-negative kinases indicated an ASK1/JNK1 pathway phosphorylated BCL-2 in vivo. Moreover, the combination of dominant negative ASK1, (dnASK1), dnMKK7, and dnJNK1 inhibited paclitaxel-induced BCL-2 phosphorylation (Fig. 5). Thus, stress response kinases phosphorylate BCL-2 during cell cycle progression as a normal physiologic process to inactivate BCL-2 at G_2/M. This inactivation of BCL-2 may be intended to lower the threshold for apoptosis in order to eliminate cells with mitotic checkpoint aberrations.

VI. Fas/TNF Signaling Activates Caspase-8 Which Cleaves and Activates BID

The best characterized signal transduction pathways that mediate apoptosis are the cell surface receptors of the tumor necrosis factor (TNF) family, including CD95 (Fas/Apo-1) and CD120a (p55 TNF-R1) (Nagata, 1996; Tartaglia et al., 1993; Wallach et al., 1998). Engagement of Fas/TNF-R1 receptor leads to formation of a protein complex known as the DISC (death-inducing signaling complex) (Boldin et al., 1996; Medema et al., 1997; Muzio et al., 1996). This complex consists of Fas/TNF-R1, FADD (MORT1) and pro-caspase-8 (MACH/FLICE/Mch5). Once caspase-8 is recruited, it is processed and released from the complex in active form to activate the downstream "effector" caspases (Medema et al., 1997; Muzio et al., 1997; Srinivasula et al., 1996).

After TNFα or Fas treatment, a "BH3-domain only" molecule, BID, is cleaved at its N-terminus (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). Cleavage of cytosolic p22 BID by caspase-8 generates a p15 C-terminal fragment that translocates to the mitochondria (Fig. 6). Truncated p15 BID (tBID) inserts into the membrane, and immunodepletion of subcellular fractions argues that tBID is required for the release of cytochrome c from the mitochondria. The TNF and Fas death signal pathways converge at BID, a shared proapoptotic effector belonging to the BH3-domain only subset of the BCL-2 family. Our studies suggest a model in which cytosolic p22 BID represents an inactive conformation of the molecule that is proteolytically cleaved to generate an active p15 BID (Fig. 6). The p15 conformation rather selectively targets mitochondria where it resides as an integral membrane protein responsible for the release of cytochrome c.

The rapid movement of p15 BID from cytosol to mitochondrial membrane suggests a specific mechanism of targeting. Why would p15 BID be of such singular importance for the release of cytochrome c? BAX, BCL-2, and BCL-X_L are able to form distinct ion conductive pores in artificial membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). P15 BID might induce the polymerization of another proapoptotic such as BAX to form a pore for the selective passage of cytochrome c (Falnes et al., 1992).

To determine whether BID has a critical role as a singularly important caspase substrate in vivo, we examined *Bid*-deficient mice (Yin et al., 1999). *Bid* −/− mice were born at the expected Mendelian frequency, had no apparent developmental abnormalities, and demonstrated grossly normal organ weights and cellularity of spleen, thymus, brain, heart, liver, lung, kidney and testes. To test whether BID was an essential in vivo substrate required for release of cytochrome c, dysfunction of mitochondria, or even the death of cells, we injected mice intravenously with anti-Fas Ab. Most wild-type (wt) mice died within ~4 hrs of acute liver failure, demonstrating massive hepatic apoptosis and hemorrhagic necrosis. Within 2 hrs of injection, the initiator Caspase-8 and effector Caspases −3/−7 were activated, and cytochrome c released in Bid +/+ mice. In contrast, most *Bid* −/− mice survived anti-Fas Ab injection and approximately half showed no apparent liver injury by gross or microscopic examination, and there was no evidence of apoptosis as assessed by immunohistochemistry. Further analysis of this subset of *Bid*-deficient livers indicated that, al-

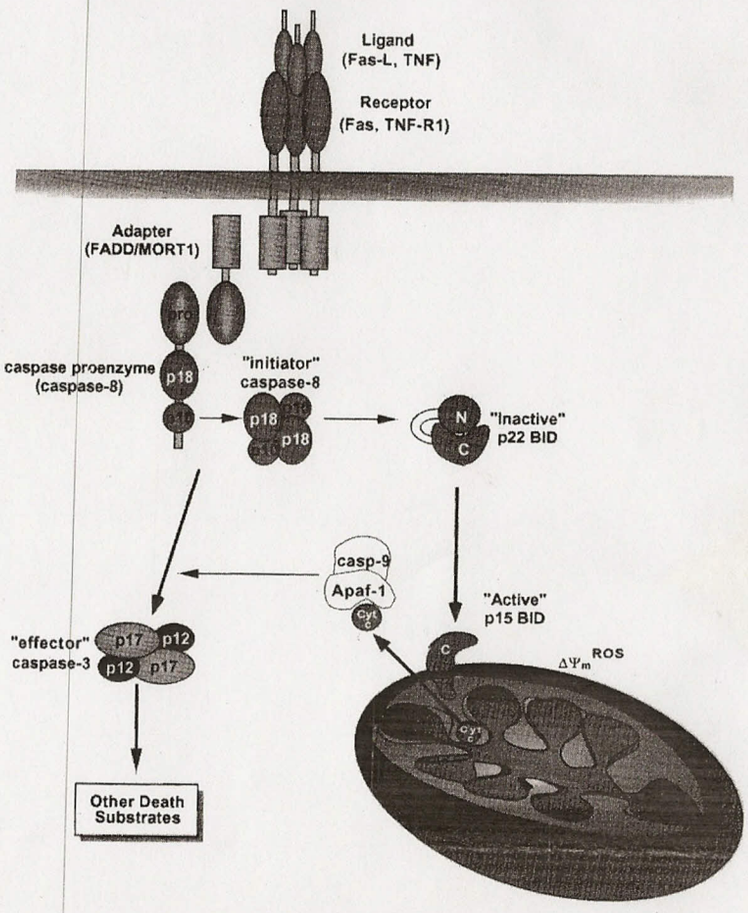

Fig. 6. Model of BID cleavage and translocation following TNF-R1/Fas engagement.

though Caspase-8 was activated substantially, strikingly, there was no substantial activation of Caspases −3/−7. Moreover, cytochrome c was not released in *Bid*-deficient hepatocytes. Thus, these unaffected mice display a requirement for BID-mediated pathway in order to mount sufficient effector caspase activity and mitochondrial dysfunction to cause any apparent apoptotic damage. Approximately half the *Bid* −/− mice displayed

a moderated pattern of liver injury, which they often survived. Their moderated pattern of damage included delayed activation of Caspases −3/−7 but preserved nuclei and no release of cytochrome c.

The BID-dependent component was examined further in cultured cell types not absolutely requiring BID for Fas or TNFα-induced death. In these *Bid* −/− cells, mitochondrial dysfunction was delayed, cytochrome c was not released, effector Caspase activity was diminished, and the cleavage of death substrates was altered. The differential cleavage of substrates may reflect the amount and more restricted distribution of effector Caspases in the absence of a Bid-induced cytochrome c/Apaf-1/Caspase-9 amplification loop designed to generate a more robust and widely distributed activation of effector Caspases. The retention of cytochrome c and the sustained transmembrane potential in *Bid* null cells further implicate mitochondria in the BID pathway. Of note, death stimuli not utilizing TNFR1/Fas death receptor engagement studied to date were not substantially affected by BID deficiency. These null mice suggest that BID evolved to trigger a mitochondrial amplification loop that insures death in cell types with limited availability of initiator or effector Caspases and/or following modest death receptor signals (Yin et al., 1999).

VII. A Structural Basis for Active and Inactive Conformers

One of the major characteristics of the BCL-2 family members is their ability to form heterodimers. Such interactions were demonstrated in yeast two-hybrid, in vitro binding assays as well as by co-immunoprecipitation from membrane-solubilized mammalian cells (Oltvai et al., 1993; Sedlak et al., 1995; Zha et al., 1996). Such interactions were also demonstrated in intact mitochondria in vivo using protein cross-linkers (Gross et al., 1998). The structure of a BCL-X_L monomer and a BCL-X_L/BAK BH3 peptide complex revealed that the BH1, 2, and 3 domains of BCL-X_L are in close proximity and create a hydrophobic pocket into which the BAK BH3 peptide binds (Muchmore et al., 1996; Sattler et al., 1997). However, a conformational change would have to occur in order for BAK to interact with BCL-X_L. Perhaps, the conformational changes that we have noted are related to this activity.

How could the N-terminal domain of proapoptotic molecules block their activity? Insight into this issue has come from solving the three-

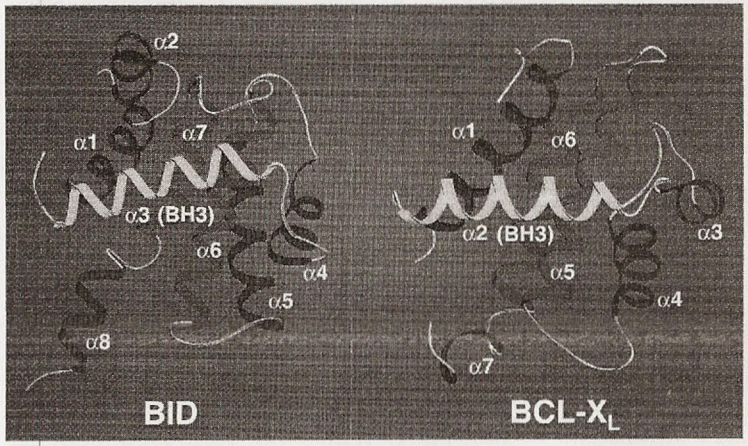

Fig. 7. Comparison of the three-dimensional structures of BID and BCL-X_L. Ribbon diagram of the averaged, minimized NMR structures of BID and BCL-X_L. BCL-2 homology (BH) 3 domain (yellow), the central hydrophobic α-helices (red), and the remaining α-helices (blue) are illustrated. Not shown are large, unstructured flexible loops between residues 31 and 70 in the BID protein and between residues 26 and 76 in the BCL-X_L protein. This image was prepared using the program SETOR. (See color plates.)

dimensional structure of p22 BID (Chou et al., 1999; McDonnell et al., 1999) (Fig. 7). Moreover, this multidimensional nuclear magnetic resonance (NMR) analysis of BID has provided a structural basis for the conformational activation of this family. BID's N-terminal first α helix makes strong hydrophobic contacts with the third α helix (which contains the BH3 domain) (Fig. 7). As testimony to the strength of this interaction, cleavage of p22 BID by caspase-8 at Asp59 (which resides in a large flexible loop between the second and the third α helices) does not result in their immediate dissociation in vitro. That dissociation does appear to occur in vivo, as p15 BID exists without an associated N-terminal fragment. Cleavage leads to the exposure of >200 Å of previously buried hydrophobic surface. These changes in hydrophobic exposure and surface charge may well contribute to the translocation and integration of tBID into mitochondria.

The structure of BID revealed that it does not possess a large hydrophobic pocket consistent with the observation that BID functions as a

TABLE I. PROPOSED STRUCTURAL SUBSETS OF THE BCL-2 FAMILY

Constitutively Inactive (Buried BH3 Domain)	BID, BCL-X_L, BAX, BAK, BCL-2, CED-9, BCL-W, MCL-1, BOK, DIVA
Active (Exposed BH3 Domain)	tBID, tBCL-2, t BCL-X_L, BCL-X_S, EGL-1, BAD, BIK, HRK, BIM, BLK, BLK, NIX, NIP 3

donor of a BH3 helix but not as an "acceptor" (McDonnell et al., 1999) (Fig. 7). Despite the very low sequence homology between BID and BCL-X_L and their opposite effects on apoptosis, it is surprising that their three-dimensional structures are quite similar (Fig. 7). Based on threading analysis, sequence and secondary structure comparisons the BCL-2 family members may be subdivided into two conformational subgroups (McDonnell et al., 1999) (Table I). A leading criteria is whether the BH3 domain, in particular its hydrophobic face, is available. After death signals, inactive or even antiapoptotic molecules can be converted to an active conformation by exposure of the BH3 domain and potentially other hydrophobic surfaces. Based on this comparison, it is likely that BAX is inherently in an inactive conformation but that, upon a death stimulus, it is modified resulting in the following: exposure of its N-terminus, availability of its BH3 domain hydrophobic surface, dimerization, and insertion into the mitochondrial membrane. A testable thesis would propose that all BCL-2 family members with a buried BH3 domain will prove to be antiapoptotic or inactive proapoptotic molecules. Upon exposure of the BH3 domain, perhaps in concert with other hydrophobic surfaces, they would convert to active proapoptotic molecules in order to accelerate the apoptotic process.

VIII. PRO-APOPTOTIC BCL-2 MEMBERS AS SENTINELS FOR CELLULAR DAMAGE

Finally, the existence of inactive conformers that must be activated by death signals plus the varied locations of proapoptotic molecules in viable cells suggests these members may serve as sentinels for cellular damage. These upstream BCL-2 family molecules may be strategically positioned to inventory cell damage at specific sites. In this model, BIM would monitor microtubule function, BID would be on the lookout for minimal caspase-8 activation, and BAX and BAD would patrol metabolic stress after loss of critical survival factors. This would be an attractive mechanism whereby a panoply of seemingly diverse injuries could rapidly converge on the common apoptotic pathway. The sudden exposure of a forbidden BH3 domain surface in response to select damage would enable cells to enter the physiologic apoptotic rather than necrotic pathway. This could prove to be a unified role, especially for the BH3-domain-only subset of molecules.

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