

BID: a novel BH3 domain-only death agonist

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The BCL-2 family of proteins consists of both antagonists (e.g., BCL-2) and agonists (e.g., BAX) that regulate apoptosis and compete through dimerization. The BH1 and BH2 domains of BCL-2 are required to heterodimerize with BAX and to repress cell death; conversely, the BH3 domain of BAX is required to heterodimerize with BCL-2 and to promote cell death. To extend this pathway, we used interactive cloning to identify *Bid*, which encodes a novel death agonist that heterodimerizes with either agonists (BAX) or antagonists (BCL-2). BID possesses only the BH3 domain, lacks a carboxy-terminal signal-anchor segment, and is found in both cytosolic and membrane locations. BID counters the protective effect of BCL-2. Moreover, expression of BID, without another death stimulus, induces ICE-like proteases and apoptosis. Mutagenesis revealed that an intact BH3 domain of BID was required to bind the BH1 domain of either BCL-2 or BAX. A BH3 mutant of BID that still heterodimerized with BCL-2 failed to promote apoptosis, dissociating these activities. In contrast, the only BID BH3 mutant that retained death promoting activity interacted with BAX, but not BCL-2. This BH3-only molecule supports BH3 as a death domain and favors a model in which BID represents a death ligand for the membrane-bound receptor BAX.

[Key Words: Apoptosis; BCL-2 family; BID; BH3 domain; cysteine protease]

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Programmed cell death plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms (Raff 1992). Genetic and molecular analysis from nematodes to humans has indicated that the pathway of cellular suicide is highly conserved (Ellis et al. 1991; Steller 1995; White 1996). Although the capacity to carry out apoptosis appears to be inherent to 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 (Oltvai and Korsmeyer 1994).

A family of ICE-like proteins are activated in apoptosis and are clearly required for aspects of cell death (Martin and Green 1995; Henkart 1996). These activated proteases have a unique recognition site that cleaves at a novel P1 aspartic acid (Thornberry et al. 1992; Lazebnik et al. 1994). Some of these target proteins may be death substrates, which upon proteolytic cleavage ensure the inevitability of death. Genetic approaches in the nematode *Caenorhabditis elegans* favor an order in which the ICE-like gene, *ced-3*, is downstream of its *Bcl-2* ho-

molog, *ced-9* (Shaham and Horvitz 1996). In addition, biochemical studies in mammalian cells demonstrated that the presence of BCL-2 blocked the activation of CPP32 (Armstrong et al. 1996; Boulakia et al. 1996; Chinnaiyan et al. 1996; Shimizu et al. 1996). Thus, the BCL-2 decisional point appears to be proximal to the irreversible damage of cellular constituents.

Recently, the family of BCL-2 proteins that control this intracellular checkpoint of apoptosis has expanded (Farrow and Brown 1996). The ratio of death antagonists (*Bcl-2*, *Bcl-x_L*, *Mcl-1*, and *A1*) to agonists (*Bax*, *Bak*, *Bcl-x_S*, and *Bad*) determines whether a cell will respond to an apoptotic signal (Oltvai and Korsmeyer 1994). This competition is mediated, at least in part, by competitive dimerization between selective pairs of antagonists and agonist molecules. For example, the death promoting molecule BAX forms homodimers and also heterodimerizes with BCL-2 or BCL-X_L (Oltvai et al. 1993). Mutagenesis studies revealed that intact BH1 and BH2 domains of the antagonists (BCL-2, BCL-X_L) were required for them to heterodimerize with BAX and to repress cell death (Yin et al. 1994; Sedlak et al. 1995). Conversely, deletion analysis has indicated that the BH3 domain of death agonists (BAK, BAX) was required for these proteins to heterodimerize with BCL-X_L or BCL-2 and to promote cell death (Chittenden et al. 1995; Zha et al. 1996). Another death promoting molecule, BIK, has BH3 but not BH1, -2, or -4 domains (Boyd et al. 1995). However, other muta-

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tions in BCL-X_L have been noted to disrupt heterodimerization with BAX, but retain death repressor activity (Cheng et al. 1996). This suggests that these molecules might also work independent of one another. Recently, the first X-ray and multidimensional NMR structure of a family member, BCL-X_L, was solved (Muchmore et al. 1996). A BCL-X_L monomer proved α -helical in which BH1–BH4 domains corresponded to α helices 1–7. A hydrophobic pocket is created by the close spatial proximity of BH1, BH2, and BH3.

Although substantial progress has been made in expanding the number of positive and negative regulatory members at each step of apoptosis, major gaps remain in linking these steps into a serial pathway. To further extend the cell death pathway we have exploited interactive cloning strategies using both agonists (BAX) and antagonists (BCL-2) as probes. The goal of this approach was to link the BCL-2 family to proximal signal transduction events and to distal death effector mechanisms. Surprisingly, we noted a frequent, common isolate that bound to both agonists and antagonists. This molecule, BID (BH3 interacting domain death agonist), will counter protection by BCL-2 and also induce the common pathway of apoptosis including cysteine proteases. BID's only homology with the BCL-2 family is a conserved BH3 domain that it requires for heterodimerization with its partners and for its death-promoting activity. BID lacks the typical carboxy-terminal signal-anchor segment and has both cytosolic and membrane localizations. Mutagenesis of BH3 indicates the importance of BID/BAX heterodimers and suggests that BID serves as a death ligand.

Results

Isolation of BID by protein interactive cloning

Murine *Bcl-2* and *Bax* cDNAs with deletions in the carboxy-terminal signal-anchor segment were used to generate GST–HMK–BCL-2 or GST–HMK–BAX fusion proteins. These purified proteins were labeled in vitro by phosphorylation of the heart muscle kinase (HMK) epitope with bovine HMK and [γ -³²P]ATP. Labeled proteins were used to screen a λ EX λ ox expression library constructed from a murine T-cell hybridoma line, 2B4. The same novel gene was identified multiple times with both BCL-2 and BAX probes. A full-length cDNA was obtained that encodes a 195-amino-acid protein with a predicted molecular weight of 21.95 kD that we have termed BID (Fig. 1A). A BLAST search identified a region within this gene that shares high sequence homology with the well-conserved BH3 domain (Chittenden et al. 1995; Han et al. 1996; Zha et al. 1996) of the BCL-2 family. However, BID does not display sequence conservation with other regions of the BCL-2 family including the BH1, BH2, or BH4 domains. Moreover, BID does not possess a carboxy-terminal hydrophobic region typical of most BCL-2 family members that serves as a signal-anchor segment for these membrane proteins (Nguyen et al. 1993).

The search also revealed two overlapping expressed sequence tag (EST) human cDNA clones (nos. 52055 and 128065) with substantial homology to *Bid*. We determined the DNA sequence of these clones, which revealed a 195-amino-acid open reading frame with 72.3% homology to murine BID (Fig. 1A), and which we pre-

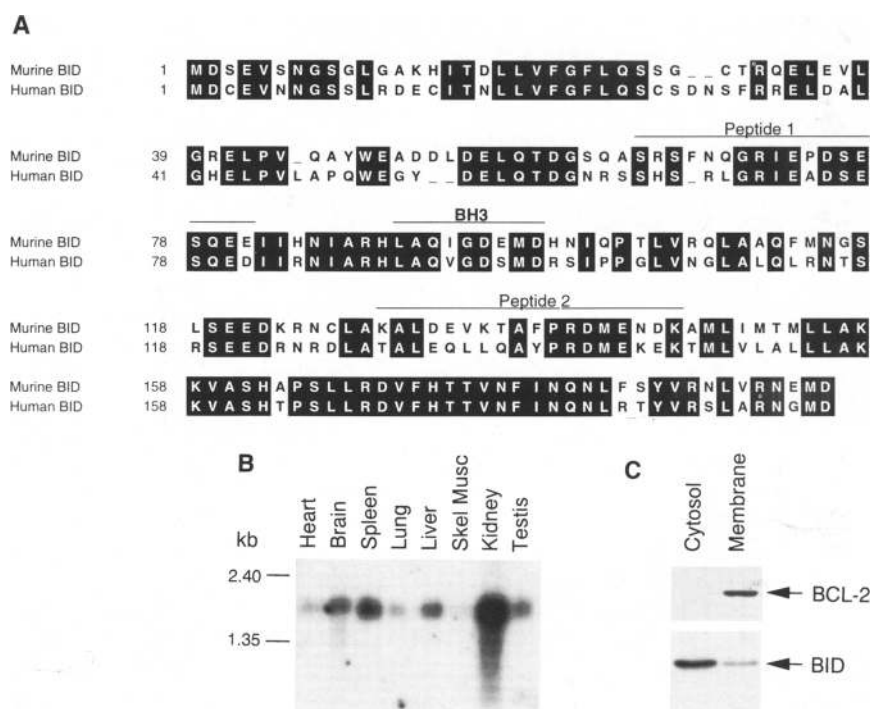


Figure 1. Amino acid sequence and distribution of BID. (A) Alignment of murine and human BID protein sequences. The nine-amino-acid motif of the BH3 domain and the two polypeptides used for Ab generation are overlined. (B) Expression of *Bid* RNA in adult mouse tissues. Northern analysis was carried out using a radiolabeled *Bid* cDNA containing the open reading frame as a probe. (C) Distribution of BID protein in the cytosol and membrane fractions of FL5.12-Bcl-2 cells. Equal amounts of protein from cytosolic and membrane fractions were size-fractionated by SDS-PAGE followed by Western blotting developed with anti-BCL-2 or anti-BID Abs.

sumed to represent the human homolog. Northern blot analysis (Fig. 1B) of RNA from adult mouse tissues demonstrated that *Bid* is most abundantly expressed in kidney, and is also present in brain, spleen, liver, testis, and lung, but little, if any, in heart and skeletal muscle.

Purified GST-BID fusion protein was used to immunize rabbits and generate a polyclonal anti-BID Ab. This Ab recognizes a 23-kD protein on Western analysis of lysates from 2B4 cells and a mouse hematopoietic cell line, FL5.12 (Fig. 1C). Antibodies generated against two BID polypeptides (Fig. 1A, peptides 1 and 2) recognize the same protein on Western blots. Subcellular fractionation revealed that BID was predominantly localized to the cytosol (>90%) with another component in the membrane fraction (Fig. 1C). This observation is compatible with the lack of a carboxy-terminal hydrophobic segment in BID.

BID is a death agonist that will counter protection by BCL-2

A mammalian expression plasmid pSFFV-Bid was constructed by placing *Bid* under the control of the Splenic Focus-Forming Virus (SFFV) long terminal repeat. Stable clones expressing BID were established following transfection of pSFFV-Bid into the interleukin (IL-3) dependent early hematopoietic cell line FL5.12 (Nunez et al. 1990). A consistent, but subtle increase in apoptosis was observed in the highest BID expressing clones following IL-3 withdrawal (Fig. 2A). The degree of cell death in individual BID clones corresponded to BID protein levels as detected by Western blot analysis (Fig. 2B).

Parental FL5.12 cells are very susceptible to apoptosis as they possess abundant BAX, but little BCL-2 or BCL-X_L. To determine whether BID could counteract the anti-apoptotic effect of BCL-2, we introduced BID into FL5.12-Bcl-2 clones. BID restored apoptosis to BCL-2 overexpressing clones with intermediate death rates between parental FL5.12 and FL5.12-Bcl-2 cells (Fig. 3A). The extent of cell death following IL-3 deprivation correlated with levels of BID protein as assessed by Western blot (Fig. 3B). When highly expressed, BID often ran as a doublet on Western blots, suggesting a post-translational modification (Fig. 3B).

Induction of BID activates an apoptotic pathway that is inhibited by zVAD

Expression of BID within FL5.12 indicated that BID could enhance apoptosis after administration of a death signal, IL-3 deprivation. To assess whether BID could induce apoptosis in the absence of an additional death stimulus we established two more assays, a doxycycline-inducible expression system in Jurkat T-cells and a transient transfection assay in fibroblasts.

Inducible Jt-Bid clones were generated by introducing the *Bid* cDNA under the control of the heptamerized *tet*-operator into a Jurkat clone stably expressing the reverse tetracycline-controlled transactivator (rtTA) (Gossen et al. 1995). Upon addition of doxycycline hydrochloride (Dox) Jt-Bid clones rapidly initiated apoptosis,

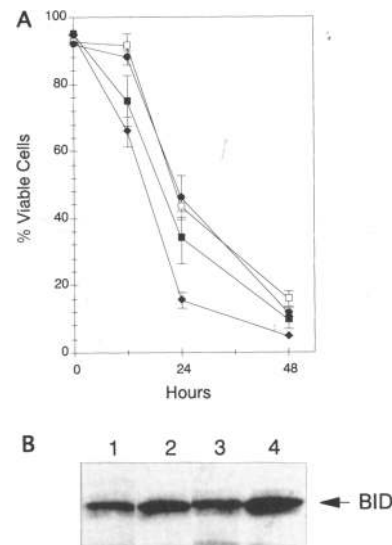


Figure 2. Overexpression of BID in FL5.12 cells leads to enhanced cell death upon IL-3 withdrawal. (A) Viability of FL5.12 clones stably expressing *Bid* following IL-3 withdrawal. Results of three independent *Bid* clones together with a Neo control clone are shown. (□) FL5.12-Neo; (■) FL5.12-Bid-14; (●) FL5.12-Bid-15; (◆) FL5.12-Bid-29. Viability was determined by trypan blue exclusion. Results represent mean \pm S.D. of duplicate assays. (B) Levels of BID in FL5.12 clones. Lysates of various FL5.12 clones were separated by 12.5% SDS-PAGE followed by a Western blot developed with an anti-BID Ab. (Lane 1) FL5.12-Neo; (lane 2) FL5.12-Bid-14; (lane 3) FL5.12-Bid-15; (lane 4) FL5.12-Bid-29.

within 12 hr, and had <40% viability at 48 hr (Fig. 4A). Western blot analysis revealed induction of BID by 12 hr and the level of expression was maintained up to 54 hr after dox treatment (Fig. 4B). We next wished to determine whether ICE-like cysteine proteases were required for BID-induced apoptosis. The small peptide-based molecule benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) is an irreversible inhibitor particularly effective against the CPP32-like subset of cysteine proteases (Fearnhead et al. 1995; Armstrong et al. 1996; Jacobson et al. 1996). Treatment of Jt-Bid cells with 50 μ M zVAD-fmk retarded BID induced apoptosis (Fig. 4A). In contrast, treatment of Jt-Bid cells with 35 μ M YVAD-cmk, an ICE inhibitor (Thornberry et al. 1992; Lazebnik et al. 1994), had no effect. This result indicates that CPP32-like cysteine proteases are involved in the cell death pathway triggered by BID.

A transient transfection system was developed in Rat-1 fibroblasts. Preliminary studies established that expression of a luciferase reporter was eliminated in cells undergoing apoptosis (data not shown). Subsequently, apoptotic regulatory genes under the control of a CMV promoter were cotransfected with the luciferase reporter and luciferase activity assays were performed 18–20 hr later. Cotransfection of *Bid* with the luciferase reporter resulted in a fivefold decrease in luciferase activity (Fig.

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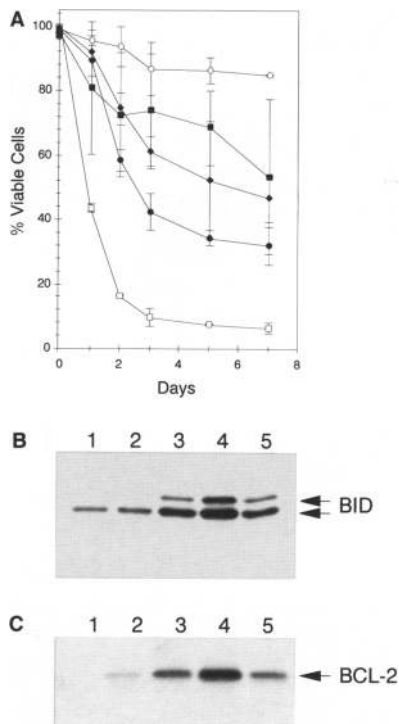


Figure 3. BID counters the death antagonist effect of BCL-2 in FL5.12 cells. (A) Viability of FL5.12 clones expressing BCL-2 and BID following IL-3 withdrawal. Results of three independent Bcl-2/Bid clones are shown. (□) FL5.12; (○) FL5.12-Bcl-2; (■) FL5.12-Bcl-2/Bid-1; (●) FL5.12-Bcl-2/Bid-3; (◆) FL5.12-Bcl-2/Bid-8. Viability was determined by trypan blue exclusion. Results represent mean \pm s.e. of two independent experiments. (B) Levels of BID in clones of FL5.12 cells determined by a Western blot developed with anti-BID Ab. (Lane 1) FL5.12; (lane 2) FL5.12-Bcl-2; (lanes 3–5) FL5.12-Bcl-2/Bid-1, -3 and -8. Lanes 1 and 2 demonstrate the endogenous level of BID in FL5.12 cells. (C) Amount of BCL-2 coprecipitating with BID in FL5.12 clones. Cell lysates were immunoprecipitated with an anti-BID Ab and separated on SDS-PAGE, followed by a Western blot developed with an anti-BCL-2 mAb (6C8). Lanes identical to B.

4C). Bcl-2 resulted in a threefold increase, protecting cells from transfection-induced cell death. Simultaneous transfection of Bid and Bcl-2 showed an intermediate luciferase activity confirming the capacity of BID and BCL-2 to counter each other. Similar to what was observed in the Jurkat-inducible system, the induction of apoptosis by BID was also repressed by zVAD-fmk, in a dose-dependent fashion (Fig. 4C).

BID heterodimerizes with both death antagonists (BCL-2 and BCL-X_L) and death agonists (BAX)

To further characterize the interactions of BID with BCL-2 family members in vivo, a vaccinia virus-mediated transient transfection assay was utilized. Both BCL-X_L and BCL-2 coprecipitated with the T7-gene-10-BID fusion protein, whereas a BH1 domain mutant, BCL-2-mI-4 (G145E), did not (data not shown). However, we

could not further assess the interaction between BID and BAX in this system because their coexpression markedly reduced the level of T7-gene-10-BID expression, apparently by inducing apoptosis of NIH-3T3 cells.

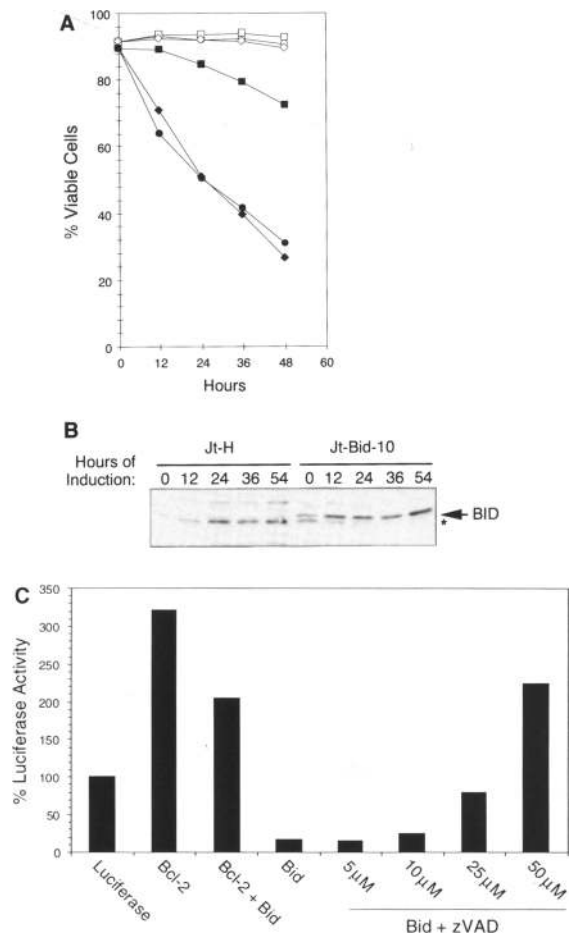


Figure 4. BID induces apoptosis in Jurkat and Rat-1 cells. (A) Induction of BID in Jurkat cells results in apoptosis that is zVAD-sensitive. Jurkat clones with the rtTA inducible system for BID expression were established by transfecting *tet*-operator driven *Bid* into Jt cells bearing the TetR^r-VP16 transactivator (see Materials and Methods). BID expression was induced by addition of doxycycline (1 μ g/ml) into the culture media at time 0 and cell viability was determined by annexin V/PI staining assessed by flow cytometry. Either 50 μ M zVAD-fmk or 35 μ M YVAD-cmk was added to the media at the beginning of induction. (○) Jt-H + Dox; (□) Jt-H + Dox + zVAD; (◇) Jt-H + Dox + YVAD; (●) Jt-Bid + Dox; (■) Jt-Bid + Dox + zVAD; (◆) Jt-Bid + Dox + YVAD. (B) BID protein at 0, 12, 24, 36, and 54 hr after doxycycline treatment as determined by Western blot of cell lysates from Jurkat clones developed with an anti-BID Ab. Asterisk indicates a nonspecific band. (C) BID induces apoptosis in Rat-1 fibroblasts which is inhibited by BCL-2 or zVAD-fmk. Bid and a luciferase reporter gene were cotransfected into Rat-1 cells with LipofectAMINE. Luciferase activity of transfected cells was assayed 18–20 hr later. Cell viability is reflected by the percent of luciferase activity compared to the control, in which the luciferase reporter was cotransfected with an empty vector. zVAD-fmk was added to the media at the indicated concentrations 5 hr after transfection.

To confirm the *in vivo* interaction noted in transient transfections, we assessed whether BID would dimerize with BCL-2 at protein levels obtainable in stably transfected cells. BID was immunoprecipitated from a series of FL5.12 clones, and the immunoprecipitates were size-fractionated by SDS-PAGE followed by immunoblotting with an antihuman BCL-2 mAb (Fig. 3C). Parental FL5.12 cells contain no human BCL-2 (Fig. 3C, lane 1), whereas FL5.12-Bcl-2 cells (Fig. 3C, lane 2) demonstrated human BCL-2 bound to endogenous BID. Substantially more BCL-2 was heterodimerized in clones that overexpressed BID (Fig. 3C, lanes 3–5).

To further assess the sites of interaction between BID and BCL-2 family members we established an *in vitro* binding assay. GST-BID fusion protein was incubated with *in vitro* translated BCL-X_L, BCL-2, BAX, or BID radiolabeled proteins. All except BID bound to GST-BID, but not GST (Fig. 5A). This data was confirmed in a yeast two-hybrid system where BID was assayed in the DNA-binding domain as well as the activation domain. BID interacted with BCL-X_L but failed to homodimerize with itself in yeast two-hybrid assays (data not shown). Altogether these data indicate that BID exists as a monomer and can form dimers or perhaps multimers with both death agonists and antagonists.

To identify the domains within antagonists (BCL-2) and agonists (BAX) that interacted with BID, mutants of BCL-2 and BAX were tested in the *in vitro* binding assay.

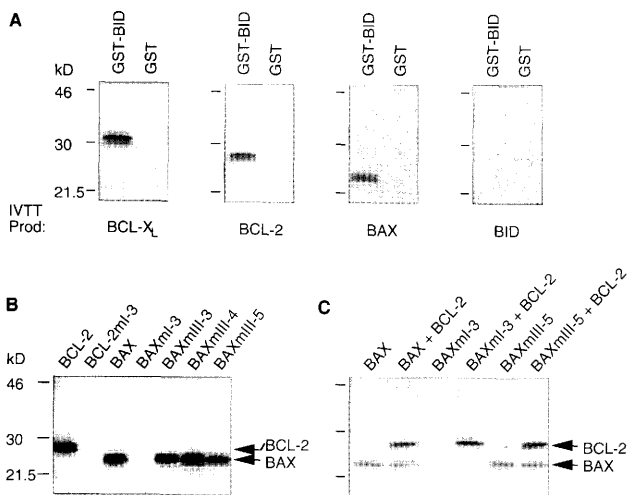


Figure 5. Interactions between BID and BCL-2 family member proteins *in vitro*. (A) *In vitro* binding assays. Equal amounts of *in vitro* translated, [³⁵S]methionine-labeled BCL-X_L, BCL-2, BAX, or BID were mixed with purified GST-BID or GST. After the incubation, GSH-agarose beads were added, and labeled products bound to GST-BID or GST were separated on SDS-PAGE followed by autoradiography. (B) Binding assays of BCL-2 and BAX mutants with BID. *In vitro* translated BCL-2 and BAX mutants were mixed with GST-BID, followed by steps described in B. (C) Independent binding of BID to BCL-2 and BAX. *In vitro* translated BCL-2 wt and BAX mutants were preassociated and then mixed with GST-BID, followed by steps described in B.

Both BCL-2mi-3 (G145A) and BAXmi-3 (G108A) which have substitutions in the conserved Gly in the BH1 domain, failed to bind BID (Fig. 5B). BAXmi-3 (G67A), mIII-4 (G67E) and mIII-5 (M74A), which bear mutations in the BH3 domain, still interacted with GST-BID (Fig. 5B). These results implicated the BH1 domain but excluded the BH3 domain as the site of interaction within the partner proteins that bind BID.

BID can interact with either BCL-2 or BAX, and BCL-2 heterodimerizes with BAX, raising the question of whether BID selectively interacts with BCL-2/BAX heterodimers or with each individual molecule. To address this, *in vitro* translated BCL-2 and BAX were preassociated before being admixed with GST-BID (Fig. 5C). Compared with BAX alone, the presence of BCL-2/BAX heterodimers did not augment the binding to GST-BID (Fig. 5C). We next used BAXmi-3, a BH1 mutant which does not bind GST-BID, but still forms heterodimers with wild-type BCL-2 (data not shown). BAXmi-3 would not bind GST-BID even when heterodimerized with BCL-2 (Fig. 5C). These data suggest that BID does not form a trimolecular complex with BCL-2/BAX heterodimers. To confirm this result we performed the converse experiment using BAXmi-5, a BH3 mutant, which does not form heterodimers with BCL-2 (K. Wang and S.J. Korsmeyer, unpubl.). The amount of BAXmi-5 or wild-type BAX that bound GST-BID was similar and was unaffected by the presence of BCL-2 (Fig. 5C). In total these observations argue that BID interacts with monomeric or perhaps homodimeric BCL-2 or BAX, but not with BCL-2/BAX heterodimers.

BH3 domain of BID is essential for its death agonist activity and its interaction with BCL-2 or BAX

The only conserved domain that BID possesses is BH3, prompting a mutational assessment of its functional importance (Fig. 6A). BH3 mutants of BID were tested for their binding to BCL-2 and BAX *in vitro* (Fig. 6B). All four mutants tested disrupted BID's interaction with either BCL-2 or BAX. However, the mutants did display different specificities: BIDmIII-1 (M97A,D98A) bound to BAX but not to BCL-2, BIDmIII-3 (G94A) bound to BCL-2 but not BAX, whereas BIDmIII-2 and mIII-4 did not bind to either (Fig. 6B). To determine whether this *in vitro* binding data accurately reflected interactions of the BID mutants *in vivo*, we introduced each BID mutant into FL5.12-Bcl-2 cells and selected stable expressing clones (Fig. 7). The expression level of BID mutants was comparable to that of a wild-type BID transfectant (Fig. 7B). The ability of each mutant to interact with BCL-2 or BAX was assessed by immunoprecipitation with an anti-BID Ab followed by an anti-BCL-2 or anti-BAX immunoblot (Fig. 7C). Wild-type BID (lane 2) and BIDmIII-3 (lane 5) interacted with BCL-2, whereas wild-type BID and BIDmIII-1 (lane 3) interacted with BAX *in vivo*, confirming the *in vitro* binding data. BIDmIII-1 was the only mutant which still interacted with BAX, albeit in decreased amounts similar to the *in vitro* assay (Fig. 7C).

The capacity of BID mutants to counter protection by

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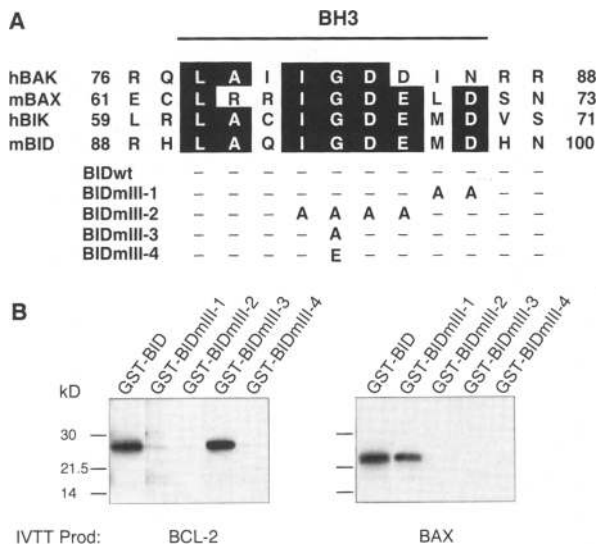


Figure 6. BH3 mutants of BID and their binding to BCL-2 and BAX. (A) Alignment of BH3 domains of death-promoting molecules and schematic representation of mutations introduced into BID. Identical amino acids are shaded. (B) In vitro binding assays. In vitro translated BCL-2 or BAX was mixed with purified GST-BID wt or mutants followed by steps described in Fig. 5B.

BCL-2 was assessed in the stably transfected FL5.12-Bcl-2 clones deprived of IL-3 (Fig. 7A). Of note, all BH3 mutants of BID were impaired in their capacity to counter protection by BCL-2. Even BIDmIII-3 (G94A) which still avidly heterodimerized with BCL-2 was less effective than wild-type BID. This dissociated the capacity of BID to form heterodimers with BCL-2 from its reversal of BCL-2 protection (Fig. 7). This result prompted further assessment of the BID mutants in the inducible system that does not require another apoptotic signal (Fig. 8). Moreover, Jurkat cells do not express substantial amounts of BCL-2. Despite substantial levels of protein (Fig. 8B), BIDmIII-2, -3, and -4 displayed no meaningful death promoting effect (Fig. 8A). Only BIDmIII-1 demonstrated substantial killing that was somewhat less than wild-type BID (Fig. 8A), perhaps reflecting its weaker binding to BAX (Figs. 6B and 7C). This BID mutant was also analyzed in the transient transfection death assay in Rat-1 fibroblasts. Once again, BIDmIII-1 demonstrated strong killing activity, whereas the activity of BIDmIII-3 and -4 was substantially impaired (Fig. 8C). Thus, the BH3 mutations in BID scored differently in stable transfectants with high levels of BCL-2 that require an external death stimulus (IL-3 deprivation, Fig. 7A) when compared with systems that induce expression of BID and do not require another signal (Fig. 8A,C). Of note, the only BID mutant (mIII-1) still active (M97A,D98A) bound BAX but not BCL-2 (Figs. 6B and 7C).

Discussion

We have identified a novel partner, BID, that interacts in

vivo and in vitro with both death agonists and antagonists of the BCL-2 family. An in vitro binding assay indicated that BID can bind BCL-2 or BAX monomers or perhaps homodimers, but suggested that it does not bind to BCL-2/BAX heterodimers. This provided an instructive exception to a generalized scheme in which BCL-2 family molecules tended to heterodimerize with agonists or antagonists, but not both (Sato et al. 1994; Sedlak et al. 1995). Prior mutagenic approaches to assess whether the death agonists or antagonists were dominant in regulating apoptosis provided mixed results. Select mutations within the BH1 and BH2 domains of BCL-2 or BCL-X_L led to simultaneous loss of BAX binding and antiapoptotic activity (Yin et al. 1994; Sedlak et al. 1995; Cheng et al. 1996). This suggested that death inhibitors protect cells by binding and neutralizing death agonists like BAX. However, several BCL-X_L mutations that lost interaction with BAX or BAK still retained 70–

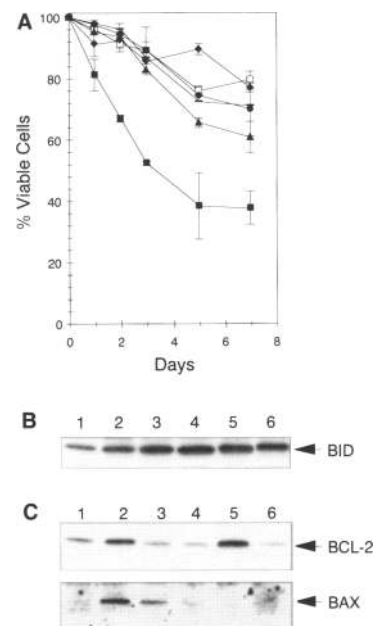


Figure 7. Effect of BH3 mutations in BID on its interaction with BCL-2 and BAX, and its ability to counter protection. (A) Viability of FL5.12-Bcl-2 clones expressing wt or mutant BID following IL-3 withdrawal. Nomenclature of BID mutants is indicated in Fig. 6A. Results represent mean \pm S.D. of duplicate assays. Two clones of each mutant with comparable protein levels were assessed with similar results. (□) FL5.12-Bcl-2/Hygro; (■) FL5.12-Bcl-2/Bid-8; (◆) FL5.12-Bcl-2/BidmIII-1.15; (▲) FL5.12-Bcl-2/BidmIII-2.10; (●) FL5.12-Bcl-2/BidmIII-3.1; (▼) FL5.12-Bcl-2/BidmIII-4.1. (B) BID in various FL5.12 clones detected by Western blot. (Lane 1) FL5.12-Bcl-2/Hygro; (lane 2) FL5.12-Bcl-2/Bid-8; (lane 3) FL5.12-Bcl-2/BidmIII-1.15; (lane 4) FL5.12-Bcl-2/BidmIII-2.10; (lane 5) FL5.12-Bcl-2/BidmIII-3.1; (lane 6) FL5.12-Bcl-2/BidmIII-4.1. (C) In vivo association of wt or mutant BID with BCL-2 and BAX. Lysates of FL5.12 clones noted above were immunoprecipitated with an anti-BID Ab, size fractionated by SDS-PAGE and a Western blot was developed with an anti-BCL-2 or anti-BAX Ab. Lanes 1–6 represent the same FL5.12 clones as indicated in B.

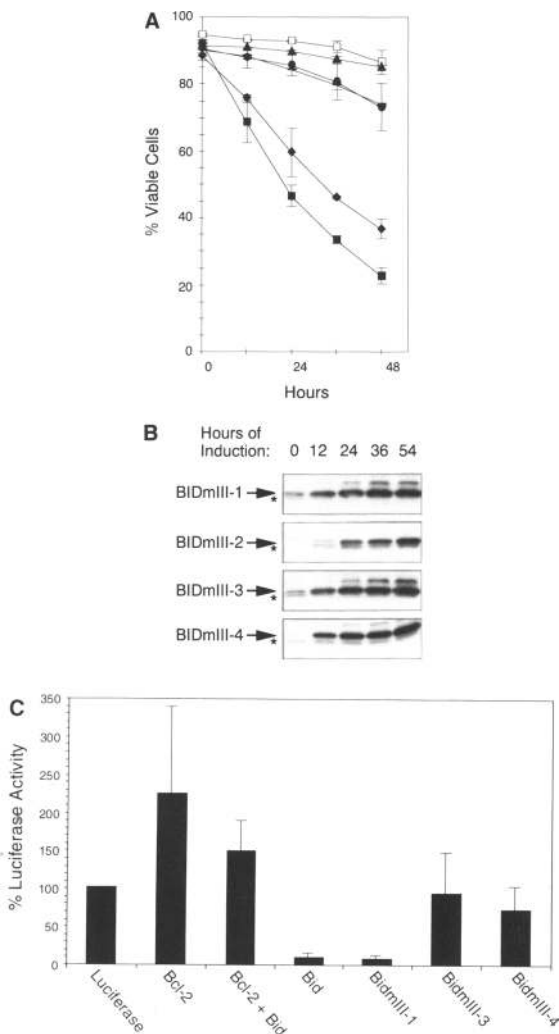


Figure 8. Death agonist activity of BID mutants in Jurkat and Rat-1 cells. (A) Death-promoting activities of wt and mutant BID in Jurkat cells. Experiments were performed as described in Fig. 4A. Results represent mean \pm S.E. of two independent experiments. (□) Jt-H; (■) Jt-Bid-1; (◆) Jt-BidmIII-1.2; (▲) Jt-BidmIII-2.7; (●) Jt-BidmIII-3.2; (▬) Jt-BidmIII-4.11. (B) BID protein levels in inducible Jurkat clones at 0, 12, 24, 36, and 54 hr after induction by doxycycline. BID was assessed by a Western blot developed with an anti-BID Ab. Asterisk indicates a nonspecific band. (C) Apoptotic activities of wt and mutant BID in a transient transfection assay of Rat-1 fibroblasts. Experiments were carried out as described in Fig. 4C. Results represent mean \pm S.E. of two independent experiments.

80% of their antiapoptotic activity (Cheng et al. 1996), suggesting that the death antagonist and agonist members of the family can function independently of one another. The characteristics of BID suggest yet another model in which agonists (BAX) or antagonists (BCL-2) represent membrane bound receptors that compete for a common ligand, BID.

BID, a functional death agonist, contains only the BH3 domain of the BCL-2 family. The death agonist BIK also

possesses BH3 and the carboxy-terminal signal-anchor segment but lacks recognizable BH1, -2, or -4 domains (Boyd et al. 1995). Of note, BID and BIK share eight of nine amino acids in BH3 (Fig. 6A), but show no homology beyond this domain. In distinction, BID does not possess a carboxy-terminal signal-anchor segment and resides in cytosolic as well as membrane fractions.

Site-specific mutagenesis of BID revealed that BH3 was required for death-promoting activity. This included the capacity to counter protection by BCL-2 as well as to induce apoptosis when expressed in Jurkat T cells or Rat-1 fibroblasts (Table 1). This is consistent with prior deletion constructs in which 12 amino acids, including BH3, were eliminated from BAK, BAX, and BIK (previously Bip1), resulting in reduced killing activity (Chittenden et al. 1995). In addition, a swap of a 23-amino-acid segment surrounding BH3 from BAX into BCL-2 converted it to a death agonist (Hunter and Parslow 1996). The point mutations in BH3 of BID determined that the central glycine of BH3 was critical to apoptotic activity.

The point mutants of BID indicated that the BH3 domain is also required for interacting with BCL-2, BCL-X_L, or BAX (Figs. 5–7; Table 1). Conversely, point mutations of BCL-2 and BAX suggest that the BH1 domain (but do not exclude BH2) of partner proteins binds to BH3 (Fig. 5). The conservation of predicted α -helical regions in BCL-2 and BAX suggests that their three-dimensional structure will be similar to that of BCL-X_L, with the reservation that a BCL-X_L monomer was solved (Muchmore et al. 1996). The point mutation information here argues that the BH3 domain of BID, an amphipathic α helix, would bind the pocket of the hydrophobic cleft contributed by the BH1 domain of partner proteins.

Instructively, the various BH3 mutants of BID did not score identically in interactions with BCL-2 and BAX or in death agonist assays. BIDmIII-3 (G94A), which binds BCL-2, but not BAX, lost its capacity to counter BCL-2 and induce apoptosis. In contrast, BIDmIII-1 (M97A,D98A) still bound BAX, but not BCL-2, and retained death agonist activity. A model consistent with all of the available data would embrace BID/BAX, but does not firmly exclude BID/BCL-2 heterodimers as the critical, functional pair (Fig. 9). Furthermore, the capacity of BIDmIII-1 to counter BCL-2 whereas mIII-3 would not, dissociates the capacity of BID to reverse BCL-2 protection from its binding to BCL-2. This provides evidence that BID restores apoptosis in FL5.12-Bcl-2 cells by its death-promoting activity that is independent of binding BCL-2 (Table 1). This could also explain why a weakened BIDmIII-1, which binds less avidly to BAX (Figs. 6B and 7C), is unable to promote apoptosis in the more stringent assay in which cells are loaded with BCL-2 (FL5.12-Bcl-2, Table 1).

BCL-2 family members that possess carboxy-terminal signal-anchor segments are integral membrane proteins predominantly localized to the mitochondrial outer membrane and nuclear envelope (Monaghan et al. 1992; Krajewski et al. 1993; Nguyen et al. 1993; de Jong et al. 1994). These data endorse a model in which BAX or

Table 1. Summary of BID mutants within BH3 domain

	BIDwt	BIDmIII-1	BIDmIII-2	BIDmIII-3	BIDmIII-4
Yeast two-hybrid interactions with BCL-x _L	+	–	–	+	–
In vitro and in vivo BCL-2 binding	+	–	–	+	–
Counter BCL-2 in FL5.12-Bcl-2 ^a	+	–	–	–	–
In vitro and in vivo BAX binding	+	+	–	–	–
Death agonist activity					
Jurkat ^b	+	+	–	–	–
Rat-1 ^c	+	+	N.D. ^d	–	–

^aAbility to counteract BCL-2's death-inhibiting effect in FL5.12-Bcl-2 cells following IL-3 withdrawal.

^bAbility to induce cell death in Jurkat cells following induction of BID expression by Doxycyclin treatment.

^cTransient cotransfection of both Bid and Luciferase plasmids into Rat-1 cells assessed by Luciferase assay.

^dNot determined.

BCL-2 would represent a membrane bound receptor and BID a death ligand that translocates between a free cytosolic and a membrane-bound receptor site (Fig. 9). For example, if BAX operates upstream of BID, a BID/BAX interaction might be required to activate BID to kill. Although we have not completely excluded the possibility, to date we have not identified any protein modifying activity by BAX. Alternatively, we favor a model in which BID is upstream of BAX or BCL-2. The interaction of BID's BH3 domain with the hydrophobic pocket of a membrane-bound receptor might activate BAX or inhibit BCL-2 functions. One possibility would be the regulation of a conformational change, reminiscent of the molten globule transition by which structurally similar molecules, colicin and diphtheria toxin B chain, form voltage-dependent pores (London 1992; Cramer et al. 1995). Ligand-receptor interaction is a major paradigm in biology by which critical signals are transduced to initiate cellular responses including apoptosis (Nagata and Golstein 1995). Programmed cell death may also be regulated by internal ligand-receptor interactions as illus-

trated by a death ligand, BID, and a membrane-bound receptor, BAX.

Materials and methods

Isolation of murine and human Bid gene

Murine *Bid* was cloned from a cDNA expression library of a murine T-cell hybridoma line, 2B4. First-strand DNA synthesis was primed with oligo(dT) and completed using SuperScript Choice system (Gibco BRL). Size-fractionated cDNA (≥ 500 bp) was directionally cloned into λ EXlox vector (Novagen), which allowed the expression of cloned cDNA in-frame with the T7-gene-10 product under the T7 promoter. Novagen's PhageMaker system was used for packaging and the cDNA library underwent one round of amplification. Screening was performed as described previously (Blaner and Rutter 1992). Briefly, GST-HMK-mBCL-2 or GST-HMK-mBAX fusion protein was labeled in vitro by phosphorylation of the HMK motif with bovine heart muscle kinase and [γ -³²P]ATP. A total of 1×10^6 plaques were plated. Plaques were transferred overnight to IPTG-immersed nitrocellulose filters. Filters were subsequently blocked and incubated with the probes overnight. After washing filters were subjected to autoradiography. Positive clones were identified and further purified. *loxP*-cre recombination was used to excise the plasmid. Sequencing was performed by standard procedures.

Two human clones containing homology to *Bid* sequences were identified when the EST division of the GenBank was searched with our newly determined murine *Bid* sequence. We obtained and completely sequenced clone no. 52055, which contains a 1.1-kb insert.

Cell lines

Cell lines used included FL5.12, an IL-3-dependent murine pro-B lymphocyte line, Jurkat, a human T-cell line, Rat-1a, a subline of the rat fibroblast cell line Rat-1 (Evan et al. 1992), and NIH-3T3, a murine fibroblast line.

FL5.12 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Gibco BRL), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% WEHI-3B-conditioned medium as a source of IL-3. Jurkat cells were maintained in RPMI 1640 (Gibco BRL) supplemented with 10% fetal calf serum, 10 mM HEPES buffer at pH 7.4, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Rat-1a and NIH-3T3 cells were maintained in Iscove's modified Dulbecco's medium

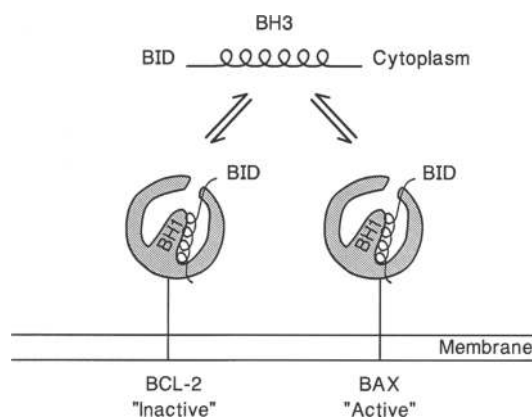


Figure 9. Theoretical model of the cytoplasmic death ligand BID binding to membrane bound BCL-2 or BAX receptors. Amphipathic BH3 domain of BID interacts with BH1 domain sites within the hydrophobic pocket of BCL-2 or BAX (adapted from Muchmore et al. 1996). Mutational analysis suggests that BID/BAX may be the key interaction.

supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml).

Plasmid construction

Murine *Bcl-2* and *Bax* cDNAs deleted of the carboxy-terminal 22 or 19 amino acids, respectively, were amplified by PCR and inserted into the *EcoRI* site of a modified pGEX-3X vector (pGEX-HMK) to generate pGEX-HMK-Bcl-2 or -Bax.

Bid cDNA was cloned into the *EcoRI* and *Sall* sites of the yeast two-hybrid DNA binding domain vector pBTM to generate pBTM-Bid. *Bid* cDNA was also cloned into the *EcoRI* site of the yeast two-hybrid activation domain vector, pACTII, to generate pACTII-Bid. The same BID *EcoRI* fragment was also cloned into pSFFV, pcDNA3 (Invitrogen), pUHD10-3, and pGEX-HMK.

Mutant Bid constructs were generated in two steps. First, the 5' portion of the molecule was PCR amplified. The 5' primer added an *EcoRI* site, whereas the 3' primer ended at the *NheI* site 324 bp into the open reading frame. Second, the amplified *EcoRI*–*NheI* fragment plus the 3' *NheI*–*EcoRI* fragment were ligated into the *EcoRI* site of pBTM. Subsequently, the entire insert was subcloned into pSFFV, pcDNA3, pUHD10-3, and pGEX-HMK.

The EXlox-Bid plasmid that encodes a T7-gene-10-BID fusion protein was generated through loxP-cre mediated recombination.

Antibodies

Purified GST–BID fusion protein as well as two polypeptides were used as immunogens to generate polyclonal anti-BID Abs. Protein A purified and delipidized anti-GST–BID Ab was later used for both Western blotting (1:2000) and immunoprecipitations. Anti-human–BCL-2 mAb 6C8 and biotinylated anti-murine–BAX polyclonal Ab 651 were used for Western blotting analyses (1:2000 and 1:500, respectively). Horseradish peroxidase-conjugated secondary Abs (CALTAG) or streptavidin (ZYMED) were used at 1:2000 and 1:4000, respectively.

Northern blot analysis

A *Bid* cDNA open reading frame probe was hybridized against a poly(A)⁺ RNA blot of adult mouse tissues (Clontech) and washed by standard protocol.

Subcellular fractionation

FL5.12-Bcl-2 (10⁷) cells were collected by centrifugation, washed twice with PBS, and resuspended in hypotonic Buffer A (10 mM Tris at pH 7.5, 25 mM NaF, 5 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.15 U/ml aprotinin, 20 mM leupeptin, and 1 mM PMSF). After incubation on ice for 15 min, cells were homogenized in a Dounce homogenizer for 50 strokes. Cells were checked under microscope to monitor the degree of lysis. Nuclei and nonlysed cells were removed by centrifugation at 500g for 10 min. The supernatant was transferred and centrifuged at 315,000g for 30 min to separate cytosolic and membrane fractions. Equal amounts of protein from each fraction were used for Western analysis.

Stable transfection system

For each transfection, 10⁷ FL5.12 cells were resuspended in 1 ml of RPMI 1640 with 10 mM HEPES at pH 7.4. Ten to twenty micrograms of linearized pSFFV construct (plus 1 µg of pGK-

Hygro in case of cotransfection into FL5.12-Bcl-2 cells) was added. After 5 min on ice, cells were electroporated at 200 V, 900 µF for 5 sec (Transfector 300, BTX). After 10 min on ice cells were transferred to 6–7 ml of nonselective medium in a 25-cm² flask. Two days later cells were put under selection in 96-well plates with 2 µg/ml G418 Sulfate (Gibco BRL) or 2 µg/ml Hygromycin B (CalBiochem).

IL-3 deprivation assays were performed as described (Oltvai et al. 1993). Briefly, cells cultured at 1 × 10⁶ to 2 × 10⁶ cells/ml were collected by centrifugation, washed two times with serum-free medium and once with IL-3-free medium. Cells were resuspended at 10⁶ cells/ml in IL-3-free medium and 200 µl aliquots were seeded in 96-well plates. At each time point, a 25-µl sample was taken from two separate wells and mixed with an equal volume of 0.08% trypan blue. Cell viability was calculated as the percentage of unstained cells counted under a microscope.

Inducible expression system

Jurkat cells were transfected with pUHD172-1neo which encodes the rtTA transactivator consisting of a fusion between TetR⁺ and VP16 (Gossen et al. 1995) under the control of a CMV promoter/enhancer. Stable transfectants were screened by transient transfection of pUHC13-3, which encodes a luciferase reporter gene driven by a CMV minimal promoter with heptamerized *tet*-operators, followed by quantitation of luciferase activity after induction with doxycycline. The clone with the highest degree of induction, Jt1, was selected and used in later studies. cDNAs encoding wild-type or mutant BID proteins were put under the control of heptamerized *tet*-operators by cloning into pUHD10-3, cotransfected into Jt1 cells with pGK-Hygro, and selected under 2 µg/ml Hygromycin B. Hygro-resistant clones were screened for BID expression by Western blot of cell lysates before and after Dox induction. Two or more inducible clones were obtained and kept for each mutant and wild-type Bid. Electroporations were performed at 250 V, 960 µF with Electro Cell Manipulator 600 (BTX).

For viability assays, 1 µg/ml of doxycycline (Sigma) was added to 2-ml cultures of each clone. At each time point, cells were collected from 0.3 ml of cell culture, resuspended in 1:4000 solution of annexin V/FITC (Bender MedSystems) and 1 µg/ml propidium iodide (PI). Flow cytometry was performed (FACScan, Becton Dickinson) and cell populations negative for both annexin V and PI were scored as viable.

Transient transfection system

This assay was modified from several similar systems described previously (Hunter and Parslow 1996; Chittenden et al. 1995; Boyd et al. 1995). All cDNA constructs were cloned into pcDNA3 (Invitrogen) under the CMV immediate early promoter. Rat-1a cells were allowed to grow to ~80% confluence in 12-well plates before transfection. The reporter luciferase plasmid (0.1 µg) was mixed with 0.05 µg of various constructs as indicated and 3 µl of LipofectAMINE (Gibco BRL) in a volume of 0.5 ml per transfection for 5 hr. Cells were lysed 18–20 hr later and a luciferase assay was carried out using luciferase substrates provided by Promega. Luciferase activity was detected by a luminometer (Optocomp II, MGM Instruments Inc.).

Cell viability was estimated as the relative luciferase activity of a cotransfection of a test construct compared with the control in which the luciferase reporter was cotransfected with an empty pcDNA3 plasmid.

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Immunoprecipitation and Western blotting analysis

Cells (5×10^6 to 10×10^6) were used in each sample for immunoprecipitation. Cells were lysed in 100 μ l of NP-40 isotonic lysis buffer with freshly added protease inhibitors (142.5 mM KCl, 5 mM MgCl₂, 10 mM HEPES at pH 7.2, 1 mM EDTA, 0.25% NP-40, 0.2 mM PMSF, 0.1% aprotinin, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin), incubated on ice for 30 min, and centrifuged at 15,000g for 10 min to precipitate nuclei and nonlysed cells. Anti-BID Ab (20 μ g) was added to the supernatant of each sample, mixed, and incubated on ice for 30 min. Subsequently 400 μ l of NP-40 buffer was added to each sample along with 25 μ l of protein A-Sepharose and incubated at 4°C with nutation for 1–2 hr. Immunoprecipitates were collected by a brief spin, washed three times with 1 ml of NP-40 buffer, and solubilized with 1 \times SDS-PAGE sample buffer.

For Western blotting, either immunoprecipitates or direct cell lysates were separated on 12.5 or 16% Tris-Glycine gels (NOVEX) and transferred to PVDF membranes (BioTrace, GelmanSciences). Filters were blocked overnight at 4°C with Tris-buffered saline plus 0.1% Tween 20 (TBST) containing 6% non-fat milk. Filters were incubated with primary and secondary Abs for 1 hr, each followed by washing three times in TBST for 5 min, and developed by ECL (Amersham).

Yeast two-hybrid assay

pBTM-Bid wt and mutants were cotransformed into yeast strain L40 [MATa his3D200 trp1-901 leu2-3,112 ade2 LYS2::[lexAop]₄-HIS3 URA3::[lexAop]₈-lacZ] with Bcl-x_L, Bcl-2, Bax, or Bid in pACTII. Transformants were grown on -Trp -Leu plates and assayed for β -gal activity as described previously (Sedlak et al. 1995).

In vitro binding assay

Equal amounts of in vitro translated, ³⁵S-labeled products of BAX, BCL-2, BCL-X_L, and BID were incubated with 1 μ g of purified GST-BID fusion protein (wt or mutant) on ice for 30 min. NP-40 buffer (500 μ l) with protease inhibitors was added to each binding mixture plus 25 μ l of GSH-agarose and nutated at 4°C for 1–2 hr. Materials bound to GSH-agarose were precipitated, washed three times in 1 ml of NP-40 buffer, and solubilized in 25 μ l of 1 \times SDS-PAGE sample buffer. Electrophoresis on 12.5% SDS-PAGE was followed by autoradiography.

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Note added in proof

The sequence data described in this paper have been submitted to GenBank under accession no. U75506.

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