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BH3 Domains of BH3-Only Proteins Differentially Regulate Bax-Mediated Mitochondrial Membrane Permeabilization Both Directly and Indirectly

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Summary

Using a Bax-dependent membrane-permeabilization assay, we show that peptides corresponding to the BH3 domains of Bcl-2 family "BH3-only" proteins have dual functions. Several BH3 peptides relieved the inhibition of Bax caused by the antiapoptotic Bcl-x_L and/or Mcl-1 proteins, some displaying a specificity for either Bcl-x_L or Mcl-1. Besides having this derepression function, the Bid and Bim peptides activated Bax directly and were the only BH3 peptides tested that could potently induce cytochrome c release from mitochondria in cultured cells. Furthermore, Bax activator molecules (cleaved Bid protein and the Bim BH3 peptide) synergistically induced cytochrome c release when introduced into cells along with derepressor BH3 peptides. These observations support a unified model of BH3 domain function, encompassing both positive and negative regulation of other Bcl-2 family members. In this model, the simple inhibition of antiapoptotic functions is insufficient to induce apoptosis unless a direct activator of Bax or Bak is present.

Introduction

Bcl-2 family proteins regulate mitochondrial outer-membrane permeabilization, often a critical early step in apoptosis (Gross et al., 1999; Kuwana and Newmeyer, 2003; Vander Heiden and Thompson, 1999). These globular, α-helical proteins are classified according to their anti- or proapoptotic effects. The antiapoptotic family members, including Bcl-2, Bcl-x_L, Mcl-1, and A1, possess four conserved Bcl-2 homology domains, BH1 through BH4. The proapoptotic family members are divided into two categories: "multidomain" or "BH1–3" (containing BH1, BH2, and BH3 regions) and "BH3-only" proteins.

Exactly how Bcl-2 family proteins regulate outer-membrane permeabilization is still unclear. Genetic and biochemical studies suggest that the BH1–3 proteins (Bax, Bak, and by analogy, perhaps Bok) are essential for many forms of apoptosis and mitochondrial outer-membrane permeabilization (Lindsten et al., 2000; Wei et al., 2001). Moreover, the BH3-only proteins appear to act upstream of the multidomain proteins (Bax, Bak) to induce apoptosis (Cheng et al., 2001).

The BH3 regions of the BH3-only proteins are essential for their proapoptotic function (Cheng et al., 2001; Huang and Strasser, 2000). Synthetic peptides corresponding to certain of these domains can induce the release of cytochrome c from mitochondria to activate caspases in *Xenopus* egg cytoplasmic extracts, highlighting mitochondria as a site of action for BH3-only proteins (Cosulich et al., 1997). BH3 domains presumably achieve their proapoptotic function through binding to other Bcl-2 family members. Such binding was indeed observed for a peptide corresponding to the Bak BH3 region, which fits into a hydrophobic pocket in Bcl-x_L (Sattler et al., 1997). The binding affinities of various BH3 peptides for Bcl-x_L appear to correlate with their ability to inhibit that protein (Sattler et al., 1997). Furthermore, small molecules that can competitively inhibit binding of Bak BH3 peptide to Bcl-x_L were reported to show proapoptotic activity (Degterev et al., 2001; Tzung et al., 2001), suggesting that antiapoptotic Bcl-2 family members inhibit apoptosis, at least in part, through binding to BH3 domains either in the BH1–3 or the BH3-only proteins or both.

Recent studies with BH3 peptides have suggested that there are two different subgroups in the BH3-only proteins. One group, represented by Bid and Bim, were proposed to induce mitochondrial membrane permeabilization via Bax/Bak. The other group, including Bad and Bik, was proposed to sensitize the mitochondrial outer membrane for permeabilization by opposing antiapoptotic family members, such as Bcl-x_L and Bcl-2 (Letai et al., 2002; Moreau et al., 2003). This led to the classification of BH3-only proteins as either death agonists or survival antagonists. Although these studies with cells or isolated whole mitochondria were informative, their interpretation is somewhat ambiguous because of the unknown contributions of the complex protein mixtures present in mitochondrial outer membranes. Thus, they could not show definitively that BH3 domains could act directly to activate Bax rather than through antagonizing the antiapoptotic Bcl-2-family members. A further difficulty has been that, although binding of BH3-only proteins to their antiapoptotic relatives has been well documented, direct interactions of BH3-only proteins such as Bid and Bim with Bax were only recently reported (Cartron et al., 2004; Harada et al., 2004).

Here, we address the functions of BH3 domains using a simplified system with which the functions of Bcl-2 family proteins can be directly assessed in a defined context. In this approach, peptides and recombinant proteins are incubated with synthetic liposomes that contain entrapped fluorescent dextrans. Earlier, we used this system to show that mixtures of Bcl-2 family proteins are sufficient to induce the formation of large pores in membranes, provided that the membranes contain the signature mitochondrial lipid, cardiolipin (Kuwana et al., 2002). Here, we show that peptides corresponding to the BH3 domains of Bim and Bid can directly activate Bax to permeabilize liposomes. In contrast, the other BH3 domain peptides tested lack the ability to activate Bax directly and can only act indirectly to relieve the inhibition produced by antiapoptotic Bcl-2 family

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members. These BH3 peptides (which we term "derepressor BH3s") require the presence of another molecule that can directly activate Bax, e.g., Bid or Bim.

Furthermore, we found that some of the BH3 peptides differentially counteract Bcl-x_L and Mcl-1, arguing that the derepression function of the peptides is determined essentially by their interactions with specific antiapoptotic family members. In confirmation of this idea, we found that the ability of each derepressor BH3 peptide to oppose Bcl-x_L or Mcl-1 correlates well with its binding affinity for that protein. Furthermore, we showed that the BH3 peptides differed markedly in their ability to induce cytochrome c release when loaded into HeLa cells; the only potent inducers of mitochondrial outer-membrane permeabilization were the Bid and Bim BH3 peptides, the direct Bax activators. Finally, we observed that BH3 domain peptides can act synergistically to induce cytochrome c release when both classes of BH3 domains are loaded into cells, and that a BH3 peptide can sensitize cells to death after administration of UV irradiation.

Results

BH3 Domains Can Activate Bax in Two Ways

To investigate the ability of proapoptotic Bcl-2 family members proteins to regulate mitochondrial membrane permeabilization, we synthesized 24- or 25-mer peptides corresponding to the BH3 regions of Bax and nine BH3-only proteins (Figure 1A). We first examined the effect of these peptides on cytochrome c release from *Xenopus* egg mitochondria. As shown in Figure 1B, some BH3 peptides (Bim, Bid, and Puma) released cytochrome c completely, while others showed limited releasing activity (Bik, Bad, Bmf, and Hrk) or none at all (Noxa B, Bnip3 and Bax). As a positive control, we treated mitochondria with N/C-Bid, a form of recombinant activated Bid protein that efficiently induces cytochrome c release from isolated mitochondria (Kuwana et al., 2002).

Because the Bim BH3 peptide released cytochrome c potently from mitochondria (Figure 1B), we examined whether this peptide could also permeabilize liposomes in the presence of recombinant Bax protein, as previously shown for the Bid BH3 peptide (Kuwana et al., 2002). Indeed, the Bim peptide at 10 μM synergized with full-length monomeric Bax to permeabilize the liposomes, and this activity was inhibited by Bcl-x_L (Figure 2A). The extent of liposome permeabilization in a fixed time interval depended on the concentration of Bim BH3 peptide (Figure 2B). The Bim peptide was even more potent than the Bid peptide, and so it appears that the Bim BH3 domain, like that of Bid, can directly activate Bax. The short form of Bim (BimS) also activated Bax directly as a recombinant protein (T.K. and D.D.N., unpublished data). We next examined a panel of BH3 peptides for similar activity. Only the Bid and Bim peptides were potent direct activators of Bax (Figure 2C). The Puma, Hrk, and Noxa B BH3 peptides appeared to activate Bax very modestly—mixed with Bax they permeabilized liposomes to an extent slightly greater than Bax alone. The significance of this weak activation is unclear.

As most of the BH3 peptides were unable to activate

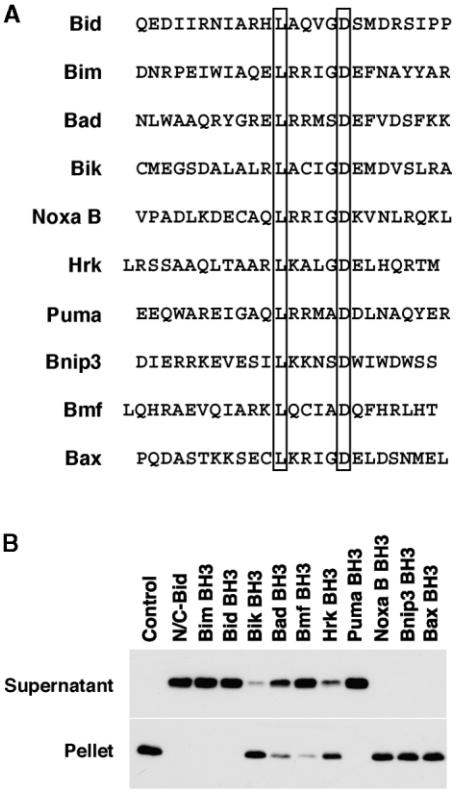

Figure 1. Several BH3 Peptides Used in the Study Induce Cytochrome c Release from Isolated *Xenopus* Egg Mitochondria

(A) Amino acid sequences of the BH3 peptides, all of which correspond to the human proteins, except for Noxa B, which corresponds to the more C-terminal of the two mouse Noxa BH3 domains.

(B) The indicated BH3 peptides (25 μM) or N/C-Bid protein (135 nM) were added to isolated *Xenopus* egg mitochondria and incubated for 3 hr at room temperature. After the incubation, the assay suspension was separated into supernatant and pellet fractions and immunoblotted for cytochrome c content. Shown is a representative of three experiments.

Bax directly, we asked whether they might function in an indirect manner by opposing the antiapoptotic family member, Bcl-x_L. Indeed, most of the peptides were able to restore Bax activity in the presence of inhibitory concentrations of Bcl-x_L (Figure 2C). The exceptions were the Noxa B, Bnip3, and Bax peptides, which were ineffective in this assay. These results show directly that active BH3 domains fall into two functional categories: direct activators (able to activate Bax directly) and derepressors (able to activate Bax indirectly, perhaps by sequestering antiapoptotic Bcl-2 relatives; see below). This was previously suggested by limited studies on BH3 peptides from four proteins and their effects on whole mitochondria (Letai et al., 2002). Our experiments now analyze these distinct functions of BH3 domains in a molecularly defined context.

A recent study reported that Bid could interact with the first α helix of Bax, leading to Bax activation as measured by the binding of the conformation-specific antibody 6A7 and cytochrome c release from mitochondria. Furthermore, a mutation in the Bid BH3 domain, R84G, was found to abrogate these effects (Cartron et

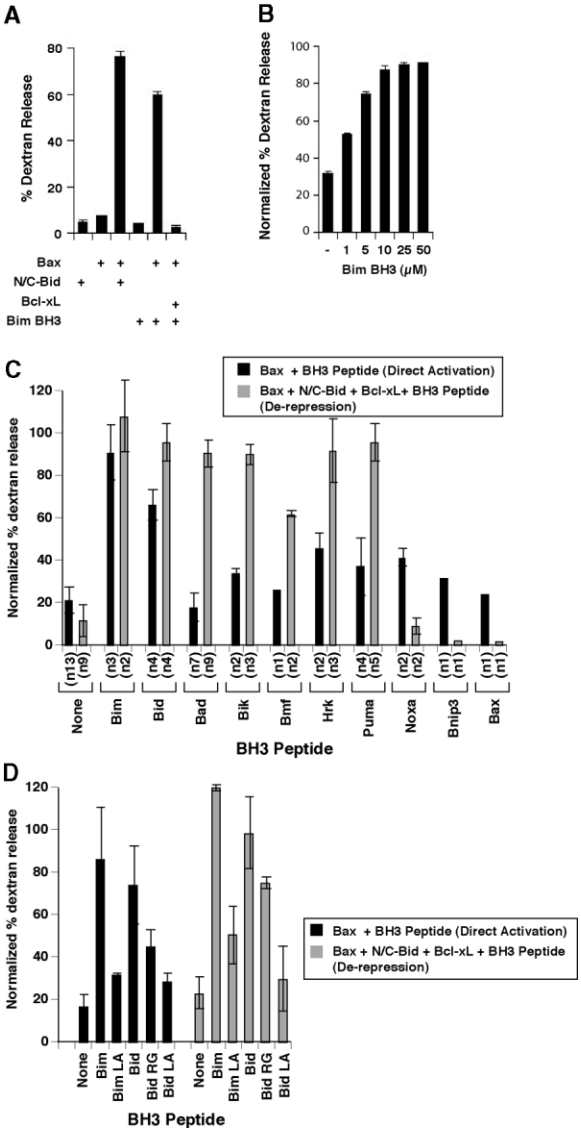

Figure 2. BH3 Peptides Stimulate Bax-Dependent Liposome Permeabilization in Direct and Indirect Ways

(A) The Bim BH3 peptide at 10 μM permeabilized liposomes in the presence of Bax (120 nM), and this permeabilization, shown as % release of fluorescein-dextran (10 kDa), was inhibited by excess Bcl-x_L (7.6 μM). N/C-Bid (45 nM) served as a positive control for efficient Bax activation.

(B) The permeabilization was dependent on Bim peptide concentration in the range of 0–25 μM. The data are normalized as a percentage of the release produced by a mixture of Bax and N/C-Bid (120 nM and 45 nM, respectively) performed within the same experiment.

(C) The indicated BH3 peptides were added individually to Bax (120 nM) or mixtures of Bax, N/C-Bid (45 nM), and Bcl-x_L (1 μM) to measure direct activation or derepression of Bax, respectively. BH3 peptides were added at 25 μM. The data are normalized as described in (B) and averaged from the indicated numbers of independent experiments.

(D) Mutant Bim (L152A), Bid (R84G), and Bid (L190A) BH3 peptides (25 μM) were examined for their direct activation and derepression activities as described in (C). Data are presented as normalized % release as in (C) and averaged from two independent experiments, where the permeabilization produced by a mixture of Bax and N/C-Bid was measured as 52% and 33%.

Error bars represent standard deviation from the mean.

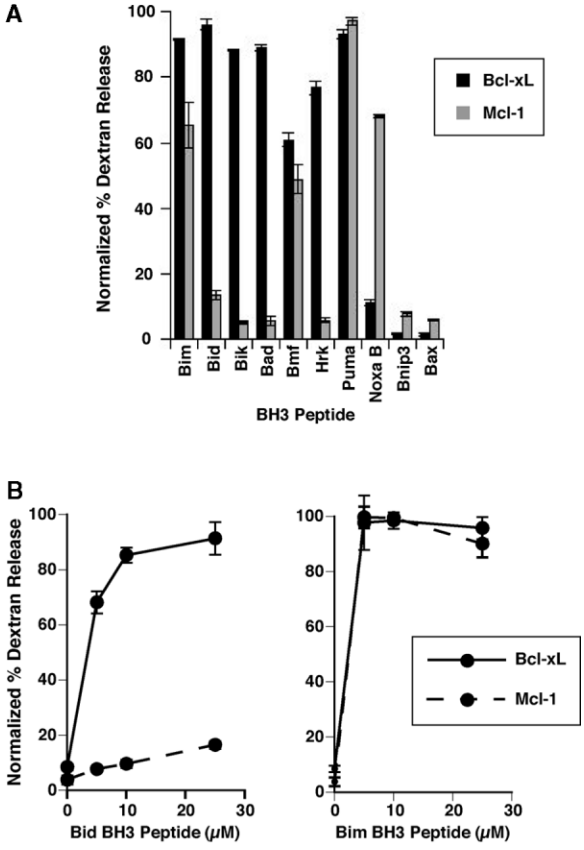

Figure 3. Opposition of Mcl-1 by BH3 Peptides

(A) BH3 peptides (25 μM) showed differential derepression activity toward Bcl-x_L and Mcl-1. Recombinant Bcl-x_L (1.3 μM) or Mcl-1 (1 μM) was added to the liposome assay to inhibit Bax/Bid-induced permeabilization, and release was measured 3 hr after addition of the indicated BH3 peptide. The data are normalized to a percentage of the release induced by Bax + N/C-Bid. All the peptides tested except Noxa B, Bnip3, and Bax inhibited Bcl-x_L, whereas only Bim, Bmf, Puma, and Noxa B inhibited Mcl-1.

(B) Bid and Bim BH3 peptides were titrated against fixed amounts of Bcl-x_L or Mcl-1 in the presence of N/C-Bid and Bax as above. The Bid peptide was ineffective against Mcl-1, whereas the Bim peptide opposed both Bcl-x_L and Mcl-1. The assay was performed as in (A).

Error bars represent standard deviation from the mean.

al., 2004). In contrast, mutations in the conserved leucine residue of the BH3 domain have been observed to disrupt binding to antiapoptotic family members. We tested the effects of the corresponding mutant BH3 peptides in the liposome assay (Figure 2D). The Bid RG mutant displayed only a partial loss of both direct Bax activation and derepression functions. On the other hand, the Bid and Bim LA mutants showed a significant loss of both direct activation and derepression activities. Thus, none of these mutations were able to clearly discriminate between the two types of activities.

We then asked whether the BH3 peptides could oppose another antiapoptotic Bcl-2 family member, Mcl-1. As shown in Figure 3A, the Bim, Bmf, and Puma BH3 peptides restored membrane permeabilization in the presence of either Bcl-x_L or Mcl-1. On the other hand, the BH3 peptides for Bid, Bik, Bad, and Hrk opposed Bcl-x_L but

were ineffective against Mcl-1, whereas the Noxa B peptide opposed Mcl-1 but not Bcl-x_L. We conclude that derepressor-type BH3 domains can have preferences for specific antiapoptotic family members and that the ability of BH3 domains to derepress Bax is determined by which antiapoptotic relative is present.

The direct activator BH3 peptides can also discriminate between Mcl-1 and Bcl-x_L with regard to derepression, as the Bid BH3 peptide was ineffective in opposing Mcl-1, whereas the Bim peptide exhibited a potent effect against both Bcl-x_L and Mcl-1 (Figure 3B). In contrast, both Bid and Bim can function as direct activators of Bax, regardless of whether permeabilization is inhibited by Bcl-x_L or Mcl-1, as long as an appropriate molecule is also present to carry out the derepression function. Interestingly, Mcl-1 could inhibit Bax-mediated permeabilization that was induced by N/C-Bid, but the Bid peptide did not reverse this inhibition. This suggests either that Mcl-1 inhibits Bax directly or that Mcl-1 can interact with the N/C-Bid protein despite being unable to bind to the Bid BH3 peptide (see Supplemental Table S1 at http://www.molecule.org/cgi/content/full/17/4/525/DC1/).

Derepressor-type BH3 peptides, such as the Bad and Puma peptides, could not induce membrane permeabilization unless another direct activator of Bax, in this case N/C-Bid protein, was also added (Figure 4). The Bid and Bim BH3 peptides displayed no requirement for N/C-Bid (Figure 4A; middle and right upper panels). Moreover, the Bad and Puma BH3 peptides did not act synergistically with suboptimal amounts of N/C-Bid in the absence of Bcl-x_L (Figure 4B). Also, in a further examination of whether Bcl-x_L or Mcl-1 have any ability to induce membrane permeabilization, we preincubated these proteins with derepressor BH3 peptides prior to the assay, but observed no permeabilization (data not shown.) We conclude from these data that the derepressor BH3 peptides can neither cooperate directly with the direct activator N/C-Bid nor convert Bcl-x_L or Mcl-1 to proapoptotic proteins, as suggested by Juin and colleagues (Moreau et al., 2003), but instead act to relieve the inhibition exerted by Bcl-x_L or Mcl-1.

It is curious that Bcl-x_L inhibited the activation of Bax when it was induced by the N/C-Bid protein more efficiently than when it was induced by the Bid (or Bim) BH3 peptides (Figures 3B and 4A). This may be because the molar concentrations of the peptides were far greater than that of the N/C-Bid protein added in these assays (10–25 μM for peptides and 45 nM for N/C-Bid). Alternatively, regions of Bid other than the BH3 domain may potentiate the inhibitory interaction with Bcl-x_L.

To explore whether interactions of BH3 peptides with Bcl-x_L or Mcl-1 were correlated with the functional results in Figures 2–4, we analyzed the binding of various BH3 peptides to Bcl-x_L, using surface plasmon resonance (BIAcore). The ability of the various peptides to bind to Bcl-x_L or Mcl-1 (Table S1) was correlated with the ability of the peptides to oppose either or both of these proteins in the Bax derepression assay (Figures 2–4). This argues that in the derepression assay, the function of these peptides is determined primarily by the antiapoptotic family members, rather than Bax.

Direct and Indirect Bax Activation Mechanisms Both Lead to Increased Bax Association with Membranes and to Bax Oligomerization

To help understand the mechanism of action of the BH3 peptides, we measured Bax association with the liposomes under the same conditions used in the permeabilization experiments. The incubations were as in Figure 2, but afterwards, the liposomes were reisolated by sucrose density float-up centrifugation and analyzed for Bax content by immunoblotting. This was a more rigorous assay for Bax membrane binding than the one we used previously (Kuwana et al., 2002) because it avoided contamination of the membranes with precipitated Bax protein (which is typically present in small amounts in the recombinant Bax preparations.) We found that, added alone, monomeric Bax showed minimal association with liposomes; however, the addition of N/C-Bid markedly increased the amount of Bax associated with the membranes. Bax membrane association was essentially abolished by coincubation with Bcl-x_L (Figure 5A). We then examined whether BH3 peptides could also influence Bax association with the membrane. The Bim BH3 peptide, a direct activator, stimulated both liposome permeabilization and Bax membrane binding (Figure 5A), both of which were inhibited by Bcl-x_L. The Bad peptide, a derepressor BH3 (Figure 2), also restored Bax association with the membrane in the presence of Bcl-x_L (Figures 5B and 5C). In every case, the amount of Bax associated with the liposomes correlated with the observed extent of permeabilization, suggesting that the recruitment of Bax to the membrane is a limiting event in the permeabilization mechanism, regardless of whether the BH3 domain acts in a direct or indirect manner. Crosslinking studies (Figures 5D and 5E) showed that the same BH3 peptides that caused Bax to be activated, either directly or through derepression, also induced Bax oligomerization. In this experiment we also found that when compared with the wild-type peptides, the ability of mutant BH3 domain peptides Bid RG, Bid LA, and Bim LA, to induce Bax oligomerization was reduced, consistent with their reduced ability to induce Bax activation in the liposome assay (Figure 2D). Thus, in general, a good correlation exists in the strengths of liposome permeabilization, Bax membrane binding, and Bax oligomerization.

Some BH3 Peptides Induce Cytochrome c Release When Loaded into Cells

To further investigate the BH3 peptides' ability to regulate multidomain pro- and antiapoptotic members in a more physiological setting, we loaded these peptides into HeLa cells expressing GFP-cytochrome c and monitored cytochrome c release directly in single cells. Cells were coloaded with BH3 peptides and AlexaFluor-conjugated dextran to mark the cells that received peptide (Figure 6A). Only the direct activator BH3 peptides, Bim and Bid, were capable of inducing GFP-cytochrome c release in the majority of loaded cells (Figures 6A and 6B). Interestingly, the Puma and Bmf peptides, which could not directly activate Bax but were the only peptides tested other than Bim that could oppose both Bcl-x_L and Mcl-1 in the derepression assay (Figure 3), consistently produced GFP-cytochrome c release in a

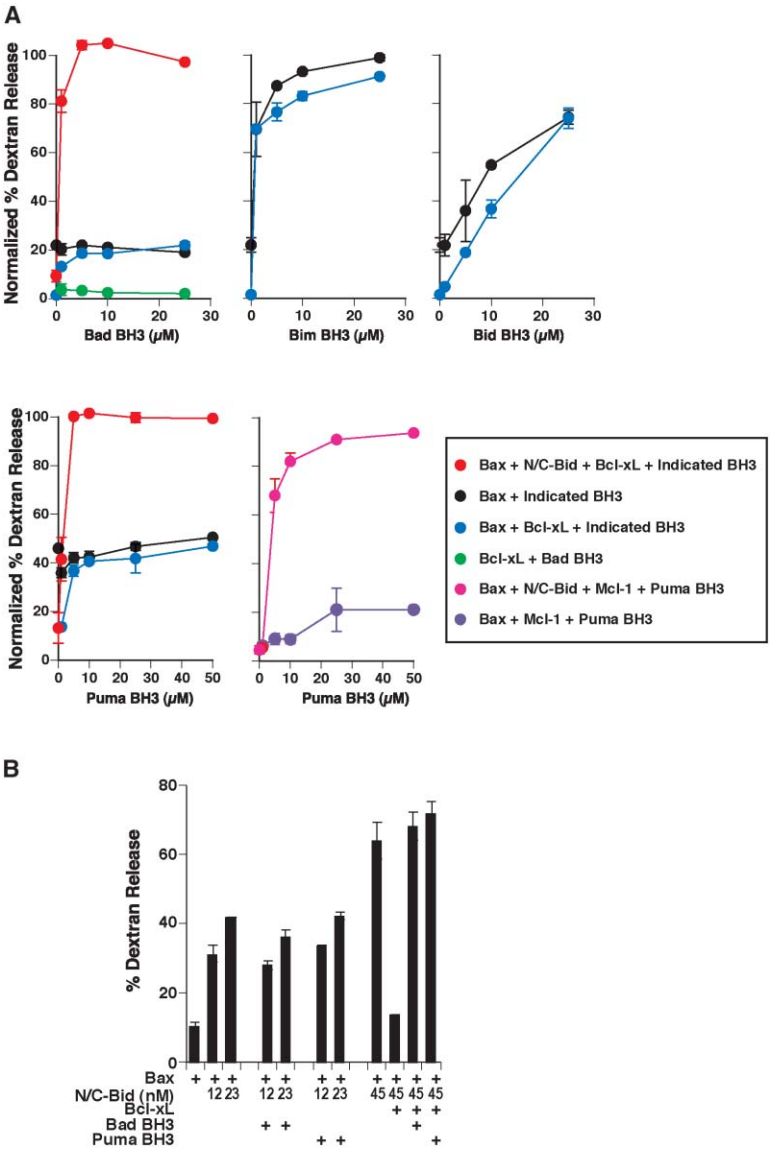

Figure 4. A Direct Activator Is Required to Induce Membrane Permeabilization

(A) Bad and Puma BH3 peptides could not induce permeabilization without N/C-Bid, whereas Bim or Bid BH3 peptides (direct activators) could. The concentrations of recombinant proteins were the same as in Figure 2C. See legend for the additions made to each sample. At the 0 μM peptide concentration, the red or magenta symbol shows the inhibition of Bax and N/C-Bid induced permeabilization by Bcl-xL or Mcl-1, respectively.

(B) Absence of synergy between N/C-Bid and derepressor peptides, such as Bad and Puma. Submaximal doses of N/C-Bid were used to determine if there was synergy between these molecules.

Error bars represent standard deviation from the mean.

small percentage of the cells. The remaining peptides did not significantly induce GFP-cytochrome c release in HeLa cells. The ability of various BH3 peptides to induce apoptosis in cells was consistent with the behavior of these peptides in the liposome assays, suggesting that this biochemical system mirrors a physiological function of BH3-only proteins in cells.

A prediction of our in vitro studies is that direct activator BH3 domains should cooperate synergistically with derepressor BH3 domains, if antiapoptotic Bcl-2 family proteins are present. We examined this in HeLa cells by first titrating the Bim BH3 peptide down to an ineffective level and then coloading the cells with this suboptimal Bim peptide concentration along with low amounts of derepressor BH3 peptides. The derepressor peptides synergized strikingly with the Bim BH3 peptide (Figure 6C), even though most of the peptides by themselves could not induce cytochrome c release measurably. Similarly, some of the peptides could act synergistically when they were coloaded into HeLa cells along with N/C-Bid. Finally, we tested the ability of BH3 peptides to sensitize cells to an apoptotic stimulus, ultraviolet (UV) irradiation. The UV dose was first titrated down to a sublethal level; then, at this minimal dose, the effect of loading various derepressor BH3 peptides was tested. Again, a synergistic induction of cytochrome c release was observed with several of the peptides, most notably Puma and Bik. The reason for the differential effects of the BH3 peptides is unknown; however, based on our in vitro findings, we can hypothesize that a critical determinant is whether a given combination of BH3 domains present in the cell can oppose all or most of the antiapoptotic Bcl-2 family proteins that are present.

Discussion

In the simple rheostat model, cell death and survival was proposed to be regulated primarily by the balance between proapoptotic and antiapoptotic classes of Bcl-2 family proteins, with death as the default (Kors-

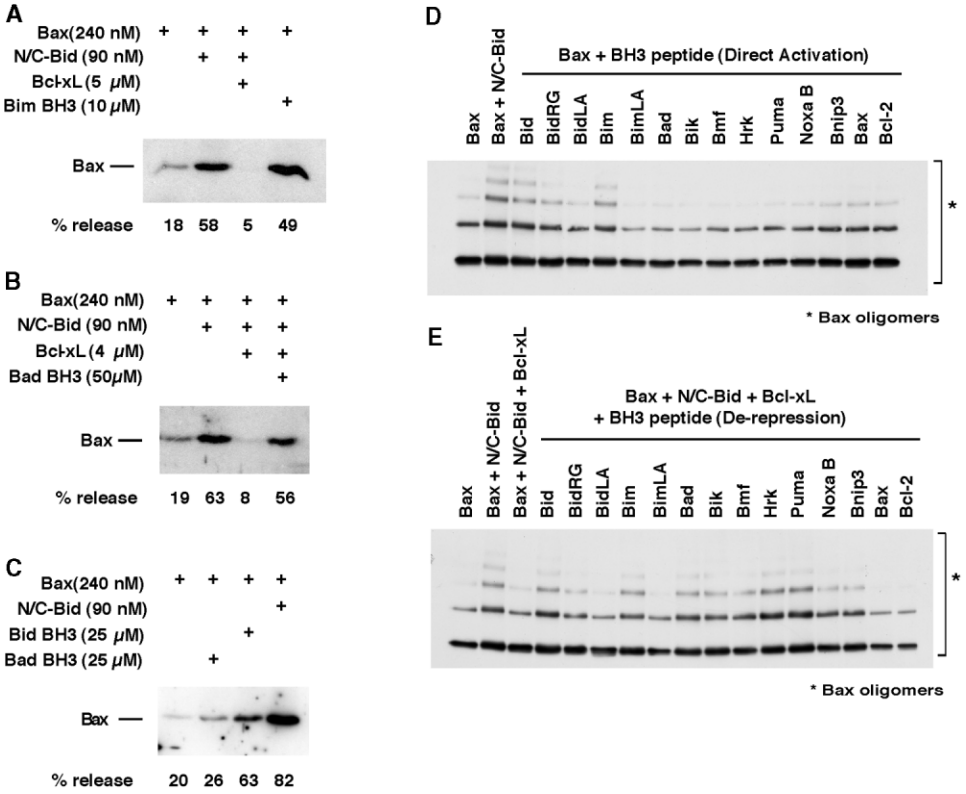

Figure 5. BH3 Peptides Induce Bax Association with Lipid Membranes and Increased Bax Crosslinking

The indicated recombinant proteins and BH3 peptides were incubated with liposomes as in Figure 2, and one part of the assay mix was subjected to sucrose float-up centrifugation to collect the membranes, which were then analyzed for Bax content by immunoblotting.

(A) Bim BH3 peptide induced Bax membrane association and membrane permeabilization to the same extent as N/C-Bid. Note that Bax association correlated with fluorescein-dextran release.

(B) Bad BH3 peptide, a derepressor, increased Bax association with the membrane as well as dextran release.

(C) The Bad peptide by itself did not stimulate Bax membrane association. The data shown are representatives of five (A), three (B), and one (C) experiments.

(D) Direct activators, Bim and Bid BH3 peptides, induce increased Bax oligomerization detected by crosslinking using BMH. The mutant peptides induced less oligomerization of Bax.

(E) Derepressors restored Bax oligomerization. Only higher molecular weight species (dimers and above) are visible because the antibody is relatively insensitive in recognizing monomeric Bax (data not shown). The data are representative of five (D) and two (E) experiments.

meyer et al., 1993). Superficially, this notion seems to be supported by observations that apoptosis can be triggered merely by downregulating or inhibiting prosurvival Bcl-2 family members, apparently shifting the balance toward the proapoptotic relatives. For example, Eμ-Myc tumor cells can undergo apoptosis when Bcl-2 is downregulated (Letai et al., 2004).

However, our results support a modified view, in which survival is the default. In this model, death must be actively induced but can be repressed by antiapoptotic Bcl-2 family proteins (Figure 7). A prediction is that the loss of antiapoptotic Bcl-2 family proteins can lead to apoptosis only if direct activator proteins, e.g., Bid and Bim, are constitutively engaged. In the example cited above, a persistent direct activation signal is indeed provided by the *myc* oncogene, which engages Bim (Egle et al., 2004). In situations like this, simultaneous but opposing mechanisms exist both to activate Bax-type proteins and to keep them inhibited. This tension between positive and negative regulators might enhance the responsiveness of the cells to death and survival stimuli. On the other hand, cells lacking positive death-inducing signals are predicted to survive upon downregulation or inhibition of antiapoptotic Bcl-2 relatives.

Bax is thought to be regulated primarily by other Bcl-2 family proteins, although regulatory partners outside the family may also be important (Chipuk et al., 2004; Cuddeback et al., 2001; Guo et al., 2003; Sawada et al., 2003). However, the detailed mechanisms of Bax activation and function are still unclear. We previously reported that the membrane-permeabilization function of Bax can be activated directly by the BH3 domain of Bid (Kuwana et al., 2002) or, surprisingly, by the p53 protein, which lacks a clearly identifiable BH3 domain (Chipuk et al., 2004). We now find that the Bim BH3 peptide (Figure 2A), as well as recombinant BimS protein (T.K. and D.D.N., unpublished data) can also directly activate Bax.

In the presence of membranes, BH3-only proteins and BH3 peptides have often been suggested to interact with Bax and Bak in a "hit-and-run" transient fashion, resulting in conformational change, oligomerization, and activation of Bax and Bak to permeabilize the mem-

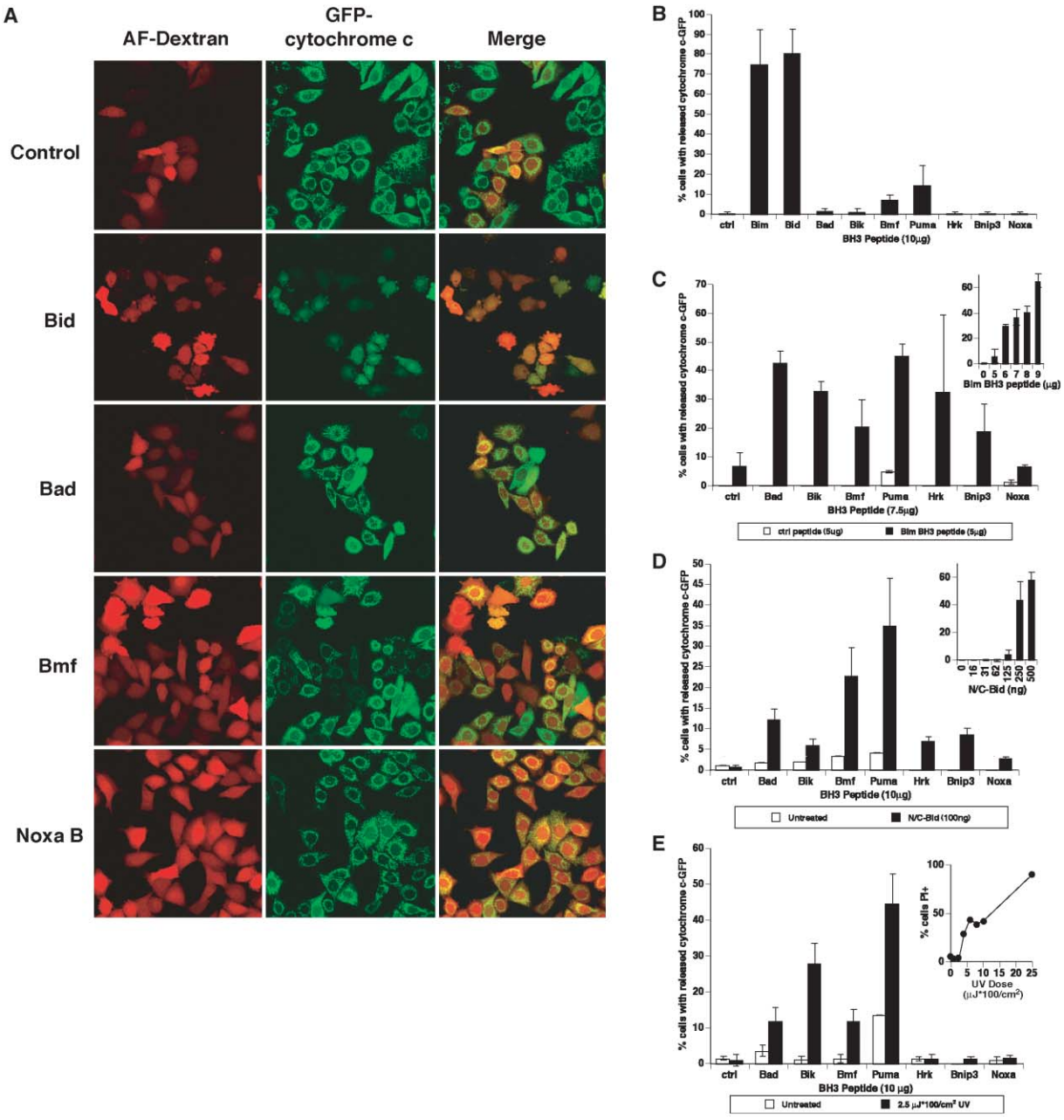

Figure 6. Cytochrome c Release Induced by BH3 Peptides Loaded into HeLa Cells Expressing Cytochrome c-GFP; Synergistic Effects Are Seen When BH3 Peptides Are Combined Or When a BH3 Peptide Is Combined with an Apoptotic Stimulus (UV Irradiation)

(A) Certain BH3 peptides induce mitochondrial permeabilization when loaded into Hela cells. Hela cells stably expressing cytochrome c-GFP (green), pretreated with zVAD.fmk (100 μM), were loaded with a control peptide (10 μg, see Experimental Procedures) or the indicated BH3 peptides (10 μg) using Chariot reagent. To observe loaded cells, fluorescently labeled dextran (red, 500 μg) was coloaded. Representative confocal micrographs are shown, taken 4 hr after treatment. Cells were examined visually for cytochrome c-GFP release.

(B) The percentages of red cells with diffuse cytochrome c-GFP localization in the experiment shown in (A) were determined from a minimum of 300 cells per well.

(C) Derepressor BH3 peptides lower the threshold for Bim BH3-mediated mitochondrial permeabilization. Cells were loaded as in (A) with each of the indicated derepressor BH3 or control peptides (7.5 μg) with or without Bim BH3 peptide at a sublethal dose (5 μg) that was determined by titration (inset). Cells were assessed for cytochrome c-GFP release after 4 hr as in (B).

(D) Derepressor BH3 peptides lower the threshold for N/C-Bid-mediated mitochondrial permeabilization. Cells were loaded as in (A) with each of the indicated derepressor BH3 or control peptides (10 μg) with or without N/C Bid protein at a sublethal dose (100ng) determined from a titration of N/C Bid on Hela cells (inset). Cells were assessed for cytochrome c-GFP release after 4 hr as in (B).

(E) Derepressor BH3 peptides lower the threshold for UV-induced mitochondrial permeabilization. HeLa cells were left untreated or pretreated with a sublethal dose of UV (2.5 μJ*100/cm^2) and zVAD-fmk (100 μM; added to prevent cell rounding and movement). Cells were cultured for 24 hr followed by loading of each of the indicated derepressor BH3 or control peptides (10 μg). Cells were assessed for cytochrome c-GFP release 4 hr after loading as in (B). To determine the sublethal UV dose, HeLa cells were treated with titrated doses of UV, cultured for 24 hr, and analyzed by propidium iodide uptake for cell death (inset).

Error bars represent standard deviation from the mean.

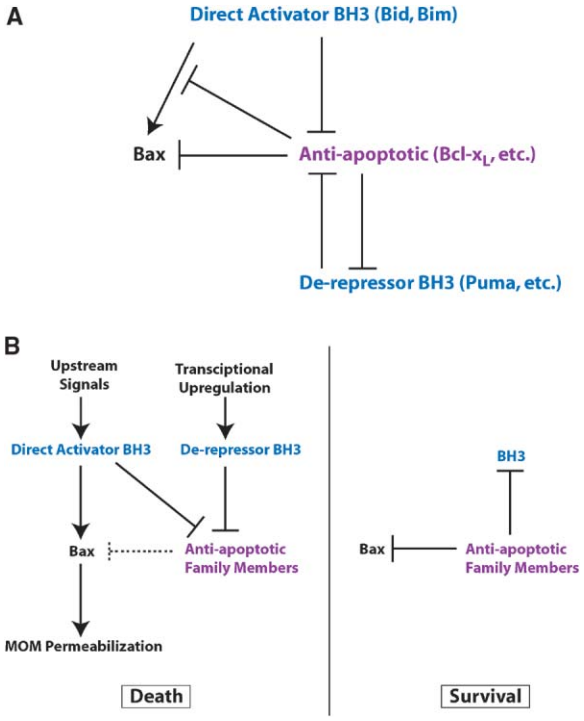

Figure 7. Model of Mitochondrial Apoptotic Regulation by Bcl-2 Family Proteins

(A) Positive and negative functional interactions. Bax is the primary effector of MOMP and is directly activated by Bid and Bim and inhibited by antiapoptotic relatives. BH3 domains of BH3-only proteins can counteract the inhibition by antiapoptotic family members, and conversely, the antiapoptotic proteins can sequester and inhibit the BH3-only proteins.

(B) Status of the regulatory networks under conditions of cell death (left) and survival (right). When the direct activator and derepressor BH3 domains outweigh the effects of antiapoptotic family members, apoptosis is the result. In contrast, when the antiapoptotic proteins predominate, cells survive.

branes. A formal possibility, however, is that the function of the direct activator BH3 peptides is not to interact with Bax per se, but rather to perturb the lipid membranes and thereby allow Bax to interact with these membranes, resulting in the changes we observe. To begin to address this possibility, we preincubated the Bim BH3 peptide with the membrane, washing out the peptide, and then adding Bax, or as a positive control, Bax + freshly added Bim BH3 peptide. We did not detect any permeabilization in this experiment except in the simultaneous presence of Bax and newly added BH3 peptide (not shown), suggesting that the peptides do not cause stable changes in the membrane. It remains formally possible, however, that the direct-activator BH3 domains and Bax act simultaneously on the membrane in order to achieve permeabilization.

The inhibitory side of the Bcl-2 family is comprised principally of antiapoptotic members, represented in this study by Bcl-x_L and Mcl-1, which inhibit Bax-dependent membrane permeabilization. Do these antiapoptotic Bcl-2 family proteins primarily oppose the multidomain Bax/Bak-type proteins or instead inhibit proteins belonging to the BH3-only class? Earlier studies suggested that Bcl-x_L and other antiapoptotic relatives probably inhibit both Bax/Bak and the BH3-only proteins (Kuwana et al., 2002; Kuwana and Newmeyer, 2003; Newmeyer and Ferguson-Miller, 2003). However, as this regulation seems to be based on stoichiometric interactions between the family members (Cheng et al., 2001; Gross et al., 1998; Sedlak et al., 1995; Yang et al., 1995; Yin et al., 1994), it is reasonable to ask the converse: can the BH3-only proteins, if present in excess, sequester and inhibit Bcl-x_L, Mcl-1, and Bcl-2? Our results show that some BH3 domains (those of Bid and Bim) can function directly to activate Bax and also indirectly to counteract the inhibition of Bax by antiapoptotic family members. Other BH3 domains lack the ability to activate Bax directly but can oppose one or more antiapoptotic family members. In cells expressing antiapoptotic proteins such as Mcl-1 and Bcl-x_L, both these direct and indirect functions of BH3-only proteins may be necessary to initiate apoptosis (Figure 7).

Supporting this idea is our observation that the direct-activator BH3 peptides (Bid and Bim), which can directly activate Bax and also sequester antiapoptotic family members, were the only ones that induced cytochrome c release effectively in HeLa cells (Figure 6). The derepressor BH3 peptides, which lack the ability to activate Bax directly, caused cytochrome c release much less efficiently, if at all. Interestingly, the Puma and Bmf peptides, which were the only derepressor BH3s that could oppose both Bcl-x_L and Mcl-1, were also the only derepressor peptides that could induce cytochrome c release in HeLa cells consistently (albeit in only a small proportion of the cells). In Figure 1, we observed a similar ranking of the peptides with regard to their ability to release cytochrome c from isolated *Xenopus* mitochondria, except that this assay may have been more sensitive and thus uncovered the activity of the Bik, Bad, and Hrk peptides, which were ineffective when loaded into HeLa cells (Figure 6). The comparatively muted response of HeLa cells to BH3 peptides could be a result of lower peptide concentrations within the cells or, alternatively, a greater abundance or diversity of antiapoptotic Bcl-2 family proteins. In the absence of detailed knowledge regarding which Bcl-2 family proteins are present in HeLa cells and *Xenopus* egg mitochondria, the differences between these two systems remain undefined.

Nevertheless, there is a general agreement between the observed activities of BH3 peptides in cells, on isolated mitochondria, and in assays with defined liposomes (summarized in Table S2). This suggests that the Bax activation and derepression functions identified in vitro reflect the real mechanisms of action of these BH3 peptides in cells. We are tempted to propose also that the behaviors of the BH3 peptides, at least for those peptides that do exhibit activity, likely mirror the potential functions of the BH3 domains in the native proteins. We found, for example, that BH3 peptides produced the same increase in Bax association with the membrane and Bax oligomerization as did recombinant activated Bid protein (Figure 5). However, the full-length BH3-only proteins could be regulated by additional mechanisms, e.g., postsynthetic modifications and conformational changes, that could hide or expose the BH3 domains or constrain them stereochemically.

The protein Bim may be a case in point. Whereas we observed that the Bim BH3 peptide, as well as recombinant BimS protein, were able to activate Bax directly, others reported that the longer forms, BimL and BimEL, possessed only the derepression activity (Terradillos et al., 2002; Terrones et al., 2004). This may suggest that BimEL and BimL are functionally different from BimS. In the longer forms of Bim, the BH3 domain might be constrained so that it can interact with antiapoptotic relatives but not the Bax-type proteins, and such constraints could conceivably be regulated by phosphorylation (Lei and Davis, 2003) or binding to dynein light chain (Puthalakath et al., 1999). Indeed, it was recently reported that in FL5.12 cells deprived of the survival factor IL-3, the readdition of IL-3 stimulated ERK-dependent Bim phosphorylation at three Ser residues; this led to a reduction in the amount of Bax that coimmunoprecipitated with Bim, whereas the association of Bim with Bcl-2 and Mcl-1 was unaffected (Harada et al., 2004). It will be important to examine the mechanistic effects of posttranslational modifications of Bim and other BH3-only proteins in defined biochemical systems like the one used here.

Our studies suggest an important corollary for apoptotic regulation by Bcl-2 family proteins: if multiple antiapoptotic members of this family are expressed in a cell, the induction of apoptosis may require the expression and activation of more than one BH3-only protein. This is because a single BH3 domain may not oppose all of the antiapoptotic family members present. For example, Bad BH3 peptide blocks Bcl-x_L but not Mcl-1, whereas the Noxa B BH3 domain opposes Mcl-1 but not Bcl-x_L. Neither of these BH3 domains alone would be predicted to activate apoptosis in cells expressing both Bcl-x_L and Mcl-1, but together they could potentially overcome both antiapoptotic proteins, thus disinhibiting Bax and Bak. We have not investigated the specificity of the other BH3 domain of Noxa, but if it differs from that of Noxa B, then the Noxa protein could have a broader set of interacting partners, and thus greater potency as an apoptotic derepressor, than either of its BH3 domains singly. Indeed, the results in Figure 6 show that BH3 domains can act synergistically in combination. Such synergy between activator and derepressor BH3 domains was not observed in the lipid vesicle system in the absence of Bcl-x_L (Figure 2B), and therefore, we find that the effects are well explained by our model (Figure 7).

Our results showed that the derepressor peptides could only stimulate Bax function in the presence of a direct activator. How then could the Puma and Bmf peptides, which are merely derepressors, induce cytochrome c release by themselves even in a small fraction of the cells in Figure 7? Perhaps the sensitive cells have constitutively mobilized a direct activator BH3-only protein, such as Bim, at levels that would be sufficient to activate Bax if not for the presence of antiapoptotic family members. The Puma and Bmf peptides could sequester these inhibitory proteins, thus freeing the endogenous direct activator BH3-only protein to activate Bax. Alternatively, in the sensitive cells, Bax could be constitutively activated via an unknown mechanism but held in check by inhibitory Bcl-2 family members. Similar possibilities may explain how derepressor BH3s can cause isolated *Xenopus* egg mitochondria to release cytochrome c (Figure 1B). Such ambiguities in the interpretation of studies done with whole cells and isolated mitochondria (Letai et al., 2002; Moreau et al., 2003) are indeed what led us in the present study to examine the functions of BH3 domains in defined mixtures of proteins and liposomes. These liposome experiments now show unambiguously that the BH3 domains of Bid and Bim can directly activate Bax, something that could not be concluded rigorously by prior studies using systems that were less well defined.

Some have suggested that antiapoptotic Bcl-2 family proteins can be converted into proapoptotic Bax-like proteins through the actions of BH3-only proteins (Moreau et al., 2003). It is conceivable that unidentified proteins in cells or mitochondria could somehow alter the function of antiapoptotic Bcl-2 family proteins when BH3 peptides are introduced. However, in our studies (Figure 4) we did not observe a direct conversion of Bcl-x_L or Mcl-1 to proapoptotic molecules by the derepressor BH3 peptides.

Our results suggest a potential mechanism for differential cellular responses to various apoptotic stimuli, as well as one possible explanation for the synergistic effects of multiple cellular stresses, e.g., in combinatorial cancer therapy. By preparing a Bid BH3 peptide with chemical modifications to increase its cell permeability and affinity for Bcl-x_L, Korsmeyer and colleagues succeeded in inhibiting the growth of leukemia cells in vivo (Walensky et al., 2004). If BH3 peptides can indeed be used to treat cancer, it will be important to know in detail how these peptides function with other Bcl-2 family members in order to design more effective therapies.

Experimental Procedures

Liposome Assays and Recombinant Proteins

We designed peptides according to Kelekar et al. (Kelekar et al., 1997), who reported that a 16-mer Bad BH3 peptide binds to Bcl-x_L with a K_D of 120 μM, whereas a 26-mer displays a K_D of 6 nM. The peptides used in this study were 24- or 25-mers, taken from similar regions to the 26-mer described by Kelekar et al. Peptides were obtained from AnaSpec (San Jose, CA, USA) at >98% purity. They were dissolved in DMSO and added to the liposome assay to give a final DMSO concentration of 2%. Liposomes were prepared, and the fluorescein-dextran release assay was performed with recombinant Bax, N/C-Bid and Bcl-x_L proteins as described (Kuwana et al., 2002). The Mcl-1 cDNA construct in pTYB1 plasmid, a gift from Xiaodong Wang, University of Texas Southwestern Medical Center, was used to transform BL21(DE3) cells. Cells were grown at 37°C to an OD_{600} of 0.8 and induced at room temperature overnight (Nijhawan et al., 2003). The protein was affinity purified using chitin beads according to the manufacturer's instructions and followed by linear NaCl gradient elution from MonoQ 5/5 (Amersham Pharmacia). The final preparation showed a doublet band with some degradation products and was 90% pure, as estimated from a Coomassie blue-stained polyacrylamide gel.

Binding Measurements

Binding between BH3 peptides and Bcl-x_L or Mcl-1 was detected by surface plasmon resonance using a BIAcore X biosensor in running buffer (10 mM HEPES, [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20). Recombinant Bcl-x_L or Mcl-1 (0.2 mg/ml) was dissolved in 20 mM Na-acetate (pH 4.5) and immobilized on the surface of CM5 sensorchips (BIAcore), which had been activated by *N*-hydroxysuccinimide (0.05 M) and *N*-hydroxysuccinimide-*N*-ethyl-*N*′-(diethylaminopropyl)carbodiimide (0.2 M). Bcl-x_L coupling yielded 1200 response units (RU) and Mcl-1, 4400 RU. The surface

of the chip was regenerated, when necessary, by injections of 12 mM 1,2-diethylamine containing 0.025% Triton X-100. Kinetic experiments were performed at 20 μl/min with peptide concentrations between 10 nM and 10 μM, depending the K_D of the peptide. Binding constants were obtained using the BIA Evaluation software (BIAcore) from four or five serial dilutions of each peptide.

These analyses were performed in the presence of small amounts of detergent to minimize nonspecific binding. A similar set of surface plasmon resonance studies with Bax gave conflicting results: in the presence of detergent, all peptides (even a control peptide from the cytochrome c sequence) bound indiscriminately, failing to fit standard binding curves; in the absence of detergent no binding was observed (not shown). Therefore this method is unlikely to prove useful for the analysis of (possibly transient) interactions of Bax with some BH3-only proteins (Cartron et al., 2004; Harada et al., 2004; Yethon et al., 2003).

Loading of BH3 Peptides into Cells

HeLa cells stably expressing GFP-cytochrome c (Goldstein et al.) were plated at 2.5×10^4 per well of an 8-chamber cover slide 24 hr prior to the experiment and preincubated with 100 μM zVAD for 2 hr before loading, to prevent them from detaching after they undergo apoptosis. Cells were loaded with one of the BH3 peptides or a control peptide (IFAGIKKKAERADLIAYLKQATAK) derived from the sequence of cytochrome c. Peptides (10 μg in DMSO) were mixed with 1 μl of Chariot protein transfection reagent (Active Motif) and 0.5 μg of 10 kDa dextran conjugated to AlexaFluor 568 (Molecular Probes) in 100 μl of serum-free medium, and the cells were incubated in this mixture for 1 hr. An equal volume of medium containing 20% fetal bovine serum was then added. Three hours later, cells were placed in fresh medium and incubated for an additional 1 hr, then analyzed for cytochrome c-GFP release by confocal microscopy. The percentage of red cells with diffuse cytochrome c-GFP staining was calculated based on a minimum of 300 red cells per well.

Bax Binding to Liposomes

Liposome assays were as described (Kuwana et al., 2002). After incubation for 3 hr at room temperature, half of the assay mixture (50 μl) was analyzed for fluorescein-dextran release and the other half was mixed with two volumes of 50% sucrose in buffer (10 mM potassium phosphate, 50 mM KCl, and 1 mM EDTA). This mixture was overlaid with 600 and 700 μl layers of 10% and 0% sucrose, respectively, in the same buffer. The sample was centrifuged in a Beckman TL-100 tabletop ultracentrifuge fitted with a TLS-55 rotor at 55,000 rpm for 2.5 hr. The liposome layer was placed into a 0.1 μm microfiltration unit (Millipore), and the vesicles were collected from the retentate after filtration. They were resuspended in SDS-PAGE loading buffer for immunoblotting, and Bax was detected using anti-Bax antibody (N20, Santa Cruz).

Bax Crosslinking

Full-length, monomeric recombinant Bax (1 ng) was diluted into HE buffer containing 5% liposomes, 50 nM N/C-BID (or indicated BH3 peptides), 1 μM recombinant Bcl-xL (where indicated), and 1 nM 1,6-bismaleimidohexane (BMH). Reactions were incubated at 37°C for 60 min before SDS-PAGE (Bio-Rad, Criterion XT Bis-Tris 4%–12% gradient, MOPS buffer) and Western blot analysis with anti-Bax (Santa Cruz, N-20).

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