Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics

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Summary

The “BH3-only” proteins of the BCL-2 family require “multidomain” proapoptotic members BAX and BAK to release cytochrome c from mitochondria and kill cells. We find short peptides representing the α-helical BH3 domains of BID or BIM are capable of inducing oligomerization of BAK and BAX to release cytochrome c. Another subset characterized by the BH3 peptides from BAD and BIK cannot directly activate BAX, BAK but instead binds antiapoptotic BCL-2, resulting in the displacement of BID-like BH3 domains that initiate mitochondrial dysfunction. Transduced BAD-like and BID-like BH3 peptides also displayed synergy in killing leukemic cells. These data support a two-class model for BH3 domains: BID-like domains that “activate” BAX, BAK and BAD-like domains that “sensitize” by occupying the pocket of antiapoptotic members.

Introduction

Diverse intrinsic death signals emanating from multiple subcellular locales all induce the release of cytochrome c from mitochondria to activate Apaf-1 and result in effector caspase activation. Proteins in the BCL-2 family are major regulators of the commitment to programmed cell death as well as executioners of death signals at the mitochondrion. Members of this family include both pro- and antiapoptotic proteins and share homology in up to four conserved regions termed BCL-2 homology (BH) 1–4 domains (Adams and Cory, 1998). The family can be divided into three main subclasses. The antiapoptotic proteins, which include BCL-2 and BCL-X L, are all “multidomain,” sharing homology throughout all four BH domains. However, the proapoptotic proteins can be further subdivided and include multidomain proteins, such as BAX and BAK, which possess sequence homology in BH1-3 domains. The more distantly related “BH3-only” proteins are to date all proapoptotic and share sequence homology within the amphipathic α-helical BH3 region, which is required for their apoptotic function (Chittenden et al., 1995; O’Connor et al., 1996; Wang et al., 1996; Zha et al., 1997).

Multidomain proapoptotic proteins such as BAX and BAK upon receipt of death signals participate in executing mitochondrial dysfunction. In viable cells, these proteins exist as monomers. In response to a variety of death stimuli, however, inactive BAX, which is located in the cytosol or loosely attached to membranes, inserts deeply into the outer mitochondrial membrane as a homooligomerized multimer (Eskes et al., 2000; Gross et al., 1998; Wolter et al., 1997). Inactive BAK resides at the mitochondrion, where it also undergoes an allosteric conformational change in response to death signals, which includes homooligomerization (Griffiths et al., 1999; Wei et al., 2000). Cells deficient in both BAX and BAK are resistant to a wide variety of death stimuli that emanate from multiple locations within the cell (Wei et al., 2001).

The BH3-only molecules constitute the third subset of this family and include BID, NOXA, PUMA, BIK, BIM, and BAD (Kelekari and Thompson, 1998). These proteins share sequence homology only in the amphipathic α-helical BH3 region, which mutation analysis has indicated is required in proapoptotic members for their death activity. Moreover, the BH3-only proteins require this domain in order to demonstrate binding to multidomain BCL-2 family members. Multiple binding assays, including yeast two-hybrid, coimmunoprecipitation from detergent-solubilized cell lysates, and in vitro pull-down experiments, indicate that individual BH3-only molecules display some selectivity for multidomain BCL-2 members (Boyd et al., 1995; O’Connor et al., 1996; Oda et al., 2000; Wang et al., 1996; Yang et al., 1995). The BID protein binds proapoptotic BAX and BAK as well as antiapoptotic BCL-2 and BCL-X L (Wang et al., 1996;
interactions. Peptides were selected from BH3-only members to test whether BH3 peptides work through an established pathway. To activate BAK or BAX, perhaps analogous to ligand/receptor binding, the BH3 domain itself from BID, or for that matter from any BH3-only member, could be responsible for directly binding and activating BAX, BAK, retained BAX, Bak.

We et al., 2000). In contrast, BAD, NOXA, and BIM as intact molecules display preferential binding to antiapoptotic members (Boyd et al., 1995; O’Connor et al., 1998; Oda et al., 2000; Yang et al., 1995). However, expression of all of these members (BID, BAD, BIM, and NOXA) results in the activation of BAX and BAK. Moreover, their expression in Bax, BAK doubly deficient cells indicates that BAX and BAK are absolutely required for their induction of cell death (Cheng et al., 2001; Zong et al., 2001). Comparison of wild-type (wt) versus mutant BCL-2, BCL-X, indicated that antiapoptotic members sequester all of these BH3-only molecules in stable mitochondrial complexes, preventing the activation of BAX and BAK (Cheng et al., 2001). However, these findings leave an important, yet unresolved issue as to whether BH3-only molecules function identically in a single pathway or whether two subsets exist based on their binding preference.

The proapoptotic activity of BH3-only molecules is apparently kept in check by either transcriptional control or posttranslational modification. Of the BH3-only members, the activation of BID is understood in the most detail. Surface Fas and TNFR1 death receptor signaling results in caspase-8-mediated cleavage within an unstructured loop of the inactive p22 BID (Li et al., 1998; Luo et al., 1998). A newly exposed N-terminal glycine is N-myristoylated, and the p7/myrBID BID complex displays increased efficiency and selectivity for targeting the outer mitochondrial membrane (Zha et al., 2000). Comparison of wt versus mutant p15 BID indicates that an intact BH3 domain is required for cytochrome c release, but not for targeting to the mitochondria. Blocking antibodies to BAK and BAK-deficient mitochondria prepared from hepatocytes both indicate that mitochondrial BAK is necessary in order for tBID to trigger release of cytochrome c (Wei et al., 2000). Coimmunoprecipitation and protein binding studies support the capacity of tBID and BAK to interact (Wei et al., 2000). Yet, it remained uncertain whether the BH3 domain itself from BID, or for that matter from any BH3-only member, could be responsible for directly binding and activating BAX, BAX.

Consequently, we turned to synthetic BH3 peptides from representative BH3-only molecules to test whether these domains, when removed from the context of intact protein, can activate BAK or BAX, perhaps analogous to ligand/receptor interactions. Peptides were selected from BH3-only members that display selective binding to antiapoptotic versus proapoptotic multidomain members to test if they function through a single or distinguishable pathways. These studies indicate that individual BH3 domains possess distinct functions. A BID-like subset “activates” proapoptotic BAK, BAX, whereas a BAD-like subset occupies the pocket of antiapoptotic BCL-2, thus “sensitizing” to the availability of other activating BH3 domains. These peptides represent two classes of tool compounds that initiate cell death using genetically defined pathways.

### Results

**BH3 peptides from BID and BIM, but not all BH3-only members, release cytochrome c similar to myristoylated BID**

Recombinant p15tBID, and even more efficiently the p7/myrBID, myristoylated BID complex (myrBID), initiate BAK oligomerization and cytochrome c release in a mitochondrial in vitro system that appears to recapitulate the mitochondrial pathway of apoptosis in vivo. Since the proapoptotic activity of BID in vitro and in vivo requires an intact BH3 domain, we tested the ability of peptides derived from this BH3 domain to initiate this activity. A 20-mer of BIDBH3 (aa 80–99) at 10 μM (Table 1) proved capable of initiating cytochrome c release, as did myrBID (Figure 1A). Next, we compared the activity of other BH3 domain peptides. While BIMBH3 (Table 1) demonstrated cytochrome c release, peptides derived from other BH3-only members BAD, BIK, and NOXA (Table 1) even at 100 μM did not display this activity (Figure 1B). A peptide derived from the BH3 domain of antiapoptotic Bcl-XL, did not cause cytochrome c release (Figure 1B). Circular dichroism studies indicate that while the relative \( \alpha \)-helical content of these peptides varies, the percent \( \alpha \)-helicity does not solely dictate the activity of the peptides. While NOXABH3 and BCL-XL BH3 have relatively low \( \alpha \)-helical content, BADDH3 demonstrates the highest \( \alpha \)-helical content and is still inactive in this assay (Table 1). Likewise, a peptide derived from the BH3 domain of BID, but containing substitutions (L90A, D95A) at two residues highly conserved throughout the family, retained \( \alpha \)-helicity but did not cause cytochrome c release (Table 1, Figure 1B).

**BAK is required for BH3 peptide-induced cytochrome c release**

To test whether BH3 peptides work through an established mitochondrial pathway of apoptosis, we examined whether they...

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**Table 1. Peptide sequence, BCL-2 affinity, BID displacement, and \( \alpha \)-helicity**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Kᵤ (nM), BCL-2 binding</th>
<th>SD ±</th>
<th>IC50 (nM), BID displacement</th>
<th>SD ±</th>
<th>% helicity (222 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIDBH3</td>
<td>EDIIRINHHALQVGDSMDR</td>
<td>220</td>
<td>30</td>
<td>838</td>
<td>192</td>
<td>19.5</td>
</tr>
<tr>
<td>BIMBH3</td>
<td>MRPEIWIQLRRLRRDPGFA</td>
<td>74</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>15.8</td>
</tr>
<tr>
<td>mBADBH3</td>
<td>LWAAQRYGRLRMDDEFPGKGL</td>
<td>39</td>
<td>6</td>
<td>173</td>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>BADDH3</td>
<td>NLAAQRYGRLRMSDFVDSFKX</td>
<td>41</td>
<td>11</td>
<td>ND</td>
<td>-</td>
<td>23.9</td>
</tr>
<tr>
<td>BIKBH3</td>
<td>MRSDDALALLACIDBBMD</td>
<td>485</td>
<td>272</td>
<td>4920</td>
<td>1648</td>
<td>8.5</td>
</tr>
<tr>
<td>NOXAABH3</td>
<td>AELPPPERAAQLRKKQYVC</td>
<td>&gt;1000</td>
<td>-</td>
<td>&gt;30,000</td>
<td>-</td>
<td>5.1</td>
</tr>
<tr>
<td>NOXABBH3</td>
<td>PADKDECAQLRIGDKVNL</td>
<td>&gt;1000</td>
<td>-</td>
<td>&gt;30,000</td>
<td>-</td>
<td>11.6</td>
</tr>
<tr>
<td>BCLXBH3</td>
<td>VIPMAAVQAQLRBDQFEFL</td>
<td>&gt;1000</td>
<td>-</td>
<td>&gt;30,000</td>
<td>-</td>
<td>3.9</td>
</tr>
<tr>
<td>BIDBH3 mut</td>
<td>EDIIRNHARHAAQVGAEDR</td>
<td>&gt;1000</td>
<td>-</td>
<td>&gt;30,000</td>
<td>-</td>
<td>16.4</td>
</tr>
</tbody>
</table>

BH3 peptide sequence were derived from human or mouse (m) where listed. NOXA has two BH3 domains, noted as A and B. Dissociation constants (K₀) for binding of fluorescein-tagged peptides to GST-tagged BCL-2 protein were determined by fluorescence polarization. IC50 refers to the concentration of unlabeled peptide required to displace 50% of the fluoresceinated BIDBH3 bound to 1 μM GST-BCL-2. Percent \( \alpha \)-helicity in aqueous solution was determined by circular dichroism.
required the multidomain member BAK to be present. One hallmark of cytochrome c release by native tBID is that it requires the presence of multidomain BAK or BAX in intact cells or BAK on purified mitochondria (Wei et al., 2000, 2001). Immunoblots of mitochondria isolated from the liver of Bak−/− mice confirmed that neither multidomain proapoptotic BAX nor BAK was present (Figure 2). Moreover, there is no compensatory alteration in the levels of antiapoptotic BCL-2 members in the absence of BAX and/or BAK (not shown). Comparison of 100 μM BIMBH3 or BIDBH3 peptide on Bak+/+ versus Bak−/− mitochondria indicated that BAK is required for the release of cytochrome c (Figure 3). This requirement for BAK argues that these α-helical BH3 peptides function through the genetic pathway of mitochondrial apoptosis rather than by an autonomous permeabilization of membranes that nonspecifically damages mitochondria.

Peptides that induce cytochrome c release induce BAK oligomerization

Previous work demonstrated that the translocation of tBID to the mitochondrion results in allosteric conformational activation of BAK, which includes its homooligomerization followed by the release of cytochrome c (Wei et al., 2000). We find that the BIDBH3 peptide (like tBID or the p7/myr15BID complex) induces BAK oligomerization as detected with the crosslinker BMH (Figure 4A). Moreover, there is a temporal relationship between BAK oligomerization and the release of cytochrome c induced by BIDBH3 peptide (Figures 4A and 4B). While BIMBH3 also induces BAK oligomerization, BADBH3 peptide, which lacks the ability to cause cytochrome c release, is unable to induce BAK oligomerization (Figure 4C). We found that BIMBH3 and BIDBH3, but not BADBH3, could also induce oligomerization of BAX in mitochondria isolated from cultured FL5.12 cells, which contain both BAX and BAK (Figures 2 and 4C). Note that while BIDBH3 induces more prominent crosslinking of BAK than does BIMBH3, BIMBH3 induces more prominent crosslinking of BAX than does BIDBH3. We tested a mutant BID peptide BIDBH3mut (L90A, D95A), and it lacked the ability to induce either cytochrome c release (Figure 1B) or BAX, BAK oligomerization.
BAK and/or BAX oligomerization accompanies peptide-induced cytochrome c release

A: Wild-type liver mitochondria were treated with 100 μM BIDBH3 at room temperature for the times indicated. Following incubation, reactions were treated with the irreversible crosslinker BMH. Twenty-five micrograms protein from each reaction was separated using denaturing electrophoresis. Monomeric and multimerized BAK species were detected by immunoblots following SDS-PAGE.

B: Wild-type liver mitochondria were treated as in A, and cytochrome c release was measured by ELISA.

C: Mitochondria from FL5.12 cells were treated for 30 min with 100 μM of indicated peptides. BAK immunoblot demonstrates BMH-crosslinked BAK oligomers. Markers 1, 2, 3, and 4 correspond to size of monomer, dimer, trimer, and tetramer as determined in prior studies (Wei et al., 2000, 2001).

D: BAX immunoblot demonstrates BMH-crosslinked BAX oligomers (Gross et al., 1998) in same treated FL5.12 mitochondria as in C.

BCL-2 inhibits mitochondrial release of cytochrome c by BH3 peptides

Mitochondria bearing protective levels of antiapoptotic BCL-2 do not release cytochrome c following treatment with 25 ng tBID in vitro, apparently because tBID is bound and sequestered by BCL-2 in stable complexes that prevent tBID from activating BAK (Cheng et al., 2001). Similarly, mitochondria with overexpressed BCL-2 proved resistant to 10 μM BIDBH3, 1 μM BIMBH3, as well as 30 nM myrBID, failing to release cytochrome c (Figure 5A). Furthermore, the presence of BCL-2 is coordinate with the loss of BAK oligomerization following exposure to BH3 peptide, suggesting that BCL-2 inhibits upstream of BAK activation (Figure 5B). These findings support a model wherein a major component of BCL-2’s role in inactivating tBID is to specifically sequester the BH3 domain, thus preventing BH3 itself from activating multidomain proapoptotic members.

BADBH3 binds BCL-2 and restores cytochrome c release by BID

We tested BH3 peptides that lack the intrinsic ability to activate BAK and cause cytochrome c release for their capacity to interfere with the antiapoptotic protection by BCL-2. We reasoned that this subset of BH3 peptides might occupy the hydrophobic pocket of BCL-2 and consequently displace proapoptotic BIDBH3 or BIMBH3 peptides. The BADBH3 peptide most prominently demonstrates the capacity to overcome BCL-2 protection of mitochondria treated with a subliminal concentration of myrBID (30 nM), while BIKBH3 shows significant, but less, potency (Figure 6A). The remaining BH3 peptides derived from NOXA and BCL-XL did not demonstrate the capacity to overcome BCL-2 protection (Figure 6A). Since even 100 μM BADBH3 in and of itself cannot activate BAK or release cytochrome c, this suggests that BADBH3 sensitizes mitochondria to BIDBH3 or BIMBH3 by successfully competing with these peptides for binding to BCL-2. At 100 μM, BADBH3 was able to restore the cytochrome c release of BCL-2-overexpressing mitochondria in vitro, apparently because tBID is bound and sequestered by BCL-2 in stable complexes that prevent tBID from activating BAK (Cheng et al., 2001). Similarly, mitochondria with overexpressed BCL-2 proved resistant to 10 μM BIDBH3, 1 μM BIMBH3, as well as 30 nM myrBID, failing to release cytochrome c (Figure 5A). Furthermore, the presence of BCL-2 is coordinate with the loss of BAK oligomerization following exposure to BH3 peptide, suggesting that BCL-2 inhibits upstream of BAK activation (Figure 5B). These findings support a model wherein a major component of BCL-2’s role in inactivating tBID is to specifically sequester the BH3 domain, thus preventing BH3 itself from activating multidomain proapoptotic members.

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BADBH3 enables cytochrome c release by BIDBH3, BIMBH3, and myrBID by overcoming the inhibitory effect of BCL-2.

Mitochondria isolated from parental and FLS.12-BCL-2 cells were treated for 30 min at room temperature with (A) 30 nM myrBID complex or 30 μM of the indicated peptides, or a combination of 30 nM myrBID and 30 μM peptide, and release of cytochrome c was measured. The indicated mitochondria were treated with a range of concentrations of (B) BIDBH3, (C) BIMBH3, and (D) myrBID, with or without BADBH3 peptide. E: Dose response of BADBH3 as well as BIKBH3 enabling myrBID-induced release of cytochrome c from mitochondria of FLS.12-BCL-2 cells.

in a dose-response fashion to BIDBH3 (Figure 6B) and BIMBH3 (Figure 6C) to levels observed for wt mitochondria. We also observed an increase in the sensitivity of wt mitochondria treated with BADBH3. This would be expected, since the source of wt mitochondria, FLS.12 cells, express some murine BCL-2. We also noted that the restoration of cytochrome c release by BADBH3 was accompanied by restoration of BAK oligomerization (not shown).

We next tested whether eliminating BCL-2 protection by BADBH3 would enable the more physiologic ligand, myristoylated BID complex (p7/myr15BID). Addition of 200 μM BADBH3 to BCL-2-overexpressing mitochondria markedly restores their sensitivity to even 1 nM myrBID. These BADBH3-treated mitochondria are more sensitive than wt mitochondria, probably reflecting the capacity of BADBH3 to inhibit the endogenous murine BCL-2 and BCL-XL resident on the mitochondria (Figure 6D). We next examined the dose-response range of BADBH3, revealing that it had measurable activity at concentrations as low as 1 μM in inhibiting BCL-2 (Figure 6E) and enabling cytochrome c release by myrBID. At 100 μM, BIKBH3 can also
BADDH3 displaces BIDBH3 from BCL-2 by fluorescence polarization analysis

To directly test whether BADDH3 could displace BIDBH3 from BCL-2, we utilized fluorescence polarization analysis. BADDH3 peptide bound full-length BCL-2 with approximately 5-fold greater affinity than BIDBH3 peptide (average of 41 versus 220 nM; Table 1, Figure 7A). Moreover, BADDH3 can efficiently displace prebound BIDBH3 peptide from BCL-2 (Figure 7B). However, to compete with prebound BIDBH3, an excess of BADDH3 is required, despite the 5-fold greater affinity of BADDH3 for BCL-2 in solution. This finding suggests that a conformational change takes place in either BCL-2 and/or a BH3 peptide upon binding. In contrast, BIDBH3 does not effectively displace BADDH3 from BCL-2. Testing the remaining peptides reveals that those peptides that cause cytochrome c release by themselves (BIDBH3 and BIKBH3) or those that enable cytochrome c release by counteracting BCL-2 (BADDH3 and BIKBH3) all bind to BCL-2 with affinities in the 50–500 nM range. BADDH3 and BIKBH3 demonstrate the ability to displace BIDBH3 from the BCL-2 protein (Table 1). The remaining peptides (NOXABH3, NOXABBH3, BCLXBH3, BIDBH3mut) that were unable to overcome BCL-2 inhibition did not bind detectably to BCL-2 or displace BIDBH3 from BCL-2 (Table 1). These results are consistent with the capacity of sensitizing BH3 domains (e.g., BADDH3 or BIKBH3) to displace activating BH3 domains (e.g., BIDBH3 or BIMBH3) from the pocket of antiapoptotic BCL-2. Once free, activating BH3 domains by this model would trigger BAK oligomerization with subsequent cytochrome c release (Figure 7C).

Transduced BADDH3 and BIDBH3 synergize in killing leukemic cells

We next explored whether we could trigger apoptosis of cancer cells by transduction of such BH3 peptides. Prior studies utilizing internalization moieties including decanoic acid, antenepedia (ANT), or HIV Tat have noted confounding issues of cellular and mitochondrial toxicity. For example, when we linked various BH3 domains to a Tat 11-mer, both wt and mutant transduced peptides rapidly killed cells. Moreover, many conjugates did not appear to work through the genetic pathway, as they displayed no inhibition by BCL-2 and readily killed Bax, Bak doubly deficient cells (not shown). Linking a polyarginine (8 aa) stretch to our BH3 peptides appears more promising. Polyarginine tags have been shown to facilitate the transport of peptides across the plasma membrane (Rothbard et al., 2000). r8BIDBH3 was capable of killing Jurkat leukemic cells, whereas r8BIDBH3mut was ineffective. Moreover, the addition of nontoxic 10 μM r8BADDH3 was able to sensitize Jurkat cells to subliminal concentrations (10 μM) of r8BIDBH3 (Figure 8). Both r8BIDBH3 and r8BADDH3 failed to kill Bax, Bak doubly deficient cells. Thus, this appears to provide an initial proof of concept experiment that sensitizing and activating BH3 domains will also synergize in vivo to initiate apoptosis of cancer cells.

Discussion

The use of synthetic peptides coupled with genetically defined mitochondria indicate that the BH3 peptide domain itself, ex-
BCL-2 or BCL-XL, but would require a second apoptotic signal. BIDBH3 peptide appears similar to native myrBID complex mimetics would possess several attractive characteristics. They states of a native BIM molecule during cell death in vivo. defined here. it is conceivable that the standard protein interaction assays molecules, will be assignable to these functional classes and critical pying the pocket of antiapoptotic BCL-2. The latter displace (Li et al., 2001; Terradillos et al., 2002). Here, the isolated BIM domains activate multidomain proapoptotic BAX, BAK, whereas Previous reports testing the capacity of intact BIM protein to from the context of an entire BH3-only molecule, can was able to bind antiapoptotic BCL-X, was it capable of promot- cised from the context of an entire BH3-only molecule, can function as a specific death ligand. The activity of BH3 peptides supports a ligand/receptor model in which “BID-like” BH3 do- mains are sufficient to trigger allosteric conformational activation of BAX, BAK, their respective receptors. Activation by BIDBH3 peptide was qualitatively indistinguishable from the myrBID protein in that either requires BAK, results in BAK oligomerization followed by cytochrome c release, and can be bound and sequestered by CBL-2 with resultant protection of BAK.

The synthetic peptides also indicate that BH3 regions are true domains rather than merely conserved sequence motifs, as the peptide domain itself has inherent functional activity. Comparison of various α-helical peptides from BH3-only proteins reveals evidence for two functional classes of BH3 domains. BID-like domains activate multidomain proapoptotic BAX, BAK, whereas BAD-like domains sensitize mitochondria for apoptosis by occup- ying the pocket of antiapoptotic BCL-2. The latter displace BID-like domains, which even at subliminal levels can now initi- ate cytochrome c release. This predicts that therapeutics that mimic a BH3 domain, whether they be peptidomimetics or small molecules, will be assignable to these functional classes and should be classified utilizing the genetic and molecular reagents defined here.

From a therapeutic vantage point, BAD-like sensitizing BH3 mimetics would possess several attractive characteristics. They might be predicted to reset susceptibility of cells protected by BCL-2 or BCL-X, but would require a second apoptotic signal to initiate an activating BH3-only protein. This implies that as a single agent, sensitizing mimetics might prove nontoxic, espe- cially to normal cells. The need for a second signal provides the opportunity to utilize cancer cell-selective pathways that could also spare normal cells.

Evidence here for a sensitizing subset of BAD-like BH3 pep- tid es provides an explanation for previous, apparent discrepan- cies concerning the mechanism of action of these proteins. Most BH3-only intact proteins including BAD, NOXA, and BIK display a marked binding preference for antiapoptotic members BCL-2, BCL-X, in interaction assays of yeast two-hybrid, pull- down, or communoprecipitation from detergent-solubilized lys- sates (Boyd et al., 1995; Oda et al., 2000; Yang et al., 1995). Moreover, mutational analysis suggested that only when BAD was able to bind antiapoptotic BCL-X, was it capable of promot- ing death (Kelekar and Thompson, 1998). Yet, BAD, NOXA, and BIK all require the multidomain proapoptotic BAX, BAK proteins to kill as evidenced in Bax, Bak doubly deficient cells (Cheng et al., 2001; Zong et al., 2001). The ability of BAD-like BH3 peptides to mediate a displacement reaction from the antiapoptotic BCL-2 pocket provides a mechanism of action that would accommodate all observations. The cooperating protein displaced from antiapoptotic pockets within intact cells would in- clude, but not be restricted to, BID-like activating BH3-only members. While helping to resolve this issue, the analysis of the BIMBH3 peptide proved provocative. Prior interaction assays indicate that the intact BIM protein displays preferential binding to antiapoptotic BCL-2, BCL-X, over proapoptotic BAX or BAK. Previous reports testing the capacity of intact BIM protein to release cytochrome c from mitochondria gave differing results (Li et al., 2001; Terradillos et al., 2002). Here, the isolated BIM BH3 domain when removed from the context of the entire pro-tein scored as BID-like, capable of activating BAX, BAK. Several potential explanations can be envisioned. It is possible that the critical α-helical face of the BH3 domain that recognizes BAX, BAK may not be exposed in the intact BIM protein. Alternatively, it is conceivable that the standard protein interaction assays used to measure binding may not reflect all of the conformational states of a native BIM molecule during cell death in vivo.

The mechanistic pathway to cytochrome c release for BIDBH3 peptide appears similar to native myrBID complex, yet the efficiency of triggering release varies greatly. Near total release of cytochrome c from mitochondria requires 10 nM myrBID complex, but 10 μM BIDBH3 peptide. Myristoylation increases the efficiency of BID targeting to mitochondria and could conceivably help focus its location on the outer mitochon- drial membrane (Lutter et al., 2001; Zha et al., 2000). It is also possible that an integrated myr15BID protein may more effec- tively present the BH3 domain to the BAK pocket. Of note, the sources of mitochondria vary in their response to individual BH3 domains. BIDBH3 is more potent than BIMBH3 for liver mitochondria, whereas BIMBH3 is more effective on the FL5.12 mitochondria. This may reflect the presence of BAX on FL5.12 but not liver mitochondria. The efficiency of oligomerization (Fig- ure 4) supports a preference of BIDBH3 for BAK and BIMBH3.

Figure 8. r8BADBH3 sensitizes Jurkat cells to r8BIDBH3 killing

Peptides were synthesized with sequence identi- cal to that found in Table 1 with the addition of eight d-arginine residues at the amino terminus followed by a glycine linker residue. Apoptotic cells were detected by Annexin V staining. Re- sults of an experiment representative of four simi- lar experiments are shown.
for BAX. A hypothesis that BIMBH3 prefers BAX would be consistent with the finding that BIM functions upstream of BAX in neuronal cell death following NGF deprivation (Putcha et al., 2001). The binding affinity of individual BH3 domains for BCL-2 members varies considerably (Figure 7; Sattler et al., 1997), providing a measurement for selectivity. Assessment of BH3 peptides by circular dichroism indicates that α-helical content is not the sole determinant of differential binding affinity, nor of the ability to induce BAX, BAK oligomerization and cytochrome c release. The specificity noted suggests a model in which distinct BH3 domains have select multidomain partners, which provides a rationale for the large number of both BH3-only and multidomain antiapoptotic members.

Whether the BH3 domains of multidomain members can initiate apoptosis is less certain. The BH3 domain isolated from BCL-X₀ studied here showed no activity, while BH3 peptides from BAX have generated mixed results. Addition of a BH3 peptide from BAK to a Xenopus cell free system induced release of cytochrome c and caspase activity, although the site of action was unknown (Cosulich et al., 1997). Addition of BAXBH3 to mammalian mitochondria has been reported to release cytochrome c without inducing permeability transition (Polster et al., 2001), consistent with the mechanistic pathway dissected here, whereas others report BAXBH3 peptides that do induce permeability transition and loss of transmembrane potential as an explanation for cytochrome c release (Narita et al., 1998). This may be inherent to α helices themselves or to hybrid proteins that can damage organelle membranes.

A substantial challenge for the future is to effectively transduce BH3 peptides or BH3 peptide mimetics into cells and assure that the induction of apoptosis is through the genetic pathway. Several studies (Holinger et al., 1999; Wang et al., 2000) including the initial polyarginine transduction approach presented here suggest this warrants further efforts. However, caution exists as a number of amphipathic α-helical peptides, especially if they are cationic, can be attracted to negatively charged membranes, including mitochondrial membranes, where they can nonspecifically disrupt the lipid matrix and membrane barrier function (Ellerby et al., 1999; Matsuzaki, 2001; Westerhoff et al., 1989). Others utilizing ANT-BH3BAD found toxicity was independent of the BCL-2 pathway and also killed yeast, which tolerate expression of the BH3-only proteins (Schimmer et al., 2001; Vieira et al., 2002). Alternative methods of internalization including receptor-mediated pathways should also be considered for BH3 peptidomimetics. The work here provides a proof of concept that BH3 mimetics can be designed that initiate apoptosis correctly, at definable points in the genetic pathway. Moreover, it provides a paradigm and reagents to dissect the mechanism of action of future BH3 mimetics.

**Experimental procedures**

**Peptide stocks**

Peptides were synthesized by Tufts University Core Facility and purified by HPLC, and identity was confirmed by mass spectroscopy. Stock solutions were 10–20 mM DMSO.

**Isolation of mitochondria**

Mouse liver mitochondria were isolated from age-matched wt or Bak<sup>−/−</sup> mice. Livers were diced, subjected to Dounce rotary Teflon pestle disruption, and then homogenized using a Kinematica Polytron homogenizer. Following suspension in isolation buffer (250 mM sucrose, 10 mM Tris-HCl [pH 7.4], 0.1 mM EGTA), mitochondria were isolated by differential centrifugation steps, followed by two washes in isolation buffer. Mitochondria from FL5.12 cells were isolated by cell disruption followed by differential centrifugation and washing as above. Cell disruption was performed either by a Kinematica Polytron homogenizer or by a combination of Dounce homogenization followed by 6–10 expulsions through a 27-gauge needle.

**Cytochrome c release**

Mitochondria at a protein concentration of 0.5 mg/ml were treated at room temperature in experimental buffer (125 mM KCl, 10 mM Tris-MOPS [pH 7.4], 5 mM glutamate, 2.5 mM malate, 1 mM KPO₄, 10 μM EGTA-Tris [pH 7.4]). Percent release was quantitated using a colorimetric ELISA (MCTC0, R&D Systems). In all experiments, treatments with DMSO were used as a control for solvent activity.

**BMH crosslinking**

1,6-bismaleimidohexane was obtained from Pierce (#22330). A 10 mM stock solution in DMSO was added to treated mitochondrial suspensions at a 1:11 dilution. Crosslinking took place for 30 min at room temperature, followed by centrifugation to pellet mitochondria. Pellets were dissolved in NuPAGE loading buffer (Invitrogen).

**Binding assays**

To determine Kₛₜ for peptide binding to BCL-2, a GST-BCL-2 fusion protein lacking the C-terminal transmembrane domain was utilized. Peptides were synthesized with a fluorescein amino terminus using an AHA linker. Peptides at 25 nM were mixed with titrations of GST-BCL-2 in binding buffer (140 mM NaCl, 10 mM Tris [pH 7.4]) at 37°C. An increase in fluorescence polarization measured on a Perkin-Elmer LS 50B luminescence spectrophotometer was quantitated to calculate binding. A nonlinear fit to a sigmoidal dose-response curve utilized the program Origin 6.0 to determine Kₛₜ. For quantitative BIDBH3 displacement assays, 25 nM fluoresceinated BIDBH3 was mixed with 1 μM GST-BCL-2 in binding buffer. Increasing amounts of unlabeled BIDBH3 peptides were titrated in, with loss of fluorescence polarization as a measurement of displacement of BIDBH3. Data were fitted to a sigmoidal curve as above, and IC₅₀ determined.

**GST-BCL-2 production**

GST-BCL-2Δ C21 fusion proteins were induced in BL21 DE3 by 0.1 mM IPTG. The bacterial pellets were resuspended in lysis buffer (1 mg/ml lysozyme, 1% Triton X-100, 0.1 mg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A in PBS) and sonicated. After centrifugation at 20,000 × g for 20 min, the supernatant was applied to glutathione-agarose beads (Sigma). The beads were washed with PBS and treated with 50 mM glutathione, 50 mM Tris-HCl (pH 8.0) to elute protein. Eluate was dialyzed against binding buffer and concentrated using Amicon centrifugal concentrating devices.

**Circular dichroism**

Circular dichroism (CD) spectra were obtained on a Jasco J-710 spectropolarimeter at 20°C using the following standard measurement parameters: wavelength, 190–280 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulation, 10; response, 1 s; bandwidth, 1 nm; path length, 0.1 cm. Stock solutions of peptide were dissolved in deionized water and concentrations determined by amino acid analysis. Samples were then diluted in 50 mM potassium phosphate (pH 7) to a calculated final concentration of 50 μM. The CD spectrum of each sample was measured in triplicate and a background spectrum of diluent alone was subtracted. For comparison, the subtracted CD spectra were normalized to 35 μM based on repeat peptide concentration determination by amino acid analysis of the diluted peptide solutions. The α-helical content of each peptide was calculated by dividing the mean residue ellipticity [θ]222° by the reported [θ]222° for a model helical deca-peptide (Yang et al., 1986).

**Immunoblot analysis**

Antibodies used for immunoblot analysis included anti-cytochrome c (7A681A, Pharmingen), anti-BAX (Upstate Biotechnology), and anti-BAX (N-20, Santa Cruz). Antibody detection was accomplished using enhanced chemiluminescence (Western Lightning, Perkin-Elmer).
Jurkat cell death experiments
Jurkat cells were grown in RPMI 1640, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 50 μM β-mercaptoethanol. Cells were treated with peptide for 5 h followed by staining with fluoro- cently-tagged Annexin V according to manufacturer’s protocol (BD Biosciences 556547). Death was quantitated by FACS, followed by analysis using FlowJo software (Tree Star, Inc.).

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