Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity


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Introduction

Apoptosis, the conserved cell suicide process by which multicellular organisms remove unwanted cells, is critical for development and tissue homeostasis, and its abnormal regulation can lead to cancer, degenerative disorders, and autoimmunity. The demise of the cell is brought about when the cysteine proteases, called caspases, cleave vital cellular substrates. All caspases are synthesized in a latent form and activated to mediate cell death. In response to most developmentally programmed cytotoxic cues or stress stimuli, caspase activation is governed by opposing members of the Bcl-2 protein family (Cory and Adams, 2002). As well as members that promote cell survival, such as Bcl-2, Bcl-xL, and Bcl-w, the family includes two groups of proapoptotic proteins: those like Bax and Bak that share three of the four Bcl-2 homology (BH) domains, and the structurally diverse BH3-only proteins (e.g., Bim, Bid, and Bad), which have in common with Bcl-2 only the small BH3 protein interaction domain. The BH3-only proteins are key initiators of the cell death process, but precisely how their association with prosurvival relatives promotes apoptosis remains unclear (Huang and Strasser, 2000; Cory and Adams, 2002). One consequence, dependent on Bax or Bak, is the release of cytochrome c from mitochondria, which serves as a cofactor for activation of caspase-9 by Apaf-1, thereby unleashing a proteolytic cascade. The Bcl-2 family may, however, also regulate other initiator caspases (Cory and Adams, 2002; Marsden et al., 2002).

Most Bcl-2 family members either normally reside on organelles or congregate there during apoptosis. Many possess a COOH-terminal hydrophobic domain thought to target them to the outer mitochondrial membrane and/or the nuclear envelope/ER. Bcl-2, for example, resides predominantly on...
the cytoplasmic face of the ER, with smaller amounts on the outer membrane of mitochondria and the nuclear envelope (Monaghan et al., 1992; Krajewski et al., 1993; Lithgow et al., 1994), whereas Bcl-xL is more prominent on the mitochondrial membrane (Gonzalez-Garcia et al., 1994; Conus et al., 2000; Kaufmann et al., 2003). Notably, Bax, which is essential for many forms of cell death, is normally cytosolic but undergoes a conformational change during apoptosis and translocates to the outer mitochondrial membrane (Hsu et al., 1997; Wolter et al., 1997; Hsu and Youle, 1998). The translocation and activation of Bax probably involves displacement of its COOH-terminal domain from a groove on its surface (Suzuki et al., 2000) and appears to be critical to its function (Wolter et al., 1997; Nechushtan et al., 1999).

It may, for example, allow Bax to oligomerize in the membrane and form pores (Cory and Adams, 2002; Kuwana et al., 2002).

As prosurvival Bcl-2 proteins probably require correct subcellular localization for their biological activity (Cory and Adams, 2002), we have explored how the membrane association of Bcl-w, a close structural and functional homologue of Bcl-2 and Bcl-xL, is related to its prosurvival function. In contrast to Bcl-2, which has been reported to be an integral membrane protein (see Discussion), we unexpectedly found that Bcl-w was only loosely associated with the membranes in healthy cells, but that engagement of a BH3-only protein markedly strengthened that association. A plausible explanation is provided by the recently determined 3D structure of Bcl-w (Denisov et al., 2003; Hinds et al., 2003): the Bcl-w COOH-terminal hydrophobic residues fold across the groove to which BH3 ligands bind (see Discussion). Hence, engagement of a BH3 ligand by the prosurvival protein must displace the hydrophobic tail, presumably then allowing it to interact tightly with membranes, as proposed to occur with Bax (Suzuki et al., 2000). To clarify how BH3 engagement is related to Bcl-w function, we have also engineered chimeric proteins that have a BH3 domain tethered to the NH2 terminus of Bcl-w, mimicking the ligand-bound state. Their behavior has allowed us to assess whether the binding of a BH3 domain is sufficient to promote membrane integration and to determine how BH3 engagement regulates the function of Bcl-w.

Results
Damage signals enhance the membrane association of Bcl-w
Confocal microscopy of HeLa cells has indicated that Bcl-w is located on the mitochondria (O’Reilly et al., 2001), and we have confirmed that observation with both HeLa cells and FDC-P1 myeloid cells (unpublished data). In view of its clear mitochondrial localization in microscopic studies (see next section in Results), we were surprised to find that Bcl-w did not behave like an organelle-associated protein on sub-cellular fractionation. Irrespective of the method used, only an insignificant amount of Bcl-w was recovered from membrane fractions. For example, none was detectable in purified mitochondria (Fig. 1 A). Moreover, Bcl-w behaved entirely as a cytosolic protein on sub-cellular fractionation of healthy FDC-P1 or HeLa cells lysed in low digitonin buffer (Fig. 1 B, 0 h; Fig. 1, C and D, control), as did the endogenous protein in several other cell types, NIH3T3, Jurkat, and T cells (unpublished data). We also fractionated lysates made without detergents, either by Dounce homogenization, needle (26 gauge) lysis, freeze/thawing, or nitrogen cavitation. In every case, rather than appearing in the pellet fraction, where proteins of the mitochondrial membrane (voltage-dependent anion channel [VDAC]/Porin) and intermembrane space (cytochrome c) were found, Bcl-w fractionated with soluble proteins such as Bax (Fig. 1 A; unpublished data). In contrast, Bcl-2, which is reported to be an integral membrane protein (Chen-Levy et al., 1989; Janiak et al., 1994), was found exclusively in the pellet fraction (Fig. 1 D, control). Thus, in healthy cells, Bcl-w appears to be only weakly bound to membranes.

We next examined whether cytotoxic signals affected the association of Bcl-w with membranes. Indeed, after FDC-P1 cells expressing FLAG–Bcl-w were treated with diverse cytotoxic stimuli, including γ irradiation (Fig. 1 B), growth factor withdrawal, or cytotoxic drugs (unpublished data), subcellular fractionation revealed a significant proportion of the protein in the membrane (pellet) fraction. This occurred before any release of cytochrome c was discernible (Fig. 1 B). Endogenous Bcl-w of FDC-P1 (Fig. 1 C) or of HeLa cells (Fig. 1 D) also underwent the transition when they were exposed to diverse cytotoxic stimuli. The pellet-bound Bcl-w was not released by alkali treatment (Fig. 1 C) and, hence, is probably integrated into the membrane. Interestingly, almost all the endogenous protein had shifted into the pellet fraction (Fig. 1, C and D), whereas only a portion of the overexpressed protein had done so (Fig. 1 B), suggesting that the binding capacity of membranes for Bcl-w is finite.

Although caspases have been reported to target some prosurvival Bcl-2–like proteins (Cheng et al., 1997), apoptosis did not alter the size of Bcl-w. Nevertheless, we tested whether caspase activation somehow induced its higher affinity for membranes by treating the cells with the broad-spectrum caspase inhibitor zVAD.fmk at a concentration that delayed cell death. Bcl-w was still recruited into the membrane fraction in response to death signals (Fig. 1 B). Hence, the increased affinity of Bcl-w for membranes probably reflects a step before caspase activation.

We next analyzed the protein complexes that Bcl-w might form by gel filtration chromatography. When lysates were prepared from healthy cells in the absence of any detergent (Fig. 1 E) or the presence of 1% digitonin (Fig. 1 F), FLAG–Bcl-w eluted primarily as a soluble monomer consistent with its predicted size of 22 kD. However, damage signals, such as γ irradiation, induced the formation of larger Bcl-w–containing complexes (Fig. 1 F). Their size inside cells may be even larger, because detergent was required to solubilize the complexes away from the membrane (pellet) fraction.

Damage signals do not alter the localization of Bcl-w
To resolve the apparent discrepancy between the predominance of Bcl-w in the soluble fraction after subcellular frac-
tionation (Fig. 1) and its clear mitochondrial localization in confocal microscopic studies, we reevaluated its localization by immunogold EM. In accord with confocal studies, a well-characterized rat monoclonal antibody raised against Bcl-w (13F9) (O’Reilly et al., 2001) revealed most (66%) of the FLAG–Bcl-w molecules around the mitochondria of both FDC-P1 (Fig. 2 A) and NIH3T3 (Fig. 2 B) cells, and an anti-FLAG monoclonal antibody gave an equivalent staining pattern (unpublished data). Little FLAG–Bcl-w was found on the nuclear and ER membranes, whereas FLAG–Bcl-2 was prevalent on those membranes (Lithgow et al., 1994) (Fig. 2 D). As expected, the anti–Bcl-w 13F9 antibody revealed far fewer endogenous Bcl-w molecules in parental FDC-P1, NIH3T3, or HeLa cells (Fig. 2 C; unpublished data), but examination of a number of fields confirmed that the endogenous protein was mainly mitochondrial in all three lines.

Our combined results suggest that Bcl-w in healthy cells is loosely associated with mitochondria and readily dislodged after cell lysis (Fig. 1), in contrast to the tightly associated Bcl-2, all of which appeared as expected in the membrane (pellet) fraction (Fig. 1 D; unpublished data).

The mitochondrial localization of Bcl-w was not changed by death signals. EM revealed a similar proportion of Bcl-w on the mitochondria of FDC-P1 cells after irradiation (Fig. 1) and its clear mitochondrial localization in confocal microscopic studies, we reevaluated its localization by immunogold EM. In accord with confocal studies, a well-characterized rat monoclonal antibody raised against Bcl-w (13F9) (O’Reilly et al., 2001) revealed most (66%) of the FLAG–Bcl-w molecules around the mitochondria of both FDC-P1 (Fig. 2 A) and NIH3T3 (Fig. 2 B) cells, and an anti-FLAG monoclonal antibody gave an equivalent staining pattern (unpublished data). Little FLAG–Bcl-w was found on the nuclear and ER membranes, whereas FLAG–Bcl-2 was prevalent on those membranes (Lithgow et al., 1994) (Fig. 2 D). As expected, the anti–Bcl-w 13F9 antibody revealed far fewer endogenous Bcl-w molecules in parental FDC-P1, NIH3T3, or HeLa cells (Fig. 2 C; unpublished data), but examination of a number of fields confirmed that the endogenous protein was mainly mitochondrial in all three lines.

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The mitochondrial localization of Bcl-w was not changed by death signals. EM revealed a similar proportion of Bcl-w on the mitochondria after subcellular fractionation. FLAG-Bcl-w–expressing FDC-P1 cells were separated into heavy membrane, cytosolic (C), and mitochondrial (mit) fractions. Equal cell numbers of the total cell lysate (Lys), homogenate (H), or the fractions were resolved by SDS-PAGE. (B) Tighter association of FLAG–Bcl-w with membranes after γ irradiation (10 Gy). Healthy or irradiated FD/FLAG–Bcl-w cells were fractionated into the soluble (s) and pellet (p) fractions using lysis buffer containing 0.025% digitonin. (C) Endogenous Bcl-w is soluble in healthy FDC-P1 cells but damage signals induce membrane association resistant to alkali treatment. Lysates prepared from healthy cells (control) or cells exposed to 100 nM staurosporine (STS) or γ irradiation (10 Gy) or deprived of IL-3 were fractionated into the soluble (s, top) or pellet (p, bottom) fractions. (D) Damage signals trigger membrane association of endogenous Bcl-w in HeLa cells. Soluble (s) and pellet (p) fractions of healthy HeLa cells (control) or 24 h after treatment with 50 J m⁻² UV irradiation (UV), 1.0 μg/ml etoposide (VP16), or 10 nM staurosporine (STS). (E and F) Bcl-w is a soluble monomeric protein in healthy cells, but damage signals cause its oligomerization. Lysates from FD/FLAG–Bcl-w cells were fractionated by gel filtration chromatography after lysis without detergent (E) or with 1% digitonin (F). Equivalent portions of the indicated fractions were loaded. By comparison with the elution of standard size markers as indicated, FLAG–Bcl-w appears to be a soluble, monomeric protein in healthy cells (E and top panel of F) but forms larger complexes after damage signals (10 Gy γ irradiation; F, bottom). The blots were probed for FLAG–Bcl-w with the anti–FLAG 9H1 or for the indicated proteins.

**Figure 1.** Apoptotic signals trigger membrane integration of Bcl-w and its oligomerization. (A) FLAG–Bcl-w is absent from the mitochondria after subcellular fractionation. FLAG-Bcl-w–expressing FDC-P1 cells were separated into heavy membrane, cytosolic (C), and mitochondrial (mit) fractions. Equal cell numbers of the total cell lysate (Lys), homogenate (H), or the fractions were resolved by SDS-PAGE. (B) Tighter association of FLAG–Bcl-w with membranes after γ irradiation (10 Gy). Healthy or irradiated FD/FLAG–Bcl-w cells were fractionated into the soluble (s) and pellet (p) fractions using lysis buffer containing 0.025% digitonin. (C) Endogenous Bcl-w is soluble in healthy FDC-P1 cells but damage signals induce membrane association resistant to alkali treatment. Lysates prepared from healthy cells (control) or cells exposed to 100 nM staurosporine (STS) or γ irradiation (10 Gy) or deprived of IL-3 were fractionated into the soluble (s, top) or pellet (p, bottom) fractions. (D) Damage signals trigger membrane association of endogenous Bcl-w in HeLa cells. Soluble (s) and pellet (p) fractions of healthy HeLa cells (control) or 24 h after treatment with 50 J m⁻² UV irradiation (UV), 1.0 μg/ml etoposide (VP16), or 10 nM staurosporine (STS). (E and F) Bcl-w is a soluble monomeric protein in healthy cells, but damage signals cause its oligomerization. Lysates from FD/FLAG–Bcl-w cells were fractionated by gel filtration chromatography after lysis without detergent (E) or with 1% digitonin (F). Equivalent portions of the indicated fractions were loaded. By comparison with the elution of standard size markers as indicated, FLAG–Bcl-w appears to be a soluble, monomeric protein in healthy cells (E and top panel of F) but forms larger complexes after damage signals (10 Gy γ irradiation; F, bottom). The blots were probed for FLAG–Bcl-w with the anti–FLAG 9H1 or for the indicated proteins.

**Figure 2.** (A) FLAG–Bcl-w is absent from the mitochondria after subcellular fractionation. FLAG-Bcl-w–expressing FDC-P1 cells were separated into heavy membrane, cytosolic (C), and mitochondrial (mit) fractions. Equal cell numbers of the total cell lysate (Lys), homogenate (H), or the fractions were resolved by SDS-PAGE. (B) Tighter association of FLAG–Bcl-w with membranes after γ irradiation (10 Gy). Healthy or irradiated FD/FLAG–Bcl-w cells were fractionated into the soluble (s) and pellet (p) fractions using lysis buffer containing 0.025% digitonin. (C) Endogenous Bcl-w is soluble in healthy FDC-P1 cells but damage signals induce membrane association resistant to alkali treatment. Lysates prepared from healthy cells (control) or cells exposed to 100 nM staurosporine (STS) or γ irradiation (10 Gy) or deprived of IL-3 were fractionated into the soluble (s, top) or pellet (p, bottom) fractions. (D) Damage signals trigger membrane association of endogenous Bcl-w in HeLa cells. Soluble (s) and pellet (p) fractions of healthy HeLa cells (control) or 24 h after treatment with 50 J m⁻² UV irradiation (UV), 1.0 μg/ml etoposide (VP16), or 10 nM staurosporine (STS). (E and F) Bcl-w is a soluble monomeric protein in healthy cells, but damage signals cause its oligomerization. Lysates from FD/FLAG–Bcl-w cells were fractionated by gel filtration chromatography after lysis without detergent (E) or with 1% digitonin (F). Equivalent portions of the indicated fractions were loaded. By comparison with the elution of standard size markers as indicated, FLAG–Bcl-w appears to be a soluble, monomeric protein in healthy cells (E and top panel of F) but forms larger complexes after damage signals (10 Gy γ irradiation; F, bottom). The blots were probed for FLAG–Bcl-w with the anti–FLAG 9H1 or for the indicated proteins.

**BH3 ligands cause tight membrane association of Bcl-w**

Apoptosis seems to be initiated when BH3-only proteins, unleashed by damage signals, bind to prosurvival relatives (Huang and Strasser, 2000; Cory and Adams, 2002). As the enhanced membrane association of Bcl-w appeared to involve a step before caspase activation, we hypothesized that it was triggered by engagement of Bcl-w by a BH3-only protein such as Bim. When FLAG–Bcl-w was immunoprecipitated from lysates of FD/FLAG–Bcl-w cells that had been exposed to a damaging agent, the complex included both the major endogenous Bim isoforms (BimL and BimEL), but neither was detectable in parallel immunoprecipitates from untreated cells (Fig. 3 A), confirming that apoptosis induces their association.

As the tighter membrane association of Bcl-w correlated with the recruitment of Bim to Bcl-w, we wished to test whether the Bim BH3 domain could induce the transition
in lysates from healthy cells. To validate the use of BH3 peptides, we first measured the affinity for Bcl-w of a BimL polypeptide, or a BH3 peptide from it, using an optical biosensor (Fig. 3, B and C). Purified recombinant BimL lacking its COOH-terminal hydrophobic 27 amino acids (BimL/H9004C27) binds purified Bcl-w with high (nM) affinity (Fig. 3 B; Hinds et al., 2003). Notably, a 26-mer peptide spanning the BH3 domain of Bim (denoted \( \text{wt\ BH3} \)) bound Bcl-w as avidly as BimL/H9004C27, and it competed effectively for the binding of BimL/H9004C27 (Fig. 3, B and C). Consequently, we focused on this peptide and two derivatives that have reduced affinity for Bcl-w, due to replacement of one or more of the four hydrophobic BH3 residues that mediate interaction with the hydrophobic groove of the prosurvival proteins (Fig. 4 A) (Sattler et al., 1997; Petros et al., 2000; Hinds et al., 2003). Replacement of the invariant BH3 leucine (L94 of the mouse BimL) with alanine (\( \text{L94A\ BH3} \)) reduced the binding over 50-fold, whereas a glutamate replacement of all four key residues (\( \text{4E\ BH3} \)) abolished binding altogether (Fig. 3 B).

To test whether the BH3 peptides enhanced membrane association of Bcl-w, lysates prepared from healthy cells were incubated with \( \text{wt\ BH3} \), the weakly binding \( \text{L94A\ BH3} \), or the non-binding \( \text{4E\ BH3} \). Subcellular fractionation showed that the wild-type peptide caused both overexpressed FLAG–Bcl-w and the endogenous protein to shift into the pellet fraction, whereas the \( \text{L94A\ BH3} \) mutant peptide did so less efficiently and \( \text{4E\ BH3} \) not discernibly (Fig. 3, D and E). Endogenous Bcl-w in lysates of HeLa cells was affected similarly (unpublished data).

Importantly, alkali treatment did not release the membrane-bound Bcl-w isolated from dying cells (Fig. 1 C) or that tightly associated due to addition of a BH3 peptide to lysates (Fig. 3 E). This result suggests that BH3 engagement converts Bcl-w into an integral membrane protein (Janiak et al., 1994), presumably by inducing a conformational change in Bcl-w that allows integration.
Fusing a BH3 domain to Bcl-w abrogates binding of BH3-only proteins

We wished to determine whether the engagement of a BH3 domain by Bcl-w is sufficient in itself to cause tight membrane association, and also to assess how the biological activity of Bcl-w is affected (see next section in Results). We therefore sought to mimic a BH3-ligated conformer of Bcl-w by fusing the 26-mer BH3 region of Bim to its NH₂ terminus via a 30-residue flexible linker (Gly₇Ser)₆ (Bird et al., 1988), generating BH3/Bcl-w (Fig. 4 A). Control Bcl-w constructs had mutant forms of the BH3 with reduced binding affinity fused to Bcl-w (Table I).

In the chimeras, the BH3, but not the BH3, peptide would be expected to occupy the binding groove of Bcl-w and displace its COOH-terminal residues (Fig. 4, C and D, left). To determine whether the binding groove of the chimeras remained accessible, we tested their ability to bind to BimEL or Bmf, another BH3-only protein (Puthalakath et al., 2001), in coimmunoprecipitation assays (the BimEL isoform was used rather than BimL, because BimL was more readily resolved by size from Bcl-w). As expected, the BH3/Bcl-w chimera still bound BimEL and Bmf (Fig. 4 D; unpublished data), showing that its groove remained free, whereas the BH3/Bcl-w chimera did not (Fig. 4 C), presumably because its groove was already occupied by the tethered BH3.

BH3 binding to Bcl-w triggers membrane association and neutralizes prosurvival activity

As the fusion constructs behaved as expected, to allow tests of their function, we stably expressed them in FDC-P1 cells and derived at least three independent clones of each construct expressing comparable levels of the chimeras (Fig. 5 A). First, we confirmed that the tethered BH3 domain did not affect the predominant mitochondrial localization of Bcl-w (Fig. 5, B and C). Next, we examined the membrane binding properties of the chimeras (Fig. 5 D and Table I). The BH3/Bcl-w construct was predicted to bind membranes more tightly, because its COOH terminus should be displaced, mimicking the conformation of wild-type Bcl-w with a BH3 protein bound. Indeed, fractionation of healthy cells revealed that a substantial proportion of the BH3/Bcl-w appeared in the pellet fraction, as observed with Bcl-w only after cell death induction (Fig. 1). In contrast, the BH3/Bcl-w chimera, as expected, appeared almost entirely in the soluble fraction, like wild-type Bcl-w (Fig. 5 D). Significantly, the translocation of BH3/Bcl-w requires its COOH-terminal domain, because the chimera with that domain excised no longer firmly attached to the membranes (Fig. 5 E). Hence, it is the displaced “tail” that mediates tight membrane binding.

Finally, we tested whether the conformational change mediated by the bound BH3 domain induces a latent killing activity.
activity in Bcl-w. That conformer might, for example, upon integration into the membrane, disturb its integrity, as translocated Bax is thought to do (see Discussion). wtBH3/Bcl-w did not, however, affect the viability of untreated FDC-P1 cells, and when they were subjected to cytotoxic stress, it did affect the loss of mitochondrial transmembrane potential ($\Delta \Psi_m$), which sometimes precedes irreversible commitment to apoptosis (Castedo et al., 1996). In contrast, 4E BH3/Bcl-w inhibited the loss of $\Delta \Psi_m$ as effectively as wild-type Bcl-w (Fig. 6 A). Importantly, the 4E BH3/Bcl-w chimera promoted survival as effectively as wild-type Bcl-w (Fig. 6 and Table I), showing that the addition of an NH2-terminal peptide does not in itself impair the biological activity of Bcl-w. Moreover, a chimera in which the four critical BH3 hydrophobic residues were replaced with alanines rather than glutamates (4ABH3/Bcl-w) could not be coimmunoprecipitated with BimEL. (D) Fusing a BH3 with the key BH3 hydrophobic residues mutated (filled circle) did not affect binding to BH3-only proteins. In the 4EBH3/Bcl-w fusion, the groove should be unoccupied (left), and that chimera readily associated with BimEL.

Discussion

The association of the related prosurvival proteins Bcl-2, Bcl-xL, and Bcl-w to intracellular membranes is likely to be closely coupled to their activity. Because all three possess similar hydrophobic COOH-terminal hydrophobic domains, it was widely assumed that all would be integral membrane proteins distributed similarly between the cytoplasmic faces of the nuclear/ER and outer mitochondrial membranes. Notable differences have, however, appeared. The majority of the Bcl-2 molecules reside on the nuclear/ER membrane, with a smaller portion on that of mitochondria, whereas Bcl-xL is more prominent on the latter (Gonzalez-Garcia et al., 1994; Conus et al., 2000; Kaufmann et al., 2003), where we also find most of the Bcl-w in healthy cells (Fig. 2). The precise mechanism by which the Bcl-2–related proteins are targeted to these membranes remains unknown (Wattenberg and Lithgow, 2001; Borgese et al., 2003).

Bcl-2 can be readily isolated from membrane fractions (Hsu et al., 1997; Hausmann et al., 2000) and seems to be an integral membrane protein (Chen-Levy et al., 1989; Janiak et al., 1994). Although one study suggests that Bcl-xL may be an integral membrane protein (Kaufmann et al., 2003), a substantial portion of the protein is readily displaced from membranes and thus cannot be integrated (Hsu et al., 1997; Hausmann et al., 2000; Nijhawan et al., 2003). We found that Bcl-w binds very weakly to membranes in healthy cells but that its affinity is dramatically enhanced in dying cells, where it appears to become an integral membrane protein (Fig. 1). Rather than a late event requiring caspase activation, this transition seems to be mediated by the binding of a BH3-only protein, such as Bim, and is thus associated with the initiation of cell death (Huang and Strasser, 2000). Engagement of the BH3 domain alone must suffice, because addition of a Bim BH3
peptide to lysates induced a change in the membrane association of Bcl-w indistinguishable from that observed in cells induced to die (compare Figs. 1 and 3). Similar conclusions have been reached regarding Bcl-xL (Youle, R., personal communication).

As the BH3-only proteins bind the prosurvival proteins with high specificity and affinity, we propose that their ligation promotes membrane integration by altering the conformation of the prosurvival protein. Although the Bcl-xL structure does not exhibit a large conformational change when a BH3 peptide is bound (Sattler et al., 1997; Petros et al., 1997).
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Model for the inactivation of prosurvival Bcl-2–like proteins by the BH3-only proteins. It is proposed that prosurvival family members, like Bcl-w, normally bind and sequester a membrane-bound effector protein (X) required for Bax/Bak activation. The engagement of a BH3-only protein (Bim here) frees X and allows it, directly or indirectly, to activate Bax and Bak, thereby initiating apoptosis.

2000; Liu et al., 2003), a caveat is that those structures were derived from a Bcl-xL protein lacking the hydrophobic COOH-terminal domain. Interestingly the structure of full-length proapoptotic Bax, a cytosolic protein, revealed that its hydrophobic COOH-terminal residues forms an α helix that nestles into a hydrophobic groove that is very similar to that used by prosurvival relatives to bind BH3 peptides (Suzuki et al., 2000). It has been proposed that the orientation of the COOH-terminal hydrophobic residues alters Bax localization, because damage signals induce Bax to translocate from the cytosol to mitochondrial membranes, where it forms oligomers (Nechushtan et al., 1999; Antonsson et al., 2001; Mikhailov et al., 2001; Nechushtan et al., 2001). As Bax, or its close relative Bak, is essential to signal many forms of cell death (Lindsten et al., 2000), the conformational alteration in Bax and Bak may represent a critical step in apoptosis.

Bcl-w may also undergo a conformational switch of the COOH terminus, like that proposed for Bax. Our recent solution structure of Bcl-w lacking only the last 10 residues (Hinds et al., 2003) unexpectedly revealed that its COOH-terminal hydrophobic residues, like that of Bax, are tucked into the hydrophobic groove, occupying the site where a BH3 domain binds in Bcl-xL (Sattler et al., 1997; Petros et al., 2000; Liu et al., 2003). Therefore, a BH3 ligand must displace the sticky hydrophobic residues and thus make it available to insert into a lipid environment. The membrane integration of Bcl-w after a death signal (Fig. 1) probably reflects insertion of the COOH-terminal domain into the membrane after its displacement from the groove by the engagement of a BH3-only protein. It is unlikely that membrane integration requires an independent signal, or is an indirect consequence of apoptosis, because the transition could be triggered simply by BH3 peptides added to lysates of healthy cells (Fig. 3), and a Bcl-w chimera having a tethered wild-type BH3 domain was integrated even in healthy cells (Fig. 5).

Previous analysis of the binding of BH3-only proteins to prosurvival relatives has not clearly established whether their association simply neutralizes the function of the latter or instead converts the Bcl-2–like protein into a Bax-like “killer.” The similarity of the structure of Bcl-xL to the pore-forming domain of bacterial toxins (Muchmore et al., 1996) has stimulated interest in the hypothesis that Bcl-2–like proteins form pores in the mitochondrial membranes (Vander Heiden and Thompson, 1999; Tsujimoto and Shimizu, 2000; Zamzami and Kroemer, 2001) and thereby control the release of proapoptotic factors such as cytochrome c (Green and Reed, 1998; Gross et al., 1999; Martinou and Green, 2001). In such a model, binding of a BH3-only protein to a Bcl-2–like protein might well initiate pore formation, leading to cell death. Our studies, however, indicate that this is unlikely, because a chimera that mimics a BH3-bound conformer of Bcl-w had no deleterious effects on the cell (Fig. 6). Genetic studies also argue against the idea that the mammalian prosurvival proteins harbor a latent killing activity because their deletion in the mouse leads to tissue degeneration and loss of cell viability, rather than the hyperplasia that would be predicted if these proteins mediate killing (Cory and Adams, 2002).

The finding that the “BH3/Bcl-w chimera is inert rather than proapoptotic is compatible with other mechanisms for the function of Bcl-2–like proteins. For example, if Bcl-2–like proteins simply sequester BH3-only proteins until their capacity is exceeded, as has been proposed (Lindsten et al., 2000; Cheng et al., 2001; Zong et al., 2001), this chimera would be inert because it can no longer bind BH3-only proteins. A second, more radical, hypothesis would be that insertion into the membrane actually inactivates the prosurvival function of Bcl-w, and hence the weakly membrane attached form of Bcl-w might be the active form.

We favor a model in which Bcl-2–like proteins normally sequester a postulated downstream effector, needed for activation of Bax/Bak and/or caspases (Fig. 7) (Cory and Adams, 2002). In this model, the chimera might be inactive because it can no longer bind the effector. If so, such an effector (X) might be bound only by the conformer of Bcl-w with the COOH-terminal residues in the groove but displaced upon BH3 binding of Bcl-w. Interestingly, although the COOH-terminal residues of Bcl-w are dispensable for binding to BH3-only proteins, they are essential for the biological activity of Bcl-w (Hinds et al., 2003). That observation, together with the data presented here, prompts us to predict that the COOH-terminal residues of prosurvival family members are required for binding to a membrane-bound effector protein akin to that proposed in Fig. 7.

Materials and methods

Expression constructs

Expression vectors for Bcl-w, BimΔN, or Bmf have been previously described (O’Connor et al., 1998; Moriishi et al., 1999; Puthalakath et al., 2001). These are based on the pEF PGKpuro or pEF PGKhygro vectors incorporating NH2-terminal FLAG and Glu-Glu (EE) epitopes, respectively (Huang et al., 1997a,b, 1998). To make the BH3/Bcl-w fusions, oligonucleotides corresponding to the 26 residues spanning wild-type or mutant BH3 of Bim were joined to wild-type Bcl-w by a flexible (Gly4Ser1)6 linker. Bacterial expression vectors to make recombinant Bcl-w or Bim proteins are described elsewhere (Hinds et al., 2003). Proofreading Pfu polymerase (Stratagene) was used for PCR, and the constructs were verified by automated sequencing. Details of the oligonucleotides and the constructs are available from the authors.
Tissue culture, transfection, and cell survival assays

The culture conditions of the cell lines used and the transfection conditions have been previously described (Strasser et al., 1995; O’Reilly et al., 1996; Huang et al., 1997a,b, 1998; O’Connor et al., 1998; Puthalakath et al., 1999; Hausmann et al., 2000; Puthalakath et al., 2001). Cell death was induced in FDC-P1 cells by IL-3 deprivation, 10 Gy γ irradiation, or 1–100 nM staurosporine (Sigma-Aldrich); in HeLa cells with 50 μM 2′-UV irradiation, 1.0 μg/ml etoposide (VP-16) Δelta West, or 10 nM staurosporine. Cell viability was quantified by flow cytometric analysis of cells excluding 5 μg/ml propidium iodide (PI; Sigma-Aldrich) using a FACScan® (Becton Dickinson). Each time point was performed in triplicate on at least three independent clones of each genotype, and the experiments were repeated at least three times. In some experiments, the cells were cultured in the presence of a broad-spectrum caspase inhibitor, 50 μM ZVAD.fmk (Bachem).

Cytolourometric determination of mitochondrial transmembrane potential

To assess mitochondrial transmembrane potential (ΔΨm), healthy or dying cells were incubated for 15 min at 37°C in buffer containing 40 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC₃[3]; Molecular Probes) before adding 5 μM of PI. The cells were kept on ice until flow cytometric analysis. After compensation to exclude nonviable cells, fluorescence was recorded at 525 nm (FL-1) for DiOC₃[3] and 600 nm (FL-3) for PI on a FACScan® (Becton Dickinson).

Subcellular fractionation, gel filtration, immunoprecipitation, and immunoblotting

Fractionation of whole cell lysates into the soluble and pellet fractions was previously described (Ramsby et al., 1994; Hausmann et al., 2000). In brief, cells lysed in HMKEE buffer (20 mM Hepes, pH 7.2, 5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors) containing 250 mM sucrose and 0.025% digitonin (Calbiochem) were left on ice for 10 min, and then the organelles, cytoskeleton, and membranes were pelleted by centrifugation (13,000 rpm, 2 min at 4°C). The pellet was solubilized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, and protease inhibitors). The protease inhibitors used include Pefabloc SC, soybean trypsin inhibitor, leupeptin, aprotinin, E64, and pepstatin (Sigma-Aldrich or Roche).

Mitochondria were isolated by swelling cells in hypotonic RSF buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5) at 4°C for 5–10 min (Lithgow et al., 1994; Spector et al., 1998). The cells were lysed using Dounce homogenization, and the osmotic balance was restored by adding 5 mM propidium iodide (PI). The cells were kept on ice until flow cytometric analysis. After compensation to exclude nonviable cells, fluorescence was recorded at 525 nm (FL-1) for DiOC₃[3] and 600 nm (FL-3) for PI on a FACScan® (Becton Dickinson).

Immunoblotting

Cells for gel filtration were swollen in HMKEE buffer and lysed by nitrosonedavation. Adding sucrose to 250 mM restored the osmolality. After removing insoluble material by centrifugation, lysates from 5 × 10⁶ cells were loaded onto an analytical Superdex™ 75 column (Amersham Biosciences) and run at 0.5 ml/min, and 0.2-ml fractions were collected. Alternatively, lysates from a similar number of cells, lysed in HMKEE buffer containing 250 mM sucrose and 1% digitonin, were resolved using an analytical Superose™ 12 column (Amersham Biosciences) under similar conditions. The columns were calibrated using standard protein markers (Amersham Biosciences).

Lysates from transiently transfected and [³⁵S]methionine/cysteine (NEN)-labeled cells were prepared and communoprecipitation experiments were performed as previously described (Huang et al., 1997a,b, 1998; O’Connor et al., 1998; Morishii et al., 1999; Puthalakath et al., 1999, 2001). Total cell lysates, immunoprecipitates, or fractionated samples were resolved by SDS-PAGE (Invitrogen) and electroblotted onto nitrocellulose membranes (Amersham Biosciences). Immunoblotting was performed as previously described (Huang et al., 1997a,b, 1998; O’Connor et al., 1998; Morishii et al., 1999; Puthalakath et al., 1999, 2001; Hausmann et al., 2001). The following antibodies were used as monoclonal anti–FLAG M2 (Sigma-Aldrich), anti–voltage-dependent anion channel (VDAC)/Porin (Calbiochem), anti–cytochrome c (TH;2C1; BD Biosciences), anti–Bcl-2–100 (Pezzella et al., 1990), anti–Bax SB7 (Sigma-Aldrich), anti–EE (Glu-Glu) (CRP), or anti–HA.11 (CRP); rat monoclonal anti–FLAG 9H1, anti–Bcl-w 13F9, or anti–Bcl-w 16H12 (O’Reilly et al., 2001); and rabbit polyclonal anti–caspase-9 (BD Biosciences). The rat monoclonal anti–FLAG antibody was generated by immunizing rats with FLAG-tagged recombinant human Apaf-1 protein (Hausmann et al., 2000) and screened using a FDC-P1 cell line overexpressing FLAG–Bcl-2 (O’Reilly et al., 1998).

Regulated targeting of Bcl-w

Regulated Bcl-w and Bim, proteins, expressed as GST fusion proteins in Escherichia coli BL21(DE3), were prepared as previously described (Day et al., 1999; Hinds et al., 1999, 2003). Bcl-w ΔC10, harboring the mutations C29S and A128E, was prepared by affinity purification using a nickel chelate column according to the manufacturer’s instructions (Qiagen) and size exclusion chromatography. Peptides corresponding to the following sequences were purchased from Mimotopes: BH3, DLURPEIRAEQERRCGDEFETTRRY; BH3, DLURPEIRAEQERRCGDEFETTRRY; and BH3, DLURPEIRAEQERRCGDEFETTRRY. The altered amino acids are in bold.

Analysis of protein interactions was performed on a Bicore 2000 biosensor (Biacore) as previously described (Lackmann et al., 1997; Hinds et al., 2003). The ability of Bim-derived BH3-domain peptides to compete for Bim binding in solution was examined by incubation of a constant concentration of Bcl-w (50 nM) ligand with increasing amounts of Bim ΔC27 or BH3 peptides before analysis on a Bim-derivatized sensor chip.

To test if BH3 peptides induce tight membrane binding of Bcl-w, cell lysates prepared in HMKEE buffer (with 250 mM sucrose and 0.025% digitonin) were incubated with 1–100 μg/ml of the peptide for 30 min at room temperature before fractionation.

Immunogold EM

Cell pellets, frozen using a Leica EM high-pressure freezer, were freeze substituted in 0.1% uranyl acetate in acetone at −90°C for 72 h, and the temperature was raised to −50°C at 6°C/h. The samples were infiltrated with a graded series of Lowicryl HM20 low temperature resin (Polysciences) in acetone consisting of 25% resin (8 h), 50% resin overnight, 75% resin (8 h), and 100% resin overnight. The infiltrated samples were polymerized under UV light for 48 h at −50°C and brought to room temperature at 6°C/h. The sample blocks were further hardened under UV light for 24 h at room temperature. Embedded cell blocks were sectioned with a diamond knife (Leica Ultracut R microtome), and 90-nm sections were collected onto Formvar-coated 200-mesh hexagonal copper grids. Prior to immobilization, sections were blocked in PBS containing 0.8% BSA and 1% horse serum for 20 min. The grids were incubated for 4 h at room temperature on 20-μl droplets containing 10 μg/ml rat anti-Bcl-w 13F9, rat anti–FLAG 9H1, mouse anti–Bcl-2–100, or isotype control (BD Biosciences) antibodies diluted with blocking agent. After three washes in blocking agent, the grids were incubated overnight on 20-μl droplets of 18-nm gold–conjugated goat anti–rat secondary antibody (1:40; Jackson Immunoresearch Laboratories) or 20-nm gold–conjugated antimate–mouse secondary antibody (1:40; British Biocell) at 4°C. Labeled grids were washed three times, immersed in distilled water, and allowed to air dry. They were then sequentially stained with saturated uranyl acetate for 15 min and triple lead stain for 10 min (Sato, 1968) and viewed on a Philips CM120 Bioimt transmission electron microscope at 120 kV. Quantification for mitochondrial-associated staining was performed on at least 36 negatives and scored independently.

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