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

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Prosurvival Bcl-2–like proteins, like Bcl-w, are thought to function on organelles such as the mitochondrion and to be targeted to them by their hydrophobic COOH-terminal domain. We unexpectedly found, however, that the membrane association of Bcl-w was enhanced during apoptosis. In healthy cells, Bcl-w was loosely attached to the mitochondrial membrane, but it was converted into an integral membrane protein by cytotoxic signals that induce binding of BH3-only proteins, such as Bim, or by the addition of BH3 peptides to lysates. As the structure of Bcl-w has revealed that its COOH-terminal domain occupies the hydrophobic groove where BH3 ligands bind, displacement of that domain by a BH3 ligand would displace the hydrophobic COOH-terminal residues, allowing their insertion into the membrane. To determine whether BH3 ligation is sufficient to induce the enhanced membrane affinity, or to render Bcl-w proapoptotic, we mimicked their complex by tethering the Bim BH3 domain to the NH_2 terminus of Bcl-w. The chimera indeed bound avidly to membranes, in a fashion requiring the COOH-terminal domain, but neither promoted nor inhibited apoptosis. These results suggest that ligation of a proapoptotic BH3-only protein alters the conformation of Bcl-w, enhances membrane association, and neutralizes its survival function.

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-x_L, 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

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Abbreviations used in this paper: BH, Bcl-2 homology; PI, propidium iodide.

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-x_L 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-x_L, 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 NH_2 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 subcellular 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 stress 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-

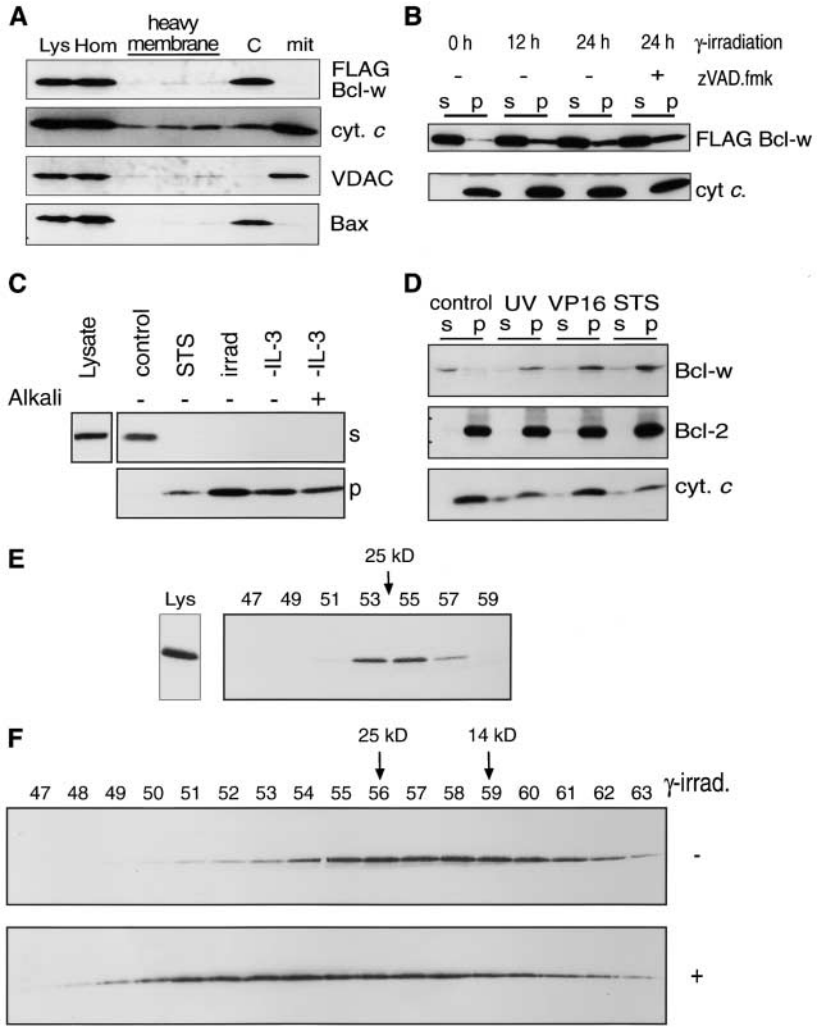

Figure 1. **Apoptotic signals trigger membrane integration of Bcl-w and its oligomerization.** (A) FLAG–Bcl-w is absent from the mitochondria after subcellular fractionation. FLAG-Bcl-w–expressing FDC-P1 cells were separated into heavy membrane, cytosolic (C), and mitochondrial (mit) fractions. Equal 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 Jm^{-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.

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. 2 E) or after deprivation of growth factor (Fig. 2 F). More of the Bcl-w molecules did appear to be clustered after the cytotoxic signals, consistent with the gel filtration experiments (Fig. 1 F). However, the biological significance of Bcl-w oligomers will require more investigation.

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 (Bim_L and Bim_{EL}), 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

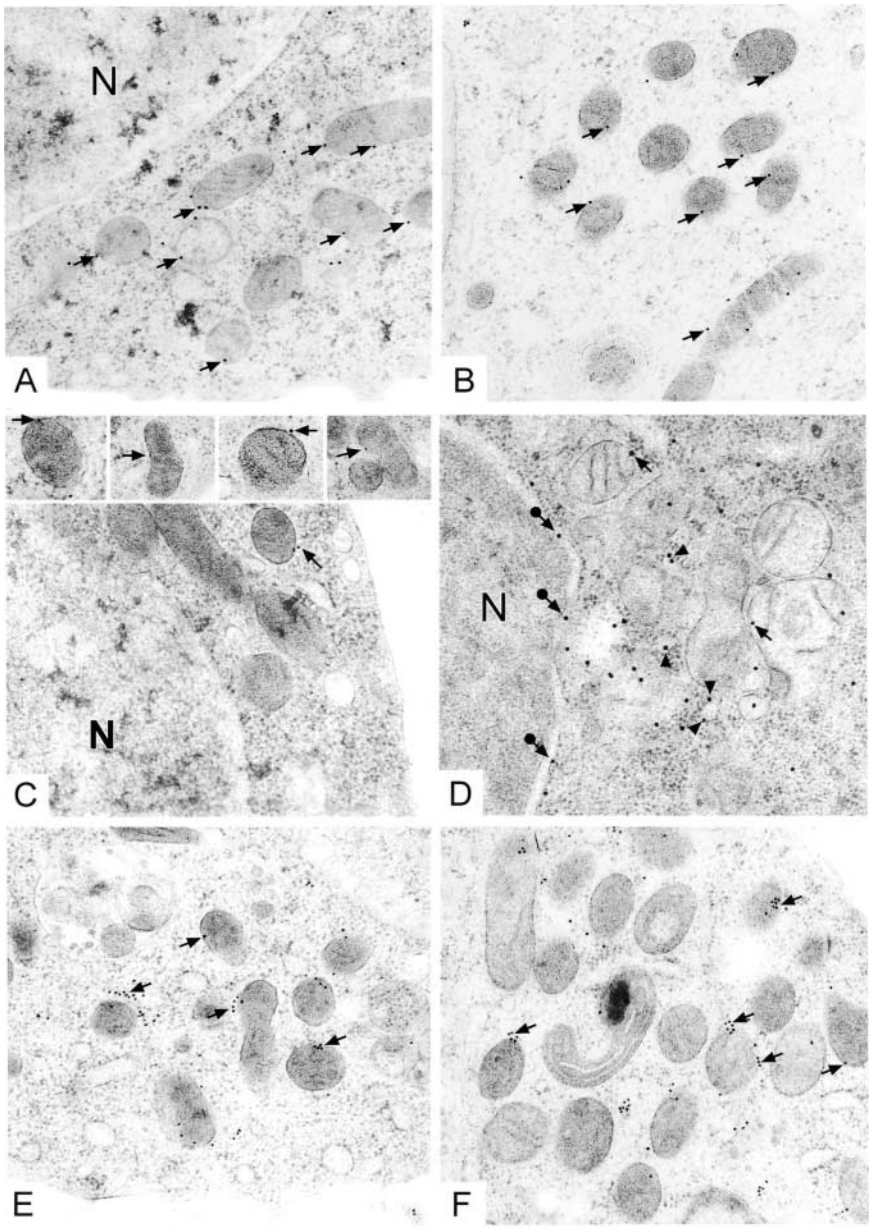

Figure 2. **Bcl-w is predominantly a mitochondrial protein in healthy and dying cells.** (A and B) Overexpressed FLAG–Bcl-w in FDC-P1 (A; ×52K) or NIH3T3 (B, ×37K) cells is mainly mitochondrial. Representative immunogold electron microscopic images of cells stained with the anti–Bcl-w 13F9 antibody were detected with 18-nm gold–conjugated goat anti–rat antibody. (C) Scanty staining for endogenous Bcl-w around the mitochondria in parental FDC-P1 cells. The four insets show Bcl-w staining around mitochondria (×27.5K). (D) Bcl-2 is present on the nuclear/ER membranes as well as the outer mitochondrial membranes of FDC-P1 cells. Overexpressed Bcl-2 detected with the mouse monoclonal anti–Bcl-2-100 antibody and revealed using 20-nm gold–conjugated goat anti–mouse antibody. (E and F) Bcl-w remains closely associated with the mitochondria after damage signals. Staining of FD/FLAG–Bcl-w cells 24 h after (E) 10 Gy γ irradiation or (F) IL-3 deprivation (×37K). Arrowhead, ER; arrow, mitochondria; arrow with ball on end, nuclear membrane. N, nucleus.

in lysates from healthy cells. To validate the use of BH3 peptides, we first measured the affinity for Bcl-w of a Bim_L polypeptide, or a BH3 peptide from it, using an optical biosensor (Fig. 3, B and C). Purified recombinant Bim_L lacking its COOH-terminal hydrophobic 27 amino acids ($Bim_L\Delta C27$) 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 ^{wt}BH3) bound Bcl-w as avidly as $Bim_L\Delta C27$, and it competed effectively for the binding of $Bim_L\Delta C27$ (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 Bim_L) with alanine (^{L94A}BH3) reduced the binding over 50-fold, whereas a glutamate replacement of all four key residues (^{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 ^{wt}BH3, the weakly binding ^{L94A}BH3, or the nonbinding ^{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 ^{L94A}BH3 mutant peptide did so less efficiently and ^{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.

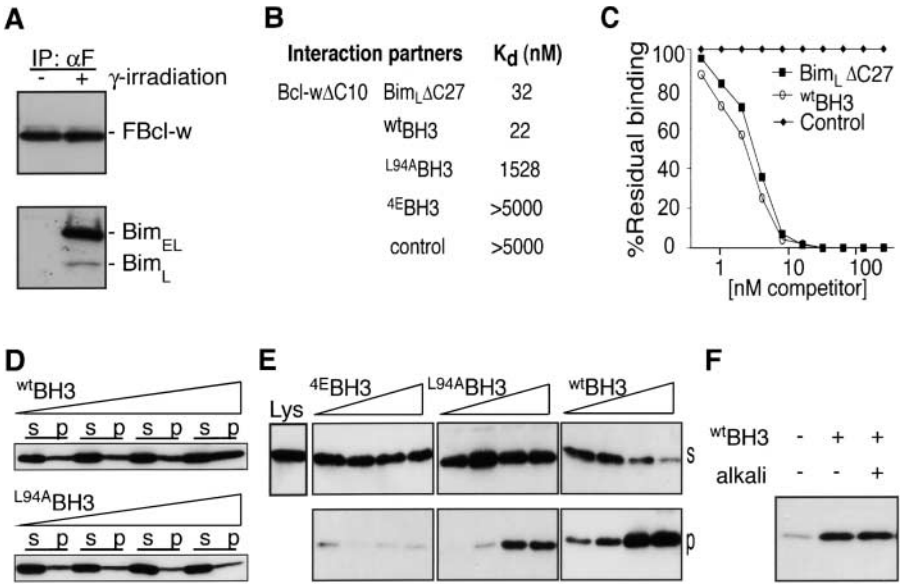

Figure 3. **BH3 peptides induce tight membrane association of Bcl-w.** (A) Damage signal (10 Gy γ irradiation) to FD/FLAG–Bcl-w cells provokes Bim_{EL} and Bim_L isoforms to associate with FLAG–Bcl-w. (B) Wild-type Bim_L protein and its BH3 peptide bind tightly to Bcl-w. The affinities of recombinant $Bim_L\Delta C27$ or 26-mer Bim BH3 peptides to recombinant Bcl-w ΔC10 determined in optical biosensor. (C) Bim BH3 peptide (^{wt}BH3) binds Bcl-w as well as Bim_L ΔC27. The ability of free $Bim_L\Delta C27$ or ^{wt}BH3 peptide to compete with immobilized $Bim_L\Delta C27$ for Bcl-w binding was assessed in biosensor experiments. (D) Addition of wild-type, but not mutant, BH3 peptide induces tight membrane association of FLAG–Bcl-w. Lysates of healthy cells were incubated with increasing amounts (0–100 μg/ml) of ^{wt}BH3 (top) or ^{L94A}BH3 (bottom) peptides before fractionation. The blots were probed with rat anti–FLAG 9H1 to detect FLAG–Bcl-w. (E) Bim BH3 peptide induces tighter association of endogenous Bcl-w from FDC-P1 cells. Lysates of healthy cells were incubated with increasing amounts of BH3 peptides and fractionated. In D and E, the blots were probed for endogenous Bcl-w. (F) BH3 peptide induces membrane integration of endogenous Bcl-w. Lysates prepared from healthy FDC-P1 cells and incubated with ^{wt}BH3 peptide were fractionated without or with alkali treatment.

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_L to its NH_2 terminus via a 30-residue flexible linker $(Gly_4Ser_1)_6$ (Bird et al., 1988), generating ^{wt}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 ^{wt}BH3, but not the ^{4E}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 Bim_{EL} or Bmf, another BH3-only protein (Puthalakath et al., 2001), in coimmunoprecipitation assays (the Bim_{EL} isoform was used rather than Bim_L, because Bim_{EL} was more readily resolved by size from Bcl-w). As expected, the ^{4E}BH3/Bcl-w chimera still bound Bim_{EL} and Bmf (Fig. 4 D; unpublished data), showing that its groove remained free, whereas the ^{wt}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 ^{wt}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 ^{wt}BH3/Bcl-w appeared in the pellet fraction, as observed with Bcl-w only after cell death induction (Fig. 1). In contrast, the ^{4E}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 ^{wt}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

Table I. **Properties of BH3/Bcl-w fusions**

Construct	BH3 binding	Membrane attachment	Mitochondrial localization	Inhibition of apoptosis
Wild-type Bcl-w	+++	−	Yes	+++
^{wt}BH3/Bcl-w	−	++	Yes	−
^{4E}BH3/Bcl-w	+++	−	Yes	+++
^{4A}BH3/Bcl-w	+	+	ND	+

Chimeras having the BH3 peptide from Bim fused to Bcl-w were compared with wild-type Bcl-w.

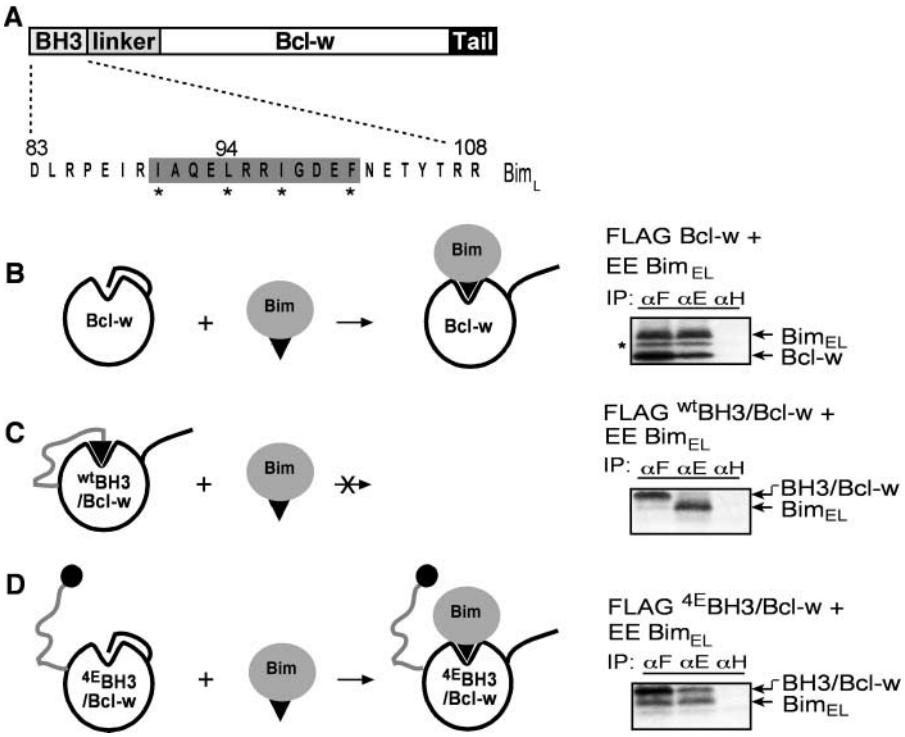

Figure 4. **Fusion of Bim BH3 to Bcl-w blocks engagement of BH3-only proteins.** (A) Representation of the constructs fusing Bim BH3 to the NH_2 terminus of Bcl-w. The shaded box below represents the core BH3 domain, and asterisks mark the hydrophobic BH3 residues critical for Bcl-w binding and altered to alanine (A) or glutamate (E) in the mutant peptides. (B) Bcl-w interacts avidly with Bim. ^{35}S metabolically labeled lysates prepared from 293T cells overexpressing FLAG–Bcl-w and EE Bim_{EL} were immunoprecipitated with the anti–FLAG M2 (αF), anti-EE (αE), or anti-HA (αH) antibodies, and the immunoprecipitations were size fractionated on SDS-PAGE gels. (C) Fusion of wild-type Bim BH3 peptide to Bcl-w blocks its binding to Bim. ^{wt}BH3 (filled triangle), linked to Bcl-w via a flexible linker (in gray), is likely to bind onto the groove of Bcl-w, thereby displacing the COOH terminus (left). As expected, FLAG–^{wt}BH3/Bcl-w could not be coimmunoprecipitated with Bim_{EL}. (D) Fusing a BH3 with the key BH3 hydrophobic residues mutated (filled circle) did not affect binding to BH3-only proteins. In the ^{4E}BH3/Bcl-w fusion, the groove should be unoccupied (left), and that chimera readily associated with Bim_{EL}.

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). ^{wt}BH3/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 NH_2-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 (^{4A}BH3/Bcl-w) was partially active (Table I); as would be expected, because this BH3 peptide should present a weakly hydrophobic face for interaction with Bcl-w. In striking contrast, the ^{wt}BH3/Bcl-w chimera neither conveyed any protection from death stimuli nor sensitized the cells to them. Thus, this chimera, which mimics a BH3-bound Bcl-w, was functionally inert, neither inhibiting nor promoting cell death (Fig. 6 and Table I).

Discussion

The association of the related prosurvival proteins Bcl-2, Bcl-x_L, 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-x_L 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-x_L 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

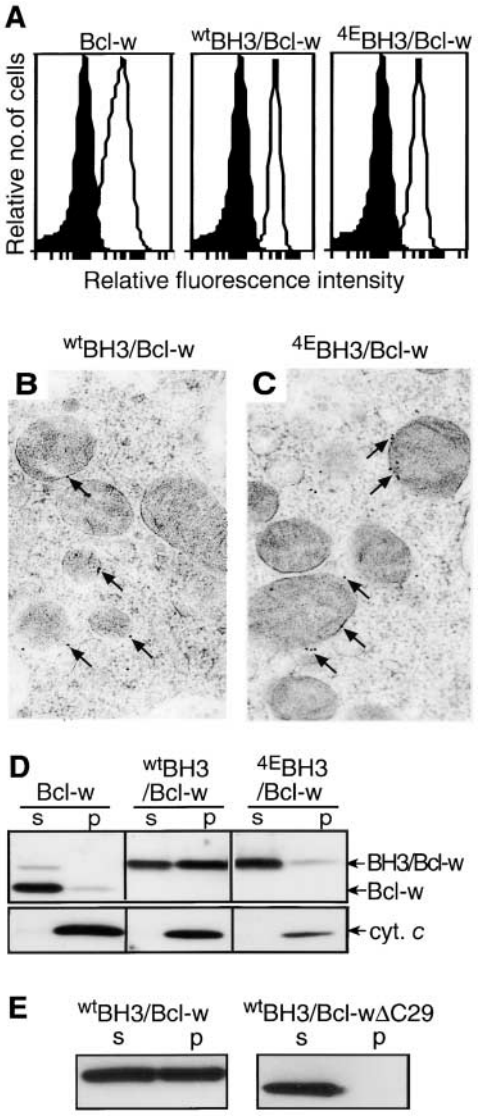

Figure 5. **Bim BH3 fusion drives Bcl-w into the membrane fraction.** (A) Expression of FLAG-tagged Bcl-w, ^{wt}BH3/Bcl-w, or ^{4E}BH3/Bcl-w fusion proteins in FDC-P1 cells. Representative clones (unfilled histograms) were stained with the anti–FLAG M2 followed by FITC-conjugated goat anti–mouse antibody and analyzed by flow cytometry. Filled histograms indicate control staining of the parental FDC-P1 cells. (B and C) BH3/Bcl-w fusions are localized at the mitochondria. Immunogold EM revealing ^{wt}BH3/Bcl-w (B) or ^{4E}BH3/Bcl-w (C) in FDC-P1 cells (×37K). Arrows indicate some gold particles located on mitochondria. (D) ^{wt}BH3/Bcl-w, but not ^{4E}BH3/Bcl-w, associates with the membrane fraction of healthy cells. The cells were lysed in buffer containing 0.025% digitonin before fractionation. (E) The COOH-terminal domain of Bcl-w is essential for targeting Bcl-w into the membrane fraction. Fractionation of cells transiently expressing ^{wt}BH3/Bcl-w (left) or ^{wt}BH3/Bcl-w ΔC29 (right). Equivalent fractions were resolved by SDS-PAGE and then blotted.

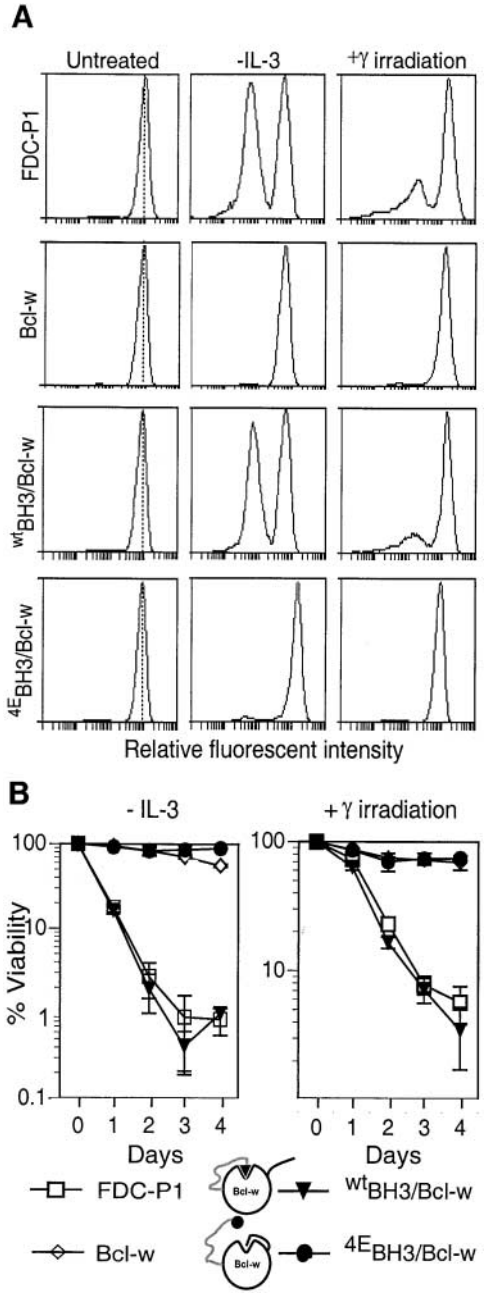

Figure 6. **Fusing wild-type BH3 to Bcl-w does not perturb mitochondrial transmembrane potential ($\Delta\Psi_m$) but inactivates its prosurvival activity.** (A) Flow cytometric analysis of untreated (left), IL-3–deprived (middle), or γ-irradiated (right) parental FDC-P1 cells or representative clones stably expressing Bcl-w, ^{wt}BH3/Bcl-w, or ^{4E}BH3/Bcl-w stained with the fluorochrome $DiOC_6(3)$ and PI. $DiOC_6(3)$ staining is shown after appropriate compensation for PI to exclude dead cells. (B) Parental FDC-P1 cells or representative clones of cell lines stably expressing Bcl-w, ^{wt}BH3/Bcl-w, or ^{4E}BH3/Bcl-w fusion proteins were deprived of their growth factor IL-3 or γ-irradiated (10 Gy). Cell viability was quantified daily by PI staining revealed by flow cytometric analyses. The data are means ± 1 SD of three or more experiments.

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-x_L (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-x_L structure does not exhibit a large conformational change when a BH3 peptide is bound (Sattler et al., 1997; Petros et al.,

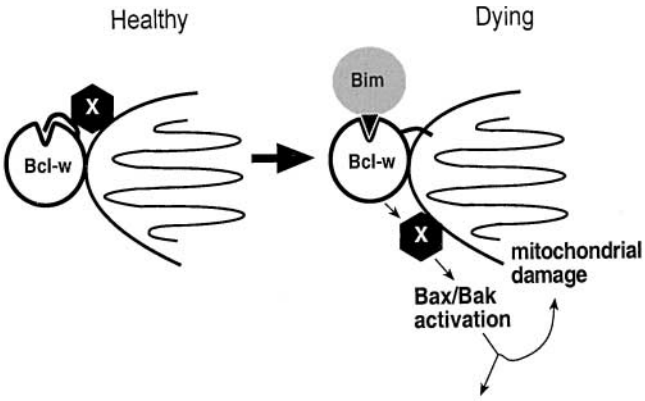

Figure 7. **Model for the inactivation of prosurvival Bcl-2–like proteins by the BH3-only proteins.** It is proposed that prosurvival family members, like Bcl-w, normally bind and sequester a membrane-bound effector protein (X) required for Bax/Bak activation. The engagement of a BH3-only protein (Bim 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-x_L 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-x_L (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-x_L 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 proapoptogenic 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 ^{wt}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, Bim_{EL}, 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 NH_2-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 $(Gly_4Ser_1)_6$ linker. Bacterial expression vectors to make recombinant Bcl-w or Bim_L 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 staurosprorine (Sigma-Aldrich); in HeLa cells with 50 Jm^{-2} UV irradiation, 1.0 μg/ml etoposide (VP-16; Della 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).

Cytofluorometric determination of mitochondrial transmembrane potential

To assess mitochondrial transmembrane potential ($\Delta\Psi_m$), healthy or dying cells were incubated for 15 min at 37°C in buffer containing 40 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$[3]; Molecular Probes) before adding 5 μg/ml 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 $DiOC_6$(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 $MgCl_2$, 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 RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 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 addition of 2.5× MS buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 7.5). Mitochondria were then isolated by differential centrifugation: 1,300 *g* for 5 min (removing nuclei and large membrane fragments), and the supernatant was centrifuged three times at 17,000 *g* for 15 min to isolate mitochondria. The final mitochondrial pellet was resuspended in 1× MS buffer. Other mitochondrial isolation procedures used include those described by Antonsson et al. (2001), Hagenbüchle and Wellauer (1992), and Wei et al., (2000). Alkali (0.1 M Na carbonate, pH 11.5) treatment of membrane pellets was performed as previously described (Janiak et al., 1994).

Cells for gel filtration were swollen in HMKEE buffer and lysed by nitrogen cavitation. Adding sucrose to 250 mM restored the osmolarity. After removing insoluble material by centrifugation, lysates from 5×10^7 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 [^{35}S]methionine/cysteine (NEN)-labeled cells were prepared and coimmunoprecipitation experiments were performed as previously described (Huang et al., 1997a,b, 1998; O'Connor et al., 1998; Moriishi 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; Moriishi et al., 1999; Puthalakath et al., 1999, 2001; Hausmann et al., 2000). The following antibodies were used: mouse monoclonal anti–FLAG M2 (Sigma-Aldrich), anti–voltage-dependent anion channel (VDAC)/Porin (Calbiochem), anti–cytochrome *c* (7H8.2C1; BD Biosciences), anti–Bcl-2-100 (Pezzella et al., 1990), anti–Bax 5B7 (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).

Recombinant proteins, peptides, and binding experiments

Recombinant Bcl-w and Bim_L 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 subcloned into the vector pQE-9 (QIAGEN) to allow NH_2-terminal tagging with the His_6 epitope. The mutations did not adversely affect the structure of Bcl-w or its binding properties (Hinds et al., 2003; not depicted) but prevented nonspecific dimerization and aggregation. This protein, referred to as Bcl-w ΔC10, 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: ^{wt}BH3, DLRPEIRIAQELRRIGDEFNETYTRR; ^{L94A}BH3, DLRPEIRIAQE**A**RRIGDEFNETYTRR; and ^{4E}BH3, DLRPEIR**E**AQE**E**RR**E**GDE**E**NETYTRR. The altered amino acids are in bold.

Analysis of protein interactions was performed on a Biacore 2000 biosensor (Biacore) as previously described (Lackmann et al., 1997; Hinds et al., 2003). Bim_LΔC27 or BH3 peptides were immobilized to Biacore CM 5 sensor chips using N-hydroxysuccinimide coupling. The binding kinetics were derived from the sensorgrams after subtraction of baseline responses by "global analysis" using the BIA Evaluation software (version 3.02; Biacore). 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_LΔC27 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 immunolabeling, sections were blocked in PBS containing 0.8% BSA/0.01% Tween 80 for 30 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 anti–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 Biotwin 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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