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Bim: a novel member of the Bcl-2 family that promotes apoptosis

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Certain members of the Bcl-2 family inhibit apoptosis while others facilitate this physiological process of cell death. An expression screen for proteins that bind to Bcl-2 yielded a small novel protein, denoted Bim, whose only similarity to any known protein is the short (nine amino acid) BH3 motif shared by most Bcl-2 homologues. Bim provokes apoptosis, and the BH3 region is required for Bcl-2 binding and for most of its cytotoxicity. Like Bcl-2, Bim possesses a hydrophobic C-terminus and localizes to intracytoplasmic membranes. Three Bim isoforms, probably generated by alternative splicing, all induce apoptosis, the shortest being the most potent. Wild-type Bcl-2 associates with Bim *in vivo* and modulates its death function, whereas Bcl-2 mutants that lack survival function do neither. Significantly, Bcl-x_L and Bcl-w, the two closest homologues of Bcl-2, also bind to Bim and inhibit its activity, but more distant viral homologues, adenovirus E1B19K and Epstein–Barr virus BHRF-1, can do neither. Hence, Bim appears to act as a 'death ligand' which can only neutralize certain members of the pro-survival Bcl-2 sub-family.

Keywords: apoptosis/Bcl-2/BH3/Bim/protein–protein interaction

Introduction

Apoptosis, the physiological process of cell death, is critical for modelling tissues and maintaining homeostasis in multicellular organisms (Kerr *et al.*, 1972; Jacobson *et al.*, 1997). The mechanism of this intrinsic suicide programme is under intense scrutiny. The executioners are a set of cysteine proteinases, termed caspases, that degrade critical cellular substrates (Nicholson and Thornberry, 1997). The regulatory machinery that governs the activation of the caspases is less well understood, but a central role is played by the Bcl-2 family (Cory, 1995; Korsmeyer, 1995; White, 1996; Jacobson, 1997; Kroemer, 1997; Reed, 1997). Bcl-2 itself was the first intracellular regulator of apoptosis to be identified (Vaux *et al.*, 1988), and high levels enhance cell survival under diverse cytotoxic conditions (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Other cellular homologues, such as Bcl-x_L (Boise *et al.*, 1993) and Bcl-w (Gibson *et al.*, 1996), also enhance cell survival, as do more distantly related viral homologues, such as the adenovirus E1B19K protein (White *et al.*, 1992) and Epstein–Barr virus (EBV) BHRF-1 (Henderson *et al.*, 1993). Remarkably, the family also includes members such as Bax (Oltvai *et al.*, 1993) and Bak (Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Kiefer *et al.*, 1995) which antagonize the activity of the pro-survival proteins and provoke apoptosis when expressed at high concentrations.

The ability of the pro-survival and anti-survival family members to form heterodimers makes it possible that each type might titrate the other, potentially accounting for their opposing actions. The relative concentrations of the opposing sub-family members would then determine whether the cell lives or dies (Oltvai *et al.*, 1993; Oltvai and Korsmeyer, 1994). Mutagenesis of Bcl-2 initially suggested that its ability to inhibit cell death required binding to a pro-apoptotic family member (Yin *et al.*, 1994), but Bcl-x_L mutants have been identified that do not bind Bax or Bak yet still block apoptosis (Cheng *et al.*, 1996). Thus, it remains unclear whether the ability to associate with other family members is central to regulating apoptosis.

The homology between members of the Bcl-2 family is greatest within four small segments, designated Bcl-2 homology (BH) regions (Yin *et al.*, 1994; Chittenden *et al.*, 1995a; Gibson *et al.*, 1996; Zha *et al.*, 1996), some of which contribute to the interactions between Bcl-2 family members. The N-terminal BH4 domain is restricted to some antagonists of apoptosis, while BH1, BH2 and BH3 can be found in both sub-families. Association of a pro-survival with a pro-apoptotic protein, such as Bcl-2 (or Bcl-x_L) with Bax (or Bak), requires the BH1 and BH2 domains of the former (Yin *et al.*, 1994; Hanada *et al.*, 1995; Sedlak *et al.*, 1995) and the BH3 domain of the latter (Chittenden *et al.*, 1995a; Simonian *et al.*, 1996; Zha *et al.*, 1996). In the tertiary structure of Bcl-x_L, the BH1, BH2 and BH3 domains form an elongated hydrophobic cleft (Muchmore *et al.*, 1996) along which the amphipathic helix formed by BH3 domains of the pro-apoptotic proteins can bind (Sattler *et al.*, 1997). The importance of the BH3 region for facilitating apoptosis has been underscored by the discovery of several BH3-containing proteins: Bik/Nbk (Boyd *et al.*, 1995; Han *et al.*, 1996), Bid (Wang *et al.*, 1996) and Hrk/DP5 (Imaizumi *et al.*, 1997; Inohara *et al.*, 1997), which are otherwise unrelated to the Bcl-2 family but are potent activators of apoptosis when overexpressed.

To search for additional regulators of apoptosis, we have screened a cDNA expression library with a Bcl-2 protein probe. This interaction screen has yielded a novel BH3-containing protein, which we have denoted Bim. Three Bim isoforms all promote apoptosis but differ in potency. Bim interacts with some but not all Bcl-2 family

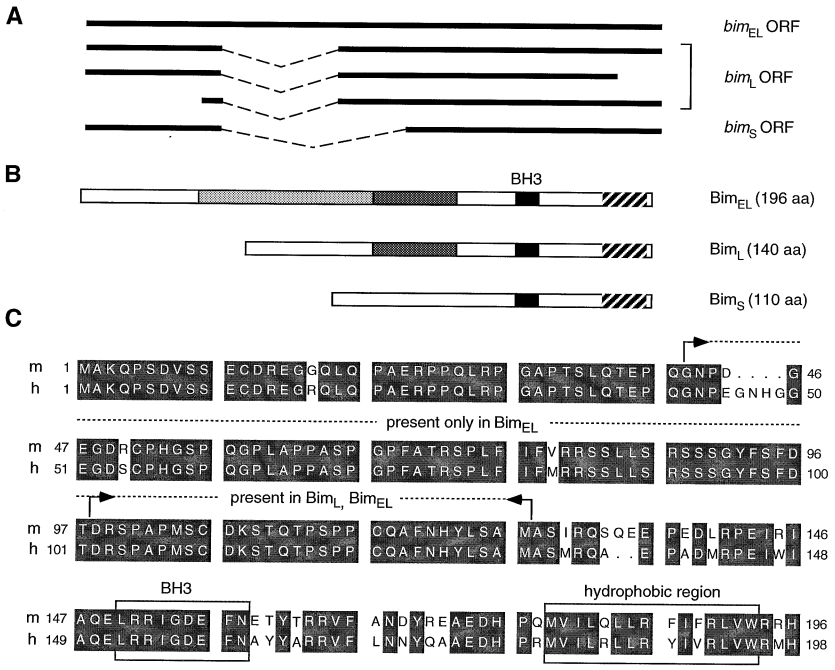

Fig. 1. Isolation of cDNAs encoding three isoforms of Bim: Bim_{EL}, Bim_L and Bim_S. (**A**) Open reading frames of five independent clones isolated by screening a cDNA expression library with recombinant Bcl-2 protein. Dotted lines indicate putative splices. (**B**) Relationship of the three Bim isoforms. The black box denotes the BH3 homology region and the hatched box the predicted hydrophobic region. Regions specific to the larger splice variants are shaded. (**C**) Sequence alignment of the mouse and human Bim_{EL} polypeptide sequences using the GCG 'BESTFIT' program, identical residues appear on a dark background. The BH3 homology region and the C-terminal hydrophobic region predicted by the Kyte–Doolittle algorithm are boxed. The arrows indicate residues present only in the longer isoforms. Since the nucleotide sequences of the mouse and human cDNAs diverged 5′ of the predicted initiating ATG and there are stop codons in all three reading frames upstream of the human open reading frame, that start codon is likely to be correct.

members that promote cell survival, and only those pro-survival relatives that bind to it can neutralize its cytotoxicity. Bim therefore appears to represent a death ligand with a specificity restricted to certain pro-survival members of the Bcl-2 family.

Results

Isolation of a novel gene encoding a Bcl-2-binding protein

In an attempt to identify novel proteins that bind to Bcl-2, we used recombinant human Bcl-2 protein, labelled with ^{32}P (Blanar and Rutter, 1992), to screen a bacteriophage λ cDNA expression library constructed from the $p53^{-/-}$ T lymphoma cell line KO52DA20 (Strasser *et al.*, 1994). A screen of 10^6 clones yielded five independent clones that encoded the same novel protein, which we named Bim, for Bcl-2 interacting mediator of cell death. Sequence analysis of the *bim* cDNAs revealed three variants of the coding region, apparently produced by alternative splicing, that we designated bim_{EL}, bim_L and bim_S (Figure 1A). RT–PCR on mRNA from KO52DA20 cells gave PCR products of the sizes expected for each of these transcripts, although bim_S was in low yield (data not shown). The predicted Bim_{EL}, Bim_L and Bim_S proteins comprise 196, 140 and 110 amino acid residues (Figure 1B). Hybridizing human fetal spleen and peripheral blood cDNA libraries with a mouse *bim* cDNA yielded human cDNAs encoding Bim_L and Bim_{EL}. Human Bim_{EL} is a protein of 198 residues, 89% identical to its mouse counterpart (Figure 1C), and human Bim_L (138 residues) is 85% identical to mouse Bim_L.

Bim has no substantial homology with any protein in current databases. However, scrutiny of its sequence (Figure 1C) revealed a stretch of nine amino acids corresponding to a BH3 homology region (Boyd *et al.*, 1995; Chittenden *et al.*, 1995a). The BH3 region of Bcl-x_L and a peptide corresponding to this region of Bak have each been shown to form part of an amphipathic α helix (Sattler *et al.*, 1997). Plotting the BH3 region of Bim as a helical wheel revealed that it was also amphipathic (data not shown). Apart from the BH3 region, the Bim sequence is unrelated to that of any other BH3-containing protein; it contains no other BH region, nor indeed any other known functional motif. The protein does, however, have a C-terminal hydrophobic region (Figure 1C). Such regions are found in most members of the Bcl-2 family and appear to be important for their localization to intracytoplasmic membranes (Kroemer, 1997).

Northern blot analysis showed that *bim* was expressed in a number of B- and T-lymphoid cell lines, although not in the myeloid line FDC-P1(Figure 2). A major transcript of 5.7 kb and minor transcripts of 3.8, 3.0 and 1.4 kb were detected. Neither the level nor relative abundance of these transcripts changed significantly in KO52DA20 cells induced to undergo apoptosis by treatment with dexamethasone (Figure 2, compare lanes 1 and 2, and lanes 3 and 4) or exposure to γ-radiation (compare lanes 3 and 5). In addition, overexpression of *bcl*-2 in several of the lines did not affect *bim* mRNA levels (Figure 2).

Bim localizes to cytoplasmic membranes

The presence of the C-terminal hydrophobic domain in Bim prompted us to investigate its subcellular localization.

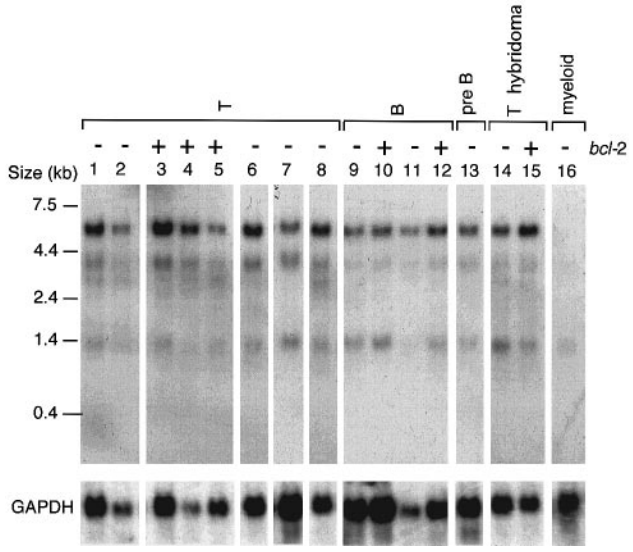

Fig. 2. Expression of *bim* RNA in haemopoietic cell lines. Northern blot analysis of poly(A)$^+$ RNA, using a mouse *bim* cDNA probe. The RNAs were derived from the following mouse lines: T-lymphomas KO52DA20 (lanes 1–5), WEHI 703 (lane 6), WEHI 707 (lane 7) and WEHI 7.1 (lane 8), B-lymphomas CH1 (lanes 9 and 10) and WEHI 231 (lanes 11 and 12), pre-B-lymphoma WEHI 415 (lane 13), T-hybridoma B6.2.16 BW2 (lanes 14 and 15) and myeloid progenitor FDC-P1 (lane 16). Those lines that harbour a *bcl*-2 expression vector or transgene are indicated by a plus sign. Certain RNAs were isolated from cells exposed to cytotoxic conditions: 1 μM dexamethasone (lanes 2 and 4), γ-irradiation (10 Gy) (lane 5). Lanes from a single autoradiograph have been electronically arranged in order.

L929 fibroblasts were transiently transfected with an expression vector encoding Bim_L tagged with an N-terminal EE epitope, and the permeabilized cells were stained with an anti-EE monoclonal antibody. Confocal microscopy revealed that Bim_L had a granular cytoplasmic distribution, consistent with an association with intracellular membranes (Figure 3A). When we also introduced the bim_L vector into L929 cells stably expressing human Bcl-2, the similarity of the anti-EE staining pattern of these cells (Figure 3C) to that of those expressing Bim_L alone (Figure 3A) demonstrated that high concentrations of Bcl-2 did not perturb the localization of Bim_L. The pattern of Bim_L staining was similar to that reported for Bcl-2 (Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994), and overlaying the images obtained from the same cells stained with anti-Bcl-2 (Figure 3B) and anti-EE (Figure 3C) antibodies showed that the two proteins co-localized (Figure 3D). This co-localization does not simply reflect binding of Bim to Bcl-2 since a mutant form of Bim lacking the BH3 region, and therefore incapable of binding to Bcl-2 (see below), localized similarly (Figure 3E).

Subcellular fractionation studies in FDC-P1 cells (see below) were consistent with these observations. Immunoblotting of fractionated lysates obtained from cell lines overexpressing human Bcl-2 and either EE-tagged Bim_L or EE-tagged Bim_L lacking the BH3 region showed that all three proteins were present in the nuclear and membrane fractions but not the cytosolic fraction (Figure 3F and data not shown). Together with the microscopic data, these results suggest that Bim localizes to intracytoplasmic membranes, independently of its association with Bcl-2.

Overexpression of Bim kills cells by a pathway requiring caspases

Other known 'BH3-only' proteins (Bik/Nbk, Bid and Hrk/DP5) provoke apoptosis when highly expressed (Boyd *et al.*, 1995; Han *et al.*, 1996; Wang *et al.*, 1996; Imaizumi *et al.*, 1997; Inohara *et al.*, 1997). We therefore tested whether Bim is cytotoxic by transiently transfecting 293T human embryonal kidney cells with a plasmid encoding EE-Bim_L. The viability of the transfected cells was determined by flow cytometric analysis of permeabilized cells stained with anti-EE antibody and the DNA-intercalating dye propidium iodide (PI). Whereas almost all untransfected cells or those transfected with an empty vector remained viable after 24 h, many of those expressing Bim (i.e. EE antibody-positive) contained sub-diploid DNA (Figure 4A). Indeed, by 3 days, 90% of the cells expressing Bim_L were dead (Figure 4B). The extent of cell death increased with the amount of *bim* DNA transfected (black bars, Figure 4C).

The cells expressing Bim appeared to die by apoptosis, as assessed by cell morphology and the generation of sub-diploid DNA (Figure 4A). As expected, the death process required activation of caspases, because co-expression of baculovirus p35, a competitive inhibitor of many types of caspases (Bump *et al.*, 1995), antagonized Bim-induced cell death, whereas an inactive (D87E) mutant p35 (Xue and Horvitz, 1995) did not (Figure 4C). Since CrmA, a potent inhibitor of caspases 1 and 8 (ICE and FLICE) (Orth *et al.*, 1996; Srinivasula *et al.*, 1996), was not effective (Figure 4C), these particular caspases do not appear to play an essential role.

Numerous failed attempts to generate lines that stably express Bim suggested that it is toxic to diverse cell types. Those repeatedly tested include haemopoietic lines (FDC-P1, CH1, Jurkat, SKW6 and B6.2.16BW2), fibroblastoid lines (Rat-1, NIH-3T3 and L929) and an epithelial line (293). The cells were electroporated with a vector encoding antibiotic resistance and either EE- or FLAG-tagged Bim_L, but no drug-selected line that expressed Bim emerged. A vector encoding untagged Bim also failed to generate viable clones. We quantified the cytotoxicity of Bim by colony assays on transfected L929 fibroblasts. Cells transfected with the EE-Bim_L vector yielded only one-fifth as many antibiotic-resistant colonies as those transfected with the control vector, and when six of the EE-Bim_L-transfected, drug-resistant colonies were expanded, only one contained any Bim and the level was very low (Table I and data not shown). Thus, Bim suppresses clonogenicity, and expression above a relatively low threshold is incompatible with prolonged cell viability.

Bim cytotoxicity can be abrogated by wild-type Bcl-2 but not inactive mutants

Co-expression experiments established that Bcl-2 could block cell death induced by Bim_L (Figure 4D). In 293T cells transiently transfected with both the *bcl*-2 and bim_L plasmids, relatively few cells died, even with a high concentration of bim_L DNA (compare the fourth bar in Figure 4C with the third in Figure 4D). The cytotoxicity of Bim, however, could not be countered by mutant forms of Bcl-2 rendered inactive by deletion of the BH4 homology region (ΔBH4) (Borner *et al.*, 1994; D.C.S.Huang, J.M.Adams and S.Cory, submitted), or by

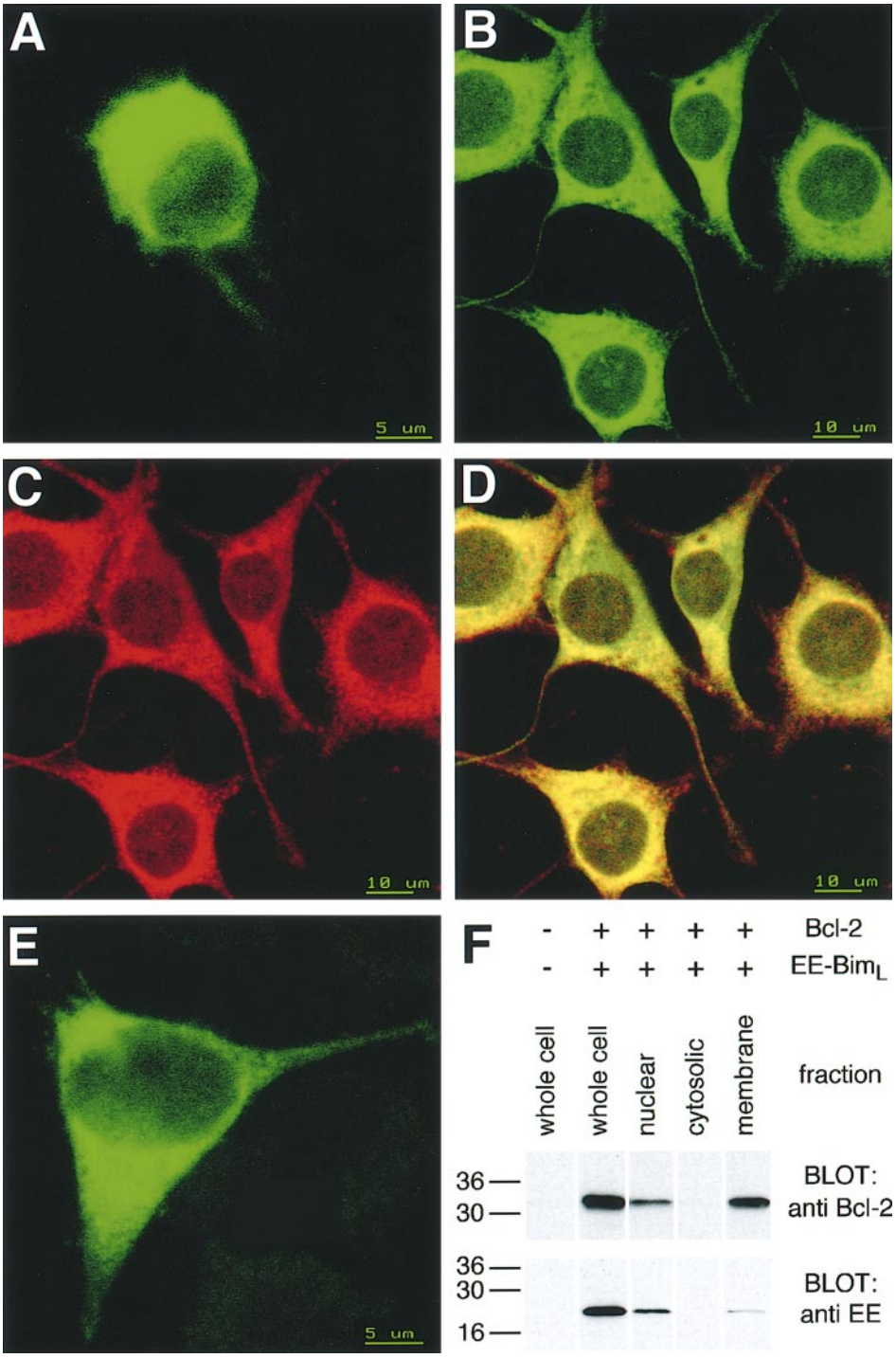

Fig. 3. Localization of Bim protein to intracellular membranes. (**A**) Single optical sections of L929 fibroblasts transiently co-transfected with EE-tagged Bim_L and baculovirus p35, then stained with the anti-EE antibody. (**B**) and (**C**) Confocal images of L929 cells stably co-expressing human Bcl-2 and EE-tagged Bim_L stained with anti-human Bcl-2 antibody (B) or anti-EE antibody (C). (**D**) Overlay of images (B) and (C). Co-localization of antibody staining is indicated by yellow fluorescence. (**E**) Confocal image of L929 cells transiently expressing human Bcl-2 and EE-tagged Bim ΔBH3 stained with anti-EE antibody. (**F**) Subcellular fractionation of lysates from FDC-P1 cells expressing EE-Bim_L and Bcl-2. Lysates from equivalent numbers of unfractionated cells (whole) and of subcellular fractions (nuclear, cytoplasmic or membrane), were resolved by SDS–PAGE and immunoblotted using the anti-human Bcl-2 (upper panels) or anti-EE (lower panels) monoclonal antibodies.

a point mutation in its BH1 (G145E) or BH2 (W188A) region (Yin *et al.*, 1994) (Figure 4D). Thus the ability to antagonize Bim-induced cell death required a functional Bcl-2 molecule.

High levels of Bcl-2 allowed stable expression of Bim_L. Indeed, when retrovirally infected L929 clones overexpressing Bcl-2 were transfected with the EE-Bim_L vector, the frequency of antibiotic-resistant colonies approached that obtained with the control vector, and four of six colonies analysed contained moderate to high levels of Bim (Table I and data not shown). Similarly, using FDC-P1 clones overexpressing wild-type Bcl-2 (but not

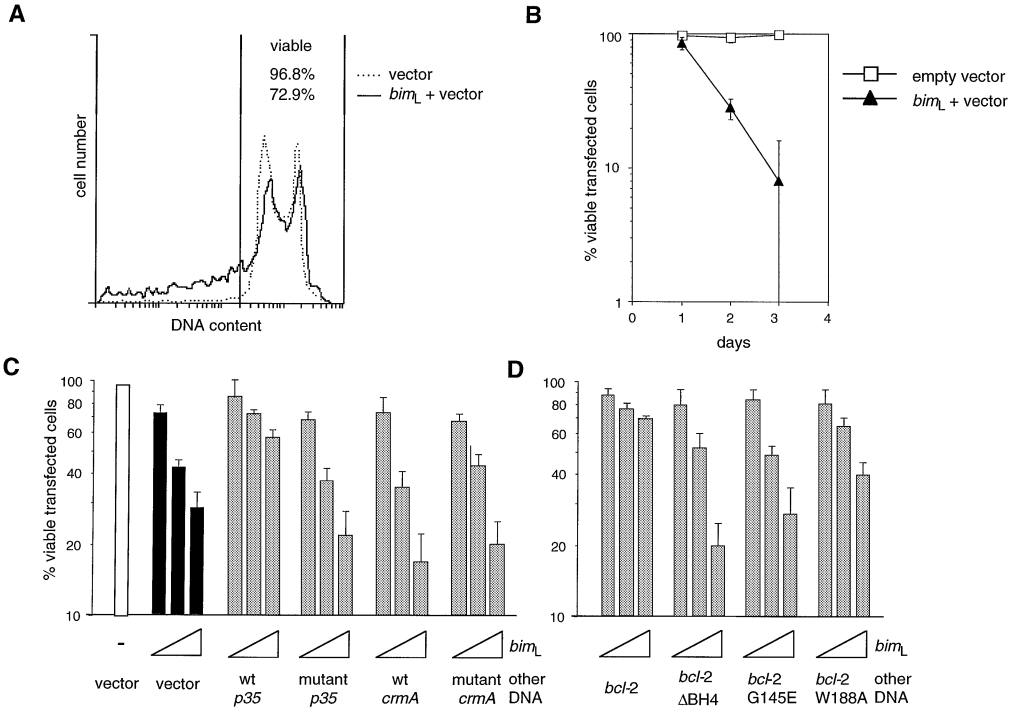

Fig. 4. Bim induces apoptosis which can be inhibited by the general caspase inhibitor p35 and Bcl-2 but not by CrmA. (**A**) Flow cytometric DNA analysis (see Materials and methods) of 293T cells transfected 24 h previously with EE-*bim*$_L$ plasmid (0.5 μg). (**B**) Kinetics of apoptosis elicited by EE-*bim*$_L$ plasmid (0.5 μg), assessed as in (A). (**C**) Cell viability 48 h after transfection with 0.1, 0.2 or 0.5 μg of EE-*bim*$_L$ plasmid alone (black bars) or together with 0.5 μg of wild-type or mutant *p35* or *crmA* plasmid (grey bars). (**D**) Cell viability 48 h after transfection with 0.1, 0.2 or 0.5 μg of EE-*bim*$_L$ plasmid together with 0.5 μg of the indicated wild-type or mutant *bcl*-2 plasmids. (C) and (D) show the percentage of viable Bim-expressing cells, determined by DNA FACS analysis, as in (A), and are the means ± SD of three or more independent experiments.

Table I. Bim inhibition of L929 colony growth is abrogated by Bcl-2

Cell line	Construct	Cloning efficiency	No. of antibiotic-resistant clones expressing Bim
L929	control	1.0	0/6
	bim$_L$	0.21 ± 0.04	1/6
	bim$_{EL}$	0.19 ± 0.05	1/6
	bim$_S$	0.11 ± 0.03	0/6
	bim$_L$ ΔBH3	0.69 ± 0.07	6/6
L929 *bcl*-2	control	1.0	0/6
	bim$_L$	0.64 ± 0.07	4/6

Parental L929 fibroblasts and a cloned derivative that stably expresses human Bcl-2 (L929 *bcl*-2) were co-transfected with a plasmid conferring antibiotic resistance with or without encoding various forms of Bim. After 48 h, antibiotic selection was added and the number of colonies were scored after 14–18 days. The data shown are means ± SD of at least four independent experiments.

mutant Bcl-2), we could readily establish sub-clones expressing intermediate to high levels of Bim$_L$ (Figure 5A). When grown in the presence of interleukin-3 (IL-3), all were indistinguishable in growth characteristics and morphology from the parental FDC-P1 cells or those bearing Bcl-2 alone. However, when deprived of IL-3 or irradiated, cells expressing Bcl-2 and a moderate or high level of Bim died more rapidly than those expressing Bcl-2 alone (Figure 5B). Since each clone had the same level of Bcl-2 (not shown), their sensitivity to apoptosis presumably reflects the ratio of the pro-death protein Bim to the pro-survival protein Bcl-2.

The three isoforms of Bim all interact with Bcl-2 in vivo but vary in cytotoxicity

We next explored whether all isoforms of Bim were equivalent. An FDC-P1 clone expressing human Bcl-2 was transfected with vectors expressing Bim$_{EL}$, Bim$_L$ or Bim$_S$, and antibiotic-resistant clones that expressed the same amount of each isoform were selected for further analysis (Figure 6A). To test for association with Bcl-2, immunoprecipitates prepared from cell lysates using a monoclonal antibody specific for human Bcl-2 were fractionated electrophoretically and blotted with anti-EE antibody. Each of the Bim isoforms clearly bound to Bcl-2 (Figure 6B). However, when the transfectants were deprived of IL-3 or subjected to γ-irradiation, it became evident that Bim$_S$ antagonized Bcl-2 more effectively than Bim$_L$, while Bim$_{EL}$ was the least potent (Figures 6C). In addition, Bim$_S$ suppressed L929 colony formation more effectively than Bim$_L$ or Bim$_{EL}$ (Table I). Thus, although all three Bim isoforms can bind to Bcl-2, they vary in cytotoxicity, Bim$_S$ being the most potent.

Bim binds to and antagonizes Bcl-x$_L$ and Bcl-w but not viral Bcl-2 homologues

Bim also associates with certain other anti-apoptotic Bcl-2 family members. Immunoprecipitation of lysates from ^{35}S-labelled 293T cells transiently co-transfected with the relevant vectors revealed binding to Bcl-x$_L$, although not to a mutant (mt 7) that lacks pro-survival activity, nor to two mutants (mt 1 and mt 15) that retain significant anti-apoptotic activity but cannot bind to Bax (Cheng *et al.*, 1996) (Figure 7A). Binding to Bcl-x$_L$ and Bcl-w was

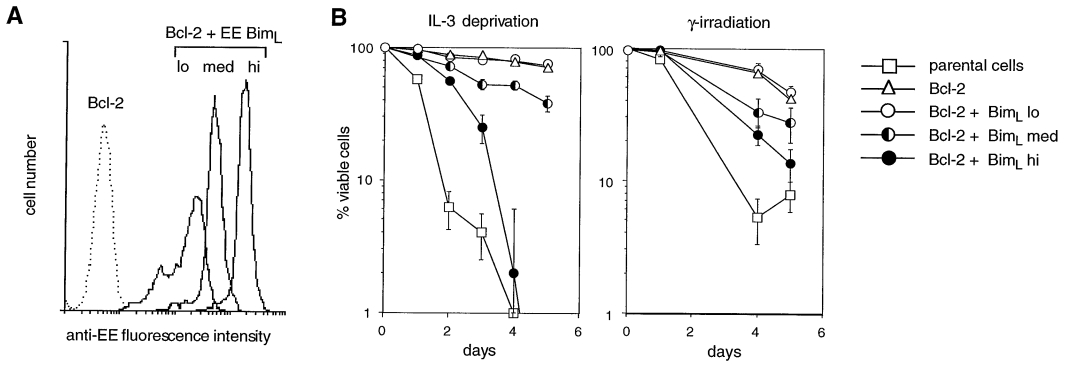

Fig. 5. Bim antagonizes the anti-apoptotic activity of Bcl-2 in a dose-dependent fashion. (**A**) Immunofluorescence staining of cloned FDC-P1 cell lines stably expressing Bcl-2 alone (dashed line) or co-expressing Bcl-2 and varying levels of EE-Bim$_L$ (solid lines). (**B**) Viability of these clones when cultured in the absence of IL-3 or after exposure to γ-irradiation (10 Gy). Cell viability was assessed by vital dye exclusion. Data shown are means ± SD of at least three experiments and are representative of results obtained with at least three independent lines of each genotype.

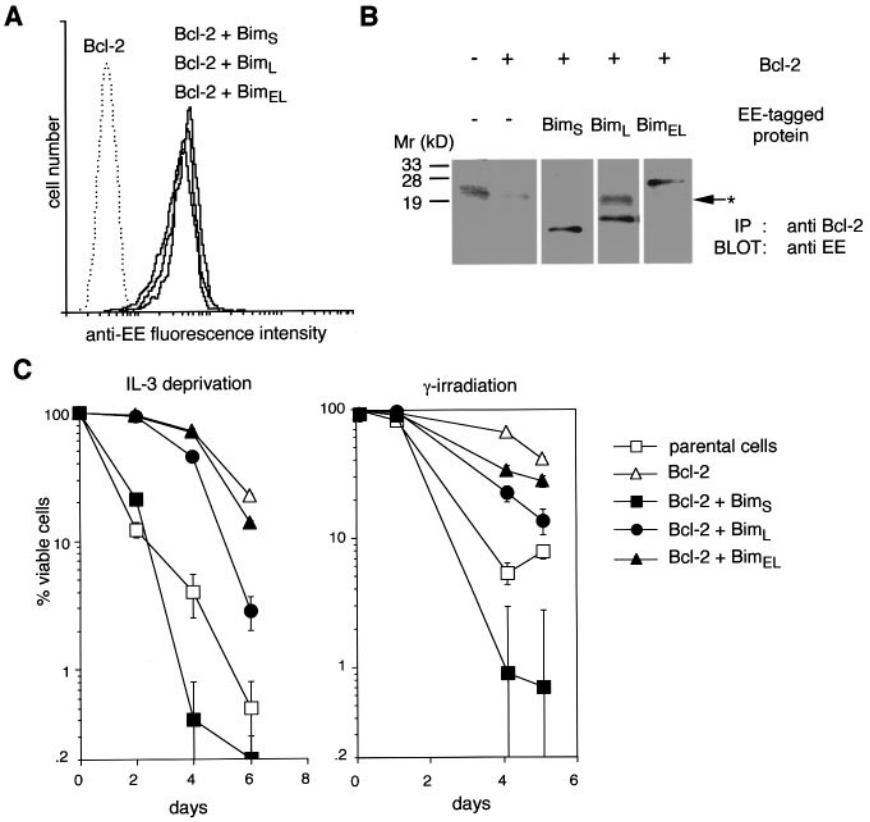

Fig. 6. Different killing activities of the three Bim isoforms. (**A**) Immunofluorescence staining, performed in parallel, of cloned FDC-P1 lines expressing Bcl-2 alone (dotted) or Bcl-2 plus EE-tagged Bim$_L$, Bim$_{EL}$ or Bim$_S$ (solid lines). (**B**) Association of EE-tagged Bim$_S$, Bim$_L$ and Bim$_{EL}$ with Bcl-2 demonstrated by anti-EE immunoblots of immunoprecipitates prepared with anti-human Bcl-2 monoclonal antibody from FDC-P1 cells expressing the indicated proteins. The 25 kDa non-specific band in the fourth lane, indicated by an asterisk, was not consistently seen. (**C**) Effect of Bim isoforms on viability of FDC-P1 cells expressing Bcl-2, after removal of growth factor or exposure to irradiation. All data were obtained on lines that expressed equivalent levels of the introduced proteins (see A). Those shown are means ± SD of at least three experiments representative of the results obtained with at least two independent lines of each genotype.

confirmed by immunoprecipitation followed by Western blot analysis (Figure 7B). Not all mediators of cell survival associate with Bim, however. Under the same conditions, Bim did not bind to either of two virally encoded Bcl-2 homologues, the adenovirus E1B19K protein and the EBV BHRF-1 protein (Figure 7B).

Functional tests mirrored the binding properties of the various Bcl-2 homologues. When transiently co-expressed with Bim in 293T cells, Bcl-x$_L$ and Bcl-w countered Bim toxicity as effectively as Bcl-2 (compare Figures 7C and D with Figure 4D). In contrast, little inhibition was observed with comparable levels of the mutant Bcl-x$_L$ proteins (Figure 7C) or the adenovirus E1B19K protein (Figure 7D). These data suggest that Bcl-2-like inhibitors of apoptosis must bind to Bim to inhibit its action.

Bim does not interact with any pro-apoptotic family member tested. No interaction of Bim with Bax could be observed under conditions in which Bim–Bcl-x$_L$ association was readily detectable (Figure 7E). In other experiments (not shown) in which we co-expressed FLAG-

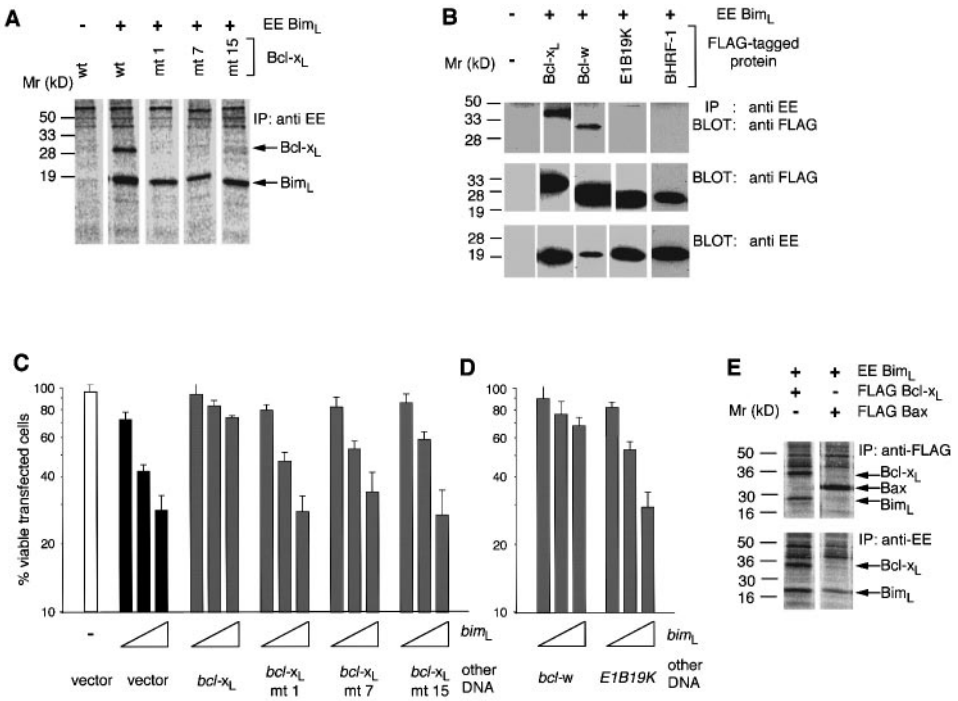

Fig. 7. Bim binds to and antagonizes Bcl-x_L or Bcl-w but not E1B19K. (**A**) Lysates of ^{35}S-labelled 293T cells transiently co-transfected with the plasmids encoding the indicated proteins were immunoprecipitated with anti-EE antibody, and the EE-Bim$_L$-containing complexes were fractionated by SDS–PAGE. (**B**) Lysates from parental 293T cells or 293T cells co-expressing EE-tagged Bim$_L$ and FLAG-tagged Bcl-x_L, Bcl-w, E1B19K or BHRF-1 were immunoblotted directly or after immunoprecipitation, as indicated. (**C** and **D**) 293T cells were transiently transfected with a vector control (unfilled bar) or with 0.1, 0.2 or 0.5 μg of EE-Bim$_L$ plasmid, either alone (black bars) or together with 0.5 μg of plasmids encoding wild-type or mutant Bcl-x_L (C), Bcl-w or E1B19K protein (D) (grey bars). The flow cytometric analysis was as described in the legend to Figure 4. Data shown are means ± SD of three or more independent experiments. (**E**) Lysates of ^{35}S-labelled 293T cells transiently co-transfected with plasmids encoding the indicated proteins were immunoprecipitated with either anti-FLAG or anti-EE antibody, and the resulting complexes were fractionated by SDS–PAGE.

tagged Bim with a range of other EE-tagged pro-apoptotic family members, we failed to detect any Bim homodimers or any interaction of Bim with Bak, Bad, Bik or Bid.

The BH3 region is essential for interaction of Bim with pro-survival Bcl-2 family members and for most of its ability to promote apoptosis

Since the BH3 region of several death-promoting proteins is essential for their activity (see Introduction), we tested a *bim*$_L$ mutant lacking the BH3 region. In transiently transfected 293T cells, the mutant protein (ΔBH3) was readily detected by immunoblotting but it did not bind to Bcl-x_L (Figure 8A), or to Bcl-2 or Bcl-w (data not shown).

In some biological assays, the ΔBH3 mutant of Bim appeared inert. In contrast to wild-type Bim, it was easy to establish lines expressing Bim$_L$ ΔBH3 from FDC-P1 (Figure 8B) or L929 cells (Table I and data not shown). Moreover, Bim$_L$ ΔBH3 did not impair the viability of the FDC-P1 cells in either the presence or absence of Bcl-2 (Figure 8C). Finally, 293T cells transiently transfected with Bim$_L$ ΔBH3 exhibited high viability (not shown). These results indicate that the BH3 region is critical for Bim to promote apoptosis and suggest that Bcl-2 blocks this activity of Bim by binding to that domain. Importantly, however, Bim$_L$ ΔBH3 was not completely inactive. In the L929 clonogenicity assay, it still markedly suppressed colony formation (Table I). Thus, regions of Bim other than BH3 may promote apoptosis or interfere with clonogenicity in another way, such as by blocking cell growth.

Discussion

Our screen for proteins that interact with Bcl-2 *in vitro* yielded the novel protein Bim. When overexpressed, Bim proved to be highly cytotoxic for diverse cell types. Indeed, Bim probably is a more potent inducer of cell death than any other known Bcl-2 family member, since stably overexpressing cell lines readily could be derived from the others (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Boyd *et al.*, 1995; Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Kiefer *et al.*, 1995; Yang *et al.*, 1995; Han *et al.*, 1996; Wang *et al.*, 1996; Inohara *et al.*, 1997; and our unpublished observations). Bim-induced cytotoxicity could be diminished by co-expression of Bcl-2, Bcl-x_L or Bcl-w, all of which bound to Bim *in vivo*. On the other hand, no protection was provided by mutant forms of Bcl-2 and Bcl-x_L that did not bind to Bim, including two Bcl-x_L mutants that provide substantial protection against other apoptotic stimuli (Cheng *et al.*, 1996). Thus, the pro-apoptotic action of Bim must, at least in part, reflect its ability to complex with the anti-apoptotic members of the family.

The small BH3 domain is the only region of Bim exhibiting homology with other members of the Bcl-2 family (Figure 9A). As this region was essential for most of its cytotoxic action (see below), Bim can be considered together with Bik/Nbk, Bid and Hrk/DP5 as a group of 'BH3-only' pro-apoptotic proteins. As demonstrated for Bak, the BH3 region of each may form an amphipathic helix that interacts with the elongated hydrophobic cleft

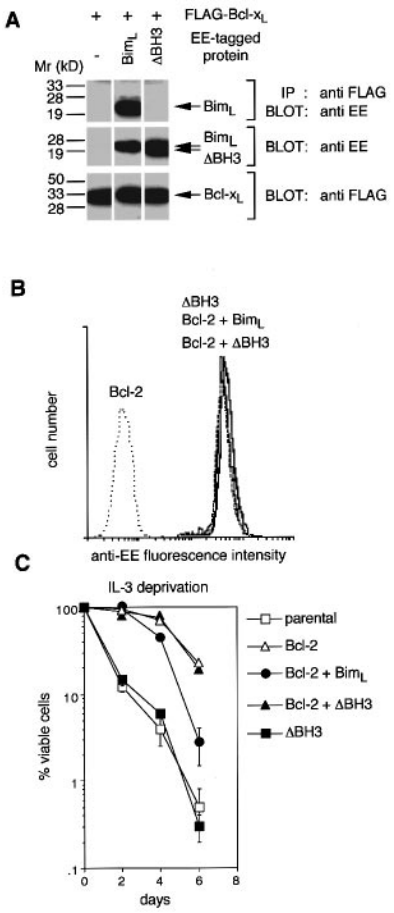

Fig. 8. The BH3 homology region of Bim is required for binding to and inhibiting pro-survival Bcl-2 homologues. (**A**) Immunoblot showing that Bcl-x_L associates with wild-type Bim_L but not with the ΔBH3 mutant. (**B**) Immunofluorescence staining of cloned FDC-P1 lines expressing Bcl-2 alone (dotted) or with EE-Bim_L or EE-Bim ΔBH3 (solid lines), and of EE-Bim ΔBH3 in the parental FDC-P1 cells (broken line). (**C**) Viability of FDC-P1 clones expressing the indicated proteins (see B) was assessed by vital dye exclusion. Data shown are means ± SD of at least three experiments and are representative of results obtained with at least three independent lines of each genotype.

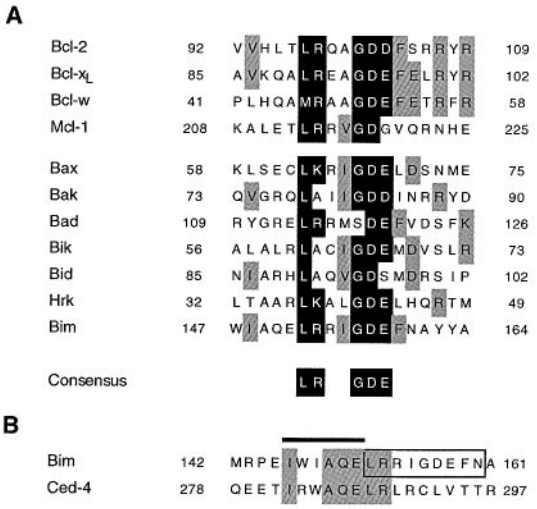

Fig. 9. BH3 homology regions in the Bcl-2 family. (**A**) Amino acid sequences of the human proteins were aligned with the modified method of Feng and Doolittle (1987) used by the GCG 'PILEUP' program. Residues that are identical or very similar (K and R, D and E, V and I, and M and L) in more than eight of the 11 proteins are shaded in dark grey, while less conserved residues (present in >5/11 proteins) are shaded in light grey. (**B**) Short stretch of amino acid homology between Bim and *C.elegans* CED-4; this region overlaps with the BH3 region of Bim, indicated by the box. The Bim residues deleted to destroy the CED-4 homology are shown by the solid line.

formed by the BH1, BH2 and BH3 regions of pro-survival molecules such as Bcl-x_L (Sattler *et al.*, 1997). Curiously, however, no residues within the BH3 region clearly distinguish the pro- and anti-apoptotic sets (Figure 9A). Presumably the BH3 regions of the latter are held in a different conformation.

It should be stressed that the four BH3-only proteins are otherwise unrelated and therefore are likely to be regulated by distinct upstream signals. They may also have somewhat different effector functions. For example, neither Bik (Han *et al.*, 1996) nor Bim (Figure 7E) binds to Bax, whereas Bid does—indeed mutagenesis of Bid linked its pro-apoptotic activity more closely to association with Bax than with Bcl-2 (Wang *et al.*, 1996). Another notable difference is that Bid lacks the hydrophobic C-terminal domain found in Bim, Bik and Hrk. As found for several other Bcl-2 family members, that domain probably features in the localization of Bim to intracellular membranes (Figure 3).

To date, Bim is the only BH3-only protein for which splice variants have been described (Figure 1). All three isoforms interacted *in vivo* with Bcl-2 and Bcl-x_L, but cell death and colony-forming assays revealed clear differences in their cytotoxicity. Since the short form was the most potent inducer of cell death (Figure 7) and suppressor of colony formation (Table I), the regions specific to Bim_L and Bim_{EL} may have a negative regulatory role. Whether the Bim isoforms are expressed differentially in different tissues or developmental stages or in response to particular apoptotic stimuli is currently under study.

Bim can interact with Bcl-2, Bcl-x_L, Bcl-w (Figures 4 and 7) and also with two more distantly related family members that promote cell survival, Mcl-1 (Kozopas *et al.*, 1993) and A1 (Lin *et al.*, 1993) (data not shown). It did not, however, interact with two viral homologues of Bcl-2, adenovirus E1B19K and EBV BHRF-1. In this regard, Bim differs significantly from at least one other BH3-only protein, since Bik/Nbk was isolated by virtue of its binding to E1B19K (Boyd *et al.*, 1995; Han *et al.*, 1996). Unlike Bcl-2, Bcl-x_L and Bcl-w, E1B19K was also an ineffectual antagonist of Bim-induced cell death (Figure 7D). These findings point towards some specificity of interaction between pro- and anti-survival members of the family. Such preferential pairings, where each partner could be regulated independently, may fine-tune the response to multiple survival or death signals. Consistent with this notion, the expression of various pro-survival mediators is induced by distinct signals: Mcl-1 and A1 by myeloid growth factors (Kozopas *et al.*, 1993; Lin *et al.*, 1993), Bcl-2 by IL-7 (von Freeden-Jeffry *et al.*, 1997) and Bcl-x_L by activation of co-receptors such as CD28 (Boise *et al.*, 1995). Regulation of the pro-apoptotic family members is less well studied, but Bax expression can be induced by genotoxic stress via the tumour suppressor p53 (Miyashita *et al.*, 1994). While the levels of Bim mRNA did not change in a T-cell line induced to undergo

apoptosis by two stimuli (Figure 2), it may be induced in other cell types or with different cytotoxic insults.

How might binding of a BH3-containing protein to Bcl-2 trigger apoptosis? In *Caenorhabditis elegans*, the Bcl-2 homologue CED-9 is thought to act by restraining the activity of CED-4, which is required for activation of the caspase CED-3 (Hengartner and Horvitz, 1994). Recent evidence that CED-9, CED-4 and CED-3 associate directly (Chinnaiyan *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997), and that Bcl-x_L can bind to CED-4, has raised the possibility that the pro-survival Bcl-2 family members function by sequestering CED-4-like molecules, precluding activation of the caspases (Chinnaiyan *et al.*, 1997; Seshagiri and Miller, 1997; Wu *et al.*, 1997). If this is so, the pro-apoptotic Bcl-2 family members may function by binding to pro-survival relatives and displacing CED-4-like proteins. The recent identification of the first mammalian CED-4 homologue, Apaf-1 (Zou *et al.*, 1997), is expected to facilitate tests of such models. It may be relevant that Bim exhibits a small but striking region of similarity to CED-4 (Figure 9B); in a nine amino acid region partially overlapping BH3, seven (in human Bim) or eight residues (in mouse Bim) are identical to CED-4. A loss-of-function missense mutation in this region of CED-4 (Yuan and Horvitz, 1992) suggested that it might be important for CED-4 function. However, a deletion in Bim that destroyed the homology (Figure 9B) did not interfere with Bim function (not shown). Moreover, the region is not conserved in Apaf-1 (Zou *et al.*, 1997), so the significance of this similarity is unclear.

An alternative hypothesis accounting for the apoptotic activity of BH3-containing proteins is that they directly induce cell death unless complexed by their pro-survival relatives. The inability of E1B19K or Bcl-x_L mutants mt 7 or mt 15 to protect against Bim-induced death is consistent with this hypothesis. Furthermore, genetic experiments have shown that Bax can induce cell death in the absence of Bcl-x_L or Bcl-2 (Simonian *et al.*, 1996; Knudson and Korsmeyer, 1997) and seemingly in the absence of caspase activation (Xiang *et al.*, 1996). The ability of a mutant Bim lacking the BH3 region to suppress colony formation (Table I), albeit not as potently as the wild-type protein, may indicate that Bim retains some pro-apoptotic activity independent of its interaction with other Bcl-2 family members. We currently are testing this hypothesis.

Materials and methods

Expression library screening and isolation of mouse and human bim cDNAs

Polyadenylated RNA prepared from p53$^{-/-}$ KO52DA20 T-lymphoma (Strasser *et al.*, 1994) cells subjected to γ-irradiation (10 Gy) was reverse-transcribed, using a combination of oligo(dT) and random oligonucleotide primers, and ligated to *Eco*RI adaptors using standard procedures. The cDNA was then ligated with *Eco*RI–*Xho*I-digested λ ZapExpress (Stratagene) arms and packaged *in vitro* according to the supplier's instructions. The resulting expression library was screened using radiolabelled Bcl-2 lacking the hydrophobic membrane localization region. To prepare this probe, cDNA encoding amino acids 1–210 of human Bcl-2 was subcloned into the vector pARΔR1 (Blanar and Rutter, 1992), and recombinant protein (FLAG-HMK-Bcl-2ΔC30) produced in isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *Escherichia coli* BL21pLysS (DE3) cells (Novagen) was purified on anti-FLAG M2 affinity gel (IBI Kodak) and then kinased *in vitro* using bovine heart muscle kinase (Sigma) and [γ-^{32}P]ATP (Amersham) (Blanar and Rutter, 1992). Approximately 10^6 plaques were screened with ~10^7 c.p.m. of the radiolabelled probe using the protocol of Blanar and Rutter (1992). To reduce non-specific background, lysates from induced parental BL21pLysS (DE3) cells and excess unlabelled ATP were included with the probe. Plaques that were positive on duplicate lifts were picked for two rounds of further screening. Positive clones were excised *in vivo* by co-infection with filamentous ExAssist (Stratagene) helper phage and sequenced by automated sequencing (ABI Perkin Elmer).

The human *bim* cDNA clone was isolated by screening human fetal spleen (Stratagene) and peripheral blood leukocyte (Clontech) λ cDNA libraries with an ~800 bp mouse *bim* cDNA probe, using standard techniques. The cDNAs were fully sequenced, analyzed using GCG or DNASTAR software and compared with sequences in the GenBank (including dbEST) and EMBL databases using the BLAST algorithm (Altschul *et al.*, 1990).

Expression constructs and site-directed mutagenesis

cDNAs were cloned into the expression vectors pEF PGKpuro (Huang *et al.*, 1997a) or pEF PGKhygro (Huang *et al.*, 1997a), or derivatives of these incorporating N-terminal FLAG (DYKDDDDK) (Hopp *et al.*, 1988) or EE (EYMPME) (Grussenmeyer *et al.*, 1985) epitope tags. The *bim*ΔBH3 mutation was generated by deleting the DNA encoding amino acids 94–100 (LRRIGDE) and replacing this with DNA corresponding to a *Hin*dIII site (encoding AL). Mutations in *bcl*-2 (ΔBH4, G145E, W188A) (O'Reilly *et al.*, 1996) were generated by PCR via splice overlap extension (Horton *et al.*, 1993) using the proof-reading *Pfu* DNA polymerase (Stratagene) (details of oligonucleotides used will be supplied on request). The sequences of derived clones were verified by automated sequencing.

Cell culture and transfection

Cell lines used were: mouse IL-3-dependent promyelocytic line FDC-P1, mouse T-hybridoma B6.2.16BW2, mouse B-lymphoma lines CH1 and WEHI 231, mouse pre-B lymphoma line WEHI 415 (derived from a tumour which arose in an Eμ-*myc* transgenic mouse), human B-lymphoblastoid line SKW6, human T-lymphoma line Jurkat, mouse T-lymphoma lines WEHI 703 and WEHI 707 (both derived from tumours which arose in Eμ-N-Ras transgenic mice) and WEHI 7.1, rat fibroblastoid line Rat-1, mouse fibroblastoid line NIH-3T3, mouse fibroblastoid line L929 subline LM(-TK), human embryonal kidney cell line 293 (ATCC CRL-1573) and SV40-transformed 293 cells, 293T (see Lithgow *et al.*, 1994; Strasser *et al.*, 1994, 1995; Huang *et al.*, 1997a). The procedures for culture and stable transfection are described elsewhere (Strasser *et al.*, 1995; Huang *et al.*, 1997a,b). Drug-resistant transfectants were cloned using the cell deposition unit of a FACStarPlus (Becton Dickinson) and clones expressing high levels of the protein of interest were identified by immunofluorescence staining of fixed and permeabilized cells followed by flow cytometric analysis.

Cell death assays

Cytokine deprivation, exposure to ionizing radiation and treatment with staurosporine (Sigma) were the principal cell death assays used to assess the sensitivity of FDC-P1 cells stably transfected with the various expression vectors. Cells were cultured in medium lacking cytokine or (in complete medium) after exposure to 10 Gy γ-radiation (provided by a ^{60}Co source at 3 Gy/min) and their viability determined over several days by vital dye (0.4% eosin) exclusion, as assessed by visual inspection in a haemocytometer, or by flow cytometric analysis of cells that excluded propidium iodide (5 μg/ml, Sigma).

Cell death assays in 293T cells were performed after transient transfection of ~5×10^5 cells using 6 μl of Lipofectamine® (Gibco BRL) and a total of 1 μg of DNA in 2 ml of medium in 6 cm dishes; for co-transfections, *bim* plasmid (0.1, 0.2 and 0.5 μg) was co-transfected with 0.5 μg of the other recombinant (e.g. *bcl*-2) plasmid plus 0.4 or 0.3 μg or none of the empty vector. At 48 h after transfection, the cells were harvested, fixed for 5 min in 80% methanol, permeabilized with 0.3% saponin (which was reduced to 0.03% in all the subsequent steps) and stained with 1 μg/ml anti-EE monoclonal antibody (BabCO), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 μg/ml, Southern Biotechnology) as the secondary agent and by 69 μM propidium iodide in 38 mM sodium citrate pH 7.4 containing 5 μg/ml RNase A (Huang *et al.*, 1997b). Analysis was performed on a FACScan II (Becton Dickinson), the proportion of dead cells being taken as the proportion of EE-positive cells with less than 2C DNA content (Nicoletti *et al.*, 1991).

L929 fibroblast colony assays were performed in triplicate by scoring

the numbers of colonies in 10 cm dishes grown for 14–18 days with appropriate antibiotic selection. These cells previously had been split (1:3) from ~10^6 cells which had been transfected in 6 cm dishes 2 days earlier with 1 μg total DNA and 12 μl of Lipofectamine®.

Immunofluorescence, subcellular fractionation, immunoprecipitation and immunoblotting

Immunofluorescence staining of cytoplasmic proteins with the monoclonal antibodies Bcl-2-100 [mouse anti-human Bcl-2 (Pezzella *et al.*, 1990)] or mouse anti-EE (BabCO) followed by FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) was performed as previously described (Strasser *et al.*, 1995; Huang *et al.*, 1997a). Cells were analysed using a FACScan (Becton Dickinson) after exclusion of dead cells on the basis of their forward and side scatter characteristics.

To investigate the subcellular localization of EE-tagged Bim_L, L929 fibroblasts transfected by lipofection (see above) were grown in chamber slides (Erie Scientific Company, New Hampshire), fixed in 4% paraformaldehyde for 10 min at room temperature and the slides were then allowed to dry and stored at –20°C. Prior to analysis by confocal microscopy, the cells were rehydrated and then permeabilized for 15 min at room temperature in 0.5% Triton-X 100 in phosphate-buffered saline (PBS). EE-Bim_L was detected by incubating the cells with anti-EE monoclonal antibody for 30 min, washing several times in PBS containing 1% fetal calf serum and 0.05% Tween-20, and then incubating for 30 min with either FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) or lissamine–rhodamine-conjugated goat anti-mouse IgG (Jackson Immunoresearch); all steps were performed at room temperature. Human Bcl-2 was detected similarly, using hamster anti-human Bcl-2 (6C8) (Veis *et al.*, 1993) followed by FITC-conjugated mouse anti-hamster IgG (Pharmingen). Untransfected cells served as negative controls. Samples were analyzed with a Leica confocal laser scanning microscope using SCANware software (Leica Lasertechnik, Heidelberg, Germany).

For subcellular fractionation, lysates were made with a Dounce homogenizer (Hsu *et al.*, 1997) from ~10^6 cells in lysis buffer: 10 mM Tris–HCl pH 7.4, 0.5 μg/ml Pefabloc, 1 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 5 mM NaF and 2 mM Na_3VO_4 (Sigma or Boehringer Mannheim). The lysates were centrifuged at 900 *g* for 10 min to obtain the nuclear pellet and the supernatant centrifuged at 130 000 *g* for 60 min to obtain the membrane fraction.

To test for protein–protein interactions *in vivo*, co-immunoprecipitation followed by immunoblotting was performed on stably transfected FDC-P1 cells or transiently transfected 293T cells as described previously (Huang *et al.*, 1997a,b). Briefly, lysates prepared from 10^5–10^6 cells were incubated with ~5 μg of antibody (anti-human Bcl-2, anti-FLAG M2 (IBI Kodak) or anti-EE monoclonal antibody), followed by protein G–Sepharose (Pharmacia), and then pelleted, washed, fractionated by SDS–PAGE and transferred to nitrocellulose membranes by electroblotting. The filters were incubated with mouse anti-human Bcl-2, anti-FLAG or anti-EE antibodies followed by affinity-purified rabbit anti-mouse IgG (Jackson Immunoresearch). Bound antibodies were detected with ^{125}I-labelled staphylococcal protein A, except in the subcellular fractionation experiments, where bound antibodies were revealed with horseradish peroxidase-conjugated sheep anti-mouse Ig (Silenus) and enhanced chemiluminescence (Amersham). In some experiments, the cells were metabolically labelled with 100–200 μCi/ml of [^{35}S]methionine (NEG-072 from NEN), and equivalent trichloroacetic acid-precipitable counts (5×10^7 c.p.m.) were used for each immunoprecipitation.

Accession numbers

The sequences described here have been deposited with GenBank, the accession numbers are: human bim_{EL} (AF032457), human bim_L (AF032458), mouse bim_{EL} (AF032459), mouse bim_L (AF032460) and mouse bim_S (AF032461).

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