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Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members

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Abstract

Certain Bcl-2 family members promote cell survival, whereas others promote apoptosis. To explore further how heterodimerization of opposing members affects survival activity, we have compared the abilities of the anti-apoptotic Bcl-w and A1 to bind to the pro-apoptotic Bax, Bak, Bad and Bik and to protect cells from their cytotoxic action. Bcl-w co-immunoprecipitated from cell lysates with Bax, Bak, Bad and Bik, but A1 bound only Bak and Bik. Mutation of A1 at a highly conserved glycine within the BH1 domain prevented binding, but the comparable Bcl-w mutant still bound Bak, Bad and Bik, indicating that the glycine is not essential for all heterodimerization. Bcl-w and A1 protected against apoptosis induced by over-expression of Bax or Bad but not that induced by Bak or Bik. With several gene pairs, binding and protection were discordant. The results may reflect critical threshold affinities but also suggest that certain pro-apoptotic proteins may also contribute to apoptosis by a mechanism independent of binding pro-survival proteins.

Keywords: apoptosis; Bcl-2 family; heterodimerization; mutagenesis

Abbreviations: wt, wild-type; IL-3, interleukin-3; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; FCS, fetal calf serum; DME, Dulbecco's Modified Eagle's; FACS, fluorescence-activated cell sorter; MT-PBS, mouse tonicity phosphate buffered saline; RIPA, radioimmunoprecipitation assay; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate

Introduction

Because apoptosis is essential for normal embryonic development, tissue homeostasis and cellular defence mechanisms and contributes to many pathological states,[1,2] this evolutionarily conserved cell death program is of considerable interest. A central regulatory role is played by the Bcl-2 family of cytoplasmic proteins (reviewed by[2,3]), some of which favor cell survival and some cell death. Their biochemical functions remain uncertain but ultimately they must control the activation of the special proteases (caspases) which dismantle the cell by cleavage of many protein targets.[4] As reviewed elsewhere,[3,5] the pro-survival family members act directly on adaptors such as Apaf-1, which is required (along with cytochrome c) for activation of caspase-9,[6,7] and/or prevent caspase activation indirectly by preserving the integrity of mitochondria, thereby preventing release of pro-apoptotic co-factors such as cytochrome c.

Mammalian Bcl-2 family members that promote survival include Bcl-2 itself, its close relatives Bcl-x_L[8] and Bcl-w,[9] and the more distant A1 (also known as Bfl-1 or GRS)[10–12] and Mcl-1.[13] Three conserved domains (BH1, BH2 and BH3) in these proteins are also found in one pro-apoptotic sub-family, comprising Bax, Bak and Bok.[14–18] Only the BH3 domain, however, is present in the other, much more divergent, pro-apoptotic group, which includes Bik/Nbk, Bad, Bid, Hrk/DP5, Bim, Bik and Blk.[19–25]

Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate one another's function, suggesting that their relative concentration may set the threshold for activation of the suicide program.[14,26] Structural studies on Bcl-x_L revealed the basis for heterodimerization: Its BH1, BH2 and BH3 regions fold into a hydrophobic groove, to which a BH3 amphipathic helix can bind.[27,28] But how critical is heterodimerization within the Bcl-2 family to the control of cell survival? A key role is favored by numerous mutagenesis studies demonstrating that the death-enhancing activity in both the Bax and BH3 sub-families largely depends upon their BH3 domain.[29,30] Moreover, replacement of a highly conserved glycine residue within BH1 of Bcl-2[31] or Bcl-x_L[32] prevents binding to Bax and abrogates survival function. On the other hand, other BH1 mutants that cannot bind to Bax or certain other pro-apoptotic proteins retain significant anti-apoptotic activity.[33,34] There is also evidence (reviewed in[3]) that members of the Bax group can kill in a manner independent of the pro-survival molecules.

Thus, whether heterodimerization is essential for survival function, particularly in countering over-expressed pro-apoptotic proteins, is not yet clear. We have explored this issue further using Bcl-w and A1, two of the less well-characterized pro-survival Bcl-2 family members. Although Bcl-w is very similar to Bcl-2 and Bcl-x_L, it lacks most of the poorly conserved region between BH4 and BH3;[9] this region forms a flexible loop in Bcl-x_L[27] and is implicated in its negative regulation.[35,36] Bcl-w is expressed in a wide range of tissues[9] and is essential for spermatogenesis.[37,38] The gene for the more divergent A1, which also lacks the unstructured loop region, is rapidly induced in myeloid cells in response to cytokines that induce differentiation[10,39] and

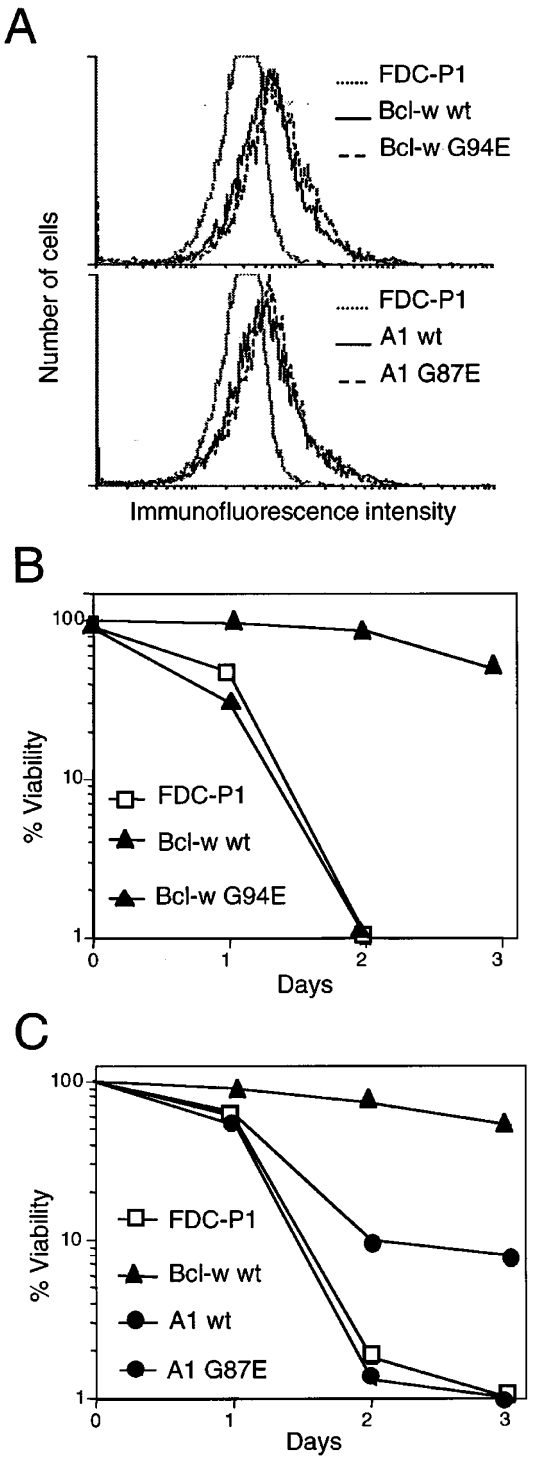

Figure 1 A point mutation in the BH1 domain of Bcl-w and A1 destroys ability to protect cells against cytokine withdrawal. (**A**) Expression of FLAG-tagged Bcl-w (clone D3B5) and A1 (clone 2E12) proteins and BH1 mutant proteins Bcl-w G94E (clone 6.22.1) and A1 G87E (clone 1C7). (**B, C**) Survival assay for these Bcl-w- and A1-expressing clones, respectively, after withdrawal of IL-3 (see Materials and Methods). Results are representative of two experiments and equivalent results were obtained with independent clones

in endothelial cells by phorbol ester, TNF-α and IL-1β.[40] It has also been implicated in B lymphocyte selection.[41]

We describe here studies on the heterodimerization potential of Bcl-w and A1 and the consequences of mutating the conserved glycine residue in BH1 of both. We have compared the ability of these wild-type and mutant proteins to inhibit apoptotis of cells over-expressing four death-promoting family members: two representatives of the Bax sub-family, Bax and Bak, and two of the BH3-only domain sub-family, Bad and Bik. The results suggest that the ability of pro-survival proteins to counter apoptotis elicited by over-expression of a pro-apoptotic protein cannot be assessed simply by their ability to form heterodimers with the opposing family members.

Results

A point mutation in the BH1 domain of Bcl-w and A1 abolishes their ability to inhibit apoptosis induced by cytokine deprivation

BH1 is the region most strongly conserved between Bcl-w, A1 and Bcl-2, and the glycine residue within an NWGR motif is conserved in all members of the Bcl-2 and Bax sub-families isolated to date, including CED-9, the Bcl-2 homolog of *C. elegans*. Conversion of this glycine to glutamate destroys survival function in Bcl-2[31] and Bcl-x_L,[32] although, paradoxically, the equivalent change in CED-9 is an activating mutation.[42] To examine the consequences of this mutation in Bcl-w and A1, the equivalent glycine codons were converted to glutamate, generating the Bcl-w G94E and A1 G87E (see Materials and Methods). To compare the biological activity of the wild-type (wt) and mutant proteins, stable transfectants were made in the interleukin-3 (IL-3)-dependent promyelocytic cell line FDC-P1,[43] and clones expressing comparable levels of the FLAG-tagged proteins were selected by anti-FLAG staining and fluorescence-activated cell sorter (FACS) analysis (e.g. Figure 1A).

To test survival activity, the lines were deprived of IL-3 and their viability assessed daily by staining with propidium iodide followed by flow cytometry. Nearly all parental FDC-P1 cells died within 48 h, whereas those expressing wt Bcl-w retained high viability for at least 3 days (Figure 1B), as reported previously.[9] A1 also enhanced the survival of FDC-P1 cells, as reported for the myeloid cell line 32D c13,[39] although A1 appeared somewhat less effective than Bcl-w (Figure 1C). In contrast, both of the mutant proteins were completely inactive in this assay (Figure 1B and C). Thus, mutation of the conserved glycine residue in the BH1 domain renders Bcl-w and A1 unable to inhibit apoptosis provoked by cytokine deprivation.

Interaction of wild-type and mutant Bcl-w and A1 proteins with death-enhancer proteins

We next determined the ability of the wt and mutant Bcl-w and A1 proteins to interact with Bax, Bak, Bad and Bik. COS cells were co-transfected with vectors encoding the FLAG-tagged Bcl-w or A1 proteins and each of the EE-tagged pro-apoptosis proteins. After 36 h, the cells were

lysed and the cytoplasmic fraction incubated with anti-FLAG antibody conjugated to agarose beads. Bound proteins were then eluted with FLAG peptide, fractionated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to filters. EE-tagged proteins that bound to the beads by virtue of their association with the FLAG-tagged proteins were revealed by incubating the filter with anti-EE antibody.

Figure 2 shows typical results obtained for Bcl-w and Bcl-w G94E. The wt protein bound to all four pro-apoptotic proteins (Figure 2A). The mutant protein was unable to bind to Bax, as previously observed for the Bcl-2 mutant,[31] but, surprisingly, still bound to Bad, Bak and Bik (Figure 2A). Thus, this mutation does not abolish the ability of Bcl-w to heterodimerize with all pro-death family members.

The binding of A1 was more selective (Figure 3). Wild-type A1 interacted strongly with Bak and moderately with Bik, but not detectably with either Bax or Bad (Figure 3A). Mutation of the conserved glycine residue destroyed its ability to bind to Bak and Bik. Immunoprecipitation of the pro-survival proteins (Figure 3B) and expression of all the pro-apoptotic ones (Figure 3C) was confirmed. The comparable levels of expression of all the pro-apoptotic ones (Figure 3C) was confirmed. The comparable levels of expression achieved by transfecting the various pairs of plasmid DNAs and the positive interactions detected exlude the possibility that the negative results were artefactual.

Ability of pro-survival proteins to inhibit apoptosis induced by death enhancer proteins

We next investigated the capacity of the wt and mutant pro-survival proteins to inhibit apoptosis provoked by over-expression of the death enhancer proteins. 293T cells were transiently transfected with vectors encoding EE-tagged Bax. Bak, Bad or Bik, together with either an empty vector or one encoding Bcl-w, A1, Bcl-2 or Bcl-x_L, using lipofectamine treatment. After 36 h, a flow cytometric assay was used to identify the transfected cells expressing the EE-tagged protein, and to determine the proportion that contained degraded DNA in the presence or absence of each of the pro-survival proteins. Figure 4 presents a typical experiment

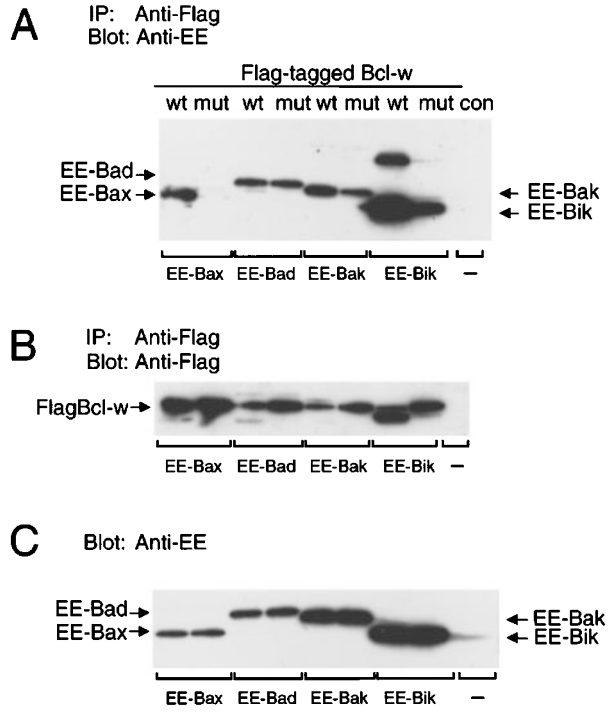

Figure 2 Interaction of Bcl-w and Bcl-wG94E with death enhancer proteins. Lysates were prepared from COS cells co-transfected with vectors expressing FLAG-tagged wt or mutant Bcl-w and EE-tagged Bax, Bak, Bad or Bik, as indicated. Control lysates (–) were mock-transfected. (**A**) Lysates were incubated with anti-FLAG-conjugated agarose beads, and bound proteins eluted with FLAG peptide, separated by SDS–PAGE, transferred to membranes and stained using anti-EE antibody. (**B**) Immunoblot from (**A**) was washed and re-probed with anti-FLAG antibody to ascertain the level of FLAG-tagged protein in the immunoprecipitates. (**C**) Western blot of fractionated lysatees stained with anti-EE monoclonal antibody to confirm expression of the tagged pro-apoptotic proteins. The upper band seen in lane 7 of (**A**) is artefactual, probably due to aggregation, and was not observed in two other experiments. Otherwise, the data shown are representative of at least three similar experiments

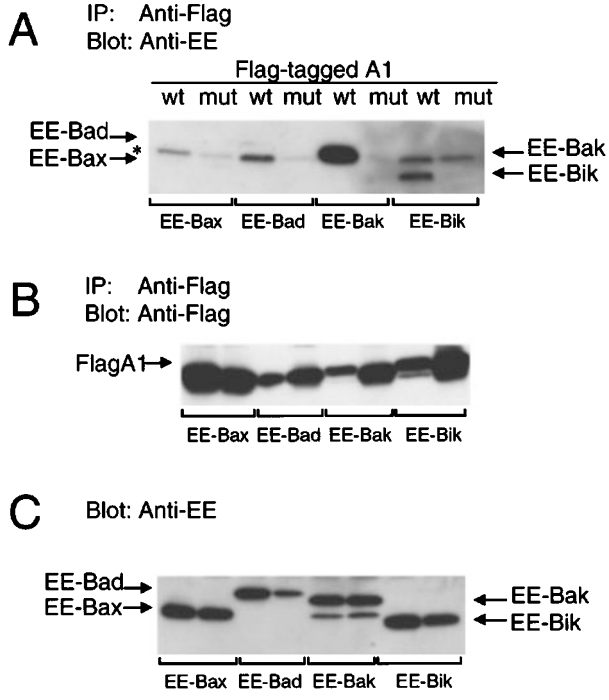

Figure 3 Interaction of A1 and A1G87E with death enhancer proteins. Lysates were prepared from COS cells co-transfected with vectors expressing either FlagA1 or FlagA1G87E and EE-tagged Bax, Bak, Bad or Bik. (**A**) The lysates were incubated with anti-FLAG-conjugated agarose beads and bound proteins eluted with FLAG peptide were separated by SDS–PAGE, transferred to filters and stained using anti-EE monoclonal antibody. The asterisk indicates immunoglobulin light chain. (**B**) The immunoblot from (**A**) was re-probed with anti-FLAG monoclonal antibody to ascertain the level of FLAG-tagged proteins. (**C**) Lysates were fractionated by SDS–PAGE and stained with anti-EE monoclonal antibody to confirm expression of the pro-apoptotic proteins. The lower bands in the EE-Bak lanes of (**C**), observed in several experiments, are probably partial breakdown products. Data shown are representative of three similar experiments

for cells expressing Bax in the presence or absence of Bcl-w. With Bax alone (Figure 4A), the cells expressing transfected Bax (EE^+ cells; Figure 4A, left panel) could be readily gated (R1 fraction), and nearly half of these proved to have $<2N$ DNA (Figure 4A, right panel). In contrast, when Bcl-w was co-expressed (Figure 4B), only a few per cent of the transfected cells had fragmented DNA.

The wild-type pro-survival proteins varied in their ability to inhibit apoptosis provoked by over-expression of the death promoters, as shown in Figure 5. Each enhanced the survival of Bax-expressing cells, although Bcl-w and Bcl-x_L were more potent than A1 or Bcl-2. In contrast, only Bcl-x_L provided significant protection against Bak. Furthermore, while Bcl-w and A1 both protected against Bad, they were ineffectual against Bik, in contrast to Bcl-2 and Bcl-x_L. Lack of protection is unlikely to reflect poor expression because the same plasmid preparations yielded comparable levels of each of the pro-survival proteins (e.g. Figures 2B and 3B). As expected, the BH1 mutant Bcl-w and A1 proteins offered no protection against Bax, Bik, or Bak (Figure 5). Surprisingly, both did enhance the viability of cells overexpressing Bad. Taken together, these findings indicate that protection and heterodimerization do not always correlate.

Discussion

The initial objective of this study was to compare the heterodimerization properties of Bcl-w and A1 and to determine the effect of mutating the highly conserved glycine residue in the BH1 domain. The wt and mutant proteins were each co-expressed in COS cells with four pro-apoptosis relatives: Bax, Bak, Bik and Bad. Wild-type Bcl-w interacted with all four (Figure 2), as found for Bcl-x_L and Bcl-2[19, 21, 29,32], although Bcl-2 is relatively poor at binding Bak.[29] Mutagenesis of the conserved glycine residue in BH1 of Bcl-w abrogated its ability to bind to Bax but, somewhat surprisingly, did not prevent binding to Bak, Bik or Bad (Figure 2). Thus, while this glycine residue is critical for certain interacting pairs of pro- and anti-survival Bcl-2 family members, it is much less important for others. Similarly, certain BH1 mutants of Bcl-x_L that are unable to bind Bax[33] can still bind Bad.[44]

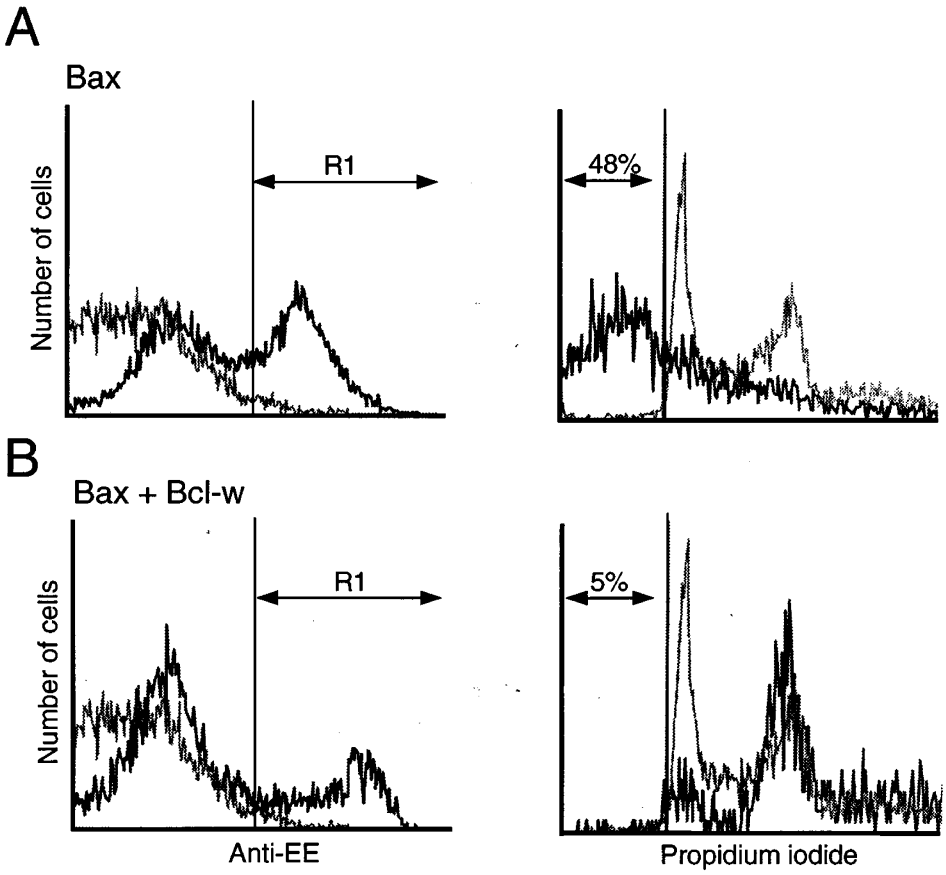

Figure 4 Flow cytometric assay demonstrating that Bcl-w inhibits apoptosis-inducing effect of Bax. 293T cells transfected with (**A**) empty vector and a vector encoding EE-Bax, or (**B**) with vectors encoding FLAG-Bcl-w and EE-Bax, were fixed and permeabilized 36 h later, stained with anti-EE antibody and FITC-labeled anti-mouse Ig antibody, and then stained with propidium iodide (see Materials and Methods). 10^4 transfected cells were analyzed by flow cytometry: the solid line in the left panels indicates the level of FITC fluorescence in EE-Bax expressing cells, and the broken line shows the background staining of cells transfected with empty pEF pGK puro vector. Cells expressing high levels of EE-tagged protein were gated (region R1), and analyzed for propidium iodide staining (solid line, right panels). The broken line in the right panel indicates propidium iodide staining of 293T cells transfected with empty pEF pGK puro vector and indicates cells in G1, S and G2 stages of the cell cycle. The percentage of apoptotic cells (those having $<2N$ DNA) is indicated

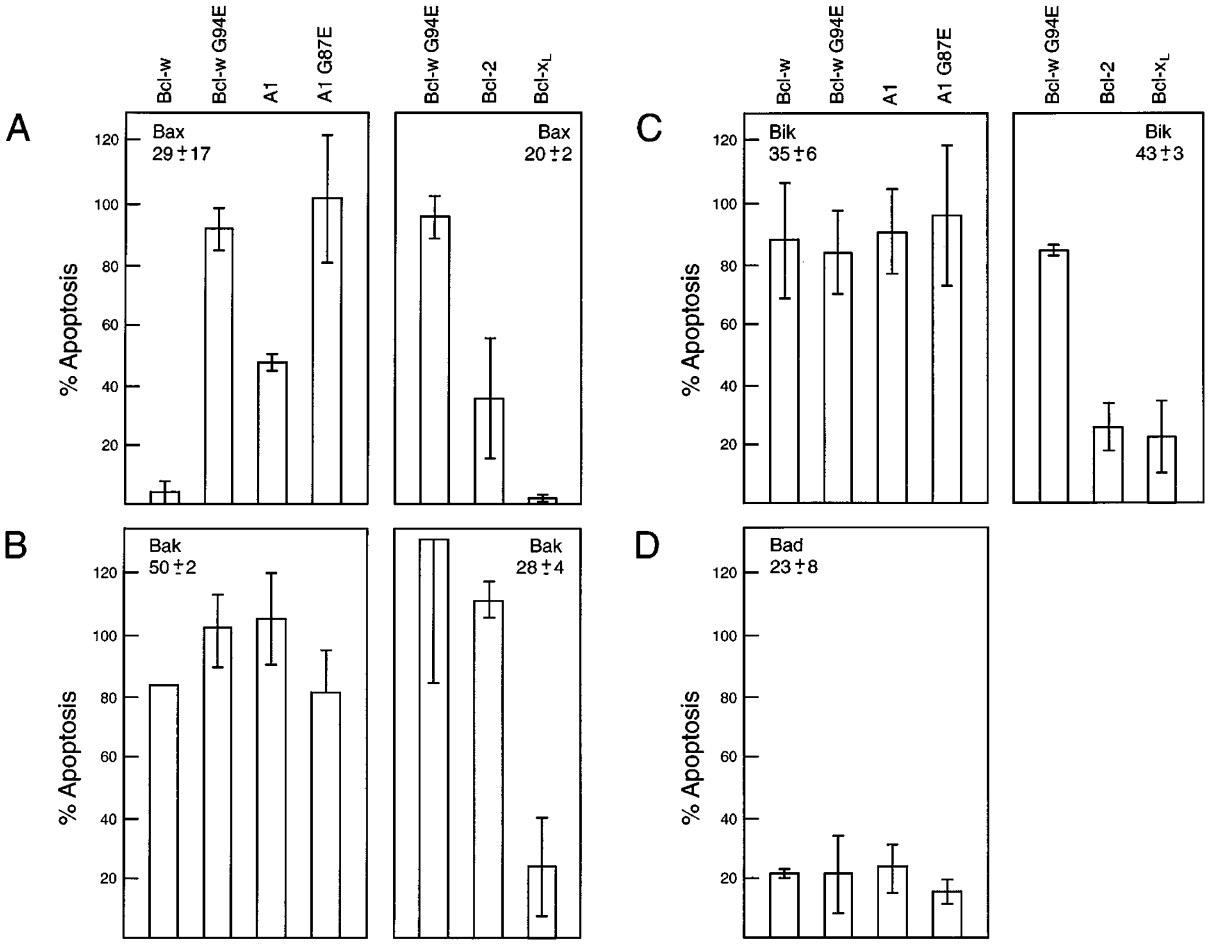

Figure 5 Effect on cell viability of co-expressing death-repressor and death-enhancer proteins. 293T cells were transiently transfected in parallel with vectors encoding EE-tagged Bax (**A**), Bak (**B**), Bik (**C**), or Bad (**D**), either alone or together with FLAG-tagged Bcl-w, Bcl-wG94E, A1 or A1G87E. The effects of co-expressing FLAG-tagged Bcl-wG94E or Bcl-x_L, or untagged Bcl-2, with the death enhancer proteins were compared separately. The level of apoptosis was determined by FACS analysis (see Materials and Methods and Figure 4). For Bax, Bak and Bad, we determined the percentage of EE-positive cells having $<2N$ DNA. Because Bik killed the cells more rapidly, too few EE-positive cells could be gated for lysis, so the percentage of cells having $<2N$ DNA was determined for the total population. The number shown in each panel indicates the mean level of apoptosis ($\pm$standard deviation) obtained when each death-enhancer was expressed alone. The bars represent the mean level of apoptosis observed in the presence of each death-repressor protein, expressed as a percentage of that obtained with the death-enhancer alone. At least two (usually three) independent experiments were performed

A1 had a rather different interaction pattern. It bound both Bak and Bik, and this binding required the conserved glycine residue in BH1 (Figure 3). However, no binding was detected wtih either Bax or Bad (Figure 3). Other examples of preferential pairing of particular pro- and anti-survival proteins have appeared recently (reviewed in[3]). Curiously, A1 and Bax have been reported to interact in the yeast two-hybrid system.[18,32] This discrepancy may reflect the greater sensitivity of the two-hybrid system.

Consistent with earlier reports,[9,39] Bcl-w and A1 both enhanced the survival of myeloid cells deprived of IL-3. However, neither of the BH1-mutated derivatives was protective (Figure 1). The greater affinity of Bcl-w than A1 for Bad (Figure 2) may account for its greater ability to protect against cytokine deprivation (Figure 1). Recent findings suggest that IL-3 can promote cell survival by inducing phosphorylation of Bad that allow its sequestration in the cytoplasm by 14-3-3 proteins, precluding its inactivation of the pro-survival proteins.[45]

We also compared the ability of the pro-survival proteins to protect 293T cells against apoptosis induced by over-expression of the four pro-apoptotic proteins. Bcl-w very effectively inhibited Bax-induced death, and this survival activity was negated by the glycine mutation (Figure 5). A1 also protected against Bax-induced death, although not as well as Bcl-w, and the mutant protein was inactive. Protection against Bad was also provided by both proteins – surprisingly even when mutated in BH1. However, neither could counter apoptosis induced by over-expression of Bak and Bik. Bcl-2 also failed to protect the cells from over-expression of Bak, although it was relatively effective against Bax and Bik (Figure 5). Finally, Bcl-x_L

protected cells over-expressing each of the three death promoters tested with it (Bax, Bak and Bik).

As summarized in Table 1, for 12 of 22 pairs tested, the ability of a pro-survival protein to bind to a pro-death protein co-related well with its ability to prevent apoptosis provoked by over-expression of the latter. For example, Bcl-w, Bcl-2 and Bcl-x_L all bound Bax and were effective inhibitors of Bax-induced death. However, for nine other pairs (shown in bold in Table 1), binding and protection were discordant. For example, while Bak bound Bcl-w, Bcl-w G94E, A1, Bcl-2 and Bcl-x_L, only Bcl-x_L was an effective antidote to Bak-induced death. Similarly, Bik interacted with all four pro-survival proteins but only Bcl-2 and Bcl-x_L countered cell death provoked by Bik over-expression. The converse discrepancy was also observed. For example, no interaction was detected between A1 and either Bax or Bad, but A1 still proffered moderate protection against death induced by high expression of these two proteins.

Some of these discrepancies might be accounted for by significant variation in the relative affinities of particular pro- and anti-apoptotic proteins for each other. On the other hand, it may also be relevant that the Bax group apparently can kill by a mechanism independent of the pro-survival proteins, perhaps related to disturbance of the mitochondrial outer membrane (reviewed in[3]). Similarly, the results of Bad with A1, and of Bik with Bcl-w and A1 (Table 1), raise the possibility that these two BH3-only proteins may also contribute to apoptosis in part by a mechanism that is independent of binding to and neutralizing pro-survival proteins. Intriguingly, mutagenesis results have suggested that the BH3 domain may be insufficient to account entirely for the death-inducing activity of Bik[46] or of Bim, another BH3-only protein.[24] Thus, significant questions remain regarding the relationship of the opposing family members to the apoptotic machinery and the importance of heterodimerization with other family members to their function.

Materials and Methods

Plasmid construction

The vectors, based on pEF BOS,[47] utilized for selection and expression in mammalian cells were derivatives of pEF pGK puro, pEF pGK hygro or pEF MC1 neo[48,49] which incorporate the N-terminal epitope tags FLAG (DYKDDDDK),[50] GluGlu (EYMPME)[51] or HA (YPYDVPDYA),[52] to facilitate detection of expressed proteins. Construction of the plasmids expressing *bcl-2*, *bcl-x_L*, *bax*, *bad*, *bik* and *bak* are described elsewhere.[24,49,53]

Mouse *bcl-w* cDNA[9] was subcloned by polymerase chain reaction (PCR), using *Bg1*II and *Xba*I restriction sites (underlined) incorporated at the ends of the primers. The 5′ primer (wI) was 5′-GC AGA TCT TTC GAA GAG CTG CCA TCC AG-3′, from the sequence −29 to −14 nucleotides upstream from the initiating ATG, while the 3′ primer (wII) was 5′-T GTC TAG ACT TTC TCA CTT GCT AGC AAA AAA GG-3′, which derives from the 3′ portion of the *bcl-w* coding region, including the five C-terminal amino acids (FFASK). Bcl-w G94E was generated from the wt cDNA by PCR, using *Sty*I and *Xba*I restriction sites (underlined) incorporated at the ends of the primers. The 5′ primer (wIII) was 5′-TTC CAA GGG GGC CCT AAC TGG G**AG** CG-3′, which encodes GGPNW**E**R, rather than the wt GGPNWGR, and the 3′ primer was wII, as above.

The full length cDNA encoding mouse A1,[10] a generous gift from Dr. M Prystowsky, was subcloned into pEF FLAG pGK puro by PCR. The 5′ primer (AI) was 5′-CG GGA TCC ATG GCT GAG TCT GAG-3′, corresponding to *Bam*HI (underlined) followed by the sequence encoding the N-terminal residues of A1 (MAESE). The 3′ primer (AII) was 5′-CGCGCTCTAGA TTA CTT GAG GAG AAA GAG C-3′, the reverse complement of the region encoding the C-terminal residues of A1 (LFLLK) followed by an *Xba*I restriction site (underlined). A1 G87E was generated from the wt cDNA by PCR and splice-overlap-extension,[54] in which two overlapping PCR products carrying the point mutation are joined together by another round of PCR. The first PCR product (A) was generated using the 5′ primer AI described above and a 3′ primer (AIII) which corresponds to residues 83 to 93 (G I I N W **E** R I V T I F), with the point mutation in bold. The second PCR product (B) was generated using the 5′ primer (AIV) 5′-GC ATC ATT AAC TGG G**AG** AGG ATT GTG ACT ATA TTT G-3′ corresponding to sequence (GIINW**E**RIVTIF) and complementary to the 3′ end of PCR product A, and the 3′ primer was AII, as above. PCR products A and B were then used as the 5′ and 3′ templates respectively in the second PCR, generating the full length sequence encoding A1G87E. PCR was performed using proof reading *Pfu* DNA Polymerase (Stratagene) and the sequences of all the clones were verified by automated sequencing (ABI Perkin Elmer).

Transfection of mammalian cells and cell death analysis

FDC-P1, a mouse promyelocytic cell line,[43] was cultured in medium containing 1000 U ml^{-1} of IL-3 unless stated otherwise. For transfection, 2.5×10^6 cells in 0.5 ml mouse tonicity-phosphate buffered saline (MT-PBS) were electroporated with 10 μg DNA using a Gene-pulser (Bio-Rad), then split into two 10 ml cultures, selected in puromycin (2 μg ml^{-1}; Sigma), geneticin (800 μg ml^{-1}; Sigma) or hygromycin (2 mg ml^{-1}; Boehringer Mannheim) and cloned by limiting dilution. Expression levels were assessed by immunofluorescence staining of permeabilized, fixed cells and flow cytometric analysis,[55] or by Western blot analysis, using anti-FLAG M2

Table 1 The ability of pro-survival proteins to bind to pro-apoptotic proteins and inhibit their cytotoxic action*

Pro-apoptotic	Bcl-w		Bcl-wG94E		A1		A1G87E		Bcl-2		Bcl-x_1	
protein	binds	inhibits	binds	inhibits	binds	inihibits	binds	inhibits	binds	inhibits	binds	inhibits
Bax	yes	yes	no	no	**no**	**yes**	no	no	yes	yes	yes	yes
Bak	**yes**	**no**	**yes**	**no**	**yes**	**no**	no	no	yes#	no	yes	yes
Bad	yes	yes	yes	yes	**no**	**yes**	**no**	**yes**	yes	nd	yes	nd
Bik	**yes**	**no**	**yes**	**no**	**yes**	**no**	no	no	yes	yes	yes	yes

nd=not determined. *Correlation of binding data (Figures 2 and 3 and unpublished data of DCSH) with protection data (Figure 5). The cases where heterodimerization did not correlate with protection are shown in bold. #Binding is weak (Chittenden *et al*., 1995b)[29]

monoclonal antibody (IBI, Eastman-Kodak, New Haven, CT, USA), anti-HA 12CA5 monoclonal antibody (Boehringer Mannheim) or anti-EE monoclonal antibody (BabCO, Richmond, CA, USA). For survival assays, parental and FDC-P1 cells transfected with vectors encoding FLAG-tagged wt and mutant proteins were washed three times by resuspension in 10 ml MT-PBS, followed by centrifugation through 1 ml of 10% fetal calf serum (FCS). The cells were then cultured at 2×10^5 cells ml^{-1} in Dulbecco's Modified Eagle's (DME) medium containing 10% FCS but no IL-3. Aliquots were harvested periodically and viability was assessed flow cytometrically by determining the proportion of cells which excluded propidium iodide (5 μg ml^{-1}; Sigma) using a FACScan (Becton Dickinson).

Transient transfection of 293T cells was used to assess the ability of wt and mutant Bcl-w and A1 proteins to inhibit apoptosis induced by death promoting Bcl-2 homologs. Plasmids (0.25 μg of each DNA) encoding the EE-tagged death enhancer protein (Bax, Bak, Bak or Bik) and a FLAG-tagged death repressor protein (Bcl-2, Bcl-x_L, Bcl-w, Bcl-wG94E, A1 or A1G87E) were added to 200 μl RPMI (without antibiotics) containing 3 μl lipofectamine (Gibco LTI) and incubated at room temperature for 45 min. The DNA/lipofectamine mix was then made up to 1 ml with RPMI and added to a well containing washed (293T cells which had been plated 24 h earlier in 6-well costar plates (Nunc) (2×10^5 cells in 1 ml RPMI containing 10% FCS). After 6 h at 37°C, 1 ml of RPMI containing 20% FCS was added and the cells incubated for a further 36 h.

To assess viability, the cells were harvested, fixed in 80% ice-cold methanol for 8 min, washed with FACS buffer (a buffered balanced salts solution[56] containing 2% FCS), resuspended in 100 μl of FACS buffer containing 0.03% saponin (FACS/saponin buffer) and anti-EE antibody and placed on ice. After 30 min, the cells were washed again in FACS/saponin, resuspended in 100 μl of FACS/saponin buffer containing fluorescein isothiocynate (FITC)-labeled goat anti-mouse IgG (Southern Biotechnology), incubated on ice for 30 min, then resuspended in propidium iodide solution (69 μM propidium ioide, 38 mM sodium citrate, pH 7.4, 2.5 μg ml^{-1} RNase A) and incubated at 37°C for 15 min. The cells were then analyzed on a FACScan (Becton Dickinson) to determine the proportion of dead EE-positive cells, i.e. transfected cells having a DNA content of <2N.[57]

Protein interactions *in vivo*

Transient co-expression in COS M6 cells was used to test for protein-protein interaction *in vivo*. After exposure for 5 min at 4°C to 15 μg of each plasmid DNA in 1 ml MT-PBS, 2×10^6 cells were electroporated, incubated at room temperature for 10 min and then cultured for 36 h in 10 ml DME containing 10% FCS. The cells were then harvested, washed in ice-cold MT-PBS and lysed in 500 μl radioimmunoprecipitation assay (RIPA) buffer (50 mM TrisCl, 150 mM NaCl; 1 mM EGTA, 0.1% NP-40 pH 7.4) containing protease inhibitors.[24] After centrifugatiaon to remove cellular debris, the lysate was incubated at 4°C for 1 h with 20 μl anti-FLAG M2 affinity matrix (IBI Eastman-Kodak). The matrix was then washed three times with 1 ml ice-cold RIPA buffer, and incubated at 4°C for 15 min with 20 μl RIPA buffer containing 2 μg of FLAG peptide [DYKDDDDK] (IBI Eastman-Kodak) to elute the bound proteins. The eluate was fractionated by SDS–PAGE using 4–20% gels (Novex) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) using a Bio-Rad semidry transfer cell. The membrane was then incubated for 1 h at room temperature in 1% blocking solution (Boehringer Mannheim) followed by 1 h with the primary antibody (either anti-EE or anti-FLAGM2), diluted in 0.5% blocking solution containing 0.05% Tween-20. After three washes for 5 min each in MT-PBS containing 0.05% Tween-20, the filters were then incubated for 20 min with horseradish peroxidase (HRP)-conjugated secondary antibody (sheep anti-mouse Ig (Silenius)), followed by three washes with 0.05% Tween-20/MT-PBS. The interacting proteins were then revealed using enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham).

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