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A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions

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Regulation of the cell death program involves physical interactions between different members of the Bcl-2 family that either promote or suppress apoptosis. The Bcl-2 homolog, Bak, promotes apoptosis and binds anti-apoptotic family members including Bcl-2 and Bcl-x_L. We have identified a domain in Bak that is both necessary and sufficient for cytotoxic activity and binding to Bcl-x_L. Sequences similar to this domain were identified in Bax and Bip1, two other proteins that promote apoptosis and interact with Bcl-x_L, and were likewise critical for their capacity to kill cells and bind Bcl-x_L. Thus, the domain is of central importance in mediating the function of multiple cell death-regulatory proteins that interact with Bcl-2 family members.

Keywords: apoptosis/Bcl-2 homolog/Bik/programmed cell death

Introduction

Disruption of apoptosis, the intrinsic cell death program, may contribute to the onset and/or evolution of human tumors (Williams, 1991; Fisher, 1994). A particularly well-characterized genetic lesion in the apoptotic pathway is the t(14:18) translocation that activates expression of the *bcl-2* oncogene in human B cell follicular lymphomas (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985). Bcl-2 functions as a potent suppressor of cell death and can suppress apoptosis triggered by diverse stimuli (reviewed by Reed, 1994). There is also a remarkable degree of conservation of Bcl-2 function across species, suggesting that Bcl-2 is intimately connected with an evolutionarily conserved cell death pathway (Vaux *et al.*, 1991, 1994; Hengartner and Horvitz, 1994).

Bcl-2 is a member of an emerging family of proteins defined by the conservation of two domains, referred to as Bcl-2 homology domains 1 and 2 (BH1 and BH2) (Williams and Smith, 1993; Oltvai and Korsmeyer, 1994). Of the known Bcl-2 homologs, Bcl-x_L bears the highest degree of homology to Bcl-2 and, like Bcl-2, functions to suppress apoptosis (Boise *et al.*, 1993). In contrast, a second Bcl-2 homolog, Bax, forms heterodimers with Bcl-2 and Bcl-x_L and was shown to counteract Bcl-2 function and accelerate apoptosis (Oltvai *et al.*, 1993). The family of Bcl-2 relatives includes proteins from DNA viruses: Epstein–Barr virus BHRF-1 and African swine fever virus LMW5-HL (Pearson *et al.*, 1987; Neilan *et al.*, 1993). The adenovirus E1B 19 kDa protein also functions as a strong suppressor of apoptosis under diverse conditions (Rao *et al.*, 1992; White *et al.*, 1992; Subramanian *et al.*, 1993). Although E1B 19 kDa lacks sequences resembling the canonical BH1 and BH2 elements that define the Bcl-2 family, it may function in a manner similar to Bcl-2, since both proteins interact with a common set of cellular proteins (Boyd *et al.*, 1994). Thus, several viruses have independently evolved to encode structural and/or functional homologs of Bcl-2.

A complex array of protein–protein interactions among different Bcl-2 family members contributes to the regulation of the cell death program (Oltvai and Korsmeyer, 1994). The anti-apoptotic activity of Bcl-2, for example, may be dependent upon its interaction with death-promoting proteins such as Bax. Mutations in Bcl-2 that eliminate heterodimerization with Bax, abrogate the ability of Bcl-2 to suppress cell death (Yin *et al.*, 1994). A combinatorial interplay between interacting proteins may also play a significant regulatory role. It was suggested that the interaction of Bad, a Bcl-2-related protein, with Bcl-x_L, promotes cell death due to the competitive displacement of Bax from Bcl-x_L (Yang *et al.*, 1995).

We and others recently described a Bcl-2 relative, Bak, that opposes Bcl-2 function and triggers apoptosis under certain conditions (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). Bak interacts with both Bcl-2 and (more strongly) Bcl-x_L (Chittenden *et al.*, 1995), as well as E1B 19 kDa (Farrow *et al.*, 1995). These data have raised the possibility that proteins like Bcl-x_L, Bcl-2 and E1B 19 kDa that suppress apoptosis may operate by binding and inactivating Bak, and/or other death-promoting proteins such as Bax.

In view of the importance of these interactions for the regulation of cell death, we sought to understand in greater detail the molecular requirements for a prototypical interaction involving a cell death promoter, Bak, with a cell death suppressor, Bcl-x_L. We report here the identification of a domain in the Bak protein, distinct from BH1 and BH2, that is uniquely required for both its cell killing and Bcl-x_L binding functions, and is itself sufficient for these activities. Moreover, sequences homologous to this domain were identified in Bax, and in an otherwise unrelated protein, Bip1 (Boyd *et al.*, 1995), that also promotes cell death and binds to Bcl-x_L. The homologous sequence motifs were shown to be important for the cell killing and protein binding functions of these proteins, indicating that this domain is critical to the actions of multiple cell death-promoting proteins.

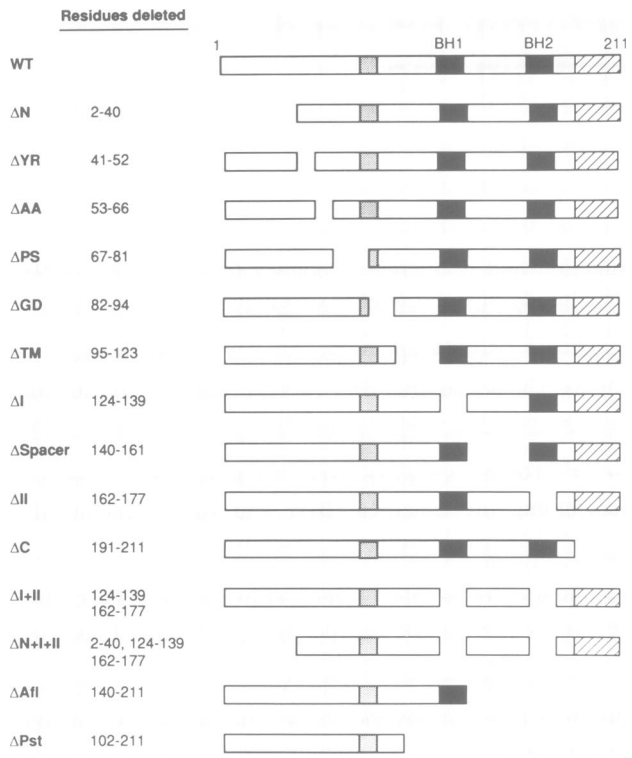

Fig. 1. Structures of Bak deletion mutants. The structures of wild-type (WT) Bak and Bak mutants are illustrated schematically. Black rectangles denote Bcl-2 homology domains 1 and 2; the cross-hatched boxes indicate the hydrophobic tail and the shaded boxes show the location of Bcl-2 homology domain 3 (amino acids 78–86, see text and Figure 4 for description). Numbers refer to the amino acid region(s) of Bak deleted in the corresponding mutant. All Bak mutants contained an amino-terminal HA epitope tag.

Results

Identification of Bak domains required for cell killing function

Previous experiments demonstrated that enforced Bak expression induces apoptosis in stably transfected Rat-1 cell lines (Chittenden *et al.*, 1995). To identify regions of the molecule that are necessary to induce apoptosis, a series of deletion mutants spanning the Bak protein were constructed (Figure 1) and tested for cell killing activity by a transient transfection assay in Rat-1 cells. This assay is similar to that previously described for detecting cell death induced by interleukin (IL)-1β-converting enzyme (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994). Rat-1 cells were transfected with a marker plasmid encoding β-galactosidase (β-gal), in combination with either an expression plasmid encoding Bak, or various control plasmids. Transfected cells were stained with X-gal 24 h after transfection to detect β-gal-expressing cells. The cell killing activity of Bak in this assay is manifested by a large reduction in the number of blue (β-gal-expressing) cells obtained relative to co-transfection of the β-gal plasmid with a control expression vector (Figure 2A; compare WT Bak and Vector). The elimination of blue cells indicates that transfected cells are killed by Bak, an interpretation supported by the measurable reduction in total cell number in wells transfected with Bak expression plasmids (not shown). Furthermore, IL-1β-converting enzyme, previously shown by a similar assay to induce apoptosis in Rat-1 cells, also eliminated blue cells in this assay when expressed from the vector employed here (Boyd *et al.*, 1995). Control expression plasmids harboring the *bak* cDNA in the anti-sense orientation, or various unrelated cDNAs, did not eliminate β-gal-positive cells. In addition, the number of blue cells could be partially restored by co-transfection of Bak with Bcl-x_L or E1B 19 kDa (not shown), suggesting that the elimination of blue cells is a reflection of an apoptotic (Bcl-x_L-inhibitable) function of Bak.

Analysis of the Bak deletion mutants revealed that much of the Bak molecule is dispensable for its cell death function detected by this assay (Figure 2A; summarized in Table I). The non-essential regions include BH1 and BH2, the two domains in the carboxy-terminal half of the protein that show the highest degree of homology to other Bcl-2 family members. Deletion of the carboxy-terminal hydrophobic stretch of amino acids (residues 191–211; mutant ΔC) slightly diminished the cytotoxic function of Bak (Figure 2A). By analogy to the role of similar sequences in other Bcl-2 relatives (Tanaka *et al.*, 1993), this hydrophobic 'tail' probably serves as a membrane anchor sequence in Bak. While the carboxy-terminal hydrophobic tail is not required for the cell killing function of Bak, it may contribute to activity by ensuring proper subcellular localization of the protein.

A segment of the Bak protein removed by the ΔGD deletion (residues 82–94) is absolutely essential for cell death function, since this mutant exhibited no cell killing activity in the transient transfection assay (Figure 2A). Deletion of adjoining residues (amino acids 67–81) immediately N-terminal to this region also significantly reduced cell death activity (Bak mutant ΔPS). The loss of activity was not related to an obvious defect in protein expression since both ΔGD and ΔPS were expressed at levels similar to wild-type Bak in transfected cells (i.e. Figure 2C, and data not shown). All other deletion mutants tested were unaltered, or slightly diminished, in their capacity to kill Rat-1 cells (Figure 2A and Table I). Taken together, these results indicate that a co-linear segment, defined by the deletion mutants ΔGD and ΔPS (residues 67–94), is uniquely required for the cytotoxic function of Bak detected in the transient assay.

Identification of Bak sequences that mediate the interaction with Bcl-x_L

Physical interaction with other Bcl-2 family members, such as Bcl-x_L, may be essential for Bak to exert its cell death function, or may regulate its activity. We therefore sought to identify elements within Bak that are necessary for its Bcl-x_L binding activity. The interaction of Bak with Bcl-x_L was detected both by an *in vitro* protein binding assay, and by co-immunoprecipitation from transfected cells. *In vitro* translated ^{35}S-labeled Bak bound to a purified, bacterially expressed glutathione S-transferase (GST)–Bcl-x_L fusion protein (Figure 2B). The specificity of this *in vitro* interaction was demonstrated by the failure of Bak to bind to purified GST alone (Figure 2B) and by the failure of GST–Bcl-x_L to bind to non-relevant proteins such as luciferase, α-factor or β-lactamase (not shown). A specific Bak–Bcl-x_L interaction was also detected by co-transfecting epitope-tagged forms of Bak and Bcl-x_L into COS cells. Bak was immunoprecipitated from trans-

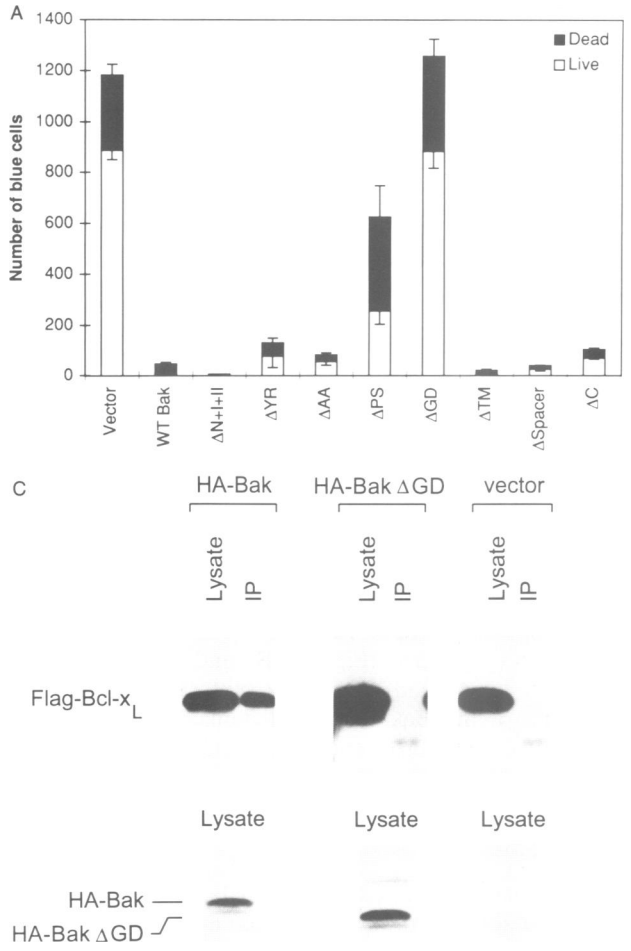

Fig. 2. Identification of a Bak domain required for cell killing and Bcl-x_L binding. (**A**) Detection of the cell killing activity of Bak in a transient transfection assay. Rat-1 cells were co-transfected with a β-gal marker plasmid and either a control plasmid (vector), or a plasmid expressing HA epitope-tagged Bak (WT Bak) or the indicated Bak deletion mutants (see Figure 1 for description of mutants). Cells were fixed and stained with X-gal 24 h after transfection, and the number of blue cells (β-gal-positive) counted by microscopic examination. The data shown are the number of dead (filled bars) and live (open bars) blue cells for each condition (mean ± SD from triplicate transfections). (**B**) Interaction of Bak with Bcl-x_L *in vitro*. *In vitro* translated ^{35}S-labeled HA-Bak or HA-Bak mutants (IVT input) were mixed either with GST–Bcl-x_L or GST. The complexes were captured on glutathione–agarose beads (GSH-beads), and bound ^{35}S-labeled protein was detected by SDS–PAGE and autoradiography. (**C**) Interaction of Bak with Bcl-x_L in transfected cells. COS cells were co-transfected with plasmids expressing Flag epitope-tagged Bcl-x_L (Flag-Bcl-x_L) and either HA-Bak, HA-Bak mutants or a control plasmid (vector). HA epitope-tagged proteins were immunoprecipitated (IP) from transfected cell lysates and associated Bcl-x_L was detected by immunoblotting with anti-Flag antibody (top panel). Expression of HA-tagged proteins was confirmed by immunoblotting with anti-HA antibody (bottom panel). The results for Bak and Bak ΔGD are shown; see Table I for summary of results for other mutants.

fected cell lysates and associated Bcl-x_L was detected by Western blot analysis of co-precipitated proteins (Figure 2C). Bcl-x_L was not detected in immunoprecipitates in the absence of co-expressed Bak, demonstrating that binding is specific.

The series of Bak deletion mutants described above (Figure 1) were tested for their Bcl-x_L binding capacity both *in vitro*, and in transfected COS cells (Figure 2B and C). Deletion of residues 82–94 (Bak mutant ΔGD) completely eliminated the ability of Bak to interact with Bcl-x_L. Interaction with Bcl-x_L was also greatly diminished by deletion of adjoining amino acids 67–81 (Bak mutant ΔPS). All other deletion mutants tested, encompassing the entire Bak open reading frame, retained the ability to bind Bcl-x_L (summarized in Table I). These results identify a contiguous Bak domain encompassed by the ΔGD and ΔPS mutants (maximally, amino acids 67–94) as uniquely important in mediating the interaction with Bcl-x_L. The same region of Bak was uniquely required for the cell killing function of Bak (see above).

A Bak domain is sufficient for cell killing activity and interaction with Bcl-x_L

The mutational analysis demonstrated the importance of Bak residues 67–94, however, it remained to be determined if this domain was itself sufficient for the biological activities of Bak. To explore this possibility, truncated Bak protein derivatives encompassing sequences within this region were tested for cytotoxic activity in Rat-1 cells and for their ability to bind Bcl-x_L (Figure 3). QVG (Bak amino acids 58–103; see Figure 3A) significantly reduced the number of blue cells when co-transfected with β-gal, indicating that it retained cell killing activity (Figure 3B). PEM (amino acids 73–123) showed little, if any, cytotoxic activity. However, PEM (and QVG) lacks the carboxy-terminal hydrophobic element and might exhibit reduced cell killing function due to altered subcellular localization. In an effort to improve the cell killing capacity of the truncated Bak species, the hydrophobic tail element (amino acids 187–211) was fused to the carboxy-termini of both PEM and QVG (to form PEM+C and QVG+C, respectively; Figure 3A). Attachment of the hydrophobic tail dramatically improved the ability of PEM to eliminate blue cells in the transfection assay, and resulted in cytotoxicity similar to wild-type Bak (Figure 3B). These results suggest that a protein domain shared by both PEM and QVG (amino acids 73–103) is sufficient for the cell killing function of Bak, in the context of the putative membrane anchor sequence.

We next examined whether QVG and PEM retained the ability to bind Bcl-x_L *in vitro* and in transfected cells (Figure 3C and D). *In vitro* translated QVG and PEM bound specifically to GST–Bcl-x_L and, in a reciprocal experiment, *in vitro* translated Bcl-x_L interacted with a

Table I. Summary of cell killing and protein binding activity of Bak deletion mutants

Bak mutant	Residues deleted	Cell killing activity	Bcl-x_L binding activity
WT	–	+	+
ΔN	2–40	+	+
ΔYR	41–52	+	+
ΔAA	53–66	+	+
ΔPS	67–81	±	±
ΔGD	82–94	–	–
ΔTM	95–123	+	+
ΔI	124–139	+	+
ΔSpacer	140–161	+	+
ΔII	162–177	+	+
ΔC	191–211	+	+
ΔI+II	124–139, 162–177	+	+
ΔN+I+II	2–40, 124–139, 162–177	+	+
ΔAfl	140–211	n.d.	(+)
ΔPst	102–211	n.d.	(+)

Cell killing activity (detected in the transient transfection assay) and Bcl-x_L binding activity (detected both *in vitro* and in transfected cells) of the Bak mutants relative to wild-type Bak is summarized as follows: +, equivalent or slightly diminished; ±, greatly diminished; –, defective. n.d. indicates experiment not done. (+) indicates binding was tested *in vitro* only.

GST–PEM fusion protein (Figure 3C). Both PEM and QVG were co-immunoprecipitated with Bcl-x_L from transfected cell extracts (Figure 3D), indicating that each contains a domain sufficient for mediating the interaction with Bcl-x_L. In addition, this domain was sufficient to mediate binding of the viral cell death suppressor, E1B 19 kDa, as shown by the specific interaction of *in vitro* translated E1B 19 kDa with GST–PEM (Figure 3C). Together with the analysis of the deletion mutants described above, these results demonstrate that amino acid sequences spanning residues 73–94 comprise a key functional domain in Bak that mediates cell killing and protein binding functions.

Sequences homologous to the Bak functional domain are present in Bax and Bip1 and are critical for their activities

Mutational analysis of Bak revealed that the same region, maximally residues 67–94, was uniquely involved in both the cell killing and Bcl-x_L binding activities of Bak. Two other Bcl-2 binding proteins, Bax and Bip1 (cloned by virtue of its interaction with Bcl-2; Boyd *et al.*, 1995), have biological activities that closely resemble those of Bak. Both Bax and Bip1 eliminate blue cells when co-transfected with β-gal in Rat-1 cells, indicating that they also are cytotoxic in this context. Bax and Bip1 also interact specifically with Bcl-x_L both *in vitro* and in transfected COS cells (Boyd *et al.*, 1995). These functional similarities prompted us to examine whether there were any structural features shared by the three proteins that contribute to their similar biological functions. Specifically, in light of the results described above, we examined whether there are sequences in Bax and Bip1 that resemble the functional domain in Bak (residues 67–94) and are likewise important for their biological activities.

Bax shows extensive homology to Bcl-2 family members (including Bak), with the highest degree of sequence homology centered around BH1 and BH2 (Oltvai *et al.*, 1993). A stretch of amino acids in Bax (residues 59–77) is homologous to sequences within the Bak functional domain (residues 74–94; Figure 4). In contrast to Bax, the primary sequence of Bip1 does not closely resemble the known Bcl-2 relatives and most notably lacks sequences matching BH1 and BH2, the defining elements of the Bcl-2 family (Boyd *et al.*, 1995). However, inspection of the Bip1 sequence identified a region (amino acids 57–69) that shows similarity to the same homologous element within Bak and Bax (Figure 4).

We tested if the homologous elements within Bax and Bip1 are also critical for the respective cell killing and protein binding functions of these proteins. Deletion mutations that removed the conserved sequence motifs were introduced into Bax and Bip1 (Figure 4), and the resulting mutants were analyzed for their ability to kill Rat-1 cells and to bind Bcl-x_L (Figure 5). Like Bak ΔGD, the Bax ΔGD and Bip1 ΔGD mutants were substantially impaired in their ability to eliminate blue cells when co-transfected with β-gal in Rat-1 cells (Figure 5A). In addition, both mutants had lost the capacity to interact with Bcl-x_L *in vitro* (Figure 5B) and no longer bound Bcl-2 in transfected COS cells (not shown). Thus, the structure of the Bak functional domain is conserved in Bak, Bax and Bip1, and is critical to the biological activities of all three proteins.

Discussion

A systematic structure/function analysis of Bak revealed that its ability to promote cell death and bind to Bcl-x_L is mediated primarily by a single domain located in the central portion of the Bak molecule. Small, truncated forms of Bak encompassing this region were cytotoxic to Rat-1 cells, and retained the capacity to bind Bcl-x_L, indicating that the domain is sufficient for these activities. Sequences homologous to a motif within the Bak functional domain are present in the central portions of Bax and Bip1, two other cell death-promoting proteins that interact with Bcl-x_L. The presence of a similar sequence in Bip1 is particularly noteworthy since, apart from this homology, the primary sequence of Bip1 does not overtly resemble that of Bak or Bax. Thus, all three proteins interact with Bcl-x_L, promote cell death and share this sequence motif. Mutations in the homologous sequences in Bax and Bip1 greatly diminished their cell death-promoting and protein binding functions, implicating the importance of this domain in the activity of diverse cell death regulatory proteins.

The conserved sequence elements within the Bak functional domain are not exclusively present in the cell death-promoting proteins Bak, Bax and Bip1. Alignment of Bcl-2 and Bcl-x_L with Bax and Bak has previously noted homologies within this region (residues 97–105 and 90–98 of Bcl-2 and Bcl-x_L, respectively) (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Williams and Smith, 1993; Chittenden *et al.*, 1995). In view of the homology of these sequences among Bcl-2 family members, we propose to label this conserved region BH3 (see also Boyd *et al.*, 1995). The role of BH3 in anti-apoptotic proteins such as Bcl-2 and Bcl-x_L remains to be elucidated; however, it may also prove to be functionally important. Substitution of two

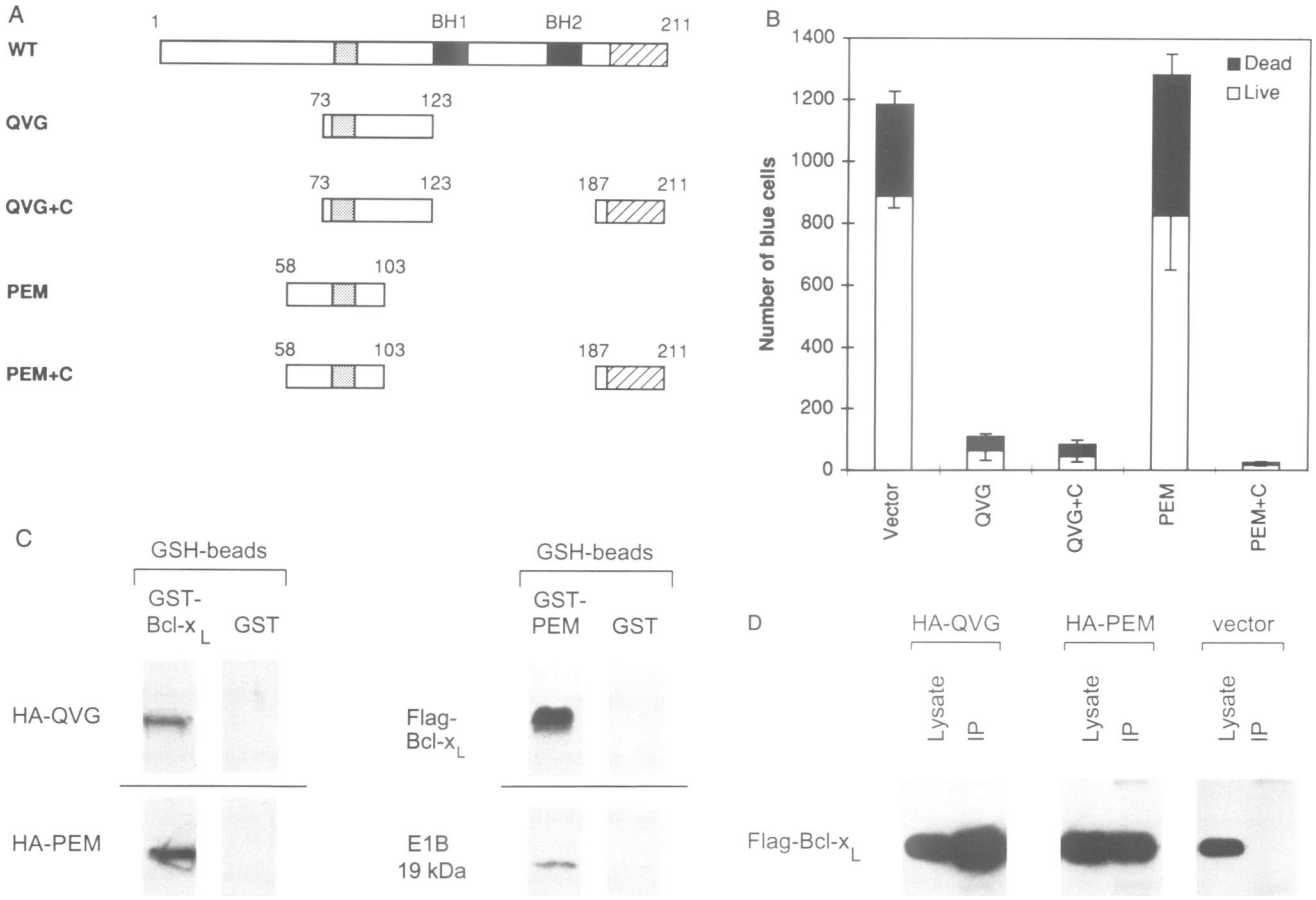

Fig. 3. A domain in the central portion of Bak is sufficient for cell killing and protein binding. (**A**) Schematic representation of truncated Bak derivatives. Bcl-2 homology domain 3 (see text and Figure 4) is shown as a shaded box; the hydrophobic tail is indicated by the cross-hatched rectangle. Numbers refer to the endpoints of Bak amino acids retained in the truncated species. All Bak derivatives contained an amino-terminal HA epitope tag. (**B**) Cell killing activity of truncated Bak derivatives in Rat-1 cells. Truncated Bak species with (QVG+C and PEM+C) or without (QVG and PEM) the carboxy-terminal hydrophobic tail were tested for cell killing activity by co-expression with β-gal in Rat-1 cells, as described in Figure 2A. (The vector control from Figure 2A is reproduced here since these data were all derived from the same transfection experiment.) (**C**) Interaction of truncated Bak derivatives with Bcl-x_L and E1B 19 kDa *in vitro*. Binding of *in vitro* translated ^{35}S-labeled QVG and PEM (HA epitope-tagged) to GST–Bcl-x_L (left panel) was assayed as described in Figure 2B. Interaction of *in vitro* translated ^{35}S-labeled Flag epitope-tagged Bcl-x_L and E1B 19 kDa with a GST fusion protein encoding residues 58–103 of Bak, GST–PEM (right panel), was assayed as described in Figure 2B for binding assays with GST–Bcl-x_L. (**D**) Interaction of truncated Bak derivatives with Bcl-x_L in transfected cells. The interaction of truncated Bak species (HA epitope-tagged) with Bcl-x_L (Flag epitope-tagged) was analyzed in transfected COS cells, as described in Figure 2C.

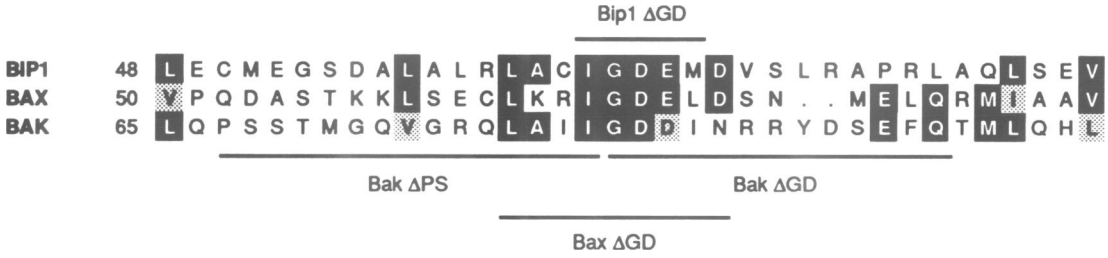

Fig. 4. Bip1 and Bax share sequences homologous to a motif within the functional domain of Bak. Numbers refer to the amino acid position of the portions of the Bip1, Bax and Bak sequences shown. Solid black shading indicates sequence identity between at least two of the proteins, and gray shading shows conservative amino acid substitutions. Solid lines delineate the amino acids deleted in the Bip1 ΔGD, Bax ΔGD, Bak ΔGD and Bak ΔPS mutants.

amino acids within the BH3 element of Bcl-2 (residues 97 and 98) abrogated the anti-apoptotic function of Bcl-2 (T.Subramanian and G.Chinnadurai, unpublished). Moreover, deletion of BH3 sequences from Bcl-x_L (GDE; amino acids 94–96) eliminated its ability to bind to Bak and suppress cell death in the assays described here (T.Chittenden, C.Flemington, R.G.Ebb and R.J.Lutz, unpublished results).

The regions of Bak that are the most conserved among Bcl-2 homologs, BH1 and BH2, were not required for cell death function or Bcl-x_L binding. Similarly, deletion of BH2 in Bax did not detectably alter its capacity to

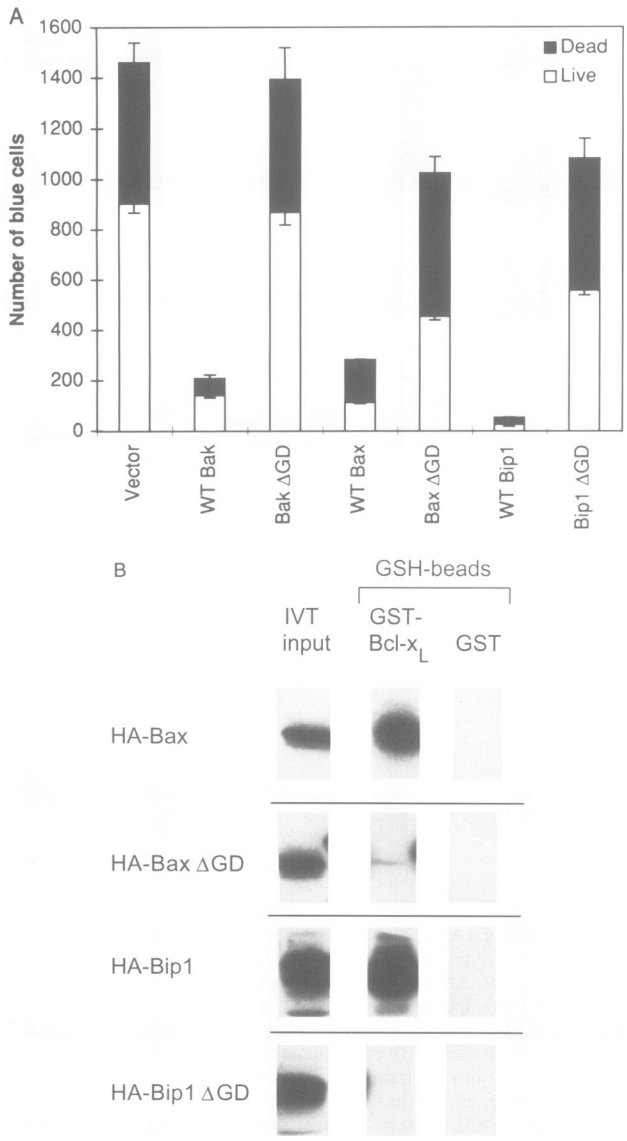

Fig. 5. The conserved domains in Bax and Bip1 are critical for their cell killing and protein binding functions. (**A**) Transient transfection of Bax and Bip1 mutants in Rat-1 cells. Cell killing activity of wild-type (WT) and ΔGD mutants of Bax and Bip1, upon co-expression with β-gal in Rat-1 cells, was detected as described in Figure 2A. Wild-type Bak and Bak ΔGD were included for comparison. (**B**) Interaction of Bax and Bip1 mutants with Bcl-x_L *in vitro*. ^{35}S-labeled HA epitope-tagged Bax, Bip1 and their respective ΔGD mutants were synthesized *in vitro* and assayed for specific binding to GST–Bcl-x_L as described in Figure 2B.

promote cell death or bind to Bcl-x_L (not shown), and Bip1 lacks canonical BH1 and BH2 domains. In contrast to these cell death promoters, Bcl-2 requires the integrity of both BH1 and BH2 to suppress apoptosis and bind to Bax (Yin *et al.*, 1994). It appears that BH1 and BH2 are similarly required for Bcl-x_L function, since Bcl-x_S, which lacks BH1 and BH2, no longer binds Bak or Bax and fails to suppress apoptosis (Boise *et al.*, 1993). These results point to significant differences between cell death promoters and cell death suppressors with respect to the sequence elements required for heterodimerization. For the death promoters (at least Bak, Bax and Bip1), sequences encompassing the BH3 domain appear to mediate the interaction. For the anti-apoptotic partner (i.e. Bcl-2) the requirements appear more complex and involve a combination of domains including BH1, BH2, BH3 and perhaps others (Boyd *et al.*, 1994; Sato *et al.*, 1994; Yin *et al.*, 1994).

While we have focused primarily on binding of Bak to Bcl-x_L in this study, this may not represent the only significant interaction governed by the BH3 domain. This region of Bak is required for interaction with Bcl-2 *in vitro* (not shown), and evidence presented here indicates that the BH3 domain will likewise mediate binding to E1B 19 kDa. It is therefore likely that other viral and cellular Bcl-2 homologs may also interact with Bak via its BH3 sequences. The apparent conservation of BH3 structure and function in Bak, Bax and Bip1 suggests that these proteins heterodimerize with anti-apoptotic proteins such as Bcl-x_L and E1B 19 kDa in a mechanistically similar fashion. However, the BH3 domain may not be the only domain involved in mediating protein interactions with Bak. While BH1 and BH2 were not required for the activities of Bak measured here, these conserved domains may contribute to the regulation of Bak or facilitate interactions with Bcl-2 homologs and/or as yet unidentified proteins. A role for BH1 and BH2 in facilitating cell killing and protein binding can also not be excluded, since the assays used in this study are unlikely to detect small differences in efficacy or affinity.

Thus far, the cell killing activity of Bak could not be separated clearly from its Bcl-x_L binding activity by the mutational analysis (see Table I). Bak mutants that lost cell killing activity (ΔGD and ΔPS) correspondingly lost Bcl-x_L binding activity, indicating that these two functions may be closely linked. One of the truncated Bak molecules tested, PEM, appeared to retain Bcl-x_L binding capacity but did not exhibit detectable cell killing activity. However, cytotoxic function was restored upon addition of the hydrophobic tail element to PEM (PEM+C), suggesting that the reduced cytotoxicity of PEM was related to its subcellular localization. This interpretation does not explain why the truncated Bak molecule QVG and the Bak mutant ΔC, which both lack the tail element, retained significant cell killing activity.

The link between Bcl-x_L binding and cell killing can be reconciled, in light of the available data, with Bak operating as either a regulator or as an active component of the cell death pathway. One possibility is that Bak may kill cells by binding, via its BH3 domain, and inhibiting molecules like Bcl-x_L that actively promote cell survival. Cell death would result from the suppression of essential survival functions provided by Bcl-x_L or related anti-apoptotic proteins. A second possibility is that Bak, Bax and Bip1 actively promote cell death, and are themselves suppressed by their interactions with Bcl-x_L, Bcl-2 and E1B 19 kDa. By this view, the BH3 domain comprises a critical cell death effector domain that may be masked by the binding of survival proteins like Bcl-x_L or E1B 19 kDa. Either scenario distinguishes the BH3 domain as an important molecular target and suggests that molecules developed to mimic or block the activity of this functional domain may prove to have interesting cell death regulatory properties and potential therapeutic utility.

Materials and methods

Plasmids and DNA manipulations

All recombinant DNA procedures were performed following standard protocols (Sambrook *et al.*, 1989). cDNA clones corresponding to the Bax and Bcl-x_L open reading frame were generated by PCR amplification using primers based on the published DNA sequences (Boise *et al.*, 1993; Oltvai *et al.*, 1993); cloning of Bip1 is described elsewhere (Boyd *et al.*, 1995). Deletions in the *bak*, *bax* and *bip1* cDNAs were introduced by single step or two-step PCR mutagenesis methods (White, 1993). The amino acids deleted in the various mutants are listed in the text (Figures 1 and 4). Bak mutants ΔAfl and ΔPst were generated by digestion of the Bak cDNA with *Afl*I and *Pst*I, respectively, and were used for *in vitro* translation reactions (see below). The truncated Bak species QVG and PEM were generated by PCR amplification of cDNA segments encoding Bak residues 73–123 and 58–103, respectively. In each case, a *Cla*I site was introduced just prior to the stop codon in the 3′ primer used for amplification. This resulted in the addition of a single aspartate residue at the carboxy-terminus of QVG, and the addition of isoleucine–aspartate at the carboxy-terminus of PEM. A segment encoding the hydrophobic tail portion of Bak (residues 187–211) was amplified by PCR, with a *Cla*I site introduced within the 5′ primer. The hydrophobic tail segment was ligated, in-frame, to the 3′ ends of QVG and PEM via the *Cla*I sites, to create QVG+C and PEM+C. The additional residue(s) introduced by the *Cla*I site was retained at the junction sites in these Bak derivatives.

The sequences of mutations and all segments of cDNA amplified by PCR were confirmed by DNA sequence analysis. The construction of the hemagglutinin (HA) epitope-tagged Bak was described previously (Chittenden *et al.*, 1995). All Bak deletion mutants and truncated species were similarly tagged at the amino-terminus with the HA epitope (Kolodziej and Young, 1991) and cloned into the cytomegalovirus (CMV) enhancer/promoter expression plasmids pcDNA-1/Amp, pRcCMV or pcDNA-3 (Invitrogen, Inc.). These plasmids were used for transfection experiments and for *in vitro* transcription/translation reactions (see below).

GST fusion proteins were generated by cloning the Bcl-x_L cDNA and the truncated Bak species, PEM, in-frame with GST in pGEX2TK (Kaelin *et al.*, 1992). The Flag epitope (Kodak) was introduced at the amino-terminus of Bcl-x_L with synthetic oligonucleotides and cloned into pcDNA-3. The E1B 19 kDa encoding plasmid used for *in vitro* translation was described previously (Boyd *et al.*, 1994).

Transient transfection assay

Transient transfection assays to detect cell killing activity were performed essentially as described previously (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994). One day prior to transfection, Rat-1 cells were plated in 24-well dishes at a density of 3.5×10^4 cells/well. The following day, the cells were transfected with a marker plasmid encoding β-galactosidase (0.16 μg), in combination with an expression plasmid encoding Bak, or the vector alone (0.42 μg), by the Lipofectamine procedure (Life Technologies). At 24 h post-transfection, cells were fixed and stained with X-gal to detect β-galactosidase expression in cells that received plasmid DNA (Miura *et al.*, 1993). The number of blue cells was counted by microscopic examination and scored as either live (flat blue cells) or dead (round blue cells). The cell killing activity of Bak in this assay was manifested by a large reduction in the number of blue cells obtained relative to co-transfection of the β-gal plasmid with a control expression vector (see text).

Detection of protein–protein interactions in vitro

GST and GST fusion proteins were expressed in *Escherichia coli* and purified by affinity chromatography using glutathione–agarose (GSH-beads) (Smith and Johnson, 1988). [^{35}S]Methionine-labeled proteins were synthesized *in vitro* using a coupled transcription/translation system (Promega) in rabbit reticulocyte lysates following the supplier's protocol. *In vitro* translation reactions were pre-cleared by mixing with 20 μl of bovine serum albumin (BSA)-washed GSH–agarose beads (50% slurry) at 4°C for 1 h in 0.1 ml 10 mM HEPES buffer, pH 7.2 containing 0.25% NP-40, 142.5 mM NaCl, 5 mM $MgCl_2$ and 1 mM EGTA (NP-40 lysis buffer). The beads were removed by centrifugation and the cleared supernatants were incubated either with GST or GST fusion proteins (final concentration 1 μM) at 4°C for 1 h. Protein complexes were recovered by incubation for 1 h with an additional 20 μl of GSH–agarose beads. The beads were washed twice with NP-40 lysis buffer followed by two washes with the same buffer without NP-40. Proteins were eluted from the beads by incubation in SDS–PAGE sample buffer at 100°C for 5 min and loaded onto 4–20% SDS–polyacrylamide gels. Following electrophoresis, gels were fixed and incubated in a fluorography enhancing solution (Amplify; Amersham). The gels were dried and subjected to autoradiography at −70°C.

Detection of protein–protein interactions in transfected cells

COS cells were seeded in 35 mm wells at a density of 2.0×10^5 cells/well. The cells were transfected with expression plasmids the following day by the Lipofectamine procedure (Life Technologies). Bak derivatives contained the HA epitope at their amino-termini; Bcl-x_L was tagged at its amino-terminus with the Flag epitope (Flag; Kodak). At 24 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and lysed in NP-40 lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin and 1 μg/ml leupeptin. The lysates were incubated with anti-HA antibody (12CA5, Boehringer Mannheim) for 1 h and 20 μl of BSA-washed protein A–agarose beads (50% slurry) for an additional hour. The beads were washed twice with NP-40 lysis buffer followed by two washes with buffer lacking NP-40. Proteins were eluted from the beads by incubation in SDS–PAGE sample buffer at 100°C for 5 min and loaded onto 14% SDS–polyacrylamide gels. Following electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore) and the membranes were blocked by incubation for 1 h with a 1% milk solution in PBS. Primary antibody (1 μg/ml 12CA5, Boehringer Mannheim; 28 μg/ml DAKO-bcl-2, 124, DAKO; 10 μg/ml anti-FLAG M2, Kodak) was incubated with the membranes for 1 h, followed by secondary antibody (0.8 μg/ml horseradish peroxidase-conjugated goat anti-mouse IgG; Jackson Laboratory) for an additional 1 h. Proteins were detected by enhanced chemiluminesence (ECL, Amersham) as described by the supplier.

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Note added in proof

The name of the protein referred to as Bip1 here has been changed to Bik (Boyd *et al.*, 1995). The GenBank accession number for Bik is U34584.