A Peptide Sequence from Bax That Converts Bcl-2 into an Activator of Apoptosis*

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Bcl-2 and Bax are members of a family of cytoplasmic proteins that regulate apoptosis. The two proteins have highly similar amino acid sequences but are functionally opposed: Bcl-2 acts to inhibit apoptosis, whereas Bax counteracts this effect. The antagonism appears to depend upon dimerization between Bcl-2 and Bax, but its mechanism is otherwise unknown. Here we report that overexpressing Bax induces apoptosis in a mammalian fibroblast cell line, and we identify a novel, short "suicide domain" in Bax that is required for this effect. Inserting this domain in place of the corresponding, divergent sequence in Bcl-2 converts Bcl-2 from an inhibitor into an activator of cell death. These findings imply that a specific region in Bax confers an active propensity for apoptosis in mammalian cells and support the view that Bcl-2 may block death primarily by suppressing Bax activity.

The form of programmed cell death known as apoptosis is controlled in part by a family of cytoplasmic proteins whose prototype is Bcl-2 (reviewed in Ref. 1). Members of this family share highly conserved amino acid sequences and the ability to dimerize with themselves and with one another but, despite these similarities, can have widely divergent biological effects (1-8). Overexpression of Bcl-2, for example, potently inhibits the apoptotic response induced by many different stimuli and in many different species and cell types. By contrast, the Bax protein, whose sequence is 52% similar to and 28% identical with that of Bcl-2, acts as a functional antagonist that can counteract the protective effects of Bcl-2. The reciprocal antagonism between Bcl-2 and Bax appears to depend upon their ability to form heterodimers, giving rise to the hypothesis that the relative abundance of homo- or heterodimers of these proteins regulates activity of a critical, distal step in the apoptotic pathway (8). However, the mechanism by which this interaction controls apoptosis is unknown, and, in particular, it has not been determined whether Bax plays an active role or merely functions as an inhibitor of Bcl-2.

Here we demonstrate that overexpression of Bax induces rapid apoptosis in the human fibroblast cell line GM701. By making a series of Bax/Bcl-2 chimeric proteins and testing their effect on these cells, we were able to isolate a region of Bax that is responsible for this effect. Furthermore, transfer of this region into a functional mutant of Bcl-2 converts Bcl-2 from an apoptotic antagonist into an apoptotic agonist. This region of Bax, which we term the "suicide domain," appears to mediate an active function in the induction of apoptosis.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmids—Human GM701 fibroblasts were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For light microscopy, 2 × 105 cells were plated onto glass coverslips in 35-mm plates and transfected with 10 μg of a plasmid encoding β-gal1 under control of the SV40 early promoter and either Bcl-2 (pS-fvBcl2-β-gal) or Bax (pS-fvBax-β-gal) under control of the spleen focus-forming virus (SFFV) long terminal repeat, in a Bluescript KS+ (Pharmacia Biotech Inc.) backbone. Cells were transfected by the calcium phosphate method for 6 h, washed twice with PBS containing 25 mM HEPES, then fed with fresh medium. At 24 h post-transfection, cells were fixed and stained as described (9), washed 3 times with PBS and once with distilled water, mounted with Aquaamount, and photographed with Nomarski optics. For quantitation of apoptosis, 2 × 105 cells were plated in 2 ml of media on Nunc 35-mm gridded plates and transfected in triplicate as above. After staining with X-gal, 200 blue cells per plate were counted and scored as normal or apoptotic (small with conspicuous blebbing). Plasmids encoding chimeric proteins were made using variants of pSFFVβ-gal-β-gal that encoded the constructs shown. These were prepared from a series of β2 deletion mutants (12) in which deletion residues had been replaced by a unique in-frame BglII site encoding the dipeptide Arg-Ser; the βax fragmenst were generated by PCR using primers that introduced flanking BglII ends, which were then ligated into the deletants. BaxA8 was created by PCR mutagenesis and contains an in-frame BglII site (encoding Arg-Ser) in place of the deletion. In some cases, an oligonucleotide encoding the epitope for anti-hemagglutinin monoclonal antibody 12CA5 (Boehringer Mannheim) was ligated onto the 3' ends of these constructs to facilitate Western blotting (12). BaxA8 was created by PCR mutagenesis and contains an in-frame BglII site (encoding Arg-Ser) in place of the deletion. All constructs were confirmed by DNA sequencing.

Western Blot Analysis—For Western blot analysis, 20 μg of plasmid was transfected into the quail QT6 fibrosarcoma line, which lacks endogenous immunoreactive β2-2, and extracts were prepared 40 h later and blotted as described (10), with all samples on a given gel containing extract from an equivalent number of cells. Blots were probed for 3 h at room temperature with either anti-Bcl-2 (Dako; specific for residues 41–54) at 1:200, or the anti-HA antibody 12CA5 at 1:2 in PBS containing 25 mM HEPES, then fed with fresh medium. At 24 h post-transfection, cells were fixed and stained as described (9), washed 3 times with PBS and once with distilled water, mounted with Aquaamount, and photographed with Nomarski optics. For quantitation of apoptosis, 2 × 105 cells were plated in 2 ml of media on Nunc 35-mm gridded plates and transfected in triplicate as above. After staining with X-gal, 200 blue cells per plate were counted and scored as normal or apoptotic (small with conspicuous blebbing). Plasmids encoding chimeric proteins were made using variants of pSFFVβ-gal-β-gal that encoded the constructs shown. These were prepared from a series of β2 deletion mutants (12) in which deletion residues had been replaced by a unique in-frame BglII site encoding the dipeptide Arg-Ser; the βax fragmenst were generated by PCR using primers that introduced flanking BglII ends, which were then ligated into the deletants. BaxA8 was created by PCR mutagenesis and contains an in-frame BglII site (encoding Arg-Ser) in place of the deletion. In some cases, an oligonucleotide encoding the epitope for anti-hemagglutinin monoclonal antibody 12CA5 (Boehringer Mannheim) was ligated onto the 3' ends of these constructs to facilitate Western blotting (12). BaxA8 was created by PCR mutagenesis and contains an in-frame BglII site (encoding Arg-Ser) in place of the deletion. All constructs were confirmed by DNA sequencing.

Stable expression of Bax is permissive for apoptosis, but does not necessarily induce death in vertebrate cells (2). In yeast, however, expression of human Bax triggers acute cell death which can be prevented by Bcl-2 (6, 7). We have observed a similar phenomenon in certain mammalian cell lines, including the human fibroblast line GM701, after transient transfection with an active bax gene. For these studies, GM701 cells were transfected with a β-galactosidase (β-gal) reporter gene together with either bax or bcl-2 on a single expression plasmid; 24 h after transfection, plates were stained for β-gal activity in order to identify transfected cells, which were then examined

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The abbreviations used are: β-gal, β-galactosidase; SFFV, spleen focus-forming virus; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; PCR, polymerase chain reaction; HA, influenza hemagglutinin epitope.
**FIG. 1.** *Overexpression of Bax induces apoptosis in GM701 fibroblasts.* A, X-gal staining of GM701 cells 24 h after transfection with a vector encoding β-gal along with either Bcl-2 (left) or Bax (right). Cells transfected with Bax show changes characteristic of apoptosis, including cell shrinkage and membrane blebbing. B, this effect of Bax is specifically inhibited by co-transfection with Bcl-2. 1 μg of pSffvBax-β-gal was co-transfected with 7 μg of pUC118 and 2 μg of either pSV40-Neo (left) or pSV40-Bcl2 (right). pSV40-Bcl2 encodes full-length human Bcl-2; the pSV40-Neo plasmid was used to control for promoter competition between the SV40 ori and the Sffv long terminal repeat. The data indicate the mean percentage of cell death ± S.D. for triplicate determinations.

**FIG. 2.** *Induction of apoptosis in GM701 cells by chimeric Bcl-2/Bax proteins.* A, schematic of the constructs tested. The Bax residues (black rectangles) in each construct are listed at left; numbers at right denote boundaries of Bcl-2 sequences (white rectangles). B, frequency of apoptosis in GM701 cells transfected with these constructs. Each was tested in two triplicate assays, both performed in a blind fashion. Data shown indicate the mean ± S.D. (n = 6). C, Western blot of constructs containing the Bcl-2 epitope. Bcl2Δ1, C2, and C4 are expressed at levels comparable to C8, demonstrating that their lack of effect on GM701 cells is not due to protein instability. None = untransfected QT6 cells. D, Western blot of HA-epitope-tagged derivatives of chimeras that lacked the Bcl-2 epitope, with C8-HA included for comparison. The ability of these constructs to kill cells was not affected by addition of the HA tag (data not shown) and likely accounts for their apparently low expression. The most potent apoptotic agonist, C3-HA, is not detectable. QT6 = untransfected QT6 cells.
under light microscopy. In repeated experiments, we found that 40–50% of β-gal-positive cells in Bax-transfected plates showed the typical morphologic features of apoptosis, including cell shrinkage, distinct membrane blebbing, and nuclear condensation (Figs. 1A and 2B). By contrast, these features were seen in only 3–5% of cells transfected with wild-type Bcl-2 (data not shown) or with Bcl2 Δ17, a severely truncated, nonfunctional Bcl-2 mutant (Fig. 2, A and B). This effect of Bax was greatly reduced by co-transfection of Bcl-2 (Fig. 1B). Thus, transient, high-level Bax expression in these cells specifically induces apoptosis that can be blocked by Bcl-2.

This phenomenon was exploited as an assay to map the peptide sequences responsible for the divergent effects of Bax and Bcl-2. Although the two proteins have closely related sequences, Bax lacks sequences corresponding to a highly conserved region at the N terminus of Bcl-2 (2). This region (residues 1–29) is absolutely required for Bcl-2 function (11), and its deletion results in a mutant which can dimerize with, and dominantly inhibit the activity of, wild-type Bcl-2 (12). When we tested this dominant negative Bcl-2 mutant (construct Bcl2 Δ11) in the GM701 assay, we found that it failed to induce death (Fig. 2), suggesting that cell killing did not result simply from inhibition of any endogenous Bcl-2-like proteins. Moreover, fusion of Bcl-2 residues 1–29 onto the N terminus of Bax (construct Bcl2/Bax) did not eliminate its cytotoxicity (Fig. 2).

We therefore asked whether sequences from Bax could alter the activity of Bcl-2. Using the GM701 assay, we tested six vectors encoding chimeric proteins in which various regions throughout Bcl-2 were replaced by their approximate counterparts from Bax (Fig. 2A, constructs C1–C10). The results demonstrate that a specific subset of Bax sequences can confer death-agonist activity when substituted into Bcl-2: chimeras C2 and C4 gave only background levels of cell killing, whereas C1, C3, C8, and C10 each induced death at or near the frequency seen with Bax (Fig. 2B). Western blots with anti-Bcl-2 antibody confirmed that constructs C2, C4, and Bcl2 Δ11 were expressed at levels comparable to that of C8 (Fig. 2C). Expression of C1, C3, and C10 was more difficult to compare directly, since they lacked the Bcl-2 epitope and killed the cells that expressed them, but a Western blot of epitope-tagged derivatives of these chimeras, expressed in QT6 cells and detected with anti-HA antibody, because some of these constructs induce apoptosis, expression appears inversely related to activity, and the most potent apoptotic agonist (Δ2C3-HA) is not detectable.

**Fig. 3.** The suicide domain of Bax is necessary for function. a, comparison of the C8 region in Bax with the corresponding region in Bcl-2, based on the Genetics Computer Group "Gap" program. b, a Bax mutant lacking residues 53–74 failed to induce apoptosis in GM701 cells (column 1) and also failed to inhibit function of full-length Bax in trans (column 3). pSffvBaxαC8-β-gal was transfected at 10 μg/plate (column 1). For the trans-inhibition assay, 1 μg of pSffvBax-β-gal was co-transfected with 7 μg of pUC118 and 2 μg of pSV40 (column 2), pSV40-BaxαC8 (column 3), or pSV40-Bcl2 (column 4). Data indicate mean percentage of cell death ± S.D. for triplicate determinations.

**Fig. 4.** Fragments of Bax cloned into a fully functional Bcl-2 mutant convert it into an apoptotic agonist. a, selected regions of Bax were cloned into Bcl2 Δ12, a 48-amino acid deletion mutant of Bcl-2 which blocks staurosporine-induced apoptosis of GM701 cells as efficiently as wild-type Bcl-2 (12). b, death-promoting activity of the resulting chimeras, and of the parental Bcl2 Δ12, assayed in GM701 cells as described for Fig. 1. Data indicate mean percentage of cell death ± S.D. for triplicate determinations. c, Western blot of HA-tagged variants of these chimeras, expressed in QT6 cells and detected with anti-HA antibody. Because some of these constructs induce apoptosis, expression appears inversely related to activity, and the most potent apoptotic agonist (Δ2C3-HA) is not detectable.
that induced cell death also included this sequence (Bax residues 55–77), which corresponds to a region that is minimally conserved between Bax and Bcl-2 (Fig. 3A). To determine whether this region is essential for the death-promoting effect of Bax, we tested the properties of a Bax mutant lacking residues 53–74 in the GM701 assay. As shown in Fig. 3B, this mutant, termed BaxΔC8, completely lacked Bax activity. When co-transfected along with full-length Bax, however, BaxΔC8 did not prevent cell killing, indicating that it is an inactive, recessive mutant and not a functional equivalent of Bcl-2.

We extended these results by inserting the active Bax sequences into a fully functional variant of Bcl-2. For this purpose, we utilized the deletion mutant Bcl2Δ2, which lacks the nonconserved residues 30–79 but inhibits apoptosis as potently as wild-type Bcl-2 in two well-characterized model systems, including staurosporine-treated GM701 cells (12, 13). As shown in Fig. 3, we found that Bcl2Δ2 did not cause cell death in our assay, but that insertion of the Bax sequences from C3, C7, or C10 in place of the deletion conferred potent death-promoting activity. Interestingly, the 23-residue Bax sequence from C8 did not confer death-agonist function onto Bcl2Δ2, implying that its activity is somewhat context-dependent. However, the C7 and C10 sequences, which each comprise C8 along with 22–23 additional Bax residues downstream or upstream, respectively, each produced chimeras that induced significant levels of cell death. Thus, inserting as few as 45 amino acid residues from Bax into a fully functional Bcl-2 completely reversed its effect on cell survival.

The family of proteins structurally related to Bcl-2 includes some members that inhibit, and others that promote, apoptotic death. The overall propensity of a cell to undergo apoptosis reflects the relative expression levels of these two protein classes (2, 4), whose functional antagonism appears to depend upon their ability to bind one another (4, 5). Because most previous studies have focused on the ability of Bax to counteract the protective effects of Bcl-2, it has not been clear whether Bax-like proteins have intrinsic death-promoting activity or simply function by blocking Bcl-2-like activity. Here we have demonstrated that Bax can induce killing of at least one vertebrate cell line directly; this killing does not result simply from inhibition of endogenous Bcl-2, since expression of a dominant negative Bcl-2 mutant fails to kill these cells (Fig. 2, construct Bcl2Δ1). Using this assay system, we have identified a short region within Bax that is essential for its killing activity and which, when substituted into an antiapoptotic or nonfunctional Bcl-2 mutant, converts these proteins into potent apoptotic agonists. These observations strongly indicate that this domain subserves an active function that is permissive for apoptosis in many cellular systems, and which may be required for Bcl-2-dependent induction of apoptosis in yeast (6, 7), as well as in the GM701 cells studied here. We designate this region, which may span as few as 23 amino acids (residues 55–77), as the suicide domain of Bax.

In another recent report, Chittenden et al. (16) show that a homologous region in the pro-apoptotic protein Bak (17–19) mediates the induction of programmed cell death in Rat-1 cells. The authors demonstrate that this region of Bak, as well as similar regions in Bax and Bik (20), are necessary for their ability to both bind other Bcl-2 family members and promote apoptosis. Of particular interest in this region is the BH3 domain, which is the only peptide sequence within Bik which shares high homology with that of Bax and Bak (20). Although Bcl-2 residues 97–105 resemble the BH3 domain closely enough to suggest a common function (see Fig. 3A), our finding that the activity of Bcl-2 variants containing residues 97–105 can be reversed by inserting the Bax domain (Fig. 4) suggests that the two sequences are not biologically equivalent.

Although the molecular function of the suicide domain remains to be determined, one possibility is that it serves to mediate the functional interaction of Bax, Bak, Bik, and perhaps other agonist proteins with a common distal factor in the apoptotic pathway. Moreover, our data are consistent with the proposal (2, 6) that Bcl-2 could promote survival simply by inhibiting Bax function, and they further suggest specific mechanisms by which this might occur. For example, if homodimerization of Bax (2, 4, 5) is necessary for activity of the suicide domain, Bcl-2 might inactivate this domain by competitively inhibiting Bax dimerization. Alternatively, this domain may be active in monomeric form but masked or inactivated when dimerized with Bcl-2 (16). In either case, our results suggest a molecular basis for the observation (14, 15) that apoptosis is the default state of many cell types, and that such cells survive by holding in check an active prodivicity for suicide.

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