

A Splicing Variant of the Bcl-2 Member Bok with a Truncated BH3 Domain Induces Apoptosis but Does Not Dimerize with Antiapoptotic Bcl-2 Proteins *in Vitro**

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Bok (Bcl-2-related ovarian killer) is a proapoptotic Bcl-2 family protein identified in the ovary based on its dimerization with the antiapoptotic protein Mcl-1. In addition to the Bcl-2 homology (BH) domains 1 and 2 and the transmembrane sequence, Bok also has a BH3 domain believed to be important for dimerization with selective antiapoptotic Bcl-2 proteins and cell killing. We identified a splicing variant of Bok mRNA with a deletion of 43 residues from the full-length protein (Bok-L), leading to the fusion of the N-terminal-half of its BH3 domain to the C-terminal-half of the BH1 domain. Genomic analysis indicated that the Bok has five exons, and the short form of Bok (Bok-S) represents the splicing out of exon three during transcription. Although Bok-S retains the apoptosis-inducing activity in transfected cells, it has lost the ability to dimerize with antiapoptotic proteins *in vitro*. Additional BH3 domain mutations of Bok-L also led to defective heterodimerization without affecting its proapoptotic action. Furthermore, similar deletions for the related channel-forming proapoptotic Bax and Bak did not impair their cell killing ability. Thus, the naturally occurring Bok-S variant represents a new form of proapoptotic protein that induces cell killing without heterodimerization with antiapoptotic Bcl-2 proteins. This variant appears to contain the minimal module spanning BH1 and BH2 domains and the transmembrane sequence for apoptosis induction by channel-forming Bcl-2 proteins.

Apoptotic cell death is critical for the maintenance of tissue homeostasis in a healthy organism as well as for pathogenesis during diseased states including cancer, neurodegenerative disorders, autoimmune diseases, and viral infection (1). Although the precise mechanisms by which apoptosis is regulated remain unknown, it is clear that programmed cell death in diverse animals is controlled by an evolutionarily conserved cellular machinery involving a set of genes including ced-3/caspase, ced-4/Apaf-1, and ced-9/Bcl-2 (1–4).

Bcl-2 family proteins, the mammalian ced-9 counterparts, share conserved Bcl-2 homology (BH)¹ domains and a C-terminal hydrophobic transmembrane (TM) region. Bcl-2 and its

related homologues, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1, have been shown to prevent apoptosis triggered by a diverse array of stimuli (5). Unlike these antiapoptotic Bcl-2 members, several proapoptotic Bcl-2 family members (Bax, Bak, Bik, BAD, BID, Bim/BOD, Hrk, and Bok) not only antagonize the survival action of antiapoptotic Bcl-2 proteins but also actively trigger apoptosis in transfected cells (5–14). Because many Bcl-2 family proteins are known to heterodimerize with other Bcl-2 proteins, it has been proposed that apoptosis is regulated by the balance of the action of anti- and proapoptotic Bcl-2 proteins present in a given cell (6). Recent studies using crystallography, computer modeling, and membrane potential recording have established that the amphipathic BH3 domain in some proapoptotic Bcl-2 proteins might regulate apoptosis by binding to a hydrophobic cleft formed by the conserved BH1, 2, and 3 domains in the antiapoptotic Bcl-2 proteins, represented by Bcl-2 and Bcl-xL (15, 16). Furthermore, the region spanning BH1 and BH2 domains of Bcl-2 proteins is important for pore formation in the artificial membrane and could function as ion channels in the mitochondria and other subcellular membrane organelles (2, 16–19).

Recently, we isolated a new proapoptotic Bcl-2 family member, Bok, found to be highly expressed in the ovary, testis, and uterus (20). Bok shares the conserved domains BH1, 2, and 3 with other proapoptotic proteins but lacks the BH4 domain found in some antiapoptotic proteins. Unlike several proapoptotic Bcl-2 proteins, Bok does not interact with Bcl-2 and Bcl-xL but preferentially dimerizes with antiapoptotic proteins Mcl-1, Bfl-1, and Epstein-Barr virus (EBV)-derived BHRF1. Although the mechanism by which Bok promotes cell death is unknown, its distinct heterodimerization property and tissue distribution pattern is consistent with the hypothesis that apoptosis is regulated by tissue-specific Bcl-2 protein pairs (20). Because Bok is homologous to Bax and Bak in BH domain arrangement and secondary structure, the Bok protein could regulate apoptosis through dimerization with antiapoptotic proteins, a mechanism originally proposed for the proapoptotic proteins Bax and Bak (6–9).

During the analysis of Bok mRNA and gene structure, we found a Bok splicing variant in which the region encoded by exon three is absent, suggesting the existence of a truncated short form (Bok-S) of the full-length Bok protein (Bok-L). The skipping of exon three maintained the original reading frame and retained the BH2 and the C-terminal membrane-anchoring domains; however, parts of the BH3 and BH1 domains were deleted. Of interest, functional analysis indicated that Bok-S is still capable of inducing apoptosis, suggesting that an intact BH3 domain of Bok is not essential for apoptosis regulation. In

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¹ The abbreviations used are: BH, Bcl-2 homology; Bok, Bcl-2-associated ovarian killer; Bok-L, Bok-long; Bok-S, Bok-short; PCR, polymerase chain reaction; TM, transmembrane; AD, activation domain; BD,

binding domain; ORF, open reading frame; bp, base pair(s); CHO, Chinese hamster ovary.

addition, the truncated Bok has lost its ability to heterodimerize with Mcl-1, BHRF-1, and Bfl-1, suggesting that the proapoptotic activity of this variant could not be mediated by its binding to antiapoptotic Bcl-2 proteins. Similar to Bok-S, mutants of related proapoptotic proteins Bax and Bak with their homologous BH3-BH1 region deleted also retained proapoptotic activity, suggesting a common mechanism of action shared by this subgroup of proapoptotic proteins. Our results define a novel splicing form of the Bok gene and demonstrate that this naturally occurring variant without a conserved BH3 domain could induce apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins *in vitro*.

EXPERIMENTAL PROCEDURES

Reverse Transcription-PCR of the Bok-S Transcript—Total RNA from different tissues was isolated from 27-day-old Sprague-Dawley rats using an anion exchange resin chromatographic column (Qiagen, Chatsworth, CA) before reverse transcription with oligo(dT)18 as primer in a reaction containing RNase H-free reverse transcriptase from Moloney murine leukemia virus (CLONTECH). For PCR amplification of Bok cDNAs, aliquots of DNA equivalent to 0.5 μ g total RNA were used in each reaction (50 μ l). To minimize contamination during PCR, control reactions containing a single primer or RNA without reverse transcriptase were routinely performed. All PCR was carried out under high stringency conditions (94 °C 45 s, 68 °C 45 s, 72 °C 4 min) for 30 cycles.

Isolation of Bok Genomic DNA, Southern Blot Hybridization, and Genomic Analysis—A genomic DNA fragment was isolated from a mouse BAC genomic DNA library (Genome Systems Inc., St. Louis, MO) using the full-length Bok cDNA probe. The Bok genomic fragment was first analyzed by restriction enzyme mapping, followed by subcloning into the pUC18 vector for dideoxy sequencing analysis of both DNA strands. Overlapping clones were isolated to define the direction of individual clones and to facilitate assignment of intron-exon junctions. For Southern blot hybridization analysis, genomic DNA (10 μ g) was digested with indicated restriction enzymes, separated by electrophoresis on a 0.8% agarose gel and then transferred onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). Hybridization was performed in the QuickHyb buffer (CLONTECH) at 60 °C with ³²P-labeled cDNA probes. The filters were washed with 0.1 \times SSC and 0.5% SDS at 65 °C before exposure.

Generation of Mutant Constructs—Specific mutations in the BH3 domain of Bok-L were generated by a two-step PCR mutagenesis method using a Bok cDNA template as described previously (21). The resulting PCR products were evaluated for correct size on a 1% agarose gel, purified, and subcloned into the *Eco*RI site of the pcDNA3 vector for mammalian cell expression (Invitrogen, Inc., San Diego, CA). Truncated Bax and Bak constructs (Bax-S and Bak-S) with homologous deletion of the BH3-BH1 region found in Bok-S were also generated using two-step overlapping PCR and subcloned into the *Eco*RV site of the pcDNA3 vector for eukaryotic cell expression (21). For the yeast two-hybrid assay, mutant Bok-L cDNAs were subcloned into the pGADGH expression vector. Restriction mapping and dideoxy sequencing confirmed proper orientation and the authenticity of the inserts.

Yeast Two-hybrid Assay—To study dimerization between different Bcl-2 family proteins and variants or mutants of Bok, cDNAs for Bok-L, Bok-S, or Bok mutants were fused to the activation domain (AD) of GAL4 in a yeast shuttle vector pGADGH. Complementary DNAs encoding different Bcl-2 proteins were fused to the GAL4-binding domain (BD) of pGBT9. After transformation of yeast cells, colonies containing different protein pairs were selected on plates lacking tryptophan and leucine (22). To test for specific protein-protein interactions, positive transformants were further selected for growth in media deficient for tryptophan, leucine, and histidine but containing 5–30 mM 3-aminotriazole to inhibit endogenous histidine production. A minimum of five independent transformants containing each pair of fusion cDNAs were routinely analyzed.

Analysis of Apoptosis in Transfected CHO Cells—Apoptosis was monitored following transfection of different cDNAs as described previously (20). CHO cells (2×10^5 cells/well) were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine. One day later, cells were transfected using the LipofectAMINE procedure (Life Technologies, Gaithersburg, MD) with the pcDNA3 expression vector with or without different cDNA inserts, together with $\frac{1}{10}$ to $\frac{1}{50}$ amounts of an indicator plasmid pCMV- β -gal to allow the identification of transfected cells. Inclusion of a 10-fold excess of expression vectors as

compared with the pCMV- β -gal reporter plasmid ensured that most of the β -galactosidase-expressing cells also expressed the protein(s) under investigation. Cells were incubated with plasmids in a serum-free medium for 4 h, followed by the addition of fetal bovine serum to a final concentration of 5% and further incubation for 14 h. After an additional culture in fresh medium for 18 h, cells were fixed by using 0.25% glutaraldehyde and stained with X-gal to detect β -galactosidase expression (20). The number of blue cells was counted by microscopic examination. Data are expressed as the percentage (mean \pm S.E.) of viable cells as compared with the control group.

In Vitro Direct Protein-binding Assay—To further demonstrate the specificity of interactions between Bok variants and antiapoptotic proteins, direct protein-protein interactions were studied using recombinant Bok and FLAG-tagged BHRF-1 translated *in vitro*. [³⁵S]methionine labeled or nonlabeled proteins were generated using the TNT coupled reticulocyte lysate system (Promega, Madison, WI). Pairs of proteins were incubated in the binding buffer (phosphate-buffered saline, 0.2% Nonidet P-40, and protease inhibitor mixture; Sigma) for 2 h at 4 °C followed by incubation with 1.5 μ g of M2 antibody against the FLAG tag (Kodak, Rochester, NY) under gentle agitation. The complexes formed between the antibody and recombinant proteins were precipitated with Protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and resolved using 12–15% SDS-PAGE. Following fixation, gels were treated with Amplify fluorographic agents (Amersham Pharmacia Biotech) before exposure to x-ray films.

Statistical Analyses and Sequence Analysis—One-way analysis of variance followed by Scheffe's F-test was used to determine the statistical significance of cell viability employing the STATVIEW software (Abacus Concepts, Inc., Berkeley, CA). The hydropathicity of protein sequence was analyzed using Biology Workbench version 2.0.²

RESULTS

Existence of Long and Short Bok Splicing Variants in Reproductive Tissues—We amplified Bok cDNA from a rat ovarian cDNA library using primers flanking the open reading frame (ORF) of Bok. A 513-bp PCR product was obtained in addition to the predicted 642-bp band. DNA sequencing of the lower molecular weight product indicated it was identical to that of the Bok cDNA except that nucleotides encoding amino acid 76–118 were missing. This short transcript (Bok-S) encoded a 170-amino acid polypeptide, and the deletion of 43 amino acids from the full-length 213 amino acid protein (Bok-L) led to the fusion of the N-terminal half of the BH3 domain to the C-terminal half of the BH1 domain (Fig. 1A). To confirm the authenticity of this variant, reverse transcription-PCR was performed using total RNA from different tissues. Electrophoresis analysis confirmed the presence of a PCR product of 642 bp in the ovary, uterus, and testis, tissues known to express the Bok transcript (Fig. 1B). In addition, a lower band of 513 bp was found in the ovary, less in the uterus, and absent from the testis. Negative control reactions using only a single primer or RNA without reverse transcription did not generate any products. Subsequent subcloning and sequencing confirmed that the 642- and 513-bp bands encode the expected Bok-L and Bok-S transcripts, respectively.

The deduced amino acid sequence of Bok-S showed that a presumptive alternative splicing led to the disruption of both BH1 and BH3 domains of Bok-L, changing the original BH3 sequence ⁷¹LLRLGDELEQIR⁸² to ⁷¹LLRLGITWGVV⁸² (Fig. 1C). However, Kyte-Doolittle hydropathicity analysis suggested that the hydropathicity profile of the BH3/BH1 fusion region found in Bok-S did not differ substantially from that of the original BH1 domain in Bok-L (Fig. 1D). Furthermore, the α 5 and α 6 regions predicted, based on their homology to similar regions in Bax and Bak (19), were unaltered in the truncated Bok-S (Fig. 1E). These regions have been postulated to be important for channel formation in the mitochondria by different Bcl-2 proteins.

Bok Gene Arrangement and the Derivation of Alternative

² Obtained from <http://biology.ncsa.uiuc.edu/BW/BW.cgi>.

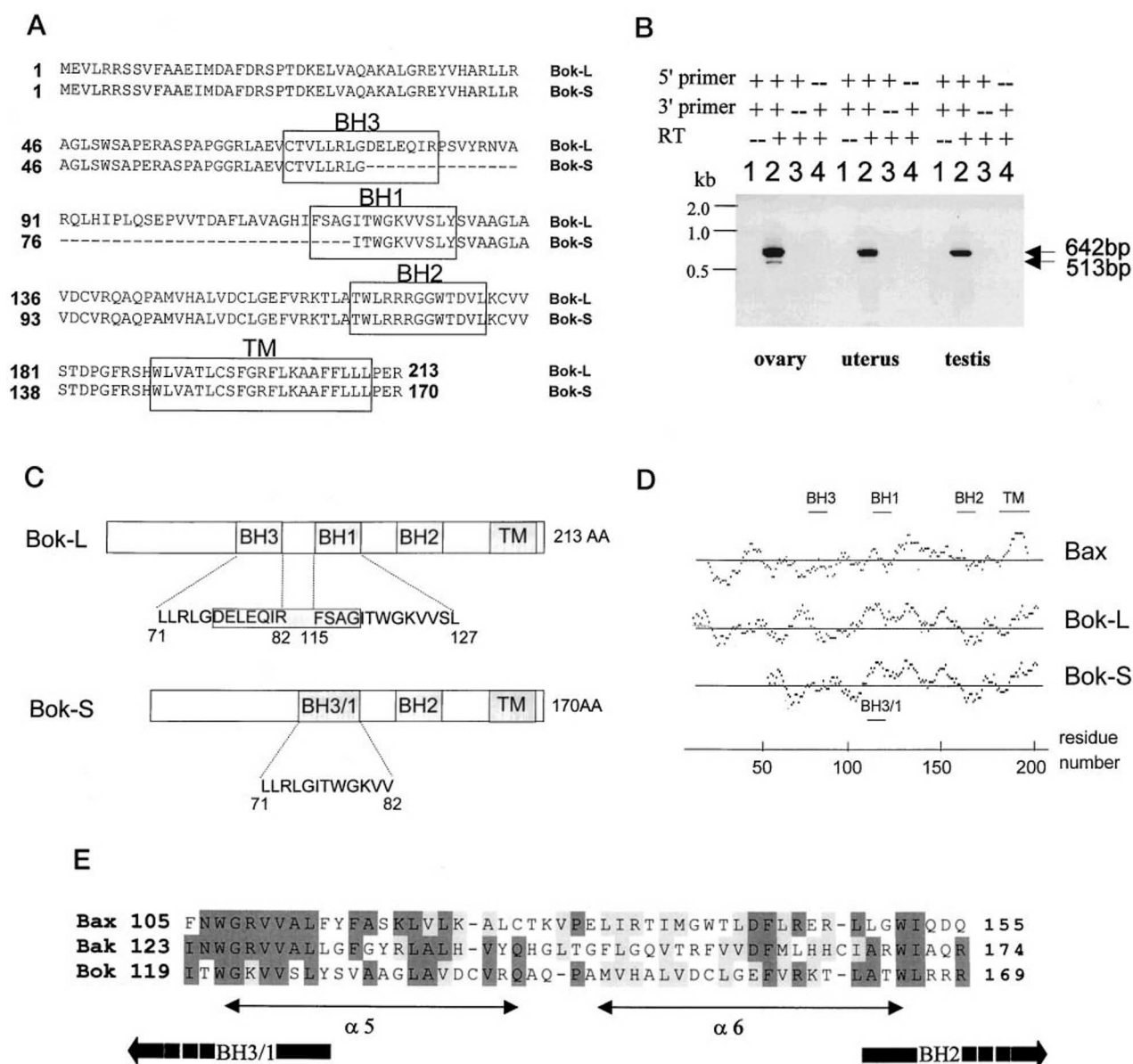


FIG. 1. Identification of long and short forms of Bok transcripts in the rat ovary and uterus. *A*, amino acid sequence alignment of Bok-L and Bok-S. The 43 amino acids deleted in Bok-S are represented by *dashes*. Numbering of amino acid residues is indicated on the *left*, whereas the BH and TM domains are *boxed*. *B*, expression of Bok splicing variants based on reverse transcription-PCR from ovarian, uterine, and testicular RNA preparations. The cDNA fragment corresponding to Bok-L (642 bp) and Bok-S (513 bp) are indicated by *arrows*. Reactions with a single primer or without the reverse transcriptase (RT) enzyme are included as negative controls. *C*, changes in the BH3 domain following alternative splicing. The new motif derived from the fusion of BH1 and BH3 domains is designated as BH3/1. Numbering of amino acid in the BH1 and BH3/1 domains are indicated at the *bottom* of amino acid residues. *D*, hydropathicity profile of Bax, Bok-L, and Bok-S. *Horizontal bars* indicate regions corresponding to the putative BH domain. Numbering of amino acid residues are shown below the profile charts. *E*, alignment of the channel-forming region in proapoptotic Bcl-2 proteins Bax, Bak, and Bok. The putative $\alpha 5$ and $\alpha 6$ helical regions are indicated (19). Numbering of amino acid residues for each protein is indicated, and conserved residues are *shaded*.

Splicing Variants—To elucidate the mechanism by which two Bok isoforms were generated, the Bok gene and its exon/intron junctions were analyzed. Following the screening of a bacterial artificial chromosome-based mouse genomic DNA library using a mouse Bok cDNA fragment, one genomic clone for Bok was isolated. The amino acid sequence of the coding region for the mouse clone was found to be identical to its rat counterpart. Southern blot hybridization of mouse genomic DNA digested with different restriction enzymes using cDNA probes corresponding to two different regions of the Bok gene demonstrated the presence of a single Bok gene in the mouse (Figs. 2, *A* and *B*). Further characterization of the genomic clone indicated that the entire Bok gene spanned 11 kilobases and consisted of five exons (Fig. 2*C*). The Bok ORF was encoded by sequences in

exons II–V, whereas exon I contained only untranslated sequences. Comparison of the ORF of Bok-S with genomic sequences indicated that Bok-S was derived following the splicing out of exon III.

Bok-S Promotes Cell Death in Transfected Cells—Because the fusion of BH3 and BH1 domains in Bok-S eliminated the conserved BH3 domain known to be important for apoptosis induction in a variety of proapoptotic Bcl-2 proteins (2), the resultant polypeptide could lose its apoptosis-inducing activity and function as a dominant-negative regulator. To study the role of Bok-S in apoptosis regulation, expression vectors containing Bok-S in either sense or antisense orientation were constructed. As shown in Fig. 3*A*, transfection of CHO cells with either Bok-S or Bok-L, but not the reverse construct,

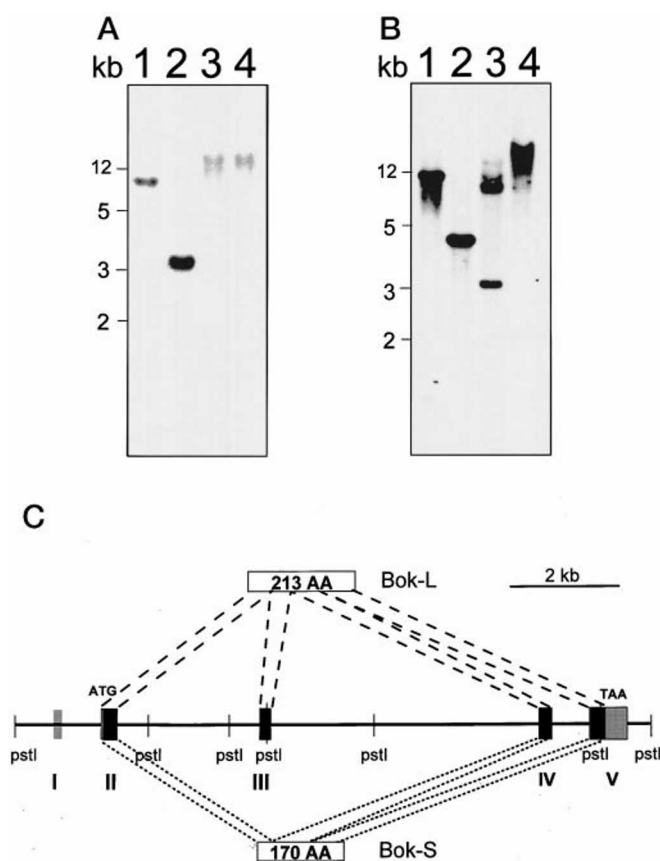


FIG. 2. **Genomic arrangement of Bok in the mouse.** In Southern blot analysis, mouse genomic DNA (10 μ g) was digested with the indicated restriction enzymes and probed with the *Pst*I fragment containing exon III of Bok gene (A) or a 345-bp cDNA fragment (Bok-L nucleotide 298 to 642) (B). Lanes 1, 2, 3, and 4 represent samples digested with *Bam*HI, *Nco*I, *Xba*I, and *Xho*I, respectively. The positions of the DNA marker are indicated at left. C, exon/intron arrangement of the Bok gene. The exons are numbered as Roman numerals, and the coding regions are indicated by dark filled boxes. The untranslated sequences in exons I and V are indicated by gray filled boxes. Bok-S (170 amino acid long) is likely derived from exon III skipping. Cleavage sites for the *Pst*I restriction enzyme are indicated.

significantly reduced the number of transfected cells, suggesting that Bok-S retained its ability to induce apoptosis despite the loss of the BH3 sequence. In addition, cell killing induced by either Bok-S or Bok-L was antagonized by cotransfection with P35, a baculovirus-derived caspase inhibitor (23).

Bok-S Does Not Heterodimerize with Antiapoptotic Bcl-2 Proteins—Because Bok was isolated based on its ability to bind Mcl-1 and the dimerization between pro- and antiapoptotic Bcl-2 proteins has been suggested to be important in apoptosis (2, 5), we analyzed whether Bok-S that maintained its cell killing ability could still dimerize with antiapoptotic Bcl-2 proteins. In the two-hybrid assay, interactions between Bok-S and different Bcl-2 family members were tested. As shown in Fig. 3B, Bok-S did not interact with any Bcl-2 proteins tested, whereas Bok-L interacted strongly with Mcl-1, Bfl-1, and BHRF-1, as previously reported (20). To further confirm findings in yeasts, a direct protein-protein interaction assay was performed using *in vitro* translated recombinant Bok variants and the antiapoptotic protein BHRF-1 that exhibited strongest interaction with Bok-L in yeast. As showed in Fig. 3C, BHRF-1 interacted strongly with Bok-L *in vitro* but showed negligible interaction with Bok-S. Thus, heterodimerization of Bok-S with antiapoptotic Bcl-2 proteins is probably not needed for apoptosis induction.

BH3 Mutants of Bok Defective in Heterodimerization Still

Retain Proapoptotic Activity—Because Bok-S lacking a BH3 domain still retained its cell killing potential, we hypothesized that the BH3 domain might be dispensable for the proapoptotic activity of Bok-L. Bok-L cDNAs with alanine or glycine substitutions in the BH3 domain were constructed, and the ability of these mutants to promote apoptosis was studied. As shown in Fig. 4A, the mutants included alanine substitutions at the highly conserved glycine 75 or glycine 75 plus flanking aspartic acid 76 and glutamic acid 77 (BokADE: G⁷⁵A and BokAAA: G⁷⁵AAA⁷⁷). In addition, a glycine substitution was made for leucine 71 to leucine 74 (BokGGGG: G⁷¹LLRL⁷⁴ to G⁷¹GGGG⁷⁴). As shown in Fig. 4A, transfection of these Bok-L mutants reduced the number of viable CHO cells as compared with the group with cells transfected with the pcDNA3 vector without an insert. In contrast, constructs with mutant cDNAs in reverse orientation had no effect on cell survival. These data suggested that the BH3 domain in Bok-L is dispensable for apoptosis induction. We further tested the ability of these BH3 domain mutants of Bok-L to dimerize with antiapoptotic Bcl-2 proteins in the yeast two-hybrid assay. As shown in Fig. 4B, substitution of residues in the BH3 domain of Bok-L abolished its interaction with Mcl-1 or Bfl-1. In addition, the ability of Bok-L to interact with BHRF-1 was also abated by glycine substitution at residues 71–74 of Bok-L. Similar to findings using the two-hybrid assay, *in vitro* translated BokGGGG mutant also lost its ability to interact with Bok-L effectively in the direct protein-protein interaction test (Fig. 4C). These data suggested that the cell killing ability of these BH3 mutants is not correlated to their ability to dimerize with antiapoptotic Bcl-2 proteins.

Mutants of Bax and Bak with Deletion of Their BH3 Domain Resembling Bok-S Also Retain Proapoptotic Activity—Because Bok-L is similar in structure to two other proapoptotic proteins, Bax and Bak, deletion mutants with truncation of the BH3-BH1 region similar to that found in Bok-S were constructed for these proteins and named as Bax-S and Bak-S (Fig. 5A). As shown in Fig. 5B, full-length Bak and Bax, like Bok-L and Bok-S, effectively reduced cell viability in the CHO cell transfection assay. Of interest, overexpression of Bax-S or Bak-S also significantly reduced the viability of transfected cells, suggesting that the BH3-BH1 regions deleted in these two proapoptotic proteins are not essential for apoptosis induction.

DISCUSSIONS

We have identified a naturally occurring variant of Bok with proapoptotic activity but exhibiting negligible dimerization with antiapoptotic Bcl-2 members. Bok-S with a 43-amino acid deletion between the BH3 and BH1 domains was likely the result of alternative mRNA splicing, leading to the skipping of exon three during post-transcriptional modification. Analysis of Bok variants and Bok mutants with alterations in the BH3 domain indicated that the BH3 domain of Bok-L is critical for heterodimerization but dispensable for apoptosis induction. Likewise, similar deletions between BH3 and BH1 domains of the homologous proapoptotic proteins Bax and Bak also retained cell killing ability. Thus, Bok-L could promote apoptosis independent of heterodimerization, and Bok-S represents a novel proapoptotic Bcl-2 member capable of inducing cell death without binding to or interference by antiapoptotic Bcl-2 partners. This functional Bok variant with retention of the region spanning BH1 and BH2 domains and the TM sequence provides a unique model for further studies of apoptosis mechanisms regulated by Bcl-2 family proteins.

The bifunctional antiapoptotic Bcl-2 proteins play a pivotal role in the decision step of apoptosis (2). These proteins, represented by Bcl-xL, maintain a channel structure important in the control of mitochondrial membrane potential and volume

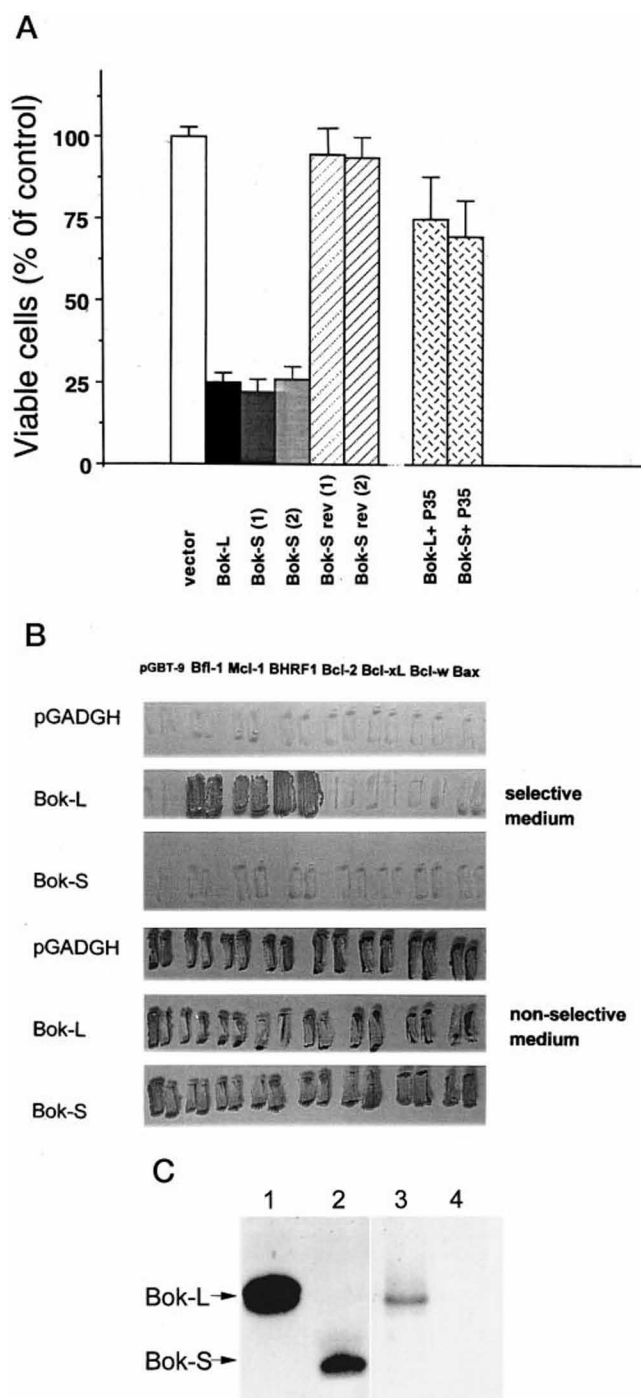


FIG. 3. Comparison between short and long forms of Bok in apoptosis induction. A, Bok-S, like Bok-L, induces apoptosis following overexpression in CHO cells. The number of viable cells were determined following transfection of the pcDNA3 vector with or without an insert encoding Bok-L, Bok-S or Bok-S in reverse orientation (*rev*). Some cells were transfected with Bok variants together with a plasmid encoding the baculoviral antiapoptotic protein P35. All values represent the mean \pm S.D. from triplicate cultures. The result shown is representative of at least three independent experiments. B, yeast two-hybrid assay of protein-protein interactions between Bok variants and different Bcl-2 family proteins. No interaction was observed between Bok-S and all Bcl-2 proteins studied. Top and bottom panels showed the growth of yeast on selective and nonselective media, respectively. C, direct interaction between BHRF-1 and Bok-L but not BHRF-1 and Bok-S *in vitro*. *In vitro* translated FLAG-BHRF-1 protein binds strongly with radiolabeled Bok-L but shows negligible interaction with Bok-S *in vitro*. Signals for Bok proteins are indicated by arrows. Lanes 1 and 2 show the migration of *in vitro* translated radiolabeled Bok-L and Bok-S, respectively. Lanes 3 and 4 indicate coprecipitation of Bok-L, but not Bok-S, with FLAG-BHRF-1 using the M2 antibody.

homeostasis (2, 24). Regulation of these channels controls the release of cytochrome C, essential for the activation of Apaf-1, and caspases, important for apoptosis execution (25–28). The antiapoptotic Bcl-2 proteins also function as docking proteins for proapoptotic Bcl-2 members (2). Because several mutants of Bcl-2 and Bcl-xL lost both antiapoptotic activity and the ability to bind proapoptotic Bcl-2 proteins, it is believed that dimerization of Bcl-2 protein pairs mediated by the consensus BH domains is important in apoptosis regulation. Crystallographic studies and computer modeling showed that the conserved BH1, BH2, and BH3 domains of Bcl-xL and related proteins constitute an elongated hydrophobic cleft (15, 29) capable of interaction with the amphipathic helix formed by BH3 domains of proapoptotic partners. Upon heterodimerization, anti- and proapoptotic Bcl-2 partners antagonize the actions of the other (29). It is likely that one of the mechanisms by which Bok-L exerts its proapoptotic action is through dimerization with antiapoptotic partners (20).

Mammalian proapoptotic Bcl-2 proteins can be divided into two subgroups based on domain arrangement. Together with Bax and Bak, Bok-L belongs to the first subgroup showing the conserved BH1, BH2, BH3, and TM domains. In contrast, members of the second subgroup (BAD, BID, Hrk/DP5, Bik/Nbk, and Bim/BOD) possess only the BH3 domain, with or without the TM region (2, 9–14, 30). Earlier studies suggested that proapoptotic proteins function by antagonizing the action of antiapoptotic proteins mediated by BH3 domains. Mutations in the BH3 domain of proapoptotic proteins abolished their dimerization with antiapoptotic partners and cell killing activity (10–14, 31–33). In addition, polypeptides containing minimal BH3 domain sequences bind antiapoptotic proteins (29, 31, 34) and induce apoptosis in transfected cells or cell-free systems (31, 35). More recent studies, however, demonstrated that Bax, like Bcl-xL and Bcl-2, also shows intrinsic ion channel activity in the artificial membrane (19, 36). In addition, mutations in the BH1, 2, or 3 domains of Bax do not affect its ability to promote apoptosis (37, 38). Likewise, Bak mutants accelerate chemotherapy-induced apoptosis independent of its heterodimerization property (39). These data suggest that the first subgroup of proapoptotic proteins, including Bax, Bak, and Bok, could induce apoptosis through channel formation in addition to their role as ligands for antiapoptotic Bcl-2 proteins (2, 16–19, 36). Because the second BH3-only subgroup members lack the region spanning BH1 and BH2 domains important for pore formation and mainly reside in the cytoplasm, they are believed to serve as ligands or facilitators of the pore forming Bcl-2 proteins.

Our findings that substitution of conserved residues in the BH3 domain of Bok-L abates its ability to dimerize with antiapoptotic proteins are in accord with studies on the BH3 domain of its proapoptotic homologues (31, 33, 40–42). Similarly, truncation of the conserved BH3 domain in the naturally occurring Bok-S variant also disrupted heterodimerization but retained cell killing ability, indicating the BH3 domain is dispensable for apoptosis induction. Thus, Bok-S represents a new form of proapoptotic protein consisting of only minimal functional modules and manifesting proapoptotic action without direct interactions with antiapoptotic proteins. As shown in Fig. 1, C–E, truncation of the region between BH3 and BH1 from Bok-L does not affect the homologous $\alpha 5$ and $\alpha 6$ regions proposed to be important for channel formation in Bax (19). In addition, the hydrophobicity property between the 5'-end of the BH1 region and the C-terminal TM domain is not altered by the truncation found in Bok-S. It is likely that the BH3/1, BH2, and TM domains found in Bok-S comprise a module sufficient for mediating apoptosis through a heterodimerization-inde-

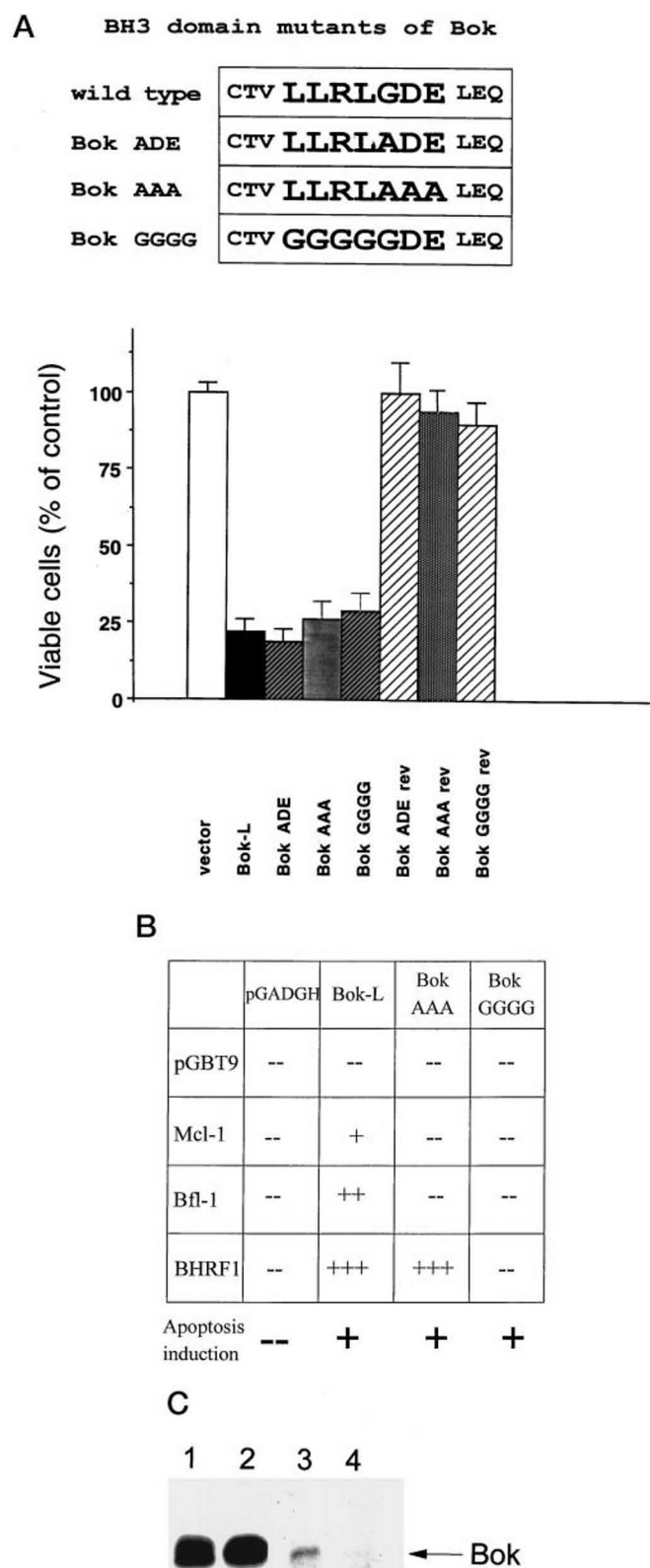


FIG. 4. Mutations in the BH3 domain of Bok-L did not affect its cell killing ability but prevented dimerization with antiapoptotic Bcl-2 proteins. A, apoptosis induction by wild type Bok-L and its BH3 domain mutants in CHO cells. Schematic representation of BH3 domain sequences in wild type Bok-L and different mutants (top panel). The alanine or glycine substitution in the BH3 domain mutants is indicated. Cell viability represents the percentage of viable transfected cells in each treatment group as compared with controls (vector alone). All values represent the mean \pm S.D. from triplicate cultures. The result shown is representative of at least three independent experiments. B, loss of heterodimerization between Bok-L mutants and the

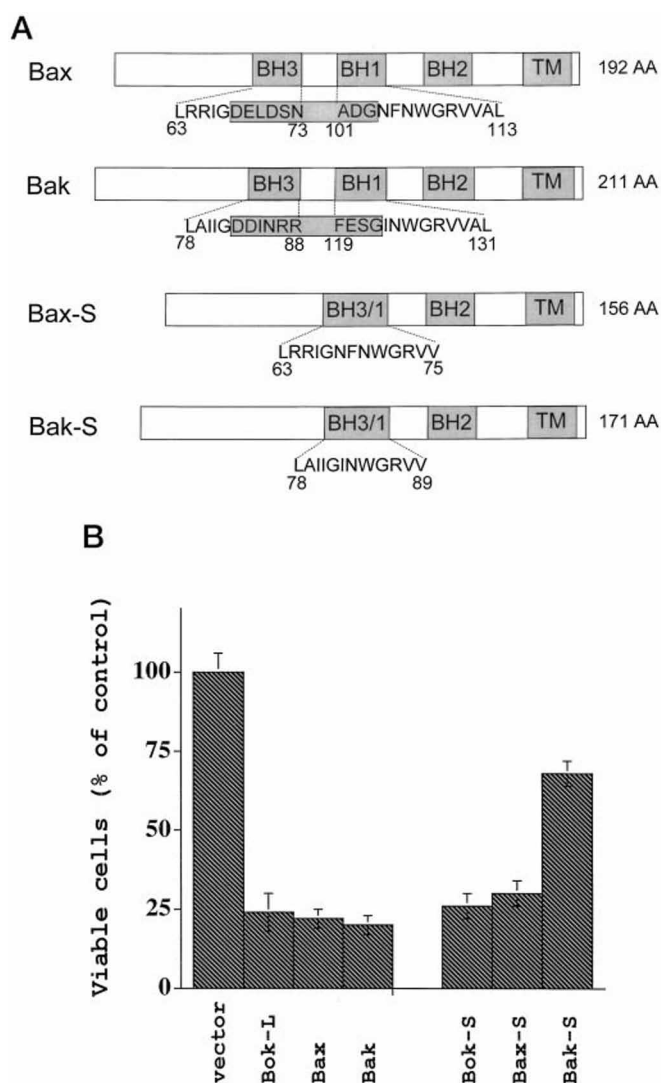
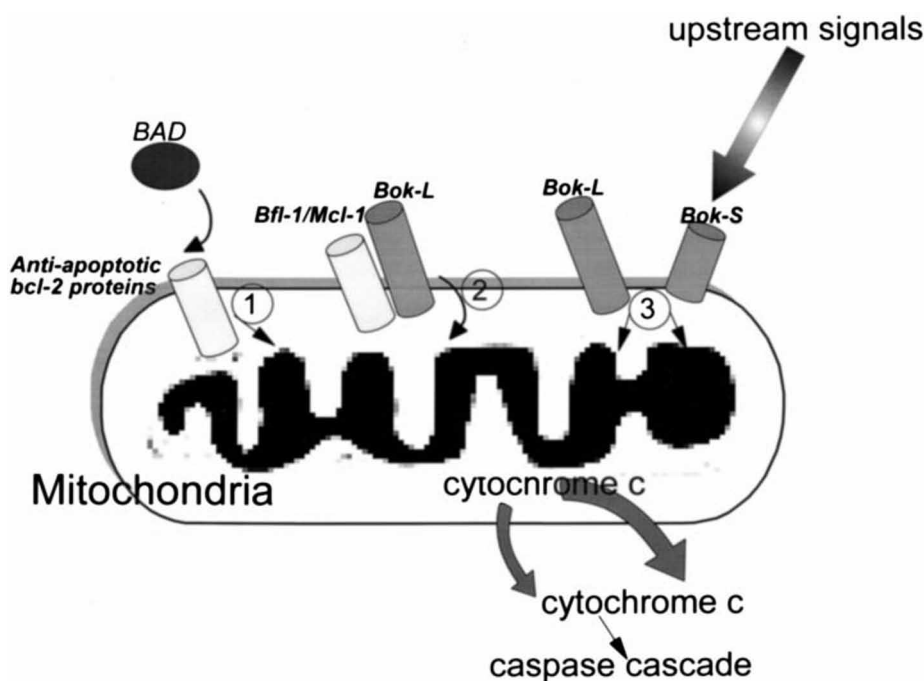


FIG. 5. Bax-S and Bak-S with deletion of the BH3-BH1 region retain proapoptotic activity. A, schematic representation of wild type Bax and Bak together with Bax-S and Bak-S constructs with BH3-BH1 deletions similar to that found in Bok-S. The BH domains are boxed, and the junctional sequences derived from the fusion of BH3 and BH1 domains (BH3/1) in the mutants are also shown. Numbering of amino acid in the BH1, BH3, and BH3/1 domains are indicated at the bottom of amino acid residues. B, apoptosis induction by wild type Bax and Bak as well as Bax-S and Bak-S in CHO cells. Cell viability represents the percentage of viable transfected cells in each treatment group as compared with vector controls or cells expressing Bok-L or Bok-S. All values represent the mean \pm S.D. from triplicate cultures. The result shown is representative of three independent experiments.

pendent pathway. Future studies on the channel-forming property of the naturally occurring Bok-S and other channel-forming Bcl-2 proteins are important for understanding the mechanisms of apoptosis. The channel-forming hypothesis is

antiapoptotic proteins Bfl-1, Mcl-1, and BHRF1 in the yeast two-hybrid assay. The results are summarized as strong (+ signs) or negligible (– signs) interactions. C, mutations in the BH3 domain of Bok-L interrupted its interaction with BHRF-1. *In vitro* translated Bok-L coprecipitated with FLAG-tagged BHRF-1 *in vitro*, whereas the BokGGGG mutant showed negligible interaction with BHRF-1. Signals for radio-labeled Bok proteins are indicated by the arrow. Lanes 1 and 2 showed *in vitro* translated labeled Bok-L and BokGGGG, respectively. Lane 3 indicated coprecipitation of Bok-L with FLAG-BHRF-1 using the M2 antibody. In contrast, BokGGGG was not coprecipitated with FLAG-BHRF-1 (lane 4).

FIG 6. Model of apoptosis regulation by proapoptotic Bcl-2 family proteins. At least three mechanisms could be involved in the action of proapoptotic Bcl-2 proteins. 1) The subgroup of proapoptotic Bcl-2 proteins with only the BH3 domain, represented here by the soluble BAD protein, heterodimerizes with membrane-bound antiapoptotic proteins to regulate apoptosis; 2) the subgroup of membrane-bound proapoptotic Bcl-2 proteins containing BH1, BH2, BH3, and TM domains, represented by Bok-L here, heterodimerizes with antiapoptotic proteins (Mcl-1 or Bfl-1) or functions as mitochondrial channels to regulate apoptosis; and 3) the unique Bok-S does not dimerize with antiapoptotic proteins but probably forms mitochondrial channels to regulate apoptosis. The regulation of Bok-S levels by upstream signals could rapidly induce apoptosis without direct interference by antiapoptotic proteins present in the same cell.



further supported by the finding that Bax-S and Bak-S with truncation at the BH3-BH1 regions homologous to that of Bok-S also retain proapoptotic activity. Recent studies also indicated that, during apoptosis, activated caspases cleave the N-terminal BH4 domain of antiapoptotic proteins Bcl-2 and Bcl-xL to yield truncated molecules resembling the proapoptotic Bax, Bak or Bok in terms of the BH domain arrangement (28, 43). Of interest, deletion of the BH4 domain from these antiapoptotic proteins confers proapoptotic activity and mitochondrial release of cytochrome C, presumably mediated through the C-terminal channel-forming region (28, 43, 44).

Like Bok, splicing variants have been reported for Bcl-2, Bcl-x, and Bax genes. The Bcl-xL gene encodes three different variants, each with a distinct function; the long form of Bcl-x (L) exhibits antiapoptotic activity, whereas Bcl-x-short and Bcl-x- β are proapoptotic (45, 46). Also, Bcl-2 variants lacking the TM domain show decreased antiapoptotic activity (47–50). The proapoptotic Bax gene also encodes a number of splicing variants with unknown function (6, 51). Although we have shown that the artificial Bax-S and Bak-S are capable of inducing apoptosis, these mutants are unlikely to exist based on the genomic arrangement of these two genes (6, 51, 52).

At least three mechanisms could be proposed for the action of proapoptotic Bcl-2 proteins (Fig. 6). 1) The subgroup of proapoptotic proteins with only the BH3 domain (e.g. the soluble BAD protein) heterodimerizes with membrane-bound antiapoptotic proteins to regulate apoptosis (41, 53, 54); 2) the subgroup of membrane-bound proapoptotic proteins with BH1, BH2, BH3, and TM domains, represented by Bok-L, heterodimerizes with antiapoptotic proteins (Mcl-1/Bfl-1) or functions as mitochondrial channels to regulate apoptosis; and 3) the unique Bok-S variant does not dimerize with antiapoptotic proteins but probably forms mitochondrial channels to regulate apoptosis. Because Bok-S does not interact with antiapoptotic proteins, apoptosis mediated through Bok-S may be important in situations when unwanted cells need to be eliminated quickly despite the presence of antiapoptotic proteins in the same cell. In the ovary and uterus known to express high levels of Bok transcripts, Bok-S expression could provide a short circuit to promote cell demise in hormone-dependent cell populations that express abundant antiapoptotic proteins (such as

Mcl-1) but have to be removed swiftly because of cyclic cell turnover during reproductive cycles. The search for novel death promoters that interact specifically with proapoptotic Bcl-2 proteins could also be simplified based on the lack of interaction between Bok-S and other Bcl-2 proteins. Further characterization of this unique proapoptotic protein would allow a better understanding of intracellular mechanism of apoptosis, particularly for hormone-regulated cell death.

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