Bcl-B, a Novel Bcl-2 Family Member That Differentially Binds and Regulates Bax and Bak

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A novel human member of the Bcl-2 family was identified, Bcl-B, which is closest in amino acid sequence homology to the Boo (Diva) protein. The Bcl-B protein contains four Bcl-2 homology (BH) domains (BH1, BH2, BH3, BH4) and a predicted carboxyl-terminal transmembrane (TM) domain. The BCL-B mRNA is widely expressed in adult human tissues. The Bcl-B protein binds Bcl-2, Bcl-XL, and Bax but not Bak. In transient transfection assays, Bcl-B suppresses apoptosis induced by Bax but not Bak. Deletion of the TM domain of Bcl-B impairs its association with intracellular organelles and diminishes its anti-apoptotic function. Bcl-B thus displays a unique pattern of selectivity for binding and regulating the function of other members of the Bcl-2 family.

Bcl-2 family members play a central role in apoptosis regulation in metazoan species. In humans, over 20 members of this family have been identified to date, including proteins that suppress (Bcl-2, Bcl-XL, Mcl-1, Bfl-1/A1, Bcl-W) and proteins that promote (Bax, Bak, Bok, Bad, Bid, Bik, Bin, Nip3, Nix) cell death (reviewed in Refs. 1 and 2). Many Bcl-2 family proteins are capable of physically interacting, forming homo- or heterodimers, and functioning as agonists or antagonists of each other (1–3). Specificity for interaction partners and tissue-specific patterns of expression combine to endow each mammalian Bcl-2 family protein with a unique physiological role in the cell resulting for example in highly diverse phenotypes when members of this multigene family are individually knocked out in mice (reviewed in Ref. 4). Thus, a need exists to identify comprehensively the members of the Bcl-2 family and to elucidate their functional characteristics. In this report, we describe the molecular cloning and initial characterization of a new human member of the Bcl-2 family, Bcl-B.

MATERIALS AND METHODS

Cloning of BCL-B cDNAs—TBLASTN searches of the human expressed sequence tag (EST) database using the amino acid sequence of the mouse Bov/Diva as a query resulted in the identification of homologous partial cDNAs. A human EST clone (GenBank™ accession number AA098865) was obtained (Research Genetics) and sequenced in its entirety, revealing an open reading frame (ORF) encompassing the last 151 residues of a protein with homology to Boo (Bcl-B) (submitted to GenBank™ with accession number AF326964). The corresponding genomic sequence for this cDNA was identified in the human genome data base (clone CTD-2184D3), which was derived from human chromosome 15q21. Because the EST clone lacked a candidate start codon, the corresponding 5′-end of Bcl-B cDNAs was cloned by a reverse transcriptase polymerase chain reaction (RT-PCR) approach, using the forward primer NKO118 (5′-CGGCCCAGGAAAAAACCGCAGG-3′), which was designed to hybridize to a region upstream of the Bcl-B ORF as predicted from the genomic data, and the reverse primer NKO121 (5′-CACCTCAAGAGAGCCTTGTGAT-3′), which is complementary to a region downstream of the predicted Bcl-B ORF corresponding to the 3′-unsaturated region of the putative mRNA. PCR amplification using human liver cDNA (CLONTECH) as a template with the above primers yielded a single ~0.9-kb product, which was cloned into pCR2.1-TOPO (Invitrogen, following the manufacturer’s instructions) to generate TOPO-Bcl-B (pNK254) and sequenced.

RT-PCR Analysis—Expression of BCL-B mRNA in various tissues was examined by RT-PCR, using oligo(dT)-primed first-strand cDNA derived from multiple adult human tissues (CLONTECH) as templates. PCR products were amplified following the manufacturer’s instructions using the forward primer NKO120 (5′-GTTGTTGAGCCTGAGCTGAGGTGG-3′) and reverse primer NKO103 (5′-CGCCCTGAGCTGATTAAGAATGCTGAGGA-3′). The PCR products were digested with EcoRI and XhoI and cloned into the EcoRI and XhoI sites of pcDNA3-Myc (Stratagene), and the EcoRI and SalI sites of pcl-Neo-FLAG (Invitrogen) and pEGFP-C2 (CLONTECH). A plasmid encoding Bcl-B lacking its COOH-terminal transmembrane domain (Bcl-BΔTM) was constructed by PCR-based mutagenesis using primers NKO101 and NKO121 (5′-CGCCCTGAGCTGATTAAGAATGCTGAGGA-3′ and reverse primer NKO103) with a single XhoI site introduced. The resulting PCR product was digested with EcoRI and XhoI and cloned into pcDNA3-Myc.

Plasmid Construction—The ORF encoding Bcl-B was PCR-amplified from TOPO-Bcl-B (pNK254) using the forward primer NKO101 (5′-GTTGTTGAGCCTGAGCTGAGGTGG-3′) and the reverse primer NKO103 (5′-CGCCCTGAGCTGATTAAGAATGCTGAGGA-3′). The PCR products were digested with EcoRI and XhoI and cloned into the EcoRI and XhoI sites of pcDNA3-Myc (Stratagene), and the EcoRI and SalI sites of pcl-Neo-FLAG (Invitrogen) and pEGFP-C2 (CLONTECH). A plasmid encoding Bcl-B lacking its COOH-terminal transmembrane domain (Bcl-BΔTM) was constructed by PCR-based mutagenesis using primers NKO101 and NKO121 (5′-CGCCCTGAGCTGATTAAGAATGCTGAGGA-3′ and reverse primer NKO103) with a single XhoI site introduced. The resulting PCR product was digested with EcoRI and XhoI and cloned into pcDNA3-Myc.

Cell Culture, Transfection, and Apoptosis Assays—HEK293, COS7, HT1080, and PCC1 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen Scientific) supplemented with 10% fetal bovine serum, 1 mm l-glutamine, and antibiotics. For transient-transfection apoptosis assays, cells (5 × 10^5) in six-well dishes were co-transfected using Superfect (Qiagen) with 0.5 μg of pcDNA3-Bax plus 0.5 μg of green fluorescence protein (GFP) marker plasmid pEGFP-C2 (CLONTECH) or 0.5 μg of pEGFP-Bak, and 1 μg of pcDNA3, pcDNA3-Myc-Bcl-B, pcDNA3-Myc-Bcl-BΔTM, or pcDNA3-FLAG-Bcl-B. The total amount of DNA was normalized to 3 μg per transfection using pcDNA3. After 24 h post-transfection, both adherent and floating cells were collected, fixed, and stained with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). The percentages of apoptotic cells were determined by counting the GFP-positive cells having nuclear fragmentation and/or chromatin condensation (mean ± S.D.; n = 3).
For stable transfections, HeLa cells in 100-mm dishes were transfected with pcDNA3 (control), pcDNA3-Myc-Bcl-B, or pRC-CMV-Bcl-2 plasmids using LipofectAMINE plus (Life Technologies, Inc.). Two days later, complete medium containing G418 (800 μg/ml) (Omega Scientific Inc.) was used to select stably transfected cells. Several of the resulting G418-resistant clones were recovered using cloning cylinders and individually expanded. G418-resistant clones were screened for the expression of desired genes by immunoblotting with antibodies. For apoptosis assays, stably transfected clones (5 × 10⁶ cells) in six-well dishes (30 mm diameter) were cultured in medium containing various concentrations of staurosporine (Calbiochem) (0.2–1 μM) or of recombinant TRAIL (Biomol) (10–100 ng/ml) for 8–10 h. Both floating and adherent cells were collected, fixed, and subjected to DAPI staining, enumerating the percentage apoptosis cells by UV microscopy. 

**Immunofluorescence and Subcellular Fractionation**—The intracellular location of Bcl-B was examined using fluorescence confocal microscopy and subcellular fractionation methods, essentially as described (6, 7).

**Co-immunoprecipitation and Immunoblotting Assays**—293T cells (5 × 10⁶ cultured with 50 μM benzoyl-Val-Ala-Asp-fuoromethylketone (Bachem) were co-transfected with 1.5 μg of pcDNA3-Myc-Bcl-B, pcNeo-FLAG-Bcl-B, pcDNA3-human calcynin-binding protein (used as a control), or pcDNA3-FLAG-Bcl-Xₐ together with 1.5 μg of pEGFP, pEGFP-Bcl-B, pcDNA3-HA-BAG1, pcDNA3-HA-Bax, pcDNA3-FLAG-Bcl-Xₐ pcDNA3-CMV-Bcl-2, or pEGFP-Bak. At 24 h post-transfection, cells were collected and resuspended in lysis buffer (142.4 mM KCl, 5 mM MgCl₂, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.2% Nonidet P-40) containing 12.5 mM β-glycerophosphate, 2 mM NaF, 1 mM Na₃VO₄, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche Molecular Biochemicals). Soluble lysates were incubated with 10 μl of anti-Myc (Santa Cruz) or anti-FLAG (Sigma) antibody-conjugated Sepharose beads overnight at 4 °C. Beads were then washed four times in 1.5 ml of lysis buffer and boiled in Laemmli gel-loading solution before performing SDS-PAGE/immunoblotting using the following polyclonal or monoclonal antibodies: polyclonal rabbit anti-hu Bcl-XL, rabbit anti-hu Bax, rabbit anti-hu Bcl-2, rabbit anti-hu Bcl-Xₐ, rabbit anti-hu Bax, or rabbit anti-hu Bak (8).

**RESULTS**

During TBLASTN searches of the publicly available EST data bases using the amino acid sequence of the mouse Boo/Divα as a query, we discovered an EST clone (GenBank™ accession number AA088865) encoding a predicted polypeptide harboring a BH1 domain. PCR methods were used to obtain cDNAs containing the complete ORF corresponding to a 204-amino acid protein (Fig. 1A). The predicted ORF was initiated by an AUG start codon within a favorable Kozak context. The predicted protein contains regions resembling the BH1, BH2, BH3, and BH4 domains typical of anti-apoptotic members of the Bcl-2 family, as well as a putative carboxyl-terminal TM domain (Fig. 1B). Comparisons of the sequence of this predicted protein with all known Bcl-2 family members by BLAST search indicated that it is most similar to the murine Bcl-2 family protein Boo (also known as Diva) (9, 10), sharing 47% amino acid sequence identity, and thus prompting the moniker “Bcl-2 family protein resembling Boo” (Bcl-B). The BCL-B gene is located on chromosome 15 (map 15q21), as determined by in silico screening of the human genome data base at NCBI. Comparison of the BCL-B cDNA sequence with genomic data indicates a two-exon structure, in which the region encoding residues Trp1⁶⁵ and Asp1⁶⁶ (within the BH2 domain) of the Bcl-B protein are interrupted by an ~2.3-kb intron. PCR analysis suggested that the BCL-B mRNA is widely expressed in adult human tissues (Fig. 1C).

The Bcl-B protein was tested for interactions with other Bcl-2 family proteins by co-immunoprecipitation experiments, wherein Bcl-B was expressed in HEK293T or HT1080 cells with various NH₂-terminal epitope tags. These studies indicated that Bcl-B is capable of associating with itself, Bax, Bcl-2, and Bcl-Xₐ, but not with Bak (Fig. 2).

The function of the Bcl-B protein was explored by transient transfection in a variety of cell lines, including HEK293T, COS7, HT1080, and PPC1. Overexpression of Bcl-B did not induce apoptosis, nor did it negate suppression of apoptosis caused by overexpression of Bcl-2 or Bcl-XL (not shown), suggesting that Bcl-B is not a pro-apoptotic protein. We therefore tested the possibility that Bcl-B is a cytoprotective protein by ascertaining its effects on apoptosis induced by the pro-apoptotic proteins Bax and Bak. Co-expressing Bcl-B markedly suppressed apoptosis induced by Bax but not Bak (Fig. 3), thus correlating with protein binding data demonstrating that Bcl-B associates with Bax but not Bak (Fig. 2). This suppression was not due to reduced levels of Bax protein, as determined by immunoblotting. In contrast to Bcl-B, co-expression of Bcl-Xₐ suppressed apoptosis induced by either Bax or Bak (Fig. 3).

To further explore the effects of Bcl-B on apoptosis, HeLa cells were stably transfected with a plasmid encoding Myc-tagged Bcl-B, versus control (empty) plasmid. Several stably transfected clones were tested for Bcl-B expression by immunoblotting, and their responses to apoptosis induced by staurosporine (STS) or TRAIL were compared. Comparisons were also made to HeLa cells stably transfected with a Bcl-2-encoding plasmid. Fig. 9, C—E, show representative results, where control transfected (vector) cells were compared with two Bcl-B-transfected clones. The Bcl-B-expressing clones shown here (clones 9 and 16) produced different relative amounts of Myc-Bcl-B protein, as determined by immunoblotting, with clone 16 containing ~5 times higher levels of Bcl-B than clone 9. HeLa cell clones such as clone 16, which contained higher amounts of Myc-Bcl-B, displayed resistance to apoptosis induced by STS and TRAIL, compared with control (vector)-transfected cells. In contrast, HeLa cell clones such as clone 9, which contained

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**Fig. 1.** Sequence analysis and the expression of Bcl-B cDNA. A, the predicted Bcl-B amino acid sequence is presented with the TM underlined (GenBank™ accession number AP326964). B, alignments of BH1, BH2, BH3, and BH4 domains of Bcl-B and other Bcl-2 family proteins are shown. Numbers on the left indicate the position of the amino acid in each protein based on GenBank™ accession numbers AAD08703 (murine Boo), Q90343 (chicken Bcl-2), or Q29158 (C. elegans CED9). Identical and similar residues are indicated by black and gray boxes, respectively. Asterisks under the BH2 alignment indicate the intron junction for hu BCL-B, BCL-2, and BCL-X genes. C, expression of BCL-B in adult human tissues. First-strand cDNAs made from RNA samples from various adult human tissues were PCR-amplified using BCL-B-specific primers. The reverse primer was downstream of the intron, thus avoiding amplification of contaminating genomic DNA. PCR products were size-fractionated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were also used for PCR as a positive control.
lower levels of Myc-Bcl-B, demonstrated only slight resistance to these apoptotic stimuli (Fig. 3, D and E). These data thus demonstrate that Bcl-B can suppress apoptosis induced by exogenous stimuli if expressed at sufficient levels. However, even HeLa cell clones with higher levels of Bcl-B did not manifest the profound resistance to apoptosis seen in Bcl-2-overexpressing cells (Figs. 3, D and E).

Many Bcl-2 family proteins associate with mitochondria in cells (reviewed in Refs. 1 and 2). Expression of GFP-tagged Bcl-B in cells revealed a punctate cytosolic pattern and partial colocalization with a mitochondria-specific dye (MitoTracker), as determined by two-color confocal microscopy (Fig. 4A). Crude subcellular fractionation analysis revealed that Myc-tagged Bcl-B protein resides predominantly in the mitochondria-containing HM fraction, similar to Bcl-2, as determined by immunoblot analysis of the cellular fractions (Fig. 4, B and C). In contrast to full-length Bcl-B, a truncation mutant of Bcl-B lacking the carboxyl-terminal TM domain (Bcl-B ΔTM) targeted less efficiently to the HM fraction (Fig. 4D). The Bcl-BΔTM protein also was ineffective at blocking Bax-induced apoptosis (Fig. 4E), even though this protein was produced at comparable levels with the full-length Bcl-B protein. Thus, efficient organelar targeting appears to be required for optimal function of Bcl-B.

**DISCUSSION**

We describe a new member of the human Bcl-2 family protein, Bcl-B. This protein is most similar in amino acid sequence to the murine Boo (Diva) protein and the most similar among all human Bcl-2 family proteins to the CED9 protein of *Caenorhabditis elegans*. The Boo (Diva) protein interacts selectively with some Bcl-2 family proteins but not others, although controversy exists as to the details (9, 10). Interestingly, one report has suggested that the Boo protein can bind Bak but not Bax, and accordingly provided evidence that Boo suppresses apoptosis induced by overexpression of Bak but not Bax (10). Conversely, we observed that Bcl-B selectively binds and suppresses apoptosis induction by Bax, but fails to interact with or negate apoptosis triggered by Bak overexpression.

The murine Boo (Diva) protein has been variably reported to either suppress or promote apoptosis (9, 10). In transient transfection assays performed in four different human tumor cell lines, we consistently observed an anti-apoptotic action of Bcl-B. Stable overexpression of Bcl-B in HeLa cells also resulted in increased resistance to diverse apoptotic stimuli. However, because Bcl-B is capable of associating with either the anti-apoptotic proteins Bcl-2 and Bcl-XL or with the pro-apoptotic protein Bak, it is possible that Bcl-B could display different phenotypes under some circumstances depending on cellular context. A similar phenomenon has been reported for some other Bcl-2 family proteins. For example, Bcl-2 can reportedly promote apoptosis in photoreceptor cells of the retina, while Bax can suppress cell death in some types of neurons.
induced apoptosis also suggests that Bcl-B does not play a significant role in suppressing Apaf1. Moreover, the observation that Bcl-B suppresses apoptosis induced by Bax but not Bak also argues against a role for Bcl-B as an Apaf1 suppressor, given that both Bax and Bak induce mitochondrial release of the Apaf1 activator, cytochrome c (14, 15).

The correlation between membrane targeting and function is reminiscent of some other Bcl-2 family proteins and suggests that the site of action of Bcl-B is close to the intracellular organelles, including mitochondria, with which it associates. Although roughly half of the Bcl-BΔTM protein was associated with the HM membrane fraction in cells, this may be due to its dimerization with other resident Bcl-2 family proteins. A membrane site of action for Bcl-B would be consistent with evidence that several Bcl-2 family proteins are capable of forming ion channels or pores in membranes (reviewed in Ref. 16). Indeed, molecular modeling of Bcl-B on the structure of Bcl-Xs suggests that it possesses a similar overall fold and that it contains amphiphatic α-helices similar to the putative pore-forming α5 and α6 of Bcl-Xs (not shown).

The differences observed in the functions and protein interaction partners of murine Boo and human Bcl-B proteins suggest that Bcl-B does not represent the human orthologue of mouse Boo/Diva. Also consistent with this interpretation is the difference in the patterns of expression of Bcl-B and Boo. Whereas Boo (Diva) is expressed predominantly in ovary, testis, and epididymis in adult mice (9, 10), RT-PCR analysis suggests that the BCL-B mRNA is widely expressed in adult human tissues. Comparisons of the sequence of BCL-B cDNAs with human genome sequence data indicate that the BCL-B gene is comprised of two exons interrupted by a ~2.3-kb intron. Interestingly, the location of this intron corresponds precisely to the intronic interruption in the coding region of the anti-apoptotic BCL-2 and BCL-X genes (corresponding to the motif GGW′D or GGVWD in BH2 (see Fig. 1B). The genomic sequence of murine boo/diva is unfortunately unavailable for comparison.

In contrast to BCL-B, the pro-apoptotic genes BAX and BAK have more complicated exon-intron organizations, in which the coding regions of the gene are spread over 5 (Bak) or 6 (Bax) exons. The similar genomic organization of the BCL-2, BCL-XL, and BCL-B genes thus suggests they evolved from a common ancestor and indirectly implies a similar mechanism of action for their encoded proteins.

Addendum—While this manuscript was in preparation, the cDNA sequence of Bcl-B was deposited into GenBank™ (accession number AF285092) by L. H. H. Zhang (unpublished data).

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Fig. 4. The COOH-terminal TM domain of Bcl-B is required for efficient membrane targeting and function. A, for confocal microscopy analysis, a plasmid encoding GFP-Bcl-B was transfected into COS7 cells. At 24 h after transfection, cells were incubated with Mito-tracker Red CMXRos, then fixed and imaged. Cells transfected with GFP control protein produced diffuse cellular fluorescence (not shown), in contrast to GFP-Bcl-B. B, for subcellular fractionation studies, HEK293T cells were transfected with plasmids encoding Myc-Bcl-B (B), Bcl-2 (C), and Myc-Bcl-B or Myc-Bcl-BΔTM (D). At 24 h post-transfection, cells were collected and post-nuclear lysates prepared (Total). An aliquot of these lysates was then fractionated by differential centrifugation at 10,000 × g to pellet HM. The resulting supernatant (Sup) was then either analyzed directly (D) or subjected to centrifugation at 150,000 × g to pellet LMs and achieve a cytosolic supernatant (B, C). Proteins from each fraction were normalised relative to cell numbers and subjected to SDS-PAGE/immunoblot analysis using antibodies specific for Myc or Bcl-2. E, HEK293T cells were co-transfected with a plasmid encoding GFP (used as a marker for transfection) and either pcDNA3 (control (CNTL)) or pcDNA3-Bax, in combination with a 2-fold excess of pcDNA3 (control), pcDNA3-Myc-Bcl-B, or pcDNA3-Myc-Bcl-BΔTM. Cells were collected and stained with DAPI after 24 h. The percentage of green cells with apoptotic morphology was determined (mean ± S.D.; n = 3).

Although stably transfected clones of HeLa cells, which contained higher levels of Bcl-B, exhibited resistance to exogenous apoptotic stimuli, the resistance afforded by Bcl-B was not as profound as that observed for Bcl-2 overexpression. This difference in potency of Bcl-B could be due to variations in the relative amounts of Bcl-B and Bcl-2 produced in transfected cells, or it could reflect a fundamental difference in the mechanisms of these proteins. In this regard, because Bcl-2 blocks cell death induced by both Bax and Bak, whereas Bcl-B inhibits apoptosis induced only by Bax but not Bak, it seems likely that Bcl-B may be less efficacious under circumstances where both Bax and Bak contribute to apoptosis induction. Bcl-B therefore may provide a mechanism for selectively inhibiting Bax-dependent apoptotic processes in vivo, while allowing Bak-dependent cell death to proceed normally.

The mouse Boo (Diva) protein was reported to associate with the caspase-activating Apaf1 protein (a homologue of C. elegans CED-4) (9, 10). Although we have observed weak interactions of Bcl-B with Apaf1 in co-immunoprecipitation assays, functional analysis has failed to reveal an effect of Bcl-B on Apaf1-induced apoptosis (not shown). Since Apaf1 is a soluble cytosolic protein (13), the inability of Bcl-BΔTM to suppress Bax-
