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Identification of a Human cDNA Encoding a Novel Bcl-x Isoform

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Alternative splicing has been shown to generate two isoforms of the apoptosis regulator bcl-x, Bcl-x_L and Bcl-x_S, in humans. Here we describe the identification and characterization of a third splice variant of the human bcl-x gene. It differs from previously described bcl-x transcripts in two respects: (1) a novel facultative intron is spliced out at the 5′ untranslated region and (2) the open reading frame arises from a continuous genomic sequence extending over the splice donor sites utilized by the bcl-x_L and bcl-x_S transcripts. Since the resulting molecule has an organisation homologous to mouse and rat Bcl-x_β we suggest calling this novel protein human Bcl-x_β. Northern blot analysis revealed that bcl-x_β mRNA is expressed in numerous cell lines. Like Bcl-x_L, h-Bcl-x_β binds to the pro-apoptotic protein Bax, suggesting a functional activity in vivo.

Key Words: **apoptosis; bcl-2 homologue; bcl-x_L; bax.**

Apoptosis is the consequence of a genetically determined cell death program which is controlled, in part, by a complex interplay between members of the Bcl-2 family proteins. Apoptotic activity depends on the ratio of death antagonists (e.g. Bcl-2, Bcl-x_L, Mcl-1, and A1) to death agonist (e.g. Bcl-x_S, Bax, Bad, and Bak) molecules (reviewed in References 1 - 6). Bcl-2, the prototype founding member of this family, and Bcl-x are the best characterized of these proteins. In humans, two isoforms of Bcl-x have been found. The 29 kD Bcl-x_L contains the highly conserved BH1 and BH2 domains which are essential for the anti-apoptotic function of proteins of the bcl-2 family (7). In addition, it contains a hydrophobic stretch of amino acids, representing a transmembrane domain essential for membrane anchorage and correct subcellular targeting. The conserved BH1 and BH2 domains are absent.in the 18 kD Bcl-x_S isoform. The difference between these two proteins is due to alternative splicing of a common pre-mRNA. In bcl-x_L, two complete coding exons are present, whereas bcl-x_S lacks the 3′ terminal part of the first coding exon due to utilization of a facultative splice donor site. In vivo, bcl-x_L is expressed mainly in tissues containing long-lived postmitotic cells such as adult brain, whereas bcl-x_S is found at high levels in cells that undergo a high rate of turnover, such as developing lymphocytes (8). Alternative splicing has also been observed in several other members of the bcl-2 family (9,10), and in members of the two other cell death regulators, namely the caspases (11,12) and ced-4 (13). This strongly suggests that differential RNA splicing is of general importance for the regulation of apoptosis. In the present study we have identified and characterized a novel bcl-x variant isolated from a human placental cDNA library.

MATERIALS AND METHODS

Cloning and DNA sequence analysis. A human placenta cDNA library cloned into the lambda ZAPII vector (Stratagene) was screened for bcl-2 homologues using the PstI - BamHI fragment of a human bcl-2 cDNA (10; nucleotide sequence 1671-2021) as a probe. cDNA fragments from positive clones were cloned into pBluescript SK-phagemid vectors. Double stranded cDNAs were sequenced from purified plasmid DNA by a Taq DyeDeoxy Terminator Cycle Sequencing method with an ABI 373 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Southern and Northern hybridization. Genomic DNA of the T-cell line H9 and total cellular RNA from several other cell lines were hybridized to different bcl-x cDNA fragments. For Southern hybridization, the DNA probes PS1 (fragment of the cDNA from the 5′ terminal BglII to BamHI site) and PS2 (fragment of the cDNA from the BamHI to 3′ terminal BglII site) were used. To specifically detect mRNAs containing the open reading frame and the 3′ untranslated region of the novel

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Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); cDNA, DNA complementary to RNA; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; kbp, kilobase pair; kD, kilodaltons.

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cDNA by Northern hybridization, the probe PN1 was generated by PCR using the primers "PN1s" (sense, 5′-TAAGAACCAGCCCCTTGTGTG-TCC-3′) and "PN1as" (anti-sense, 5′-CCAGTCAGGTTTCCTCAACTA-TCA-3′). For detection of bcl-x_L or bcl-x_S mRNAs, the probe PN2 was generated by PCR using primers "PN2s" (sense, GATACTTTTGTG-GAACTCTATGGG-3′) and "PN2as" (anti-sense 5′-CTGGTGGCAATG-GCGGCTGGACGG-3′). The exact localization of the probes is depicted in Fig. 1 of the Results section. Probes were radioactively labeled with α-^{32}P dCTP by megaprime DNA labeling according to the manufacturer's protocol (Amersham, UK).

Expression of recombinant proteins. For expression of the recombinant novel Bcl-x and Bax proteins the respective cDNAs were ligated into the cloning sites of the prokaryotic expression vector, pMal-c2 creating an in-frame fusion protein with Maltose Binding Protein (MBP) according to the manufacturer's protocol (New England BioLabs, Beverly,MA). Standard PCR mutagenesis was used to add the restriction sites for EcoRI at the 5′ terminus and XbaI at 3′ terminus of novel bcl-x and bax coding regions. *E. coli* XL-1 Blue were transformed with the expression constructs and screened for positive clones according to standard protocols (14). Transformed *E. coli* were grown in LB media containing 100 μg/ml ampicillin in a incubation shaker at 37°C. When the density of the culture reached 0.6 at A_{600}, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 2 hours induction, bacteria were harvested and lysed by resuspending in 1× gel-loading buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The lysates were heated to 100° C for 3 minutes and examined by SDS-polyacrylamide gel (PAGE) as described by Laemmli (15). Gels were stained for proteins with Coomassie Brilliant Blue. To prepare fusion proteins for Western blot analysis and Bcl-x-Bax binding assays, bacteria were resuspended in PBS and lysed by sonication. Cell debris was then removed by centrifugation at 15000 rpm and supernatants were stored at −80°C until further use. Western blot analysis was performed as described elsewhere (16). Briefly, lysates from bacteria expressing MBP proteins or MBP alone were size-fractioned and electroblotted onto a nitrocellulose membrane. Filters were incubated with either rabbit anti-human Bcl-x_L antiserum (Transduction Lab, Lexington, KY), or with a rat anti-mouse Bax monoclonal antibody (Pharmingen, San Diego, CA). As second step reagents a goat anti-rabbit antiserum (Pierce, Rockford, IL) or rabbit anti-rat antiserum conjugated with peroxidase (Dako, Denmark) were used. The final immunocomplex was detected by chemiluminescence using the ECL detection reagents (Amersham, Buckinghamshire, England).

Bcl-x-Bax binding assay. Microtiter plates were coated with lysates from cells producing MBP alone or MBP-Bax proteins at a concentration of 500 ng of total cellular proteins/well. After saturation of wells with 5% BSA, the lysate containing MBP-Bcl-x_β protein was added. Subsequently, wells were washed extensively and incubated with anti-Bcl-xL rabbit antiserum (Transduction Lab, Lexington, KY). The bound immunoglobulins were detected with a peroxidase labeled goat anti-rabbit immunoglobulin-antiserum (Pierce, Rockford, IL). Binding of anti-serum was visualised by addition of the reagents o-phenylendiamine according to the manufacturer's instructions (Abbot Lab, Abbot Park, IL), and microtiter plates were read at OD_{450}nm using a Microelisa Autoreader. A similar approach was used to detect binding of Bax to Bcl-xβ. We used rat anti-mouse Bax monoclonal antibody (Pharmingen, San Diego,CA), and as second step reagent a rabbit anti-rat antiserum conjugated with peroxidase (Dako, Denmark) was used.

RESULTS AND DISCUSSION

Isolation of a Novel Human bcl-x cDNA Clone

To search for new members of the bcl-2 family we screened a human placental cDNA library with a DNA probe containing the BH1 and BH2 regions of bcl-2.

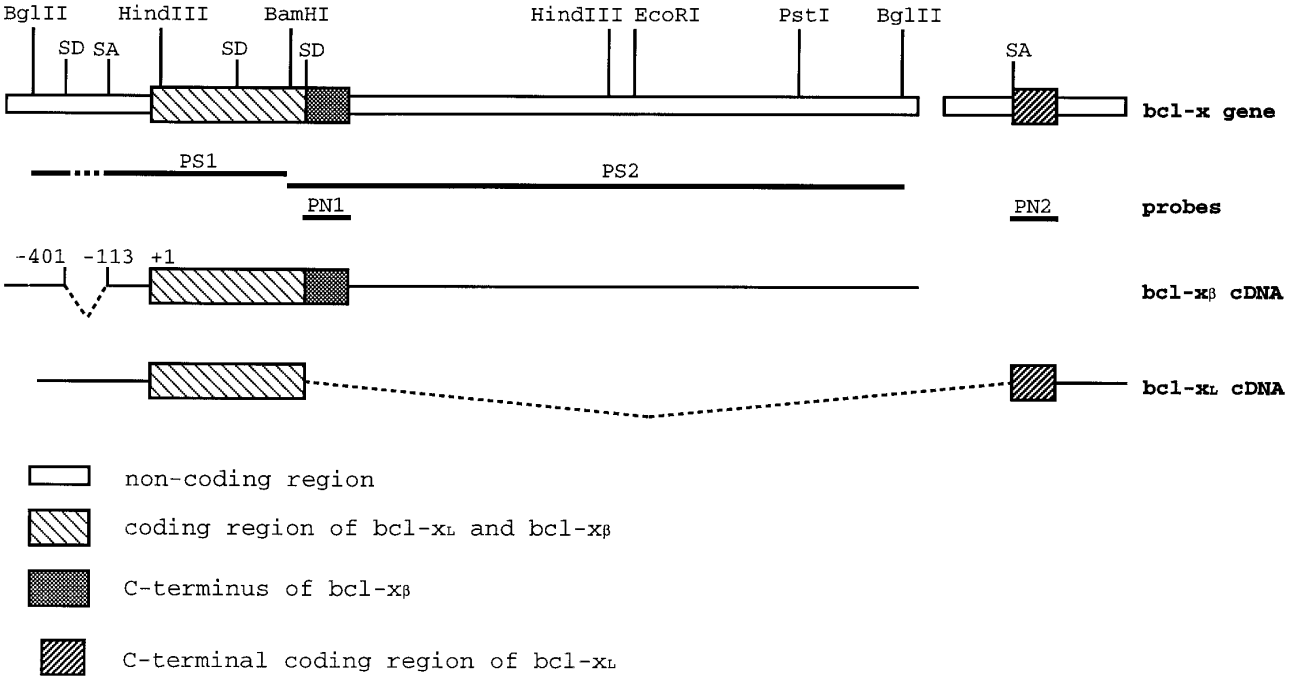

FIG. 1. Genomic organization of the bcl-x-gene and schematic representation of human bcl-x_β cDNA. Fragments of the cDNA used as probes for further experiments are shown as black bars and are designated PS1, PS2, PN1, and PN2. Restriction sites for several enzymes as well as facultative splice donor (SD) and splice acceptor (SA) sites are indicated.

A.

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ATG TCT CAG AGC AAC CGG GAG CTG GTG GTT GAC TTT CTC TCC TAC AAG CTT TCC    54
  M   S   Q   S   N   R   E   L   V   V   D   F   L   S   Y   K   L   S   18

CAG AAA GGA TAC AGC TGG AGT CAG TTT AGT GAT GTG GAA GAG AAC AGG ACT GAG   108
  Q   K   G   Y   S   W   S   Q   F   S   D   V   E   E   N   R   T   E   36

GCC CCA GAA GGG ACT GAA TCG GAG ATG GAG ACC CCC AGT GCC ATC AAT GGC AAC   162
  A   P   E   G   T   E   S   E   M   E   T   P   S   A   I   N   G   N   54

CCA TCC TGG CAC CTG GCA GAC AGC CCC GCG GTG AAT GGA GCC ACT GGC CAC AGC   216
  P   S   W   H   L   A   D   S   P   A   V   N   G   A   T   G   H   S   72

AGC AGT TTG GAT GCC CGG GAG GTG ATC CCC ATG GCA GCA GTA AAG CAA GCG CTG   270
  S   S   L   D   A   R   E   V   I   P   M   A   A   V   K   Q   A   L   90

AGG GAG GCA GGC GAC GAG TTT GAA CTG CGG TAC CGG CGG GCA TTC AGT GAC CTG   324
  R   E   A   G   D   E   F   E   L   R   Y   R   R   A   F   S   D   L  108

ACA TCC CAG CTC CAC ATC ACC CCA GGG ACA GCA TAT CAG AGC TTT GAA CAG GTA   378
  T   S   Q   L   H   I   T   P   G   T   A   Y   Q   S   F   E   Q   V  126

GTG AAT GAA CTC TTC CGG GAT GGG GTA AAC TGG GGT CGC ATT GTG GCC TTT TTC   432
  V   N   E   L   F   R   D   G   V   N   W   G   R   I   V   A   F   F  144

TCC TTC GGC GGG GCA CTG TGC GTG GAA AGC GTA GAC AAG GAG ATG CAG GTA TTG   486
  S   F   G   G   A   L   C   V   E   S   V   D   K   E   M   Q   V   L  162

GTG AGT CGG ATC GCA GCT TGG ATG GCC ACT TAC CTG AAT GAC CAC CTA GAG CCT   540
  V   S   R   I   A   A   W   M   A   T   Y   L   N   D   H   L   E   P  180

TGG ATC CAG GAG AAC GGC GGC TGG GTA AGA ACC AAG CCC CTT GTG TGT CCC TTT   594
  W   I   Q   E   N   G   G   W   V   R   T   K   P   L   V   C   P   F  198

TCT TTG GCC TCT GGT CAG AGA TCC CCA ACA GCC CTT CTT CTG TAT CTC TTT CTG   648
  S   L   A   S   G   Q   R   S   P   T   A   L   L   L   Y   L   F   L  216

TTG TGT TGG GTG ATT GTT GGA GAC GTT GAT AGT TGA GGA AAC CTG ACT GG        698
  L   C   W   V   I   V   G   D   V   D   S   *                          227
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B.

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                181 ▼                                                  227
h Bcl-x beta    WIQENGGWVR TKPLVCPFSL ASGQRSPTAL LLYLFLLCWV IVGDVDS
m Bcl-x beta    WIQENGGWVR TTPLVCPPLA CVSLLCEHP
r Bcl-x beta    WIQENGGWVR TTPLVCPPLV CLSSVEIPNC PFWSPGMVVE DIDYSGDIPG LL
ch Bcl-x        WIQENGGWVR TALP
h Bcl-2 beta    WIQDNGGWVG ASGDVSLG
h Bax beta      WIQDQGGWVR LLKPPHPHHR ALTTAPAPPS LPPATPLGPW AFWSRSQWCP LPIFRSSDVV
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FIG. 2. (**A**) Nucleotide and the deduced amino acid sequence of human bcl-x_β open reading frame. Nucleotides and amino acids are numbered from the first letter of the initiation codon and shown at right. Amino acid residues that are different from human bcl-x_L are underlined. The stop codon is indicated by "*". (**B**) Alignment of the C-terminal sequences of different beta Bcl-2 homologous proteins. Amino acid sequences were retrieved from Genbank and EMBL data banks. Identical matches between compared amino acid residues are shown on shaded background. Numbers represent amino acid positions in human Bcl- x_β. A black inverted triangle indicates the beginning of the β-form specific aa sequences.

Several recombinant clones hybridizing with different intensity were identified and purified. DNA sequencing revealed that cDNAs from plaques yielding a strong hybridization signal represented bcl-2. In contrast, a clone which yielded only a weak hybridization signal showed partial sequence identity with a previously described Bcl-x_L cDNA (8). Sequence comparison of the 5′ untranslated region (5′ UTR) of the novel bcl-x cDNA with a human genomic bcl-x clone (17) revealed that a stretch of 289 bp of the genomic sequence flanked by classical splice motifs (from nucleotide −401 to −113, with respect to the open reading frame) is missing in the novel cDNA (Fig.1). Splicing of a homologous intron has been found for mouse bcl-x (17,18), whereas in rat facultative splicing of a shorter intron terminating at the 3′ splice acceptor site described here has been shown (19). Splicing of the human 5′ UTR intron defined by the novel cDNA must be facultative because it is not spliced out in a Bcl-x_L cDNA described previously (8). The evolutionary conservation of this facultative splicing argues for its biological importance either in regulating mRNA stability or translation efficiency.

The full open reading frame of the novel cDNA clone

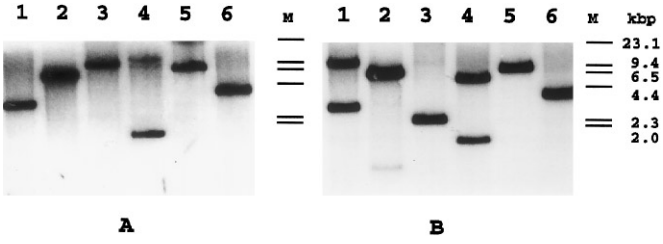

FIG. 3. Southern analysis of human genomic DNA (H9) with different portions of bcl-x cDNA. (**A**) Hybridization with a BglII-BamHI cDNA fragment containing the 5′-end of bcl-x_β which represents the common DNA sequence for both cDNAs (bcl-x_L and novel bcl-x; DNA probe PS1). (**B**) Hybridization with the 3′-end of the novel bcl-x cDNA specific sequence (DNA probe PS2). DNA was digested with EcoRI (lane 1), PstI (2), BamHI (3), HindIII (4), XhoI (5), and BglII (6).

encodes a protein of 227 amino acids with a calculated molecular mass of about 26 kD (Fig.2*A*). Homology comparison of the amino acid (aa) sequence confirmed that the N-terminal 188 aa were completely identical with the N terminus of Bcl-x_L. Eight of the next 9 aa are identical to the ones in the Bcl-x_β isoforms identified in mouse and rat (18,19). The last 30 aa differed from all previously reported sequences of Bcl-2 family genes (Figure 2*B*). However, a stretch of 18 highly hydrophobic aa within this C terminus resembles the membrane anchor region found in Bcl-x and Bcl-2 (data not shown).

When we used partial sequences of the 3′ untranslated region of the novel bcl-x cDNA to search the expressed sequence tag (EST) data base, we found identity with several ESTs derived from different tissues (accession number: D20136/HUMGS01109; AA48972; AA513204; AA507670; W23679; AA127228; AA400706; H77308; W35112; H20787; and others). No function has been attributed to these sequences previously.

Genomic Organization

To determine the localization of the novel bcl-x sequence within the bcl-x locus we performed Southern blot analysis. The same pattern of bands was obtained with genomic DNA from the T-cell line H9 (Fig. 3) and from several other human cell lines as well as from primary tissue yielded (data not shown). Southern hybridization with either DNA probe PS1 or PS2 detected identical EcoRI (3.5 kilobase pairs, kbp), PstI (7.7 kbp), and HindIII (1.8 kbp) DNA fragments (Fig. 3*A* and 3*B*). Moreover, both probes hybridized to a single fragment of around 8.1 kbp and 4.1 kbp after XhoI and BglII digests, respectively. These results are compatible with the assumption that the complete cDNA has arisen from the bcl-x gene which has been localized to a single locus in the pericentric region of chromosome 20 (20). To investigate whether an intron is spliced out during the generation of the ORF we performed PCR using primers annealing within the coding sequence and the 3′-untranslated region of the new cDNA. The fact that identical products were amplified from both genomic DNA and cDNAs derived from various cell lines (data not shown) confirmed that the ORF was generated without splicing. Based on Southern blot hybridization and sequencing data, the structure of the bcl-x gene with respect to the new cDNA is illustrated in Fig. 1. Since the organization of the ORF is homologous to that of bcl-x_β isoforms identified in mouse and rat, we suggest calling the novel form of bcl-x human bcl-x_β (h-bcl-x_β).

Expression of h-bcl-x_β mRNA

To examine the expression pattern of h-bcl-x_β, Northern blot analysis was performed on total RNA isolated from different human cells with a probe specific for h-bcl-x_β (PN1) (Fig. 4). Expression of bcl-x_β varied among

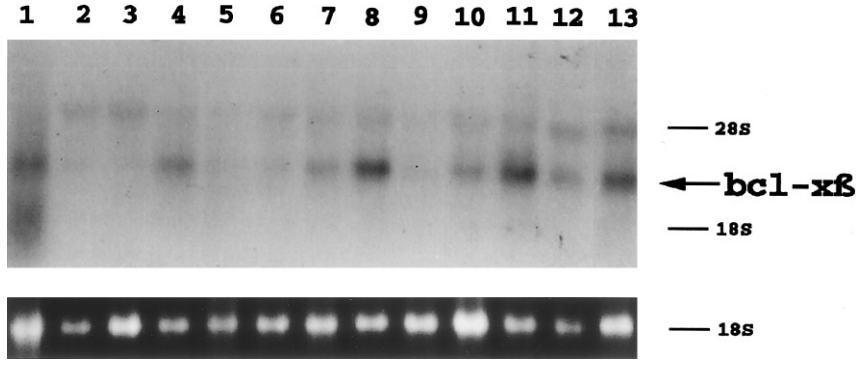

FIG. 4. Expression of bcl-x_β mRNA in different human cell lines. Total RNA from H9 (T-lymphoid, lane 1), AA-2 (B-lymphoid, lane 2), RL7 (B-lymphoid, lane 3), HMC1 (human mast cells, lane 4), HL60 (promyelocytic, lane 5), HeLa (epithelial carcinoma, cervix, lane 6), A431 (keratinocyte, lane 7), A375 (melanoma cell line, lane 8), RHTP49 (melanoma cell line, lane 9), SHT3 (melanoma cell line, lane 10), 8814 (melanoma cell line, lane 11), SK-Mel5 (melanoma cell line, lane 12), and U937 (myeloid, 13) was tested for bcl-x_β expression. To detect bcl-x_β specific mRNAs by Northern blot hybridization, the cDNA probe PN1 specific only for bcl-x_β cDNA (100 bp cDNA fragment) was generated by PCR.

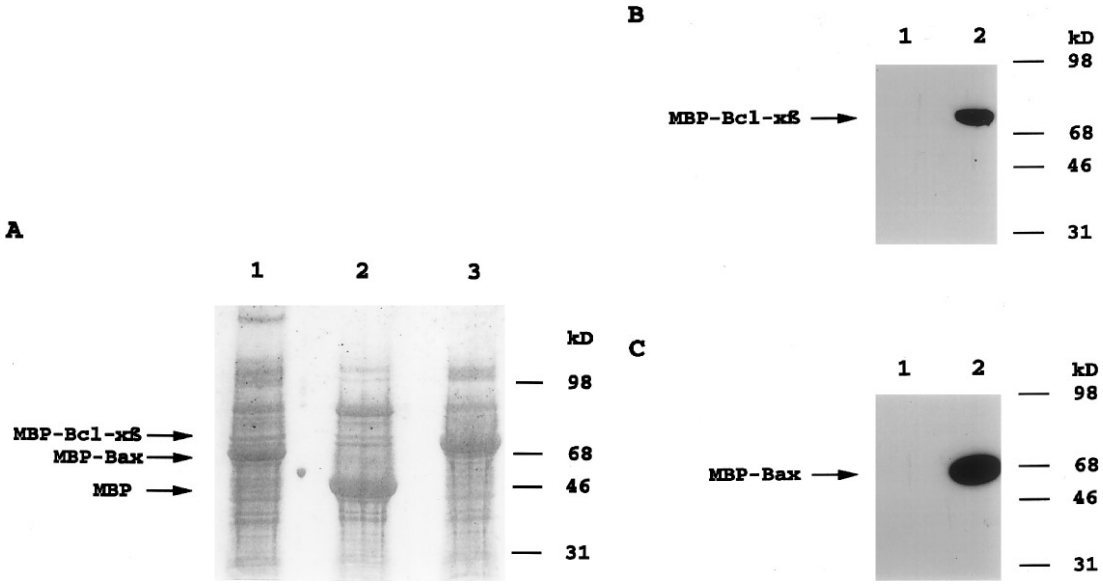

FIG. 5. (**A**) PAGE showing expression of Bcl-x_β and Bax in E. coli strain XL-1 Blue. Lanes: 1, cell lysate of XL-1 Blue cells expressing the fusion protein Maltose Binding Protein (MBP) and Bax; 2, cell lysate of XL-1 Blue cells expressing the MBP alone; 3, cell lysate of XL-1 Blue cells expressing the fusion protein of MBP and Bcl-x_β. (**B**) Western blot showing the detection of expression of Bcl-x_β in E. coli strain XL-1 Blue. Lanes: 1, cell lysate of XL-1 Blue cells expressing MBP alone; 2, cell lysate of XL-1 Blue cells expressing the fusion protein of MBP and Bcl-x_β. (**C**) Western blot showing the detection of expression of Bax in E. coli strain XL-1 Blue. Lanes: 1, cell lysate of XL-1 Blue cells expressing MBP alone; 2, cell lysate of XL-1 Blue cells expressing the fusion protein of MBP and Bax.

different cell lines and primary cells. High level of bcl-x_β was detected in the T lymphoid cell line H9, in the U937 myeloid cell line, and in the two melanoma cell lines A375 and 8814. Its expression was low in two B lymphoid cell lines tested (AA-2 and RL7). In the majority of samples investigated, the expression of bcl-x_β correlated with that of bcl-x_L as determined by hybridization with a probe specific for the second coding exon of that splice variant (data not shown). The ratio of bcl-x_L to bcl-x_S has been suggested to determine the propensity of a cell to undergo apoptosis and to play a role during murine mammary gland involution (21). Whether the relative expression level of Bcl-x_β compared to the other splice variants of the bcl-x gene is subject to regulation in physiological situations remains to be investigated.

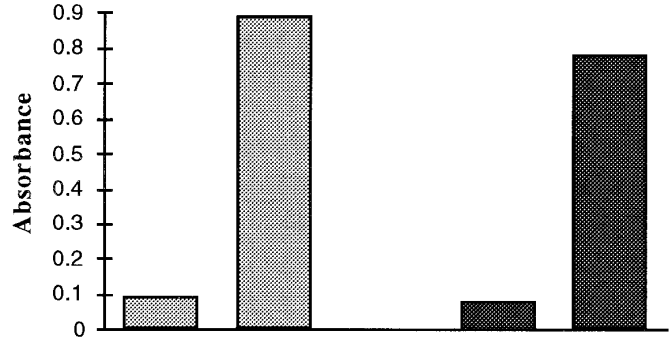

plate coated with:	MBP	MBP-Bax		MBP	MBP-Bcl-xβ
incubation with:	MBP-Bcl-xβ	MBP-Bcl-xβ		MBP-Bax	MBP-Bax
detection with:	anti-Bcl-x_L	anti-Bcl-x_L		anti-Bax	anti-Bax

FIG. 6. Detection of interaction between Bcl-x_β and Bax *in vitro*. Microtiter plates were coated with lysates from cells producing MBP alone, MBP-Bcl-x_β, or MBP-Bax. After saturation of wells, the lysates containing MBP-Bcl-x_β or MBP-Bax proteins were added. Subsequently, final immunocomplexes were detected with monoclonal antibodies against human Bcl-x_L or human Bax as described in Materials and Methods.

Interaction of Recombinant Bcl-x_β and Bax Proteins

Since apoptosis is regulated by interactions between different Bcl-2 homologs, we investigated whether human Bcl-x_β can bind to the human death inducer Bax. The bacterial expression vector pMAL-c2 was used to express both bcl-x_β and bax cDNA, as fusion proteins with the maltose-binding-protein (MBP). The identity of the expressed fusion proteins was confirmed by Western blot analysis using antisera against Bcl-x_L and Bax (Fig. 5*A*, 5*B* and 5*C*). The interaction of Bcl-x_β with Bax was investigated by an *in vitro* binding assay using the respective MBP-fusion proteins (Materials and Methods) (Fig. 6). MBP-Bcl-x_β was found to bind to MBP-Bax, as well as MBP-Bax to MBP-Bcl-x_β, but not to MBP alone. These results suggest that Bcl-x_β is able to form a complex with Bax and therefore might also be involved in regulating apoptosis. Whether Bcl-x_β acts to enhance or inhibit apoptosis is at present unclear, and may depend on the experimental system used. For instance, mouse Bcl-x_β was found to act anti-apoptotically in neurons (22), rat Bcl-x_β promoted apoptosis in promyeloid cells (19).

In summary, human bcl-x_β represents a widely expressed splice variant of the bcl-x gene that is capable of interacting with at least one of the major pro-apoptotic proteins. Further experiments with h-Bcl-x_β will clarify its physiological role.

ACKNOWLEDGMENTS

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Note added in proof. During the preparation of this manuscript, we found that Inohara N. and Ohta S. submitted to GenBank a nucleotide sequence of the coding region of human Bcl-x_β gene which is completely identical to our h-bcl-x_β (Accession number U72398).

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