Novel BNIP1 variants and their interaction with BCL2 family members

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Abstract By PCR and EST database searches we have identified three novel BNIP1 splice variants, and found that one of them, BNIP1-b, contains a highly conserved BH3 domain. The BNIP1 gene has been assigned to chromosome 5q33–34. Using in vitro protein-protein interaction assays, all BNIP1 variants were shown to interact with BCL2 and also with BCL2L1 (previously Bcl-xL). These interactions are BH3-independent. Furthermore, the BNIP1 variants cannot interact with BAX. The results suggest that the BNIP1 variants are novel members of the BCL2 family but function through a mechanism different from other BH3-only members.

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Key words: BNIP1; Variant; BCL2; BCL2L1; BAX; Interaction

1. Introduction

Programmed cell death or apoptosis plays an important role in the development of multicellular organisms [1] and in pathological processes [2]. Apoptosis is under tight control of rather complex regulatory circuits which are evolutionarily conserved from the nematode *Caenorhabditis elegans* to man. Proteins of the BCL2 family turned out to be important regulators of apoptosis [3].

The BCL2 family consists of a growing number of proteins, some of them protect cells from apoptosis, such as BCL2, BCL2L1, BCL2A1, and MCL1, but others promote apoptosis, such as BAX, BAK, and BAD. Several domains in the BCL2 family proteins are evolutionarily conserved, which are described as BCL2 homology (BH) domains. BH1 and BH2 domains are common throughout the family [4]. Anti-apoptotic members share BH3 and BH4 domains [5,6], whereas pro-apoptotic members of the family, such as BAX and BAK, share the BH3 domain, which, however, has different features from that of the anti-apoptotic members [5–8]. Within the family, protectors and promoters of cell death interact with each other by forming homodimers or heterodimers.

BCL2 interacts with members of the BCL2 family, but it can also associate with other proteins as shown by yeast two-hybrid methods, by protein interaction cloning techniques or immunoprecipitation from human cell extracts [9–14]. However, the precise function in apoptosis has not been determined yet for the majority of these proteins.

In the present study, we report the isolation of three novel BNIP1 variants, one of them contains a highly conserved BH3 domain. All BNIP1 variants are able to interact with BCL2, BCL2L1 but not with BAX.

2. Materials and methods

2.1. Cloning of human BNIP1 variants and chromosomal location of BNIP1

PCR was performed to clone BNIP1 and BNIP1-a from a human prostate λ-gt11 cDNA library (Clontech). The 5′ primer was designed according to the previously published BNIP1 cDNA sequence [10] and contains an *Eco*RI site (underlined) flanking the initiation codon (bold) (5′-GG<u>AATTCC</u>AT**ATG**GCGGCTCCCCAAGAC-3′). The 3′ reverse primer is complementary to sequences downstream of the BNIP1 stop codon (bold) (5′-GG<u>AATTC</u>GTCGAC**ACT**GTGTTC-CATCCAACCTG-3′). The PCR products were cloned in the *Eco*RI site of vector pZero 1-1 (Invitrogen) and sequenced. Searching the GenBank database of human expressed sequence tags (ESTs) with the BNIP1 cDNA sequence, a 351 nt EST sequence (GenBank accession number H11663) was found and sequenced, its gene was named BNIP1-b. A 495 bp EST (GenBank accession number W95979) was also identified and termed BNIP1-c. The search of the GenBank database for STSs using BNIP1-b as a template revealed that a human STS WI-6822 (accession number G06277) matches 100% to the nucleotides +941 to +1229 of the BNIP1-b sequence. BNIP1 was located on chromosome 5 using the NCBI Unigen program. EMBL/GenBank/DDBJ accession numbers for BNIP1-a, BNIP1-b and BNIP1-c are AF083956, AF083957 and AF083958, respectively.

2.2. Plasmid constructs and in vitro transcription/translation

cDNA of bacterial chloramphenicol acetyltransferase (CAT), human BCL2L1, human BCL2, human BAX, and the BNIP1 variants were sub-cloned into pcDNA3 (Invitrogen) using standard cloning procedures. All constructs were confirmed by sequencing. For coupled in vitro transcription/translation, 2 μg of plasmids were linearized (creating 5′ overhangs) and used with TNT wheat germ lysates (Promega, Inc.) in the presence of [^{35}S]methionine according to the supplier's manual.

2.3. In vitro interaction assay

6His-BCL2, 6His-BCL2L1, 6His-BAX, and GST-BCL2 fusion proteins were purified from bacterial extracts (provided by Dr. G. Fendrich, Novartis). Fusion proteins (10 μg) were prebound to Ni^{2+}-TNA chelation resins (Qiagen) or glutathione-Sepharose beads (Qiagen), mixed with 10 μl of in vitro translated, ^{35}S-labeled proteins for 1 h at 4°C on a rotator, and then washed 5 times in NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40). The resin or beads were resuspended in Laemmli buffer, heated to 100°C for 5 min, vortexed, and the resulting supernatants were applied to 12% SDS-PAGE. Gels were dried and exposed to an X-ray film or phosphate image screen (Molecular Dynamics).

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Abbreviations: PCR, polymerase chain reaction; EST, expressed sequence tag; STS, sequence tagged site; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; BH3, BCL2 homology region 3; BNIP1, BCL2/adenovirus E1B 19 kDa interacting protein 1 (previously Nip1); BNIP1vs, BNIP1 splice variants, including BNIP1-a, BNIP1-b and BNIP1-c (previously: Nip1s1, Nip1s2 and Nip1s3); BCL2A1, BCL2-related protein A1 (previously Bfl-1); BCL2L1, BCL2-like 1 (previously Bcl-xL)

2.4. Northern blot analysis

The mRNA expression pattern of the BNIP1 variants was determined by Northern blots on a multiple tissue membrane from Clontech. Probes were prepared from PCR products using primer set a (5′-CCAGTTCTCTATCAAAGGGC-3′ and 5′-ACTGAAGGTAA-CAGGT-3′), which amplifies an 129 nt DNA fragment encoding domain B, and primer set b (5′-AGCAATCAGGCCTCATG-3′ and 5′-TTGCCTTAAGAGATC-3′), which amplifies a 102 nt DNA fragment encoding domain D of BNIP1vs.

3. Results

3.1. BNIP1 and its variants

BNIP1 has been suggested as a target for BCL2 interaction [10]. During the course of cloning BNIP1, we further identified three novel, alternatively spliced forms of BNIP1 by use of PCR and by search of the EST database (Fig. 1A). Briefly, PCR was performed on phage DNA of a prostate cDNA
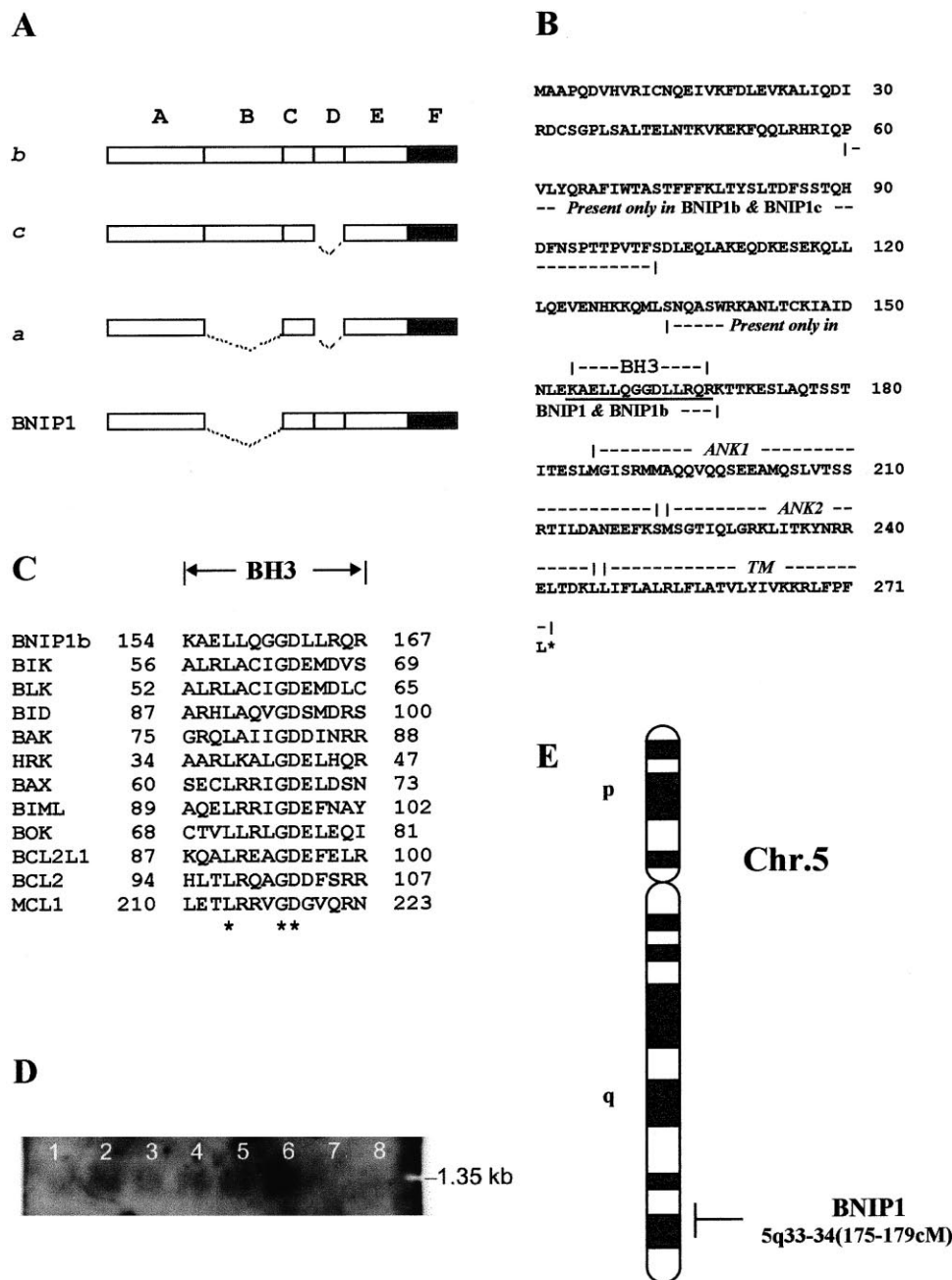
A

B

C

D

E

Fig. 1. Isolation of cDNAs encoding three novel isoforms of BNIP1: BNIP1-a, BNIP1-b and BNIP1-c. A: Schematic representation of the BNIP1 variants. Domains A–F were defined in relation to the deduced amino acid sequence of BNIP1-b. The black box represents the putative transmembrane domain (domain F). Dotted lines indicate putative splice regions. B: Relationship of the four BNIP1 variants showing the predicted amino acid sequence of BNIP1vs. The BH3 domain is underlined. 'ANK1', 'ANK2' and 'TM' represent ankyrin repeat-like motifs and the putative transmembrane domain, respectively. C: Alignment of the putative BH3 domain of BNIP1 with the BH3 domain of other members of the BCL2 family. Asterisks indicate conserved amino acids. D: Distribution of BNIP1b and BNIP1c in human tissues. A 129 nt DNA probe labeled with digoxigenin was hybridized to poly(A)$^+$ RNA from the following human tissues: pancreas (1), kidney (2), skeletal muscle (3), liver (4), lung (5), placenta (6), brain (7) and heart (8). The same membrane was reprobed with β-actin for calibration (not shown). E: Schematic illustration of the chromosomal localization of the BNIP1 gene. The search of the GenBank database for STSs was done with nucleotides +941 to +1229 of the BNIP1-b sequence. The NCBI Unigen program was used for chromosomal localization of BNIP1.
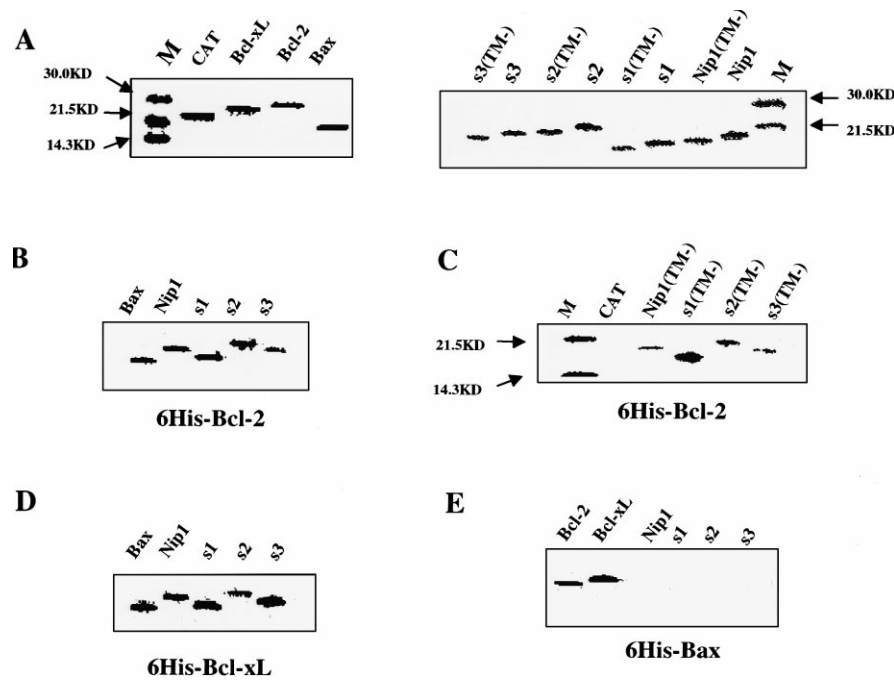
Fig. 2. Analysis of interaction of BNIP1vs with BCL2 family proteins by in vitro binding assays. A: Protein expression by the TNT translation system (Promega) in the presence of [35S]methionine. Results from two independent experiments are presented in the right and left panels. (TM−) represents proteins lacking the C-terminal transmembrane domain. 'a', 'b' and 'c' denote BNIP1-a, BNIP1-b and BNIP1-c, respectively. Fusion proteins for 6His-BCL2 (B and C), 6His-BCL2L1 (D) and 6His-BAX (E) were purified from bacterial expressions. Fusion proteins were prebound to Ni2+-TNA chelation resins, mixed with in vitro translated, 35S-labeled BNIP1vs proteins. After extensive washes, the resin was resuspended in Laemmli buffer for SDS-PAGE. Gels were dried and exposed to a phosphor image screen (Molecular Dynamics).

library to amplify the entire coding region of BNIP1 [10]. Two bands were observed as PCR products, each band was cloned and subjected to sequence analysis (not shown). The sequence of these PCR products demonstrated that the longer one corresponds to BNIP1 cDNA and the shorter one is a splicing variant of BNIP1 which contains a 102 nt in-frame deletion and was named BNIP1-a. Subsequently, a Blast Search of the GenBank database ESTs was performed using the cDNA sequence of BNIP1 and we identified two other forms of BNIP1, termed BNIP1-b and BNIP1-c. BNIP1-b was found in an infant brain cDNA library and contains a 129 nt in-frame insertion. BNIP1-c was isolated from a fetal heart cDNA library and has the same 129 nt in-frame insertion but also the same 102 nt in-frame deletion as in BNIP1-a. Based on the splicing events, we define six regions, A–F, for the deduced amino acid sequence of BNIP1-b (Fig. 1A). We refer to BNIP1 and its variants as BNIP1vs. The predicted

amino acid sequence of BNIP1vs showed no significant homology to known proteins. However, alignment of the C-terminus of BNIP1vs (Fig. 1B) with proteins in the database revealed an interesting region with similarities to the ankyrin repeats of Notch 2 [15] and ICP4 [16], which is a repeated sequence motif present in a variety of proteins whose common function involves binding to other proteins [17]. Most interestingly, region D, which is present in BNIP1 and BNIP1-b, contains a fully conserved BH3 domain of 16 amino acids (Fig. 1C). Therefore, the BNIP1vs could be novel members of the BCL2 family.

Northern blot hybridization of the RNA from various human tissues revealed hybridizing bands of about 1.35 kb (Fig. 1D). Messages were detected in placenta, lung, liver, skeletal muscle, kidney, pancreas, testis and small intestine with a probe prepared from the 129 nt DNA sequence encoding domain B, which is present in BNIP1-b and BNIP1-c. Low or no

Table 1
Differential expression of BNIP1 variants

Probe	Heart	Brain	Placenta	Lung	Liver	Skeletal muscle	Kidney	Pancreas
a	+/−	+/−	+	+	+	+	+	+/−
b	++	++	+	+	++	++	+	++
	Spleen	Thymus	Prostate	Testis	Ovary	Small intestine	Colon	Peripheral blood leukocytes
a	+/−	−	+/−	++	−	++	−	−

The mRNA expression pattern of BNIP1 variants was determined by Northern blots on a multiple tissue membrane from Clontech. Probes were prepared from PCR products using primer set a which amplifies a 129 nt DNA fragment encoding domain B and primer set b which amplifies a 102 nt DNA fragment encoding domain D of BNIP1vs (see Section 2). +/− stands for low gene expression, ++ for high expression and − for no expression.

expression was seen in other tissues (Table 1). With the 102 nt probe present in BNIP1 and BNIP1-b (encoding domain D), messages were detected in heart, brain, liver, skeletal muscle and pancreas (Table 1). At this stage it is not possible to find proper probes to further distinguish the expression patterns of individual BNIP1 variants yet.

A computer search with the BNIP1-b cDNA sequence of a STS database revealed a human STS WI-6822 (GenBank accession number G06277) matching nucleotides +941 to +1229 of the BNIP1-b sequence. The genomic locus of BNIP1 could be mapped to human chromosome 5 between the markers D5S504 and D5S425, thus localizing the BNIP1 gene to chromosome 5 at 5q33–34 (175–179 cM) (Fig. 1E).

3.2. Interaction of BNIP1vs with BCL2 family proteins

Boyd et al. [10] have previously demonstrated that the BNIP1 protein can interact with E1B 19K protein and BCL2 in a yeast two-hybrid assay. The interaction was confirmed by immunoprecipitation. It is therefore interesting to examine whether the BNIP1vs are capable of interacting with BCL2 family members. Further comparison of the binding of different BNIP1 variants may allow us to define the structural regions which are required for interaction with BCL2. We initially determined their interaction with BCL2 in an in vitro protein-protein interaction assay. BCL2 was expressed as a 6His fusion protein in *Escherichia coli* and immobilized on a Ni^{2+}-TNA chelation resin before incubation with ^{35}S-labeled BNIP1vs that had been prepared by in vitro transcription/ translation (Fig. 2A) using wheat germ extracts. After extensive washing, proteins which interacted physically with BCL2 were collected and detected after SDS-PAGE. In vitro translated ^{35}S-labeled BAX and CAT proteins served as positive and negative controls (Fig. 2B,C). As shown in Fig. 2B, BNIP1, BNIP1-a, BNIP1-b and BNIP1-c could interact with 6His-BCL2. BAX interacted with BCL2 but the CAT protein did not (Fig. 2C).

As seen in Fig. 1, BNIP1-a has a 34 amino acid (domain D) in-frame deletion with respect to BNIP1 and BNIP1-b has a 43 amino acid (domain B) in-frame insertion. The comparison of the interactions of BNIP1, BNIP1-a and BNIP1-b with BCL2 (Fig. 2B) does not show major differences. This suggests that the interaction of BNIP1vs with BCL2 is not dependent on domains B and D. The membrane domain (F) is also not essential for the interaction of BNIP1vs with BCL2 (Fig. 2C).

Since all members of the BCL2 family share some homology in conserved BH1, BH2 or BH3 domains [18,19], we examined whether BNIP1vs could interact with BCL2L1 and BAX. As described above, BCL2L1 and BAX were expressed as 6His fusion proteins. Fig. 2D shows that BNIP1vs could interact with BCL2L1, but they were not able to bind to BAX (Fig. 2E). The specificity of the interactions was verified by the use of CAT protein which failed to bind to BCL2L1 and BAX (data not shown).

4. Discussion

The experimental data characterize BNIP1 and its variants as proteins interacting with BCL2 and BCL2L1. We show that the BNIP1 gene encodes four alternatively spliced proteins, called BNIP1, BNIP1-a, BNIP1-b and BNIP1-c (Fig. 1). They are expressed differentially in various tissues (Table 1).

Interestingly, all BNIP1vs can interact with BCL2 (Fig. 2B,C) and BCL2L1 (Fig. 2D), but not with BAX (Fig. 2E), implying that the binding sites for BNIP1vs are conserved in BCL2 and BCL2L1 but are absent in BAX.

An interesting finding is that BNIP1-b and BNIP1 contain a conserved BH3 domain. It is located in section D of the sequence which is missing in BNIP1-a and BNIP1-c. A closer sequence comparison of BNIP1-b and BNIP1 with members of the BCL2 family indicates that the BNIP1vs do not share BH1 and BH2 domains. The BH3 domain has been demonstrated, in many studies, as a stretch of amino acids that is required for interaction between pro-apoptotic and anti-apoptotic members, and to promote cell death (for review see [20,21]). Therefore, BNIP1-b and BNIP1 may be novel BH3-only members of the BCL2 family.

What could be the function of the BNIP1 variants in vivo? Since BNIP1-b and BNIP1 contain a BH3 domain but BNIP1-a and BNIP1-c do not, they may have opposite functions in apoptosis similar to that observed in Bcl-xL vs. Bcl-xS [22] and CED-4L vs. CED-4S [23]. However, our preliminary data on overexpression of BNIP1vs in both rat REF52 and mouse FL5.12 cells show that they do not function as direct promoters or inhibitors of apoptosis (data not shown). Moreover, the interaction of BNIP1vs with BCL2 and BCL2L1 is independent of the BH3 domain (Fig. 2), suggesting that BNIP1vs may function through a mechanism different from other BH3-only members. One possibility is that the BH3 domain in BNIP1vs is hidden, therefore the exposure of the BH3 domain is required for their function. Additional experiments are necessary to verify these possibilities and to explore the function of BNIP1vs.

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