# Bcl-rambo beta, a special splicing variant with an insertion of an Alu-like cassette, promotes etoposide- and Taxol-induced cell death

Peng Yi[1], Wei Zhang[1], Zhiwei Zhai, Lin Miao, Yi Wang, Mian Wu*

*Department of Molecular and Cell Biology, Key Laboratory of Structural Biology, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, PR China*

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**Abstract** **The exonization of an Alu-like element into a coding sequence is unique to primates and this phenomenon distinguishes our genome from other mammals. Here, we report the presence of a special splicing variant of a proapoptotic protein Bcl-rambo in human lymph node, designated as Bcl-rambo beta. This variant contains a 98 bp Alu-like sequence which acts as an exon. There exists an in-frame stop codon within this inserted Alu-like cassette, resulting in generation of a premature protein of 104 amino acid residues. Unlike the Bcl-rambo, Bcl-rambo beta is lacking of the BH1, BH2 and BH3 motifs and becomes a BH4-only protein. Bcl-rambo beta is detected in several adult human tissues such as heart, lymph node and cervix but is absent in human brain tissue. In addition, Bcl-rambo beta is found not to be associated with mitochondria due to the absence of its C-terminal membrane anchor region. Nevertheless, this cytosol-localized protein is capable of promoting etoposide- and Taxol-induced cell death. Although the exact function of the Alu sequence is not fully characterized, the Alu element within the Bcl-rambo beta appeared to contribute to the pro-apoptotic capability, since removing of the Alu sequence from Bcl-rambo beta abrogates its ability to induce cell death. Our data support the speculation that the Alu element insertion during the splicing process may play an important role in the generation of protein diversity in primate cells by a yet uncharacterized mechanism.**



*Key words:* Mitochondrion; Bcl-rambo; BH4-only; Alu sequence; Primate

## 1. Introduction

Apoptosis, also known as programmed cell death (PCD), is a genetically regulated cellular suicide mechanism that plays a vital role in development and in the defense of homeostasis [1]. One of the major components of cell apoptosis machinery is the Bcl-2 family. Up to now, more than 20 members of this family have been characterized in mammals [2,3]. The Bcl-2 family includes both anti-apoptotic and proapoptotic proteins containing one or more Bcl-2 homology (BH) domains. The anti-apoptotic subfamily includes e.g. Bcl-2 and Bcl-$X_L$ and the proapoptotic subfamily consists of e.g. Bax, Bid and Bak. In addition, the proapoptotic Bcl-2 subfamily can be further divided into two groups. One includes proteins that contain three Bcl-2 homology domains (BH1–3) such as Bax and Bak, while the other group involves proteins that contain the BH3 domain only, which can be represented by Bad and PUMA [2,3].

Recently Kataoka et al. have identified a novel Bcl-2 homolog Bcl-rambo which shows high homology to the proteins of the Bcl-2 family containing BH domains 1–4. However, unlike Bcl-2, Bcl-rambo possesses a unique 250 amino acid long C-terminal extension. Bcl-rambo is localized to mitochondria and possesses proapoptotic capability that can be specifically blocked by the caspase inhibitors IAPs. Interestingly, the induction of cell death by Bcl-rambo is dependent on its unique C-terminal extension rather than its BH motifs. Thus, Bcl-rambo forms a new type of proapoptotic Bcl-2 member that promotes cell death independently of its BH motifs [4].

In the course of studying the function of the Bcl-rambo, we cloned a splicing variant Bcl-rambo beta from the human lymph node cDNA library. Surprisingly, this variant contains a 98 bp Alu-like insertion as an exon and there is an in-frame stop codon within this inserted sequence, resulting in expression of a 104 amino acid protein, which contains only a BH4 domain. We report here the gene structure of Bcl-rambo beta and propose its splicing mechanism for exonization of the Alu sequence. By expressing GFP-Bcl-rambo beta fusions in living cells, we were able to demonstrate that Bcl-rambo beta has a diffused localization throughout the cells. It is interesting to note that lacking of the C-terminal extension of Bcl-rambo beta does not compromise its function in promoting apoptosis induced by etoposide and Taxol as expected. Deletion of the Alu sequence from the Bcl-rambo beta abolished its proapoptotic activity, indicating that the Alu sequence may be involved in the proapoptotic function of Bcl-rambo beta.

## 2. Materials and methods

### 2.1. Reagents and antibodies

The following antibodies were used in this study: monoclonal antibody mAb-GFP was purchased from MBL (Japan) and monoclonal antibody mAb-Flag was from Sigma. Etoposide and Taxol used in this study were of GCP grade. Restriction enzymes were purchased from New England Biolabs (USA) and Takara (Japan). Medium compounds for culturing of *Escherichia coli* were ordered from Oxoid (UK). Majority of biochemical reagents were purchased from Sigma. Mitotracker Red CMXRos was ordered from Molecular Probes (USA).

### 2.2. Oligonucleotides

The sequences of the oligonucleotides used in this study are as

*Corresponding author. Fax: (86)-551-3606264.
*E-mail address:* wumian88@yahoo.com (M. Wu).

[1] These two authors contributed equally to this paper.

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follows (all primers are read from 5′ to 3′): P1: CCGGAATTCATGGCGTCCTCTTCTACTGT; P2: CTGTAATCCTGGCTACTCAGG; P3: CCGTCGACCTATTTCTTTCTCAGAGCCA; P4: CGGTCGACCTAAGACTTGCCCTCAGACAGCGG (the reverse primer for Bcl-rambo-ΔMA); P5: CGCTCGAGCTATTCAGAAATTTCTTTGTC (the reverse primer for Bcl-rambo-BH4).

### 2.3. Polymerase chain reaction (PCR) conditions

One microgram of primer was added to a 20 ul reaction volume containing 2 mM $MgCl_2$, 250 uM of each of deoxynucleotides, and 25 U of Taq polymerase. The mixture was incubated first at 94°C for 10 min, followed by 30 cycles of 94°C denaturing for 1 min, 54°C annealing for 1 min, and 72°C for 3 min. After 30 cycles, another 10 min were added to ensure the completion of all the extension at 72°C.

### 2.4. Plasmid construction

The full-length gene fragments of Bcl-rambo (1458 bp) and Bcl-rambo beta (1342 bp) were both cloned into the *Eco*RI/*Xho*I sites of plasmid pcDNA3-FLAG (gift from Dr. Sun, Stanford University) and the *Eco*RI/*Sal*I sites of plasmid pEGFP-C1 (gift from Dr. Yao, UC Berkeley) respectively to generate four recombinant expression vectors pcDNA3-FLAG-Bcl-rambo, pcDNA3-FLAG-Bcl-rambo beta, pEGFP-C1-Bcl-rambo and pEGFP-C1-Bcl-rambo beta. The fragments of Bcl-rambo-ΔMA (membrane anchor, 1377 bp) and Bcl-rambo-BH4 (231 bp) were cloned into the *Eco*RI/*Sal*I sites to generate recombinant plasmids pEGFP-C1-Bcl-rambo-ΔMA and pEGFP-C1-Bcl-rambo-BH4, respectively. All cloned gene fragments were verified by DNA sequencing using ABI Prism automated sequencing method.

### 2.5. Cell culture and transfection

The 293A cells were purchased from QBI (USA). The human cervical HeLa cancer cell line was kindly provided by Dr. Yao (UC Berkeley, USA). Cells were maintained in Dulbecco's minimal Eagle's medium (DMEM) containing 10% heat-inactivated bovine serum, 1 mM glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin (Life Technologies, Inc., Grand Island, NY, USA) at 37°C under an atmosphere of 5% $CO_2$ in air. Transfection of cultured cells with various mammalian expression vectors by lipofectamine (Life Technologies, Inc.) was according to the methods provided by the manufacturer's specification.

### 2.6. Cell viability assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. Briefly, cells were plated at $1\times10^4$ cells/well in 96-well microtiter plates overnight and then transfected with indicated plasmids by lipofectamine. Cells were then treated with 100 ul fresh medium containing 60 ug of etoposide, cultured for 5 h followed by an additional 3 h incubation with 250 ug/ml MTT solution. After incubation, the medium was removed and replaced with 100 ul dimethyl sulfoxide (DMSO) and the data were analyzed using a microplate reader at a wavelength of 570 nm. The formula used to translate the cell viability to the percentage of cell death is: $\%_x = (V_x - V_{empty})/V_{empty} \times 100\%$ (%, percentage of cell death; $x$, transfected gene of interest; $V$, the cell viability value; empty, vector alone.)

### 2.7. Manipulation of nucleic acids and miscellaneous methods

Standard methods are used for the transformation of *E. coli*, preparation of recombinant plasmids from *E. coli*, restriction enzyme mapping, and isolation and ligation of restriction fragments [5].

## 3. Result

### 3.1. Identification of the Bcl-rambo beta

In the course of investigating the function of Bcl-rambo, we performed a PCR amplification using the human lymph node cDNA library (Clontech, USA) as template. Two oligonucleotides P1 and P3 (see Section 2 and Fig. 4A) were used as primers. Unexpectedly, two prominent amplified fragments with molecular sizes of 1.4 and 1.3 kb were visualized on 1% agarose gel. The longer PCR product (1.4 kb) was confirmed to be the full-length Bcl-rambo and the shorter form (1.3 kb) was later identified as a variant of Bcl-rambo by DNA sequencing determination. The short form of Bcl-rambo was then designated as Bcl-rambo beta by us and its complete nucleotide sequence has been deposited in GenBank with an assigned accession number of AF539453. Different from Bcl-rambo, Bcl-rambo beta has an additional 98 bp insertion after exon 2, but skips two exons corresponding to exons 4 and 5 of the Bcl-rambo (Fig. 1A). The amino acid sequences of Bcl-rambo and Bcl-rambo beta were aligned in Fig. 1B. Comparison of the two protein sequences indicated that the Bcl-rambo beta only contains the BH4 domain and its C-terminal expressed by Alu insertion is completely different from that of the Bcl-rambo.

### 3.2. The 98 bp insertion of Bcl-rambo beta derives from an Alu element

BLAST homology search of the nucleotide sequence of the inserted 98 bp in the Bcl-rambo beta with the GenBank database revealed a remarkable homology (91%) with human Alu repeat sequences. The nucleotide sequence of this Alu insertion is shown in Fig. 2A. This sequence was used to compare with several reported sequences of Alu subfamilies and they displayed high similarities to each other (Fig. 2B).

The sequence of the Bcl-rambo was mapped to the human genome (22q11) and at least eight Alu repeat sequences were identified in the total of five introns. Sequence alignment analysis showed that the 98 bp Alu sequence of Bcl-rambo beta perfectly matched with an intron sequence segment corresponding to the nucleotides 77554–77653 between exons 2 and 3 of Bcl-rambo (GenBank accession number AC006285, deposited by Fang, F. et al.), indicating that the Bcl-rambo beta is generated by a unique alternative splicing mechanism. There were conserved 5′ donor and 3′ acceptor sites in these spliced intron sequences. Based on these conserved splice sites, we hypothesized a model for the splicing mechanism of Bcl-rambo beta as depicted in Fig. 3.

### 3.3. The tissue distribution of Bcl-rambo beta

There exist three stop codons within the inserted Alu cassette, one of which serves as the terminal signal for the translation of Bcl-rambo beta (Fig. 2A). The resulting premature open reading frame (ORF) of Bcl-rambo beta contains only 104 amino acids. To examine whether the Bcl-rambo beta naturally exists in the tissue and what the distribution pattern for the Bcl-rambo beta is, we performed PCR reactions using four different cDNA libraries made from adult tissues of human heart, lymph node, brain and cervix (HeLa cells) as templates. Primers P1 and P2 (sequence-specific primers designed to base-pair with the sequence (nucleotides 307–327) within the Alu cassette) were used in the PCR amplification to detect the ORF of Bcl-rambo beta (320 bp) and primers P1 and P3 were used to amplify the full length of Bcl-rambo (Fig. 4A). An amplified band with expected size of approximately 320 bp of the Bcl-rambo beta was obtained, which is consistent with the observation that two adjacent bands were amplified when P1 and P3 were used as primers. This result indicates that Bcl-rambo and Bcl-rambo beta coexist in the same tissues. It is interesting to note that both the Bcl-rambo and the Bcl-rambo beta were hardly detected in brain tissue indicating that their transcription and/or splicing may be tissue selective (Fig. 4B). An extra band of approximately 600 bp was also generated by

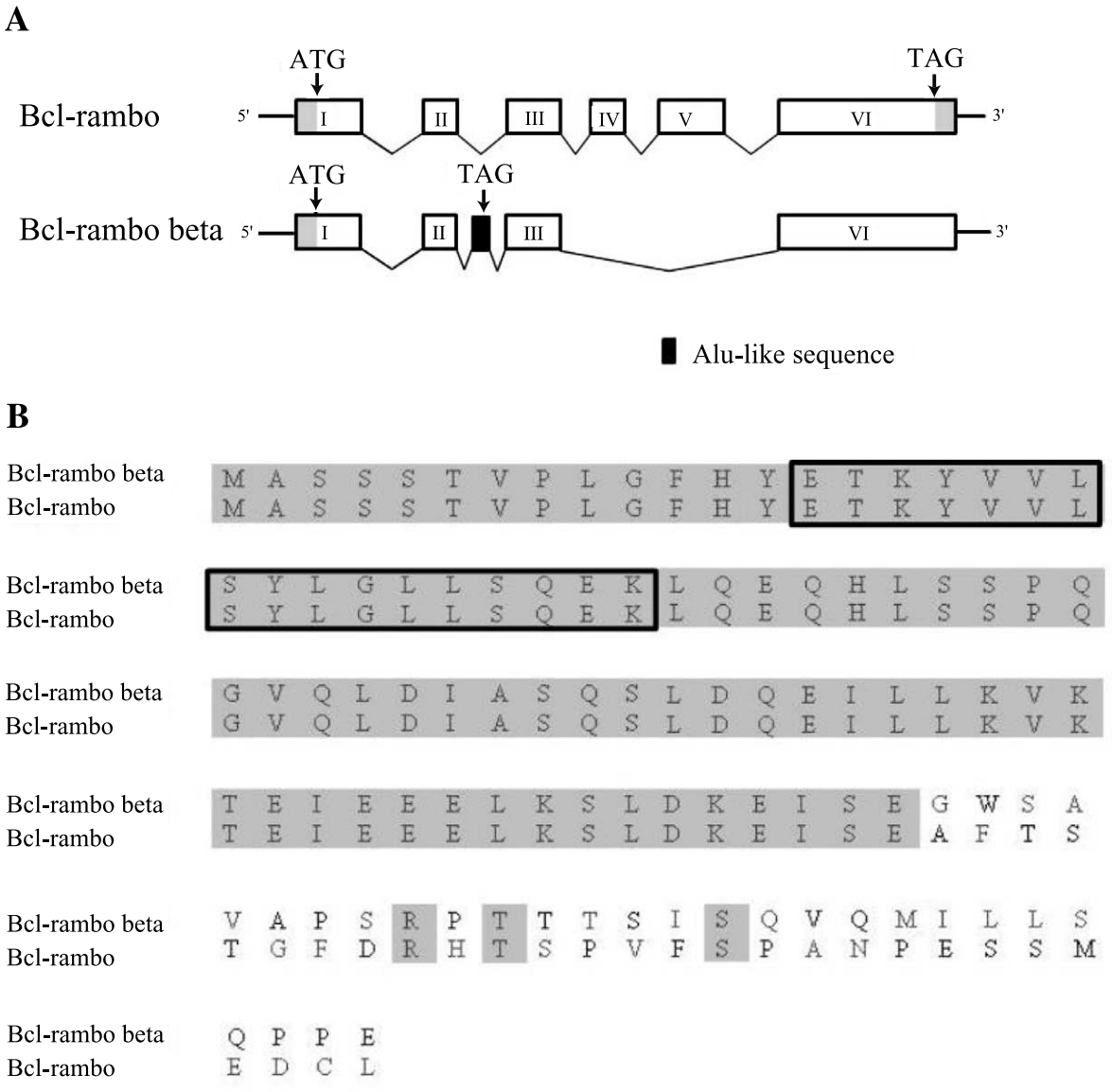



Fig. 1. The structural comparison between Bcl-rambo and Bcl-rambo beta. A: The schematic diagram of intron/exon structure of Bcl-rambo and Bcl-rambo beta. Exons numbered I–VI are represented as open boxes. The Alu-like sequence is represented as black box. The shaded parts within the boxes I and VI represent the 5′ and the 3′ untranslated regions, respectively. The initiate start ATG and the termination TGA are also denoted by arrows. B: Alignment of the protein sequence of Bcl-rambo beta to the first 104 amino acids of Bcl-rambo. The identical amino acids are shaded and the BH4 domain is boxed.

the PCR using human lymphocyte as template (Fig. 4B), which may represent an unknown isoform of Bcl-rambo. The exact nature of this PCR product is currently under investigation.

### 3.4. The Bcl-rambo beta displays a diffused localization throughout the cell

The Bcl-rambo beta lacks the membrane anchor (MA) domain in its C-terminal extension due to the premature translation termination. The MA region is believed to be essential for the localization of Bcl-rambo in the mitochondria. To examine the subcellular localization of Bcl-rambo beta, GFP-Bcl-rambo beta recombinant fusion construct was made and transfected into HeLa cells. The location of the GFP-Bcl-rambo beta was imaged by fluorescent microscopy and the result is shown in Fig. 5B. GFP-Bcl-rambo beta displayed an evenly diffused localization throughout the transfected HeLa cells when compared with Mitotracker Red staining control (Fig. 5B, c and c′). To further compare its subcellular localization patterns with GFP-Bcl-rambo and GFP-Bcl-rambo-ΔMA, two GFP fusion plasmids pEGFP-C1-Bcl-rambo and pEGFP-C1-Bcl-rambo-ΔMA were transfected into the HeLa cells and the fluorescence images are shown in Fig. 5B, b and d. As expected, the GFP-Bcl-rambo was localized in the mitochondria (Fig. 5B, b), when compared with its corresponding locations stained with the mitochondrial dye Mitotracker Red (Fig. 5B, b′). The location of expressed GFP-Bcl-rambo-ΔMA in HeLa cells was found to be mainly in the cytosol. This result is somehow different from what Kataoka et al. reported previously [4], they claimed Bcl-rambo lacking the MA region exhibited a diffused nuclear localization. At present, this apparent discrepancy cannot be

**A**

5'>GCTGGAGTGCAGTGGCACCATCTCGGCCCACCACAACCTCCATCTCCC

AGGTTCAAATGATTCTCCTGTCTCAGCCTCCTGAGTAGCCAGGATTACA

G<3'

**B**

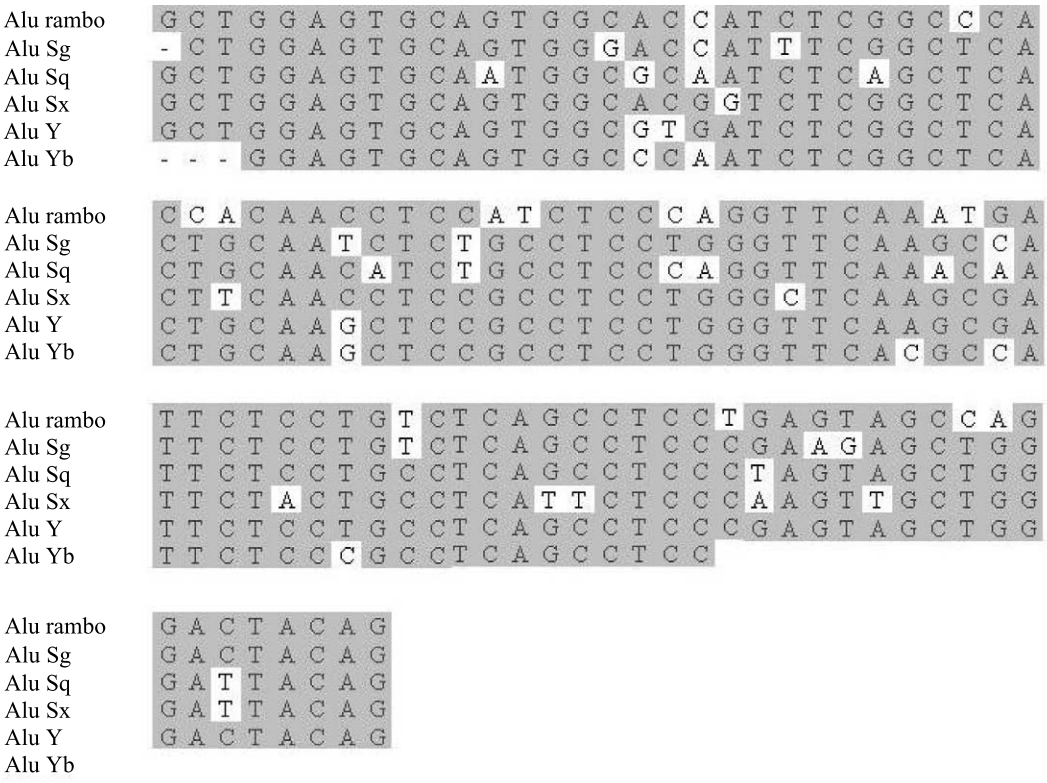



Fig. 2. DNA sequence alignment of the Alu sequence in Bcl-rambo beta with five Alu subfamilies. A: The nucleotide sequence of the Alu insert in Bcl-rambo beta. Three potential stop codons are boxed. The TGA in shaded box is the real stop codon for Bcl-rambo beta. B: Sequence alignment of the 98 bp Alu sequence of Bcl-rambo beta with five reported Alu repeat sequences. The identical nucleotides are shaded. AluSg, AluSq, AluSx, AluY, AluYb (U82670) are five different subfamilies of the Alu repeat element. The sequences were aligned by DNAstar MegAlign program.

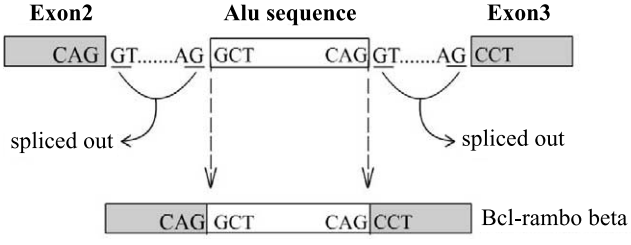



Fig. 3. The proposed splicing mechanism for Bcl-rambo beta. Bcl-rambo beta is generated by splicing of Bcl-rambo at the junctions between exons 2 and 3. Conserved 5′ donor site (GT) and 3′ acceptor site (AG) that can be recognized by spliceosome are underlined. Exons are represented as boxes.

explained clearly, however the possibility of utilization of different host cell types for transfection resulting in a different subcellular localization pattern can not be dismissed. To clarify whether the Alu sequence per se contributes to the diffused localization of Bcl-rambo beta, the Alu sequence (98 bp) was deleted from the Bcl-rambo beta gene and the resulting fragment containing BH4 domain was cloned into pEGFP-C1 to generate the recombinant plasmid pEGFP-C1-Bcl-rambo-BH4 (shown in Fig. 5A). After transfection the localization of GFP-Bcl-rambo-BH4 was shown in Fig. 5B, e and e′. The Bcl-rambo beta without the Alu sequence at the C-terminal exhibited a similar subcellular localization pattern as that of Bcl-rambo beta (Fig. 5B, c and c′), indicating the Alu sequence alone probably has no effect on the subcellular distribution. Western blotting analysis was also performed to verify the expression of all transfected genes in the HeLa cells and the results are shown in Fig. 5C.

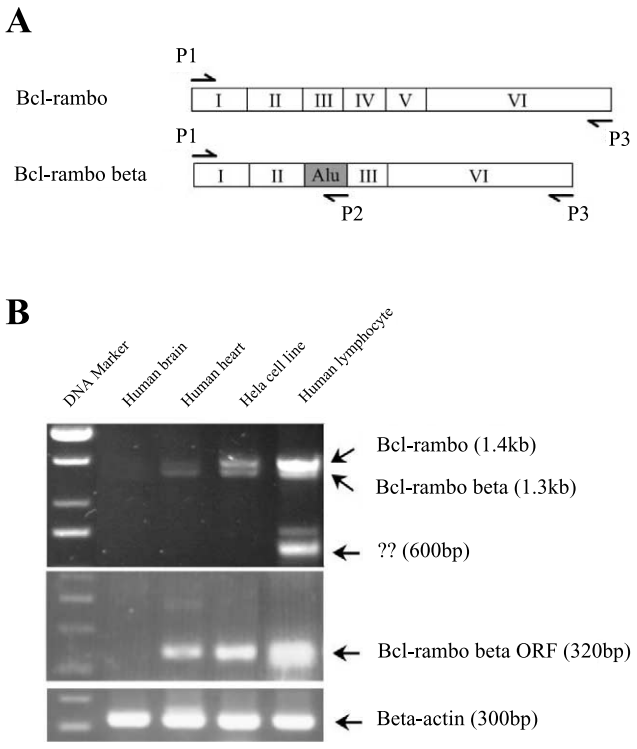



Fig. 4. Tissue distribution of Bcl-rambo beta. A: The primers P1, P2 and P3 are indicated at their proper positions. P1 and P2 were used to amplify the coding region of Bcl-rambo beta. P1 and P3 can amplify both Bcl-rambo and Bcl-rambo beta when using human tissue cDNA libraries as template. P2 was specifically designed to complement the 3′ end of the inserted Alu sequence from nucleotides 307–327. B: Results from PCR amplification using four adult human tissue cDNA libraries as template (human brain, human heart, human lymph node and HeLa cells). The upper panel shows the PCR result using P1 and P3 as primers and the middle panel is the PCR result using P1 and P2 as primers. In the lower panel, beta-actin was used as an internal control to ensure equal amount of the template used.

### 3.5. *The Bcl-rambo beta promotes etoposide- and Taxol-induced cell death*

Bcl-rambo contains a unique C-terminal extension that is reported to be essential for its proapoptotic capability [4]. Since Bcl-rambo beta lacks this C-terminal extension, we then examined whether Bcl-rambo beta still functions in cell apoptosis. HeLa cells and 293A cells were transfected with pcDNA3-Flag empty vector, pcDNA3-Flag-Bcl-rambo and pcDNA3-Flag-Bcl-rambo beta respectively and were then treated with Taxol (100 nM) or etoposide (8 ug/ml) as apoptotic stimuli. Results from the MTT cell death assay were shown in Fig. 6A. To our surprise, the Bcl-rambo beta was found to promote Taxol- or etoposide-induced cell death even more strongly than Bcl-rambo (Fig. 6A, a and b). In addition, the proapoptotic effect triggered by the Bcl-rambo beta can be partially blocked by Bcl-$X_L$, suggesting that the Bcl-rambo beta-induced apoptosis is dependent on the mitochondrial pathway (Fig. 6A, b).

HeLa cells were transfected with pEGFP-C1 control vector, pEGFP-C1-Bcl-rambo, pEGFP-C1-Bcl-rambo beta and pEGFP-C1-Bcl-rambo-BH4. 36 h after the transfection the cells were observed under fluorescent microscopy. A majority of the cells transfected with pEGFP-C1-Bcl-rambo beta became rounded and detached from the culture dish (Fig. 6B, c), whereas cells transfected with the other three plasmids, especially the pEGFP-C1-Bcl-rambo-BH4, remained morphologically unchanged (Fig. 6B, a, b and d). Consistent with this observation, GFP-Bcl-rambo beta protein was barely detected in the Western blotting experiment (Fig. 5C). These results indicate that overexpression of the Bcl-rambo beta induced the cell death in HeLa cells and the Alu sequence per se may contribute to the proapoptotic ability of Bcl-rambo beta.

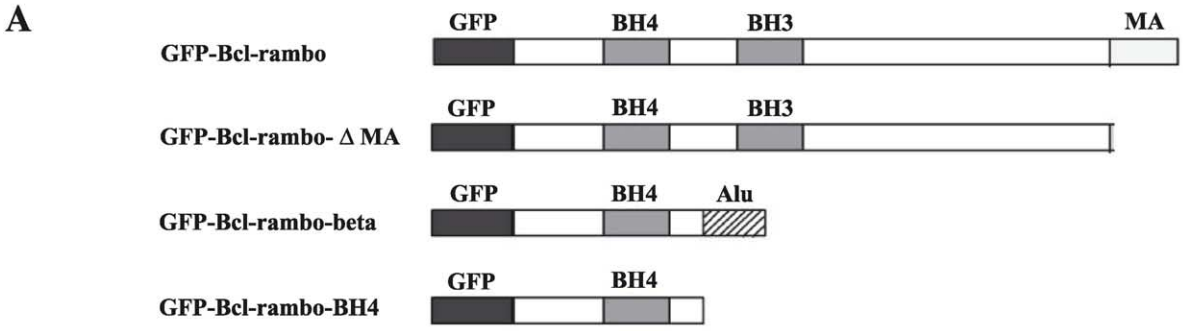
## 4. Discussion

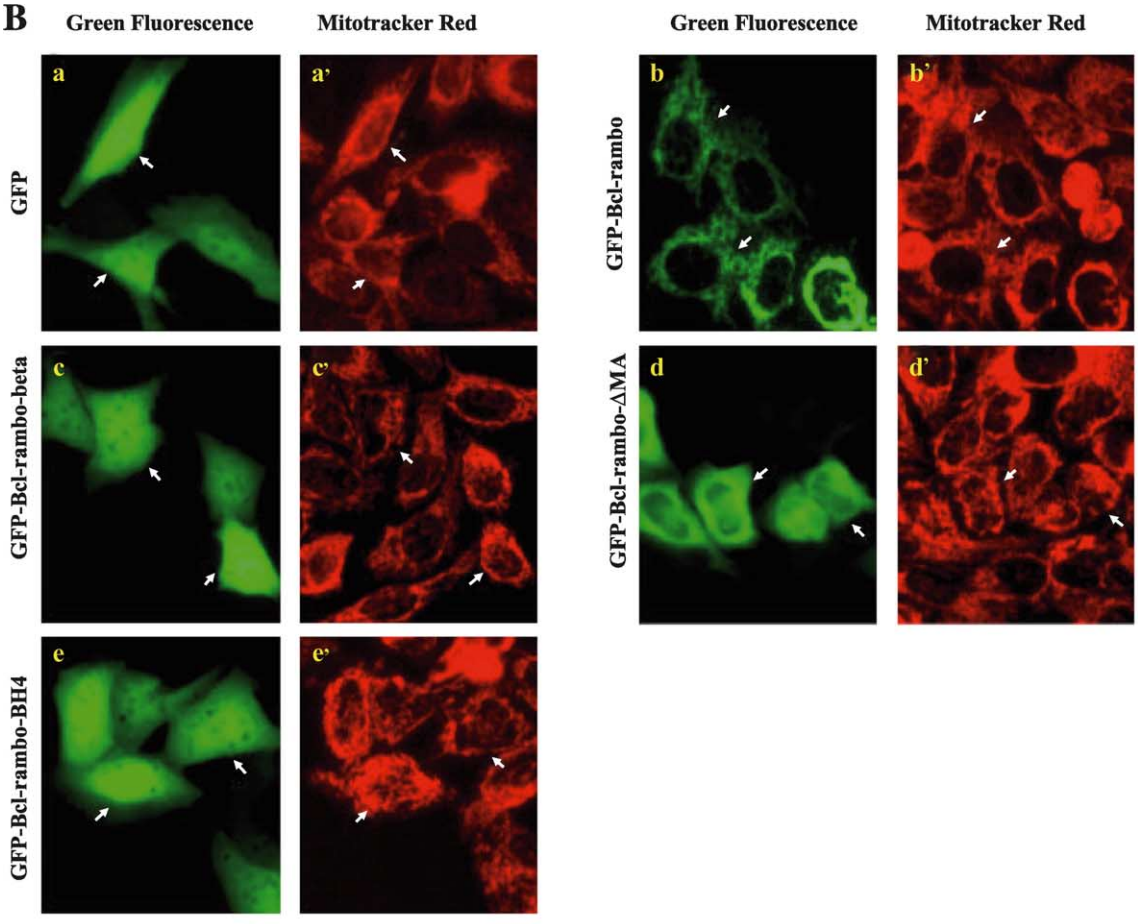
The insertion by an Alu-like element in mature mRNA is not unusual [6,7]. Sorek et al. [8] and Hide et al. [9] have analyzed about 150 genes that all contain Alu insertion by aligning a database of ESTs and cDNAs to the human genome. They have concluded that all the Alu exons are alternatively spliced. In addition, it has been reported recently that transposable elements are found in protein-coding regions of about 4% human genes, and Alu elements account for one-third of these insertions [10]. Some of these insertions are correlated with genetic diseases. For example, Ferlini et al. have identified a genomic sequence with features characteristic of Alu-like mobile elements rearranged into the dystrophin gene in a family affected by X-linked dilated cardiomyopathy [11]. Mitchell and coworkers have also found that there exists a 142 bp Alu sequence insertion in mature mRNA that associates with the deficiency of ornithine delta-aminotransferase [12]. At present, it is not clear whether an Alu sequence insertion into Bcl-rambo is connected to some specific diseases. Bcl-rambo beta is not the only example for the inclusion of an Alu sequence into Bcl-2 family genes. For example, by computational blast search, we have found for the first time that Bim-beta3, a variant of Bcl-2 family protein, also contains an Alu sequence at the C-terminal similar to Bcl-rambo beta [15]. The finding that exonization of the Alu sequence occurs in two Bcl-2 family members may be explained by that there exists a unique device in primate cells to increase the variability and versatility of Bcl-2 family proteins.

Based on the statistic data obtained by Sorek et al., the insertion of an Alu sequence on the mRNA is random. Over 80% of Alu sequences are inserted in the coding region, whereas less than 20% are inserted either in the 5′ or 3′ untranslated region. In the case of Bcl-rambo beta, the Alu cassette is inserted into the coding region of Bcl-rambo. Theoretically, there will be three effects caused by Alu sequence insertion into the protein-coding region, namely, to add a new domain, to create an alternative translational start codon and to cause a premature termination [8]. As shown in Fig. 2A, there exist three stop codons in all three possible reading frames within the Alu sequence inserted in the Bcl-rambo, therefore, translation from the ATG start of Bcl-rambo will be terminated anyway at one of these three stop codons, giving rise to a premature form of Bcl-rambo beta.

Another unusual phenomenon of Bcl-rambo beta splicing is that the inclusion of the Alu exon leads to the skipping of two downstream consecutive exons. A similar example was found that insertion of an Alu repeat also causes the skipping of the following exon in human beta-glucuronidase gene, which was reported to be associated with MPS VII in some patients [16]. The exact molecular mechanism underlying this exon skipping is not fully understood.

The Bcl-rambo beta is distinctive from the Bcl-rambo and


A
GFP
BH4
BH3
MA
GFP-Bcl-rambo
GFP-Bcl-rambo- Δ MA
Alu
GFP-Bcl-rambo-beta
GFP-Bcl-rambo-BH4


B
Green Fluorescence
Mitotracker Red
GFP
GFP-Bcl-rambo
GFP-Bcl-rambo-beta
GFP-Bcl-rambo-ΔMA
GFP-Bcl-rambo-BH4
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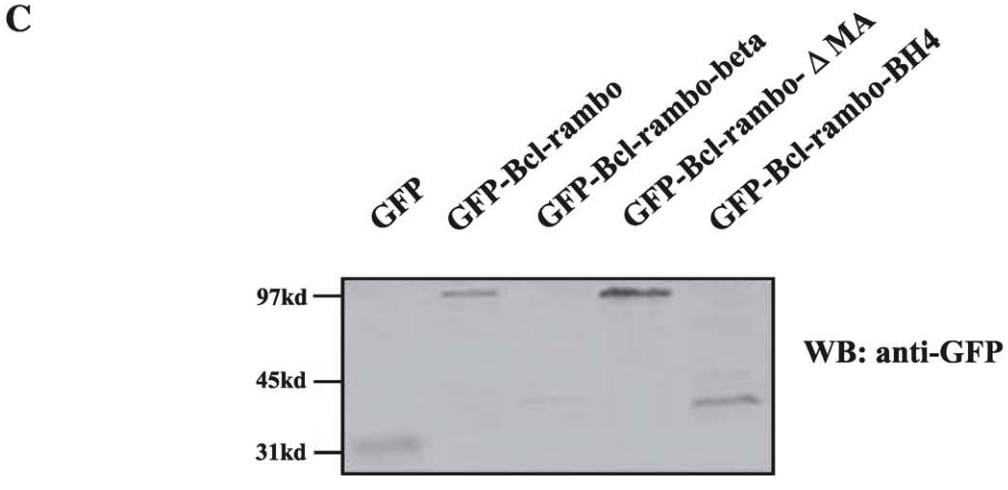
a'
b
b'
c
c'
d
d'
e
e'


C
GFP
GFP-Bcl-rambo
GFP-Bcl-rambo-beta
GFP-Bcl-rambo- Δ MA
GFP-Bcl-rambo-BH4
97kd
45kd
31kd
WB: anti-GFP

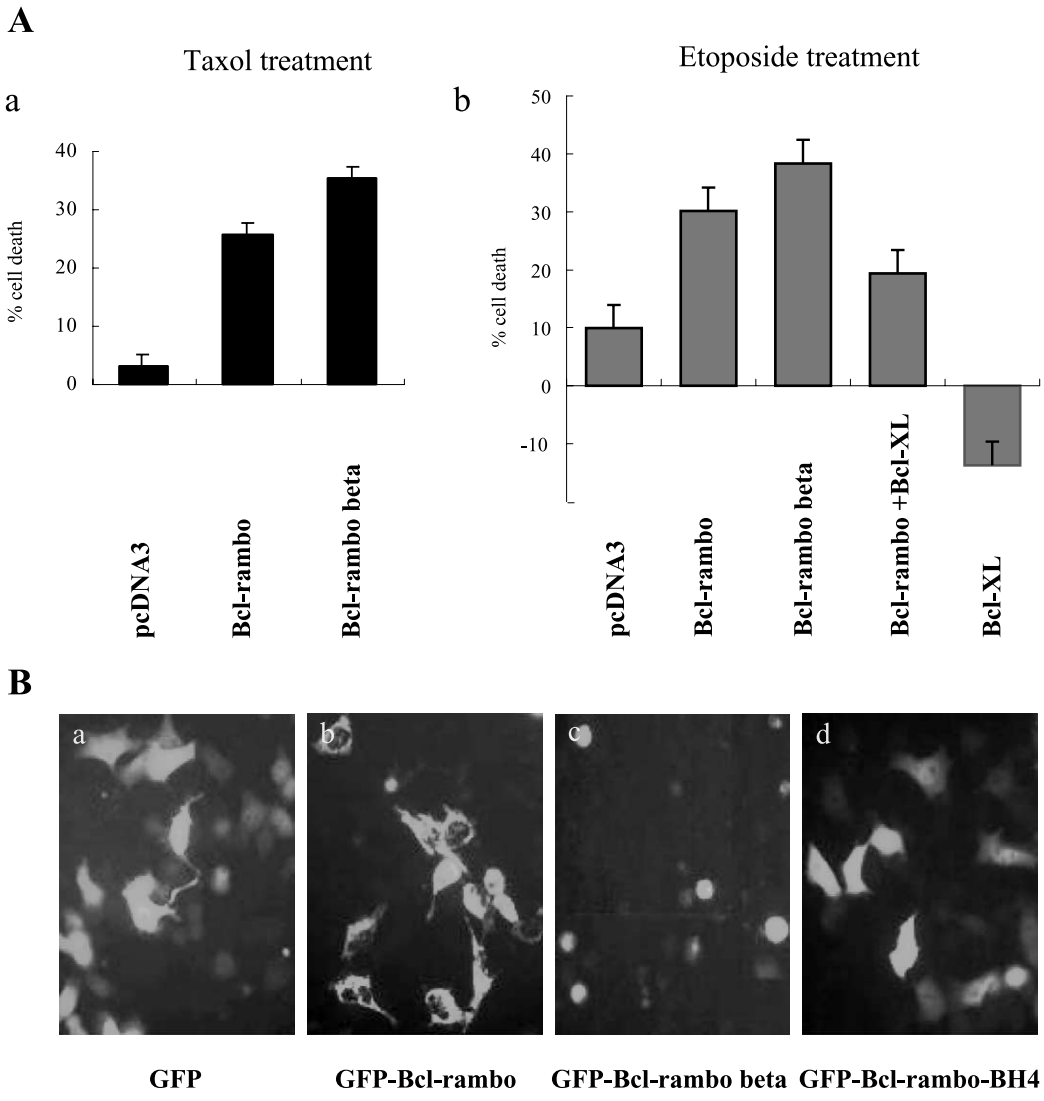



Fig. 6. Bcl-rambo beta potentiates etoposide- and Taxol-induced cell death. A: 293A cells (a) and HeLa cells (b) were plated separately onto a 96-well plate and were transfected with pcDNA, pcDNA-Bcl-rambo and pcDNA-Bcl-rambo beta and the viability of the cells was determined by the MTT method. The 100% viable cells were defined by measurement obtained from the untransfected cells. Values shown are means ± S.D. of triplicate samples. B: HeLa cells were transfected with control vector pEGFP-C1 (a), pEGFP-C1-Bcl-rambo (b), pEGFP-C1-Bcl-rambo beta (c) and pEGFP-Bcl-rambo-BH4 (d). Most of the Bcl-rambo beta transfected cells become rounded and detached from the plate at 36 h after transfection (c).

some other Bcl-2 family members in several aspects. First of all, unlike Bcl-rambo, which is present in the mitochondria, Bcl-rambo beta is found in the cytosol due to the lack of the MA region [4]. Secondly, different from Bad or Bax, the absence of the MA region in Bcl-rambo beta eliminates the possibility of translocation of this variant from cytosol to the mitochondria when the cells are undergoing apoptosis [13]. Last but not least, although Bcl-rambo beta does not contain the BH3 domain, which is required for some Bcl-2 family members such as Bax or Bid in inducing apoptosis [2], still it retains proapoptotic capability. Nevertheless, Bcl-rambo beta is not the sole example to possess that property. As an analogy, BNIP3, a member of the Bcl-2 family, when deleting both BH3 domain and membrane anchor (MA), was shown

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Fig. 5. Subcellular localization of Bcl-rambo beta, Bcl-rambo, Bcl-rambo-ΔMA and Bcl-rambo-BH4. A: The diagram of four recombinant plasmids, GFP inserted. The conserved Bcl-2 homology motifs (BH3 and BH4) are shaded in gray; the membrane anchor domain (MA) is shaded in light gray; the Alu sequence is slashed and GFP is shaded in black. B: HeLa cells were transfected with pEGFP-C1 control vector (a and a′), pEGFP-C1-Bcl-rambo (b and b′), pEGFP-C1-Bcl-rambo beta (c and c′), pEGFP-C1-Bcl-rambo-ΔMA (d and d′) and pEGFP-C1-Bcl-rambo-BH4 (e and e′). 15 h after transfection cells were stained with Mitotracker Red (100 nM) for 30 min. The subcellular localization of each GFP fusion protein was determined by fluorescent microscopy. Cells positive for both GFP and Mitotracker Red are indicated by arrows. C: Western blotting analysis was performed using the anti-GFP monoclonal antibody (MBL, Japan) to verify the expression of all transfected genes in the HeLa cells.

to be unaffected in its proapoptotic capability [14], indicating the mechanisms by which Bcl-2 family proteins function in apoptosis may be flexible.

To our knowledge, the Bcl-rambo beta is the first gene identified containing an Alu sequence insertion which may be correlated with the proapoptotic activity. The detail mechanism of how the Bcl-rambo beta and its Alu sequence are involved in the apoptosis awaits further investigation.

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