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Overexpression of BimSs3, the novel isoform of Bim, can trigger cell apoptosis by inducing cytochrome *c* release from mitochondria✪

Lingfeng Liu, Jinzhong Chen, Jiayi Zhang, Chaoneng Ji, Xiaomeng Zhang, Shaohua Gu, Yi Xie and Yumin Mao✉

State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai, P.R. China

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Bim is defined as the pro-apoptotic BH3-only protein of the Bcl-2 family, which is a critical sensor and mediator in the mitochondrial-dependent apoptosis. In a previous work, we have cloned a novel transcript of Bim (GenBank accession number: AY305716) from the fetal brain cDNA, which is widely expressed in some carcinoma tissues and normal human tissues. According to the sequence analysis and the newly-defined nomenclature system of Bim isoforms (Adachi *et al.*, 2005, *Cell Death Differ* 2: 192), we term it BimSs3 according to its characteristic structure. The subcellular location analysis indicated that the fused protein GFP-BimSs3 is distributed in the whole cell, mainly to the nucleus. Overexpression of BimSs3 in HEK293 cells causes apoptosis (28.16±1.55%) compared to the negative control (5.44±2.63%). It also causes cytochrome *c* release from the mitochondrial fraction to the cytosolic fraction during apoptosis. Western blotting assay indicates the molecular mass of GFP-BimSs3 is approximately 31.0 kDa (GFP: 27 kDa). Hence the open reading frame of BimSs3 may initiate at the second ATG and encodes a 36 amino-acid peptide with BH3 domain.

Keywords: BimSs3, apoptosis, cytochrome *c*

INTRODUCTION

Apoptosis plays a fundamental role in the development and maintenance of the immune system (Rathmell & Thompson, 2002). Deficiency in immunocyte apoptosis would have striking effects on the immune system development, homeostasis and activation, which leads to a variety of diseases, such as lymphoproliferative disorder and autoimmunity (Jaruga *et al.*, 2000; Straus *et al.*, 1999; Navratil *et al.*, 2006; Fadeel & Orrenius, 2005). Numerous studies have indicated that apoptosis can be divided into two general categories: the "intrinsic" and the "extrinsic" pathways according to the signaling cascades of apoptotic events (Liu *et al.*, 1996; Zou *et al.*, 1997; Du *et al.*, 2000; Putcha *et al.*, 2002). The "intrinsic" pathway is the mitochondria-dependent pathway with characteristic of mitochondrial membrane permeabilization (MMP) and release of the cytochrome *c* from mitochondria in apoptosis (Wakabayashi, 1999; Wang, 2001). In the healthy cells, the protein is located in the space between the inner and outer mitochondrial membranes. In mitochondria-dependent apoptosis, the apoptotic stimulus induces the release of cytochrome *c* from the mitochondria into the cytosol where it binds to Apaf-1. The cytochrome *c*–Apaf-1 complex activates caspase-9, which then activates caspase-3 and other downstream caspases (Cereghetti & Scorrano, 2006).

✉**Corresponding author:** Yumin Mao, State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai, P.R. China 200433; tel: (86 21) 6564 2502; fax: (86 21) 6564 2502; e-mail: ymmao@fudan.edu.cn

✪GenBank accession number of BimSs3: AY305716 and AAT34900.

Abbreviations: Bak, Bcl-2 homologous antagonist/killer; Bax: Bcl-2 interactive cell death susceptibility regulator; Bcl-2, B-cell lymphoma protein 2 beta isoform; BH, Bcl-2 homology domain; Bim, Bcl-2-like 11 isoform; DAPI, 4,6′-diamidino-2-phenylindole; GFP, green fluorescent protein.

The members of the Bcl-2 family have been defined as the dominant regulators in the mitochondria-dependent apoptosis (Adams & Cory, 1998; Gross *et al*., 1999). Structural and functional analyses revealed that the pro-apoptotic homologues could be subdivided into the Bax subfamily and the growing BH3-only subfamily (Daniel *et al*., 2003; Kuwana *et al*., 2005). The members of the Bax subfamily, such as Bax and Bak, have been shown to be the downstream apoptotic executor and to induce mitochondria membrane permeabilization in apoptosis (Letai *et al*., 2002). BH3-only proteins act as mediators that link various upstream signals, including death receptors and DNA damage signaling, to the mitochondrial and the endoplasmic reticulum (ER) pathway. The BH3 domain mediates BH3-only proteins to interact with the anti-apoptotic or pro-apoptotic Bcl-2 family members and plays an essential role in apoptosis (Daniel *et al*., 2003). Bim, one of most important BH3-only proteins, acts as a vital sensor to initiate apoptosis in response to intracellular damage or death stimuli. Three classic isoforms BimEL, BimL and BimS were initially detected and defined as potent inducers of apoptosis (O'Connor *et al*., 1998). With the BH3 domain, these isoforms are able to interact with anti-apoptotic members of the multidomain Bcl-2 family such as Bcl-2, Bcl-xL and antagonize their function by releasing the sequestered Bax or Bak. They could also activate multi-domain pro-apoptotic members of the Bcl-2 family, such as Bax, Bak and cause these proteins to undergo allosteric conformation and form the lethal pore in the mitochondrial outer membrane which leads to cytochrome *c* release and irreversible apoptosis (Letai *et al*., 2002; Moreau *et al*., 2003; Chen *et al*., 2005). With the DLC binding domain, BimEL and BimL are maintained in the inactive conformation through binding to the microtubule-associated dynein motor complex. BimS without the dynein binding domain is not sequestered by microtubules and induce apoptosis more potently than BimEL and BimL (Chen *et al*., 2004). Up to now, at least eighteen transcripts of human Bim mRNA have been detected, generated by alternative splicing (O'Connor *et al*., 1998; Gross *et al*., 1999; Mami *et al*., 2001; Bouillet *et al*., 2001; Liu *et al*., 2002; Idogawa *et al*., 2003; Chen *et al*., 2004). Some of them have BH3 domain, dynein light chain (DLC) binding domain or hydrophobic C-terminal region. These various isoforms may differ in size and pro-apoptotic activity. To facilitate the dissemination of information concerning these isoforms, Adachi and colleagues proposed the nomenclature system of Bim isoforms according to their domain structure (Adachi *et al*., 2005). In the newly-defined nomenclature system, Bim isoforms are divided into six groups: BimS, BimL, BimEL, BimD, BimDd and BimEDd. These group names describe the domain structure of the isoforms. Bim EL, Bim L, and Bim S represent three classical kinds of Bim isoforms which all contain the BH3 domain and induce cell apoptosis. BimD has no BH3 domain and acts as a kind of decoy. BimDd has a dynein-binding domain, whereas BimED has exon 3. Exon 3 is also the characteristic fraction of BimEL.

In a former work, we have cloned a novel transcript of Bim (GenBank accession number: AY305716) from the pre-made human fetal brain cDNA. Here we present some further work on the isoform. The comparison between its cDNA sequence and the genomic sequences (AC096670) (from NCBI website) by GeneDoc software indicates that it contains intact exon 8 (22–125 bp), exon 10 (126–250 bp), exon 11 (251–349 bp) and part of exon 2 (1–20 bp) (Fig. 2B). However, it contains an exceptional structure of two exons fused together, part of exon 2 and intact exon 8. According to its characteristic features and the nomenclature system of Bim isoforms (Adachi *et al*., 2005), we named it BimSs3. The flow cytometry assay and cytochrome *c* releasing apoptosis assay confirmed its pro-apoptotic activity through the mitochondria-dependent pathway. The western blotting assay indicates its molecular mass is about 4 kDa which suggests its open reading frame initiates at the second ATG and encodes a 36 amino-acid peptide with a BH3 domain.

MATERIALS AND METHODS

Bioinformatic analysis. To identify the gene structure, multiple alignments were performed by the GeneDoc program (http://www.psc.edu /biomed/genedoc/). The sequence of Bim isoforms from Genbank was also used. The associated software for analysis includes Gene Runner, Primer Premier 5.0.

Cell culture. The HEK293 cell line was maintained in DMEM medium (Gibco) supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator.

Subcellular location of BimSs3. The Bim-fused T-vectors were digested with *Eco*RI and *Sal*I and the gel-purified fragments of BimL and BimSs3 were cloned into pEGFP-C1 (Clontech). HEK293 cells were transfected with pEGFP-C1, pEGFP-C1-BimSs3 and pEGFP-C1-BimL plasmids (1 μg/ml) by Lipofectamine™ 2000 according to the standard protocol (Invitrogen). At 24 h post-transfection, the cells were observed under a fluorescent microscope (Olympus). The cells transfected with pEGFP-C1-BimSs3 were fixed in 4% paraformaldehyde for 30 min followed by staining with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 30 min before observation. The fluo-

rescence of GFP and DAPI were observed with wave length of: 488 nm, 405 nm for excitation and 510 nm, 461 nm for emission, respectively. Then the pictures were merged with the associated software.

Flow cytometry apoptosis assay. The pEGFP-C1, pEGFP-C1-BimSs3 and pEGFP-C1-BimL were transfected into HEK293 cells by Lipofectamine^{TM} 2000 according to the standard protocol (Invitrogen) The apoptotic rate of cells was detected using APO-BrDU kit (fluorescein labeled anti-BrDU antibody for labeling DNA breaks and propidium iodide (PI)/RNase A solution for counter staining the total DNA) (Becton Dickinson). The experiments were repeated at least three times.

Cytochrome *c* releasing apoptosis assay. Cytochrome *c* releasing apoptosis assay kit was purchased from Biovision (Moutain View, CA, USA). HEK293 cells are divided into three groups and transfected with the pEGFP-C1, pEGFP-C1-BimS and pEGFP-C1-BimSs3 plasmids (1 μg/ml), respectively. After 18 h incubation at 37°C in a 5% CO_2 incubator, the transfected cells are divided into the mitochondrial and cytosolic fraction according to the standard protocol provided by Biovision. Purity of both fractions is determined by Western blot analysis using anti-Cox antibody (a mitochondrial protein) and calpain (a cytoplasmic protein). Cytochrome *c* is detected in two fractions by Western blotting using anti-cytochrome *c* antibody. Endogenous β-actin protein expression in the cells is also detected as a control of sample quantity by Western blotting using anti-β-actin antibody (Santa Cruz).

Western blotting assay. The transfected cells were washed twice with phosphate-buffered saline and lysed in 0.5 ml lysis buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, and protease inhibitor tablets from Roche) for 15 min at 4°C. After cell fractionation, the lysate was centrifuged at 14000 r.p.m. for 15 min at 4°C. The supernatant was used for subsequent Western blotting assay. The proteins were subjected to SDS/PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk in TBS-T (20 mM Tris/HCl, 137 mM NaCl, and 0.1% (v/v) Tween 20) and detected with rabbit anti-human GFP polyclonal antibody (Santa Cruz) or mouse anti-cytochrome *c* antibody (Biovision) followed by horseradish peroxidase conjugated anti-rabbit antibody (Sigma). The proteins were visualized by using enhanced chemiluminescence immunoblotting detection reagents (Amersham). The protein marker was provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS.

RESULTS AND DISCUSSION

The mitochondria dependent apoptosis is tightly regulated by the Bcl-2 family of proteins, all of which contain the Bcl-2 homology (BH) domain and function to either inhibit or promote apoptosis (Willis & Adams, 2005). The anti-apoptotic subfamily of the Bcl-2 family is composed of multi-BH domain anti-apoptotic members such as Bcl-2, Bcl-xL and MCL-1. The pro-apoptotic members of the Bcl-2 family are further subdivided into the multidomain pro-apoptotic proteins, which include Bak and Bax, and the BH3-only proteins, which induce activation of Bak or Bax directly and indirectly (Kuwana *et al*., 2005). BH3-only proteins are defined as the central regulators or mediators of the mitochondrial-dependent apoptosis in the pro-apoptotic members of Bcl-2 family (Willis & Adams, 2005). Bim, one of most important BH3-only proteins, acts as a vital sensor and executor to initiate apoptosis in response to intracellular damage or death stimuli (Marani *et al*., 2002; Zhang *et al*., 2006). It counteracts and antagonizes the anti-apoptotic muti-domain proteins of the Bcl-2 family, such as Bcl-2, Bcl-xL (Cheng *et al*., 2001).

In a former work, we have cloned a novel mRNA transcript of Bim (AY305716) from the fetal brain cDNA library. Pre-made human normal and pathological tissues cDNA panel based PCR revealed this novel transcript is expressed widely in cancer tissues and some normal human tissues (Chen *et al*., 2004). The novel isoform's cDNA contains three potential ATGs (Fig. 1). Starting from the second ATG, the putative protein may com-

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ATG GCA AAG CAA CCT TCT GAT CTT CCA TGA GG CAG GCT GAA
 M   A   K   Q   P   S   D   L  P M/& R   Q   A   E
CCT GCA GAT ATG CGC CCA GAG ATA TGG ATC GCC CAA GAG TTG
 P   A   D  M   R   P   E   I   W   I   A   Q   E   L
CGG CGT ATC GGA GAC GAG TTT AAC GCT TAC TAT GCA AGG AGG
 R   R   I   G   D   E   F   N   A   Y   Y   A   R   R
TTA  GAG AAA TAG AGG AAG TTG TCG TGT AGT TGT CAT GTA TTC
 L   E   K   &
AGTCCACTTAAGGGCAGTGGGGAAGCGTTTGAGACGGAGCTGTGGAG
GCTGAATCCTTGAAGGAGGAGGTGAGAGAGGCACAGGTATTTTTGAAT
AATTACCAAGCAGCCGAAGACCACCCACGAATGGTTATCTTACGACTGT
TACGTTACATTGTCCGCCTGGTGTGGAGAATGCATTGA
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Figure 1. Nucleotide sequence and putative amino-acid sequence of the novel Bim transcript.
The potential ATG is boxed; the putative BH3 domain is underlined.

prise 36 amino acids with a molecular mass of 4.424 kDa.

Exon structure and nomenclature of the novel Bim isoform

Using GeneDoc software, we performed the multiple alignments between BimS, BimSs, BimSs', BimSs2 and the novel isoform (Fig. 2). The data indicate that this novel isoform comprises the intact exon 8 (22–125 bp), exon 10 (126–250 bp) and exon 11 (251–349 bp) matching completely with other Bim alternative-splicing transcripts. However, it has an aberrant structure of two exons (exon 2 and exon 8) fused together (The first 21 bp fragment belongs to exon 2 and the 22–125 bp fragment is intact exon 8 which encodes the BH3 domain). Hence the result suggested that the new transcript should belong to the BimS group of the newly defined nomenclature system. Based on the sequence alignment and newly determined nomenclature system of Bim, we then termed this novel isoform "BimSs3" for it contains all the same exons of BimSs' except its incomplete exon 2.

Subcellular location of human BimSs3

Through fluorescent microscopy, the EGFP-BimSs3 fusion protein was found to distribute to the

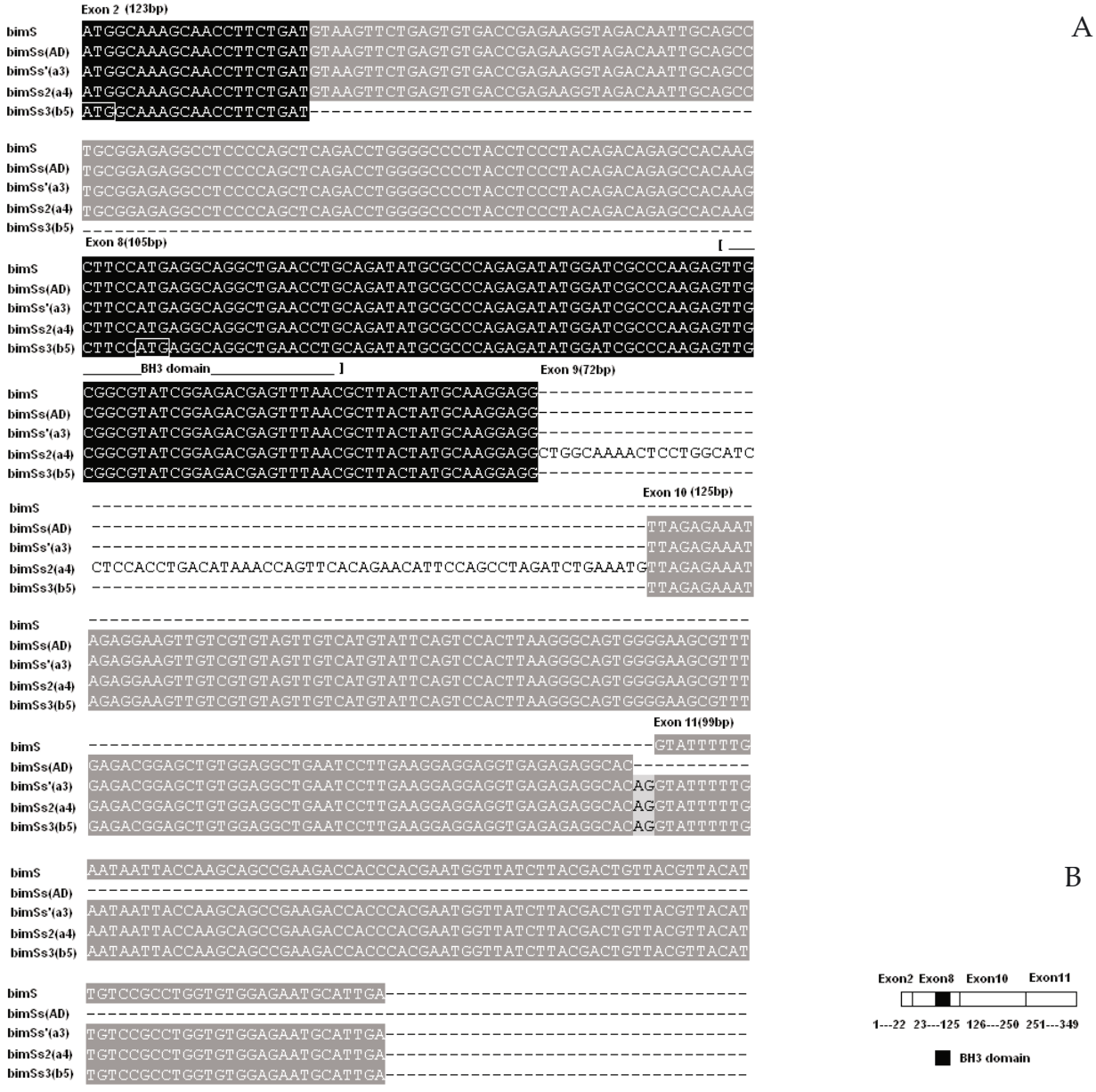
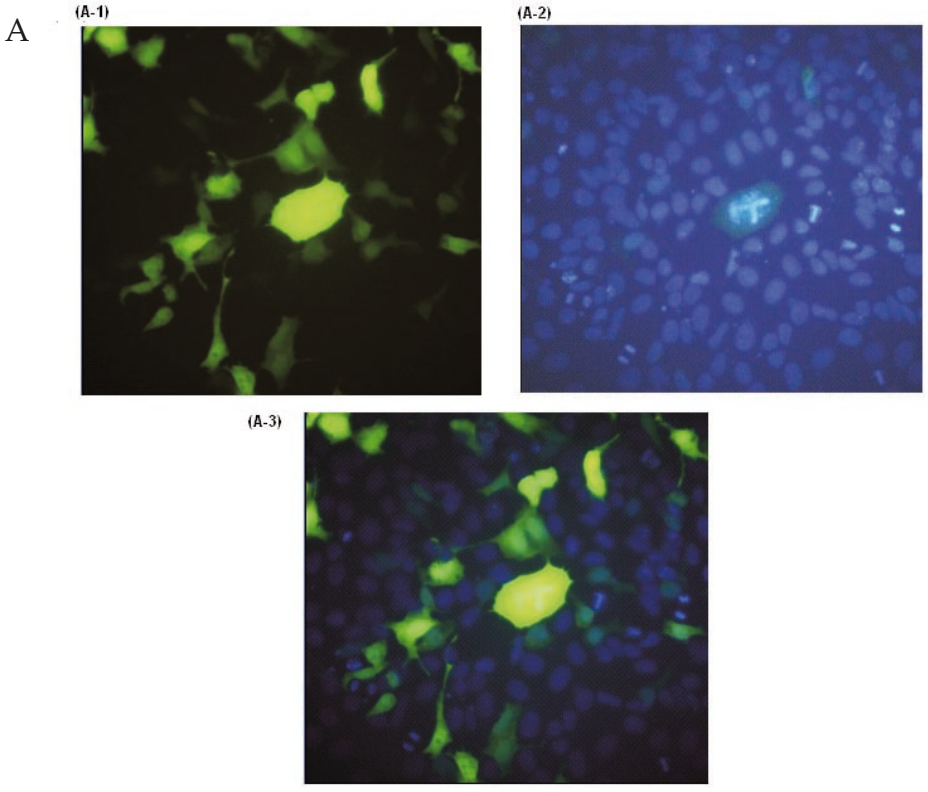


Figure 2. Sequence analysis of BimSs3.
(A) Multiple sequence analysis of Bim isoforms: BimS, BimSs, BimSs', BimSs1, BimSs2 and BimSs3. The exon starting site and its nucleotide number are marked on the top of every sequence. (B) Diagram of the exon structure of BimSs3.



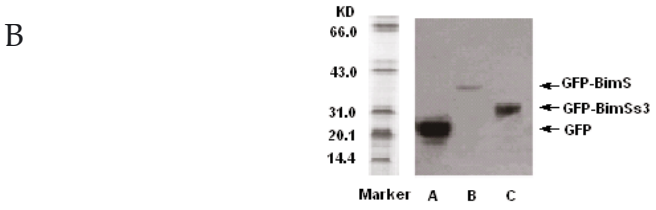


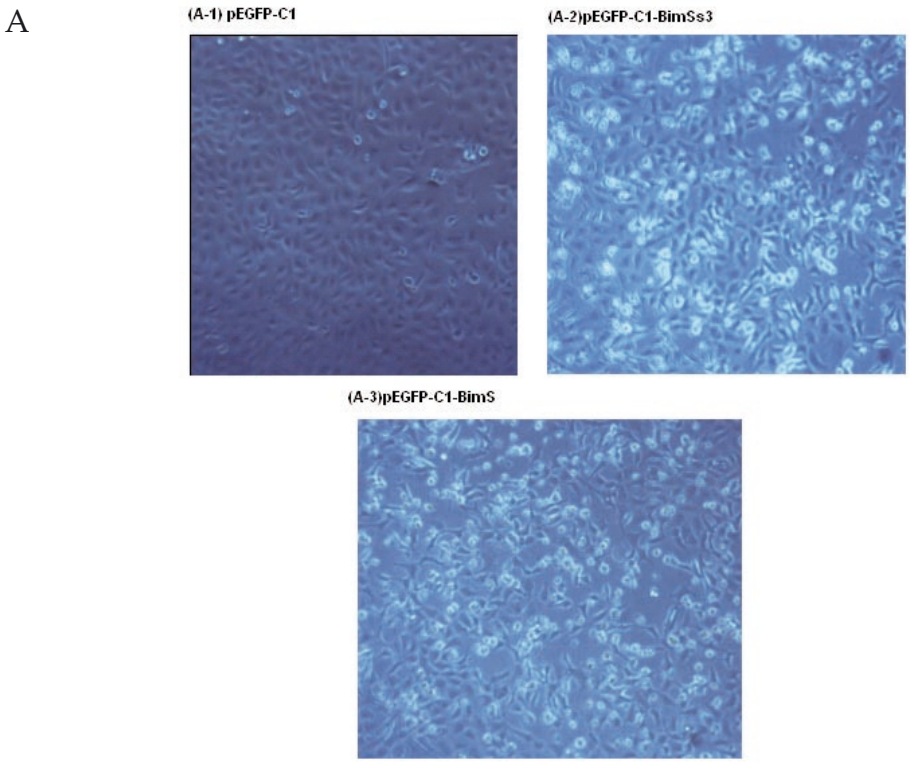
Figure 3A. Subcellular location analysis of BimSs3.
A. pEGFP-C1; B. pEGFP-C1-BimS; C. pEGFP-C1-BimSs3). The HEK293 cells expressing GFP-BimSs3 were detected using fluorescent microscopy (nuclei stained by DAPI). (A-1) GFP-Bim (green fluorescence); (A-2) nuclei (blue fluorescence); (A-3) merged pictures of (A-1 and A-2).
Figure 3B. Western blotting assay.

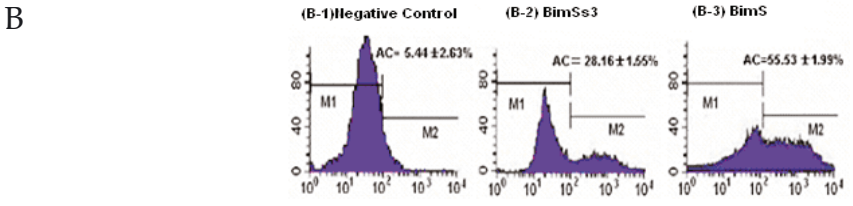
whole cell, mainly to the nucleus (Figs. 3A-1, A-2, A-3). It is different from GFP-BimL which is known to mainly localize in the cytoplasm (Chen *et al.*, 2004). This may be due to its putative peptide without the dynein light chain (DLC) binding domain.

To confirm the expression of GFP-fused protein, the transfected cells were lysed and detected by Western blotting using anti-GFP antibody. The immunoblotting assay indicated that the panel of BimSs3 is localized between the panels of GFP and GFP-BimS. According to the marker, the molecular mass of GFP-BimSs3 was approx. 31 kDa. Hence the molecular mass of the putative BimSs3 might be 4 kDa containing about 30–40 amino acids (Fig. 3B). However, there is no significant ORF in mRNA of BimSs3 due to its incomplete exon 2 and intact exon 8 fusing together. Through analysis of its sequence and putative molecular mass, we predicted the genuine ORF of BimSs3 might start at the second ATG and contain 108 nucleotides (Fig. 1).

Apoptosis detection

Under the microscope, the cells transfected with pEGFP-C1-BimSs3 presented poor growth status with many dying cells floating in the culture medium just like the cells transfected with pEGFP-C1-BimS (Fig. 4A-1, A-2). This suggests that this novel isoform of Bim might also induce apoptosis. Flow cytometry assay using an APO-BrDU kit was





AC: apoptosis rate of cell

Figure 4. BimSs3 induces apoptosis in HEK293 cells.
A. Optical microscope image. The HEK293 cells were divided into three groups and transfected with pEGFP-C1, pEGFP-C1-BimSs3 and pEGFP-C1-BimS, respectively. After 24 h incubation, the cells were observed under the optical microscope. **B. Flow cytometry apoptosis analysis**. The HEK293 cells were terated as above except that after 24 h incubation, the cells were submitted to flow cytometry apoptosis analysis using APO-BrDU kit..

carried out to detect apoptosis. Compared with the negative control of plasmid pEGFP-C1 (Fig. 4B-1: 5.44±2.63%), it presented significant pro-apoptotic activity (Fig. 4B-2: 28.16±1.55%), although its pro-apoptotic activity is weaker than BimS (Fig. 4B-3: 55.53±1.99%). Experiments were performed in triplicate and the representative results are shown.

To gain insight into the intracellular function of BimSs3, we turned to the cytochrome *c* releasing apoptosis assay to detect whether its pro-apoptotic activity is associated with the mitochondria during cell apoptosis. During apoptosis, Bim isoforms act as mediators and triggers to activate Bak/Bax which in turn form the lethal pore in the outer membrane of mitochondria and lead to release of the cytochrome *c* from mitochondria. So in the cells undergoing mitochondria-dependent apoptosis, cytochrome *c* can be detected both in the mitochondrial fraction and the cytosolic fraction, mainly in the cytosol, while in healthy cells, cytochrome *c* can only be detected in the mitochondria. Using cytochrome *c* releasing detection kit, the transfected cells were divided into the mitochondrial fraction and the cytosolic fraction.

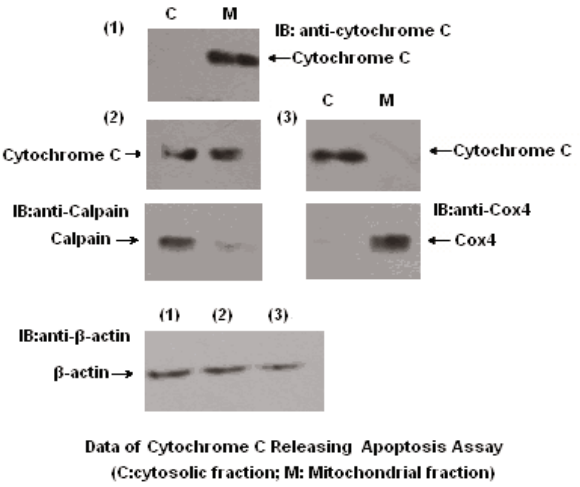


Figure 5. Cytochrome *c* releasing apoptosis assay.
(1) Negative control (the HEK293 cell transfected with pEGFP-C1); (2) The HEK293 cells transfected with pEGFP-C1-BimSs3; (3) The HEK293 cells transfected with pEGFP-C1-BimS. Western blot analysis using anti-Cox and anti-calpain antibody were carried out to confirm purity of two fractions. The endogenous β-actin protein was also detected by anti-β-actin antibody to provide loading quantity controls.

Purity of both fractions was confirmed by Western blot analysis through detecting the distribution of the Cox (a mitochondrial protein) and calpain (a cytoplasmic protein) in the two fractions by immunoblotting assay using Cox antibody and calpain antibody. In the cells transfected with pEGFP-C1-BimSs3, cytochrome *c* can be detected in the two fractions, mainly in the cytosol. In the cells transfected with pEGFP-C1, cytochrome *c* distributed only to mitochondrial fraction. In the cells transfected with pEGFP-C1-BimS, cytochrome *c* mainly localized in the cytosolic fraction (Fig. 5). The result clearly indicated overexpressing BimSs3 in the cells was able to induce cytochrome *c* release from mitochondria to the cytosol during apoptosis which was consistent with the current knowledge that Bim induced apoptosis through a mitochondria-dependent pathway.

In summary, we have cloned a novel transcript of Bim from the fetal brain cDNA and characterized its pro-apoptotic activity through flow cytometry apoptosis assay and the cytochrome *c* releasing apoptosis assay. Based on the multiple sequence alignment and the newly-defined nomenclature system of Bim isoforms, we term it BimSs3 according to its characteristic structure. Western blotting assay indicates the molecular mass of BimSs3 is about 4 kDa. The open reading frame of BimSs3 may initiate at the second ATG and encode a 36 amino-acid peptide with a BH3 domain. Hence BimSs3 is newly-defined BH3-only protein of Bcl-2 family. Further studies will be carried out to determine the potential molecular mechanisms in the BimSs3-mediated apoptosis.

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