Oncogene (2003) 22, 2457–2465

www.nature.com/onc

Bfl-1S, a novel alternative splice variant of Bfl-1, localizes in the nucleus via its C-terminus and prevents cell death

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Bfl-1 is an antiapoptotic Bcl-2 family member and a mouse A1 homologue. The mouse A1 has been reported to have three isoforms, but little is known about human Bfl-1. By reverse–transcriptase polymerase chain reaction analysis, we have identified Bfl-1S (short form), an alternative splice variant of Bfl-1. The Bfl-1S primary sequence contains four conserved Bcl-2 homology (BH) domains and a positive-charged C-terminus containing KKRK amino acids. The expression of Bfl-1S mRNA was detected predominantly in normal lymph nodes and in B-lymphoid leukemia cells. Confocal microscopic analysis using green fluorescence protein fusion proteins demonstrated that Bfl-1S is localized in the nucleus by its C-terminus as an intrinsic nuclear localization sequence. Bfl-1S acts as an antiapoptotic agent in coexpression experiments with Bax, a proapoptotic molecule. The expression of Bfl-1S provided significant resistance against staurosporine (STS) treatments in Molt-4 human T-leukemia cells. Bfl-1S also significantly inhibited the cleavage of Bid, and of caspases 3 and 8 against STS treatment. These results indicate that Bfl-1S is a novel human Bcl-2 family member that possesses antiapoptotic function.
Oncogene (2003) **22,** 2457–2465. doi:10.1038/sj.onc.1206274

Keywords: Bfl-1S; Bcl-2 family; alternative splice; apoptosis; Molt-4; nuclear localization

Introduction

It is widely believed that Bcl-2 family proteins are central regulators of apoptosis. Bcl-2 family proteins can act either as proapoptotic regulators, such as Bax, Bad, Bid, Bcl-xS and Bak, or as antiapoptotic regulators, such as Bcl-2, Bcl-xL, Bcl-w and Bfl-1 (Chao and korsmeyer, 1998; Reed, 1998). By physical interaction with each other, the Bcl-2 family proteins are capable of forming a complex network of homo- and heterodimers via molecular regions, such as the Bcl-2 homology (BH) domains, and these physical interactions sometimes play important roles in the net effects of pro- and antiapoptotic members of the family (Yin *et al*., 1994).

Several proteins of the Bcl-2 family, including Bcl-2, Bcl-x and Bax, have membrane-bound and soluble isoforms, which are generated by alternative splicing. The *BCL-2* gene contains at least two exons. Bcl-2α is the predominant isoform, which contains a carboxyl-terminal transmembrane domain that has potent death-inhibitory activity. Bcl-2β lacks the transmembrane domain and has reduced antiapoptotic activity (Hockenbery *et al*., 1990). Alternative splicing forms of Bcl-x give rise to two transcripts coding for either a long (Bcl-xL) or a short (Bcl-xS) form of the protein. Bcl-xL inhibits cell death, whereas Bcl-xS antagonizes the antiapoptotic action of both Bcl-2 and Bcl-xL (Boise *et al*., 1993). These results suggest that isoforms of the Bcl-2 family can either cooperate or antagonize each other. Therefore, it becomes important to know the function of isoforms.

Bfl-1 possessing antiapoptotic activity against various death signals was discovered by data bank searches of expressed sequence tags (EST) (Choi *et al*., 1995). Subsequently, a human Bfl-1 cDNA was identified as a tumor necrosis factor (TNF)-inducible transcript (Karsan *et al*., 1996a) and was shown to protect endothelial cells against TNF-induced cell death (Karsan *et al*., 1996b). The expression of Bfl-1 mRNA was detected in bone marrow, spleen and lung at higher levels, and in the thymus, testis and small intestine at lower levels (Choi *et al*., 1995; Karsan *et al*., 1996a). Bfl-1 appears to be induced by inflammatory cytokines such as TNF and interleukin-1 (Karsan *et al*., 1996a, b), and was found to be a direct transcriptional target of nuclear factor-κB (NF-κB) (Lee *et al*., 1999; Wang *et al*., 1999; Zong *et al*., 1999). The Bfl-1 protein shares about 72% amino-acid identity with mouse A1 protein. It has recently been demonstrated that mice have three functional isoforms of A1 (Hatakeyama *et al*., 1998), but in humans, no

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Received 11 October 2002; revised 4 December 2002; accepted 10 December 2002

report has been made about isoforms of Bfl-1, a human homologue of mouse A1.

In this study, we found that there exist at least two isoforms of Bfl-1, which result from alternative splicing of the *BFL-1* gene, and designated a new alternative splicing isoform as Bfl-1S (short). The production of the Bfl-1 and Bfl-1S transcripts was revealed to result from the exclusion or inclusion of a 56-base pair (bp) exon. Here we report the genomic structure coding for Bfl-1S, expression pattern, subcellular localization and anti-apoptotic function of Bfl-1S. The results indicated that Bfl-1S is a novel human Bcl-2 family member, which targets the nucleus via its C-terminal nucleus localization sequence (NLS).

Results and discussion

Identification and sequencing of Bfl-1S, a novel splicing variant of the human BFL-1 gene

The reverse–transcriptase polymerase chain reaction (RT–PCR) using primers specific for Bfl-1 cDNA revealed two kinds of transcripts. The shorter one was revealed to code for the Bfl-1 protein, while the longer one to be the variant containing the 56 bp exon (Figure 1a). By searching the GenBank™/EBI database using this shorter form of cDNA sequence, two expression sequence tags (EST) sequences (XM_044554 and AL110097) were found to be identical to the human Bfl-1 cDNA sequence (U27467), except for a 56-bp nucleotide added near the 3′-end. To understand the transcriptional mechanisms expressing the Bfl-1 isoforms, the *BFL-1* gene was searched for in the human genomic database of GenBank™/EBI. A 10.4 kb *BFL-1* gene located on chromosome 15 was identified from the 1688 kb Human Chromosome 15 Working Draft Sequence (the GenBank™/EBI database no. NT_010360). The splicing sites of the 10.4-kb *BFL-1* gene were predicted using NetGene2 ver. 2.4 software provided by the NetGene2 WWW Sever (Center for Biological Sequence Analysis, The Technical University of Denmark, Lyngby, Denmark). Comparisons of the cDNA sequences suggested a 3-exon structure for the *BFL-1* gene (Figure 1a). The production of transcriptional variant of Bfl-1 was found to result from the alternative exclusion or inclusion of the 56-bp exon II. The 56-bp shorter transcript was found to contain the open reading frame (ORF) for Bfl-1 of 175 amino acids, and the 56-bp longer transcript to contain the ORF for a protein of 163 amino acids by including exon II. The inclusion of the 56-bp exon II results in early translational termination by an upstream stop codon. Therefore, the longer transcript encodes for a shorter protein, which we named Bfl-1S (short). This type of splicing is

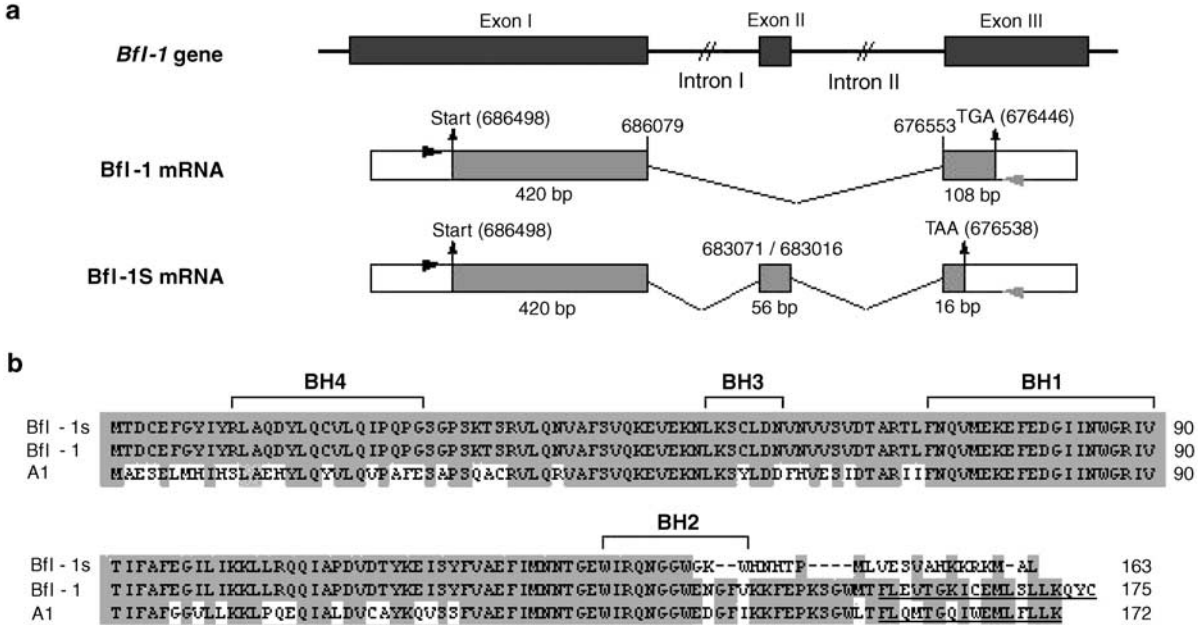

Figure 1 Sequence analysis of Bfl-1S cDNA and the genomic structure of the *BFL-1* gene. (**a**) The organization of the *BFL-1* gene is compared with mRNAs encoding Bfl-1 and Bfl-1S. A 10.4 kb of the *BFL-1* gene located on chromosome 15 was identified from 1688 kb of the Working Draft Sequence of Human Chromosome 15 (the GenBank™/EBI database no. NT_010360). Splicing sites, and start and stop positions are indicated as upper numbers at their respective genomic nucleotide positions. Ligation of exons I and III produces Bfl-1 mRNA (U27467), whereas the inclusion of the 56-bp exon II produces Bfl-1S mRNA, which encodes a 163 amino-acid protein by an open reading frame (ORF) shift, and causes an early translation termination. Positions of the RT–PCR primers are indicated by a black arrowhead for RT-primer1 and a gray arrowhead for RT-Primer2. (**b**) Comparison of primary sequences of Bfl-1S, Bfl-1 and mouse homologue A1. Identical residues are shaded. Bfl-1S mRNA (GenBank™/EBI Accession number XM_044554) encodes a protein of 163 amino acids, whereas Bfl-1 mRNA (U27467) encodes a longer protein of 175 amino acids. Bfl-1S contains four conserved Bcl-2 homology (BH) domains, BH1, 3 and 4, and a partially modified BH2 domain. Bfl-1 and A1 contain a putative transmembrane (TM) domain (underlined) in their C-termini, whereas Bfl-1S does not contain a TM domain

very similar to that of ICH-1 (CASPASE 2) gene, which also consists of three exons and produces Ich-1 (proapoptotic) or Ich-1S (antiapoptotic) by the exclusion or the inclusion of a 61-bp exon (Wang *et al.,* 1994). Bfl-1 and Bfl-1S proteins are identical over the first 140 amino acids and then differ at their C-terminal ends. A comparison of the primary sequence of Bfl-1S with those of Bfl-1 and A1 (a mouse homologue of Bfl-1) revealed that Bfl-1S contains the conserved Bcl-2 homology (BH) domains, 1, 3 and 4, and the partially modified BH2 domain (Figure 1b). Interestingly, Bfl-1S contains a highly hydrophilic stretch at its C-terminus, which consists of a consecutive series of four positively charged amino acids, KKRK, and lacks the hydrophobic transmembrane domain, which is a common domain of the antiapoptotic Bcl-2 family members including Bfl-1 (Figure 1b).

Expression of Bfl-1S mRNA in human normal tissues and cancer cells

Previously, Northern blot analysis showed the presence of high levels of Bfl-1 mRNA in the bone marrow, spleen and the lung. Since Northern blotting failed to resolve the mRNAs encoding Bfl-1 and Bfl-1S, RT–PCR assay was performed with the RT-primers 1 and 2 to amplify simultaneously Bfl-1 and Bfl-1S cDNAs as 577 and 633 bp products. Bfl-1 mRNA expression was detected in the lymph node, spleen, lung and colon at higher levels, and in the thymus and liver at lower levels (Figure 2a). In contrast, Bfl-1S was found at high levels in the lymph nodes and at a lower level in the spleen. Amplification of β-actin mRNA was used as an internal control to semiquantitatively assess the relative amounts of mRNA in each tissue. The expressions of Bfl-1 and Bfl-1S mRNAs were also investigated in 43 human cancer cell lines, established from the stomach, colon, lung, liver and blood. A high level of expression of Bfl-1 mRNA was detected in 12 cell lines including seven of 12 blood cell lines (58%), three of four lung (75%) and two of 10 stomach cancer cell lines (20%) (Figure 2b). Interestingly, Bfl-1S mRNA expression was detected in B leukemia cell lines, IM-9, Jijoye, BJAB and Wil2-NS, and the myeloid cell line KG-1 but not in T-cell lines. The Bfl-1/Bfl-1S mRNA ratio was estimated to be about 4.1 in normal lymph nodes and about 5.7 in Wil2-NS cells, by measuring the intensity of the RT–PCR band.

Previously, *in situ* hybridization showed that Bfl-1 mRNA was prominently expressed in the germinal centers of lymphoid tissues, including lymph nodes, tonsils and lymphoid nodules of the gastrointestinal tract, and was only marginally expressed in the fetal thymus (Jung-Ha *et al*., 1998). The germinal center within the lymph node and spleen is thought to be a region where B-cell proliferation and differentiation occur after interactions between B cells and antigen-presenting follicular dendritic cells (Kepler and Perelson, 1993). Recent results have also shown that Bfl-1 and Bcl-xL expressions are upregulated by NF-κB-

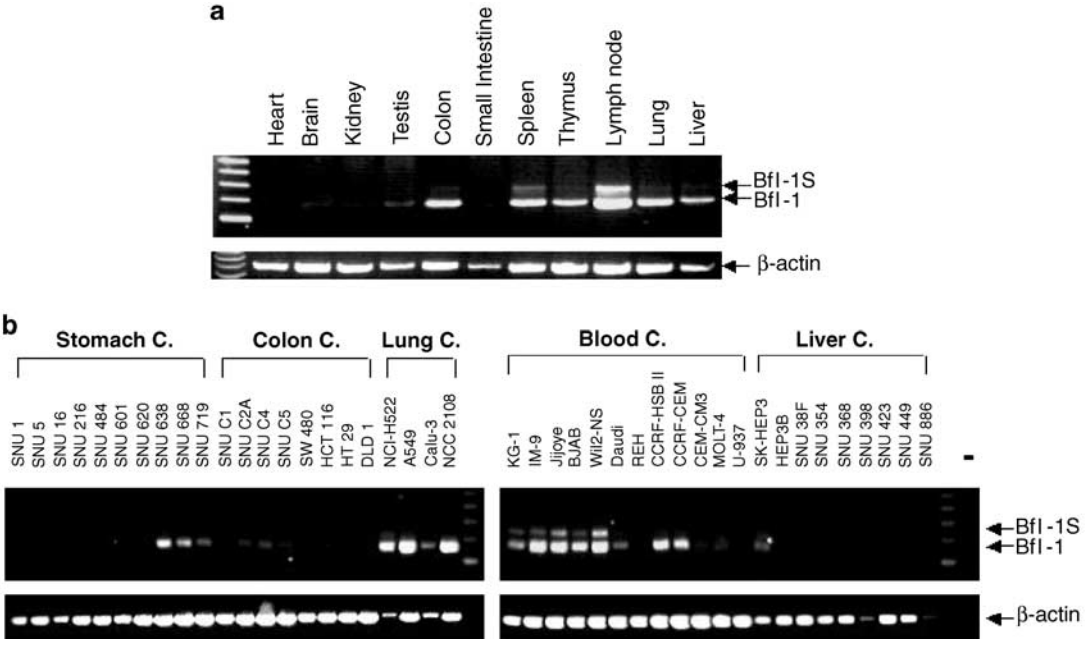

Figure 2 Expression of Bfl-1S mRNA in normal human tissues and cancer cells. (**a**) The expression of Bfl-1S mRNA in human normal tissues was analysed by RT–PCR with the Bfl-1-specific RT-primers 1 and 2. Each 1 μg of total RNA was subjected to cDNA synthesis as described in 'Materials and methods'. A total of 35 cycles of PCR amplification were performed for Bfl-1S cDNA and 23 cycles for β-actin cDNA. PCR amplification simultaneously produced Bfl-1 and Bfl-1S cDNAs as 577 and 633-bp products. PCR products were separated on 3% agarose gel and visualized by ethidium bromide (EtBr) staining. (**b**) Expression of Bfl-1S mRNA in cancer cells was analysed by the RT–PCR using cDNA templates prepared from the total RNAs of 43 human cancer cell lines. A total of 30 cycles of PCR amplification were performed for Bfl-1S cDNA and 25 cycles for β-actin cDNA

mediated CD40 signaling in B lymphocytes, Daudi and Ramos cells (Lee *et al*., 1999). In addition, mouse homologue A1 expression is significantly upregulated in mature B cells, whereas A1 expression is low in pro- and pre-B cells and in immature marrow B cells (Tomayko and Cancro, 1998). Therefore, the selective expression of Bfl-1S in lymph nodes and in B-leukemia cells suggest that Bfl-1S and Bfl-1 may contribute to cell survival during B-cell proliferation and differentiation.

Bfl-1S localizes in the nucleus via *its C-terminus, which contains a consecutively positively charged KKRK sequence*

Most antiapoptotic Bcl-2 family proteins contain a hydrophobic transmembrane domain in their C-termini, which localizes them to specific intracellular membranes. Bfl-1 also contains a hydrophobic stretch in its C-terminus and localizes to the mitochondrial membrane (D'Sa-Eipper and Chinnadurai, 1998; Zhang *et al*., 2000). On the other hand, Bfl-1S contains a hydrophilic C-terminus, which consists of 23 amino acids and includes four consecutive positively charged amino acids, a KKRK sequence. To examine the subcellular distribution of Bfl-1S, Bfl-1, and Bcl-xL, they were expressed in 293 T cells with green fluorescence protein (GFP) fused to their N-termini. Mitochondria were stained with Mitotracker as a red fluorescence and DNA with DAPI as a blue fluorescence (Figure 3a). As expected, GFP-Bfl-1 and GFP-Bcl-xL were found to predominantly localize to the mitochondria of 293 T cells (Figure 3a), which is consistent with the other reports for Bfl-1 and Bcl-xL (D'Sa-Eipper *et al*., 1996; D'Sa-Eipper and Chinnadurai, 1998). In contrast, GFP-Bfl-1S fusion proteins were found to localize to the nucleus, suggesting that Bfl-1S is a nucleus-targeting protein. Most nuclear proteins have NLS domains, which generally consist of positively charged amino acids. Therefore, we speculated that the Bfl-1S C-terminus containing KKRK sequence may be important for nuclear localization.

To investigate the role of the C-termini of Bfl-1S and Bfl-1, we expressed GFP-Bfl-1ΔC fusion protein, which contains a common sequence (amino acids 1–140) of Bfl-1 and Bfl-1S. The results showed that the GFP-Bfl-1ΔC fusion proteins mainly localized to the mitochondria as GFP-Bfl-1, although they were also detected in low intensity diffusely throughout the cytoplasm (Figure 3b). These results indicated that the C-terminus of Bfl-1 is not solely essential for the mitochondrial localization of Bfl-1, and that certain internal sequences at N-terminal end of Bfl-1 may also be involved in subcellular localization. These results are consistent with previous observations in primary rat kidney epithelial cells (D'Sa-Eipper and Chinnadurai, 1998). By BLAST searching, we found that a tumor suppressor ING1 (inhibitor of growth 1), which is known to localize to the nucleus, contains NLS motif that is similar to that of Bfl-1S, for example, the KKKK amino acids (Cheung and Li, 2001). Thus, we conclude that the C-terminus of Bfl-1S serves as an NLS domain and leads Bfl-1S to the nucleus, and that Bfl-1S is a novel and unique human Bcl-2 family protein.

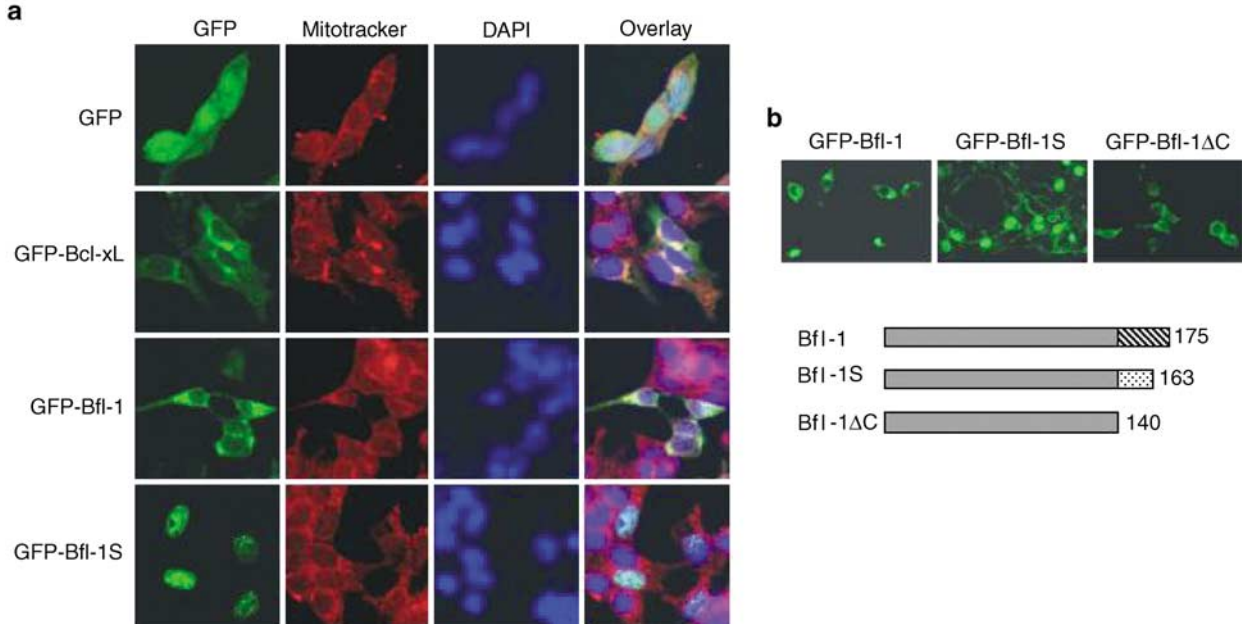

Figure 3 Subcellular localization of Bfl-1S. (**a**) Expression vectors encoding GFP, GFP-Bcl-xL, GFP-Bfl-1 and GFP-Bfl-1S were transfected into 293 T cells. At 24 h after transfection, mitochondria were stained with 150 nM Mitotracker (red), and the DNA was stained with 1 μg/ml DAPI (blue). Cells were analysed by confocal laser microscopy. (**b**) 293 T cells were transfected with plasmids encoding GFP-Bfl-1, GFP-Bfl-1S and GFP-Bfl-1ΔC (1–140) and observed under a fluorescence microscope

Antiapoptotic activity of Bfl-1S against Bax-induced apoptosis in 293 T cells

We tested whether Bfl-1S, like Bfl-1, has the ability to protect 293 T cells from Bax-induced apoptosis (Holmgreen *et al.,* 1999; Zhang *et al.,* 2000). 293 T cells were cotransfected with plasmids encoding Bcl-xL, Bfl-1, or Bfl-1S and with or without plasmid carrying Bax. At 24 h after transfection, cell viability was measured by fluorescence microscopy as described in 'Materials and methods'. Bfl-1S and Bfl-1 were able to suppress Bax-induced apoptosis although their effects were weaker than that of Bcl-xL (Figure 4). The weaker antiapoptotic activity of Bfl-1 versus that of Bcl-xL is consistent with the previous observations, which showed the antiapoptotic activity of Bfl-1 and A1 in yeast (Zhang *et al.,* 2000) and in 293 T cells (Holmgreen *et al.,* 1999). Bcl-xL is well known to interact with Bax and inhibit Bax-induced apoptosis directly (Priault *et al*., 1999). Unlike Bcl-xL, Bfl-1 could not interact with Bax but only interact with tBid (Werner *et al*., 2002). Therefore, it is possible that Bfl-1 and Bfl-1S might inhibit Bax-induced apoptosis by interacting with downstream molecules of Bax signal.

Antiapoptotic activity of Bfl-1S in Molt-4 cells against treatment with chemotherapeutic agents

Bfl-1 is known to be able to protect cells from various proapoptotic stimuli including TNF stimulation (Zong *et al*., 1999, Duriez *et al*., 2000) and chemotherapeutic treatments (Wang *et al*., 1999; Cheng *et al*., 2000; Shim *et al*., 2000). Accordingly, we investigated whether Bfl-1S could inhibit chemotherapeutic-induced apoptosis. Molt-4 cells, human T-leukemia cells that do not express endogenous Bfl-1 and Bfl-1S were transduced with retroviral vectors containing Bfl-1 or Bfl-1S. The expression levels of Bfl-1 and Bfl-1S mRNAs were confirmed by RT–PCR (Figure 5c). The established stable cells were treated with several chemotherapeutic agents including staurosporine (STS, 1 μM), 5′-5-fluorouracil (FU) (1 μM), cycloheximide (CHX, 20 μg/ml). Nonapoptotic healthy cells were counted using FACS analysis as Annexin V and propidium iodide (PI) double negative cells. The experiments were repeated at least three times and similar results were obtained. One of the representative experiments is shown in Figure 5. Bfl-1S was found to inhibit against apoptosis induced by STS and marginally against that induced by 5-Fu or CHX

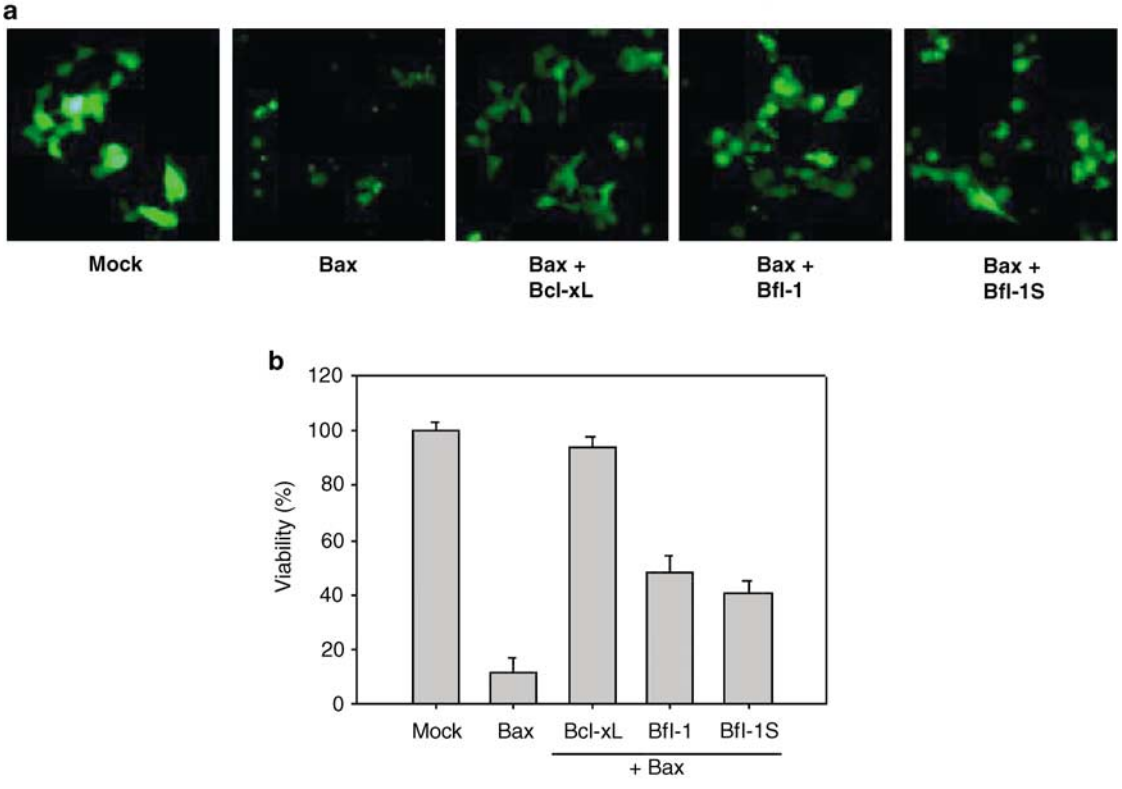

Figure 4 Antiapoptotic activity of Bfl-1S against Bax-induced apoptosis in 293 T cells. Expression vectors, pcDNA6-Bfl-1S-V5, pcDNA6-Bfl-1-V5 and pcDNA3.1-Bcl-xL were transiently cotransfected with pcDNA3.1-Bax and pEGFP-N1 into 293 T cells. At 24 h after transfection, the cells were trypsinized and stained with propidium iodide (PI), as described in 'Materials and methods'. Healthy cells showing green fluorescence, and not those showing red or yellow fluorescence were counted. (**a**) Fluorescence microphotograph of 293 T cells expressing Bfl-1S, Bfl-1, and Bcl-xL with Bax on the six-well plate. (**b**) Quantitative analysis of apoptotic cells induced by Bax overexpression. The percentage of healthy cells is expressed as means ± s.e. from three independent experiments performed in triplicate

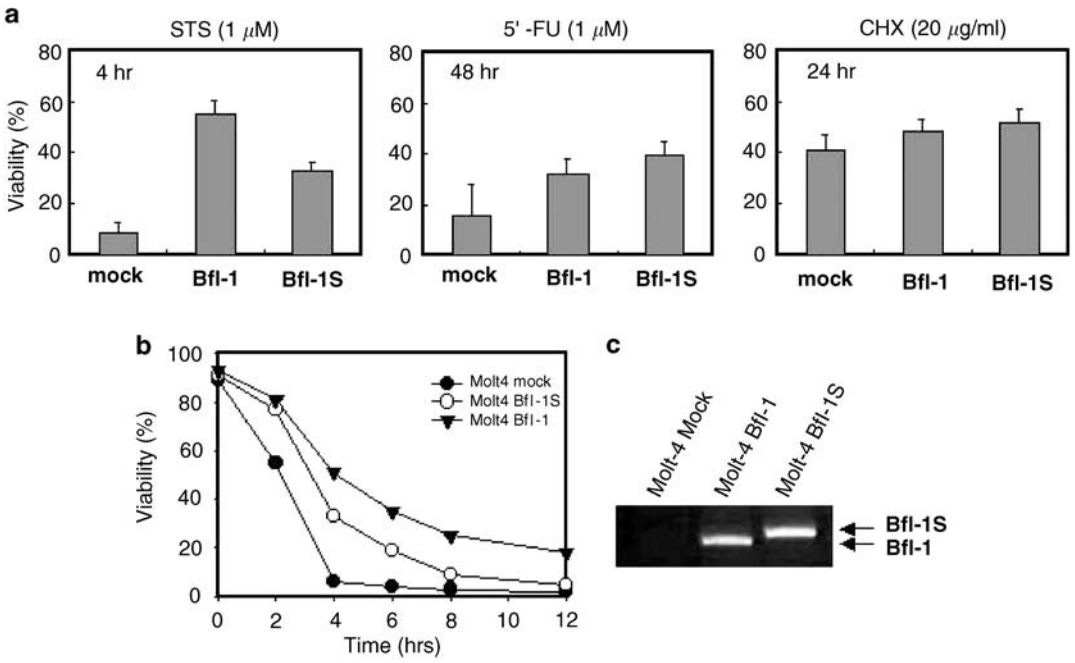

Figure 5 Antiapoptotic activity of Bfl-1S against chemotherapeutic agents-induced apoptosis. (**a**) Cell viability after treatment with 1 μM STS or 1 μg/ml of 5-FU or 20 μg/ml of cychloheximide (CHX) at the indicated time. Cell viability was determined by FACS analysis. (**b**) Time course of cell viability after treatment with 1 μM STS. Cell viability was determined by FACS analysis. (**c**) Expressions of Bfl-1 and Bfl-1S mRNAs in Molt-4 stable cells were analysed using the RT–PCR method with RT-primers 1 and 2. The cells showed similar expressions of Bfl-1 and Bfl-1S mRNAs

(Figure 5a,b). Therefore, we used 1 μM of STS treatment in Molt-4 stable tranfectants for further investigation of antiapoptotic mechanism of Bfl-1S.

Bfl-1S inhibits the cleavage of Bid, and caspases 3 and 8 but not of caspase 9 and disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$)

To explore the inhibitory effect of Bfl-1 and Bfl-1S on STS-induced apoptosis, we examined several candidates that are able to modulate apoptosis. Previously, in response to TNF-α, Bfl-1 was found to inhibit the apoptosis of human microvascular endothelial cells (HMECs) by maintaining mitochondrial function. In that study, Bfl-1 was shown to inhibit mitochondria depolarization, cytochrome *c* (Cyt c) release, cleavage of Bid, caspase 9 and poly (ADP-ribose) polymerase, but did not inhibit caspase 3 or 8 cleavage (Duriez *et al*., 2000). Recently, Bfl-1 was also found to inhibit the CD95 and the TRAIL-induced apoptosis of Jurkat T-leukemia cells by sequestering full-length Bid and truncated Bid (tBid), and by inhibiting cytochrome *c* release (Werner *et al*., 2002). Therefore, we first examined the cleavage of endogenous Bid, caspases 3, 8 and 9 in Molt-4 stable transfectants after treatment with 1 μM of STS. Interestingly, Bfl-1S significantly prevented the cleavage of Bid, caspases 3 and 8 as much as Bfl-1 (Figure 6a). On the other hand, Bfl-1S only slightly delayed the cleavage of caspase 9 whereas Bfl-1 also significantly inhibited the cleavage of caspase 9 (Figure 6a), indicating that the inhibitory target molecule may be somewhat different between Bfl-1 and Bfl-1S.

Bid is a BH-3 domain-only Bcl-2 family member and appears to be a preferred substrate for caspase 8, and a low level of caspase 8 activation is demonstrated to be sufficient to cleave Bid (Han *et al*., 1999). Bid is diffusely localized throughout the cytoplasm in healthy cells. After cleavage of N-terminus, truncated form, tBid, is translocated to mitochondria, and subsequently, it promotes Cyt *c* release and apoptosis (Li *et al*., 1998; Luo *et al*., 1998). Bfl-1 directly and selectively binds to both full-length Bid and tBid via the Bid BH-3 domain. Bfl-1S also conserves four BH domains. Thus, one could speculate that Bfl-1S delays the activation of mitochondrial apoptosis by sequestering Bid at the nucleus region and by interfering with the cleavage of Bid.

Currently, the mechanism of the inhibitory effect of Bfl-1 on caspase cleavage is not known. In human microvascular endothelial cells, Bfl-1 inhibits the cleavage of caspase 9 but not of caspases 3 and 8 in response to TNF-α (Duriez *et al*., 2000). Our results, however, show that Bfl-1 inhibited the cleavage of caspases 3, 8 and 9 (Figure 6a). One explanation for this divergence may be cell-type specific or apoptosis pathway specific. The lower inhibitory effect of Bfl-1S on the caspase cleavage may result from its nucleus localization.

In the same line, Bfl-1 that was localized at mitochondria was found to prevent the loss of $\Delta\Psi_m$ when apoptosis was induced by STS, whereas Bfl-1S failed to prevent $\Delta\Psi_m$ loss (Figure 6b). These results suggest that the localization of Bfl-1 within the

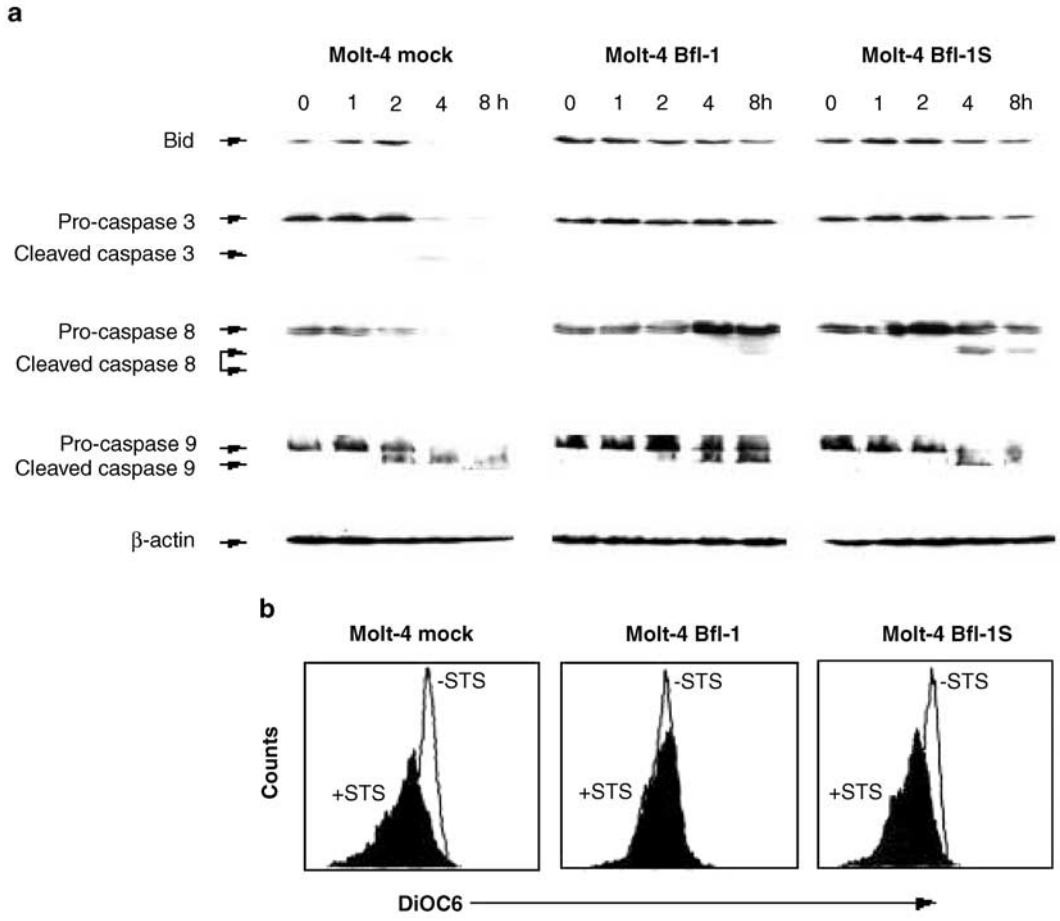

Figure 6 Bfl-1S inhibits the cleavage of Bid, caspases 3 and 8, but does not inhibit caspase 9 cleavage or the disruption of the $\Delta\Psi_m$ induced by STS. (**a**) Molt-4 cells were exposed to 1 μM of STS for various times as indicated, and the cell lysates were immunoblotted. Bid cleavage was followed by the disappearance of the intact form. Activation of procaspases 3, 8 and 9 were monitored by the appearance of the respective cleavage products. β-actin was monitored as an internal control. (**b**) Cells were incubated with $DiOC_6$ to monitor $\Delta\Psi_m$ by FACS. The loss of $\Delta\Psi_m$ was equated with decreased fluorescence and a shift to the left

mitochondria may be required to maintain $\Delta\Psi_m$. This could explain the weaker antiapoptotic activity of Bfl-1S versus that of Bfl-1.

Currently, the biological role of Bfl-1S is not fully understood. However, several results point towards the function of Bcl-2 family proteins in nucleus. A1, a mouse homologue of Bfl-1, is known to localize to the nucleus of COS-7 cells and to inhibit STS-induced apoptosis, although it does not have a known NLS (Somogyi *et al*., 2001). A recent study also shows that A1 can interact directly with mouse INGh1, which targets the nucleus and enhances the apoptosis of HC11 cells, and inhibits the proapoptotic effect of mouse INGh1 (Ha *et al*., 2002). Another viral Bcl-2 family member, E1B 19 K protein, can localize to the nucleus membranes by associating with nuclear lamins in spite of lacking a membrane targeting domain (Rao *et al*., 1997). The antiapoptotic mechanisms of E1B 19 K protein involves the sequestration of functional proteins such as Ced-4, the *Caenorhabditis elegans* APAF-1 homologue, from cytosol to the nucleus (Han *et al*., 1998), and of FADD, a mammalian caspase recruiting protein that functions in Fas-mediated apoptosis (Perez and White, 1998) and of Btf, a transcription factor that induces apoptosis upon overexpression (Kasof *et al*., 1999).

Further studies on the contributions of Bfl-1 and Bfl-1S to cell survival will provide important information about the biological function of the Bcl-2 family members in the nucleus.

Materials and methods

Cloning of Bfl-1S cDNA, a novel splicing variant of human Bfl-1 cDNA

In a previous study (Shim *et al*., 2000), the RT–PCR products of different sizes of cDNA derived from the mRNA of human B-lymphoblastic Wil2-NS cells were identified using Bfl-1-specific primers, RT-primer1 5′-AGCTCAAGACTTTGCTC TCCACC (sense) and RT-primer2 5′-TGGAGTGTCCTTT CTGGT CAACAG (antisense). The RT–PCR products were subcloned into pCR2.1-TOPO vector using a TOPO™ TA Cloning kit (Invitrogen, Groningen, Netherlands), and the DNA sequences were analysed.

Total RNA preparation and RT–PCR assay

Bfl-1S mRNA was detected by RT–PCR. Total RNA was isolated from lysates of human tissues obtained by surgical resection at the Seoul National University Hospital and from cancer cell lines using Tri-reagent (Sigma, St Louis, MO, USA), according to the manufacturer's instructions. Total RNA (1 μg) was used in a 20 μl cDNA synthesis reaction using oligo $(dT)_{18}$ primer and moloney-murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). cDNA mixture (2 μg) was used for PCR amplification. For the amplification of the Bfl-1 and Bfl-1S cDNAs, RT–PCR was carried out with the Bfl-1-specific RT-primers 1 and 2, as previously described. These primers simultaneously amplified the 577 and 633-bp PCR products of Bfl-1 and Bfl-1S. As a control, human β-actin cDNA was amplified to determine the integrity of the RNA and the efficiency of the cDNA synthesis using primers 5′-GGAAATCGTGCGTGACATTAAGG (sense) and 5′-GGCTTTTAGG ATGGCAAGGGAC (antisense).

Cell lines

Human cancer cell lines were obtained from the Korean Cell Line Bank (KCLB, Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea). A total of 43 cancer cell lines were examined, which included stomach cancer (10 cell lines), lung cancer (four cell lines), colon cancer (eight cell lines), liver cancer (nine cell lines) and leukemia (12 cell lines). These cells were cultured in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD, USA) containing 10 μM HEPES and 10% fetal bovine serum (Life Technologies, Inc.). 293 T cells were cultured in the DMEM medium (Life Technologies, Inc.) containing 10% fetal bovine serum.

Plasmids

cDNAs containing the ORFs of Bfl-1 and Bfl-1S were generated by PCR using Bfl-1 and Bfl-1S cDNAs on pCR2.1-TOPO plasmid as templates and the following primers: 5′-GAATTCGATGACAGACTGTGAATTTGGATAT, sense for both Bfl-1 and Bfl-1S; 5′-GGATCCTCAACAGTATTGCTTCAGGAGAGA, antisense for Bfl-1; and 5′-GGATCCTTACAAAGCCATTTTCCTCTTCTTG, antisense for Bfl-1S. The resulting PCR products were digested with restriction endonucleases and subcloned into the *Eco*RI and *Bam*HI sites of pEGFP-C1 (Clontech, Palo Alto, CA, USA). The PCR product of Bfl-1 obtained using sense primer 5′-GAATTCGATGACAGACTGTGAATTTGGATAT and antisense primer 5′-AAGAATTCACCAGCCTCCGTTTTGCCT was subcloned into the *Eco*RI site of pEGFP-C1 to construct pEGFP-Bfl-1ΔC. To construct retroviral expression vectors, whole cDNA fragments of Bfl-1 and Bfl-1S on pCR2.1-TOPO plasmid were isolated after digestion with *Eco*RI restriction enzyme and inserted into the *Eco*RI site of pLXIN, retroviral vector (Clontech). To construct the mammalian expression plasmids, the PCR products of Bfl-1 and Bfl-1S were digested with restriction endonucleases and subcloned into the *Bam*HI and *Eco*RI sites of pcDNA6 V5-His version C (Invitrogen). The primers used were 5′-GGATCCATGACAGACTGTGAATTTGGATAT-3′, sense for both Bfl-1 and Bfl-1S; 5′-GAATTCCACAGTATTGCTTCAGGAGAGA, antisense for Bfl-1; and 5′-GAATTCCCAAAGCCATTTTCCTCTTCTTG, antisense for Bfl-1S. The expression plasmids of Bax and Bcl-xL (pcDNA3.1-Bax and -Bcl-xL) were gifts from Dr HT Kim (Catholic Research Institute of Medical Science, The Catholic University of Korea). All the constructs were confirmed by DNA sequencing.

Gene transfer

Transfections were carried out in either six-well plates or 6 cm culture dishes using Lipofectamine (GIBCO-BRL, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. At the time of transfection, the cells were about 40% confluent. pLXIN-Bfl-1, pLXIN-Bfl-1S and pLXIN were transiently transfected into the packaging cell line, PA317, using Lipofectamine. Viral supernatants were obtained and used to transduce Molt-4 human T-leukemia cells in the presence of 4 μg/ml of Polybrene. Stable cell lines were obtained by selection in 400 μg/ml of G-418 (Life Technologies, Inc.) for 4 weeks.

Confocal laser scanning microscopy

Transfected 293 T cells were prepared for microscopy by staining with 150 nM of a mitochondria-specific dye, Mitotracker Red CMXRos (Molecular probes, Eugene, Oregon, USA) for 30 min in the cell culture medium. The cells were then washed twice with PBS and fixed with 4% PFA. Fixed cells were counterstained with 1 μg/ml of DAPI (Calbiochem, San Diego, CA, USA), and placed on microscope slides. Images were obtained using an LSM510 (Carl Zeiss, Co, Ltd, Germany) confocal microscope.

Apoptosis assay

To monitor apoptosis by Bax, 293 T cells were cotransfected with 0.5 μg of pcDNA3.1-Bax plasmid with 1.0 μg of pcDNA6-Bfl-1-V5, pcDNA6-Bfl-1S-V5 or pcDNA3.1-Bcl-xL plasmids, together with 0.1 μg of the pEGFP-N1 to allow transfected cells to be identified in the six-well plates. Both floating and adherent cells post-trypsinization were collected 24 h after transfection, stained with PI at a final concentration of 2.5 μg/ml using the Live-Dead Cell Staining Kit (BioVision, CA, USA), and then observed under a fluorescence microscope. Healthy transfected cells showed only green fluorescence by EGFP and dead cells (or apoptotic cells) showed red fluorescence by PI or yellow fluorescence by the overlaying of green and red fluorescence. The cells were scored for the presence (yellow) or absence (green) of apoptosis in each transfection.

To monitor apoptosis in Molt-4 cells, 5×10^5 cells were treated with 1 μM STS or 1 μM 5′-FU or 20 μg/ml CHX for the indicated time. Cells were harvested and washed once in ice-cold 10 mM PBS supplemented with 0.5% BSA and then stained with FITC- labeled Annexin V (2.5 μg/ml) and PI (5 μg/ml) for 20 min at room temperature. Flow cytometric analysis was performed using an Epic XL flow cytometer (Coulter, Marseille, France).

Loss of mitochondrial transmembrane potential ($\Delta\Psi_m$)

To measure $\Delta\Psi_m$ disruption, 5×10^5 cells were incubated with 20 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, OR, USA) for 15 min at 37°C and their fluorescence was determined by using a flow cytometer.

Immunoblot analysis

The following antibodies were used: mouse anti-Bid, anticaspase-3, -8, -9, anti-goat HRP antibody (Santa Cruz, CA, USA), anti-β-actin antibody (Sigma) and anti-mouse HRP antibody (Amersham Pharmacia, Buckinghamshire, England). For immunoblot analysis, 50 μg of protein was subjected to SDS–PAGE and transferred onto a nitrocellulose membrane, which was blocked with 5% skimmed milk, probed with

primary antibodies and visualized using an ECL chemiluminescence kit (Amersham Pharmacia).

Abbreviations

RT–PCR, reverse–transcriptase polymerase chain reaction; PI, propidium iodide; GFP, green fluorescence protein; BH domain, Bcl-2 homology domain; NLS, nuclear localization sequence; STS, staurosporine; CHX, cycloheximide; 5-FU, 5-fluorouracil; $\Delta\Psi_m$, mitochondrial transmembrane potential.

Acknowledgements

This work was supported by grants from the Korea Ministry of Health and Welfare (01-PJ1-PG3-20700-0033) and Seoul National University Hospital (03-2002-051). We thank Dr Masao Seto for a critical reading of this manuscript and helpful discussions.

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