Published in final edited form as:

Biochem Biophys Res Commun. 2008 October 17; 375(2): 190–193. doi:10.1016/j.bbrc.2008.07.116.

Alternative splicing generates a short BCL6 (BCL6S) isoform encoding a compact repressor

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Abstract

BCL6 gene transcribes two main mRNA isoforms, variant 1 and variant 2, which have distinct transcription start sites. However, these two isoforms encode identical BCL6 protein. Recently, a third variant was sequenced from a human hippocampus cDNA library (DB465062). In this study, we cloned and sequenced a novel BCL6 spliced isoform (BCL6S) which lacks exon 7 that encodes the first two zinc fingers of BCL6. We found a splicing isoform, BCL6S, that excludes exon 7 but retains the last four of the six zinc fingers (ZFs) coding region of the BCL6 gene. BCL6S mRNA and protein were expressed in human cell lines and tissues that expressed BCL6, but accounted for a minor portion of BCL6 transcripts or protein. BCL6S protein was also detectable in BCL6 positive cells. BCL6S could form homodimers or heterodimers with BCL6 and could bind to classical BCL6 binding sites. Luciferase reporter assays demonstrated that BCL6S could effectively repress typical BCL6 target genes. BCL6S is a compact repressor that may have a functional role in normal and neoplastic germinal center B cells.

Keywords

BCL6; zinc finger; alternative splicing; transcription repressor; germinal center

The *BCL6* proto-oncogene encodes a BTB/POZ zinc finger (ZF) transcriptional repressor, locating on chromosome 3q27 [1] with 10 exons over 26 kilobases (kb) region. The protein contains an amino-terminal POZ motif encoded by sequence starting at exon 3 and the six C2H2-type ZFs at the carboxy-terminal is encoded by exons 7 to 10.

The BCL6 protein is a nuclear phosphoprotein that is the master regulator in germinal center (GC) formation and GC B-cell differentiation and survival. Deregulation of BCL6 has been implicated in the pathogenesis of lymphomas especially diffuse large B cell lymphoma (DLBCL). The BCL6 amino-terminal POZ domain interacts with the co-repressors N-COR, SMRT and BCOR, and the six carboxy-terminal ZF motifs recognize specific DNA sequences. The PEST domain, the second repression domain (RDII) in the middle of BCL6, interacts with the corepressor MTA3 [2] and the function of BCL6 may be modified by phosphorylation or acetylation of this domain [3]. In addition to direct DNA binding to conserved cis-elements of

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target genes, BCL6 can also repress genes indirectly through binding to other transcription factors, such as peroxisome proliferator-activated receptor (PPAR) δ [4] and MIZ-1 [5].

BCL6 transcribes two main isoforms, variant 1 and variant 2, which have distinct transcription start sites and are regulated by two discrete promoters. Recently, a third variant was sequenced from a human hippocampus cDNA library (DB465062). These observations indicate *BCL6* gene can be transcribed from alternative promoters, and there may be cell type-specific regulation and expression of these isoforms. All these isoforms do not affect the protein sequence.

Here we report a novel short BCL6 spliced isoform (BCL6S) which lacks exon 7 that encodes the first two zinc fingers of BCL6. Our investigations indicate that BCL6S is a compact, yet potent transcription repressor for many BCL6 target genes.

Methods

Cloning of the human BCL6S transcript

cDNA from DHL16 was prepared by reverse transcription from cytoplasmic RNA. BCL6 cDNA was amplified by PCR with BCL6 5' (5'-CGCGGATCCCACCATGGCCTCGCCGGCTGACAG-3') and BCL6 3' (5'-GTGCTCGAGCGGCAGGCTTTGGGGAGCTCCG-3') primers, and cloned into pcDNA3.1/His-V5 expression plasmid by double digestion of the PCR product with BamHI and XhoI.

Detection of BCL6S expression by RT-PCR and western blot

4 μ g of total RNA from the following cell lines (DHL16, Raji, Daudi, CL-01, U266, and HepG2) were reverse transcribed using random hexamer with Superscript II (Invitrogen, Carlsbad, CA, USA) at 37°C. BCL6 ZF 5' (5'-CCATGGAGCCTGAGAACCTTG-3') and BCL6 ZF 3' (5'-TGGCTGGCCGGTTGAACTGG-3') primers flanking the first two zinc fingers coding regions were designed to amplify both BCL6 and BCL6S giving rise to a 517bp and a 349bp product respectively. BCL6 and BCL6S C-terminal fragments were separated on 15% SDS-PAGE after cyanogen bromide cleavage [10] and detected by western blotting with 1:500 diluted BCL6 antibody (C-19, Santa Cruz Biotechnologies, Santa Cruz, CA).

Minigene splicing assay

A *BCL6* minigene was constructed by replacing the cDNA sequence encoding ZF 1 and 2 and flanking sequence by *BCL6* genomic DNA from exon 6 to 8 amplified from BAC RP11-213G (BACPAC Resource Center, CA). HEK293 cells were transiently transfected with the minigene plasmid; total RNA was extracted after 48 hours, reversely transcribed after random hexamer, and amplified. DHL16 cell line was transiently transfected using Nucleofector (Amaya, Gaithersburg, MD) with buffer V and program L29.

Luciferase reporter assay

BCL6 target genes (*BCL6*, *MIP1 α* , *CD23b*, and *PRDM1*) were selected for the reporter assay. *BCL6* and *MIP1 α* promoters were PCR amplified from the genomic DNA and cloned into pGL3-basic vector, while the short BCL6 binding sequences from *CD23b* or *Blimp1* genes were cloned upstream of the SV40 promoter in the pGL3-promoter vector. 300ng reporter plasmids with 100ng BCL6 or BCL6S expression plasmids were transfected into HEK293 cells using ExGen 500 (Fermentas, Hanover, MD). Luciferase activity was measured and normalized according to the manufacturer's instruction (Promega Co, Madison, WI) after 48 hours.

Immunoprecipitation

V5 and FLAG tagged BCL6 constructs were transfected into HEK293T cells using ExGen 500. The cells were lysed, sonicated and precleared. 1.5µg anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA) was added and incubated overnight at 4°C. The precipitated proteins were blotted with anti-FLAG monoclonal antibody (Sigma, St. Louis, MO) and cell lysates from transfected cells were blotted with anti-V5 and anti-FLAG respectively following SDS-PAGE separation.

Biotinylated oligonucleotide pull-down assay

Biotin labeled DNA oligonucleotides were synthesized, one containing the two tandem BCL6 binding site identical to the sequence in wild type (WT) BCL6 exon 1 (5'-AACTGGGGT**TTCTTAGA**AGTGGTGATGCAAGAAGTT**TCTAGGAA**AGGCCGGA-3') and another with the two BCL6 binding sites mutated (Mut) (5'-GCGAATTCTGGGGT**GTCTCAGA**AGTGGTGATGCAAGAAGTT**TCCTGGGA**AGGCCGGA-3') (BCL6 binding sites are shown in bold and the mutated nucleotides are underlined). FLAG-tagged BCL6 and BCL6S plasmids were transfected into HEK 293T cells, and lysed in HKMG buffer. The oligonucleotide pull-down assay was performed as described by Bond et al [11].

Results and discussion

Identification of the novel BCL6 splice variant

cDNA representing the full-length open reading frame of BCL6 from the B cell lymphoma cell line DHL16 was amplified and cloned into pcDNA3.1/His-V5 expression vector. PCR amplification of the inserts demonstrated two different sized products and sequence analysis revealed that the longer product represented the regularly spliced BCL6, whereas the shorter product, BCL6S (GenBank accession no.EU883531), lacked the 168 nucleotides from exon 7 resulting from alternative splicing. The BCL6S transcript had the same open reading frame and encoded a 650aa protein whereas the full-size BCL6 protein has 707aa (Fig. 1A).

RT-PCR with primers flanking the ZF region showed 517bp and 349bp bands corresponding to BCL6 and BCL6S respectively in all BCL6 mRNA positive cells (Fig. 1B). However, BCL6S could not be detected in mouse A20 lymphoma cell line using a primer pair that could amplify both human and mouse BCL6 ZF. To confirm that BCL6S was generated by alternative splicing rather than through a PCR artifact, a BCL6 minigene was transiently transfected into HEK293T cells. This minigene was expected to generate two different sized BCL6 transcripts, giving rise to two different molecular weight fusion proteins (Fig. 2A). Minigene splicing assay demonstrated two distinct sizes BCL6 transcripts by RT-PCR and cyanogen bromide cleavage clearly showed that BCL6 and BCL6S C-terminal fragments corresponding to the two spliced isoforms were detected in transfected HEK293T cells (Fig. 2B). BCL6S protein was detectable in B cell lymphoma cell lines, and the BCL6 minigene also gave rise to BCL6 and BCL6S forms in transiently transfected DHL16 B cells (Fig. 2C). Unexpectedly, the BCL6 minigene gave rise to relatively more BCL6S mRNA and protein than did the endogenous gene in B-cell lines; this may be due to differences in the regulation of splicing of the precursor mRNA from the minigene and the endogenous gene. These results strongly supported that BCL6S was normally spliced by exon skipping. By immunofluorescence analysis, we found that BCL6S was located in the nucleus (data not shown), consistent with a report that the nuclear localization signal of BCL6 is embedded in the last four ZFs [6].

BCL6S displays similar transcriptional repression, DNA binding and protein-protein interaction to BCL6

Four known BCL6 target genes were tested by luciferase assay in HEK 293 cells. Fig. 3A showed that BCL6 and BCL6S could repress BCL6 target genes *BCL6*, *CD23b*, *Blimp1*, and *MIP1a* with similar efficacy. BCL6 DNA binding was detected by a pull-down assay using a biotinylated oligonucleotides containing the well-defined tandem BCL6-binding sites within BCL6 exon 1. This revealed that both BCL6 and BCL6S bind to the DNA (Fig. 3B) and thus indicated that the first two zinc fingers of BCL6 were not absolutely required for its DNA binding.

An immunoprecipitation assay using FLAG and V5 tagged BCL6 expression constructs transfected into HEK 293T cells showed that BCL6S could form homodimers and also heterodimers with BCL6 (Fig. 4). We speculated that BCL6S, like BCL6, could recruit the same corepressors to its POZ domain, which when dimerized, forms the platform for NCOR, SMRT, and BCOR binding. Our data imply that BCL6S, like BCL6, could be a powerful transcriptional repressor.

Previous studies have shown that the zinc finger region of BCL6 has multiple functions. In addition to binding DNA, it is involved in the nuclear targeting of BCL6, the recruitment of the class II HDACs (HDAC4, HDAC5, HDAC7) [7], and the interaction with PLZF [8], and ETO [9]. Point mutation studies of BCL6 ZFs have demonstrated that the two N-terminal ZFs are not necessary for cognate DNA-binding, nuclear localization or transcriptional repression [6]. The above functions also seem to be intact in BCL6S and the precise role of the first two zinc fingers is not clear. It is striking that the closely related POZ-ZF protein, BCL6B (BAZF), which has apparently identical DNA sequence specificity, contains only 5 ZFs. The two genes have very similar exon organization, but BCL6B exon 6, homologous to BCL6 exon 7, encodes only a single ZF.

Conclusion

we have identified an alternative splicing isoform of *BCL6* gene coding a smaller protein BCL6S. Our findings indicate that BCL6S is a compact, potent transcription repressor. The first two ZFs of BCL6 are not absolutely required for its DNA binding or nuclear localization. However, the proteins interacting with the first two ZFs of BCL6 and the precise affinity differences of DNA binding between BCL6 and BCL6S are yet to be determined. Genome-wide study of gene expression regulated by BCL6S may provide clues on whether BCL6S plays a redundant role or has unique functions in GC B cell or its malignant counterparts.

Acknowledgements

This work was supported in part by a NCI grant (5U01/CA114778).

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Fig. 1. Alternative spliced BCL6S is co-expressed with BCL6 in different B cell lines
 BCL6S is formed when exon 6 and exon 8 are fused together without interrupting the open reading frame. The predicted exon skipping and protein structure are illustrated in (A). The transcripts were detected from multiple cell lines by RT-PCR using primers flanking the ZFs region; β -actin (*ACTB*) was used as cDNA load control, 517bp and 349bp bands corresponding to BCL6 and BCL6S respectively. U266 is a myeloma cell line that does not express BCL6 (B).

Fig. 2. BCL6 minigene transcribes two BCL6 isoforms and encodes two different size of proteins BCL6 minigene containing exon 7 flanked by intronic sequences (A). Two BCL6 transcripts, BCL6 and BCL6S, are detected by RT-PCR in DHL16, and in BCL6 minigene transfected HEK293 T cells. Two predicted BCL6 C-terminal ZF fragments released by cyanogen bromide cleavage were detected by western blot (WB) using an antibody (C-19) against the C-terminus of BCL6. Transfectants from BCL6, BCL6S or both were used as controls (B). BCL6S was detectable in three B cell lymphoma cell lines by BCL6 antibody (C-19). BCL6 minigene is also spliced and translated into BCL6 and BCL6S in DHL16 B cells detected by anti-FLAG antibody after cyanogen bromide cleavage respectively (C).

Fig. 3. BCL6S binds to BCL6 binding sites and represses gene expression

HEK 293 cells were cotransfected with *BCL6*, *CD23b*, *PRDM1* and *MIP1α* luciferase reporters and BCL6 or BCL6S expression vector. Luciferase activities in cell extracts were analyzed by the dual-luciferase reporter assay system and normalized using pRL-TK-luciferase activity in each sample (A). Experiments were repeated thrice in duplicate and luciferase activities are expressed as mean ($\pm$ S.D.). Biotinylated double stranded oligonucleotide pull-down of BCL6-FLAG and BCL6S-FLAG proteins from transfected HEK293T cells blotted with anti-FLAG monoclonal antibody, ten-fold excess non-biotinylated wild type competitor (WT+comp.) or mutated (Mut) double stranded oligonucleotides was used as controls (B).

Fig. 4. BCL6S dimerization

The dimerization patterns of BCL6 and BCL6S were demonstrated by immunoprecipitation assay. BCL6 and BCL6S plasmids tagged with FLAG or V5 were cotransfected into HEK293T cells, cell lysates were immunoprecipitated with anti-V5 antibody and blotted with anti-FLAG antibody. The cell lysates were also blotted with anti-FLAG and anti-V5 antibodies respectively.