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The *ITM2B* (*BRI2*) gene is a target of BCL6 repression: Implications for lymphomas and neurodegenerative diseases

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ABSTRACT

The human *BCL6* gene encodes a transcriptional repressor that is crucial for germinal center B cell development and T follicular helper cell differentiation. It is involved in the pathogenesis of certain human lymphomas. In an effort to identify targets of BCL6 repression, we used a previously described cell system in which BCL6 repressive effects are inhibited, followed by subtractive hybridization, and identified the integral membrane 2B gene (*ITM2B*, formerly *BRI2*) as a potential target. Here we show that BCL6 can bind to its preferential consensus binding site within the first intron of *ITM2B* and represses its transcription. Knockdown of endogenous BCL6 in a human B cell lymphoma line increases ITM2B expression. Further, there is an inverse relationship between the expression levels of BCL6 and ITM2B proteins in 16 human B- and T-cell lymphomas studied by immunohistochemistry. Both the BCL6 and ITM2B proteins are expressed ubiquitously. Similar to some other targets of *BCL6*, a short form of the ITM2B protein generated by alternative splicing induces apoptosis in hematopoietic cell lines. Molecular alterations in the *ITM2B* gene are associated with two neurodegenerative diseases, Familial British and Familial Danish dementia. ITM2B dysfunction also may be relevant for the development of Alzheimer's disease. Our data confirm *ITM2B* as a target of BCL6 repression in lymphoma. A further understanding of the genes that function as regulators of the ITM2B protein may provide insights for the development of new molecular tools not only for targeted lymphoma therapy but also for the treatment of these dementias.

1. Introduction

The BCL6 nuclear zinc finger protein is encoded by a gene located on chromosome 3, band q27, and functions as a transcriptional repressor [1–4]. It has long been known to play an important role in the pathogenesis of diffuse large B cell lymphomas, and, more recently, its additional role in T-cell biology has been appreciated [5,6]. BCL6 has been called a "master regulator" of germinal center formation and is believed to repress the transcription of hundreds of proteins [7]. In a study of germinal center B cells and diffuse large cell lymphomas, it was found to bind to the promoters of about 3,000 genes (enhancer and intronic elements were not studied). Less frequently, BCL6 has been implicated in the regulation of the growth of other cancers, e.g., colorectal and breast cancer, as well as in the control of other disease processes, e.g., myasthenia gravis [8–10].

In an effort to identify *BCL6* target genes, we previously developed a dominant-negative cell system in which the BCL6 repressive effects are inhibited, enabling the detection of genes that are ordinarily repressed. By subtractive hybridization, we selectively amplified differentially expressed sequences, thus detecting upregulated messages [11]. With the use of this methodology, we now describe the identification of the integral membrane 2B gene (*ITM2B*, formerly called *BRI2*) as a novel target of BCL6 repression.

Like the BCL6 protein, the ITM2B protein is expressed ubiquitously [12]. A short form of the ITM2B protein, which is generated by alternative splicing, has been shown to induce apoptosis in hematopoietic cell lines [13], a function that is similar to some other targets of BCL6 [11,14]. However, ITM2B has not been studied in the context of human lymphomas. Interestingly, alterations in the *ITM2B* gene are associated with two neurodegenerative diseases, Familial British dementia (FBD) and Familial Danish dementia (FDD), and data have been presented indicating that aberrant ITM2B function also may play a role in the development of Alzheimer's disease (AD) [15–20]. Therefore, an understanding of

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the regulation of ITM2B expression by BCL6 is likely to have important implications for new therapeutic tools.

2. Materials and methods

2.1. Differential expression by Northern blotting

Preparation of constructs, cDNA subtraction, and amplification of differentially expressed sequences have been described previously [11]. Briefly, through the use of a cell system in which the BCL6 repressive effects were inhibited, we identified upregulated genes that are ordinarily its targets of repression. We converted the BCL6 zinc fingers (BCL6ZF), which bind DNA but lack repressive effects, into a transcriptional activator as described and used this construct to compete with wild-type endogenous BCL6 in a cell line (BJAB, an Epstein–Barr virus-negative Burkitt lymphoma cell line) expressing BCL6 at high levels [11]. The cells were transiently transfected with this construct or the vector in which it had been cloned. Subtractive hybridization was performed, and the inserts of the cDNA clones obtained after PCR were sequenced. Potential targets of the BCL6 repressive effects were ^{32}P-labeled and hybridized to Northern blots prepared from BJAB cells that had been transfected with the BCL6ZF construct or the vector. The blots were washed under stringent conditions, exposed to film, then the probes were stripped, and the blots were rehybridized with a ^{32}P-labeled human β-actin cDNA control probe (Clontech Laboratories, Inc, Mountain View, CA), washed under stringent conditions, and reautoradiographed. Quantitation of relative band intensity was normalized to β-actin by scanning densitometry.

2.2. Consensus binding site

To clone the sequences surrounding the preferential consensus binding site for BCL6, 0.697 kb of human genomic DNA was amplified by PCR (forward primer, 5′- GGACACTGCTGTAGCATATTGG-3′; reverse primer, 5′-CTTTCATTGTGAAGCACAGCTC-3′); 1 μl of the PCR product was blunted and ligated to 50 μg of the pJET 1.2/Blunt Cloning Vector (Thermo Scientific, Waltham, MA) per the manufacturer's guidelines. The appropriate insert was isolated from this vector with *Bgl*II restriction enzyme digests and ligated to the *Bgl*II site of the pGL3 basic luciferase reporter vector (Promega, Madison, WI) (PGL3ITM2B).

2.3. ChIP assay

Two ChIP assays were performed with an EZ-ChIP™ Chromatin Immunoprecipitation Kit (#17-371, EMD Millipore Corp, Billerica, MA). BJAB cells (Burkitt lymphoma line rich in endogenous BCL6, described above), 4.7×10^6 per sample, were cross-linked with 1% formaldehyde for 5 min at room temperature. The reaction was terminated with an excess of glycine. Cross-linked chromatin was sonicated to ~200–1000 bp with a Microson™ XL 2000 sonicator (Qsonica, LLC, Newtown, CT); cells were vortexed then sonicated four times on ice, on average, at 9 W, 7 W, and 5 W twice, for 10 s each, respectively, with a 50 s cooling period on ice between sonications, followed by immunoprecipitation with antibodies to BCL6 (sc-858 X, Santa Cruz Biotechnology, Dallas, TX) and anti-rabbit IgG as a negative control. ChIP DNA was amplified by PCR. One set of primers (described in the Consensus Binding Site section) amplified the BCL6 consensus site in the first intron of the *ITM2B* gene, and another set of primers amplified a coding region of *ITM2B*, which did not contain any putative BCL6 binding sites. These primers amplified 100 bp within the second exon of *ITM2B*: forward primer, 5′-GGTACCAG TTGGCCAAAGAA-3′; reverse primer, 5′-TTTGTACAAGTATGCTCCTCCT AGA-3′. Bands were detected on 0.8% and 0.9% agarose gels, respectively.

2.4. Transfection assays/functional analysis

NIH3T3 cells (murine fibroblasts with minimal expression of both endogenous BCL6 [21] and ITM2B [22], which we have previously used for similar studies [4,23], were grown under standard conditions and plated at 1.5 to 4×10^5 cells per well in a six-well dish. They were transfected the next day by calcium phosphate DNA-coprecipitation or by TurboFect™ Transfection Reagent (Thermo Scientific) with PGL3ITM2B (0.6 to 0.65 μg)—a construct containing the exact BCL6 consensus binding site identified in the first intron of the *ITM2B* gene, and either full-length *BCL6* cDNA (1.25 to 1.46 μg) subcloned in the pCGN expression vector (pCGNBCL6) or an equivalent amount of a truncated BCL6 expression construct (control), also subcloned in the pCGN vector. We have used this truncated construct in prior studies as a control [4,23] because it lacks the zinc finger DNA-binding region of BCL6 and, therefore, cannot bind DNA. A CMV-driven β-galactosidase expression construct was cotransfected. Cells were harvested at 47.5 h to 70 h. Luciferase levels were normalized for transfection efficiency by using the values of β-galactosidase assays as previously described [4].

Relative luciferase activity was defined as the luciferase levels obtained with the BCL6 construct divided by the luciferase levels obtained with the truncated control. A paired *t* test was used to evaluate whether the mean relative luciferase activity of the ITM2B consensus binding site in the cells transfected with BCL6 vs. those transfected with the truncated control from three independent experiments (triplicate wells in each) was significantly different from 1.

2.5. Transfection of BCL6 siRNAs and Western blotting

BJAB cells were transfected with human BCL6 siGENOME SMARTpool reagent or CONTROL nontargeting siRNA 1 (Dharmacon, LaFayette, CO) by electroporation as described previously [23]. Whole cell extracts were prepared from the transfected cells and subjected to Western blotting as described. The antibodies used included rabbit polyclonal antibodies to BCL6 (sc-858 or sc-368, Santa Cruz Biotechnology, Santa Cruz, CA), a validated affinity-isolated Prestige antibody to ITM2B produced in rabbit (Sigma-Aldrich Co. LLC, Saint Louis, MO, #HPA029292), and affinity-isolated actin antibody produced in rabbit (#A2066, Sigma-Aldrich). The membranes were washed and incubated with anti-rabbit IgG (Fc), alkaline phosphatase conjugate (Promega, Madison, WI), then washed again. Protein bands were detected with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

Calculations of relative band intensity on four Western blots were normalized to the intensity of β-actin expression by scanning densitometry. The paired *t* test was used to compare BCL6 and ITM2B protein levels, respectively, in the siRNA BCL6-transfected cells with the corresponding control cells.

2.6. Immunohistochemistry

Human paraffin-embedded lymphoma blocks were retrieved from the Surgical Pathology archives under an institutional review board-approved protocol. In each case, the sections that are stained with the different antibodies are from the same tissue block, but they are not necessarily consecutive sections. BCL6 staining was performed as described previously [24] with mouse monoclonal anti-human BCL6 (clone LN22; Novocastra). Staining for ITM2B was performed overnight at 4 °C with an affinity-isolated Prestige antibody produced in rabbit (Sigma-Aldrich, Saint Louis, MO, #HPA029292) that was diluted 1:20–1:25. Antigen–antibody binding was detected with DAB chromogen, and tissues were counterstained with hematoxylin. Images were taken with a BX41 microscope (Olympus), DP72 digital camera, and cellSens Standard imaging software (Olympus).

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