Mutations of the BCL-6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma

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Abstract

The BCL6 proto-oncogene encodes a transcriptional repressor whose expression is deregulated by chromosomal translocations in ~40% of diffuse large B-cell lymphoma (DLBCL). The BCL6 regulatory sequences are also targeted by somatic hypermutation in germinal center (GC) B-cells as well as in a fraction of all GC-derived lymphomas. However, the functional consequences of these mutations are not known. Here we report that a subset of mutations specifically associated with DLBCL cause deregulated BCL6 transcription. These mutations affect two adjacent BCL6 binding sites located within the first non-coding exon of the gene and prevent BCL6 from binding its own promoter, thereby disrupting its negative autoregulatory circuit. These alterations were found in ~16% of DLBCL cases devoid of chromosomal translocations involving the BCL6 locus, but not in normal GC B-cells. This study establishes a novel mechanism for BCL6 deregulation and reveals a broader involvement of this gene in DLBCL pathogenesis.

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Introduction

The BCL6 proto-oncogene was originally identified because of its involvement in 3q27 chromosomal translocations associated with diffuse large B-cell lymphomas (DLBCL)\textsuperscript{1-4}. BCL6 encodes a POZ/Zinc finger (ZF) transcriptional repressor expressed in mature germinal center (GC) B-cells and required for GC formation as well as for affinity maturation during T cell-dependent antibody responses \textsuperscript{5-10}. The BCL6 protein acts as a potent transcriptional repressor of promoter sequences containing its specific DNA recognition motif \textsuperscript{8,11}. This function requires DNA binding through the ZF domain and two noncontiguous transrepression domains, including its N-terminal POZ motif and a second region located in the middle of the molecule. Due to the similarity between the DNA binding sites of BCL6 and the STAT6 transcription factor, BCL6 can modulate incoming IL-4 signaling by repressing STAT6-induced transcription from the immunoglobulin (Ig) germ line \textit{e} promoter, thereby modulating isotype switching toward IgE \textit{in vitro} and \textit{in vivo}\textsuperscript{12}. Based on gene expression profiling of B cells that differentially express BCL6, it has also been proposed that BCL6 suppresses genes involved in lymphocyte activation, differentiation, cell cycle arrest and apoptosis \textsuperscript{13,14}. Thus, BCL6 represents an important modulator of B-cell responses in the GC, and its transcriptional silencing is required for GC exit and, possibly, for differentiation toward plasma cells and/or memory B-cells\textsuperscript{15}.

Consistent with its essential role in the GC reaction, BCL6 expression is regulated by a number of signals important for GC development and differentiation. At the protein level, activation of the B cell receptor induces MAP kinase-mediated phosphorylation of BCL6 which, in turn, targets BCL6 for rapid degradation by the ubiquitin proteasome pathway \textsuperscript{16}. At the transcriptional level, a variety of stimuli, including CD40 receptor engagement and mitogenic stimulation, lead to downregulation of BCL6 RNA in cultured B-cells (Niu et al, submitted). More recently, a novel mechanism has been identified which downregulates BCL6 activity by inhibiting its transrepressive function through p300-mediated acetylation \textsuperscript{17}.

In tumors, chromosomal translocations affecting the BCL6 locus at band 3q27 represent the most common and specific genetic abnormality associated with DLBCL, accounting for \textgreater 40% of the cases \textsuperscript{1,18,19}. These translocations juxtapose heterologous promoters from the partner chromosome with intact BCL6.
coding sequences, leading to deregulated expression of BCL6 by a mechanism known as promoter substitution 20,21.

In addition, the 5’ regulatory sequences of BCL6 can be altered by multiple somatic mutations in 30-40% of normal GC B-cells 22-24. These mutations cluster within ~2Kb from the transcription initiation site at average frequencies of 1.2 x 10^{-3}/bp in the mutated cells 22-24; they are often biallelic, extremely heterogeneous, and display features of the IgV-associated somatic hypermutation mechanism, including a preference for single basepair substitutions, the predominance of transitions over transversions, and a preferential (RGYW) targeting motif 22-24. Consistent with their association with the physiologic GC reaction, BCL6 mutations are also found in a fraction of all B-cell tumors that carry mutated IgV sequences and display a GC or post-GC phenotype, including B-cell chronic lymphocytic leukemia (B-CLL), Burkitt lymphoma (BL), follicular lymphoma (FL), DLBCL, and multiple myeloma (MM)25-27. The structural features of these mutations, as well as their distribution and the specificity for GC B-cells, strongly suggest that BCL6 mutations and IgV mutations are derived by the same mechanism. Nonetheless, the biological role of BCL6 mutations in normal and malignant B-cell development remains unclear.

In the present study, we investigated the functional consequences of BCL6 mutations by performing a structure-function analysis on 55 mutant alleles (a total of 371 mutations) derived from normal GC lymphocytes or from various lymphoma subtypes. We identified a subset of mutations, located within the first non-coding exon of BCL6, that disrupt the mechanism of negative autoregulation normally involved in controlling BCL6 expression levels. These mutations were found only in DLBCL cases, suggesting that they may have been selected during lymphomagenesis for their ability to deregulate BCL6 expression.

Materials and Methods

Cell lines, stable transfections and sorting of normal GC B-cells

All B-cell lines used were cultured in IMDM supplemented with 10% FCS, 100U/ml penicillin, 100 U/ml streptomycin and 2mM L-glutamine. The EBV transformed EREB cell line requires addition of 1µM β-estradiol to the culture medium. To generate the stable EREB-MT and EREB-MT BCL6 cell lines, EREB
cells were transfected by electroporation with 20µg pMEP4 (Invitrogen, Carlsbad, CA) or pMEP4-HA-BCL-6 and selected in hygromycin (150µg/ml). For induction of exogenous BCL6 expression in the EREB-MT-HA-BCL6 stable line, 2 µM CdCl₂ were added to the culture medium and cells were harvested at various time points as indicated in Figure 5b. The 293T fibroblast cell line was maintained in DMEM containing 10% FCS, penicillin/streptomycin and 2mM L-glutamine. The protocol for isolation of tonsillar GC B lymphocytes is reported in detail in Pasqualucci et al, 1998.

**DNA extraction and PCR amplification of the BCL6 promoter region**

Genomic DNA from sorted GC B-cells or lymphoma cases (n=46, including 32 DLBCL, 5 B-CLL, 4 FL and 5 BL) was extracted according to standard methods. For amplification of the ~2.7 Kb BCL6 genomic region cloned into the pLA/S5 reporter plasmids, the Expand™ High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN) and the following primers were used: 5’-AGGAGAGACACACTTCAGC-3’ (sense) and 5’-ATCGTAGAAGGGAACACAAC-3’ (antisense). For mutational analysis of the BCL6 exon 1 sequences, a 596 bp genomic fragment spanning this region was PCR amplified for 30 cycles using the primers E1.15 and E1.20C described in Migliazza et al, 1995.

**Cloning and sequence analysis**

Purified amplicons were sequenced directly from both strands as described. The 2.7 Kb BCL6 amplicons were digested with NdeI and SpeI, gel purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA), and cloned into the corresponding sites of the pLA/S5 vector (see below) using the Rapid Ligation Kit (Roche); ~100 recombinant clones from GC B-cells and 6-10 clones from each tumor case were analyzed to identify individual alleles. Full-length sequencing of the ~2.7 Kb BCL6 promoter region was then performed on selected clones to confirm the mutations observed by direct sequencing and to detect additional changes, possibly representing subclonal variants or DNA polymerase-introduced errors. The BCL6 5’ non-coding sequence has been deposited in GenBank under accession number AY189709 [note that the numbering has been corrected (+1 bp) compared to the one reported in our previous publications 23,25,27].
Plasmid constructs

The original BCL6 luciferase reporter plasmid (pLA/S14wt) was constructed in multiple steps by inserting a ~14 Kb AvrII/NcoI genomic fragment of the native BCL6 promoter (spanning the 5' flanking sequences, exon 1, intron 1 and exon 2) into the multiple cloning site of the pGL3 vector (Promega, Madison, WI). This construct displayed comparable basal activity to a deletion mutant lacking the ~7.7 Kb SspI/Bsu36I fragment at the 3' end of intron 1 (pLA/S5wt, Figure 1A). The latter one was therefore used as a backbone to derive all the mutant reporter constructs by exchanging the wild-type 2776 bp NdeI-SpeI region of the BCL6 5' sequences with the corresponding fragment PCR amplified from genomic DNA of GC B-cells and lymphoma cases. Four of these constructs (Ly1A, 93-611A, 93-611B and 93-2889A) were further dissected by swapping —in different combinations— mutant and wildtype fragments corresponding to the 5' flanking, first exon and first intron of BCL6, as defined by the restriction sites NdeI (-694), PmlI (-64), SexAI (+276) and SpeI (+2082). The single point mutants were generated by site-directed mutagenesis using the pLA/S5wt plasmid as a target and the Transformer Site-Directed Mutagenesis Kit (BD Biosciences Clontech, Palo Alto, CA). The expression vector pMT2T-HA-BCL6 and its deletion mutants pMT2T-HA-BCL6-ZF and pMT2T-HA-BCL6-∆ZF have been described previously. The metallothionein inducible expression vector pMEP4-HA-BCL6 was constructed by cloning a 2.4Kb BCL6 cDNA fragment, HA-tagged, into the BamHI site of pMEP4 (Invitrogen).

Transient transfections and reporter gene assays

The B-cell lymphoma lines Ly1, MUTU I and MUTU III (1.5 x 10^7/sample) were transfected by electroporation at 250V and 960 µF using a Bio-Rad Gene Pulser apparatus. Equimolar amounts (2 pmol) of wildtype and mutant reporter plasmids were used in each experiment, together with 0.5 pmol of a plasmid expressing the Renilla luciferase (pRL-TK) as a control. Transient transfections into 293T cells were performed by the calcium phosphate precipitation method, using 0.1 pmol of the various reporter constructs and increasing amounts (0.01, 0.02, 0.04, 0.1 pmol) of the effector plasmids (pMT2T-HA-BCL6, pMT2T-HA-BCL6-ZF and pMT2T-HA-BCL6-∆ZF). The total amount of transfected DNA was kept constant in each experiment by adding pMT2T vector sequences to a final amount of 10 µg. The pRL-TK vector (0.15 µg) was also co-transfected as an internal control for transfection efficiency. Luciferase
activities were measured 48 hours after transfection using the Dual-Luciferase™ Reporter Assay Kit (Promega), according to the manufacturer’s protocol.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts were prepared from the Ly1 cell line as described and gel shift analysis was performed using ~3 µg of extract, the oligoprobes indicated in Fig. 3B, and an anti-BCL6 antiserum (N71-1) that recognizes the N-terminus of the BCL6 protein.

**Chromatin Immunoprecipitation (ChIP) assay**

The detailed protocol for formaldehyde crosslinking and chromatin immunoprecipitation has been published. Briefly, samples (each obtained from 1-2.5 x 10^7 B-cells) were immunoprecipitated overnight at 4 °C with 2 µg of the anti-BCL6 polyclonal antibody N3 (Santa Cruz Biotechnology). In each experiment, one sample with no Ab and one sample with an irrelevant Ab (anti-IgG) were included as negative controls for nonspecific binding. Immune-complexes were recovered by adding 30 µl of blocked salmon sperm DNA/Protein A agarose beads (50% slurry, UpState Biotechnology, Lake Placid, NY) and incubating the samples for 2 hr at 4°C. Following extensive washing and reverse crosslinking, DNA was purified by phenol-chloroform extraction, ethanol precipitated and resuspended in 30 µl of TE (100 µl for the total input sample, representing 0.5% of the soluble chromatin before immunoprecipitation). Fragments containing the BCL6 exon 1 binding sites were detected by PCR amplification using 2.5 µl of the above DNA preparations as template and two different oligonucleotide pairs: 5’-ACGCTCTGCTTATGAGGA-3’ (sense) and 5’-CGGCAGCAACAGCAATAA-3’ (antisense) for the B1 fragment (position +39 to +300); 5’-GGGTTCCTAGAAGTGGTG-3’ (sense) and 5’-CAAAGCATTTGGCAAGAG-3’ (antisense) for the B2 fragment (+227 to +421). Two additional primer sets were designed to amplify the control regions A, located ~1 Kb upstream of the transcription initiation site (5’-ACTAGGACCCACAATGAA-3’ and 5’-CGTTTCAAGATCGTTGTA-3’), and D, corresponding to the coding exon 5 (5’-GTGTGCCACAGCAATATC-3’ and 5’-TGGCAGTCAGATTCTGG-3’). Following 32 cycles of amplification, PCR products were resolved on 2% agarose gels and visualized by ethidium bromide.
staining. The same amplicons were subsequently gel-purified and sequenced both directly and upon cloning into pGEM-T vector (Promega) as described.

**Northern blot, Western blot and RT-PCR analysis.**

Total RNA was prepared from various B-cell lines and lymphoma cases using the Trizol reagent (Invitrogen). Northern blot and Western blot analyses for BCL6 expression were performed as described. For cDNA synthesis, 2 µg of RNA were reverse transcribed using oligo(dT)18 primer and SuperScript II reverse transcriptase (Invitrogen). The resulting first-strand DNA was PCR amplified using primers that anneal to the BCL6 exon1 and 2 and directly sequenced.

**Results**

**Deregulated expression of DLBCL-associated mutant alleles**

To investigate the consequences of BCL6 mutations on transcriptional regulation of the BCL6 gene, we compared the transcriptional activity of mutant BCL6 alleles derived from normal (i.e., sorted GC B-cells) and lymphoma cells by using a transient transfection/reporter gene assay in two lymphoma lines (Ly1 and MUTUI I) permissive for BCL6 expression. Initial studies showed that a luciferase reporter gene under the control of the native BCL6 promoter (~14 Kb AvrII/NcoI genomic fragment spanning the 5’ flanking sequences, exon 1, intron 1 and exon 2) had comparable activity to that of a deletion mutant lacking ~7.7 Kb at the 3’ end of intron 1 (data not shown). Therefore, we used this shorter plasmid (Figure 1A) as a backbone to generate a series of mutant reporter constructs in which the wildtype 2.7 Kb NdeI/SpeI fragment was substituted with variant BCL6 sequences amplified from normal GC cells (20 mutated alleles) or from various tumor cases (6 DLBCL, 5 BL, 4 FL and 5 B-CLL). For each tumor sample, both alleles, designated as A and B, were tested: while mutations were biallelically distributed in 15 cases, the remaining cases displayed one mutated and one wildtype allele (a detailed description of the mutations present in each construct is available in Supplementary Information, Table 1). The basal expression level of the corresponding plasmids (pLA/S5 series) was then evaluated in the transfection/reporter gene assay in Ly1 and MUTUI-BL59.
The results showed that all of the mutant alleles derived from normal GC cells (N=20) have a transcriptional activity comparable to that of the wildtype allele, arbitrarily set as 1 (Figure 1B). Similarly, mutant alleles from BL (N=8), FL (N=7) and B-CLL (N=6) cases were also indistinguishable from the wildtype allele in this assay. However, four of the 12 (33%) DLBCL-associated alleles were significantly and reproducibly overexpressed (4-18 fold) in both transfected cell lines (see Figure 1B for representative data with the Ly1 cell line). Of the four deregulated alleles, two were derived from two distinct DLBCL cases (Ly1 and 93-2889), while the remaining two were derived from a single case (93-611). Interestingly, deregulation was not observed when the same reporters were transfected in a cell line (MUTU III) that lacks BCL6 expression (not shown), suggesting the involvement of the BCL6 protein itself in the observed effect (see below). These results indicate that some mutations associated with DLBCL can transcriptionally deregulate the BCL6 gene.

**DLBCL-associated deregulating mutations cluster within exon 1 of BCL6**

Each of the four BCL6 alleles displaying deregulated activity in the transient transfection assay contains multiple basepair substitutions scattered throughout the first non-coding exon, the first intron and, in two cases, the 5’ flanking sequences (see Supplementary Information). To identify the mutation(s) responsible for DLBCL-associated deregulation, we generated a series of reporter constructs in which wildtype and mutant fragments corresponding to the 5’ flanking, first exon and first intron of each mutated allele were swapped in all possible combinations. The activity of these constructs was then tested using the same transient transfection/reporter gene assay in Ly1 cells. The results in Figure 2 document that, in all four alleles, the mutated sequences responsible for transcriptional deregulation were located in exon 1 (see construct b in each panel). Notably, this region only contains a single mutation in the deregulated allele Ly1A (T257C, indicated by an asterisk) or two distinct changes in deregulated alleles 93-611A, 93-611B and 93-2889A. To ascertain which of these changes is responsible for transcriptional deregulation, each was incorporated into the pLA/S5 reporter construct by site-directed mutagenesis and their effect on transcriptional activation was tested vis-à-vis the wildtype sequence. Remarkably, a single nucleotide substitution from each deregulated allele (T257C in Ly1A, A260G in 93-611A, T231G in 93-611B, and T234C in 93-2889A) recapitulated the abnormal behavior displayed by the original mutant. These results
demonstrate that, within multiply mutated BCL6 alleles, individual mutations—all located in the first exon—were responsible for the observed deregulation.

**Deregulating mutations cluster within two BCL6 binding sites**

The four “deregulating mutations” are clustered in two sequence motifs within exon 1 of BCL6. Significantly, both motifs share extensive homology to the preferred DNA-binding sequence of BCL6 polypeptides (Figure 3A, boxed areas; active mutations in bold and underlined), suggesting that the BCL6 protein may bind and regulate its own promoter region. To test this hypothesis, we examined whether the two potential binding sites within exon 1 (called BSE1A and BSE1B) could bind BCL6 in an electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotide probes corresponding to the wildtype BSE1A (+225+244 from the minor BCL6 promoter) and BSE1B (+249+268) sequences were incubated with nuclear extracts from the BCL6-expressing cell line Ly1. Figure 3B shows that both probes were associated with a DNA-binding protein complex similar in migration to the one formed by B6BS, a probe representing the canonical BCL6 binding site (lanes 1 and 3 in each gel). Supershift analysis using antisera against the N-terminus of BCL-6 (N71-1) confirmed that these complexes contained BCL6 (lanes 2 and 4). The difference in intensities between the BSE1A- and BSE1B-associated bands may reflect differences in the abundance of these complexes and/or distinct binding affinities of the BSE1A (8/9 matches with the core domain of the canonical BCL6 binding site) and BSE1B (100% identity in the 9 bp core) sequences. Notably, oligonucleotide probes carrying the tumor-derived deregulating mutations (m231, m234, m257 and m260) failed to bind BCL6 (lanes 5-8). Also, it may be relevant that alleles bearing mutations of the “high affinity” BSE1B site (i.e., Ly1A and 93-611A) were significantly more deregulated than those harboring mutations of the “low affinity” BSE1A site (93-611B and 93-2889A)(see Figures 1A and 1B). Thus, the BCL6 protein can bind the exon1 sequences *in vitro* and this binding is abrogated by the DLBCL-associated mutations.

To confirm that endogenous BCL6 can bind to its native promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays using an anti-BCL6 polyclonal antibody (N3) in two BCL6-positive cell lines. Ly1 cells carry a mutation in the BSE1B site of allele A, which confers deregulation in
the reporter assay (Figure 2) and inactivates BCL6 binding in the EMSA (Figure 3B). The P3HR1 line was used as a control since it contains mutations in the BCL6 exon 1/intron 1 boundary (G172C, T380A, T397G) that are located outside of the BSE1A and BSE1B motifs and are not associated with deregulation. Four different oligonucleotide pairs were designed to amplify genomic fragments corresponding to the relevant exon 1 sequences (products B1 and B2) or to two distal control regions (products A and D) located ~1 Kb upstream and >10 Kb downstream of exon 1, respectively (Figure 4A). The results show that BCL6 binds specifically to regions B1 and B2, but not to the control regions A and D, in both BCL6-expressing cell lines (Figure 4B); no product was obtained using an irrelevant antibody (IgG) or a control line (CB33) that does not express BCL6 (Figure 4B, right panel). Thus, the exon 1 sequence containing the BSE1A and BSE1B sites represents a specific target for BCL6 binding in vivo.

Based on the EMSA results showing that BCL6 binds to the wildtype, but not mutant, exon 1 sequences, we investigated whether this differential binding can also be detected in vivo by examining the allelic representation of chromatin immunoprecipitates obtained from Ly1 cells (and P3HR1 cells as a control) using the anti-BCL6 antibody. Since all mutations are heterozygous in these lines, the presence of each individual allele could be uniquely identified by PCR/sequencing. Figure 4C shows that both alleles are present in the genomic DNA, as well as in the chromatin solution before immunoprecipitation (input), from both Ly1 and P3HR1 (top and middle panels). However, we consistently observed preferential amplification of the wildtype allele in the ChIP product obtained from Ly1, but not from P3HR1 cells (bottom panels). This predominance of the unmutated allele B in the immunoprecipitated chromatin from Ly1 was confirmed by cloning and sequencing the corresponding PCR amplicon, which revealed wildtype sequences in 83% of the clones as opposed to the ~50% expected and observed in the control genomic DNA and in the total input (not shown). Taken together, these results demonstrate that the BCL6 exon 1 sequence is a direct physiologic target for BCL6 binding in vivo, and that this binding is impaired by the “deregulating” mutations associated with DLBCL.

**BCL6 exon1 binding sites mediate a negative autoregulatory circuit**

Since BCL6 is a transcriptional repressor, these data suggested the existence of an autoregulatory circuit in which BCL6 binds its own promoter and inhibits its own transcription. To examine whether BCL6 genes
are subjected to this autoregulatory circuit *in vivo*, we first analyzed endogenous BCL6 expression levels in a BL cell line, Ramos, which had been engineered to constitutively express exogenous BCL6 under the control of a retroviral vector (PINCO). The results shown in Figure 5A indicate that even modest levels of exogenous BCL6 expression (Ramos cells do not tolerate higher levels) lead to a proportional reduction (~40%) in endogenous BCL6 levels.

To exclude that the observed phenomenon may be related to cell culture selection or to clonal variability, we then examined the temporal and quantitative response of endogenous BCL6 transcription in a lymphoblastoid cell line, EREB, transfected with an inducible BCL6 gene vector (pMEP4-HA-BCL6, for brevity MT-BCL6) or with an empty vector (pMEP4, for brevity MT) as control. In this system, transcripts from the exogenous BCL6 sequences are driven by the heavy-metal-inducible metallothionein promoter and can be distinguished from endogenous transcripts because of their lower molecular weight. Stably transfected EREB-MT and EREB-MT BCL6 cells were analyzed by Northern blot for BCL6 expression at different time points following addition of CdCl₂ to the cell culture medium. As indicated in Figure 5B, endogenous BCL6 transcripts can be clearly detected in the EREB-MT BCL6 clone at time 0, when very low levels of exogenous BCL6 are present, possibly due to the leaky activity of the MT promoter (line 4). However, CdCl₂-mediated induction of exogenous BCL6 caused a progressive decrease in endogenous BCL6 mRNA levels (lanes 5,6). Thus, downregulation of BCL6 gene expression in the presence of exogenous BCL6 represents a specific and dose dependent effect of increased BCL6 protein levels, fulfilling the criteria for an autoregulatory circuit.

Finally, we examined whether the autoregulatory circuit functions at the transcriptional level and whether it targets the BCL6 promoter region. To this end, the pLA/S5 reporter construct containing the wildtype BCL6 promoter was co-transfected with increasing amounts of a BCL6 expression plasmid (pMT2T-HA-BCL6) into 293T cells (Figure 5C). Luciferase activities revealed a strong and dose-dependent repression of the reporter gene in the presence of exogenous BCL6 (8% residual activity at 0.1 pmol input) (Figure 5D, solid bars). This effect was due to genuine BCL6-mediated transcriptional repression because it was not detected when the same reporter was co-transfected with BCL6 deletion mutants that lack either the DNA-binding (pMT2T-HA-BCL6-ZF) or the N-terminal transrepression (pMT2T-HA-BCL6-ZF) domain (Figure 5D, hatched bars). Taken together, these data document the
existence of an autoregulatory circuit in which BCL6 modulates its own transcription by binding to its promoter sequences in native B cells.

**BCL6 mutations disrupt the BCL6 autoregulatory mechanism**

To study whether BCL6 autoregulation targets specifically the BSE1A and BSE1B motifs and is disrupted by the four DLBCL-associated mutations, we used the same transient transfection/reporter gene assay and tested the degree of resistance of the mutated alleles to this negative feedback loop. To this end, the responses of the four “deregulated” pLA/S5 single mutant reporters (Figure 2) were compared to that of the wildtype construct upon co-transfection with increasing amounts of pMT2T-HA-BCL6 in 293T cells. Variable degrees of resistance to exogenous BCL6 repression were reproducibly observed in each of the four mutants (Figure 6). This abnormal response was especially evident in the Ly1A-, 93-611A- and 93-2889A-derived transfectants, and, to a lesser extent, in 93-611B. These data indicate that the DLBCL-associated mutations confer resistance to the autoregulatory feedback loop, and also establish the BSE1 motifs as the **in vivo** targets for autoregulation.

**Deregulated expression of BCL6 in DLBCL cases carrying BSE1 mutations**

The results obtained in the transient transfection experiments imply that “deregulated” alleles escape the negative autoregulatory circuit and are overexpressed, while the wildtype allele remains responsive and transcriptionally silent. Consistent with this hypothesis, Northern and Western blot analysis of DLBCL cell lines showed that the Ly1 cells, which carry a BCL6 allele mutated in the BSE1 site, express levels of BCL6 mRNA and protein higher than lines carrying either translocated, non BSE1-mutated or wild-type BCL6 alleles (see Figure 7A and B for representative results). Furthermore, an analysis of BCL6 allele-specific transcription by RT-PCR and sequencing revealed that only one allele –the one that carries the mutation in the BSE1 site– is transcribed in Ly1 cells (Figure 7C, top panel). In contrast, BCL6 is biallelically expressed in the BJAB (Figure 7C, bottom panel) and P3HR1 (not shown) lymphoma lines, both of which are heterozygous for “non-deregulating” mutations. The correlation between exon 1 mutations and deregulation could not be validated in primary tumors because the presence of contaminating
normal cells makes it very difficult to quantitatively analyze allelic expression in tumor cells by RT-PCR with accuracy. Nonetheless, these data demonstrate that the “deregulating” mutations alter the transcriptional regulation of endogenous BCL6 genes in native DLBCL cells.

Frequency of deregulating mutations in DLBCL

To determine the frequency of BSE1 mutations in DLBCL, we screened by PCR amplification and direct sequencing a panel of 32 DLBCL cases, all cytogenetically characterized, for the presence of mutations affecting the two BCL6 binding sites. Our survey revealed that 4/25 (16%) samples lacking 3q27 translocations (~13% of all DLBCL) carry alterations in these sequences; conversely, mutations within the BSE1 sites were not found in cases where the BCL6 gene had been disrupted by 3q27 abnormalities. These results indicate that mutations affecting the BCL6 exon 1 binding sites are associated with a sizable fraction of DLBCL and appear to be mutually exclusive with BCL6 rearrangements.

Discussion

Mutations affecting the 5′ non-coding region of the BCL6 gene are found in 30-40% of normal GC B-cells and in all GC or post-GC-derived B-cell non-Hodgkin lymphoma. Despite this high frequency, the functional consequences of the mutations in both normal and transformed B-cells are not known. The present study elucidates one mechanism by which specific BCL6 mutations associated with DLBCL can deregulate its own expression, and identifies a negative autoregulatory circuit that controls BCL6 transcriptional activity in vivo. These results have direct implications for the regulation of BCL6 expression in normal and neoplastic B-cells and for the role of BCL6 in lymphomagenesis.

The first observation emerging from this study is that the pattern of BCL6 mutations can differ among normal GC cells and transformed cells from various GC-derived lymphoma subtypes. In fact, BCL6 mutations affecting the two BCL6 binding sites in exon 1 (BSE1A and B) were found at sizable frequency among DLBCL derived alleles, but not in normal GC cells or in indolent lymphomas. Since BCL6 mutations occur physiologically in GC B-cells, most likely by the same mechanism that hypermutates IgV sequences, it is conceivable that their distribution may be stochastically determined.
in different cells. Thus, the finding of BSE1 mutations specifically restricted to DLBCL suggests a selection for these mutations in association with lymphoma development.

Our results also indicate that the basis for this selection is the ability of BSE1 mutations to disrupt the negative autoregulatory mechanism that controls BCL6 expression. The possibility that BCL6 regulates its own expression was previously proposed based on the identification of a BCL6 binding motif in exon 1 that mediates BCL6-dependent transcriptional repression of a reporter plasmid containing a heterologous promoter. The present study provides conclusive evidence for the presence of two BCL6 binding sites, one of which was not previously reported, and demonstrates that both are required for BCL6-mediated transcriptional repression in the context of the physiological BCL6 promoter region. Most notably, our results show that these two sites bind BCL6 \textit{in vivo} and that this binding is lost in DLBCL-associated mutant alleles. Taken together, these findings are consistent with the existence of a negative autoregulatory feedback loop in which BCL6 regulates its own expression by direct binding and repression of its promoter region. The existence of this mechanism is further supported by a number of independent observations: i) endogenous BCL6 expression is downregulated in cell lines (Figure 5A,B) and in transgenic mice (our unpublished results) expressing exogenous BCL6; ii) the expression of truncated BCL6 alleles is increased in mice expressing a non-functional BCL6 protein due to inactivation of the BCL6 gene by homologous recombination (Ye et al., 1997 and our unpublished results); iii) the normal BCL6 allele is not expressed in DLBCL cases where the second allele is deregulated by chromosomal translocation.

Autoregulatory circuits represent a common, evolutionarily conserved mechanism for modulation of gene expression and are typically used by genes encoding transcription factors. These circuits have homeostatic functions by limiting the range over which expression levels of a gene can fluctuate. In mammalian cells, autoregulatory circuits have been described for well-known proto-oncogenes encoding transcription factors that need to be tightly regulated (e.g., c-MYC). In the case of BCL6, loss of proper autoregulation could be especially relevant considering that this protein can repress the transcription of genes that induce cell cycle arrest, apoptosis and differentiation. For at least some of these genes, the extent of repression depends upon the relative levels of BCL6 versus other transcriptional activators, such
as STAT6, which regulate the same genes\textsuperscript{12}. Thus, aberrantly high levels of BCL6 may lead to constitutive repression of genes necessary for GC exit and further differentiation, thereby contributing to lymphomagenesis.

The results herein identify the two BSE1 motifs within BCL6 exon 1 as the critical targets for BCL6 deregulation in DLBCL. This notion is supported by several observations: i) exon 1 sequences spanning the BCL6 binding sites are highly conserved between human and mouse (100% identity over 120 bp, see also Figure 3A); ii) this region is separated from the BCL6 coding exons in most DLBCL cases that carry a 3q27 translocation (~40%); and iii) this region is lost by internal deletions in a small fraction of cases\textsuperscript{35-37}. Thus, one common consequence of most translocations and DLBCL-associated BSE1 mutations or deletions is the removal of the sequences involved in BCL6 autoregulation.

The identification of a fraction of DLBCL (~13% overall, corresponding to 16% of the non-translocated cases) in which BCL6 expression is deregulated by exon 1 mutations suggests a broader involvement of the BCL6 proto-oncogene in the pathogenesis of DLBCL than previously suspected based on the frequency of chromosomal translocations (40%)\textsuperscript{1,18,19}. In fact, the frequency of DLBCL-associated mutations that alter BCL6 expression may be even higher if we consider that the assay used here can identify deregulated alleles only if the mutations affect constitutive BCL6 expression levels. Thus, tumor-associated mutations that alter the response of the BCL6 gene to regulatory signals may also exist, but would not be identified by our assay. This possibility is supported by the observation that, analogous to cases carrying BCL6 translocations, a number of DLBCL that display BCL6 mutations, but not translocations, are resistant to the physiologic BCL6 downregulation induced by CD40 signaling (G. Cattoretti et al., in preparation). The experimental strategies used in this study can be adapted to investigate the effect of mutations on the ability of various signals to downregulate BCL6 expression.

Finally, by identifying an expanded fraction of DLBCL cases carrying deregulated BCL6 expression, these findings have diagnostic and therapeutic implications. The prognostic significance of BCL6 deregulation has been the object of intense controversy, with some studies claiming it as a favorable indicator and others disclaiming the finding\textsuperscript{38-42}. This controversy can be explained by the observation herein that the fraction of DLBCL carrying chromosomal translocations, the one considered by the above prognostic studies, does not account for the entire fraction of DLBCL carrying deregulated BCL6, and that
those with deregulating mutations should also be considered. On the therapeutic side, these results have implications for the clinical trials in which BCL6 is currently under evaluation as a therapeutic target for histone deacetylase inhibitors.\textsuperscript{17}

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References


Figure Legends

Figure 1. Abnormal expression of DLBCL-derived mutant BCL6 alleles. (A) Schematic diagram of the pLA/S5 wildtype reporter containing the BCL6 5’ non-coding region. Mutant constructs were generated by exchanging the 2.7Kb NdeI/SpeI fragment with the corresponding region of mutant BCL6 alleles derived from normal GC B-cells or various lymphoma types. (B) Ly1 cells were transfected by electroporation with equimolar amounts of the indicated pLA/S5 reporter constructs and the pRL-TK plasmid as an internal control for transfection efficiency. In each tumor case, identified by a bracket, both alleles were tested (Solid bars = mutated alleles; white bars = wildtype alleles). After forty-eight hours, cells were harvested and the luciferase activities were measured. One representative experiment out of 3-8 independent transfections performed in duplicate with similar results is shown. Average luciferase activity (± standard deviation) are expressed as relative increases respect to the wildtype construct (set as 1), after normalization for Renilla activity; differences in activity were defined as significant when >2 fold (dotted line). The DLBCL-derived alleles Ly1A, 93-611A, 93-611B and 93-2889A were found overexpressed (4 to 18 fold), while no significant changes were observed in transfectants deriving from normal GC cells or other lymphoma types. DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; B-CLL, B-cell chronic lymphocytic leukemia.

Figure 2. Mapping the “deregulating mutations”. Transient transfection assays were performed as described in Figure 1, using the reporter constructs shown on the left of each panel. Grey fragments correspond to the mutated 5’ flanking, exon1 (box) and intron 1 region of the original allele (construct “a”), which were swapped in all possible combinations to map the deregulating mutation(s). Asterisks indicate the presence of mutations (note that two distinct nucleotide changes were present in the exon 1 sequences of allele 93-611A, 93-611B and 93-2889A). Bars represent the activities of the resulting constructs (mean ± standard deviation), obtained from two independent experiments performed in duplicate. In all four cases, a single mutation located in the BCL6 first non-coding exon recapitulates the activity displayed by the original construct.
**Figure 3. In vitro binding of BCL6 to the BSE1 motifs.** (A) Sequence of the BCL6 exon 1 region where the four deregulating mutations (in bold, underlined) were mapped. The corresponding murine sequence is also aligned to show the complete sequence identity between the two species. Boxed areas represent the two BCL6 binding motifs (BSE1A and BSE1B). “Non-deregulating” mutations are shown in gray. (B) BCL6 deregulating mutations abolish binding of BCL6 to its exon1 sequences *in vitro*. EMSA were performed on Ly1 nuclear extracts using as probes the indicated oligonucleotides. The B6BS probe containing the BCL6 canonical binding site was used as a control. Arrows point to BCL6 containing complexes. Supershift analysis was performed using an anti-BCL6 (N71-1) antiserum that recognizes the N-terminus of the BCL6 protein.

**Figure 4. BCL6 binds to its exon 1 sequence in vivo (ChIP assay) and the binding is abrogated by BCL6 “deregulating” mutations.** (A) Schematic representation of the human BCL6 locus; the four genomic fragments amplified for analysis are approximately positioned below the map (B1 and B2, test region; A and D, control regions). (B) Ethidium bromide stained agarose gels of PCR products A-D obtained from two BCL6 expressing cell lines (Ly1 and P3HR1) and a control line that lacks BCL6 expression (CB33). After formaldehyde cross-linking, chromatin was immunoprecipitated using the anti-BCL6 Ab N3 or an irrelevant Ab (IgG) as control, and PCR reactions were performed on ChIP products, total chromatin before immunoprecipitation (input), and genomic DNA as a positive control for PCR. One sample was also processed with no Ab to serve as a negative control. (C) BCL6 binds to the wildtype, but not to the mutated allele in the Ly1 cell line. Direct sequencing of PCR products obtained from genomic DNA, total input, and immunoprecipitated chromatin (ChIP) in the Ly1 cell line and in P3HR1 as a control. Arrows indicate the position of the mutations (Ly1: T257C; P3HR1: T380A and T397G).

**Figure 5. The BCL6 promoter is a target for BCL6 mediated transcriptional repression.** (A) Negative regulation of endogenous BCL6 by constitutive expression of exogenous BCL6 in Ramos cells. Northern blot analysis of endogenous (endo) and exogenous (exo) BCL6 expression in Ramos cells and in Ramos clones transduced with the PINCO-HA-BCL6 retroviral vector. The signal intensity ratio between endogenous BCL6 and GAPDH, quantitated by phosphoimager analysis, is shown in the lower panel. (B)
CdCl₂-induced downregulation of endogenous BCL6 gene expression in EREB cells stably transfected with an inducible BCL6 gene. Northern blot analysis of EREB cells transfected with MT (EREB-MT) or MT-BCL6 (EREB-MT BCL6) plasmids. Cells were treated with CdCl₂ as described in Methods and collected at the indicated intervals. The Ramos cell line was included as a control for size of the endogenous BCL6 transcripts. The filters were sequentially hybridized with a radiolabeled BCL6 cDNA probe and with GAPDH to control for amount of RNA loading. (C) Schematic representation of the plasmids used in transient co-transfection experiments. POZ, protein-protein interaction domain; ZF, Zinc Finger DNA-binding domain. (D) The reporter construct indicated in (C) was transfected alone or in the presence of increasing amounts of various BCL6 expressing plasmids into 293T cells. Luciferase activities measured forty-eight hours after transfection revealed a strong and dose-dependent repression of the reporter gene when co-transfected with the wildtype BCL6 expressing plasmid (solid bars), but not with two deletion mutants that lack the DNA-binding (ΔZF) or the transrepression domain (ZF) (hatched bars). All experiments were performed in duplicate and standard deviations are indicated.

**Figure 6. Mutations within the BCL6 exon1 binding sites confer resistance to BCL6 mediated transrepression activity.** The responses of the four DLBCL-derived BSE1-mutant reporter constructs to BCL6 mediated transrepression activity were compared to that of a wildtype plasmid in co-transfection experiments in 293T cells, as described in Figure 5D.

**Figure 7. Deregulated BCL6 expression in DLBCL cases carrying BSE1 mutations.** (A) Northern blot analysis of BCL6 expression in various DLBCL-derived cell lines. Equal amounts (12 µg) of total RNA were loaded on formaldehyde-agarose gel, blotted and sequentially hybridized with a full-length BCL6 cDNA probe and with GAPDH as control for the amount and integrity of the RNA. Quantitative analysis of the data was performed by phosphorimager (bottom panel). For each sample, the presence of 3q27 chromosomal translocations [t(3q27)], mutations affecting the BSE1 sites [M (BSE1)], and mutations located outside the BSE1 sites [M(others)] is indicated. The lymphoblastoid cell line CB33, which does not express BCL6, was included as a negative control. (B) Western blot analysis of BCL6 protein expression in the same cell lines, using the anti-BCL6 (N3) Ab and an anti-β-tubulin Ab as control for protein loading.
(C) Nucleotide sequencing of RT-PCR products generated from the mutated lymphoma cell lines Ly1 and BJAB. Only the allele A, identified by the T257C mutation in the BSE1 site, but not the allele B (containing a normal sequence in exon 1) is expressed in Ly1, while BCL6 is biallelically expressed in the control line BJAB, heterozygous for “non-deregulating” mutations. Ramos cells are shown as a control for unmutated exon 1 sequences.
Figure 1
Figure 2
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Figure 7
Mutations of the BCL-6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma

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