BCL-6 Negatively Regulates Expression of the NF-κB1 p105/p50 Subunit[1]

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BCL-6 is a transcription repressor frequently deregulated in non-Hodgkin's B cell lymphomas. Its activity is also critical to germinal center development and balanced Th1/Th2 differentiation. Previous studies have suggested that NF-κB activity is suppressed in germinal center and lymphoma B cells that express high levels of BCL-6, and yet the reason for this is unknown. We report in this study that BCL-6 can bind to three sequence motifs in the 5′ regulatory region of *NF-κB1* in vitro and in vivo, and repress *NF-κB1* transcription both in reporter assays and in lymphoma B cell lines. *BCL-6*$^{-/-}$ mice further confirm the biological relevance of BCL-6-dependent regulation of *NF-κB1* because BCL-6 inactivation caused notable increase in p105/p50 proteins in several cell types. Among these, *BCL-6*$^{-/-}$ macrophage cell lines displayed a hyperproliferation phenotype that can be reversed by NF-κB inhibitors, e.g., *N*-tosyl-L-phenylalanine chloromethyl ketone and SN50, a result that is consistent with increased nuclear κB-binding activity of p50 homodimer and p50/p65 heterodimer. Our results demonstrate that BCL-6 can negatively regulate NF-κB1 expression, thereby inhibiting NF-κB-mediated cellular functions. *The Journal of Immunology,* **2005, 174: 205–214.**

BCL-6 is the most frequently targeted proto-oncogene in non-Hodgkin's lymphomas (NHL)[4] (1). One of the most common NHL subtypes is the diffuse large B cell lymphoma (DLBCL) believed to derive from germinal center (GC) B cells. Nearly 50% of these tumors constitutively express BCL-6 either as the result of chromosomal translocations or activating mutations, both of which enable BCL-6 transcription to bypass a negative autoregulation mechanism (2, 3). Recent work also suggested a strong link between BCL-6 expression and favorable DLBCL prognosis after chemotherapy (4, 5). However, the mechanism underlying BCL-6-mediated transformation remains elusive, largely because information connecting BCL-6 function with the regulation of cell growth or apoptosis has not been forthcoming. BCL-6 also plays important roles in the normal immune system, as *BCL-6*$^{-/-}$ mice fail to form GC and suffer from a severe hyper-Th2-type inflammatory disease (6, 7). Because the development of both T and B cells as well as the T cell-independent Ab response are unperturbed in these mice, the GC and Th cell defects reflect BCL-6 function at specific stages of T and B cell differentiation. On the B cell side, although it is clear that the GC phenotype in the BCL-6 KO mice is B cell autonomous (8), little is known regarding which aspects of the B cell response during GC development are dependent upon BCL-6.

A number of BCL-6 target genes have been reported that include the germline transcript Iε (9), activation markers CD69 and CD44 (10), cell cycle regulators p27Kip1 and cyclin D2 (10), regulator of plasma cell differentiation Blimp-1 (10–12), and several chemokine genes (10, 13). The link between BCL-6 and Blimp-1, a master regulator of plasma cell differentiation, suggests that BCL-6 may facilitate transformation by inhibiting differentiation of lymphoma cells. Nonetheless, none of these target genes provides a convincing explanation for lack of GC in the *BCL-6*$^{-/-}$ mice. Published work on the role of BCL-6 in proliferation and apoptosis is very controversial. Although overexpression of BCL-6 in CV-1 and U2OS cells was found to cause cell cycle block in S phase and apoptosis (14, 15), an antiapoptotic role was described for BCL-6 in differentiating mouse myocytes (16). Furthermore, although BCL-6 was reported to repress p27Kip1 and cyclin D2 expression in B cells (10), its role in regulating cell proliferation has not been directly studied in depth.

The NF-κB family of transcriptional factors plays a central role in immune, inflammatory, and acute phase responses. They are also implicated in the control of lymphocyte activation, proliferation, and apoptosis. Mammalian NF-κB is composed of dimers of five family members, namely NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), Rel (c-Rel), and RelB. In most cells, the majority of the dimers are sequestered in the cytoplasm by the inhibitory IκB proteins. Nuclear translocation of NF-κB occurs when extracellular stimuli activate the IκB kinase (IKK) complex that phosphorylates the IκB proteins, targeting them for destruction by the ubiquitination/proteasome pathway (17, 18). Many signaling pathways implicated in B cell proliferation, including those triggered by LPS, anti-CD40, and anti-IgM, converge upon and require the activity

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[4] Abbreviations used in this paper: NHL, non-Hodgkin's lymphoma; ABC, activated B cell; BCoR, BCL-6 corepressor; BCoRc, BCL-6 corepressor isoform c; ChIP, chromatin immunoprecipitation; DLBCL, diffuse large B cell lymphoma; GC, germinal center; GDI, guanine nucleotide dissociation inhibitor; IKK, IκB kinase; SMRT, silencing mediator of retinord and thyroid receptor; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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of NF-κB (19–21). In the macrophage/osteoclast lineage, a homodimeric cytokine, CSF-1, is the primary regulator of survival, proliferation, and differentiation (reviewed in Ref. 22). Major events triggered by engagement of the CSF-1 receptor, c-Fms, include activation of STAT1, STAT3, the MAPK, and the PI3K/AKT pathways (23–26). Although NF-κB function has not been directly studied in this process, its involvement is highly likely because there is strong evidence that an activated AKT pathway can trigger NF-κB via IKK-dependent and IKK-independent mechanisms (27–29). It is therefore conceivable that NF-κB not only can be activated in response to CSF-1 signaling in macrophages (30, 31), but also plays an important role in regulating CSF-1-mediated cell proliferation.

At the onset of this work, we were intrigued by the observation that activity of the NF-κB pathway is specifically down-regulated in normal GC B cells where the level of BCL-6 is at its highest (32). A similar, inverse correlation between BCL-6 and NF-κB activity was also reported for DLBCL (33). Two general hypotheses can be proposed to explain this phenomenon. First, many NF-κB target genes are individually suppressed via different mechanisms in normal GC and GC-derived tumor cells; second, activity of the entire NF-κB pathway is turned down by a negative regulator acting at an upstream step. Because BCL-6 is known to be a potent transcription repressor, we investigated the possibility that BCL-6 may repress transcription of certain member(s) of the NF-κB family. We report in the present work that the *NF-κB1* gene is a direct transcriptional target of BCL-6 in mature B cell lines. This regulatory relationship is also observed in several BCL-6 high tissue/cell types, including skeletal muscle, thymus, and macrophage cell lines from the *BCL-6*$^{-/-}$ mice. In macrophage cell lines, BCL-6 inactivation caused an increase in p105/p50 expression and enhanced nuclear κB-binding activity of p50 homodimer and p50/p65 heterodimer, which in turn results in a hyperproliferation phenotype sensitive to NF-κB inhibitors. Thus, our results indicate that BCL-6 can negatively regulate *NF-κB1* expression in vivo and modulate NF-κB-dependent cellular function in selected cell types.

Materials and Methods

Generation of bone marrow-derived macrophages and retroviral infection

Day 5 bone marrow macrophages were prepared from wild-type and *BCL-6*$^{-/-}$ mice, according to procedure described previously (34), and cultured in CSF-1 medium (α-MEM with 15% FBS and 120 ng/ml human rCSF-1 (gift of Chiron)). Macrophage cell lines dependent on CSF-1 for proliferation and survival were established from primary bone marrow-derived macrophages by infection with a retrovirus expressing the SV40 large T Ag (35). For the retroviral expression of BCL-6, a BCL-6 cDNA fragment was inserted into the *Eco*RI site of pMSCV-IRES-GFP vector (36) to make pMSCV-BCL-6-IRES-GFP. This construct and the control virus were packaged in the Phoenix-Eco packaging cell line via calcium phosphate-mediated transfection. Viral supernatants were collected after 48 h, filtered through a 0.45-μm filter, and used to infect immortalized macrophages. Briefly, SV40 T Ag-immortalized macrophages were incubated with fresh viral supernatants in the presence of 120 ng/ml CSF-1 and 4 μg/ml polybrene, centrifuged for 2 h at 1200 rpm at 25°C, and incubated at 37°C overnight. Following 2 days of culture in the CSF-1 medium, GFP-positive cells were sorted by FACSVantage SE (BD Biosciences) and used for MTT assay.

MTT assay

Cell proliferation was measured in triplicate by the MTT assay, as described previously (37). Briefly, macrophages were seeded in a 96-well plate (3×10^3 cells/well) in CSF-1 medium. Twenty-four hours after, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma-Aldrich), or peptide inhibitor SN-50 and its mutant control SN50M (Calbiochem) were added to the culture at the concentrations indicated in figure legends. For consecutive days following treatment, the MTT dye (0.5 mg/ml final concentration) was added to the cells and incubated at 37°C for 4 h before the cells were lysed in buffer containing 10% SDS and 0.01 N HCl. Absorbance at 570 nm (A_{570} nm) was measured in a microtiter well reader.

Cell culture and stable transfection

The 293 cells were maintained in DMEM with 10% FBS and transfected with Superfect (Qiagen), according to the manufacturer's instructions. All human lymphoma cell lines including Mutu III, Ramos, and Ly-1 were cultured in IMDM supplemented with 10% FBS. For stable transfections, cells were electroporated with the heavy metal-inducible BCL-6 expression vector, pMEP4-HA-BCL-6, or the empty vector control and selected in hygromycin B (Invitrogen Life Technologies) containing medium. To induce BCL-6 expression from Mutu III, IMDM supplemented with 150 nM $CdCl_2$ + 20 nM PF1070A (38) was used.

RT-PCR and Northern and Western blots

Total RNA samples were prepared from tissues and cultured cells with the TRIzol reagent (Invitrogen Life Technologies). A total of 10 μg of RNA per sample was separated on 0.9% formaldehyde-agarose gel, transferred, and hybridized using standard methods. Probes (full-length human *NF-κB1*, *NF-κB2* cDNA, and a rat GAPDH probe) were labeled with [α-^{32}P]dCTP using the Ready-to-go kit (Amersham Biosciences). The filters were exposed to phosphor imaging screens, and the signals were analyzed by the ImageQuant software. For semiquantitative RT-PCR, 3 μg of RNA was used for cDNA synthesis with the Superscript reverse transcriptase (Invitrogen Life Technologies). PCRs were performed with serially diluted cDNA input (1:5 and 1:25), and the products were separated on agarose gels. Sequences of the primers used in the PCRs are available upon request. Whole cell lysates used for Western blots were prepared by lysing cells in the radioimmunoprecipitation assay buffer supplemented with a protease inhibitor mixture (Roche). Equivalent amounts of protein were separated on 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and processed according to standard methods. The results were visualized by the ECL system (Amersham Biosciences). Images of the bands were scanned and analyzed by the ImageQuant software. Abs against p50 (H-119 and E-10), p52 (K-27), p65 (A), C-Rel (B-6), IκBα (C-21), and BCL-6 (N-3) were purchased from Santa Cruz Biotechnology. Ab to guanine nucleotide dissociation inhibitor (GDI) was used as an internal control for protein loading.

EMSAs

Methods for preparation of nuclear extracts, EMSA procedures for BCL-6, and NF-κB have been described previously (39, 40). Briefly, 10 μg of nuclear extracts was incubated for 15 min at room temperature with a ^{32}P-labeled probe in binding buffer (10 mM Tris-HCl (pH 7.7), 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol) plus 2 μg of poly(d(I-C)) (Roche). Complexes were separated in 5% polyacrylamide gels in TGE buffer (25 mM Tris-HCl (pH 7.7), 190 mM glycine, 1 mM EDTA), dried, and autoradiographed. For supershift analysis, nuclear extracts were preincubated for 15 min at room temperature with 1 μl of the corresponding Abs before addition of the radiolabeled probe. We used an NF-κB probe corresponding to an NF-κB site in the *H-2K*^{b} gene (5′-CAGGGCTGGG GATTCCCATCTCCCACAGTTTCACTTC-3′). The sequence of the canonical BCL-6 probe is 5′-GAAAATTCCTAGAAAGCATA-3′. Core sequences of the BCL-6-like sites in *NF-κB1* are given in Fig. 2. Full sequences of the probes for the *NF-κB1* and *NF-κB2* genes are available upon request. The quality of the nuclear extracts was monitored in parallel gel shift experiments using an Sp1 probe (5′-ATTCGATCGGGG CGGGGCGAGC-3′). The gels were dried and exposed to phospho-imaging screens, and the signals corresponding to specific DNA-protein complexes were analyzed by the ImageQuant software.

Reporter constructs and reporter assays

A 5.1-kb DNA fragment flanking the human *NF-κB1* exon 1 was amplified by PCR with Pfx DNA polymerase (Invitrogen Life Technologies) using BAC clones RPCI-11.347O8 (BACPAC Resources, ⟨http://bacpac.chori.org⟩) as template. Sequences of the two PCR primers are: 5′-CGCGA GGTACCTGTGAACATGAGTTTA-3′ (forward, with *Kpn*I site) and 5′-CTGCTCGAGCTTACTTCTCCCAAGTTCAAACC-3′ (reverse, with *Xho*I site). The PCR product was digested with *Kpn*I and *Xho*I and cloned into similarly digested pGL3-basic vector (Promega). Two-step PCR mutagenesis was performed to obtain the site-directed mutant Mut A. To make Mut B, a 138-bp sequence flanking the −1090 BCL-6 site was removed from the wild-type reporter using *Bst*X I and *Sal*I digestions. For reporter assays in 293 cells, 0.15 μg of the reporter, 0.05 μg of a CMV-β-gal plasmid, plus various amounts of pMT2T-BCL-6 were transfected into cells in each well of 24-well dishes by the SuperFect method (Qiagen).

When indicated in Fig. 3, corepressor expression plasmid encoding either silencing mediator of retinoid and thyroid receptor (SMRT) or BCL-6 corepressor isoform c (BCoRc) was used at 0.1 pmol per plate. All transfections were performed in duplicate and harvested 48 h later. Luciferase activities were measured with the Luciferase Assay System (Promega) and normalized by the control readings from the β-gal assay.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the ChIP assay kit (Upstate Biotechnology, Waltham, MA), following the manufacturer's instructions with the following modifications: 10×10^6 cells were used for each reaction; chromatin was sheared to an average length of 600 bp; and 2 μg of the anti-BCL-6 N3 Ab or normal rabbit IgG (both from Santa Cruz Biotechnology) was used. PCR products obtained after 29 cycles of amplification were resolved on 1.5% agarose gels. Sequences for the BCL-6 exon 1 primers were published previously (2). Sequences for the other primers are available upon request.

Results

Reciprocal expression pattern between BCL-6 and both NF-κB1 and NF-κ2 in lymphoma B cell lines

As the initial step to test our hypothesis that BCL-6 may play a role in *NF-κB* gene expression, we examined expression of *NF-κB1* and *NF-κ2* genes in three pairs of Burkitt's lymphoma cell lines (EBV latency type I and III). The two cell lines in each pair were derived from the same patient, but differ in their EBV activation status. Because the EBV oncoprotein eatent membrane protein 1 (LMP1), which can down-regulate BCL-6 (41), is only expressed in type III, but not type I Burkitt's lymphoma cells, both BCL-6 protein and mRNA are highly expressed in type I, but are very low in type III cell lines (Fig. 1, and data not shown). Northern blot results shown in Fig. 1 demonstrate that in BCL-6-positive type I cell lines, mRNA levels of both *NF-κB1* and *NF-κB2* are relatively low. Conversely, in type III cell lines in which *BCL-6* is turned off, levels of *NF-κB1* and *NF-κB2* are much higher. There is also more NF-κB1 p50 protein in the three type III cell lines compared with that in the corresponding type I cells (data not shown). These results are consistent with the idea that BCL-6 may negatively regulate transcription of the *NF-κB1* and *NF-κB2* genes.

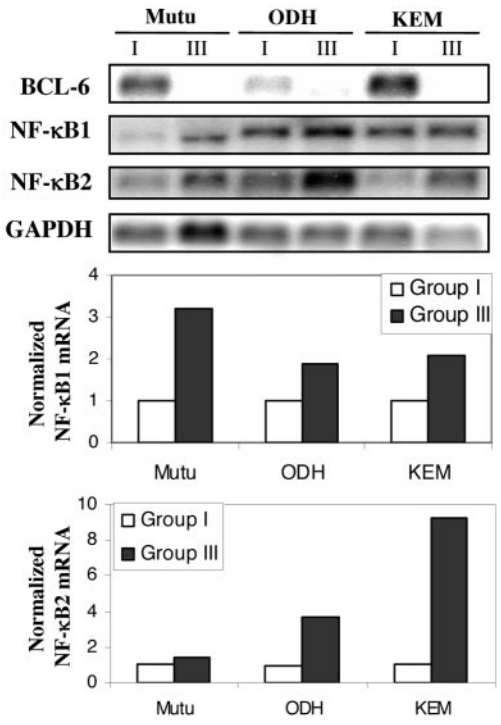

FIGURE 1. Expression pattern of *BCL-6* and *NF-κB1/NF-κB2* genes in paired type I and type III Burkitt's lymphoma cell lines. Northern blot analysis of *NF-κB1*, *NF-κB2*, and *BCL-6* transcripts in three paired (type I and III) Burkitt's lymphoma cell lines (Mutu, ODH, KEM). All signals were quantitated and normalized to that of GAPDH used as a loading control. For purpose of comparison, values for the type I cell lines in each pair were set as 1.0 in the graphs below.

BCL-6 binds to three sites within the 5′ regulatory region of NF-κB1 in vitro and represses transcription of a NF-κB1 reporter construct

Because BCL-6 is a well-known transcription repressor, we began to investigate whether *NF-κB1* and/or *NF-κB2* may be direct BCL-6 target genes. Using the MatInspector program (42), we identified three potential BCL-6 binding sites within a 1.1-kb distance 5′ to the human *NF-κB1* gene; two more sites were found within the 23.5-kb first intron (Fig. 2). To evaluate the functionality of these sites, EMSA analyses were performed using synthetic oligonucleotides corresponding to all five of these sites and nuclear extract of the BCL-6-positive Ly-1 cells. Fig. 2 shows that the sites located at −1090 bp, +2.7 kb, and +20 kb formed specific BCL-6-DNA complexes that can be competed with an excess amount of the cold consensus BCL-6 oligo probes and supershifted by an anti-BCL-6 Ab. Furthermore, these three BCL-6 binding site probes could also compete with the labeled consensus FB20 probe for binding to BCL-6, although they did so with a lower affinity when compared with the cold FB20 probe. Oligos representing the other two sites did not form specific BCL-6 complex in this assay. For the *NF-κB2* gene, only a single BCL-6-like site was predicted by the MatInspector program in the genomic sequences surrounding its exon 1. This motif, located 1.3 kb downstream of the +1 site, only formed a very weak BCL-6 complex in EMSA experiments (data not shown).

BCL-6 represses transcription of a NF-κB1 reporter construct

To determine whether the EMSA-positive sequence motifs can mediate transcription repression by BCL-6, a luciferase reporter construct containing two of these three sites and the *NF-κB1* exon 1 sequence (p105-5.1 wild type) was made and tested in reporter assays in 293 cells. Fig. 3*B* shows that BCL-6 can repress transcription from this reporter in a dose-dependent manner. When 0.3 μg of BCL-6 plasmid was used, as much as 5-fold repression can be achieved. To evaluate the relative importance of the two BCL-6 sites carried by the reporter, three mutant reporters were made that inactivate either one or both of these two sites (Fig. 3*A*). When these mutants were compared with the wild-type construct in reporter assays, it became clear that repression by BCL-6 was nearly completely lost when the −1090-bp site was removed (Mut B); in comparison, mutation of the +2.7-kb site (Mut A) had no appreciable effect, and the compound mutant (Mut C) did not confer further resistance to BCL-6 beyond Mut B (Fig. 3*B*). Although we have not studied the importance of the +20-kb site due to its remote location from exon 1, our data argue against any significant cooperativity between the −1090-bp site and the +2.7-kb site and implicate the −1090-bp site as the most critical sequence element for BCL-6 repression. Interestingly, we also found that although BCL-6 is known to be a potent transcription repressor, on its own, it has a fairly mild effect (2- to 2.5-fold repression) on the NF-κB reporter in 293 cells. Nonetheless, a much stronger repression was reproducibly observed when a recently identified BCL-6 corepressor, BCoR, was also used in the assay (Fig. 3*C*). BCoR appears to be unique in this property as another corepressor, SMRT, did not show any appreciable effect (Fig. 3*C*). Although response of the mutant panel is shown here with BCoR cotransfection, similar results were obtained when BCL-6 was used alone, except that the

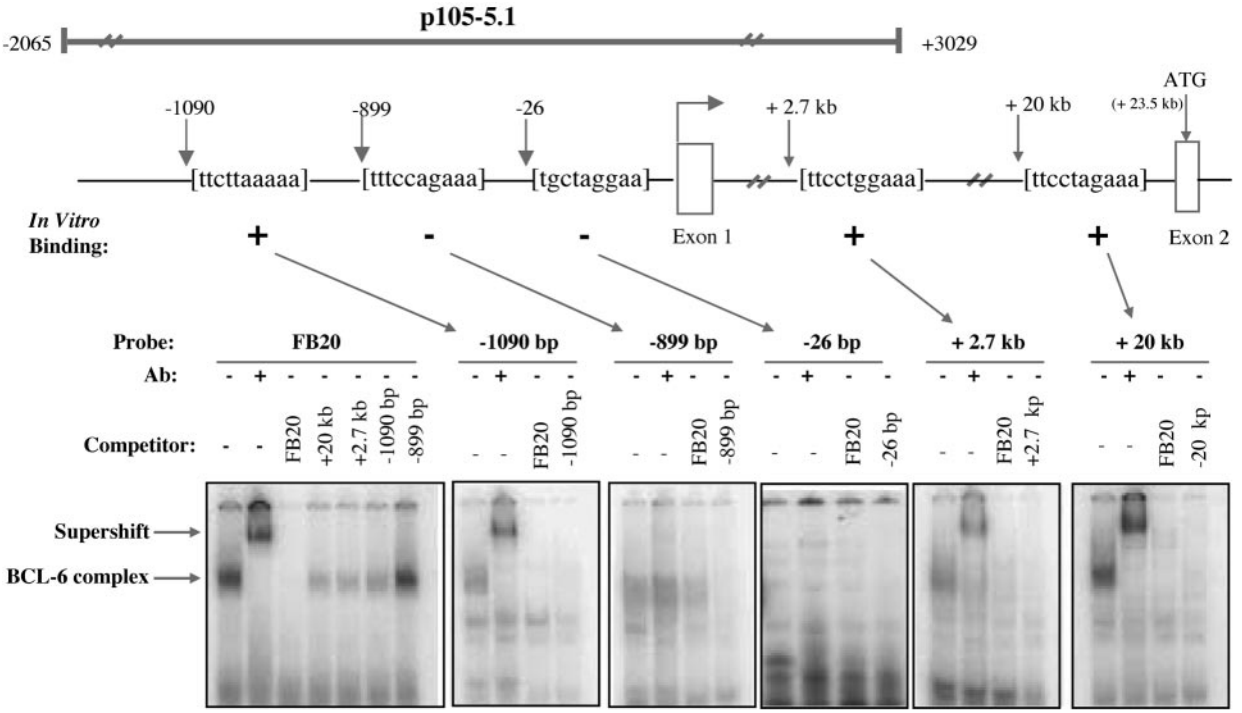

FIGURE 2. BCL-6 binds to multiple sites in the *NF-κB1* gene in EMSA. Sequences surrounding the transcriptional start site (+1) and before the starting codon (ATG) of the human *NF-κB1* gene contain five potential BCL-6 binding sites located at −1090 bp, −899 bp, −26 bp, +2.7 kb, and +20 kb. The 30-bp oligonucleotide probes corresponding to these sites were ^{32}P labeled and used with nuclear extracts from the BCL-6-positive Ly-1 cell line in EMSA. The FB20 probe containing a canonical BCL-6 binding site was used as the positive control. Specificity of the complexes was verified by supershift assay with the anti-BCL-6 Ab as well as competition with 50-fold excess of cold probes, as indicated in the figure.

magnitude of BCL-6 repression was lower (Fig. 3*C*, and data not shown). We also made a reporter construct driven by a 3.2-kb genomic fragment from the *NF-κB2* locus. As shown in Fig. 3*B*, this reporter (p100-3.2) is completely resistant to BCL-6 across a broad range of BCL-6 dosage that we have tested. In summary, results from our reporter assays strongly suggest that BCL-6 can directly repress transcription of the *NF-κB1*, but not *NF-κB2* gene.

BCL-6 down-regulates expression of NF-κB1 in vivo

In vivo, transcription regulation takes place in a chromatin context that may or may not be fully recapitulated by reporter assays. Thus, our next step was to determine whether the endogenous *NF-κB1* gene can also be repressed by BCL-6. To approach this issue in a dynamic setting, we stably transfected Mutu III cells with an expression vector that can be induced by cadmium to express exogenous BCL-6. As shown in Fig. 4*A*, expression of the exogenous BCL-6 protein was first detectable 4 h after induction, peaked at 8 h, and subsided by 24 h. As expected, levels of both NF-κB1 p105/p50 and NF-κB2 p52 started to drop after the appearance of BCL-6 and began to rebound as BCL-6 started to decrease. This reciprocal behavior of BCL-6 and NF-κB was not observed in control-transfected cells during the early phase of induction, indicating that the rapid reduction in NF-κB levels was due to BCL-6 expression, rather than cadmium treatment per se. Similar response from *NF-κB1* and *NF-κB2* mRNAs was observed based on Northern blot analyses (data not shown). We also established a BCL-6-inducible line in Ramos, an EBV-negative, Burkitt's lymphoma cell line, and obtained similar results (data not shown). Because our gel shift and reporter assay results suggested that *NF-κB2* is unlikely to be a direct target gene of BCL-6, the *NF-κB2* down-regulation observed in this study and reported elsewhere in GC B cells (32) is most likely a secondary effect, because *NF-κB2* is known to be an NF-κB target gene (43). During the entire BCL-6 induction process, the expression of another NF-κB family member, p65, remained unchanged, further demonstrating the specificity of regulation by BCL-6 (Fig. 4*A*).

To demonstrate that *NF-κB1* down-regulation was accompanied by direct binding of BCL-6 protein to the sequence motifs studied in EMSA and reporter assays, we performed ChIP experiments on the Mutu III cells 16 h after cadmium induction. The promoter region of the *BCL-6* gene itself is involved in negative autoregulation (2), and was therefore used as the positive control. The β-actin gene, not regulated by BCL-6, is used as the negative control. Data in Fig. 4*B* indicate that BCL-6 is associated with all three EMSA-positive sites in Mutu III/BCL-6 cells because the signals recovered by the BCL-6 Ab were much stronger than that from control IgG ChIP. In particular, there was no background for the positive control BCL-6 locus and the NF-κB1 +2.7-kb site, and no signals were recovered from the β-actin locus from either Ab. Thus, although the −1090 bp site seems to be the most important in reporter assays, BCL-6 can bind to all three BCL-6 binding sites in the endogenous *NF-κB1* gene in Mutu III cells.

Relationship between BCL-6 and NF-κB1 expression in non-B cell types

Genetically engineered mouse models provide valuable experimental systems to study gene regulation in a biologically relevant context. If BCL-6 is a functionally relevant repressor of NF-κB1 expression, then elevated levels of p105/p50 are expected in *BCL-6*$^{-/-}$ mice. Although Burkitt's and DLBCLs are believed to originate from GC B cells, complete lack of GC structures in *BCL-6*$^{-/-}$ mice prevented us from analyzing gene expression relationship between BCL-6 and NF-κB in GC B cells. We therefore studied expression of NF-κB1 as well as other members of the

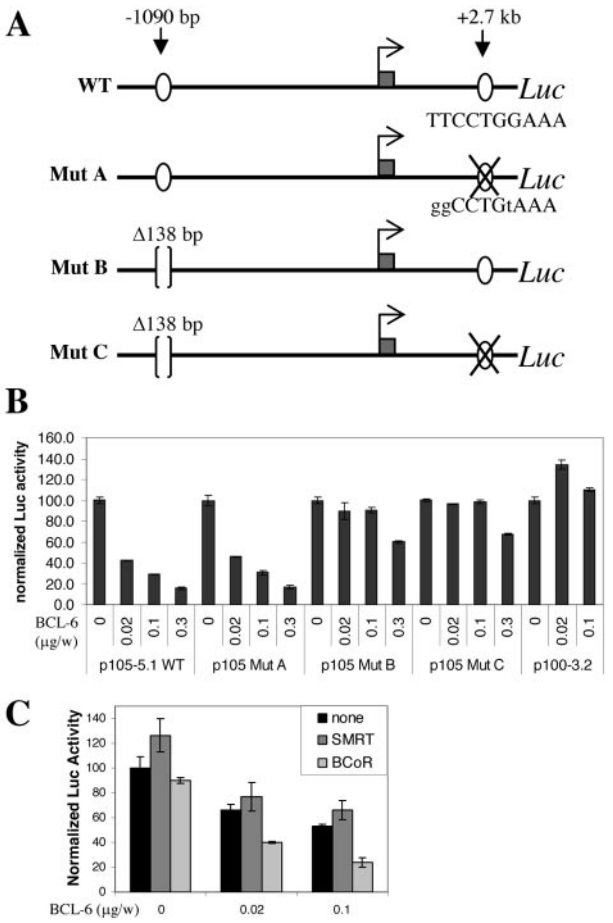

FIGURE 3. BCL-6 represses transcription from the *NF-κB1* promoter in reporter assays. *A*, Schematic representation of the wild-type and mutant *NF-κB1* reporters used in the experiments. Sequences at the +2.7-kb site before (WT) and after (Mut A) site-directed mutagenesis were shown. The −1090-bp site was inactivated by a microdeletion (Mut B). Mutations in Mut A and B were combined to generate Mut C. *B*, Reporter assays in 293 cells showing response of various NF-κB1 reporters and a NF-κB2 reporter (p100-3.2) to BCL-6. Indicated amounts of BCL-6 expression vector were used with 0.1 pmol of BCoRc plasmid. Luciferase activities were normalized to that of the basal level (defined as 100) for each reporter. *C*, Response of the wild-type NF-κB1 reporter to BCL-6 was tested in the presence or absence of corepressors SMRT or BCoR. All reporter activities were normalized to that of the basal level (set as 100) in the absence of either BCL-6 or corepressor.

NF-κB family in three non-B cell types where there is a readily detectable amount of BCL-6 protein. These include skeletal muscle, thymus, and SV40 large T immortalized macrophage cell lines. As expected, BCL-6 inactivation resulted in notable increases in both p105 and p50 in these three cell types (the p105 precursor is not detected in muscle) (Fig. 5). IκBα, a very sensitive and well-known target gene of NF-κB signaling, was also up-regulated to various degrees, most notably in thymus and muscle. This implies that BCL-6 ablation not only elevated levels of the NF-κB1 proteins, but the activity of the NF-κB pathway was also increased. Interestingly, expression of NF-κB2 p100/p52 was not significantly altered, but p65 was up-regulated. Collectively, these data indicate that BCL-6 can negatively regulate NF-κB1 expression both in lymphoma B cells and in non-B cells.

BCL-6$^{-/-}$ macrophage cell lines hyperproliferate

Up-regulation of IκBα in *BCL-6$^{-/-}$* mice implies that activity of the NF-κB pathway may also be altered in the absence of BCL-6. Thus, we next investigated the functional consequences of *NF-κB1* suppression by BCL-6. Although our results have shown that BCL-6 can suppress expression of *NF-κB1* in lymphoma cells such as Mutu III, cadmium-induced BCL-6 expression in these cell lines does not last for >24 h (Fig. 4). Thus, we turned to macrophage cell lines as our model system. SV40 large T immortalized macrophages resemble primary macrophages in their morphology and CSF-1-regulated signal transduction (our unpublished data). Due to the well-established role of NF-κB in cell cycle progression, we examined growth behavior of these cell lines. All of seven independently immortalized *BCL-6$^{-/-}$* cell lines showed a hyperproliferative phenotype when compared with wild-type controls. An example is shown in Fig. 6*A*. During 2 days of culture in CSF-1 medium, the number of *BCL-6$^{-/-}$* macrophages more than doubled, whereas the number of wild-type macrophages increased by only 36%. To directly demonstrate the causal role of BCL-6 in this hyperproliferation phenotype, we infected the *BCL-6$^{-/-}$* macrophages with either control GFP or BCL-6-IRES-GFP-expressing retroviruses, and monitored the growth behavior of sorted, GFP-positive cells. During the next 3 wk, the percentage of GFP-positive cells decreased persistently in cells reconstituted with BCL-6, but not GFP, suggesting that BCL-6 expression caused growth retardation, and thus cells with persistent BCL-6 expression had a growth disadvantage (Fig. 6*B*). As we did not detect significant apoptosis in any of the infected cell populations (data not shown), we believe that the decrease in percentage of GFP$^+$ cells in the BCL-6-reconstituted culture was due to a reduced proliferation rate rather than an increase in apoptosis. This interpretation was validated by MTT assays that directly measured proliferation of infected cells. As shown in Fig. 6*C*, proliferation of the *BCL-6$^{-/-}$* macrophages was reduced by BCL-6 reconstitution, but not by expressing GFP alone. In this experiment, although GFP$^+$BCL-6$^+$ cells were plated, significant numbers of GFP$^-$ cells with no or low BCL-6 expression were expected on day 4 (Fig. 6*B*), suggesting that the observed reduction in proliferation rate was underestimated. Supporting the role of BCL-6 as a negative regulator of NF-κB1, BCL-6 reconstitution decreased both NF-κB1 p105/p50 and NF-κB2 p100/p52, while IκBα was also affected (Fig. 6*D*). These results demonstrate that in macrophage cell lines, BCL-6 negatively regulates cell proliferation, and that BCL-6-dependent proliferation changes also correlate with alterations in NF-κB1 protein expression.

Elevated NF-κB activity is responsible for hyperproliferation of BCL-6$^{-/-}$ macrophage cell lines

Because the NF-κB transcription factors can exist both in the cytoplasm and the nucleus, yet only the nuclear forms are capable of binding to DNA and regulating gene expression, we investigated the effect of BCL-6 inactivation on nuclear κB-binding activities in *BCL-6$^{-/-}$* macrophage cell line by EMSA (Fig. 7*A*). We detected two major κB-binding complexes that are consistent with many previous reports by other investigators. Specifically, the fast migrating complex I was completely inhibited by a p50-specific Ab, and thus it is composed of p50 homodimers. The slower migrating complex II contained two bands. Both the p65 and p52 Abs weakened the bottom band, while the p50 Ab reduced the top band and significantly inhibited the bottom band of complex II. Thus, complex II represents a mixture of heterodimers between p50 or p52 and the Rel family proteins, including p65. We did not observe any notable effect of C-Rel Ab on the shift pattern most likely because mouse macrophages express very low levels of C-Rel (data not shown). It is evident from the *left panel* in Fig. 7*A* that the *BCL-6$^{-/-}$* macrophages had much more p50 homodimer κB-binding activity. A moderate increase in the abundance of complex II was

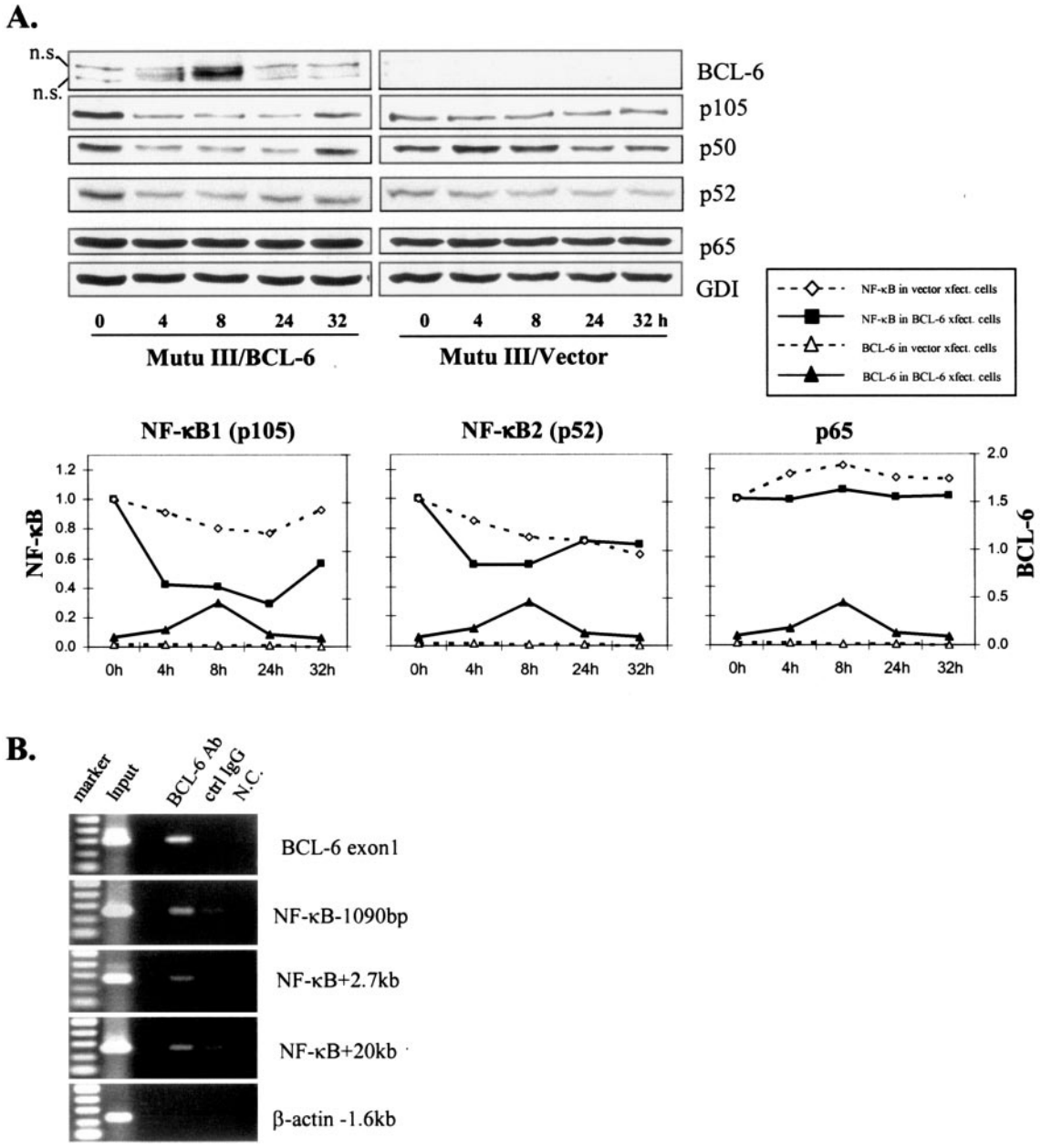

FIGURE 4. Exogenous BCL-6 protein down-regulates endogenous NF-κB1 and NF-κB2 in lymphoma cell line Mutu III. *A*, The inducible pMEP4-HA-BCL-6 vector was stably transfected into the Mutu III cell line. Cells were sampled before and at the indicated time points after induction and analyzed by Western blot for expression of NF-κB1 (p105/p50), NF-κB2 (p52), as well as p65. The NF-κB2 precursor p100 is not detectable in these cells due to rapid processing. All signals were quantitated and normalized to GDI, and the normalized data were used to generate the graphs below the protein blots. In the graphs, NF-κB and BCL-6 responses were plotted using the *left* and the *right* y-*axes*, respectively. Levels of the NF-κB proteins before BCL-6 induction were set at 1.0. n.s., Nonspecific. *B*, BCL-6 protein binds to the 5′ regulatory region of *NF-κB1* in vivo. ChIP assay was performed with BCL-6-transfected Mutu III cells 16 h after cadmium induction using either a BCL-6 Ab or control IgG (ctrl IgG). Recovered genomic DNA as well as a fraction of the total chromatin input was used in PCR to amplify sequences from the loci of interest. Marker, 100-bp DNA ladder; N.C., negative control PCR with no template. The result shown is a representative of three independent experiments.

also detected. Furthermore, such elevated κB-binding activity (both complex I and II) was reduced in a dose-dependent manner when cells were treated with TPCK, a proteasome inhibitor that blocks IκB degradation. To determine the role of elevated κB-binding activity in the hyperproliferation phenotype, we turned to a p50-specific inhibitor, SN50, which is a cell-permeable peptide designed to block p50 nuclear translocation. Because our supershift assays demonstrate that most of the κB-binding complexes in the *BCL-6*$^{-/-}$ macrophage cell lines contain p50 (Fig. 7*A*), blocking p50 nuclear translocation is expected to significantly reduce NF-κB activity in these cells. Indeed, SN50 reduced the proliferation of *BCL-6*$^{-/-}$ cells in a dose-dependent manner, while the control peptide SN50M had only a minimal effect (Fig. 7*C*). Very minor effects were observed in SN50-treated wild-type cells, possibly reflecting the role of basal level NF-κB activity in CSF-1-dependent cell proliferation. Similar results were also obtained when these macrophages were treated with 0.1 and 1.0 μM TPCK (data not shown). Thus, elevated nuclear NF-κB activity is responsible for the accelerated proliferation of *BCL-6*$^{-/-}$ macrophage cell lines. Although we have not yet determined which NF-κB

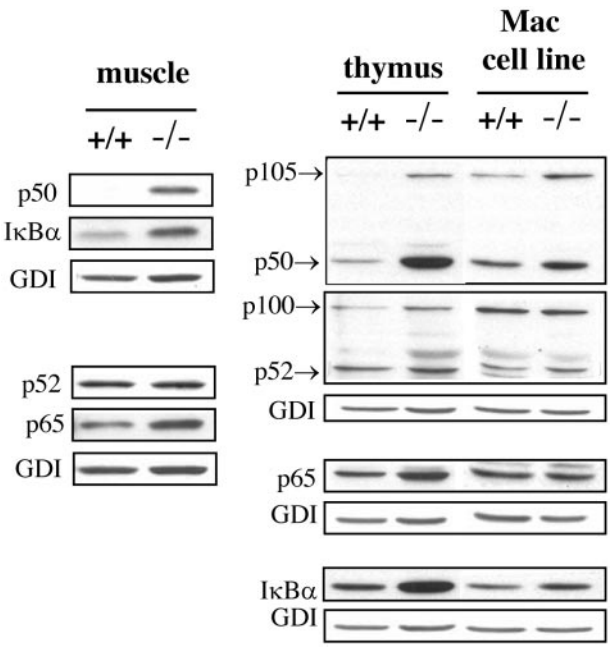

FIGURE 5. Expression of NF-κB family members in selected cell types from the *BCL-6*$^{-/-}$ mouse. Western blot analysis was performed on whole cell extracts prepared from the indicated tissue and cell types. p105 and p100 precursors were undetectable in skeletal muscle. GDI signals were shown as loading controls.

target gene(s) is directly involved, we have assessed the mRNA levels of a number of genes assumed to be regulated by NF-κB by semiquantitative RT-PCR (Fig. 7*C*). Our results demonstrate that although expression of c-Myc, cyclin D1, TNF-α, and CSF-1R was not significantly altered in the absence of BCL-6, *BCL-6*$^{-/-}$ macrophage cell line did express more IL-1β and had a dramatically elevated CSF-1 mRNA level. Similar increases in IL-1β and CSF-1 mRNAs were also observed in *BCL-6*$^{-/-}$ thymus (data not shown). Because CSF-1 is the primary growth factor for macrophage survival and proliferation, it is likely that CSF-1 overexpression is at least a contributing factor in the hyperproliferation phenotype of *BCL-6*$^{-/-}$ macrophage cell lines.

Discussion

Although several studies have shown that an inverse relationship exists between the high level of BCL-6 expression and NF-κB activity in normal GC B cells and GC-derived DLBCL (32, 33), the reason for this intriguing phenomenon was not understood. In this study, we report that the *NF-κB1* gene is a direct transcriptional target of BCL-6. We have defined the required *cis*-regulatory sequence in the *NF-κB1* gene and compared two corepressors in their ability to enhance *NF-κB1* repression by BCL-6. Our results show that overexpression of BCL-6 reduces the expression of p105/p50, while BCL-6 inactivation results in elevated p105/p50 proteins and increased nuclear κB-binding activity. Our study not only adds NF-κB1 to the growing list of BCL-6 target genes, it also demonstrates that BCL-6 can modulate NF-κB-dependent biological activity by lowering the level of NF-κB1 p50 subunit.

In this work, we have primarily focused on the tissue and cell types that express moderate to high levels of BCL-6 protein, including skeletal muscle, thymus, and macrophage cell lines in which BCL-6 inactivation leads to an increase in the expression of p105/p50. Nevertheless, we have observed that in cell types with low levels of BCL-6, such as unimmunized total spleen, the levels of p105/p50 proteins did not increase in response to BCL-6 inactivation. Although the reason for this cell-type specificity is not known, two potential explanations are suggested by our data. First, a relatively high level of BCL-6 may be needed to repress the *NF-κB1* gene efficiently. This notion is consistent with the fact that BCL-6 is expressed at moderate to high levels in all four tissue/cell types in which NF-κB1 is responsive to BCL-6 repression, but is undetectable by Western blot either in naive B220^{+} B cells or in

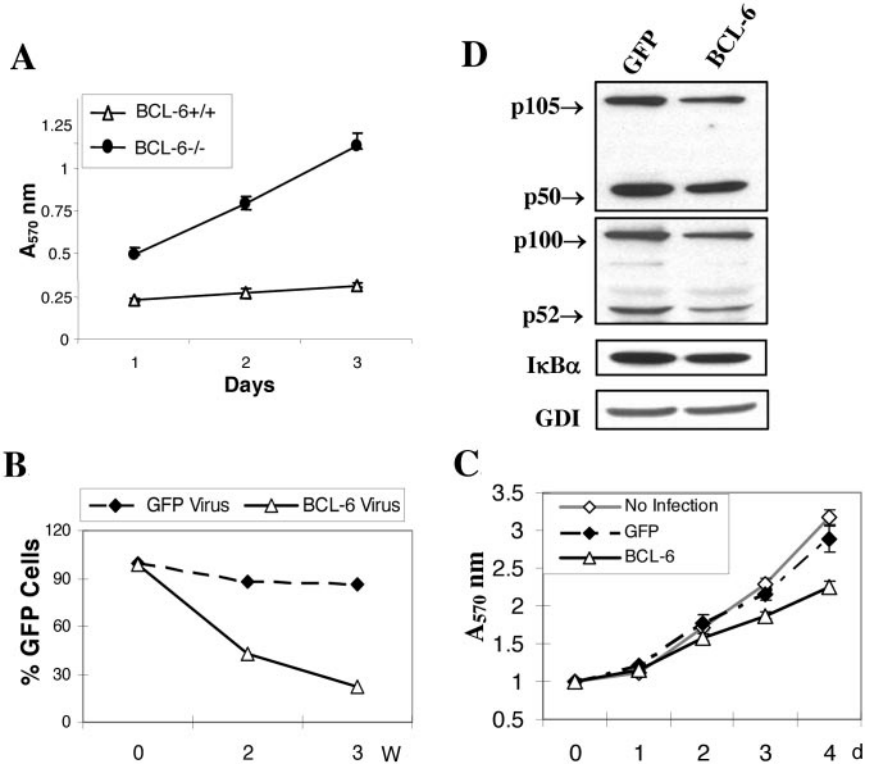

FIGURE 6. Immortalized *BCL-6*$^{-/-}$ macrophages hyperproliferate. *A*, Proliferation of cells of wild-type and *BCL-6*$^{-/-}$ macrophage cell lines. Equal numbers of SV40 T Ag-immortalized wild-type and *BCL-6*$^{-/-}$ macrophages were seeded into 96-well dishes. Numbers of viable cells were measured by the MTT assay at the indicated time points. *B* and *C*, BCL-6 reconstitution inhibited hyperproliferation. Immortalized *BCL-6*$^{-/-}$ macrophages were retrovirally infected with pMSCV-BCL-6-IRES-GFP or the empty control vector pMSCV-IRES-GFP. Forty-eight hours later, GFP-positive cells were FACS sorted and cultured in CSF-1 medium. Aliquots of cells were analyzed by flow cytometry for percentage of GFP-positive cells at the indicated time points (*B*). Proliferation of freshly sorted cells was compared with that of uninfected *BCL-6*$^{-/-}$ macrophages in a MTT assay (*C*). *D*, Whole cell lysates from BCL-6- and control GFP-virus-infected cells were analyzed by Western blot for expression of NF-κB proteins.

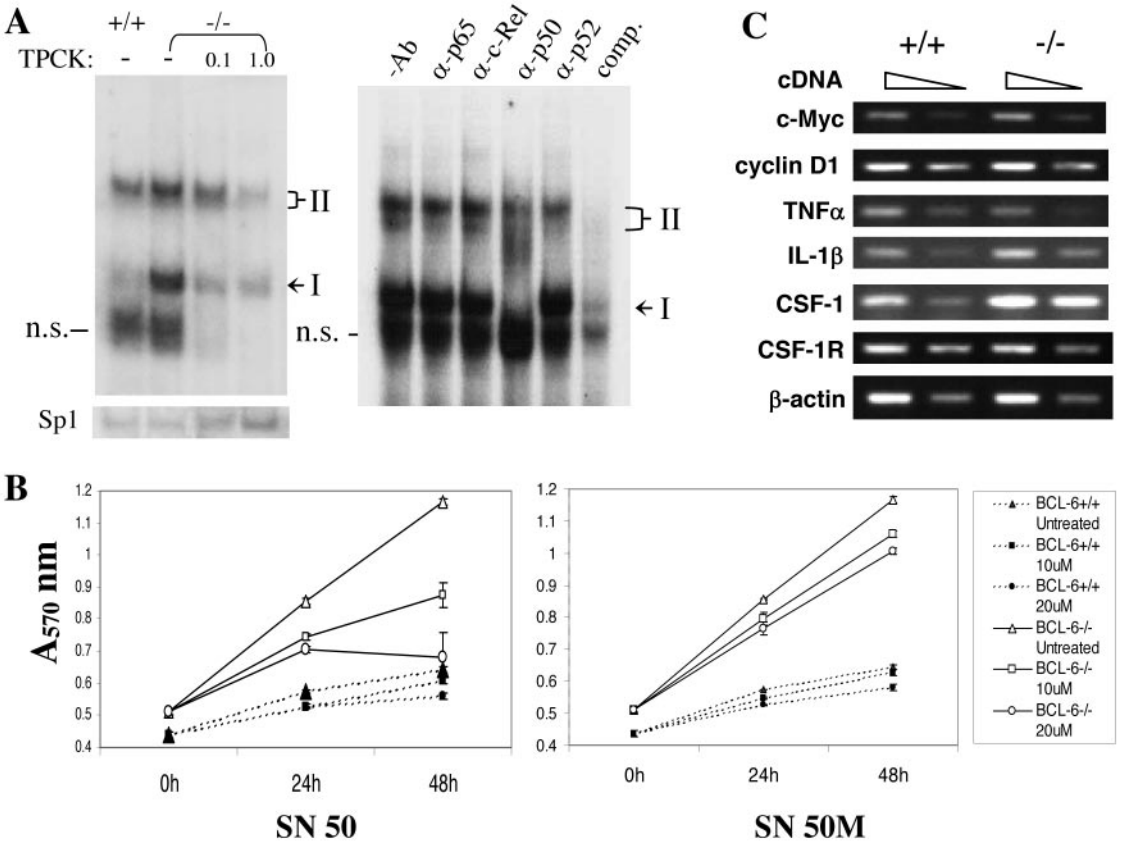

FIGURE 7. Hyperproliferation in the absence of BCL-6 was due to elevated NF-κB activity. *A*, Nuclear κB-binding activity was measured by EMSA with a labeled NF-κB probe. In the *left panel*, nuclear extracts from either wild-type or *BCL-6*$^{-/-}$ macrophages with or without TPCK treatment (0.1 and 1.0 μM) were used. Untreated *BCL-6*$^{-/-}$ extract was used in the *right panel*. The two major κB-binding complexes are designated complex I and II. Relative contribution of various NF-κB family members was assessed by use of indicated Abs. n.s., Nonspecific band. In the *left panel*, DNA-binding activity of Sp1 was also shown for purpose of loading control. *B*, Hyperproliferation of *BCL-6*$^{-/-}$ macrophages could be curbed by the NF-κB p50-specific peptide SN50 in a dose-dependent manner, while the control peptide SN50M has no specific effect. Equal numbers of macrophages were seeded into 96-well culture dishes. One day later, the cells were either left untreated or treated with SN50 or its control peptide, SN50M, at 10 and 20 μM concentrations. Numbers of viable cells were measured 24 and 48 h later by MTT assay. *C*, Semiquantitative PCRs were performed to assess expression of selected NF-κB target genes. cDNA samples prepared from either wild-type or *BCL-6*$^{-/-}$ macrophages were serially diluted 5-fold and used as PCR template. β-actin signals were shown for loading control.

total spleen (data not shown). A second possibility lies in the selective corepressor usage. Our reporter assay revealed that when BCL-6 itself was presented to the *NF-κB1* reporter, a specific, yet rather mild repression was observed. Nevertheless, this repression can be enhanced 2- to 3-fold when BCoR, but not SMRT was also cotransfected (Fig. 3*C*). BCoR is a recently identified corepressor that can selectively interact with the POZ domain of BCL-6, but not other BTB/POZ-containing transcription factors tested (44). Based on Affymetrix gene chip analysis, expression of BCoR is much more selective than either SMRT or nuclear corepressor of retinoid receptor (NCoR) (University of California at Santa Cruz Genome Browser ⟨http://genome.ucsc.edu/⟩), raising the possibility that BCoR availability in a given cell type may also contribute to the sensitivity of NF-κB1 to BCL-6. This is not the first time that cell type or situation-dependent repression by BCL-6 is observed. It was reported that MIP-1α is expressed in both B cells and macrophages, yet it is only repressed by BCL-6 in B cells, unstimulated and CSF-1-treated macrophages, but not in macrophages treated with LPS (10, 13). Thus, cell type-specific repression by BCL-6 is likely to be a common phenomenon, and thus is an interesting topic for future studies.

It is well established that the most important mechanisms governing NF-κB activity operate upstream of IKK. Many cellular events triggered by inflammatory cytokines, growth factors, and oncogenes converge on the IKK complex after multistep processes that involve protein ubiquitination and phosphorylation, and ultimately result in nuclear translocation of NF-κB complexes (reviewed in Ref. 18). In the nucleus, posttranslational modifications such as phosphorylation and acetylation have also been described to modulate transcriptional activity of p65 and p50 (reviewed in Ref. 45). Nevertheless, because many NF-κB target genes are activated by NF-κB heterodimers formed between the Rel family proteins and p50, significant fluctuation in p50 level, in theory, can alter the abundance and composition of NF-κB complexes in a cell. Consistent with the notion that proteolytic processing of the p105 precursor is largely constitutive, BCL-6 ablation in mouse causes similar increase in p105 and p50 levels (Fig. 5). At least in macrophage cell lines, this leads to notably enhanced nuclear κB-binding activity that is attributed to increase in both p50 homodimer and, to a lesser degree, p50/p65 heterodimers (Fig. 7*A*). At the moment, it is not clear whether the p50 homodimer or the p65/p50 heterodimer contributed more to the hyperproliferation phenotype of *BCL-6*$^{-/-}$ macrophage cell lines. On one hand, p50 homodimer is normally considered as a transcription repressor due to its lack of a transcription activation domain; on the other hand, there are published studies showing p50 homodimer contributing

to increased gene expression. For example, Ishikawa et al. (46) studied a mouse model in which the genetically engineered *NF-κB1* locus directly produces the mature p50 subunit instead of the p105 precursor. In these animals, a dramatic increase in the nuclear p50 homodimer was observed, and yet expression of a number of NF-κB target genes, including CSF-1, actually increased. Interestingly, CSF-1 mRNA is also overexpressed in *BCL-6*$^{-/-}$ thymus and *BCL-6*$^{-/-}$ macrophage cell lines (Fig. 7*C*, and data not shown). Thus, in the absence of BCL-6, elevated expression of *NF-κB1* can alter expression of at least some of the NF-κB target genes.

GC B cells and GC-derived lymphoma cell lines represent an opposite scenario in which BCL-6 protein is found at its highest level in vivo. Our results with lymphoma cell lines show that high level BCL-6 expression in the Mutu III and Ramos cell lines directly represses *NF-κB1* transcription, while expression of NF-κB2 is also decreased, mostly likely as a secondary effect. Consistent with the notion that the level of NF-κB proteins can play a role in NF-κB signaling, in GC B cells where the activity of NF-κB pathway is suppressed, several NF-κB proteins, including NF-κB2, c-Rel, RelB, and IκBα, are also down-regulated (32). Lack of GC in *BCL-6*$^{-/-}$ mice precludes direct analysis of NF-κB regulation by BCL-6 at this stage of B cell development. Nevertheless, our results from other cell types highly expressing BCL-6 combined with data from the Mutu III and Ramos cell lines strongly suggest that, through its ability to reduce the level of NF-κB proteins (both NF-κB1 and 2), BCL-6 is at least partially responsible for the diminished NF-κB activity in GC. Little is known regarding the importance of GC-specific NF-κB down-regulation, as previously published NF-κB knockout mice often have defects in pre-GC development of B cells (reviewed in Ref. 47). Still, behavior of the *IκBα*$^{-/-}$ B cells is worth noting. It was reported that B cell development proceeded normally in the absence of IκBα; proliferation of the *IκBα*$^{-/-}$ B cells was even accelerated and, yet, GC formation and T cell-dependent Ab response were impaired (32, 48). This observation implies that constitutive NF-κB activity is likely to be incompatible with normal GC development.

Finally, our work offers an explanation for the association of increased BCL-6 mRNA expression with a favorable prognosis of DLBCL. Based upon gene expression profiling, Staudt and colleagues (33) identified two distinct subtypes within the clinical diagnosis of DLBCL: one is the GC group with good survival after chemotherapy; the other is the activated B cell (ABC) group with poor survival. As expected, the GC subtype expresses high levels of BCL-6 with low NF-κB activity, while the ABC type has low BCL-6 expression and constitutively activated NF-κB. Their subsequent work identified three gene expression markers with the most significant prognostic value; among them was BCL-6 (5). As a single variable, the level of BCL-6 mRNA was also reported to strongly predict survival in DLBCL patients irrespective of any subclassification (4). In DLBCL, the low NF-κB activity in the GC (favorable prognosis) vs high NF-κB in the ABC (poor prognosis) type is in good agreement with the well-documented role of NF-κB in cell survival and drug-induced chemoresistance (reviewed in Ref. 49). The question remaining is why BCL-6 is tightly associated with sensitivity to chemotherapy. Our findings in this study suggest that in GC-type DLBCL, the low NF-κB activity is a direct result of high level BCL-6 expression that is frequently caused by either chromosomal translocations or activating mutations (1). In other words, while repeated chemotherapy may gradually select for NF-κB high subclones in ABC DLBCL, NF-κB activity may be genetically fixed to be low in GC DLBCL. There is evidence that ABC-type DLBCL cells depend upon constitutive NF-κB activity for survival (50). Thus, our work provides additional support for the proposal by Staudt and colleagues (50) to initiate clinical trials to evaluate the efficacy of NF-κB inhibitors as chemosensitizers in drug-resistant DLBCLs.

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