Bcl10 can promote survival of antigen-stimulated B lymphocytes

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To understand the nature of negative responses through the B-cell antigen receptor (BCR), we have screened an expression cDNA library for the ability to block BCR-induced growth arrest and apoptosis in the immature B-cell line, WEHI-231. We isolated multiple copies of full-length, unmutated Bcl10, a signaling adaptor molecule encoded by a gene found to translocate to the immunoglobulin heavy chain (IgH) locus in some mucosa-associated lymphoid tissue (MALT) lymphomas. A conditionally active form of B-cell lymphoma 10 (Bcl10) protected WEHI-231 cells from BCR-induced apoptosis upon activation. Induction of Bcl10 activity caused rapid activation of nuclear factor–κB (NF-κB) and c-Jun N-terminal kinase (JNK), but not activation of extracellular signal–regulated kinase (ERK) or p38 mitogen-activated protein (MAP) kinases. These results support genetic and biochemical experiments that have implicated Bcl10 and its binding partners Carma1 and MALT1 in mediating the ability of the BCR to activate NF-κB. The ability of Bcl10 expression to prevent BCR-induced growth arrest and apoptosis of WEHI-231 cells was dependent on NF-κB activation. Finally, overexpression of Bcl10 in primary B cells activated ex vivo promoted the survival of these cells after removal of activating stimuli. Taken together these results support the hypothesis that enhanced *BCL10* expression caused by translocation to the *IGH* locus can promote formation of MALT lymphomas. (Blood. 2005;106:2105-2112)

Introduction

Proper regulation of apoptosis is critical for the normal function of the immune system and for the prevention of bloodborne malignancies. B and T lymphocytes are normally subject to the induction of apoptotic cell death in the primary lymphoid organs, as exemplified by B-cell receptor/T-cell receptor (BCR/TCR)–induced apoptosis; and in the periphery, as exemplified by Fas- or cytokine deprivation–induced cell death.[1,2] Genetic changes that cause loss of apoptotic susceptibility are likely early events in the pathogenesis of many lymphoid cancers.

To understand the regulation of B-cell apoptosis, WEHI-231, a lymphoma-derived B-cell line with an immature B-cell phenotype, has been frequently studied because it exhibits growth arrest and essentially 100% apoptosis in response to BCR crosslinking.[3] A number of factors known to be involved in B-cell oncogenesis have been shown to relieve or prevent BCR-induced growth arrest and/or apoptosis in WEHI-231 cells. These include the cell-cycle regulator c-Myc[4] and the B-cell lymphoma-2 (Bcl-2) family member, Bcl-xL.[5,6] Another lymphoma-related gene, *BCL6*, which inhibits B-cell terminal differentiation, displays abnormality at its genomic locus in these cells.[7] BCR-induced growth arrest and apoptosis of these cells are prevented by immunologic stimuli that promote activation of normal B cells, including lipopolysaccharide stimulation via Toll-like receptor 4 (TLR4) and helper T-cell stimulation via cytokines and CD40.[8] To understand the molecular events controlling B-cell fate in this system, we have taken a function-based approach and screened a cDNA expression library for genes that can abrogate BCR-induced apoptosis of WEHI-231 cells. From this screen, we isolated multiple copies of full-length, unmutated Bcl10.

The human *BCL10* gene was initially discovered via its involvement in a chromosomal translocation associated with a fraction of extranodal marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) and was also found to be mutated in some lymphoid and nonlymphoid malignancies.[9-12] Paradoxically, overexpression of Bcl10 in cell lines causes apoptosis.[13-17] Thus, the mechanism underlying the role of Bcl10 in lymphomagenesis remains to be clarified. Surprisingly, *Bcl10*-deficient mice were not found to have a defect in apoptosis, but they were found to have decreased numbers of all 3 types of mature B cells and to be defective in antigen receptor–induced activation of lymphocytes.[18,19] Subsequent studies have indicated that Bcl10 functions downstream of lymphocyte antigen receptors in conjunction with 2 other intracellular proteins, Carma1 and MALT1 to promote ubiquitination of the inhibitor of nuclear factor–κB [NF-κB] (IκB) kinase subunit NEMO,[20] leading to activation of the transcription factor NF-κB.[21,22] These results suggest that Bcl10 function may be enhanced by the chromosomal translocation seen in some MALT B lymphomas, for example, by increasing its expression.[12,23] Here, we describe our findings that ectopic expression of Bcl10 promotes the survival of BCR-stimulated lymphoma B cells and primary activated B cells. These observations suggest that *IGH/BCL10* translocations can promote lymphomagenesis by enhancing Bcl10 function, thereby contributing to the survival and/or growth of lymphoma B cells.

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Materials and methods

Cell culture, antibodies, and reagents

WEHI-231 cells were cultured as described.[24] Phenol red–free medium was used for the culture of cells expressing the fusion proteins of Bcl10 and the ligand binding domain of the estrogen receptor (Bcl10-ER and Bcl10 CARD-ER). Other reagents and their sources are as follows: goat anti–mouse immunoglobulin M (IgM; μ-chain-specific) was from Jackson Immunological Research Laboratories (West Grove, PA); antibodies specific for activated (dually phosphorylated) forms of c-Jun N-terminal kinases (JNKs), p38 mitogen-activated protein (MAP) kinase, and p44/42 extracellular signal–regulated kinase (Erk) MAP kinase were from New England Biolabs (Beverly, MA); anti–IκB-α, anti–IκB-β, and anti-JNK1 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti–β-tubulin (KMX-1) was from Chemicon International (Temecula, CA); anti-HA (influenza virus hemagglutinin; HA.11/16B12) was from Babco (Richmond, CA); BD (N-benzyloxycarbonyl-Asp-fluoromethylketone) was from Enzyme Systems Products (Livermore, CA); polybrene and 2-estradiol were from Sigma (St Louis, MO). The NF-κB–inhibiting peptide (DRQIKIWFQNRRMKWKKTALDWSWLQTE)[25] was synthesized by Genemed Synthesis (South San Francisco, CA). For a negative control peptide, we used a synthetic peptide with a sequence ARGAVSDEEMMELREAFAYGRKKRRQRRRG, which is derived from the protein L-plastin with an S to A change at the N-terminus. The control peptide was synthesized by Quality Control Biochemicals (Hopkington, MA) and generously provided by Eric Brown, University of California, San Francisco (UCSF).

Purification, stimulation, and retroviral infection of splenic B cells

Cells were prepared from the spleens of healthy Balb/c mice (6 weeks old)[24] and allowed to bind for 1 hour at 37°C to Petri dishes coated with anti-IgM. After extensive rinses, the bound cells (greater than 95% B-cell purity by anti-CD19 staining) were stimulated for 40 hours with anti-IgM (10 μg/mL), anti-CD40 (2 μg/mL; BD Bioscience, Palo Alto, CA), and IL-4 (interleukin 4; 10 ng/mL; R&D Systems, Minneapolis, MN). For retroviral infection, cells were rinsed off the dishes, pelleted, resuspended in retroviral supernatants containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4) and 4 μg/mL polybrene, and plated on a 6-well plate. The plate was centrifuged at 700*g* for 30 minutes and left in a 37°C incubator undisturbed for 4 hours. Subsequently, cells were washed and incubated in fresh medium with stimuli for another 2 days to allow retroviral integration and expression.

cDNA library, cDNAs, and plasmids

A retroviral cDNA library from HeLa cells was purchased from BD Clontech (San Jose, CA). The cDNA inserts were recovered from the genomic DNA of apoptosis-resistant clones via polymerase chain reaction (PCR) using vector-annealing primers provided by the manufacturer. To generate the secondary virus library, PCR products from the resistant cell clones were pooled, end-filled with T4 DNA polymerase, digested partially with *Not*1 or *Sfi*1, and ligated to a vector pLIB preparation with one end blunt and the other being *Sfi*1 or *Not*1 (pLIB was digested with *Sal*1, end-filled with the Klenow fragment of the *Escherichia coli* DNA polymerase 1, and then digested with *Not*1 or *Sfi*1). The transformed bacteria were pooled to generate the secondary library for repeat screening.

Tagged Bcl10 alleles had the HA sequence (AYPYDVPDYAL, from influenza virus hemagglutinin) added to the N-terminus of Bcl10 after the initiator methionine. Human Bcl10 CARD was generated via PCR and encoded amino acids 1 to 123 of Bcl10 plus the HA tag. The estrogen receptor cDNA (ligand-binding domain)[26] was obtained from Dr J. Michael Bishop (UCSF) and fused in frame via a *Bam*H1 linker to the C-terminus of human Bcl10 or its CARD domain to generate Bcl10-ER or Bcl10 CARD-ER fusion proteins. pMXpie (pMX-puro-IRES [internal ribosome entry site]–EGFP [enhanced green fluorescent protein]), is a derivative of pMX (courtesy of Dr L. Lanier, UCSF).[27] The Bcl10 sequences were subcloned into this vector along with a 5′ cytomegalovirus (CMV) promoter.

Functional screening of retroviral cDNA library in infected WEHI-231 cells

The plasmid cDNA library or vector pLIB (15 μg each) were transfected into 1.5×10^7 Bosc23 cells using the Effectene reagent (Qiagen, Valencia, CA). Viral supernatant was collected 24 hours after transfection and 3 times at 12-hour intervals. WEHI-231 cells (2×10^7) in a 6-well plate were infected 3 times with freshly collected viral supernatant (4 mL per well, with 6 μg/mL polybrene), by a 1-hour spin-infection (700*g*, 30°C), followed by a 10-hour incubation at 37°C. Twenty-four hours after infection, cells were resuspended in fresh medium containing 10 μg/mL anti-IgM and plated into 4 24-well plates. Fresh medium with anti-IgM was added 2 weeks later. Infection with the library but not with the vector alone resulted in growth of 12 clones within 4 weeks. Using either genomic DNA or cDNA as templates, multiple PCR bands were obtained from each of the 12 primary resistant clones, presumably as a result of multiple infections. To identify the cDNAs responsible for the protection, PCR products from the 12 clones were pooled and subcloned via *Not*1 or *Sfi*1 into the retroviral vector pLIB. The secondary virus library was used to infect WEHI-231 cells in a single infection, and the infected culture was selected similarly with anti-IgM, yielding 34 resistant clones. Four of the 34 clones gave rise to single PCR bands of 0.9 to 3 kilobase (kb) in length, all of which were shown by sequencing to contain the open reading frame (ORF) of Bcl10, with variation in the length of the 3′ untranslated region. PCR products from all the clones were subjected to Southern blotting with a probe made from a PCR fragment of the Bcl10 ORF, and all the clones were found to contain 1 or 2 Bcl10-hybridizing bands.

Cell stimulation and assay for viable cells

The viable cell assay used in this study measured the number of viable cells by propidium iodide (PI) exclusion in a stimulated culture and then compared it with the number of viable cells in the identically seeded control culture (typically seeded at 1×10^5 cells/mL) as a percentage.[24] Where indicated, replicate points refer to replicate cultures, analyzed in parallel. The percentage of decrease in viable cell number resulting from BCR stimulation varied somewhat from one experiment to another, possibly because of differences in prior growth history and culture conditions that are difficult to control. Nonetheless, the relative trend among different treatments was highly consistent among different experiments. The Annexin V–staining analysis was performed as described.[24]

Cell-cycle analysis

Bromodeoxyuridine (BrdU; Sigma) was added to cell cultures to a final concentration of 10 μM. Following incubation at 37°C for 1 hour, cells were analyzed for cell-cycle status as described.[24]

Assays for NF-κB and JNK activation

Activity of NF-κB was assessed by detecting changes in the levels of IκB-α and IκB-β by immunoblotting with anti–IκB-α and anti–IκB-β. To measure the DNA binding activity of NF-κB factors in nuclei, an electrophoretic mobility shift assay (EMSA) was performed using a kit (Gel Shift Assay System) from Promega (Madison, WI). To determine JNK activity in the cells, JNK1 was immunoprecipitated and subjected to an in vitro kinase assay as described.[28]

Results

Functional cloning of Bcl10 as an antiapoptotic factor

We hypothesized that the BCR-induced cell-cycle arrest and subsequent cell death in WEHI-231 cells[3] may be due to BCR-induced down-regulation of progrowth and survival factors that are

normally present in the cells.[4,29] Enforced expression of such factors under a constitutive promoter may lend resistance to BCR-induced apoptosis. To identify these factors, we introduced into the cells a cDNA expression library and screened for resistance to BCR-induced apoptosis. WEHI-231 cells were transduced with a commercial retroviral cDNA expression library under the transcriptional control of the long terminal repeat (LTR), a promoter unresponsive to BCR stimulation (data not shown). The cells were treated with anti-IgM, and transduced cDNA sequences from surviving clones were recovered by PCR using vector sequences. After 2 rounds of infection and anti-IgM selection, 34 surviving clones were obtained (Figure 1A). Four of these clones harbored single species of differentially sized cDNA inserts. Each of these cDNA inserts was found to contain the full open reading frame of human Bcl10 and was devoid of mutations. By hybridization to a Bcl10 probe, we determined that each of the remaining 30 clones from this library screening also contained transduced Bcl10 cDNAs (data not shown). As will be described in a subsequent report, other genes were obtained in subsequent screening using this procedure.

Bcl10 as an antiapoptotic factor for BCR-stimulated WEHI-231 cells

To confirm the prosurvival effects of Bcl10, we expressed Bcl10 ectopically in WEHI-231 cells in several ways and examined BCR-induced growth arrest and apoptosis. Two Bcl10 cDNAs recovered from the library screen were subcloned into a retroviral vector conferring puromycin resistance. WEHI-231 cells were infected with the Bcl10-expressing viruses or a vector-derived virus, selected for puromycin resistance for 5 days, and tested for the ability of the BCR to induce apoptosis. The number of viable cells in anti-IgM–treated cultures, as defined by exclusion of propidium iodide (PI), was determined by flow cytometry, and compared with that of the corresponding unstimulated culture to obtain a percentage. As shown in Figure 1B, many more cells infected with the Bcl10-expressing virus were alive after the 45-hour BCR stimulation compared with cells infected with the vector virus. This measurement of protection would detect changes in either cell-cycle arrest or apoptotic death or both. Experiments described in the next section of "Results" indicate that Bcl10-infected cells were protected from both responses to BCR stimulation. Similarly, a cDNA encoding the Bcl10 protein tagged at its N-terminus with an HA epitope was cloned into a retroviral vector capable of coexpressing Bcl10 and green fluorescent protein (GFP) via an internal ribosomal entry sequence (IRES). This retrovirus was used to infect WEHI-231 cells (Figure 1C) or CH31 cells, another lymphoma-derived cell line also susceptible to BCR-induced cell death (Figure 1D). Infected cells (GFP^+) were examined for their responses to BCR-induced apoptosis 1 day after infection. As shown in Figure 1C-D, Bcl10 expression clearly provided resistance to the negative effects of BCR stimulation in both WEHI-231 and CH31 cells.

We further examined whether Bcl10 could confer resistance to Fas-induced apoptosis, using a subline of WEHI-231 that is sensitive to Fas-induced apoptosis as well as BCR-induced apoptosis.[24] We found that Bcl10-expressing cells underwent apoptosis upon Fas stimulation with the anti-Fas antibody, Jo2, similarly to vector virus-infected cells (data not shown). Therefore, Bcl10 prevents BCR-induced growth arrest and apoptosis but does not appear to regulate Fas-induced apoptosis in these cells.

Growth-inhibitory effects of Bcl10 in WEHI-231 cells

Previous studies found that overexpression of Bcl10 in nonlymphoid cells such as HEK 293 cells resulted in apoptosis,[10,11,13,15-17,30] a result we were able to reproduce (data not shown). Interestingly, despite extensive efforts, we were unable to obtain WEHI-231 cells that stably expressed the introduced HA-tagged Bcl10. To test whether Bcl10 expression might have a negative effect on the growth of WEHI-231 cells in the absence of BCR stimulation, we infected these cells with retroviruses expressing GFP or GFP plus Bcl10 (Bcl10-IRES-GFP) or a

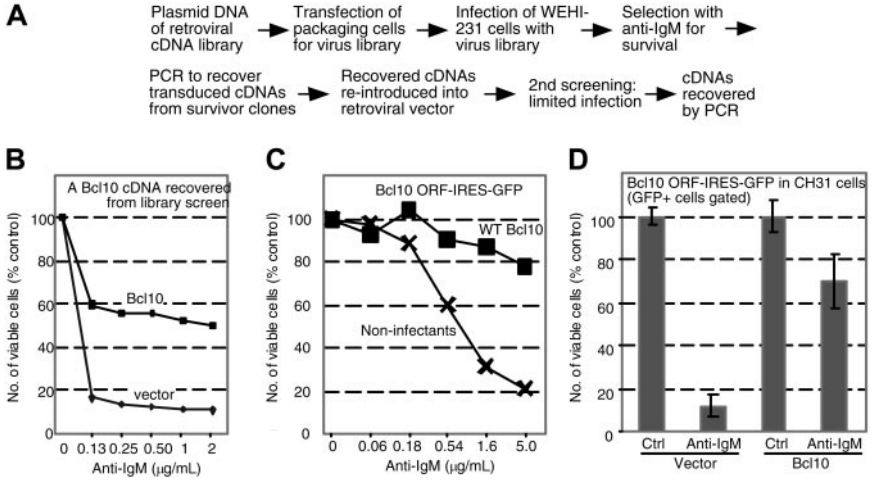

Figure 1. Functional cloning of Bcl10 as a factor preventing BCR-induced apoptosis. (A) Scheme for selection of cDNAs that protect WEHI-231 lymphoma B cells against anti-IgM–induced growth arrest and apoptotic death (also see "Functional cloning of Bcl10 as an antiapoptotic factor"). (B) A Bcl10 cDNA recovered from the screen was inserted into a puromycin-resistant retrovirus. Infected WEHI-231 cultures were selected for puromycin-resistance for 5 days and then treated with anti-IgM (45 hours) to test the prosurvival activity of the cDNA. The number of viable (PI-excluding) cells was determined by flow cytometry and compared with that of the unstimulated control culture as a percentage. Squares indicate Bcl10-expressing retrovirus; diamonds, vector only. Similar results were obtained with a second Bcl10 cDNA recovered from the screen (not shown). (C) A short-term survival assay was performed on WEHI-231 cells infected with a retrovirus encoding a bicistronic message of Bcl10 open reading frame (ORF; without untranslated regions)–IRES-GFP. Infected cells were incubated for 1 day to allow Bcl10 expression and then stimulated with varying doses of anti-IgM for 45 hours. The number of viable cells was determined as in panel B. ■ indicates GFP^+ cells; ×, GFP^- cells. The ability of the retroviral construct to express Bcl10 (HA-tagged) was verified by immunoblotting of transfected Bosc23-cell lysates (not shown). The noninfectants were GFP^- cells in the infected cultures. Infection with a vector-derived virus had no effect on BCR-induced apoptosis (data not shown). (D) Bcl10 also conferred survival advantage to CH31 cells. Cells were infected by Bcl10-IRES-GFP retrovirus and examined similarly to panel C, but with or without a saturating concentration of anti-IgM (10 μg/mL) (vector control, IRES-GFP). Values shown are averages of triplicate determinations ± SD ($P < .005$ for the comparison of anti-IgM–treated cells with Bcl10 versus anti-IgM–treated cells without Bcl10).

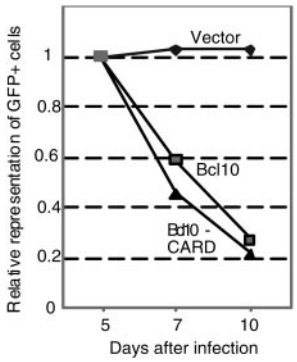

Figure 2. Growth-inhibitory effects of Bcl10 overexpression in WEHI-231 cells. WEHI-231 cells overexpressing Bcl10 rapidly lost representation in infected cultures consisting of infectants and noninfectants. Cells were infected with retroviruses coexpressing Bcl10 or its N-terminal CARD (Bcl10-CARD) and GFP via a bicistronic mRNA containing an IRES, under the transcriptional control of the CMV enhancer. The fraction of GFP^+ cells in each culture was determined in triplicate by flow cytometry over a period of 10 days. For comparison purposes, the GFP^+ fractions were all normalized to the fraction on day 5, when the fraction of GFP^+ cells was maximal or near-maximal in each culture. Actual percentage of GFP^+ cells at day 5 varied among different cultures from 3% to 25% as a result of nonidentical virus titers during infection. Diamonds indicate vector only; squares, full-length Bcl10; and triangles, Bcl10-CARD domain only.

truncated Bcl10 expressing just the N-terminal CARD region (Bcl10 CARD-IRES-GFP). The infected cultures, composed of GFP^+ (ie, infectants) and GFP^- (noninfectant) cells, were followed over a period of 10 days for changes in the relative proportion of GFP^+ cells. As shown in Figure 2, the fraction of GFP^+ cells in vector virus–infected cultures remained unchanged over this period of time, indicating that the viral infection and GFP expression did not provide any detectable advantage or disadvantage to cell growth. In contrast, the fraction of GFP^+ cells in cultures infected with Bcl10- or Bcl10 CARD-expressing viruses decreased to a small percentage over a several day period, suggesting that the constitutive overexpression of these proteins inhibited cell growth or induced apoptosis. Thus, whereas Bcl10 protected against BCR-induced apoptosis, it was also inhibitory for growth and/or survival in unstimulated WEHI-231 cells.

Conditionally active forms of Bcl10 and Bcl10 CARD

To further study the function of Bcl10, we wanted to establish a system for rapidly regulating the activity of Bcl10. For a number of proteins (including Myc and Raf), fusion to the ligand-binding domain of the human estrogen receptor results in a chimeric protein whose activity is regulated by estrogens such as β-estradiol (E2).[26] A retrovirus encoding an N-terminally HA-tagged Bcl10-ER protein and conferring puromycin resistance was used to infect WEHI-231 cells. Individual puromycin-resistant clones were obtained, and those expressing Bcl10-ER were identified by anti-HA immunoblotting. The levels of Bcl10-ER protein expression in these stable clones were generally low.

All the tested WEHI-231 clones expressing Bcl10-ER exhibited a marked E2-dependent enhancement in survival after BCR stimulation (Figure 3A; data not shown). When the Bcl10-ER–expressing cells were treated with different concentrations of E2, which presumably activated the fusion protein to varying degrees, intermediate doses of E2 (around 12 nM) were more protective than higher doses. It is possible that at the higher concentration of E2, there was a combination of a protective effect of Bcl10 superimposed with the growth-suppressive effects of Bcl10 seen in Figure 2. Indeed, Bcl10-ER activation by E2 treatment in WEHI-231 cells not stimulated via the BCR impeded cell growth moderately in optimally growing cultures (data not shown). E2 treatment of parental WEHI-231 cells or of puromycin-resistant clones not expressing Bcl10-ER, or of WEHI-231 cells expressing only the estrogen-binding domain of the estrogen receptor did not produce detectable effects in any of the conditions tested (Figure 3B; data not shown).

BCR-stimulated WEHI-231 cells arrest growth in about 24 hours and subsequently die by apoptosis in 48 hours.[31] Both of these processes reduce the number of viable cells in anti-IgM–treated cultures. To further understand the prosurvival property of Bcl10, Bcl10-ER–expressing cells were stimulated via the BCR in the presence or absence of E2 and then subjected to cell-cycle analysis by BrdU incorporation and PI staining. As shown in Figure 3B, 16-hour BCR stimulation alone substantially inhibited the

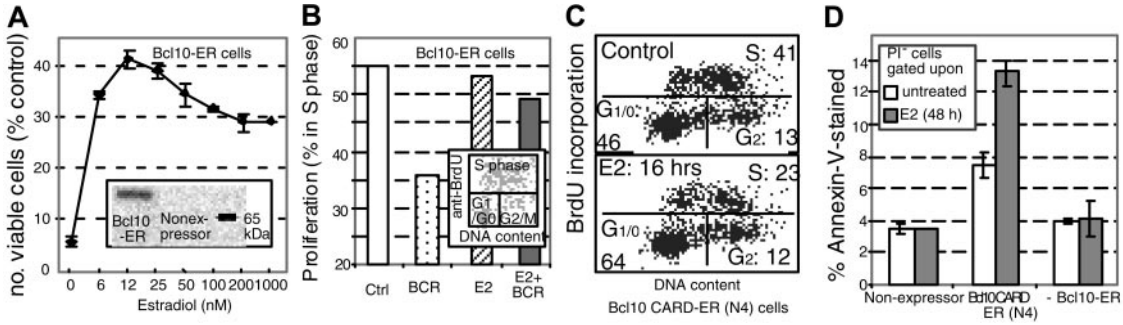

Figure 3. An inducible form of Bcl10 conditionally enhanced the survival of WEHI-231 cells. (A) Cells stably expressing a Bcl10-ER fusion protein were treated with ethanol (solvent) or varying concentrations of the inducer, estradiol (E2), and stimulated with 10 μg/mL anti-IgM for 48 hours. The numbers of viable cells (PI^-) were then determined in triplicate by flow cytometry. Examination of WEHI-231 cells expressing ER (ligand binding domain only) via short-term retroviral infection revealed no effect of E2 on BCR-induced apoptosis (data not shown). Shown in insert is an anti-HA immunoblot of the HA-tagged Bcl10-ER in the stable expressor clone of WEHI-231 and in a "nonexpressor," that is, a clone derived from drug selection of cells infected with the Bcl10-ER–expressing virus showing an undetectable level of Bcl10-ER expression. (B) Bcl10 activation prevented BCR-induced cell-cycle arrest. Bcl10-ER–expressing cells (4×10^5 cells/mL) were stimulated with anti-IgM (10 μg/mL) and/or treated with E2 (1 μM) to activate Bcl10 for 16 hours, a time prior to onset of apoptosis. The cell-cycle status of the cultures was analyzed by BrdU incorporation (1 hour), anti-BrdU/PI staining, and 2-parameter flow cytometry. The percentage of S-phase cells (ie, BrdU-incorporating cells, see insert) in a culture was used to assess the proliferation of the culture. Shown is a representative of 3 different experiments. (C) Bcl10 CARD induced growth arrest. Cells stably expressing Bcl10 CARD-ER (N4) (1×10^5 cells/mL) were treated with E2 (1 μM) for 4 or 16 hours and then subjected to cell-cycle analysis as in panel B. One of 3 representative experiments is shown. No effects of E2 treatment for 4 hours were detected (data not shown). (D) Prolonged activation of the Bcl10 CARD-ER protein led to apoptosis. Cells expressing Bcl10 CARD-ER, Bcl10-ER, and nonexpressing cells (1×10^5 cells/mL) were treated with E2 (1 μM) or ethanol for 16, 24, 48 hours and then stained with fluorescein-labeled annexin V to detect cells with externalized phosphatidylserine. PI^- cells were gated upon to reveal dying but not dead cells. Data shown are triplicate determinations from the 48-hour time point; no effects of E2 treatment on annexin-V staining were detected at the 16- or 24-hour time points. Error bars indicate SEM.

proliferation of the cells, reducing S phase cells from 55% of all cells in the control culture to 35% in the BCR-stimulated culture. Simultaneous activation of Bcl10 by E2, however, markedly but not completely reversed BCR-induced growth arrest. Thus, Bcl10 exerts its protective effects in BCR-stimulated WEHI-231 cells at least partly through its ability to block BCR-induced cell-cycle arrest.

When the N-terminal CARD domain of Bcl10 was fused to ER, the resulting fusion protein, Bcl10 CARD-ER, failed to provide protection against BCR-induced apoptosis of WEHI-231 cells (data not shown) but did inhibit growth of WEHI-231 cells in an E2-dependent fashion (Figure 3C) and, unlike full-length Bcl10, increased the fraction of apoptotic cells (Figure 3D).

Full-length Bcl10 activates both NF-κB and JNK signaling pathways

The experiments described in the preceding section of "Results" indicated that signaling events activated by Bcl10-ER but not by Bcl10 CARD-ER are responsible for the progrowth and survival effects of Bcl10. To identify such events, cells expressing Bcl10-ER or Bcl10 CARD-ER were treated with E2 to activate Bcl10 or its CARD for different periods of time and then subjected to analyses of potential signaling events.

To examine NF-κB activation, lysates of E2-treated cells were immunoblotted with anti–IκB-α or anti–IκB-β antibodies to detect the possible degradation of IκB-α or IκB-β, events necessary for NF-κB activation. Following E2 treatment, Bcl10-ER–expressing cells, but not Bcl10 CARD-ER–expressing or the nonexpressor control cells, showed rapid decreases in the protein levels of both IκB-α and anti–IκB-β (Figure 4A-B). Correspondingly, elevated levels of NF-κB–binding activity were detected by EMSA in the nuclear extracts of E2-treated Bcl10-ER–expressing cells but not in those of E2-treated Bcl10 CARD-ER–expressing or control cells (Figure 4D). The rapidity with which full-length Bcl10 activated the NF-κB pathway strongly suggests that it is a direct action of Bcl10.

Activation of the JNK pathway is often associated with NF-κB signaling and functionally with apoptotic induction.[32] Thus, we also wanted to determine whether Bcl10 or its CARD domain activates the JNK pathway. As shown in Figure 4E, E2 treatment of Bcl10-ER–expressing cells led to increased levels of active forms of JNKs, as assessed with an antibody specific for phosphorylated, activated isoforms of JNKs. In contrast, no change in the level of phospho-JNKs was detected over a 2-hour period in E2-treated Bcl10 CARD-ER–expressing or nonexpressing cells (data not shown). The same lysates of E2-treated Bcl10-ER– or Bcl10 CARD-ER–expressing cells were also immunoblotted with activation-specific antibodies for phospho-p38 MAP kinase or for phospho-p44/42 Erk MAP kinases, but no changes in the levels of phosphorylated forms of these kinases were detected upon E2 treatment in any of the 3 types of cells (data not shown). To confirm the ability of full-length Bcl10 but not its CARD domain to activate the JNK pathway, JNK1 was immunoprecipitated from cells, and an in vitro kinase assay was performed to determine its kinase activity, with CD40-stimulated nonexpressing cells as a positive control. As shown in Figure 4G, significantly higher JNK1 kinase activity was detected in Bcl10-ER–expressing cells treated with E2 for both 15 minutes and 30 minutes than in the untreated cells, further demonstrating the ability of Bcl10 to activate the JNK pathway. In contrast, neither Bcl10 CARD-ER–expressing cells nor nonexpressing cells showed any increase in JNK1 kinase activity upon E2 treatment. Thus, full-length Bcl10, but not a truncated Bcl10 containing only the CARD domain, was able to activate both the NF-κB and JNK pathways, correlating with the ability of these molecules to inhibit BCR-induced apoptosis.

Role of NF-κB in mediating the protective effect of Bcl10

NF-κB has an important antiapoptotic function in a number of different circumstances, including in tumor necrosis factor (TNF)–stimulated cells.[33] To determine whether the ability of Bcl10 to protect WEHI-231 cells from BCR-induced growth arrest and apoptosis was dependent on its ability to activate NF-κB, we used a cell-permeable peptide that interferes with assembly of the IκB kinase complex.[25] This peptide inhibited the growth of WEHI-231 cells when added at high concentrations, perhaps because some NF-κB activity is necessary for the viability or proliferation of these cells, which, like other lymphoma-derived B-cell lines, contains some active NF-κB when grown in culture. When added at a 50-μM concentration, this peptide inhibited the accumulation of viable WEHI-231 cells by about one third. Strikingly, this concentration of the inhibitory peptide nearly completely blocked the protective effect of activating Bcl10 by E2 (Figure 5). Thus, the ability of Bcl10 to protect against BCR-induced growth arrest and apoptosis was highly dependent upon its ability to activate NF-κB.

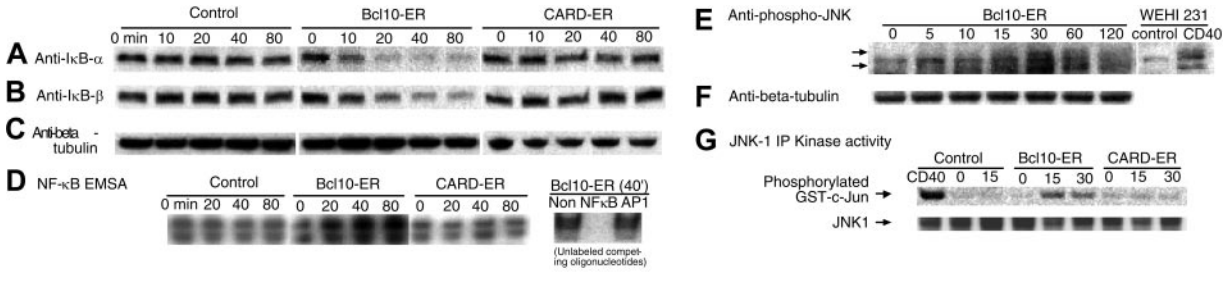

Figure 4. Rapid activation of the NF-κB and JNK pathways by Bcl10 but not by its CARD. (A-D) NF-κB activation. Bcl10-ER or Bcl10 CARD-ER–expressing cells or a nonexpressor control clone were treated with E2 (1 μM) for indicated periods of time (minute) and directly lysed with sodium dodecyl sulfate (SDS) sample buffer. Lysates were blotted with anti–IκB-α (A) and, after stripping, with anti–IκB-β (B) to detect degradation of IκB-α or IκB-β. To control for sample loading, identical gels were blotted with anti–β-tubulin (C). (D) Indicated cells were treated with E2 (1 μM) to activate Bcl10 or Bcl10 CARD. Nuclear lysates were prepared and electrophoretic mobility shift assay (EMSA) performed with a ^{32}P-labeled oligonucleotide consensus sequence for NF-κB binding. (Right) The specificity of the assay was verified in a repeat experiment in which competing nonlabeled NF-κB– or activator protein 1 (AP1)–binding oligonucleotides (the latter being a negative control) were included in 25-fold molar excess during the binding. (E-G) JNK activation by Bcl10 but not its CARD domain. Indicated cells were treated with E2 (1 μM) and analyzed for JNK activation by blotting the lysates with an antibody specific for phosphorylated JNKs (arrows) (E). CD40-stimulated (15 minutes) WEHI-231 cells were used as a positive control. To verify equal loading of samples, an identical gel was blotted with anti–β-tubulin (F). (G) Cells were treated with E2 (1 μM) and lysed. JNK1 was immunoprecipitated from the lysates and measured for its in vitro kinase activity using purified glutathione *S* transferase (GST)–c-Jun (N-terminus) as the substrate. CD40-stimulated (15 minutes) nonexpressor cells were used as a positive control for the kinase assay. The kinase reactions, once no longer radioactive, were blotted with anti-JNK1 to confirm comparable levels of immunoprecipitated JNK1 protein in the reactions (bottom).

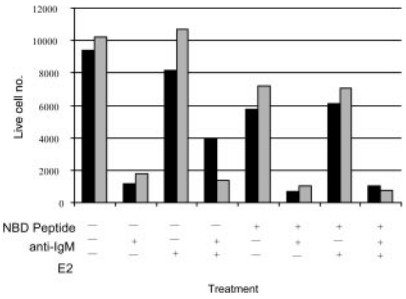

Figure 5. The ability of Bcl10 to protect from BCR-induced growth arrest and apoptosis is dependent on NF-κB. WEHI-231 cells (3×10^5/mL) expressing Bcl10-ER were treated with different combinations of β-estradiol (E2, 1μM), anti-IgM (10 μg/mL), and NF-κB inhibitory peptide (NBD, 50μM). NBD is a cell-permeable peptide containing sequences from the IκB kinase (IKK) γ subunit and from HIV-1 tat. This peptide inhibits NF-κB activation by blocking assembly of a functional IKK complex. The number of viable cells was determined in triplicate 48 hours after treatment, as in Figure 1. A control peptide containing a sequence from the L-plastin protein did not exhibit any effect on Bcl10-mediated protection against BCR-induced growth inhibition or apoptosis (not shown). ■ indicates Bcl10-ER; ▨, Nonexpressors.

Bcl10 promotes the survival of splenic B cells cultured in vitro

To assess the effect of Bcl10 on survival of activated normal B cells, primary B cells were isolated from mouse spleen and activated in vitro via the BCR (by anti-IgM), CD40 (by anti-CD40), and IL-4 receptor (by IL-4) for 2 days. The ex vivo cultures were then infected with a retrovirus encoding Bcl10 and GFP simultaneously through a biscistronic mRNA (Bcl10-IRES-GFP) or a retrovirus encoding GFP alone as a control. The cultures, containing infected cells (GFP^+) and noninfected cells (GFP^-), were maintained with the stimuli (anti-IgM, anti-CD40, and IL-4) for 2 more days. Subsequently, the stimuli were removed, and the ratios of GFP-positive cells in the cultures were analyzed at various times. Bcl10 clearly conferred a survival factor to the activated B cells after withdrawal of the activating stimuli, because the fraction of Bcl10 and GFP^+ cells increased until the GFP^- cells had all died by day 11 (Figure 6A). No such enhancement of GFP^+ cells was seen when the B cells were infected with a retrovirus encoding only GFP (Figure 6B). The cultures from vector virus infection contained few viable cells by 10 to 14 days of incubation ex vivo. Bcl10-expressing cultures had viable $CD19^+$ B cells (Figure 6C) that were also proliferating, as determined by uptake of BrdU (data not shown).

Discussion

We developed a screen to isolate genes capable of protecting the immature B-cell line WEHI-231 from BCR-induced apoptosis. From this antiapoptotic screen, we isolated full-length, unmutated Bcl10, a small adapter protein containing a CARD domain, initially discovered because the *BCL10* gene is present in chromosomal translocations juxtaposed to the Ig heavy chain locus in a fraction of extranodal marginal zone B-cell lymphomas of the mucosa-associated lymphoid tissue (MALT lymphomas).[10,11] We found that Bcl10 expression prevented BCR-induced growth arrest and apoptosis of WEHI-231 cells and also of a second murine B-cell line, CH31. By using a conditionally active form of Bcl10, we found that Bcl10 was able rapidly to activate the NF-κB transcription factor and the JNK MAP kinase, but not Erk or p38 MAP kinases. The ability of Bcl10 to prevent BCR-induced growth arrest and apoptosis was found to be dependent on its ability to activate NF-κB. Finally, we found that Bcl10 overexpression in primary activated murine B cells promoted the survival of these cells in ex vivo cultures, a condition in which normal B cells readily undergo apoptosis over time. These observations demonstrate the prosurvival function of Bcl10 in B cells in certain circumstances and suggest a mechanism for the ability of *IGH/BCL10* translocations to promote lymphoma formation.

Bcl10 and lymphomagenesis

The initial discovery of Bcl10 translocation to the immunoglobulin heavy chain (*IGH*) locus in B-cell lymphomas was paradoxic because overexpression of this gene in nonlymphocytic cell lines induced apoptosis.[10,11,13-17] Because mutated forms of Bcl10 could be found in these translocations,[10,11] it was initially proposed that Bcl10 was a tumor suppressor gene that was inactivated by translocation followed by Ig locus-directed somatic mutation. However, this hypothesis did not agree with the fact that Bcl10-deficient mice have normal apoptosis, but defective development to the mature B-cell stages and compromised lymphocyte activation via their antigen receptors.[19,34] Our observations indicate that wild-type Bcl10 can exert an antiapoptotic effect on B lymphocytes; therefore, it is likely that translocation to the IgH locus leads to increased levels of normal Bcl10, which is promoting cell survival under certain conditions.

MALT lymphomas are suspected to arise from B cells undergoing immune responses to infections, such as *Helicobacter pylori* infection for gastric lymphomas.[12,35,36] Chromosome translocations are often observed in MALT lymphomas with the most frequent being t(11;18)(q21;q21) encoding the *API2/MALT1* fusion protein and with t(1;14)(p22;q32) involving *BCL10* occurring less often.[37] MALT1 and Bcl10 interact with each other and with the molecular

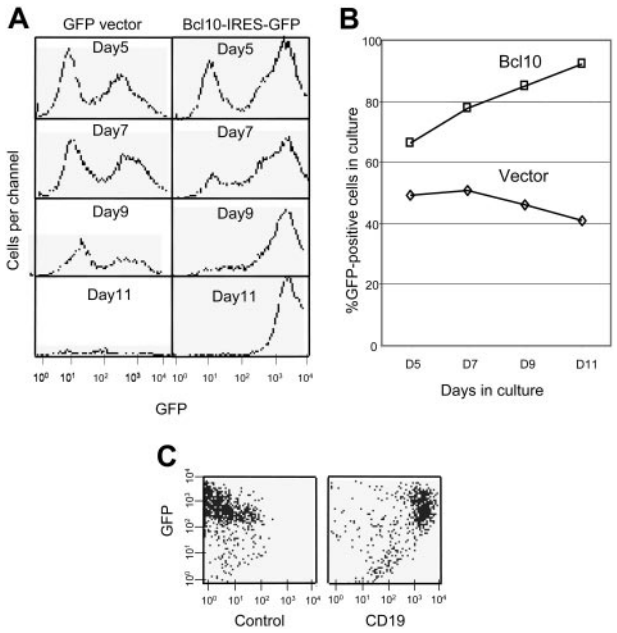

Figure 6. Bcl10 protects primary B cells from apoptosis. Primary B cells were isolated from Balb/c mouse spleens and activated for 2 days with anti-IgM, anti-CD40, and IL-4. The cells were then infected with a retrovirus encoding Bcl10-IRES-GFP or a control retrovirus expressing only GFP. After another 2 days incubation with anti-IgM, anti-CD40, and IL-4, the cells were cultured in medium without stimuli. Triplicate cultures were sampled at different times via flow cytometry (A) for GFP expression, and the percentage of positive cells was determined (B; squares indicate Bcl10-expressing retrovirus; diamonds, vector only). To confirm that the surviving cells in the Bcl10-expressing cultures were B cells, they were stained with anti-CD19 or an isotype control antibody(C).

scaffold Carma1 and participate in a signaling cascade leading from lymphocyte antigen receptors to NF-κB activation.[20,21] Our results strongly suggest that the genetic abnormalities involving *MALT1* and *BCL10* enhance MALT1 function in MALT lymphomas, promoting lymphoma growth and/or survival through activation of NF-κB and/or the JNK signaling pathway. However, we also observed that Bcl10 overexpression in WEHI-231 cells inhibited their growth in the absence of BCR stimulation (Figure 2). Therefore, it is possible that *IGH/BCL10* translocations have both positive and negative effects on MALT lymphoma formation, selecting for outgrowth of cells with mutations in the translocated *BCL10*. Alternatively, the *BCL10* mutations frequently seen in these translocations could be inadvertent events and nonconsequential. The activities that we have observed for Bcl10 in WEHI-231 cells could provide a suitable assay for assessing the effects of these mutations on the prosurvival function of Bcl10 in combination with BCR signaling and on its antiproliferative function in the absence of BCR signaling.

Bcl10 and lymphocyte function

The major phenotype of *Bcl10*-deficient mice is a complete defect in the activation of both B cells and T cells via their antigen receptors. In addition, Bcl10-deficient lymphocytes cannot activate NF-κB via their antigen receptors.[18] Correspondingly, lymphocytes from c-Rel–deficient mice cannot be activated via their antigen receptors.[38,39] Thus, activation of NF-κB via the BCR and TCR is likely to be the key physiologic function of Bcl10. Our studies provide additional support for that conclusion. We created a Bcl10-ER fusion protein that is functional only in the presence of ligands for the estrogen-binding domain. Addition of β-estradiol to WEHI-231 cells expressing this fusion protein resulted in rapid activation of NF-κB and JNK, but not activation of the Erk or p38 MAP kinases. This shows that the connection of Bcl10 to NF-κB activation downstream of antigen-receptor signaling is a reasonably direct one and does not appear to require any intervening changes in gene expression. Genetic experiments, such as the lack of response of Bcl10-deficient B cells,[18] or overexpression experiments examining NF-κB reporter construct activity,[10,13,14,17] cannot rule out less direct actions. Our data provide evidence that Bcl10 acts in a biochemical pathway between the BCR and the IKK complex that activates NF-κBs.[40-46]

The prosurvival function of Bcl10 that we observed required both the CARD domain and the C-terminal domain of Bcl10, which lacks homology to other known proteins.[10,11,13-17] The CARD domain is responsible for association with Carma1.[44,47] The C-terminal domain was necessary for downstream signaling functions in addition to the protection from BCR-mediated growth arrest and apoptosis. The C-terminal domain of Bcl10 binds to MALT1, which is likely responsible for activating NF-κB.[22] Interestingly, expression of the Bcl10 CARD domain alone had a negative effect on WEHI-231 growth and survival. The mechanism of this effect was not studied, but one possibility is that the ectopically expressed CARD domain may act as a dominant negative, lowering signaling from endogenous Bcl10, and thereby inhibiting its positive effects on the cell.

We found that the ability of Bcl10 overexpression to rescue WEHI-231 cells from BCR-induced apoptosis was dependent upon activation of NF-κB (Figure 5). This is consistent with previous reports showing that inhibition of the NF-κB pathway enhanced BCR-induced death in WEHI-231 cells,[4,48] while augmentation of NF-κB signaling provided protection.[49] BCR-induced expression of antiapoptotic factors such as Bcl-xL and A1 have been shown to be dependent upon NF-κB.[50,51] The effects of JNK activation by Bcl10 in WEHI-231 cells remains to be examined, but JNK activation is often associated with apoptotic induction.[32,52]

A striking feature of B-cell development is that immature B cells exhibit a negative response to antigen encounter, whereas mature B cells exhibit a positive response.[29,53] This dichotomy is likely important for immunologic tolerance because it provides a mechanism to remove self-reactive B cells from the immune system. In this regard, our finding that overexpression of Bcl10 can prevent the negative response of the immature B-cell line WEHI-231 to BCR stimulation is remarkable. Conversely, B cells deficient in Bcl10 or c-Rel are unable to make a positive response to antigen stimulation.[18] It is possible that the high apoptotic sensitivity of normal immature or transitional B cells to BCR stimulation is due to the inability of these cells to activate or maintain NF-κB signaling.[54,55] Thus, acquisition of the signaling capacity from the BCR to NF-κB via Bcl10 and associated signaling components may represent a functional milestone during the transition of B cells from the apoptosis-susceptible immature stage to the apoptosis-resistant mature stage. Jun and Goodnow[53] have recently proposed a similar model to explain tolerogenic signaling versus activation signaling by the BCR, namely that the former involves BCR signaling events not including Bcl10 and NF-κB activation, whereas the latter includes the incorporation of the Bcl10/Carma1 signaling complex.

In summary, the experiments reported here have revealed an antiapoptotic function of Bcl10 in B cells. These results suggest that dysregulated Bcl10 signaling, as occurs following *IGH/BCL10* translocation, confers a survival advantage and more activated status to B cells in some circumstances, predisposing them to lymphomagenesis.

Acknowledgments

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