BCL-6 Expression During B-Cell Activation

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Translocations involving the BCL-6 gene are common in the diffuse large cell subtype of non-Hodgkin's lymphoma. Invariably, the BCL-6 coding region is intact, but its 5′ untranslated region is replaced with sequences from the translocation partner. The present study shows that BCL-6 expression is regulated in lymphocytes during mitogenic stimulation. Resting B and T lymphocytes contain high levels of BCL-6 mRNA. Stimulation of mouse B cells with anti-IgM or IgD antibodies, bacterial lipopolysaccharide, phorbol 12-myristate 13-acetate plus ionomycin, or CD40 ligand led to a fivefold to 35-fold decrease in BCL-6 mRNA levels. Similar downregulation of BCL-6 mRNA was seen in human B cells stimulated with *Staphylococcus aureus* plus interleukin-2 or anti-IgM antibodies and in human T lymphocytes stimulated with phytohemagglutinin. BCL-6 mRNA levels began to decrease 8 to 16 hours after stimulation, before cells entered S phase. Although polyclonal activation of B cells in vitro invariably decreased BCL-6 mRNA expression, activated B cells from human germinal centers expressed BCL-6 mRNA at levels comparable to the levels in resting B cells. Despite these similar mRNA levels, BCL-6 protein expression was threefold to 34-fold higher in germinal center B cells than in resting B cells, suggesting that BCL-6 protein levels are controlled by translational or posttranslational mechanisms. These observations suggest that the germinal center reaction provides unique activation signals to B cells that allow for continued, high-level BCL-6 expression.

THE BCL-6 GENE HAS recently been implicated in the etiology of the subset of non-Hodgkin's lymphoma (NHL) with a large-cell component.[1-4] These lymphomas of mature B lymphocytes account for 30% to 40% of newly diagnosed NHL cases and as much as 80% of the NHL mortality.[5] The BCL-6 gene[1,3] (also designated LAZ3[4] and BCL-5[2]) is located at chromosome 3q27, a common site of translocation in NHL. Rearrangements of the BCL-6 gene have been detected in as many as 45% of diffuse large-cell lymphomas (DLCL), but they also occur frequently in lymphomas with mixed small and large-cell histology and in follicular lymphomas, particularly those that have transformed into more clinically aggressive tumors.[6-8] Likewise, in acquired immunodeficiency syndrome (AIDS)-associated NHL, BCL-6 rearrangements are detectable in 20% of cases belonging to the DLCL subgroup.[9] One intriguing report suggested that BCL-6 rearrangement defines a biologically distinct subgroup of DLCL that has a more favorable prognosis after chemotherapy,[10] although this correlation was not apparent in all studies.[7]

The BCL-6 protein has six Krüppel-like zinc fingers at its carboxy terminus and thus is presumably involved in transcriptional regulation.[2-4] In addition, BCL-6 belongs to the newly recognized subfamily of zinc finger transcription factors that share an amino terminal POZ domain.[11] One function of POZ domains is in homodimerization and heterodimerization,[11] raising the possibility that BCL-6 may act in conjunction with another POZ domain factor.

Despite these structural clues, the normal biologic function of BCL-6 remains to be elucidated. One consistent feature of the BCL-6 rearrangements in NHL is that the breakpoints cluster in a 5′ untranslated region of the gene, leaving the BCL-6 coding region intact.[3,4,8] In this regard, the BCL-6 rearrangements in DLCL are formally analogous to the c-myc rearrangements in Burkitt's lymphoma. This observation raised the possibility that deregulation of BCL-6 expression is responsible for the malignant transformation in DLCL. We therefore surmised that an important part of the biology of BCL-6 would be shown by studying the control of its expression in nontransformed lymphocytes. Indeed, recent immunohistochemistry studies of lymphoid tissues showed that BCL-6 protein was readily detectable in germinal center B cells but not in most other B cells in human lymphoid tissues.[12-14] In the present report, we investigated the regulation of BCL-6 mRNA expression during lymphocyte activation. We show that naive B lymphocytes activated by a variety of mitogenic stimuli in vitro downregulate BCL-6 mRNA, whereas activated B cells from germinal centers continue to express BCL-6 mRNA. Furthermore, we show that germinal center B cells express dramatically more BCL-6 protein than resting B cells, despite similar BCL-6 mRNA levels in the two cell populations. These findings suggest that the germinal center microenvironment delivers unique activation signals to B lymphocytes that maintain BCL-6 expression.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Cell lines. HeLa, K562, Hut78, Jurkat, Nalm6, Raji, BJAB, WIL2-NS, and ARH77 were purchased from ATCC (Bethesda, MD). The Epstein-Barr virus (EBV)-immortalized B-cell line VDSO was kindly provided by Dr G. Tosato (Food and Drug Administration, Bethesda, MD), and MBB1 was cloned by limiting dilution of the EBV-immortalized B-cell line Meade, which was provided by Dr T. Waldmann (National Cancer Institute, Bethesda, MD). The B-cell line RL was provided by Dr D. Longo (National Cancer Institute, Frederick, MD).

Reagents. $F(ab')_2$ fragments of goat antimouse IgM antibodies and fluorescein isothiocyanate (FITC) antimouse IgM were pur-

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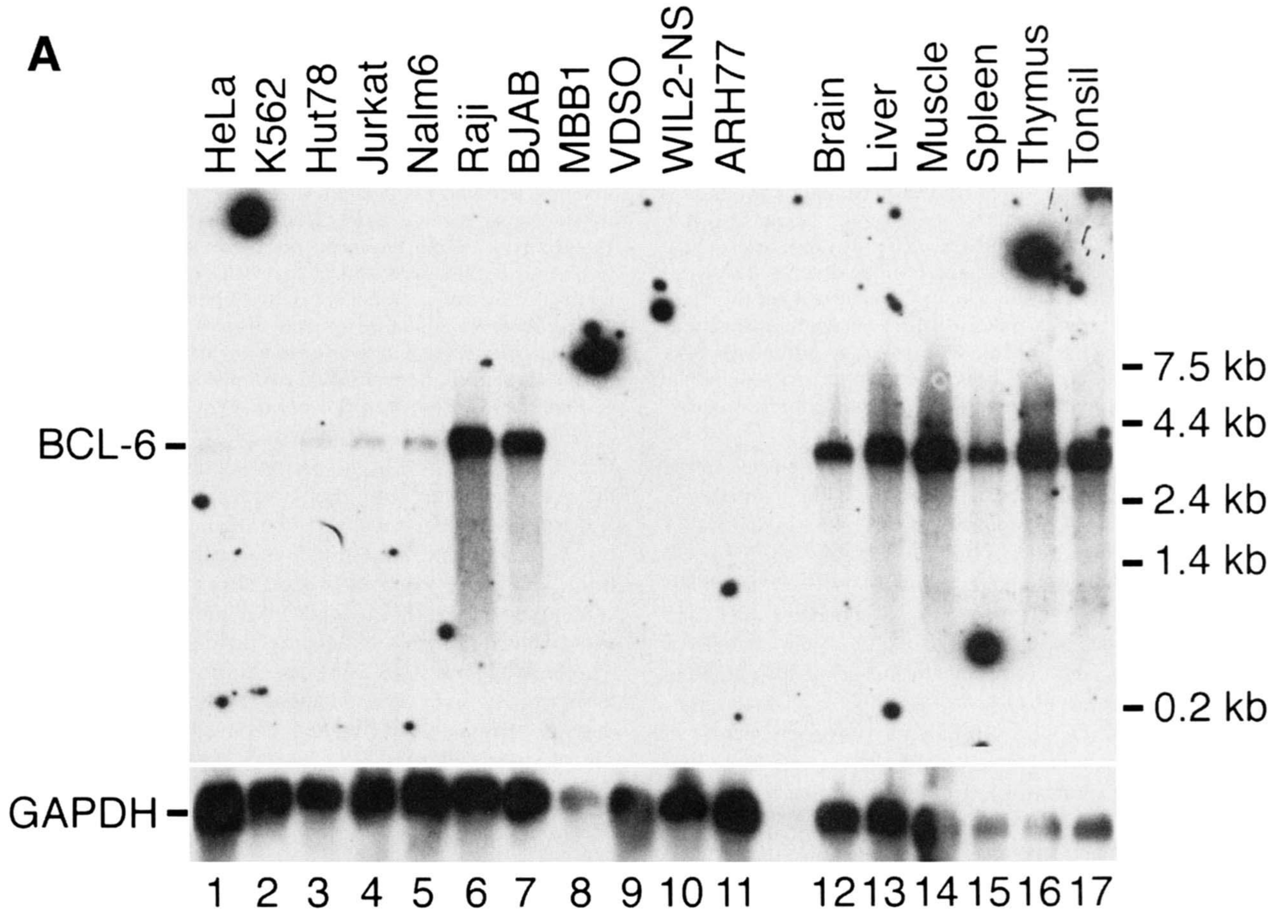

Fig 1. BCL-6 mRNA expression in human tissues and cell lines. (A) Northern blot analysis of BCL-6 and GAPDH expression using poly-A[+] RNA from the cervical carcinoma cell line HeLa; the erythroleukemia cell line K562; the T-cell lines Hut78 and Jurkat; the pre-B–cell line Nalm6; the mature B-cell lines Raji, BJAB, MBB1, VDSO, WIL2-NS, and ARH77; and the indicated human tissues.

chased from Jackson ImmunoResearch (West Grove, PA). $F(ab')_2$ goat antihuman IgM was purchased from Southern Biotechnology Associates (Birmingham, AL). Bacterial lipopolysaccharide (LPS; *Salmonella typhosa*) was obtained from Fisher (Malvern, PA). Phorbol 12-myristate 13-acetate (PMA), RNase H, and propidium iodide were obtained from Sigma Chemical Co (St Louis, MO). Ionomycin and *Staphylococcus aureus*, Cowan's strain (SAC; Pansorbin cells) were obtained from Calbiochem Corp (La Jolla, CA). Phytohemaglutinin (PHA) was obtained from Murex Diagnostics (Bartford, UK). Recombinant interleukin-2 (rIL-2) was obtained from R&D Systems (Minneapolis, MN). Rabbit complement was obtained from Pel-Freeze (Rogers, AR). The anti-Thy 1.2 antibody, J1j, was kindly provided by Dr M. Cancro (University of Pennsylvania, Philadelphia, PA). The dextran-coupled antimouse IgD antibody, $H\delta^a/1$, was provided by Dr Clifford Snapper (Uniformed Health Services, Bethesda, MD).

Cloning and sequencing of human and mouse BCL-6. A full-length human BCL-6 cDNA was obtained by screening a Raji cDNA library with a BCL-6 fragment isolated from a previously described subtracted library.[15] The full-length mouse BCL-6 cDNA was subsequently cloned from a muscle cDNA library (Stratagene, La Jolla, CA) using a polymerase chain reaction (PCR)-generated probe spanning base pairs 330 to 800 of the human BCL-6 cDNA.[3] Automated nucleotide sequencing was performed with an Applied Biosystems 373A DNA sequencer (Foster City, CA). The mouse BCL-6 mRNA sequence has been given Genbank accession no. U41465. This sequence is identical to an unpublished mouse BCL-6 sequence in Genbank (accession no. D38377) except for two nucleotide differences in the coding region that may be mouse strain polymorphisms. One nucleotide discrepancy does not change the amino acid sequence, whereas the second changes amino acid 456 from glycine to alanine.

Purification of mouse and human lymphocytes. Mouse splenic B cells from 8- to 12-week-old mice were prepared as previously described.[16] Human naive B cells were purified by positive selection from peripheral blood mononuclear cells with anti-CD19–coated magnetic beads (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions.[17] The resulting B-cell populations were routinely 95% surface IgM^+ as determined by flow cytometry. Germinal center B cells were purified as described[18] from human tonsils obtained from Holy Cross Hospital (Silver Spring, MD) and were greater than 95% $CD38^+$, $CD20^+IgD^-$. Human peripheral blood T cells were isolated as described[19] and were 70% to 95% $CD2^+$ $CD5^+$ after enrichment.

Cell cultures. Bulk cultures[20] and proliferation assays[16] with mouse splenic B cells were set up as described. Purified human B and T cells were cultured at 2×10^6 cells/mL in RPMI containing 10% fetal calf serum (FCS), 1 μg/mL glutamine, 1 μg/mL each of penicillin and streptomycin, and 5×10^{-5} mol/L 2-mercaptoethanol. Unless indicated otherwise, cultures were preincubated at 37°C for

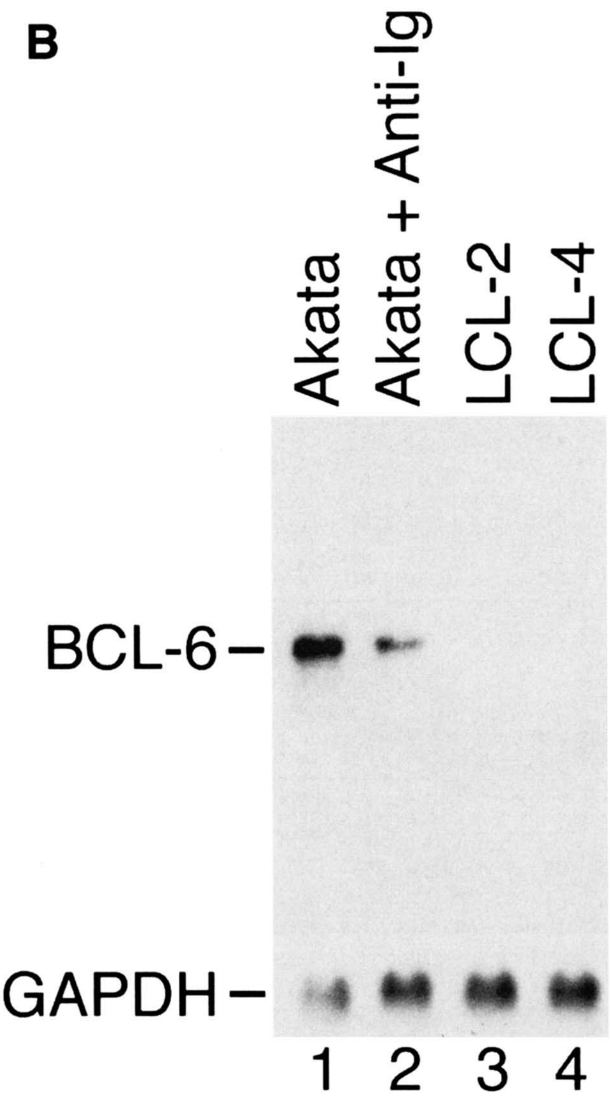

Fig 1. (cont'd). (B) Northern blot analysis of BCL-6 and GAPDH expression using total RNA from the Burkitt's lymphoma cell line, Akata, with and without stimulation with anti-Ig antibodies for 24 hours and from two EBV-transformed lymphoblastoid cell lines, LCL-2 and LCL-4.

1 hour before the addition of the indicated stimulus. Mouse splenic B cells were stimulated with 50 μg/mL anti-IgM, 50 μg/mL LPS, 10 ng/mL anti-IgD dextran, or 1 ng/mL PMA plus 1 μg/mL ionomycin. Human B cells and the AKATA B-cell line were stimulated with 50 μg/mL anti-IgM or 1:20,000 wt/vol SAC plus 5 ng/mL rIL-2, and human T cells were stimulated with 1 μg/mL PHA. Before RNA isolation, cells were washed twice in cold phosphate-buffered saline (PBS) and checked for viability by trypan blue exclusion. DNA content was determined by staining cell aliquots with propidium iodide by standard methods,[21] followed by analysis on a Becton Dickinson FACscan with CellQuest software.

CD40 ligand stimulation of B cells. CD40 ligand was expressed in insect cells using a recombinant baculovirus encoding full-length human CD40 ligand (kindly provided by K. Meek and P. Lipsky[22]). High-titer virus stocks grown in Sf9 cells or Sf21 cells using standard techniques[23] were used to infect Sf9 cells grown in suspension culture for 66 hours at 27°C (multiplicity of infection [MOI] of 20). Infected Sf9 cells were harvested by centrifugation, washed twice with ice-cold Rinaldini's salt solution (Sigma) containing 1.0 mmol/L $CaCl_2$, and membranes were prepared as described.[24,25] Membranes were washed, resuspended in Dulbecco's PBS, and titrated in a B-cell proliferation assay.[24,25] The optimal dilution of CD40 ligand-containing membranes was then used to stimulate 3×10^7 purified mouse splenic B cells in a 10-mL bulk culture.

Preparation of RNA and Northern blot analysis. Total RNA was prepared with the Stratagene RNA isolation kit (La Jolla, CA) and poly-A^+ RNA was isolated by selection on oligo-dT cellulose (Collaborative Biomedical Products, Bedford, MA). Poly-A^+ RNA from human tissues was purchased from Clontech (Palo Alto, CA). Northern blot analysis was performed using standard methods.[26] Mouse RNA blots were hybridized with a 1,200-bp *Eco*RI fragment derived from the mouse BCL-6 cDNA, stripped, and then rehybridized with a 800-bp *Pst* I fragment derived from rat GAPDH.[27] Human RNA blots were hybridized with the human BCL-6 PCR fragment described above, stripped, and then rehybridized with a human GAPDH PCR fragment. Quantitation of Northern blots was achieved on a phosphorimager or densitometer, each equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis. Two different rabbit polyclonal anti–BCL-6 antibodies were used for Western blot analysis. Antipeptide antisera (Research Genetics, Huntsville, AL) were prepared by immunizing rabbits with the peptide C-amino caproic acid-EDEIALH-FEPPNAPLNRK (derived from BCL-6 amino acids 310 through 327) coupled to keyhole limpet hemocyanin. Affinity-purified anti–BCL-6 antibodies were generated from the antisera using an affinity column prepared from the immunogen. In some experiments, an anti–BCL-6 antisera generated against BCL-6 amino acids 3 through 484 was used (Santa Cruz Biotechnology, Santa Cruz, CA).

For Western blot analysis, 2.5×10^6 germinal center or human peripheral blood B cells were resuspended in PBS, lysed with the addition of an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2-mercaptoethanol,[26] boiled for 5 minutes, and briefly sonicated. Samples were electrophoresed through a 7.5% SDS-PAGE gel, and proteins were transferred to a nitrocellulose membrane (BA-S NC; Schleicher & Schuell, Keene, NH) using a Mini-Transblot apparatus (Biorad, Hercules, CA) according to the manufacturer's protocol. Blots were incubated with anti–BCL-6 antibodies at 3.3 μg/mL in blocking solution for 1 hour at room (PBS with 5% wt/vol nonfat dry milk). Bound antibody was detected either using an ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's protocol or by incubating the blot with ^{125}I-labeled donkey antirabbit antibodies (1 μCi/mL in blocking solution; Amersham) for 30 minutes at room temperature. The blots were stripped of bound antibody by heating at 50°C for 30 minutes in a buffer consisting of 62.5 mmol/L Tris, pH 7.5, 100 mmol/L 2-mercaptoethanol, and 2% wt/vol SDS. To normalize for protein loading, blots were reprobed with anti-Sp1 antibodies (3.3 μg/mL; antibody PEP 2; Santa Cruz Biotechnology).

RESULTS

Expression of BCL-6 mRNA in cell lines and tissues. We initially cloned BCL-6 from a subtracted cDNA library that was enriched in genes expressed in the Raji B-cell line and not in the K562 erythroleukemia cell line.[15] Previous studies of the expression of BCL-6 mRNA in human cell lines and tissues have yielded conflicting results, concluding that BCL-6 is preferentially expressed in mature B cells,[3] is preferentially expressed in muscle,[4] or that BCL-6 expression is ubiquitous.[28] To resolve this uncertainty, we simultaneously

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Mouse BCL-6   M A S P A D S C I Q F T R H A S D V L L N L N R L R S R D I L T D V V I V V S R E Q F R A H K T V L   50
Human BCL-6   M A   P A D S C I Q F T R H A S D V L L N L N R L R S R D I L T D V V I V V S R E Q F R A H K T V L   50

Mouse BCL-6   M A C S G L F Y S I F T D Q L K C N L S V I N L D P E I S P E G F C I L L D F M Y T S R L N L R E G   100
Human BCL-6   M A C S G L F Y S I F T D Q L K C N L S V I N L D P E I N P E G F C I L L D F M Y T S R L N L R E G   100

Mouse BCL-6   N I M A V M T T A M Y L Q M E H V V D T C R K F I K A S E A E M A P A L K P P R E E F L N S R M L M   150
Human BCL-6   N I M A V M A T A M Y L Q M E H V V D T C R K F I K A S E A E M V S A I K P P R E E F L N S R M L M   150

Mouse BCL-6   P H D I M A Y R G R E V V E N N M P L R N T P G C E S R A F A P P L Y S G L S T P P A S Y P M Y S H   200
Human BCL-6   P Q D I M A Y R G R E V V E N N L P L R S A P G C E S R A F A P S L Y S G L S T P P A S Y S M Y S H   200

Mouse BCL-6   L P L S T F L F S D E E L R D A P R M P V A N P F P K E R A L P C D S A R Q V P N E Y S R P A M E V   250
Human BCL-6   L P V S S L L F S D E E F R D V - R M P V A N P F P K E R A L P C D S A R P V P G E Y S R P T L E V   249

Mouse BCL-6   S P S L C H S N I Y S P K E A V P E E A R S D I H Y S V P E G P K P A V P S A R N A P Y F P C D K A   300
Human BCL-6   S P N V C H S N I Y S P K E T I P E E A R S D M H Y S V A E G L K P A A P S A R N A P Y F P C D K A   299

Mouse BCL-6   S K E E E R P S S E D E I A L H F E P P N A P L N R K G L V S P Q S P Q K S D C Q P N S P T E S C S   350
Human BCL-6   S K E E E R P S S E D E I A L H F E P P N A P L N R K G L V S P Q S P Q K S D C Q P N S P T E A C S   349

Mouse BCL-6   S K N A C I L Q A S G S P P A K S P T D P K A C N W K K Y K F I V L N S L N Q N A K P E G S E Q A E   400
Human BCL-6   S K N A C I L Q A S G S P P A K S P T D P K A C N W K K Y K F I V L N S L N Q N A K P G G P E Q A E   399

Mouse BCL-6   L G R L S P R A Y P A P P A C Q P P M E P A N L D L Q S P T K L S A S G E D S T I P Q A S R L N N L   450
Human BCL-6   L G R L S P R A Y T A P P A C Q P P M E P E N L D L Q S P T K L S A S G E D S T I P Q A S R L N N I   449

Mouse BCL-6   V N R S L G G S P R S S S E S H S P L Y M H P P K C T S C G S Q S P Q H T E M C L H T A G P T F P E   500
Human BCL-6   V N R S M T G S P R S S S E S H S P L Y M H P P K C T S C G S Q S P Q H A E M C L H T A G P T F A E   499

Mouse BCL-6   E M G E T Q S E Y S D S S C E N G T F F C N E C D C R F S E E A S L K R H T L Q T H S D K P Y K C D   550
Human BCL-6   E M G E T Q S E Y S D S S C E N G A F F C N E C D C R F S E E A S L K R H T L Q T H S D K P Y K C D   549

Mouse BCL-6   R C Q A S F R Y K G N L A S H K T V H T G E K P Y R C N I C G A Q F N R P A N L K T H T R I H S G E   600
Human BCL-6   R C Q A S F R Y K G N L A S H K T V H T G E K P Y R C N I C G A Q F N R P A N L K T H T R I H S G E   599

Mouse BCL-6   K P Y K C E T C G A R F V Q V A H L R A H V L I H T G E K P Y P C E I C G T R F R H L Q T L K S H L   650
Human BCL-6   K P Y K C E T C G A R F V Q V A H L R A H V L I H T G E K P Y P C E I C G T R F R H L Q T L K S H L   649

Mouse BCL-6   R I H T G E K P Y H C E K C N L H F R H K S Q L R L H L R Q K H G A I T N T K V Q Y R V S A A D L P   700
Human BCL-6   R I H T G E K P Y H C E K C N L H F R H K S Q L R L H L R Q K H G A I T N T K V Q Y R V S A T D L P   699

Mouse BCL-6   P E L P K A C   707
Human BCL-6   P E L P K A C   706
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Fig 2. Deduced amino acid sequence and tissue expression of mouse BCL-6. Amino-terminal POZ and carboxy-terminal zinc-finger domains are boxed and positions differing between mouse and human are indicated by enclosed human residues.

compared the levels of BCL-6 in human cell lines and tissues on the same Northern blot (Fig 1A). We detected BCL-6 mRNA readily in some, but not all, mature B-cell lines (Fig 1A, lanes 6 through 11). BCL-6 was also found to be highly expressed in 3 additional mature B-cell lines and in one of two pre-B–cell lines tested (data not shown). BCL-6 mRNA levels were low in T-cell, myeloid, and erythroid and HeLa cell lines (Fig 1A, lanes 1 through 5, and data not shown). BCL-6 mRNA was detected in all tissues tested, with only minor quantitative differences between tissues (Fig 1A, lanes 12 through 17). Interestingly, the levels of BCL-6 mRNA in certain B-lymphoma cell lines (Fig 1A, Raji and BJAB) were not notably higher than tissue levels. Rather, it appears that the expression of BCL-6 mRNA is downregulated in many cell lines when compared with expression in tissues.

Although BCL-6 is generally expressed at high levels in many Burkitt's lymphoma B-cell lines (Fig 1A and B and data not shown), it was peculiarly absent from six mature B-cell lines, four of which were long-term B-lymphoblastoid cell lines (LCLs) transformed with EBV (Fig 1A, MBB1 and VDSO; and Fig 1B, LCL-2 and LCL-4). This observation provided a clue to possible regulation of BCL-6 during lymphocyte activation because previous studies of LCLs have shown that they have an activated B-cell phenotype.[29-31] EBV-encoded proteins have been shown to drive expression of cell surface markers that are upregulated after antigenic activation of normal resting B cells,[30,31] and thus it was conceivable that BCL-6 would be similarly modulated. This hypothesis led us to investigate the expression of BCL-6 in the type I Burkitt's lymphoma cell line, Akata.[32] Akata cells contain EBV in a latent state in which only the EBNA1 gene product is expressed.[32] However, when Akata cells are stimulated by cross-linking of their surface Ig receptors, the EBV lytic cycle is initiated and the cells express several of the EBV gene products that are constituitively expressed in LCLs.[32] Activation of Akata cells in this fashion led to the downmodulation of BCL-6 mRNA (Fig 1B).

BCL-6 mRNA expression during activation of resting B lymphocytes. These observations led us to investigate BCL-6 mRNA levels during activation of resting B lymphocytes. To perform this analysis with mouse as well as human B lymphocytes, we cloned the mouse BCL-6 gene by low stringency hybridization with a human BCL-6 probe. Sequencing of a 3.5-kb mouse BCL-6 cDNA clone showed striking homology to human BCL-6; the predicted amino acid sequences from the cDNAs are 94% identical (Fig 2). The regions of highest sequence conservation were the zinc finger domain (100% identity) and the POZ domain (98%

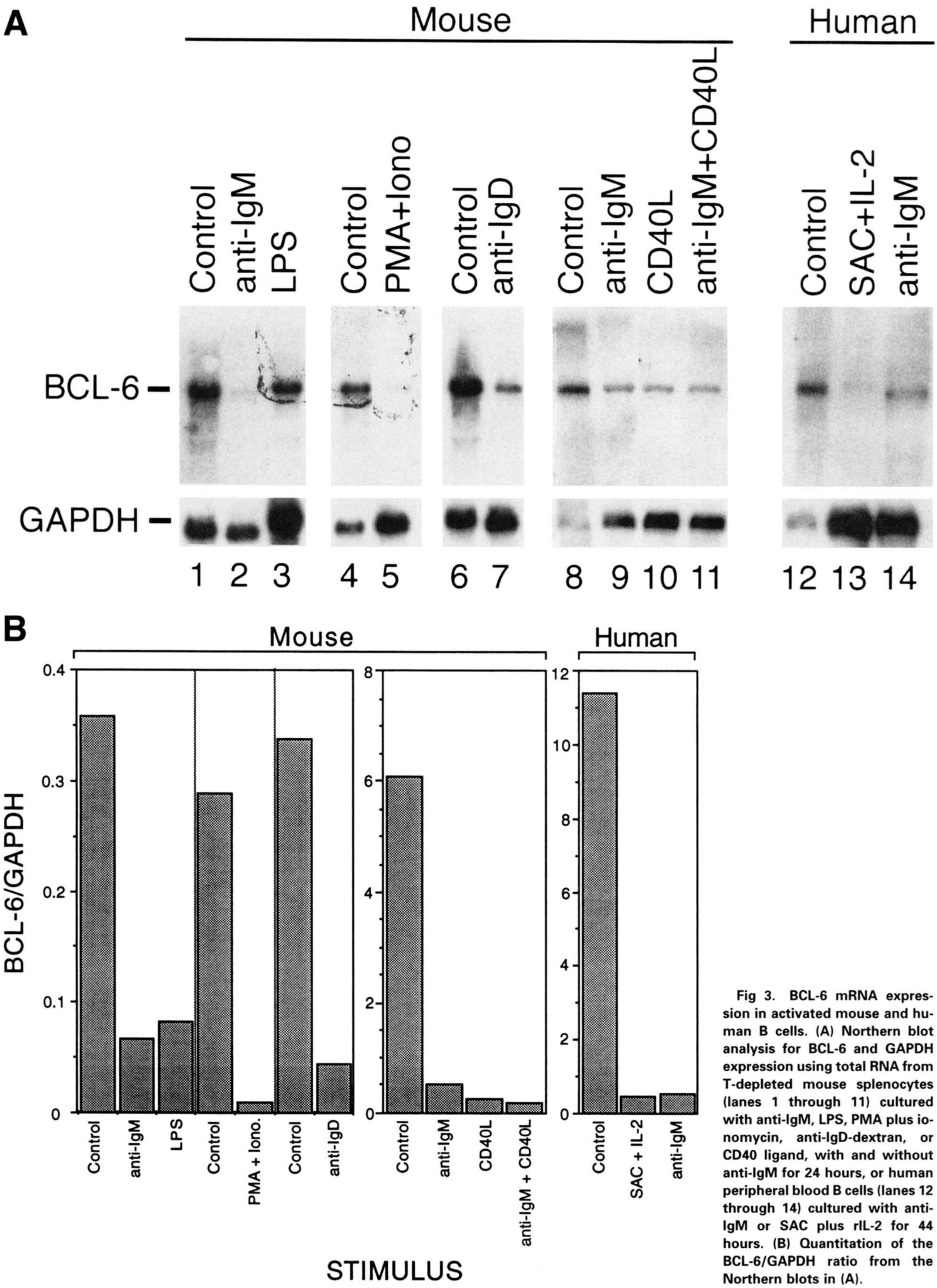

Fig 3. BCL-6 mRNA expression in activated mouse and human B cells. (A) Northern blot analysis for BCL-6 and GAPDH expression using total RNA from T-depleted mouse splenocytes (lanes 1 through 11) cultured with anti-IgM, LPS, PMA plus ionomycin, anti-IgD-dextran, or CD40 ligand, with and without anti-IgM for 24 hours, or human peripheral blood B cells (lanes 12 through 14) cultured with anti-IgM or SAC plus rIL-2 for 44 hours. (B) Quantitation of the BCL-6/GAPDH ratio from the Northern blots in (A).

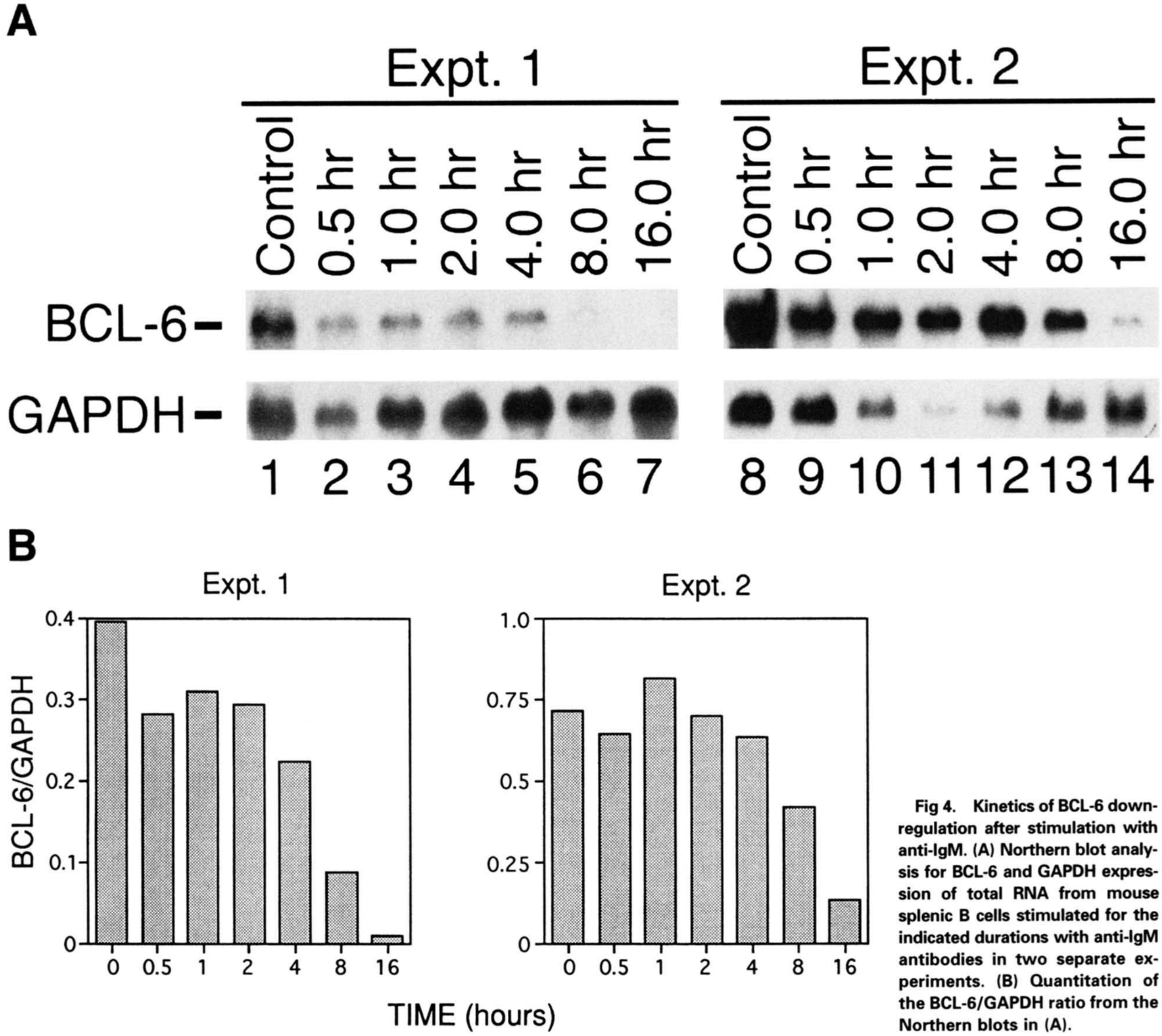

Fig 4. Kinetics of BCL-6 downregulation after stimulation with anti-IgM. (A) Northern blot analysis for BCL-6 and GAPDH expression of total RNA from mouse splenic B cells stimulated for the indicated durations with anti-IgM antibodies in two separate experiments. (B) Quantitation of the BCL-6/GAPDH ratio from the Northern blots in (A).

identity). This high degree of sequence homology suggests that the mouse and human proteins function analogously and, in particular, share equivalent sequence-specific DNA binding activity. The mouse BCL-6 cDNA was used to probe a Northern blot of mouse tissue mRNA and, as with human BCL-6, BCL-6 mRNA was detected at comparable levels in all tissues (data not shown).

To assess BCL-6 expression during B-cell activation, we purified B lymphocytes from mouse spleen and human peripheral blood, because the vast majority of lymphocytes in these sites are in the G0 phase of the cell cycle.[17,33] BCL-6 mRNA was readily detected in unstimulated mouse and human B cells (Fig 3A, lanes 1, 4, 6, 8, and 12). In vitro cultures of mouse B cells stimulated with a variety of mitogenic agents (anti-IgM antibodies, LPS, PMA plus ionomycin, anti-IgD antibodies coupled to dextran, CD40 ligand, or CD40 ligand plus anti-IgM antibodies) exhibited marked reductions in BCL-6 mRNA levels 24 hours after stimulation (Fig 3A). Quantitative analyses of these blots indicated that activation with these stimuli resulted in a 77% to 97% reduction in BCL-6 expression (Fig 3B). A similar reduction in BCL-6 expression was observed after 44 hours of stimulation of human B cells with SAC plus IL-2 or anti-IgM antibodies (Fig 3A). At this late time point after activation, the B cells should be cycling asynchronously, suggesting that BCL-6 mRNA levels remain reduced in G1, S, G2, and M phases of in vitro-activated B cells. However, we cannot rule out the possibility that BCL-6 might be transiently elevated during some phase of the cell cycle in these cultures.

To determine the kinetics of BCL-6 downregulation, we investigated BCL-6 mRNA expression at multiple time points after anti-IgM stimulation of mouse splenic B cells. BCL-6 expression declined markedly at 8 hours after stimulation and, by 16 hours, was reduced to 2% to 19% of the levels in resting lymphocytes (Fig 4A and B). The modest decrease in BCL-6 expression at the 0.5-, 1-, 2-, and 4-hour

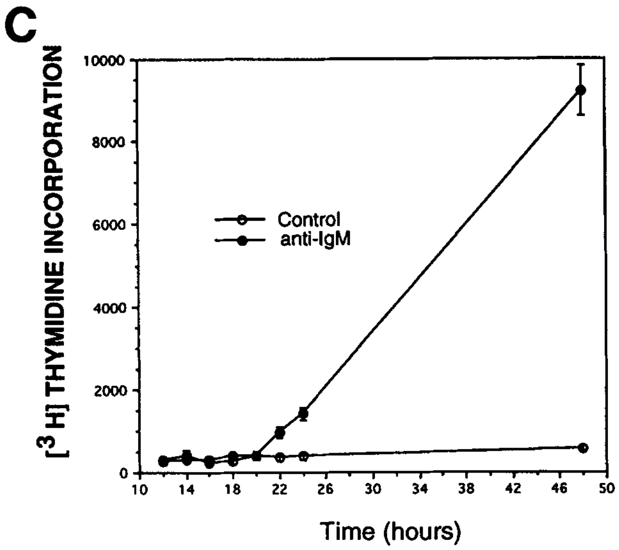

Fig 4. (cont'd). (C) DNA synthesis analysis of anti-IgM–stimulated mouse splenic B cells. B cells were cultured in quadruplicate without or with anti-IgM antibodies at 50 μg/mL for the indicated time periods. All cultures were pulsed with ^{3}H-thymidine for the final 4 hours of culture and harvested at the indicated time points.

time points in experiment 1 (Fig 4B) was not a consistent finding (Fig 4B, experiment 2). To determine the timing of the downregulation of BCL-6 relative to entry into S phase of the cell cycle, parallel cultures were pulsed for 4 hours at multiple time points after anti-IgM treatment. DNA synthesis began 20 to 22 hours after stimulation, 12 hours after BCL-6 mRNA levels began to decline, and 4 hours after BCL-6 expression was severely reduced (Fig 4C). Thus, during in vitro mitogenic activation of B cells, BCL-6 expression decreased during the mid-G1 phase of the cell cycle, before entry into S phase.

BCL-6 expression during T-lymphocyte activation. Because BCL-6 was expressed in human and mouse thymus (Fig 1A and data not shown), we considered the possibility that BCL-6 mRNA levels might be modulated after activation of T lymphocytes. To test this notion, resting human peripheral blood T cells were polyclonally activated with PHA. BCL-6 mRNA was detectable in resting T cells and persisted for 4 hours after PHA stimulation (Fig 5A). However, by 10 to 16 hours of culture with PHA, BCL-6 levels had declined markedly and remained low thereafter, up to and including 47 hours after stimulation. DNA content analysis indicated that S phase and G2/M phase were reached by 16 and 22 hours, respectively, and by 46 hours, the cells were apparently progressing through the cell cycle asynchronously (data not shown). These data show that, in T cells, as in B cells, BCL-6 was downregulated after mitogenic activation before entry into S phase.

Expression of BCL-6 mRNA and protein in activated germinal center B cells. T-cell–dependent activation of B cells leads to the formation of germinal centers.[34] Recent immunohistochemical analyses of human lymphoid tissues using anti–BCL-6 antibodies showed strong and selective expression of BCL-6 protein in germinal center B cells.[12-14] To understand the molecular basis of this selective expression, we compared BCL-6 mRNA levels in resting human peripheral blood B cells and activated germinal center B cells from human tonsils. Surprisingly, both B-cell subsets were found to express BCL-6 mRNA comparably (Fig 6B and C). Thus, antigen-stimulated B cells that enter the germinal center microenvironment do not downregulate BCL-6 mRNA expression as do B cells activated in vitro.

In contrast, germinal center B cells expressed considerably more BCL-6 protein than did resting B cells, as judged by Western blot analysis (Fig 6A, B, and C). BCL-6 protein was readily detectable in germinal center cells (Fig 6A) and in B-cell lines that express BCL-6 mRNA (Fig 6A, BJAB and RL), but not in B-cell lines that express little or no BCL-6 mRNA (Fig 6A, VDSO). The level of BCL-6 protein expression in germinal center cells and B-cell lines was roughly equivalent. The BCL-6 protein from germinal center cells migrated slower in SDS-PAGE gel than BCL-6 protein generated by in vitro translation (Fig 6A, compare lanes 5 and 6). Because phosphorylation has been shown to alter the mobility of BCL-6 protein (Onizuka et al[13] and A.D., unpublished observations), this finding suggests that BCL-6 is a phosphoprotein in germinal center B cells. However, the most interesting finding was that germinal center cells expressed threefold to 34-fold more BCL-6 protein than resting B cells, even though the BCL-6 mRNA levels were similar (Fig 6B and C). These findings showed that the elevated expression of BCL-6 protein in activated germinal center B cells is not primarily regulated by changes in steady-state mRNA levels.

DISCUSSION

We report here that expression of the proto-oncogene BCL-6 is modulated during mitogenic stimulation of lymphocytes in vitro. The highest levels of BCL-6 mRNA were observed in resting B and T lymphocytes. Stimulation of lymphocytes by diverse mitogenic agents led to profound decreases in BCL-6 expression in mid to late G1 phase. BCL-6 levels remained low thereafter, even in cultures of asynchronously dividing cells. By contrast, activated B lymphocytes derived from human germinal centers maintained high BCL-6 mRNA levels. Taken together, these findings suggest that the regulation of BCL-6 mRNA expression during lymphocyte activation depends critically on the nature of the activation signal. Furthermore, we have presented evidence that BCL-6 protein expression is regulated independently of mRNA expression to achieve selective and high-level expression of BCL-6 protein in germinal center B cells.

The first indication that BCL-6 expression might be regulated in B lymphocytes came from the observation that EBV-transformed LCLs did not express BCL-6, whereas Burkitt's lymphoma cell lines had high levels of BCL-6 mRNA. Indeed, three other independently isolated LCLs have been shown to lack BCL-6 mRNA.[2,8] LCLs differ from Burkitt's lymphoma lines in that LCLs express a set of EBV gene products that have been shown to transcriptionally activate a variety of cellular genes (for review see Klein[35]). In particular, LCLs express the EBV membrane protein LMP1 that

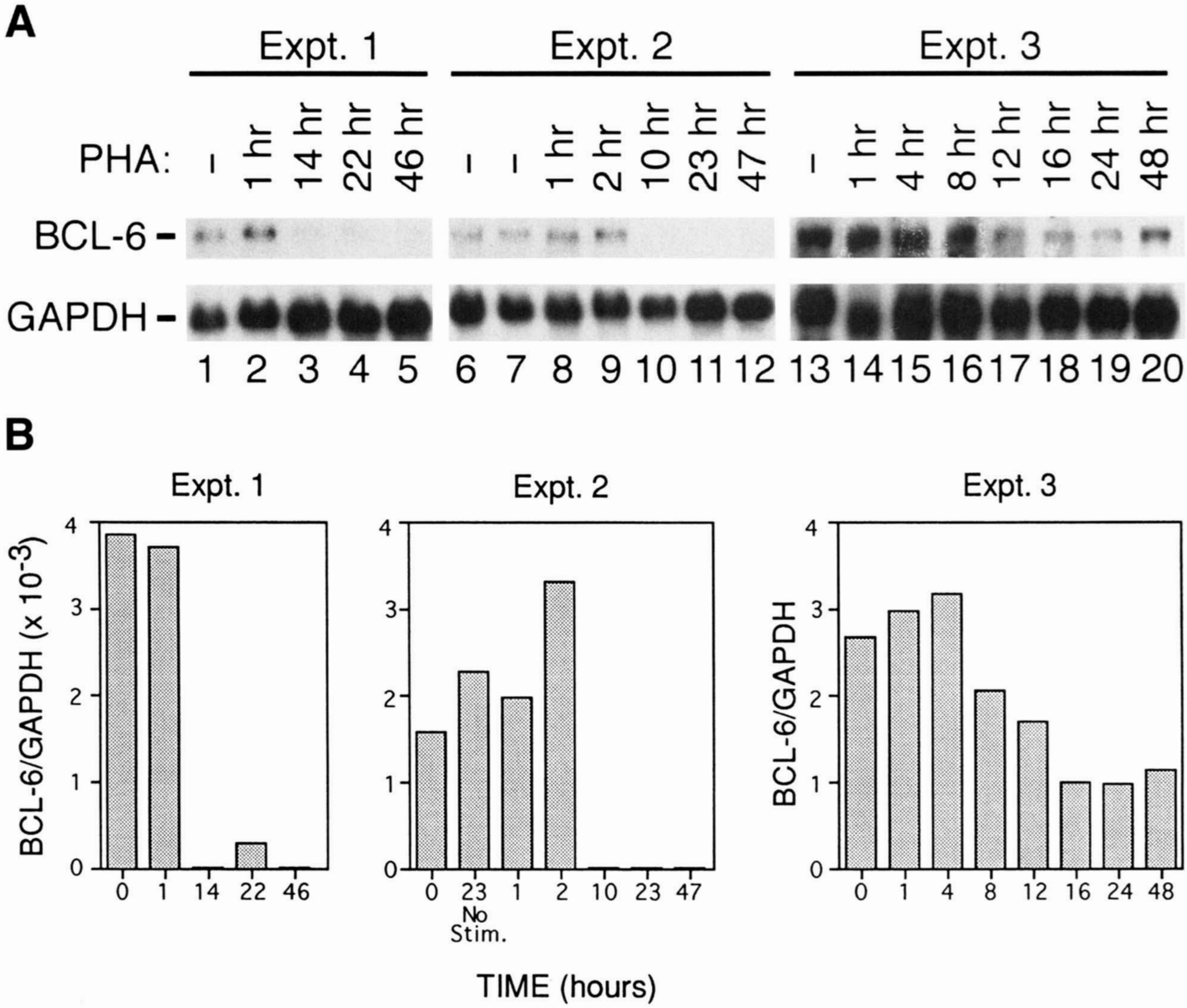

Fig 5. BCL-6 expression after stimulation of human T cells. (A) Northern blot analysis for BCL-6 and GAPDH expression using total RNA from human peripheral blood T cells cultured for the indicated durations with PHA. (B) Quantitation of the BCL-6/GAPDH ratio from the Northern blots in (A).

has been shown to induce the expression of a variety of membrane proteins that are normally found only on activated B lymphocytes.[31] LMP1 has been shown to activate NF-kB,[36,37] possibly by virtue of its association with a member of the TRAF protein family.[38] Transactivation of cellular genes in LCLs is mediated additionally by the EBNA2 protein that activates cellular promoters indirectly via a protein-protein interaction with the J_k signal recognition protein.[39,40] Additional evidence of regulated expression of BCL-6 came from the observation that anti-Ig treatment of Akata Burkitt's lymphoma cells caused BCL-6 mRNA levels to decrease. Akata cells triggered with anti-Ig antibodies activate the latent EBV genome and partially resemble LCLs in that they begin to express the latent membrane protein gene products, LMP1, LMP2A, and LMP2B.[32] However, activated Akata cells differ from LCLs in that they do not express EBNA2 and do express the several EBV gene products that initiate a lytic viral cycle.[32] Thus, it is not clear at present whether the downregulation of BCL-6 in LCLs and Akata cells proceeds by the same or distinct pathways. It will therefore be interesting to delineate which EBV gene products modulate BCL-6 expression as this may shed light on the signaling pathways that converge on the BCL-6 gene.

The stimuli that downregulated BCL-6 in lymphocytes act through distinct signaling pathways but were all mitogenic. B lymphocytes stimulated with anti-IgM, anti-IgD, PMA plus ionomycin, LPS, CD40 ligand, or SAC plus IL-2 had decreased BCL-6 levels, as did T lymphocytes stimulated with PHA. Signals induced in B cells by treatment with anti-IgM or LPS are mechanistically distinct in that anti-IgM triggering engages tyrosine kinases that are inhibited by herbimycin, whereas LPS signaling is not affected by this drug.[41] Nevertheless, anti-Ig and LPS-derived signals converge on common pathways such as activation of NF-kB.[42,43]

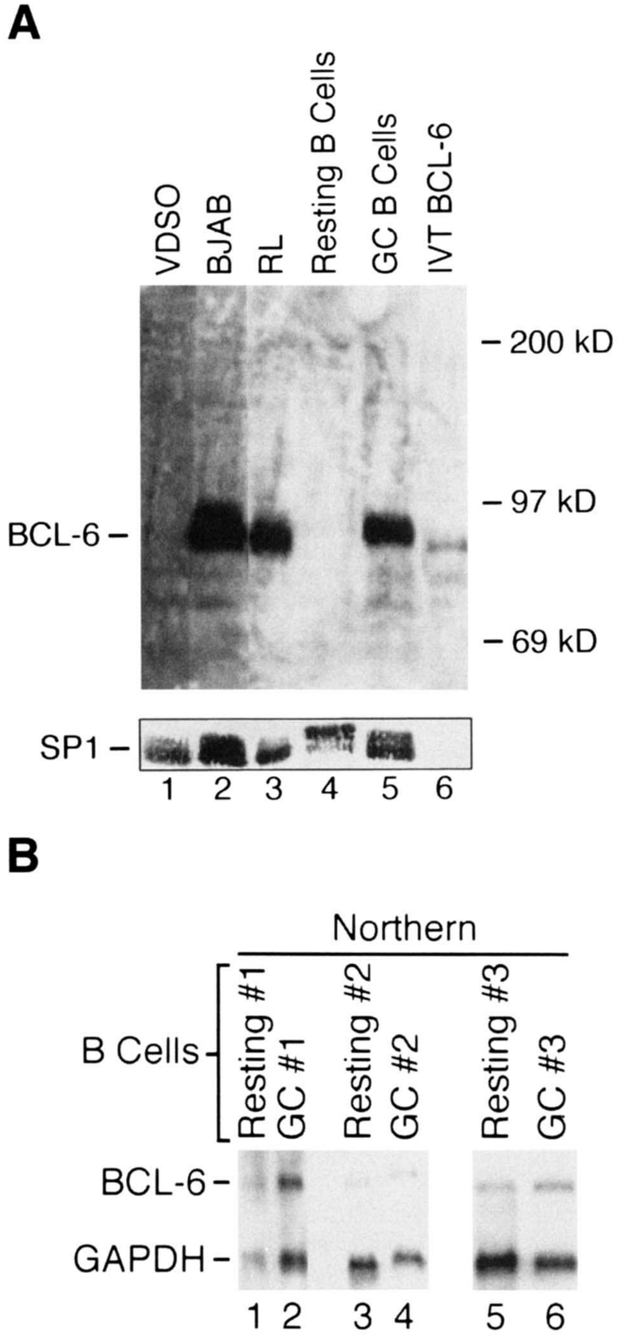

Fig 6. BCL-6 mRNA and protein levels in naive and germinal center B cells. (A) Western blot analysis of BCL-6 and Sp1 protein expression in purified human peripheral blood B cells (lane 4), human tonsillar germinal center B cells (lane 5), B-cell lines that express BCL-6 mRNA (BJAB and RL; lanes 2 and 3) or do not express BCL-6 mRNA (VDSO; lane 1), and in vitro-translated BCL-6 (lane 6). The blot was incubated with a polyclonal antibody directed against the amino-terminal 484 amino acids of BCL-6 and developed using an ECL kit. (B) Northern and Western analysis of BCL-6 mRNA and protein in purified human peripheral blood B cells (Resting #1 through 3) and human tonsillar germinal center B cells (GC #1 through 3). Each sample was derived from a different human donor. GAPDH mRNA expression was used for normalization of loading on the Northern blots and SP1 protein expression was used for normalization of loading on the Western blots. Western blots were incubated with an antipeptide antibody specific for BCL-6 and developed using radiolabeled antirabbit Ig antibodies.

The signaling pathways that are engaged on treatment of B cells with CD40 ligand have not been fully elucidated, although NF-kB is also activated.[44-46] Interestingly, both CD40 and LMP1 interact with a newly described member of the TRAF family of putative signal transduction proteins.[38,47,48] Because LMP1 is expressed in LCLs, it is conceivable that this protein may play a role in downregulating BCL-6 in LCLs as well as in CD40 ligand-stimulated cells. Finally, it is important to note that all of the signals that caused downmodulation of BCL-6 mRNA in vitro were mitogenic. The downmodulation of BCL-6 occurred 8 to 16 hours after stimulation and thus was not an immediate early event. Therefore, our working model is that BCL-6 regulation is a secondary effect of in vitro mitogenic signaling that is mediated indirectly by one or more of the immediate early transcription factors.

However, a striking dichotomy in our studies is that, although in vitro-activated B cells have low BCL-6 mRNA expression, activated B cells from human germinal centers expressed BCL-6 mRNA to the same degree as resting B lymphocytes. The germinal center is a complex microenvironment in which T-cell–dependent stimulation of B lym-

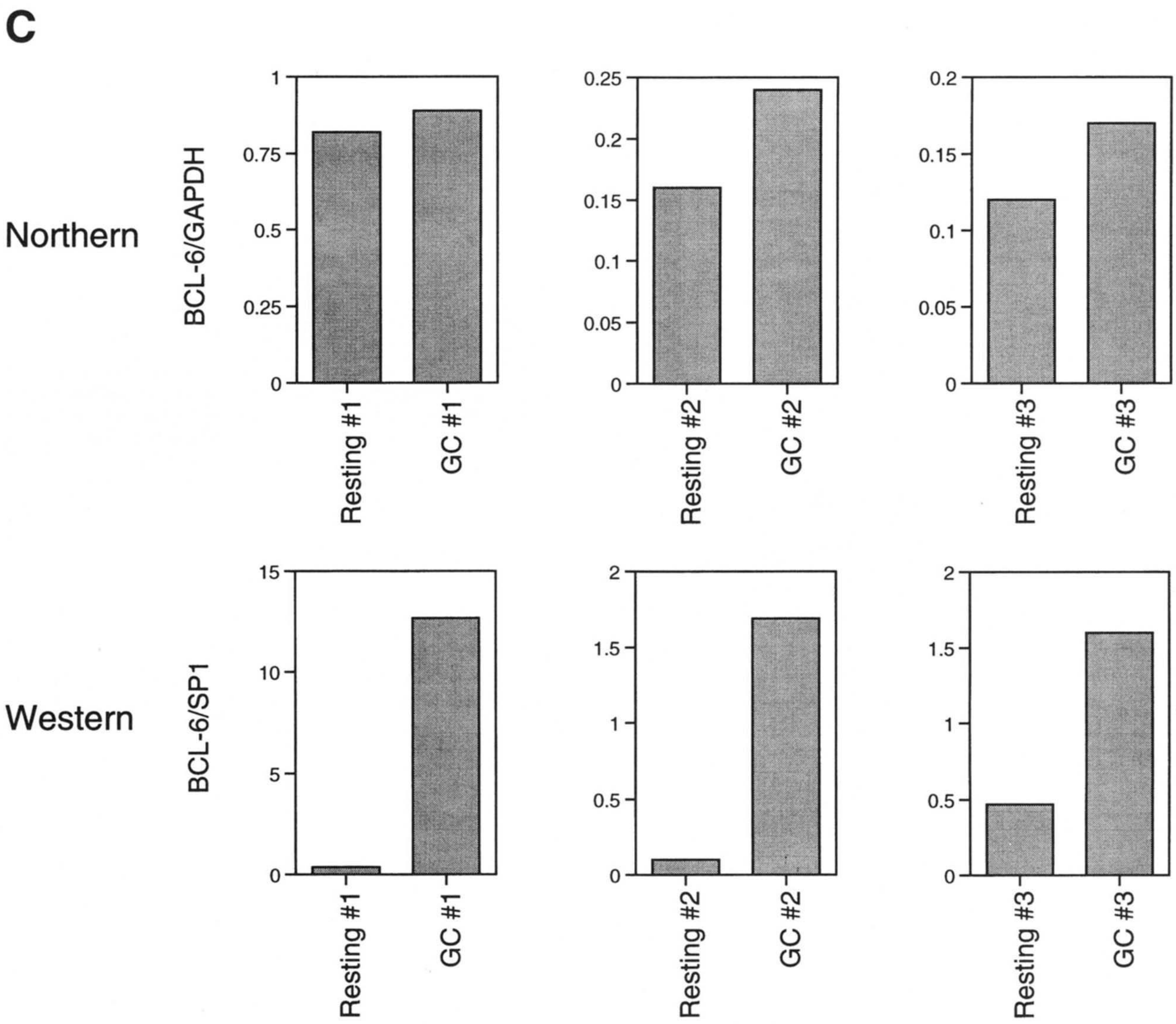

Fig 6. (cont'd). (C) Quantitative analysis of the Northern and Western blot data in (B) using a Phosphorimager.

phocytes leads to somatic hypermutation of Ig genes, Ig isotype class switching, and the generation of memory B cells and plasma cells.[34] Activation of B cells in germinal centers results from the interplay of three cell types: antigen-specific B cells, antigen-specific T cells, and follicular dendritic cells. Activated T cells display CD40 ligand on their cell surface and stimulate B cells through CD40. Indeed, ongoing CD40 signaling is necessary to maintain the germinal center response.[49] Interactions between B and T cells in the germinal center also include binding of B7-2 on the B-cell surface to CD28/CTLA-4 on T cells, and this signaling event is important for the generation of B-cell memory and somatic hypermutation of Ig genes.[49] Antigen present as immune complexes on the surface of follicular dendritic cells may signal B cells through their cell surface receptor.[34] Finally, it has been shown that soluble antigen can signal germinal center B cells through the cell surface Ig receptor to undergo apoptosis.[50,51] Thus, the signaling of B cells in germinal centers is multifactorial and not readily mimicked within in vitro cultures. Indeed, B cells stimulated in vitro through CD40 along with interleukin-4 are able to switch Ig isotype but do not allow somatic hypermutation of Ig genes.[52] In light of the complex nature of the germinal center microenvironment, it is less surprising that BCL-6 mRNA expression differed between B cells activated in vitro and B cells activated in germinal centers. Clearly, our in vitro culture conditions must lack critical coordinate signals that are present within the germinal center and serve to maintain BCL-6 mRNA expression during B-cell activation.

Quantitative analysis of BCL-6 protein levels revealed that purified human germinal center B cells expressed considerably more protein than resting human B cells purified from peripheral blood. This finding is in keeping with recent immunohistochemical studies of human lymphoid tissues that showed that BCL-6 protein was readily detectable in germinal center B cells but not in most resting B cells in the mantle and marginal zones.[12-14] However, an unanticipated finding was that the higher BCL-6 protein levels in germinal center cells could not be fully accounted for by increased mRNA expression. Thus, in some preparations of resting and germinal center B cells, BCL-6 protein levels were threefold to 34-fold higher in the germinal center B cells despite roughly comparable BCL-6 mRNA levels in the two cell populations. These data raise the possibility that regulation of mRNA levels may not be the only mechanism by which BCL-6 protein expression is upregulated in germinal center B cells. mRNA translation is regulated in many genes and can be mediated by binding of proteins to *cis*-acting RNA motifs in the untranslated regions of mRNAs.[53] In this regard, it is interesting that the untranslated regions of human and mouse BCL-6 mRNA have stretches of high sequence conservation (data not shown). Posttranslational protein modifications can affect steady-state protein levels by altering protein stability. It is therefore noteworthy that BCL-6 is a phosphoprotein,[13] because protein phosphoylation has been shown to alter the rate of degradation of some proteins.[54,55] Future analysis of BCL-6 mutants should show whether translational and posttranslational mechanisms govern the regulation of BCL-6 protein expression.

In summary, two modes of regulation are used to ensure high-level BCL-6 protein expression in germinal centers. First, in contrast to many modes of B cell activation in vitro, B-cell activation in germinal centers does not lead to the downmodulation of BCL-6 mRNA expression. Second, our data suggest that BCL-6 protein levels may be regulated independently of BCL-6 mRNA levels, resulting in higher BCL-6 protein levels in germinal center B cells than in resting B cells. Such complex regulation of BCL-6 expression during B-cell activation is consistent with a postulated important and selective function of BCL-6 during the germinal center reaction.

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