B Lymphocytes Differentially Use the Rel and Nuclear Factor κB1 (NF-κB1) Transcription Factors to Regulate Cell Cycle Progression and Apoptosis in Quiescent and Mitogen-activated Cells

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Summary

Rel and nuclear factor (NF)-κB1, two members of the Rel/NF-κB transcription factor family, are essential for mitogen-induced B cell proliferation. Using mice with inactivated Rel or NF-κB1 genes, we show that these transcription factors differentially regulate cell cycle progression and apoptosis in B lymphocytes. Consistent with an increased rate of mature B cell turnover in naive nfkb1$^{-/-}$ mice, the level of apoptosis in cultures of quiescent nfkb1$^{-/-}$, but not c-rel$^{-/-}$, B cells is higher. The failure of c-rel$^{-/-}$ or nfkb1$^{-/-}$ B cells to proliferate in response to particular mitogens coincides with a cell cycle block early in G1 and elevated cell death. Expression of a bcl-2 transgene prevents apoptosis in resting and activated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells, but does not overcome the block in cell cycle progression, suggesting that the impaired proliferation is not simply a consequence of apoptosis and that Rel/NF-κB proteins regulate cell survival and cell cycle control through independent mechanisms. In contrast to certain B lymphoma cell lines in which mitogen-induced cell death can result from Rel/NF-κB–dependent downregulation of c-myc, expression of c-myc is normal in resting and stimulated c-rel$^{-/-}$ B cells, indicating that target gene(s) regulated by Rel that are important for preventing apoptosis may differ in normal and immortalized B cells. Collectively, these results are the first to demonstrate that in normal B cells, NF-κB1 regulates survival of cells in G0, whereas mitogenic activation induced by distinct stimuli requires different Rel/NF-κB factors to control cell cycle progression and prevent apoptosis.

The outcome of lymphocyte responses to antigenic or mitogenic activation reflects a balance between the relative rates of cell division, death, and differentiation (1). Although signal transduction pathways that control cell survival and proliferation are the subject of intense investigation, in mature B cells little is known about the role of transcription factors in regulating these processes. Among the many transcription factors implicated in controlling gene expression in B lymphocytes (2), the Rel (3) and nuclear factor (NF)-κB1 (4, 5) subunits of the Rel/NF-κB family have recently been shown to be essential for mitogen-induced proliferation. Rel/NF-κB transcription factors are homo- and heterodimeric proteins comprising subunits encoded by a small multigene family related to the *c-rel* protooncogene (6). These proteins regulate transcription by binding to decameric sequences (κB motifs) located in the promoters and enhancers of many viral and cellular genes, particularly those encoding proteins involved in immune, acute phase, and inflammatory responses (6–8). The five known mammalian Rel/NF-κB proteins, NF-κB1 (p50, p105), NF-κB2 (p52, p100), RelA (p65), RelB, and Rel share a highly conserved 300–amino acid NH_2-terminal Rel homology domain that encompasses sequences required for DNA binding, protein dimerization, and nuclear localization (6). The CH_2-termini of these proteins are divergent, with those of Rel, RelA, and RelB containing transcriptional transactivation domains (6, 9). Before stimulation, in most cells, a large proportion of Rel/NF-κB is retained in the cytoplasm in an inactive form through association with a family of inhibitor (IκB) proteins (10, 11). A wide range of stimuli promote the nuclear translocation of Rel/NF-κB complexes by a mechanism involving the phosphorylation of conserved NH_2-terminal serine residues in IκBα and IκBβ (12, 13), which targets these IκB proteins for ubiquitin-dependent proteosome-mediated degradation (12–15).

To determine the physiological roles of the various Rel/

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NF-κB proteins, mice with inactivated *c-rel* (3), *nfkb1* (4), *relb* (16, 17), or *rela* (18) genes have been generated by gene targeting. rela$^{-/-}$ mice die at day 15 of embryogenesis, apparently as a result of fetal hepatocyte apoptosis (18). In contrast, Rel (3), RelB (16, 17), and NF-κB1 (4) are not essential for embryogenesis, but are important in the function of hemopoietic cells. Although differentiation of stem cells into all hemopoietic lineages appears normal in c-rel$^{-/-}$ and nfkb1$^{-/-}$ mice, Rel and NF-κB1 are involved in controlling genes induced during immune responses. Mature B cells from both mutant strains are defective in their response to mitogens and antigens (3, 4), whereas T cells and macrophages from c-rel$^{-/-}$ mice exhibit defects in the production of cytokines and immune regulatory molecules, including IL-2, IL-3, IL-6, GM-CSF, G-CSF, TNF-α, and iNOS (19, 20). RelB appears to serve a dual role. Naive RelB$^{-/-}$ mice spontaneously develop hemopoietic lesions characterized by multifocal inflammatory infiltrates, myeloid hyperplasia, and splenomegaly (16, 17). Although this phenotype suggests that RelB is important in regulating genes in hemopoeitic cells that are associated with constitutive or housekeeping functions, RelB$^{-/-}$ mice challenged with pathogens reveal that RelB is also involved in various specific and nonspecific immune responses (17).

Rel/NF-κB proteins have also been implicated in the control of apoptosis. For example, loss of RelA renders embryonic fibroblasts highly sensitive to TNF-α–induced apoptosis (21) and expression of a *trans*-dominant IκBα mutant in various cell types markedly increases the death of these cells when treated with TNF-α, ionizing radiation, or daudorubicin (22, 23). Although enforced expression of Rel can overcome TNF-α–induced apoptosis of HeLa cells expressing the *trans*-dominant IκBα mutant (24), Rel has also been implicated as an inducer of cell death. Overexpression of Rel induces apoptosis in chicken bone marrow cells (25) and high levels of c-rel messenger RNA (mRNA)[1] were found in cells undergoing programmed cell death in the developing chick embryo (25).

Although Rel and NF-κB1 have been shown to be important in mitogen-induced B cell proliferation (3, 4), it is not known whether these proteins are needed for cell cycle progression, inhibition of apoptosis, or both. Here we examine in detail defects in proliferation and cell survival of B cells from c-rel$^{-/-}$ and nfkb1$^{-/-}$ mice. The results show that different mitogenic signals require Rel and/or NF-κB1 to promote G1 progression during B cell division and that specific NF-κB–like factors are necessary for the survival of quiescent and mitogen activated B cells.

Materials and Methods

Mice. The generation of the c-rel$^{-/-}$ (3) and nfkb1$^{-/-}$ (4) mice and Eμ-bcl-2-36 transgenic mice, which express a human bcl-2 cDNA in B and T cells (26), has been described previously. All mouse strains had been backcrossed for >9 generations with C57BL/6 mice before being used for this study. c-rel$^{-/-}$bcl-2^{+} and nfkb1$^{-/-}$bcl-2^{+} mice were generated by intercrossing the two parental mouse strains. Inheritance of the *c-rel* and *nfkb1* mutant alleles and the bcl-2 transgene was determined by PCR analysis of tail biopsy DNA samples using specific oligonucleotide primers.

Purification of B Lymphocytes. Small, resting B lymphocytes were purified from spleens of 4–6-wk-old wild-type c-rel$^{-/-}$, c-rel$^{+/-}$, or nfkb1$^{-/-}$ litter-matched mice by negative cell sorting. In brief, all unwanted cells (granulocytes, macrophages, erythroid cells, and T lymphocytes) were stained with FITC-labeled surface marker specific monoclonal antibodies (8C5 anti-Gr-1, M1/70 anti-Mac-1, Ter119, T24.31.2 anti-Thy1, KT3 anti-CD3, GK1.5 anti-CD4, and 53.6.72 anti-CD8) and dead cells were stained with the vital dye propidium iodide (PI; 1 μg/ml). Viable cells that were not stained with the FITC-labeled monoclonal antibodies were purified on a FACS® II or a FACStar Plus® cell sorter (Becton Dickinson, San Jose, CA). The purity of all sorted B cells was verified by staining cells with a phycoerythrin labeled anti-B220 antibody (CALTAG Labs., S. San Francisco, CA) and ranged between 97 and 99% purity.

B Lymphocyte Activation in Tissue Culture. B lymphocytes were cultured in the high glucose version of DMEM supplemented with 13 μM folic acid, 250 μM l-asparagine, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum at an initial concentration of 3×10^5/ml. Cells were stimulated in vitro with LPS (Difco, Detroit, MI) at a concentration of 20 μg/ml, affinity purified goat anti–mouse IgM $(Fab')_2$ fragments (Jackson Immuno Research, Bar Harbor, ME) at 20 μg/ml, rat anti–mouse CD40 monoclonal antibody FGK45 (a gift of Dr A. Rolink, Basel Institute, Basel, Switzerland) at 2 μg/ml, or rat anti–mouse RP monoclonal antibody at 1 μg/ml (27). All stimulations were performed in the absence or presence of saturating concentrations of recombinant mouse interleukins, IL-2, IL-4, and IL-5.

Cell Turnover Studies. Cell turnover was determined by labeling proliferating cells in vivo with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO), which was provided continuously for 1, 3, or 7 d in the drinking water (1 mg/ml plus 2% glucose to overcome taste aversion). Drinking water bottles were shielded from light and exchanged after 3 days. Spleen cellularity did not alter significantly during the course of this treatment. BrdU incorporated into cellular DNA was detected by immunofluorescence staining with an FITC-labeled monoclonal antibody BU-1 (Becton Dickinson) and flow cytometric analysis performed according to published procedures (28). In brief, cells were fixed in 0.5% paraformaldehyde in PBS (20 min at room temperature). As the BU-1 antibody only binds to BrdU within single-stranded DNA, the DNA was denatured using 3N HCl containing 0.5% Tween-20 to permeabilize cell membranes (20 min at room temperature). The acid was neutralized with 0.1 M disodiumtetraborate and cells were then stained (30 min) with FITC–BU-1 in the presence of 0.5% Tween-20. Combined analysis of incorporated BrdU and cell surface antigen expression to identify virgin and mature B cells was performed using a modified version of published procedures (28). R-PE anti-IgM (CALTAG Labs.) and biotinylated anti-IgD (clone 11-26C) antibodies plus Tricolor-streptavidin (CALTAG Labs.) were used to identify virgin B cells (IgM^{+}IgD^{-}) and mature B cells (IgM^{+}IgD^{+}). Cells were stained with biotinylated antibodies and fluorochrome conjugates after fixation and DNA denaturation with the exception of biotinylated anti-IgD, which had to be added beforehand. Flow cytometric analysis

[1]*Abbreviations used in this paper:* BrdU, 5-bromo-2-deoxyuridine; mRNA, messenger RNA; PI, propidium iodide.

(10,000 cells/sample) was performed on a FACScan®. As a negative control for staining with FITC–BU-1 anti-BrdU antibody, we included cells from a mouse that had not received BrdU in the drinking water in all experiments. Routinely, <1% of such cells were stained above background. To verify that mice had incorporated BrdU and as a positive control for staining with FITC-BU-1, we routinely confirmed that cells with a high turnover rate, such as granulocytes within bone marrow, were strongly labeled.

Proliferation and RNA Synthesis Assays. B cells were cultured as described above in 100 μl at a starting density of 3×10^5 cells/ml in 96-well microtiter plates. Cellular proliferation and RNA synthesis were measured at various times by adding 0.5 μCi of [^{3}H]thymidine or 0.25 μCi of [^{3}H]uridine, respectively, for 6 h, after which cells were harvested onto glass fiber filters. Incorporated radioactivity was quantitated by scintillation counting.

Immunofluorescence Staining and Flow Cytometry. The expression of class II MHC, B7.2, and CD25 was analyzed on resting and anti-IgM– or anti-CD40–treated splenic B cells were stimulated for 24 h. Cells were surface stained with biotinylated monoclonal antibodies M5/114 (anti-class II MHC), GL-1 (anti-B7.2), PC16 (anti–IL-2 receptor α chain CD25), or isotype-matched control monoclonal antibodies as described previously (3). Binding of biotinylated antibodies was revealed by staining with R-phycoerythrin-streptavidin (CALTAG Labs.) as the secondary reagent. Viable (PI negative) cells (5,000–10,000) were analyzed in a FACScan® flow cytometer (Becton Dickinson). For the coculture experiments, c-rel$^{-/-}$ (Ly5.1) cells were separated from control c-rel$^{+/+}$ (Ly5.2) cells by cell sorting after staining with a biotinylated anti-Ly5.2 monoclonal antibody plus R-PE streptavidin.

Cell Cycle Analysis. Cell division in mitogen stimulated lymphocyte cultures was determined by assessing cellular DNA content using PI staining (29). Cells were first fixed (>8 h at 4°C) in 70% ethanol, treated with 0.5 μg/ml DNase-free RNase A (Boehringer Mannheim, Indianapolis, IN) for 20 min at room temperature, and finally stained with 69 μM PI in 0.1 M sodium citrate (pH 7.4) for 30 min at 4°C. Flow cytometric analysis (10,000 cells/sample) was performed on a FACScan® at a low flow rate (100–300 cells/s). Cell cycle distribution was determined with the Cellfit program (Becton Dickinson) or manual gating.

Analysis of DNA and Protein Content. Simultaneous analysis of cellular DNA and protein content was performed as described previously (30). In brief, 5×10^5 resting or mitogen-activated B cells were first fixed in ice-cold 70% ethanol (>8 h), washed once in phosphate-buffered saline, and resuspended at a cell density of $\sim 10^6$ cells/ml in PBS containing 0.5 μg/ml Hoechst 33342 (HO; Calbiochem Corp., La Jolla, CA), 1 μg/ml pyronin Y (Polysciences, Warrington, PA), and 0.1 μg/ml FITC (isomer 1; BBL Microbiology Systems, Cockeyville, MD). Cells were stained for 30 min and then analyzed by flow cytometry using a modified dual lasar FACS® II. Dead cells were excluded from analyses by gating on forward and side light scatter parameters. A total of 10,000 events were collected and analyzed using an in house computer program.

Northern Blot Analysis. 10 μg samples of total RNA isolated from resting or anti-IgM antibody-activated B cells were fractionated on 1% formaldehyde agarose gels, transferred onto Hybond C Extra (Amersham Corp., Arlington Heights, IL) and sequentially hybridized as previously described (31) with a ^{32}P-radiolabeled mouse 1.4-kb XhoI c-myc cDNA (32) and a rat 1.1kb PstI glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA (33). Both probes were labeled by random primer extension to specific activities ranging between 5×10^8 and 10^9 cpm/μg.

Results

Apoptosis of Quiescent B Cells Is Accelerated by the Loss of NF-κB1, but Not by an Absence of Rel. To assess whether Rel and NF-κB1 have a role in regulating the survival of primary B cells, we first compared the rate of B cell turnover in normal, c-rel$^{-/-}$ and nfkb1$^{-/-}$ mice. Splenic B cells isolated from mice administered BrdU in the drinking water continuously for up to 7 d were analyzed for BrdU incorporation using flow cytometry (Fig. 1 *A*). Although no significant difference was observed in the rate of BrdU incorporated by surface (s)IgM$^+$sIgD$^-$ or sIgM$^+$sIgD$^+$ splenic B cells after 2 d, by days 4 and 7 of this kinetic analysis, nfkb1$^{-/-}$ B cells had incorporated between two- and fourfold more BrdU than either normal or c-rel$^{-/-}$ B cells. To determine if the increased turnover of nfkb1$^{-/-}$ B cells in vivo could be due to enhanced cell death, the survival of resting splenic B cells from mice of each strain were examined in tissue culture (Fig. 1 *B*). As expected, normal B cells did not proliferate in the absence of mitogens (results not shown), and a significant proportion had died by apoptosis over the course of 72 h. c-rel$^{-/-}$ B lymphocytes behaved indistinguishably from control cells, whereas the frequency of apoptotic cells was higher in cultures of quiescent nfkb1$^{-/-}$ B cells. Collectively these findings suggest that the survival of resting B cells is dependent on NF-κB1, but not Rel.

Rel and NF-κB1 Are Required for Cell Cycle Progression and Survival of Mitogen-stimulated B Cells. To determine if the role of Rel and NF-κB1 in B cell proliferation involves the regulation of cell division and/or apoptosis, we performed cell cycle and cell death analysis on mitogen-activated splenic B cells from c-rel$^{-/-}$, nfkb1$^{-/-}$, and control mice. Purified B lymphocytes were stimulated with LPS or cross-linking antibodies specific for IgM or the B cell–specific membrane protein, RP (27) in the presence or absence of cytokines. Cell proliferation and apoptosis were assessed daily over a 72-h time course by cell counting, [^{3}H]thymidine incorporation, and flow cytometric analysis of cellular DNA content. The proliferation, apoptosis, and cell division data for all mitogenic stimulations is graphically summarized in Fig. 2. The first 24 h of a normal mitogenic response is characterized by apoptosis and a low level of cell division. The extent of cell death is dependent on the stimulus, varying between 15 (LPS) and 44% (anti-IgM antibody) (Fig. 2 *B*). Between 24 and 72 h, a reduction in the frequency of dead cells coincides with an increase in viable cells due to division (Fig. 2, *A* and *C*; [^{3}H]thymidine incorporation and the frequency of cells residing in S, and G2/M, respectively). Consistent with previous findings, c-rel$^{-/-}$ (3) and nfkb1$^{-/-}$ (4, 5) B cells exhibit overlapping, but distinct, defects in mitogen responsiveness. c-rel$^{-/-}$ B cells proliferate poorly in response to all single mitogens, whereas nfkb1$^{-/-}$ B cells react normally to anti-IgM antibody, but show diminished (10-fold) responsiveness to anti-RP antibody and respond poorly to LPS (3–5% of normal [^{3}H]thymidine incorporation). A combination of the cytokines IL-2, IL-4, and IL-5, which alone do not induce B cell prolifera-

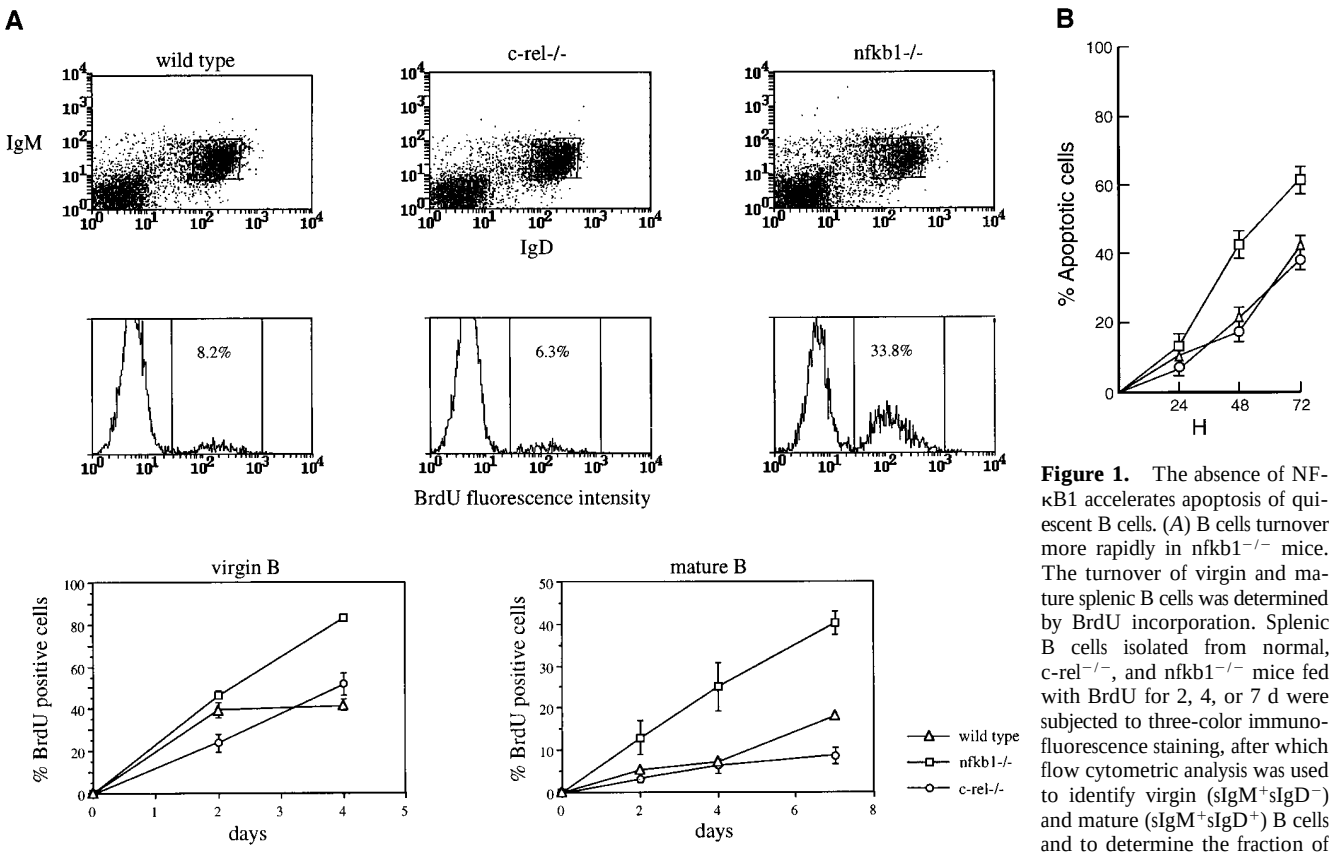

Figure 1. The absence of NF-κB1 accelerates apoptosis of quiescent B cells. (*A*) B cells turnover more rapidly in nfkb1$^{-/-}$ mice. The turnover of virgin and mature splenic B cells was determined by BrdU incorporation. Splenic B cells isolated from normal, c-rel$^{-/-}$, and nfkb1$^{-/-}$ mice fed with BrdU for 2, 4, or 7 d were subjected to three-color immunofluorescence staining, after which flow cytometric analysis was used to identify virgin (sIgM^{+}sIgD^{-}) and mature (sIgM^{+}sIgD^{+}) B cells and to determine the fraction of these cells that had incorporated BrdU. Examples from the analysis of mice fed BrdU for 4 d are shown. The top row shows two-color FACS® dot plots (*x-axis*, staining with R-PE–labeled anti-IgM antibodies; *y-axis*, staining with biotinylated anti-IgD antibodies plus Tricolor-streptavidin), with the boxed regions used for electronic gating of mature B cells. The middle rows present histograms of the anti-BrdU–labeling intensity (FITC) of these mature B cells. The percentages of BrdU^{+} cells are indicated as are the boundaries used for distinguishing them from BrdU^{-} cells. The bottom panels summarize the kinetics of BrdU labeling for virgin and mature splenic B cells over a 4- and 7-d time course, respectively. The values are arithmetic means plus SD from the analysis of three normal, c-rel$^{-/-}$, and nfkb1$^{-/-}$ mice at each time point. (*B*) Cell death in culture. Resting splenic B cells from normal (*triangles*), c-rel$^{-/-}$ (*circles*), and nfkb1$^{-/-}$ (*squares*) mice were cultured in DMEM/10% FCS without mitogen for a period of 72 h. At 24-h intervals, the frequency of dead cells was determined by trypan blue exclusion and flow cytometric analysis of fixed cells stained with PI. At the start of the experiment, >99% of cells of all genotypes were viable. The data represents the mean ±SD of five experiments.

tion, but instead maximize the response of normal B cells to mitogens (34), only rescued some c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cell mitogenic responses such as the anti-RP antibody stimulation of Rel$^{-/-}$ cells.

Impaired c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cell proliferation coincides with higher than normal levels of apoptosis (Fig. 2 *B*) and reduced cell division (Fig. 2 *C*), the extent of which both vary in a mitogen-specific manner. For example, after 72 h, 93 and 60% of anti-IgM– and anti-RP–stimulated c-rel$^{-/-}$ cells, respectively, have undergone apoptosis compared to 20 and 18% of normal B cells. Of the remaining viable B cells, <0.5% of anti-IgM or anti-RP antibody-stimulated c-rel$^{-/-}$ cells are in S or G2/M (compared with 30 and 37% of normal cells). LPS stimulation leads to the death of 34 and 44% of c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells, respectively, after 72 h (compared with 11% of normal cells), with 10- and 6-fold fewer of the viable c-rel$^{-/-}$ (3.5%) and nfkb1$^{-/-}$ (5.5%) cells, respectively, residing in S or G2/M compared to normal B cells (35%). Cytokines enhance proliferation of c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells by increasing cell division and reducing apoptosis (see Fig. 2 *C*).

Increased Apoptosis and Impaired Division of Mitogen-stimulated c-rel$^{-/-}$ B Lymphocytes Is Cell Autonomous. As previous studies have demonstrated that Rel is a crucial regulator of several growth factor and cytokine genes (3, 19, 20), we determined if the impaired cell division and increased apoptosis of mitogen-stimulated c-rel$^{-/-}$ B cells resulted from an inability to produce an autocrine growth factor or the expression of a growth inhibitory activity. This was assessed by comparing apoptosis and cell cycle progression in anti-IgM or anti-RP antibody-stimulated cocultures of Ly5.1 c-rel$^{-/-}$ and Ly5.2 c-rel$^{+/+}$ B cells. The results in Fig. 3 show that c-rel$^{+/+}$ and c-rel$^{-/-}$ cells proliferate or die by apoptosis at the same rate, regardless of whether the cells are grown in mixed or separate cultures. Reduced cell division and increased apoptosis of LPS-stimulated nfkb1$^{-/-}$ B cells was also shown not to be influenced by the presence of normal cells (Grumont, R.J., results not shown). These

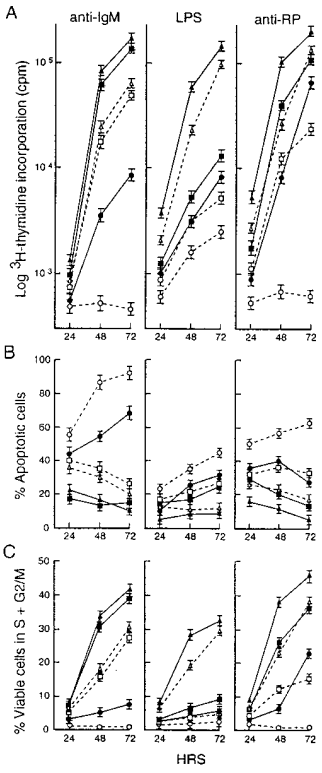

Figure 2. Cell cycle progression is impaired and apoptosis increased in mitogen activated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells. Purified, resting normal (*triangles*), c-rel$^{-/-}$ (*circles*), or nfkb1$^{-/-}$ (*squares*) B cells were stimulated for 72 h with optimal concentrations of anti-IgM antibodies, LPS, or anti-RP antibodies in the absence (*open symbols*) or presence (*closed symbols*) of IL-2, IL-4, and IL-5. (*A*) Cellular proliferation. B cell proliferation was measured at 24-h intervals over a 72-h period by [^{3}H]thymidine incorporation. (*B*) Cell death. The frequency of apoptotic cells, expressed as a proportion of the total cell number, was determined by flow cytometric analysis of permeabilized cells stained with PI. Greater than 99% of resting B cells were viable at the start of the experiment. (*C*) Cell cycle analysis. The fraction of viable B cells in the S, G2, or M phases of the cell cycle expressed as a function of the stimulation period was determined by flow cytometric analysis of permeabilized cells stained with PI. Less than 1% of cells were in G1, S, or G2/M before stimulation. All results in this figure represent the mean ±SD from eight experiments.

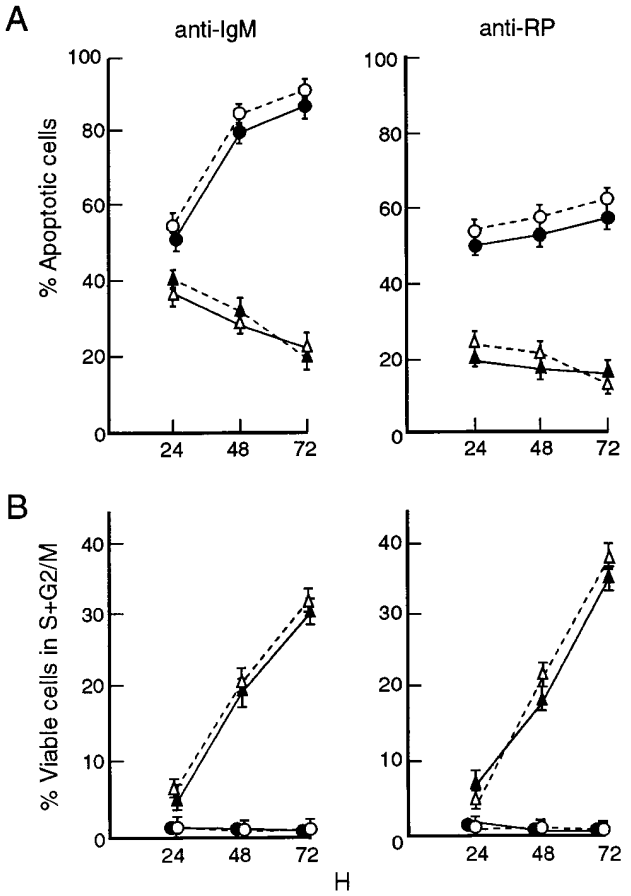

Figure 3. The cell cycle block and enhanced apoptosis in mitogen-stimulated c-rel$^{-/-}$ B lymphocytes are due to a cell autonomous defect. Equivalent numbers of resting c-rel$^{+/+}$ Ly5.2 (*triangles*) and c-rel$^{-/-}$ Ly5.1 (*circles*) B cells were stimulated separately (*open symbols*) or in mixed cultures (*closed symbols*) with anti-IgM or anti-RP antibodies over a period of 72 h. At 24-h intervals, normal and Rel$^{-/-}$ cells from individual cultures or isolated from the mixed cultures by cell sorting were fixed, stained with PI, and the DNA content determined by flow cytometric analysis. The frequency of viable normal and c-rel$^{-/-}$ cells in G0 before stimulation was >99%. (*A*) Levels of apoptosis in B cell populations from the separate or mixed cultures are equivalent. The frequency of apoptotic cells is expressed as a proportion of the total number of B cells of each genotype. (*B*) c-rel$^{-/-}$ B cell division is not influenced by normal cells. The percentage of cells in the S plus G2/M phases of the cell cycle correspond to that fraction of the total viable B cell population in each culture. These results are representative of the mean of four independent experiments.

results establish that the cell cycle block and increased cell death in mitogen stimulated c-rel$^{-/-}$ B cell cultures is due to a cell autonomous defect that does not involve autocrine growth factors.

Mitogen-stimulated c-rel$^{-/-}$ B Cells Undergo Some Early Activation Steps. Mitogenic activation of B lymphocytes is associated with early changes in several biochemical and cellular parameters, including increased cell size, elevated RNA synthesis, and modified expression of cell surface proteins (35). We investigated which of the early activation events are affected by the absence of Rel. Normal and c-rel$^{-/-}$ B cells stimulated for 24 h with mitogens and cytokines were stained with antibodies specific for various B cell surface markers and then examined by flow cytometry. A representative sample of data from cells stimulated with anti-IgM or anti-CD40 antibodies plus cytokines (IL-2, IL-4, and IL-5) is shown in Fig. 4. Forward scatter profiles indicate that although c-rel$^{-/-}$ B cells enlarge during the initial 24 h of activation, it is to a lesser extent than normal B cells. The changes in cell surface marker expression are complex. B7.2 is upregulated normally on both anti-IgM and anti-CD40 antibody-stimulated c-rel$^{-/-}$ cells, but CD25 expression is only weakly elevated on anti-IgM antibody-treated c-rel$^{-/-}$ cells. This difference appears to be mitogen-specific, as no significant difference in CD25 induction is seen on anti-CD40 antibody-stimulated normal or c-rel$^{-/-}$ cells. MHC class II expression is increased on c-rel$^{-/-}$ B cells by mitogen treatment, but to a lesser extent than on normal B cells. The expression of all other cell surface markers that were examined on c-rel$^{-/-}$ lymphocytes was normal (Strasser, A., results not shown) as were those expressed on mitogen-stimulated nfkb1$^{-/-}$ B cells (Gerondakis, S., unpublished results). These results indicate that c-rel$^{-/-}$ B cells can undergo some early events associated with mitogen-induced activation, but remain blocked at a stage characterized by a cell volume that is intermediate between the resting and the cycling state.

The Cell Cycle Block in Mitogen-activated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B Cells Occurs Early in the G1 State. The low frequency of

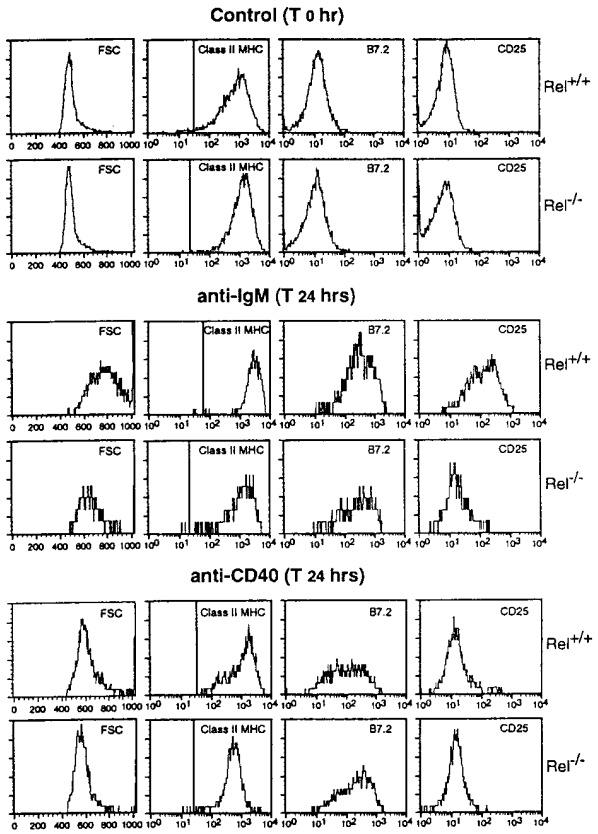

Figure 4. Mitogen stimulation of c-rel$^{-/-}$ B cells leads to changes in cell size and the expression of certain cell surface markers. Normal and Rel$^{-/-}$ splenic B cells in the resting state (*T 0*), treated with anti-IgM, or treated with anti-CD40 antibodies for 24 h were stained with biotinylated monoclonal antibodies specific for class II MHC, B7.2, and the IL-2Rα chain (CD25) and examined by flow cytometry. *FSC*, the forward light scatter profiles (x-axis: log scale), is a measure of cell size. The vertical lines shown in the class II MHC panels depict the fluorescence intensity of cells stained with a biotinylated isotype-matched control antibody. Data shown are electronically gated to exclude dead cells and are representative of three experiments.

anti-IgM or -RP antibody-treated c-rel$^{-/-}$ and LPS-stimulated nfkb1$^{-/-}$ B cells in S and G2/M indicated that these cells appear to be blocked at an earlier point in the cell proliferative cycle. Although the partial enlargement of mitogen-treated c-rel$^{-/-}$ B cells was consistent with progression to an early phase in G1 (36–38), the G1 state can be subdivided into early and late phases; the late phase being characterized by an increased rate of RNA and protein synthesis (35). To define the point in the cell cycle at which c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells arrest, RNA synthesis was monitored in mitogen-treated B cells by [^{3}H]uridine incorporation. Fig. 5 *A* shows that RNA synthesis in anti-IgM antibody, anti-RP antibody, and LPS-stimulated c-rel$^{-/-}$ and LPS-stimulated nfkb1$^{-/-}$ cells is between 10- and 30-fold lower than in normal cells. Moreover, cytokine enhanced division of anti-RP antibody-treated c-rel$^{-/-}$ B cells coincides with a >20-fold increase in RNA synthesis compared with anti-RP antibody-stimulated c-rel$^{-/-}$ B cells. The same trend is seen in mitogen-stimulated c-rel$^{-/-}$ B cells stained for protein and DNA content (Fig. 5 *B*). c-rel$^{-/-}$ cells treated with anti-RP antibody alone fail to progress to G1, whereas a significant proportion of anti-RP antibody plus cytokine-stimulated c-rel$^{-/-}$ B cells can progress via G1 into S phase and G2/M (Fig. 5 *B*). These results demonstrate that c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells are blocked at a point early in G1 that precedes increased RNA and protein synthesis and that cytokines can partly overcome this cell cycle block allowing c-rel$^{-/-}$ cells to progress through S plus G2/M.

Expression of a bcl-2 Transgene Inhibits Apoptosis but Does Not Overcome the Cell Cycle Defect. To further understand the mechanism(s) by which Rel and NF-κB1 prevent apoptosis in B lymphocytes, we tested whether the expression of a bcl-2 transgene could inhibit the apoptosis of c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells. Survival curves for nonstimulated normal, c-rel$^{-/-}$, and nfkb1$^{-/-}$ B cells expressing bcl-2 (Fig. 6 *A*) show that in addition to inhibiting the death of normal quiescent B cells, a finding consistent with previous reports (39), bcl-2 prevented the enhanced apoptosis of resting nfkb1$^{-/-}$ B cells. Analysis of apoptosis and cell division in mitogen-stimulated c-rel$^{+/+}$bcl-2^{+} and c-rel$^{-/-}$bcl-2^{+} B cell cultures reveals that bcl-2 also prevented the death of anti-IgM– and anti-RP–treated c-rel$^{-/-}$ B cells (Fig. 6 *C*), but failed to promote cell division (Fig. 6, *B* and *D*). Collectively, these findings show that the death of B cells resulting from an absence of Rel or NF-κB1 belong to that class of apoptosis that can be inhibited by bcl-2.

c-myc Expression Is Normal in Mitogen-stimulated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B Cells. Recent studies have shown that anti-IgM antibody-induced apoptosis in W231 B lymphoma cells appears to result from an inhibition of Rel/NF-κB–mediated c-myc transcription (40, 41). To determine if the loss of Rel or NF-κB1 alters c-myc expression in primary B cells, the level of c-myc mRNA in normal, c-rel$^{-/-}$, and nfkb1$^{-/-}$ B cells before and after stimulation with anti-IgM antibodies was examined by Northern blot analysis (Fig. 7). Consistent with previous findings (42), c-myc mRNA levels are upregulated in normal B cells within 2 h of stimulation (lane *5*) and remain elevated after 4 h (lane *9*). In anti-IgM antibody stimulated W231 cells, an initial increase in induced c-myc expression (1 h) is followed by a downregulation of c-myc mRNA to near prestimulation levels by 3 h (40). In contrast, c-myc mRNA levels were upregulated normally in anti-IgM antibody-activated c-rel$^{-/-}$ (lanes *6* and *10*) and nfkb1$^{-/-}$ (lanes *7* and *11*) B cells. Furthermore, expression of a bcl-2 transgene, which reduces anti-IgM antibody-induced apoptosis in c-rel$^{-/-}$ B cells, did not alter the pattern of c-myc expression (lanes *8* and *12*). These results establish that neither Rel nor NF-κB1 alone is essential for anti-IgM–induced c-myc expression in primary B lymphocytes and that the cell cycle block and enhanced apoptosis in mitogen-activated c-rel$^{-/-}$ B cells is not a consequence of reduced c-myc expression.

The c-rel$^{-/-}$ B Cell Proliferative Defect Is Partially Overcome by Certain Combinations of Mitogens and Cytokines. Despite the inability of c-rel$^{-/-}$ B cells to proliferate in vitro in response to a range of discrete mitogens, humoral responses are reduced but not abrogated in c-rel$^{-/-}$ mice (3; data not

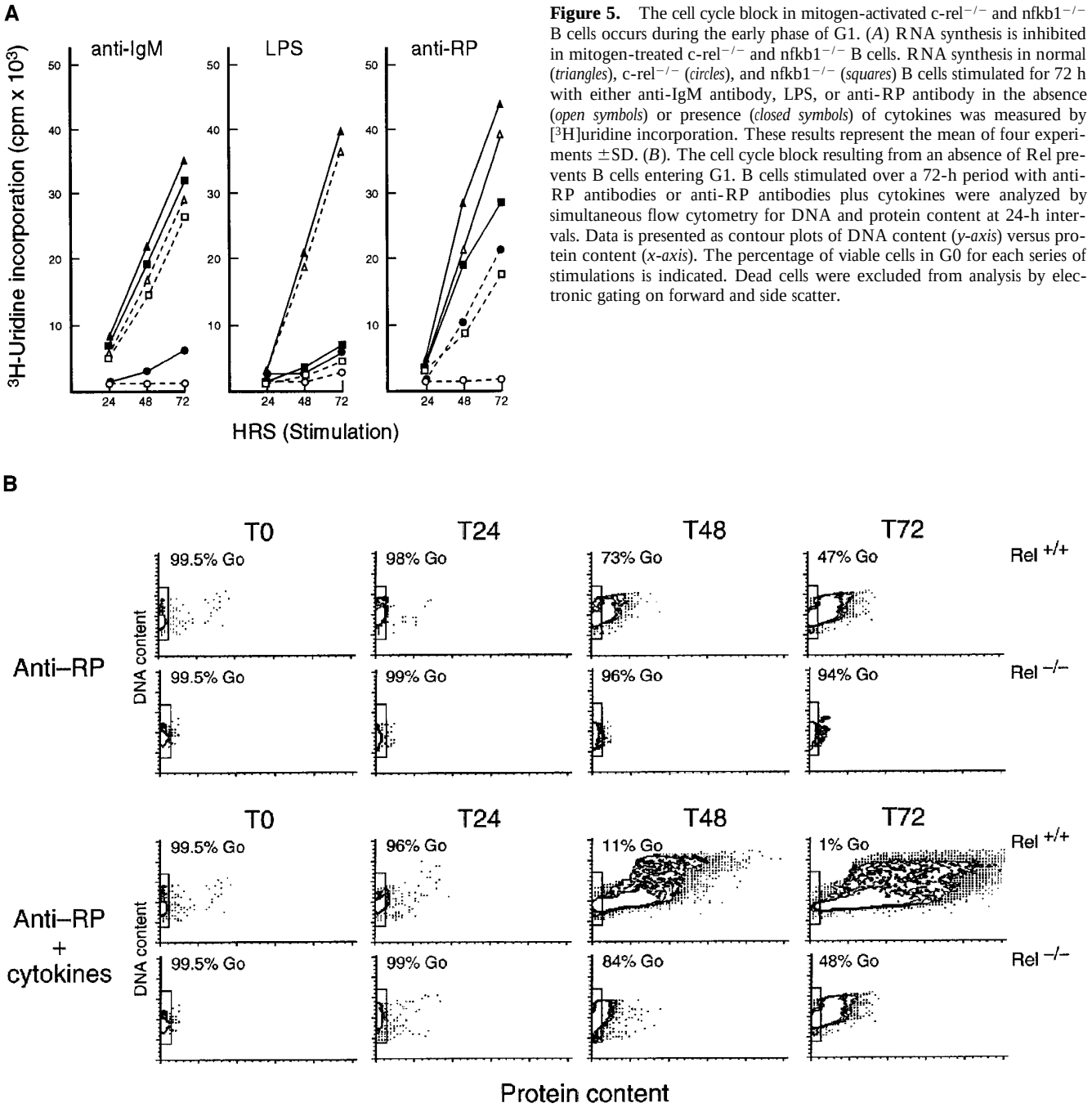

Figure 5. The cell cycle block in mitogen-activated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells occurs during the early phase of G1. (*A*) RNA synthesis is inhibited in mitogen-treated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells. RNA synthesis in normal (*triangles*), c-rel$^{-/-}$ (*circles*), and nfkb1$^{-/-}$ (*squares*) B cells stimulated for 72 h with either anti-IgM antibody, LPS, or anti-RP antibody in the absence (*open symbols*) or presence (*closed symbols*) of cytokines was measured by [^{3}H]uridine incorporation. These results represent the mean of four experiments ±SD. (*B*). The cell cycle block resulting from an absence of Rel prevents B cells entering G1. B cells stimulated over a 72-h period with anti-RP antibodies or anti-RP antibodies plus cytokines were analyzed by simultaneous flow cytometry for DNA and protein content at 24-h intervals. Data is presented as contour plots of DNA content (*y-axis*) versus protein content (*x-axis*). The percentage of viable cells in G0 for each series of stimulations is indicated. Dead cells were excluded from analysis by electronic gating on forward and side scatter.

shown). Since B cells are normally activated by multiple signals during an immune response (43, 44), we examined the proliferative response of c-rel$^{-/-}$ B cells to a range of mitogen and cytokine combinations. Table 1 summarizes these results as the amount of [^{3}H]thymidine incorporated 72 h after stimulation. In contrast to single stimuli, certain combinations of mitogens or mitogens plus cytokines could partially overcome the c-rel$^{-/-}$ B cell proliferative defect. For example, anti-IgM antibody plus LPS or anti-RP antibody plus LPS synergistically enhanced c-rel$^{-/-}$ B cell proliferation, whereas anti-RP plus anti-IgM antibodies failed to augment the response above the level achieved by either of the single mitogens. IL-4 increases anti-RP antibody-induced c-rel$^{-/-}$ B cell proliferation ~70-fold, yet this cytokine increases the response to LPS only marginally (two- to threefold). Consistent with previous findings (3), the proliferation of c-rel$^{+/-}$ B cells in response to the panel of single and multiple stimuli is intermediate between that of normal and c-rel$^{-/-}$ B cells (Table 1). Based on these results, B cell mitogens can be categorized into two groups: those which do or do not cooperate to promote cellular proliferation in the absence of Rel.

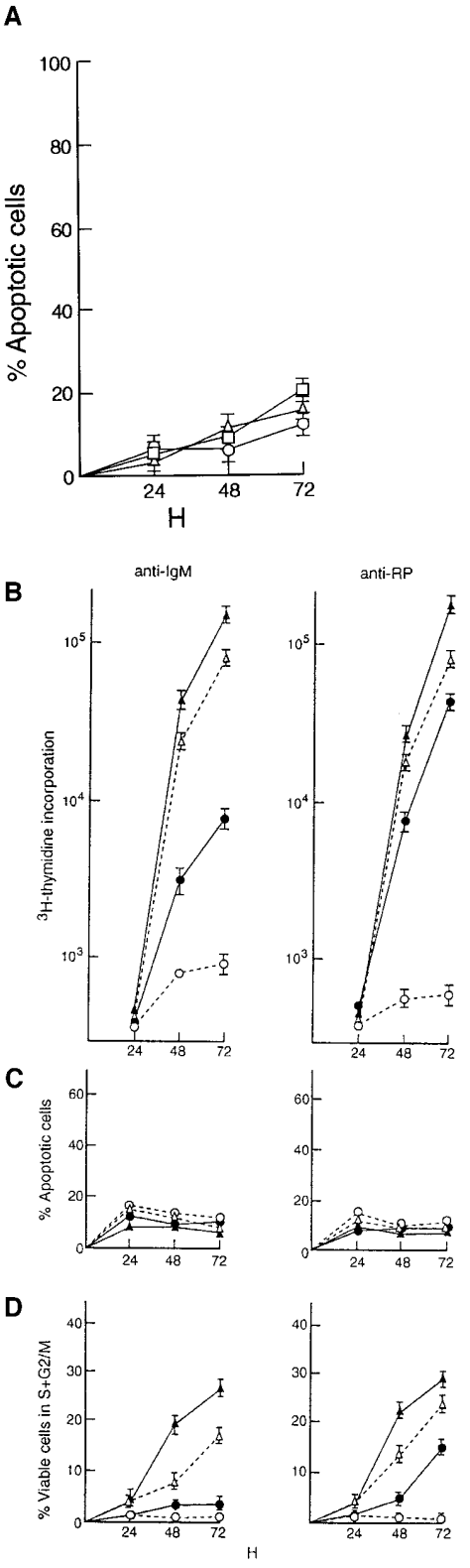

Figure 6. Bcl-2 inhibits apoptosis of mitogen-stimulated c-rel$^{-/-}$ B cells, but fails to overcome the cell cycle block. (*A*) Bcl-2 inhibits the enhanced apoptosis of nfkb1$^{-/-}$ B cells. Quiescent splenic B cells isolated from c-rel$^{+/+}$bcl-2^{+} (*triangles*), c-rel$^{-/-}$bcl-2^{+} (*circles*), and nfkb1$^{-/-}$bcl-2^{+} (*squares*) mice were cultured in DMEM/10% FCS without mitogen for a period of 72 h. At 24-h intervals, the frequency of dead cells was determined by trypan blue exclusion and by flow cytometric analysis of fixed cells stained with PI. (*B*) Proliferation of c-rel$^{-/-}$bcl-2^{+} cells is impaired. The proliferation of c-rel$^{+/+}$bcl-2^{+} (*triangles*) and c-rel$^{-/-}$bcl-2^{+} (*circles*) B cells stimulated with either anti-IgM or anti-RP in the absence (*open symbols*) or presence (*closed symbols*) of cytokines was monitored at 24-h intervals over a 72-h period by [^{3}H]thymidine incorporation. (*C*) Mitogen-induced death of c-rel$^{-/-}$ cells is rescued by bcl-2. The frequency of apoptotic cells in the mitogen-stimulated c-rel$^{+/+}$bcl-2^{+} and c-rel$^{-/-}$bcl-2^{+} B cell cultures (symbols as for *B*) was determined by flow cytometric analysis of PI-stained cells. The frequency of apoptotic cells is presented as a proportion of the total cell number. Greater than 99% of resting B cells of both genotypes were viable before mitogen stimulation. (*D*) Cell cycle progression of mitogen-stimulated c-rel$^{-/-}$bcl-2^{+} cells remains blocked. The DNA content of mitogen-stimulated c-rel$^{+/+}$bcl-2^{+} and c-rel$^{-/-}$bcl-2^{+} B cells (symbols as in *B*) was determined by flow cytometric analysis of PI-stained cells. The fraction of viable B cells in S, G2, or M is expressed as a function of the stimulation period. Less than 1% of viable cells were in G1, S, or G2/M before stimulation. The results shown represent the mean and SD from four experiments.

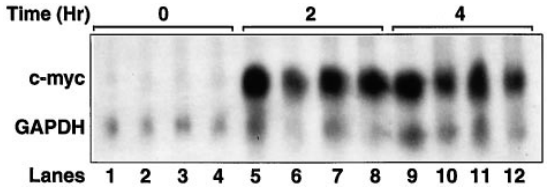

Figure 7. c-myc expression is normal in c-rel$^{-/-}$ B cells. 10-μg samples of total RNA isolated from resting (lanes *1–4*) and anti-IgM antibody-stimulated splenic B cells from normal, c-rel$^{-/-}$, nfkb1$^{-/-}$, and c-rel$^{-/-}$bcl-2^{+} mice stimulated for 2 h (lanes *5–8*) and 4 h (lanes *9–12*) were fractionated on formaldehyde denaturing gels, transferred to nitrocellulose membranes, and sequentially hybridized with radiolabeled murine c-myc cDNA (32) or glyceraldehyde-3-phosphate-dehydrogenase cDNA (33) probes. Filters were exposed for 48-h autoradiography at −70°C. Lanes *1*, *5*, and *9*, normal B cells; *2*, *6*, and *10*, Rel$^{-/-}$ B cells; *3*, *7*, and *11*, nfkb1$^{-/-}$ B cells; and *4*, *8*, and *12*, c-rel$^{-/-}$bcl-2^{+} B cells.

Discussion

We have demonstrated that Rel and NF-κB1, subunits of the Rel/NF-κB family of transcription factors, differentially regulate cell cycle progression and apoptosis in B lymphocytes. NF-κB1 prevents apoptosis in quiescent cells, whereas both Rel and NF-κB1 are important for the survival of mitogen-activated B cells. Differences in the proliferative defects exhibited by B cells lacking Rel or NF-κB1 also indicate that the requirement of one or both of these transcription factors for progression through the G1 phase of the cell cycle is dependent on the particular mitogenic signal.

Different NF-κB–like Proteins Are Required for the B Cell Proliferative Response to Specific Mitogens. Although both Rel and NF-κB1 are required for normal B cell proliferation (3–5) and Rel/NF-κB1 is the predominant NF-κB–like heterodimer in splenic B lymphocytes (45–47), the induction of B cell mitogenesis by specific stimuli appears to require different NF-κB–like complexes. This is most clearly illustrated for anti-IgM antibody-stimulated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells. Anti-IgM antibody-activated nfkb1$^{-/-}$ B cells proliferate normally despite Rel/RelA heterodimers and Rel homodimers being the only NF-κB–like factors

Table 1. *Synergistic Enhancement of c-rel$^{-/-}$ B Cell Proliferation by Certain Combinations of Stimuli*

Genotypes	Anti-CD40	Anti-IgM	Anti-RP	LPS	Anti-CD40 anti-IgM	Anti-CD40 anti-RP	Anti-CD40 LPS	Anti-IgM anti-RP	Anti-IgM LPS	Anti-RP LPS	Anti-RP IL-4	LPS IL-4
+/+	9.2 ± 0.5	112 ± 6.2	180 ± 11.1	111 ± 15.6	150 ± 9.2	222 ± 21.9	204 ± 16.4	104 ± 14.0	258 ± 29.2	339 ± 30.1	285 ± 37.6	152 ± 17.0
+/−	5.4 ± 1.0	22 ± 0.9	25 ± 4.1	53 ± 4.1	53 ± 7.2	19.3 ± 6.2	83.3 ± 20.0	18.8 ± 1.4	195 ± 16.6	308 ± 36.0	152 ± 26.9	64 ± 19.2
−/−	1.4 ± 0.5	0.39 ± 0.2	0.67 ± 0.2	4.2 ± 0.8	1.1 ± 0.6	27 ± 3.6	25 ± 6.1	0.98 ± 0.3	98.6 ± 7.2	149.9 ± 9.8	83 ± 12.7	8.6 ± 1.9

B cell proliferation with optimal concentrations of mitogens was monitored by [^{3}H]thymidine incorporation 72 h after stimulation. All data is expressed as $\times 10^3$ cpm and represents the mean values and standard deviation from five experiments.

upregulated in the nucleus (4; Grumont, R.J., results not shown). In contrast, c-rel$^{-/-}$ B cells stimulated with this mitogen fail to proliferate, even though nuclear NF-κB1/RelA heterodimers and NF-κB1 homodimers are upregulated with normal kinetics (3). Taking into consideration the recent finding that proliferation of anti-IgM antibody-stimulated rela$^{-/-}$ B cells is also impaired (48), this indicates that B cell mitogenesis triggered by engaging the antigen receptor only requires Rel/RelA heterodimers and Rel homodimers. LPS-dependent B cell proliferation, however requires Rel, RelA, and NF-κB1 (3, 4, 48).

Certain mitogen combinations such as anti-IgM antibody and LPS, anti-RP antibody and LPS, or anti-RP antibody plus cytokines synergistically enhance B cell proliferation by increasing cell division and reducing apoptosis, whereas others such as anti-IgM and anti-RP antibodies fail to increase c-rel$^{-/-}$ B cell mitogenesis above that promoted by the individual stimuli. These findings are consistent with models in which dual stimuli partly overcome the proliferative defect as a result of each mitogen activating distinct intracellular signals required for survival and/or cell cycle progression. Conversely, it would appear that non-synergizing mitogens such as anti-IgM plus anti-RP antibodies engage the same or functionally equivalent signaling pathways. If Rel controls a single pathway crucial for the division and survival of B cells, then perhaps multiple mitogens can in part bypass the need for Rel by engaging the pathway downstream of Rel. The molecular basis of mitogen synergy remains to be determined. This could involve activation of other NF-κB–like complexes, or Rel/NF-κB–independent signal transduction pathways. The second possibility is consistent with the finding that mitogen-activated protein (MAP) and stress-activated protein (SAP) kinases are activated normally in c-rel$^{-/-}$ B (results not shown).

In B Cells, Rel and NF-κB1 Regulate G1 Cell Cycle Progression. c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells undergo abortive mitogenic responses characterized by initial cell enlargement but failure to undergo RNA and protein synthesis. This indicates that cell cycle arrest occurs at a stage between early and late G1. Consistent with Rel and NF-κB1 controlling cell cycle progression is the recent finding that the cyclin-dependent kinase Cdk2 can regulate the transcriptional activation of RelA through mutual interaction with the coactivator p300 (49). Our data also shows that the involvement of particular Rel/NF-κB subunits in cell cycle regulation may be cell-type specific. Although NF-κB1 is important for B cell division and NF-κB (NF-κB1/RelA) is induced in fibroblasts during the G0 to G1 transition (50), mitogen-stimulated primary fibroblasts that lack NF-κB1 divide normally (Gerondakis, S., unpublished results).

The synergistic action of mitogen combinations in promoting c-rel$^{-/-}$ B cell proliferation (e.g., anti-RP antibodies plus cytokines) demonstrates certain signals permit near normal numbers of c-rel$^{-/-}$ B cells to progress through G1 and complete the cell cycle. Although this indicates that neither Rel nor NF-κB1 appear to be essential at other

stages of the cell cycle, it remains a possibility that a requirement for these transcription factors at a post-G1 stage of the cell cycle could also be overcome by certain mitogenic signals. Despite dual stimuli increasing the frequency of dividing c-rel$^{-/-}$ cells, an inability to completely bypass the Rel-dependent phase in G1 is consistent with only a subset of splenic B cells responding to the combined stimulus. If true, this would indicate heterogeneity in the control of cell division amongst primary splenic B cells.

Rel and NF-κB1 Are Important for Survival of Quiescent and Mitogen-activated B Cells. Our results demonstrate for the first time that NF-κB1, like Rel and RelA, is important in regulating cell survival. In normal B lymphocytes, NF-κB1 is required for the survival of quiescent cells, whereas both Rel and NF-κB1 prevent apoptosis after mitogenic stimulation. Data from a variety of experimental systems indicate that Rel/NF-κB–like transcription factors can play both anti- (21–24, 40, 41, this paper) and proapoptotic (25, 51, 52) roles. Such a dual function for particular Rel/NF-κB–like factors may depend on the cell type and the nature of the apoptotic signal. For example, the importance of cell type is emphasized with NF-κB1 protecting resting and LPS-treated B cells from apoptosis (this paper) while being dispensible for survival of TNF-α–treated embryonic fibroblasts (21). Quantitative differences in the survival of c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells treated with different mitogens also emphasizes the complex relationship between Rel/NF-κB–dependent mitogenesis and apoptosis.

It remains to be determined how Rel and NF-κB1 promote the survival of B lymphocytes and which gene(s) crucial to cell survival are regulated by these transcription factors in quiescent and mitogen-stimulated cells. Since Rel and NF-κB1 can function both as a transcriptional activators or repressors (4, 19, 20), cell survival mediated by these proteins could result either from the induction of a survival gene or repression of a cell death gene. Candidate gene(s) include those specifically dedicated to controlling cell survival, or in the case of mitogen-stimulated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells, these could be cell cycle genes if enhanced levels of death are an indirect consequence of the impaired expression of genes required for G1 progression. Support for the former model comes from the finding that even though mitogen-induced rela$^{-/-}$ B cell proliferation is impaired, apoptosis is normal (48), suggesting that the defects in mitogen-treated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells could be the result of Rel and NF-κB1 regulating two distinct groups of genes, one crucial for cell cycle progression and the other apoptosis. Moreover, it appears likely that Rel/NF-κB factors regulate the expression of more than one gene important for lymphocyte survival. Although c-myc is a crucial regulator of cell division and apoptosis (53) and anti-IgM–treated W231 B lymphoma cells undergo apoptosis due an inhibition of Rel/NF-κB–dependent c-myc transcription (40, 41), the expression of c-myc was normal in quiescent or anti-IgM–treated c-rel$^{-/-}$ and nfkb1$^{-/-}$ B cells. This difference may indicate that the regulation of c-myc transcription differs in primary and immortalized B cells. NF-κB–induced cellular inhibitor of apoptosis protein 2 (c-IAP2) expression was also shown to be important in preventing the TNF-α–mediated death of Jurkat T cells (54), yet the absence of Rel or NF-κB1 does not alter c-IAP2 expression in B cells (results not shown). Ultimately, the identification of Rel/NF-κB–regulated genes in B cells that are important for cell cycle regulation and cell survival should offer important insight into the mechanisms by which different Rel/NF-κB dimers regulate these processes in response to particular mitogenic signals.

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