CD40, a tumor necrosis factor receptor family member, is expressed on B lymphocytes. Interaction between CD40 and its ligand (CD40L), expressed on activated T lymphocytes, is critical for B cell survival. Here, we demonstrate that CD40 signals B cell survival in part via transcriptional activation of the RelB NF-κB subunit. CD40L treatment of chronic lymphocytic leukemia cells induced levels of relB mRNA. Similarly, CD40L-mediated rescue of WEHI 231 B lymphoma cells from apoptosis induced upon B cell receptor (surface IgM) engagement led to increased relB mRNA levels. Recently, we characterized a new *de novo* synthesis pathway for the RelB NF-κB subunit, induced by the cytomegalovirus IE1 protein, in which binding of p50/p65 NF-κB and c-Jun/Fra-2 AP-1 complexes to the *relB* promoter works in synergy to potently activate transcription (Wang, X., and Sonenshein, G. E. (2005) *J. Virol.* 79, 95–105). CD40L treatment of WEHI 231 cells caused induction of AP-1 family members Fra-2, c-Jun, JunD, and JunB. Cotransfection of Fra-2 with the Jun AP-1 subunits and p50/c-Rel NF-κB led to synergistic activation of the *relB* promoter. Ectopic expression of *relB* or RelB knockdown using small interfering RNA demonstrated the important role of this subunit in control of WEHI 231 cell survival and implicated activation of the anti-apoptotic factors Survivin and manganese superoxide dismutase. Thus, CD40 engagement of transformed B cells activates *relB* gene transcription via a process we have termed the *de novo* RelB synthesis pathway, which protects these cells from apoptosis.

CD40, a 48-kDa transmembrane protein belonging to the tumor necrosis factor receptor superfamily, is expressed on B lymphocytes as well as dendritic cells, macrophages, epithelial cells, and hematopoietic progenitor cells (1). Its ligand (CD40L)2 is found on activated T lymphocytes; interaction between the receptor and ligand induces B cell activation and proliferation, Ig secretion, memory cell formation, and isotype switching (2). The critical role that CD40 plays in B cell function is made evident by the absence of germinal centers and secondary immune responses in CD40-deficient mice and the association of mutations in CD40 and CD40L with the human disease X-linked hyper-IgM syndrome (3). In addition, CD40 signaling is also involved in neoplastic cell proliferation. The interaction between CD40 and its ligand can inhibit apoptosis of normal and transformed B cells induced by engagement of the B cell receptor (BCR), serum deprivation, or treatment with a chemotherapeutic agent (4). For example, triggering of CD40 by the anti-CD40 monoclonal antibody G28-5 inhibits apoptosis induced by the chemotherapeutic agent fludarabine in B cells from patients with chronic lymphocytic leukemia (CLL) (5). A variety of second messengers are activated after treatment of B cells with CD40L, including NF-κB (6). In WEHI 231 murine B lymphoma cells, a model often used to study NF-κB and its effects on B cell survival, CD40L stimulation following BCR engagement rescues cells from apoptosis (7). BCR engagement of WEHI 231 cells normally leads to an initial transient increase in p50/c-Rel NF-κB factor activity at 1 h, which is followed by a rapid decline in levels (8), and then to apoptosis (9). Studies from our laboratories have shown that CD40L-mediated rescue of WEHI 231 B cells is due in part to maintenance of NF-κB factor binding (10), although the nature of the subunits responsible was not determined.

The mammalian NF-κB family members are p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52. These subunits contain a 300-amino acid long region termed the Rel homology domain, which is involved in subunit dimerization and binding to DNA (11). For the most part, the different members can form heterodimers, which vary significantly in their transactivation potential (11, 12). As we first showed by antisense and ectopic c-Rel expression studies in splenic B lymphocytes and WEHI 231 B cells (9), NF-κB transcription factors play critical roles in proliferation control and B cell survival (13, 14). NF-κB, which regulates the c-myc, and c-myb genes (8, 15, 16), has also been implicated in promoting neoplastic transformation (13, 14). In most non-B cells, NF-κB is sequestered in the cytoplasm bound to specific inhibitory proteins (IκB [(inhibitor of κB protein)] of which IκB-α is the prototype. In B cells, NF-κB activity is constitutive, but can be further induced or modulated (17–19). Activation of classical NF-κB (p50/p65) through the canonical pathway is mediated via the IκB kinase complex, con-
taining two kinases, IκB kinase-α and β, and multiple copies of a structural protein IκB kinase-γ or NEMO (20, 21). The IκB kinase complex phosphorylates IκB-α, leading to its subsequent ubiquitination and degradation. In the alternative NF-κB activation pathway, p100/RelB complexes are sequestered in the cytoplasm via the C-terminal ankyrin repeats of p100 (20). Following activation of IκB kinase-α, p100 is phosphorylated, ubiquitinated, and clipped by the proteasome into p52, and the resulting p52/RelB complexes can translocate to the nucleus. For example, CD40L treatment of murine B lymphocytes induces p52/RelB complexes via the alternative pathway (22, 23). In addition to post-translational regulation, RelB can also be controlled at a transcriptional level. The relB promoter has two proximal κB elements and is an NF-κB target (24). Our laboratory characterized a novel de novo RelB synthesis pathway induced by the cytomegalovirus IE1 (immediate early-1) protein that occurs via binding of both classical NF-κB and AP-1 complexes containing c-Jun with either Fra-2 or Fra-1 to a distal AP-1 element, leading to synergistic transcriptional activation of the relB promoter (25).

Recently, Gricks et al. (26) used microarray analysis to begin to assess the effects of CD40L treatment on patients with CLL and noted an apparent activation of relB mRNA levels, which was absent in cells from healthy individuals. The physiological relevance of this activation was not further elucidated. Here, we confirm that CD40L induces relB mRNA levels in CLL cells as well as in WEHI 231 B lymphoma cells. Using WEHI 231 cells as a model, we show for the first time that CD40L engagement causes induction of the de novo RelB synthesis pathway, involving c-Jun, JunD, JunB, and Fra-2 AP-1 factors that work in synergy with p50/c-Rel NF-κB. Notably, RelB plays a critical role in rescue from apoptosis, indicating that transcriptional regulation of the RelB NF-κB subunit is a new mechanism whereby cell survival is controlled.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation, Culture, and Treatment Conditions**—Peripheral blood was drawn from five CLL patients with flow cytometry-confirmed B-CLL with an Institutional Review Board approved consent. Patient samples were arbitrarily labeled JM37, JM40, JM42, JM61, and JM62. Leukemic cells were isolated by centrifugation using Histopaque 1077 (Sigma). Cultures contained >90% leukemic cells, which were maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum, 2 mmol/liter l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma) at 37 °C and 5% CO2. After isolation, cells were allowed to rest for 24 h in fresh medium. For CD40L treatment, 20–30 × 106 cells were treated with supernatants containing CD40L and anti-CD8 antibody at the optimal concentrations of 1:5 and 1:40, respectively, as described previously (10). NIH 3T3 fibroblasts were grown and transfected as published previously (25).

**Plasmids**—The murine RelB expression vector and its corresponding pMxNeo empty backbone vector (EV) were kind gifts from Rodrigo Bravo (Bristol-Myers Squibb Co.) (28) and Reza Forough (Texas A&M University, College Station, TX) (27), respectively. The murine c-Rel expression vector and its corresponding pcDNA3 EV DNA were kind gifts of Tom Gilmore (Boston University, Boston, MA). The pCLEco vector, used in virus production, was obtained from Imgenex (San Diego, CA). pRelB-sense and pRelB-siRNA (containing control or RelB small interfering RNA (siRNA) oligonucleotide in the pSIREN-RetroQ vector) were a generous gift of Finn-Eirik Johansen (Rikshospitalet University Hospital, Oslo, Norway) (29). The human p1.7 relB promoter-luciferase (-1694 to +1) construct driving a luciferase reporter in the pGL3-Basic vector and the SV40-β-galactosidase reporter vector were kindly provided by Carlos V. Paya (Mayo Clinic, Rochester, MN) (25). Constructs expressing c-Jun, JunD, JunB, and Fra-2 in the pCI vector were kindly provided by Dany Chalbos (INSERM, Montpellier, France) (30). The wild-type murine pSVSport-c-Rel and pMT2T-p50 expression vectors have been described (31, 32). The pcDNA3.1 DNA (Invitrogen) was used as an EV in coexpression experiments.

**RelB and c-Rel WEHI 231 Stable Transfectants**—WEHI 231 cells were electroporated as described previously (33) with either the murine RelB or c-Rel expression vector or their corresponding EV DNAs. Mixed populations of stable transfectants were selected with 1400 μg/ml Geneticin (G418, Sigma) for 2 weeks. Single clones were obtained by serial dilution of the mixed population of transfectants.

**Retroviral Gene Delivery**—Retrovirus stock was generated using the ecotropic packaging cell line Bosc23 (34). The pCLEco vector was cotransfected with either pRelB-sense or pRelB-siRNA into Bosc23 cells using FuGene 6 (Roche Applied Science). Two days later, supernatants containing viral particles were harvested and used to infect WEHI 231 cells in the presence of 6 μg/ml Polybrene (Sigma). After 48 h, infected cells were selected with 0.4 μg/ml puromycin (Sigma). Single clones were obtained by serial dilution of the mixed population of transfectants.

**Immunoblot Analysis**—Whole cell and nuclear extracts were prepared and quantitated using the Bio-Rad Dc protein assay kit, and samples (15–70 μg) were subjected to immunoblotting as described previously (35). A Bio-Rad Precision Plus standard protein ladder was used to determine molecular mass. Antibodies against NF-κB family members p50 (catalog no. sc-7178), p65 (sc-372), p52 (sc-7386), RelB (sc-226), and c-Rel (sc-71); AP-1 family members JunB (sc-46), JunD (sc-74), c-Fos (sc-7202), Fra-1 (sc-183), and Fra-2 (sc-604); and Survivin (sc-10811), Bcl-xL (sc-8392), Bax (sc-493), Bcl-2 (sc-492), and Oct-1 (sc-232) were purchased from Santa Cruz Biotechnology. Antibody against manganese superoxide dismutase (MnSOD; catalog no. 06-984) was purchased from Upstate. Antibodies against c-Jun (catalog no. 9162) and β-actin (AC-15) were purchased from Cell Signaling Technology and Sigma, respectively.
RESULTS

CD40L Induces relB mRNA Levels in CLL Cells—To begin to measure the effects of CD40L on relB mRNA expression, B-CLL cells were isolated from three patients (coded JM37, JM40, and JM42) and left untreated or treated with CD40L for 24 h. RNA was isolated and subjected to RT-PCR analysis, and values for relB were normalized to GAPDH, which served as a loading control (Fig. 1A). Stimulation of CLL cells from patients JM37, JM40, and JM42 with CD40L resulted in increases in the relB mRNA levels of 7.1-, 6.6-, and 4.6-fold, respectively. These findings are consistent with the microarray analysis performed by Gricks et al. (26), who found that CLL cells responded to CD40L treatment via activation of relB mRNA, whereas repression of relB was seen in B cells from healthy individuals.

CD40L Increases relB mRNA Levels in WEHI 231 Cells—We next measured whether the effects of CD40L on relB mRNA levels can be extended to other transformed B cells and selected WEHI 231 B lymphoma cells, in which CD40L promotes cell survival from anti-IgM-induced apoptosis (7). WEHI 231 cells were treated for 6 h with CD40L or anti-IgM alone or in combination, and cytoplasmic RNA was subjected to Northern blot analysis (Fig. 1B). Treatment of WEHI 231 cells with CD40L either alone or in combination with anti-IgM increased relB mRNA levels, whereas anti-IgM treatment alone decreased the levels. In this and a duplicate experiment, treatment with CD40L or CD40L plus anti-IgM resulted in 3.1- and 2.9-fold increases in relB mRNA levels, respectively, whereas anti-IgM treatment resulted in a 0.6-fold decrease (Fig. 1C). Thus, CD40L treatment induces relB mRNA in WEHI 231 B lymphoma cells, as seen in CLL cells.

CD40L Causes an Increase in Nuclear Levels of RelB—To test for the expected activation of RelB protein, B-CLL cells isolated from three patients (coded JM42, JM61, and JM62) were left untreated or treated with CD40L for 24 h. Nuclear proteins were extracted and subjected to immunoblot analysis for RelB and Oct-1 (used as a loading control) (Fig. 1D). CD40L stimulation of CLL cells resulted in a dramatic induction of RelB protein, ranging from 8.7- to 11.4-fold compared with untreated samples. On average, the induction of RelB protein levels appeared to be higher than that of relB mRNA levels upon CD40L stimulation (Fig. 1A). CD40L treatment has been shown to activate p52/RelB complexes via the alternative pathway, which could explain this difference in protein and mRNA levels. To test for this pathway, the nuclear levels of p52 were assessed (Fig. 1D). In untreated CLL cells, p52 levels were not detectable (Fig. 1D). Upon CD40L stimulation, nuclear p52 levels increased in all three CLL patient samples. The data indicate that the alternative pathway is activated in CLL cells upon CD40L stimulation and could account for the observed disparity between RelB protein and relB mRNA levels. Quantitative assessment of the contribution of this pathway to the RelB increase was not possible, however, as we were unable to detect p52 in the untreated samples.

We next studied the kinetics of changes in RelB protein levels in WEHI 231 cells induced by CD40 engagement. Nuclear proteins were isolated from cells in exponential growth or following treatment for 1, 4, or 10 h with CD40L or anti-IgM alone or
in combination and analyzed by immunoblotting. An increase in the nuclear levels of RelB was detectable by 1 h of treatment with either CD40L alone or plus anti-IgM and was more substantial by 4 h and increased further by 10 h (Fig. 2A). Analysis of Oct-1 levels confirmed essentially equal loading. The immunoblots were subjected to densitometry, and the data for RelB normalized to Oct-1 are presented in Fig. 2C. By 10 h, CD40L alone or with anti-IgM increased RelB levels by 10.4- and 9.1-fold, respectively. The data at the 4-h time point from this and a duplicate experiment were plotted. Similarly to what was seen after CD40L stimulation of CLL cells, treatment of WEHI 231 cells with CD40L alone or in combination with anti-IgM resulted in 8.8- and 6.6-fold increases in RelB (Fig. 2D), respectively, which were substantially higher than the ~3-fold increase in mRNA levels observed at 6 h (Fig. 1C).
CD40L Induces a Prosurvival de Novo RelB Synthesis Pathway

Next, to test for alternative pathway activation in WEHI 231 cells, the nuclear levels of p52 were assessed (Fig. 2A). CD40L alone or with anti-IgM caused a detectable increase in p52 at 1 h, which continued to 4 h and then either remained elevated or dropped back slightly by the 10-h time point, such that levels increased by 3.6- or 2.3-fold, respectively, at the 10-h time point (Fig. 2C) and by 4.2- or 2.3-fold at 4 h (Fig. 2D). Anti-IgM treatment alone led to decreased p52 levels (Fig. 2), suggesting that the lower extent of increase in p52 upon the co-treatment probably results from anti-IgM-induced signaling. Taken together, these data suggest that CD40L leads to activation of the de novo RelB synthesis pathway, which leads to a sustained increase in relB mRNA and RelB subunit expression, and to the alternative pathway, which leads to an increase in the levels of p52/RelB complexes, which appears more transient.

Treatment with anti-IgM alone led to a slight increase in RelB levels and to substantial decreases in c-Rel and p65 levels between 1 and 4 h, which persisted to 10 h (Fig. 2B); we showed previously that this leads to apoptosis (8, 9). Whereas CD40L treatment alone caused substantial increases in the levels of c-Rel, it was insufficient to overcome anti-IgM-mediated shutoff of this subunit (Fig. 2B). CD40L treatment resulted in large increases in p65 and p50 and led to maintenance of levels equivalent to those in untreated cells upon co-treatment. Thus, CD40L-mediated rescue from anti-IgM-induced cell death also overrides the shutoff of these two NF-κB subunits.

CD40L Induces AP-1 Family Members—In the cytomegalovirus IE1-induced de novo RelB synthesis pathway, synergistic transcriptional activation of the relB promoter by binding of both classical NF-κB and AP-1 complexes containing c-Jun with either Fra-2 or Fra-1 increases relB mRNA levels (25, 38). Thus, we next tested for activation of AP-1 components that mediate de novo relB synthesis in WEHI 231 cells. Nuclear extracts were prepared from untreated WEHI 231 cells or following treatment with CD40L, anti-IgM, or anti-IgM plus CD40L for 4 h and subjected to EMSA for AP-1 binding using the AP-1 element upstream of the relB promoter as a probe. As seen in Fig. 3A, extracts from control WEHI 231 cells displayed two bands of AP-1 binding (bands 1 and 2). Whereas CD40L or anti-IgM treatment alone resulted in a slight increase in binding, a much more robust increase was seen upon anti-IgM/CD40L co-treatment. In addition, the appearance of a new faster migrating complex (band 3) was evident. Sp-1 binding confirmed essentially equal loading. The increases in AP-1 factor binding seen with either CD40L alone or the combined anti-IgM/CD40L treatment were maintained up to 10 h (Fig. 2B). Thus, CD40L-mediated rescue from anti-IgM-induced death of WEHI 231 cells leads to enhanced AP-1 factor binding.

FIGURE 3. CD40L treatment of WEHI 231 cells activates AP-1 family members. A, nuclear extracts were isolated from asynchronously growing WEHI 231 cells (0) or following treatment with anti-IgM (αIgM) or CD40L alone or in combination for 4 h. Samples (2.5 μg) were used in EMSA with the relB AP-1 site as a probe. Binding to an Sp-1 oligonucleotide was used as a control for equal loading. B, AP-1 binding to the relB AP-1 site was examined (as described for A) using extracts isolated following 10 h of treatment. C, nuclear extracts (50–70 μg) isolated from asynchronously growing WEHI 231 cells or from cells treated with anti-IgM or CD40L alone or in combination for 4 h were subjected to immunoblot analysis using antibodies against the indicated AP-1 family members. Oct-1 protein levels confirmed equal loading. The asterisks indicate a supershifting band. The brackets indicate a clearing in an AP-1 band, and the bars indicate a supershifting band.

JUNE 15, 2007•VOLUME 282•NUMBER 24 JOURNAL OF BIOLOGICAL CHEMISTRY 17479
CD40L Induces a Prosurvival de Novo RelB Synthesis Pathway

To identify the nature of the individual AP-1 subunits activated by CD40L, anti-IgM, or anti-IgM plus CD40L co-treatment, nuclear extracts were subjected to immunoblotting for c-Jun, JunD, JunB, Fra-1, Fra-2, and c-Fos (Fig. 3C). The levels of c-Jun and JunD increased upon treatment with CD40L or anti-IgM alone, and treatment with both CD40L and anti-IgM showed essentially an additive effect. JunB levels were seen to increase substantially only in cells exposed to anti-IgM/CD40L co-treatment. In the case of Fra-2, a new slower migrating band was visible upon CD40L treatment alone or in combination with anti-IgM, consistent with the appearance of a phosphorylated, more active form (39). Fra-1 levels were barely detectable in untreated cells and increased modestly with anti-IgM and somewhat more substantially with the anti-IgM/CD40L co-treatment. Very low levels of c-Fos were detected in WEHI 231 cells and increased upon treatment only with anti-IgM, but appeared unaffected by CD40L (Fig. 3C). Thus, c-Jun, JunD, JunB, and Fra-2 levels or activities increase upon treatment with CD40L alone or in combination with anti-IgM and could participate in the de novo RelB synthesis pathway.

CD40L Induces Binding of c-Jun, JunD, JunB, and Fra-2 to the relB AP-1 Site—We next tested for binding of AP-1 family members to the relB AP-1 site. Nuclear extracts isolated from WEHI 231 cells co-treated with CD40L and anti-IgM for 4 h were used in antibody gel shift analysis. Addition of antibody against c-Jun, JunD, JunB, or Fra-2 resulted in a clearing of AP-1 bands (Fig. 3D, middle and lower panels) and the appearance of supershifted bands, which were more clearly visible in the darker exposures (Fig. 3D, upper and middle panels). No changes were observed in the samples with anti-Fra-1 and anti-c-Fos antibodies. Thus, the data confirm binding of the AP-1 family members c-jun, JunD, JunB, and Fra-2 induced by anti-IgM/CD40L co-treatment.

p50/c-Rel NF-κB Transactivates the relB Promoter in Synergy with AP-1 Complexes Containing Fra-2 with c-Jun, JunD, or JunB—Previously, p50/p65 NF-κB was shown to activate the relB promoter in synergy with c-Jun/Fra-2 AP-1 complexes (25). Although p50 and p65 subunit expression is induced by CD40L treatment, the predominant NF-κB complex in WEHI 231 B lymphoma cells is p50/c-Rel (8, 40). To determine whether p50/c-Rel complexes work in synergy with AP-1, in particular, c-Jun, JunB, JunD, and Fra-2, NIH 3T3 cells were selected for their high transfection efficiency and low basal levels of NF-κB and AP-1 (25, 32). NIH 3T3 cells were cotransfected with vectors expressing c-Jun, Fra-2, p50, or c-Rel alone or in combination and the p1.7 relB promoter-luciferase reporter construct (Fig. 4A). Transfection of c-Jun or Fra-2 alone or p50/c-Rel had little effect on relB promoter activity (<2-fold increase). Whereas the combination of c-Jun and Fra-2 increased relB promoter activity by 16.2 ± 1.5-fold, a striking 133.9 ± 15.4-fold induction was seen upon cotransfection of vectors expressing c-Jun/Fra-2 and p50/c-Rel (Fig. 4A), indicating that these subunits have potent synergistic effects on relB promoter activity.

The ability of JunD and JunB to transactivate the relB promoter alone or in combination with Fra-2 and p50/c-Rel was similarly tested (Fig. 4, B and C). JunD alone was found to increase relB promoter activity by 4.6 ± 0.1-fold, whereas Fra-2 alone again showed little effect (Fig. 4B). A combination of
JunD and Fra-2 induced promoter activity by 45.3/2.8-fold, whereas a combination of JunD/Fra-2 and p50/c-Rel complexes caused an even larger increase in relB promoter activity of 115.4/12.9-fold. Finally, expression of JunB alone or in combination with Fra-2 resulted in a 10.9/1.2- or 45.7/2.3-fold induction, respectively (Fig. 4C). The combination of JunB/Fra-2 and p50/c-Rel complexes resulted in a more potent 80.4/3.8-fold increase in relB promoter activity. Taken together, these data show that Fra-2 plus any of the three Jun family members that are induced by CD40L (c-Jun, JunD, or JunB) can synergize with p50/c-Rel NF-κB to potently transactivate the relB promoter.

Knockdown of RelB Promotes Anti-IgM-induced Death of WEHI 231 Cells—To test whether endogenous RelB expression promotes WEHI 231 cell survival, an siRNA strategy and anti-IgM treatment were used. WEHI 231 cells were infected with pRelB-siRNA and control pRelB-sense retroviral vectors (expressing RelB siRNA and RelB control sense RNA, respectively) as described previously (29). Stable mixed populations of transfectants were selected using puromycin, and two individual clones were isolated by limiting dilution (WEHI-siRelB clones 8 and 10). Both WEHI-siRelB clones 8 and 10 had lower basal levels of nuclear RelB compared with the control WEHI-sense RelB population (Fig. 5A). After anti-IgM treatment for 24 h, RelB levels were seen to increase in all cells, as observed above (Fig. 2A), but remained substantially lower in the two WEHI-siRelB clones compared with untreated control cells. An analysis of cell cycle progression by flow cytometry revealed considerably more growth arrest in the RelB siRNA-expressing cells upon anti-IgM treatment compared with control cells (62.6 and 63.4% of WEHI-siRelB clone 8 and 10 cells, respectively, were in G0/G1 versus 45.1% of WEHI-sense RelB cells) (Fig. 5B). Previously, anti-IgM-treated WEHI 231 cells were shown to die via apoptosis using DNA laddering and flow cytometry analysis of sub-G1 DNA content and dead cells quantified by trypan blue exclusion (9). When anti-IgM-induced cell death was assessed by trypan blue exclusion, WEHI-siRelB clones 8 and 10 displayed substantially increased levels of cell death (40.6 and 39.1%, respectively) compared with the 18.1% seen with the control WEHI-sense RelB population after 48 h of anti-IgM treatment (Fig. 5C). These results were confirmed by flow cytometry (Fig. 5D). WEHI-siRelB clones 8 and 10 displayed a greater percentage of apoptotic cells (40.3 and 39.1%, respectively) compared with 18.0% apoptosis seen in the sense RelB population (Fig. 5D). These findings implicate RelB in the control of WEHI 231 B lymphoma cell viability.

Genes Encoding Anti-apoptotic Factors MnSOD and Survivin Are RelB Targets in WEHI 231 Cells—To identify possible targets of RelB that mediate survival, we examined the expression of MnSOD, Survivin, Bcl-2, and Bcl-xL, known NF-κB targets with anti-apoptotic functions (41–44), as well as of Bax, the pro-apoptotic Bcl-2 family member. Whole cell extracts were isolated from untreated WEHI-sense RelB cells and WEHI-siRelB clones 8 and 10 and subjected to immunoblot analysis (Fig. 6A). Knockdown of RelB was paralleled by a decrease in MnSOD and Survivin protein levels, whereas expression of Bcl-2, Bcl-xL, and Bax remained unchanged. To determine
whether the decrease in MnSOD and Survivin could be related to a decline in mRNA levels, RT-PCR was performed (Fig. 6B). Expression of the RelB siRNA vector resulted in a 50–70% repression of relB mRNA in the two clones, consistent with the RelB knockdown seen above. Of note, MnSOD and survivin RNA levels were substantially reduced in WEHI-siRelB clones 8 and 10: 56–63% for MnSOD and 35–49% for survivin. Thus, these data implicate RelB in the control of expression of the two known anti-apoptotic factors MnSOD and Survivin in WEHI 231 cells.

Ectopic RelB Expression Induces Prosurvival Gene Expression and Rescues WEHI 231 Cells from Anti-IgM-induced Death—To test whether ectopic RelB expression is sufficient to rescue cells from anti-IgM-mediated apoptosis, WEHI 231 cells were transfected with pMexNeo EV DNA or a murine relB expression vector DNA. Mixed populations of stable transfectants (WEHI-EV MP and WEHI-RelB MP cells) were selected and two clones (WEHI-RelB clones 1 and 7) were isolated from the WEHI-RelB MP cells by limiting dilution. Nuclear extracts (WEHI-EV MP and WEHI-RelB MP cells) were selected and subjected to immunoblot analysis for the levels of Survivin, MnSOD, Bax, Bcl-xL, and Bcl-2 protein levels (Fig. 7C). Ectopic RelB expression increased the levels of MnSOD and Survivin, whereas no changes in the levels of Bcl-2, Bcl-xL, and Bax were seen. Thus, RelB regulates MnSOD and Survivin. Consistent with these findings, CD40L treatment of B-CLL cells increased the mRNA levels of MnSOD and survivin (Fig. 7D). Taken together, these data indicate that ectopic RelB induces expression of prosurvival genes and that these proteins can protect WEHI 231 B lymphoma cells from anti-IgM-induced death.

Ectopic c-Rel Expression Induces Prosurvival Proteins and Rescues WEHI 231 Cells from Anti-IgM-induced Death—Previously, we demonstrated that ectopic c-Rel expression protects WEHI 231 B cells from apoptosis (9). To compare the prosurvival genes controlled by these two NF-κB subunits, WEHI 231 cells were stably transfected with either the empty backbone vector (WEHI-EV) or a c-Rel expression vector, and single WEHI-c-Rel clones 3 and 4 were isolated. Overexpression of c-Rel in the two clonal lines, which was confirmed by immunoblotting, led to increased levels of MnSOD, Survivin, Bcl-xL, and Bcl-2 (Fig. 8A) and enhanced survival upon anti-IgM treatment (Fig. 8B), consistent with our earlier findings (9). Thus, c-Rel induces a wider spectrum of prosurvival proteins compared with RelB.

CD40L stimulation of both patient B-CLL cells and WEHI 231 B lymphoma cells induced mRNA levels of the relB NF-κB subunit. In the WEHI 231 cell model system, CD40L-mediated RelB activation occurred via the newly identified de novo RelB synthesis pathway, and RelB was found to be essential for the protection of these cells from apoptosis induced by BCR engagement. Recently, we reported that cytomegalovirus IE1 protein mediates activation of p50/p65 NF-κB and c-Jun/Fra-2, which work in synergy to potently induce relB gene transcription (25). Of note, CD40 activation was found to significantly increase relB mRNA levels in B cells isolated from CLL patients in microarray analysis (26). Using RT-PCR to quantify mRNA levels, we have confirmed that CD40L treatment of B-CLL cells results in increased relB mRNA levels. This mRNA increase could be reproduced in the WEHI 231 B lymphoma cell model. Furthermore, CD40L treatment of WEHI 231 cells was found to increase the nuclear levels of c-Jun, JunD, and Fra-2 phospho-
CD40L Induces a Prosurvival de Novo RelB Synthesis Pathway

A

\[
\begin{array}{cccc}
\text{EV MP} & \text{RelB MP} & \text{RelB clone 1} & \text{RelB clone 7} \\
\hline
\text{αIgM (h)} & 0 & 24 & 0 & 24 & 0 & 24 & 0 & 24 \\
\text{RelB} & & & & & & & & \\
\text{Oct-1} & & & & & & & & \\
\end{array}
\]

B

\[
\begin{array}{ccc}
\text{EV MP} & \text{RelB MP} & \text{RelB clone 1} & \text{RelB clone 7} \\
\hline
\text{% Dead Cells} & 0 & 24 & 0 & 24 & 0 & 24 & 0 & 24 \\
\end{array}
\]

C

\[
\begin{aligned}
\text{RelB} & \quad \text{MnSOD} \\
\text{Bcl-2} & \quad \text{Bax} \\
\beta\text{-actin} & \quad \text{Survivin} \\
\beta\text{-actin} & \quad \text{Bcl-X}_L
\end{aligned}
\]

D

\[
\begin{aligned}
\text{CD40L:} & \quad \text{JM37} & \quad \text{JM40} & \quad \text{JM42} \\
\text{MNSOD} & \quad - & \quad + & \quad - & \quad + & \quad - & \quad + \\
\text{survivin} & \quad - & \quad + & \quad - & \quad + & \quad - & \quad + \\
\text{GAPDH} & \quad - & \quad + & \quad - & \quad + & \quad - & \quad +
\end{aligned}
\]

FIGURE 7. Ectopic expression of RelB induces prosurvival gene expression and reduces anti-IgM-induced death of WEHI 231 cells. A, mixed populations of WEHI 231 cells stably transfected with either the pMexNeo EV or RelB expression vector (WEHI-EV MP or WEHI-RelB MP cells, respectively) or single WEHI-RelB clones 1 and 7 were either left untreated (0) or treated with anti-IgM (αIgM) for 24 h, and nuclear extracts were prepared. Samples (20 μg) were subjected to immunoblot analysis for the levels of RelB and Oct-1 (used as a loading control). B, the stable lines in A were treated with anti-IgM for 48 h, and trypan blue analysis was performed to determine the percent dead cells in the population. C, whole cell extracts were isolated from untreated WEHI-EV MP, WEHI-RelB MP, and WEHI-RelB clone 1 and 7 cells, and samples were subjected to immunoblot analysis for RelB, MnSOD, Bcl-2, Bax, Survivin, Bcl-xL, and β-actin (used as a loading control). D, RNAs from patient JM37, JM40, and JM42 B-CLL cells either left untreated or stimulated with CD40L for 24 h (as described for Fig. 1A) were analyzed by RT-PCR for the levels of MNSOD, survivin, and GAPDH.
CD40L induces a prosurvival de novo RelB synthesis pathway.

A

![Diagram](Image)

**FIGURE 8. Ectopic expression of c-Rel induces prosurvival gene expression and reduces anti-IgM-induced cell death.** A, WEHI 231 cells were stably transfected with either the pcDNA3 EV (WEHI-EV) or c-Rel expression vector, and single WEHI-c-Rel clones 3 and 4 were isolated. Samples (10–50 μg) of whole cell extracts were subjected to immunoblot analysis for the levels of c-Rel, MnSOD, Survivin, Bcl-XL, Bcl-2, and β-actin (used as a loading control). B, the stable lines in A were treated with anti-IgM for 48 h, and trypan blue analysis was performed to determine the percent dead cells in the population.

Blood associated with both tumor survival and resistance to chemotherapy (59). Interestingly, activation of RelB by CD40L was so potent that it was able to overwhelm the reduction by RelB siRNA (data not shown). Of note, Zarnegar et al. (61) found that knockdown of RelB using siRNA technology strongly represses bcl-xL mRNA. In our studies, p50/RelB complexes failed to bind to the κB element of the bcl-xL promoter or to induce its transcription (38). Consistent with our previous data, in this study, overexpression or knockdown of RelB in WEHI 231 cells did not change the levels of Bcl-xL, whereas ectopic c-Rel induced its expression. These differences suggest that RelB may be involved in bcl-xL regulation indirectly through a still unknown factor. Bcl-2 levels in WEHI 231 cells were also unchanged by RelB, whereas they were induced with c-Rel. Interestingly, our data implicate RelB in the regulation of the antioxidant and anti-apoptotic factor MnSOD in WEHI 231 cells. This is consistent with a recent report by Josson et al. (41) indicating that MnSOD, regulated through RelB, increases the resistance of prostate cancer cells to radiation. Finally, CD40L stimulation has been shown to improve the viability of B cells isolated from patients with CLL, leading to the specific up-regulation of Survivin (64). Here, we have shown that CD40L stimulation of patient B-CLL cells increases both survivin and MnSOD mRNA levels. Consistently, our data indicate that RelB regulates the levels of Survivin and MnSOD in WEHI 231 cells. Overall, our data identify a transcriptional mechanism regulating RelB expression that plays an important role in the control of B cell survival and proliferation.

Acknowledgments—We thank J. Taiyang and S. Gurdak for generously providing CD40L and anti-CD8 antibody and R. Bravo, R. Forough, D. Chalbos, F.-E. Johansen, and C. V. Paya for cloned DNAs. We thank Xiaobo Wang and Sean Eddy for helpful comments on the manuscript and Sandy Solomon and Dave Sherr for help with flow cytometry.

REFERENCES

1. Quezada, S. A., Jarvinen, L. Z., Lind, E. F., and Noelle, R. J. (2004) *Annu. Rev. Immunol.* **22**, 307–328
2. Bishop, G. A., and Hostager, B. S. (2003) *Cytokine Growth Factor Rev.* **14**, 297–309
3. Lougaris, V., Badolato, R., Ferrari, S., and Plebani, A. (2005) *Immunol. Rev.* **203**, 48–66
4. Kehry, M. R. (1996) *J. Immunol.* **156**, 2345–2348
5. Romano, M. F., Lamberti, A., Tassone, P., Alfinito, F., Costantini, S., Chiuruazzi, F., DeFranco, T., Bonelli, P., Tuccillo, F., Turco, M. C., and Venuta, S. (1998) *Blood* **92**, 990–995
6. Berberich, I., Shu, G. L., and Clark, E. A. (1994) *J. Immunol.* **153**, 4357–4366
7. Tsutaba, T., Wu, J., and Honjo, T. (1993) *Nature* **364**, 645–648
8. Lee, H., Arsuria, M., Wu, M., Duyao, M., Buckler, A. J., and Sonenshein, G. E. (1995) *J. Exp. Med.* **181**, 1169–1177
9. Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Arsuria, M., Katz, D., FitzGerald, M. J., Rothstein, T. L., Sherr, D. H., and Sonenshein, G. E. (1996) *EMBO J.* **15**, 4682–4690
10. Schauer, S. L., Wang, Z., Sonenshein, G. E., and Rothstein, T. L. (1996) *J. Immunol.* **157**, 81–86
11. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* **9**, 2723–2735
12. Finco, T. S., and Baldwin, A. S. (1995) *Immunity* **3**, 263–272
13. Foo, S. Y., and Nolan, G. P. (1999) *Trends Genet.* **15**, 229–235
CD40L Induces a Prosurvival de Novo RelB Synthesis Pathway

14. Gilmore, T. D., Koedood, M., Piffat, K. A., and White, D. W. (1996) Oncogene 13, 1367–1378
15. Toth, C. R., Hostutler, R. F., Baldwin, A. S., Jr., and Bender, T. P. (1995) J. Biol. Chem. 270, 7661–7671
16. Duyao, M. P., Buckler, A. J., and Sonenshein, G. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4727–4731
17. Sen, R., and Baltimore, D. (1986) Cell 47, 921–928
18. Rooney, J. W., Dubois, P. M., and Sibley, C. H. (1991) Eur. J. Immunol. 21, 2993–2998
19. Liu, J. L., Chiles, T. C., Sen, R. J., and Rothstein, T. L. (1991) J. Immunol. 146, 1685–1691
20. Ruland, J., and Mak, T. W. (2003) Immuno Rev. 193, 93–100
21. Israel, A. (2006) Trends Immunol. 27, 395–397
22. Neumann, M., Wohlleben, G., Chuvpilo, S., Kistler, B., Wirth, T., Serfling, A. F., and Sherr, D. H. (1997) J. Exp. Med. 186, 989–999
23. Gricks, C. S., Zahrieh, D., Zauls, A. J., Gorgun, G., Drandi, D., Mauerer, K., Wang, X., and Sonenshein, G. E. (2005) J. Virol. 80, 3315–3324
24. Bren, G. D., Solan, N. I., Miyoshi, H., Pennington, K. N., Pobst, L. J., and Paya, C. V. (2001) Oncogene 20, 7722–7733
25. Wang, X., and Sonenshein, G. E. (2005) J. Virol. 79, 95–105
26. Ryseck, R. P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzenski, P., and Bravo, R. (1992) Mol. Cell. Biol. 12, 674–684
27. Weylie, B., Zhu, J., Singh, U., Ambrus, S., and Forough, R. (2006) J. Vasc. Res. 43, 61–69
28. Teysier, C., Belgue, K., Galtier, F., and Chalbos, A. (2007) J. Immunol. 173, 1849–1857
29. Romieu-Mourez, R., Kim, D. W., Shin, S. M., Demico, E. G., Landesman-Bollag, E., Seldin, D. C., Cardiff, R. D., and Sonenshein, G. E. (2003) Mol. Cell. Biol. 23, 5738–5754
30. Teysier, C., Belgue, K., Galtier, F., and Chalbos, A. (2007) J. Immunol. 173, 1849–1857
31. Romieu-Mourez, R., Kim, D. W., Shin, S. M., Demico, E. G., Landesman-Bollag, E., Seldin, D. C., Cardiff, R. D., and Sonenshein, G. E. (2003) Mol. Cell. Biol. 23, 5738–5754
32. La Rosa, F. A., Pierce, J. W., and Sonenshein, G. E. (1994) Mol. Cell. Biol. 14, 1039–1044
33. Wu, M., Bellas, R. E., Shen, J., and Sonenshein, G. E. (1998) J. Exp. Med. 187, 1671–1679
34. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Proc Natl. Acad. Sci. U. S. A. 90, 8392–8396
35. Weylie, B., Zhu, J., Singh, U., Ambrus, S., and Forough, R. (2006) J. Vasc. Res. 43, 61–69
36. Teysier, C., Belgue, K., Galtier, F., and Chalbos, A. (2007) J. Immunol. 173, 1849–1857
37. Romieu-Mourez, R., Kim, D. W., Shin, S. M., Demico, E. G., Landesman-Bollag, E., Seldin, D. C., Cardiff, R. D., and Sonenshein, G. E. (2003) Mol. Cell. Biol. 23, 5738–5754
38. La Rosa, F. A., Pierce, J. W., and Sonenshein, G. E. (1994) Mol. Cell. Biol. 14, 1039–1044
39. Wu, M., Bellas, R. E., Shen, J., and Sonenshein, G. E. (1998) J. Exp. Med. 187, 1671–1679
40. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Proc Natl. Acad. Sci. U. S. A. 90, 8392–8396
41. Suzuki, T., Yoshimura, L. C., Lo, P., Terry, N., Reid, P. S., and Ford, J. R. (2002) Immunity 16, 37–50
42. Furman, R. R., Asgary, Z., Mascarenhas, J. O., Liou, H. C., and Schattner, E. J. (2000) J. Immunol. 164, 2200–2206
43. Challos, A., Elpisopoulos, A. G., Holder, M. J., Burguete, A. S., Pound, J. D., Chamba, A., Grafton, G., Armitage, R. J., Gregory, C. D., Martin-Bou, H., Young, L., and Gordon, J. (2002) Blood 99, 3411–3418
44. Younes, A., Snell, V., Consoli, U., Clodi, K., Zhao, S., Palmer, J. L., Thomas, E. K., Armitage, R. J., and Andreff, M. (1998) Br. J. Haematol. 100, 135–141
45. Zarnegar, B., He, J. Q., Oganesyan, G., Hoffmann, A., Baltimore, D., and Cheng, G. (2004) Proc Natl. Acad. Sci. U. S. A. 101, 8108–8113
46. Jacques, T., Chen, S., and Baud, V. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14635–14640
47. Suhusami, M., and Pilk, R. B. (1999) Oncogene 18, 7360–7369
48. Chen, C., Edelstein, L. C., and Gelinas, C. (2000) Mol. Cell. Biol. 20, 2687–2695
49. Granzerio, L., Ghia, P., Girard, D., Strola, G., Geuna, M., Montagna, L., Piccoli, P., Golini, C., and Calgaris-Cappio, F. (2001) Blood 97, 2777–2783
50. Agarwal, S. K., Novotny, E. A., Crabtree, J. S., Weitzman, J. B., Yaniv, M., Burns, A. L., Chandrasekharappa, S. C., Collins, F. S., Spiegel, A. M., and Marx, S. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10770–10775