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Rel/NF-κB Represses *bcl-2* Transcription in pro-B Lymphocytes

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The mechanisms controlling programmed cell death (PCD) during early B cell development are not well understood. Members of both the Bcl-2 family of apoptosis-related proteins and the nuclear factor-kappa B/Rel (NF-κB/Rel) family of transcription factors are expressed differentially during B cell development. To date, however, no direct interactions between these two families have been demonstrated. The FL5.12 cell line represents a model for progenitor B cell development. Such cells reproducibly undergo PCD upon IL-3 withdrawal. The signal to enter the apoptotic pathway is mediated by a shift in the ratio of Bcl-2:Bax. While bax levels remain constant, bcl-2 transcription rate, steady-state mRNA, and protein levels decrease. Analysis of the bcl-2 promoter reveals 3 κB sites functionally able to bind κB factors from FL5.12 nuclear extracts. Cotransfection studies demonstrate that NF-κB factors can repress *bcl-2* transcription and that site-directed mutagenesis of the κB motifs abolishes this repression. These studies suggest that NF-κB mediates PCD in pro-B cells through transcriptional repression of the survival gene *bcl-2*, thus shifting the bcl-2:bax ratio in favor of death-promoting complexes.

B cell development Progenitor B cells Programmed cell death or apoptosis Transcription Nuclear factor kappa B BCL-2

IN the bone marrow microenvironment, stromal support cells secrete various cytokines and trophic factors, which maintain the survival of early lineage B cells [reviewed in (13)]. Nevertheless, between the progenitor (pro-) and precursor (pre-) stages of B-cell development, an estimated 75% of cells die by programmed cell death (PCD) (13,15,40). The molecular mechanisms regulating this process at this stage of B-cell maturation remain uncharacterized. The nontransformed interleukin-3 (IL-3)-dependent murine progenitor lymphocytic cell line, FL5.12, serves as an excellent model system in which to study apoptosis in early B-cell lineages because of its cytokine dependency, which recapitulates the maintenance of bone marrow B cells by survival factors (33). Upon cytokine withdrawal these cells reproducibly undergo apoptosis (6). Under appropriate conditions, FL5.12 can be induced to differentiate into IgM-secreting cells, demonstrating that their developmental pathway is intact (33). This tissue culture system allows for the dissection of signal transduction pathways that regulate PCD in early lineage B lymphocytes in the bone marrow microenvironment. Our studies focused on the interrelationship between two groups of proteins: the Bcl-2 family and the NF-κB/Rel family of transcription factors.

The massive apoptosis occurring between pro- and pre-B stages coincides with downregulation of Bcl-2 protein (15,29,40). Bcl-2, the prototypic member of the bcl-2 family, was first identified as part of the most common translocation in human B-cell follicular lymphoma (3,9,44). In many forms of PCD, the bcl-2 family regulates the rheostat between cellular life and death (26). The cellular ratios of "survival" bcl-2 family factors (e.g., bcl-2, bcl-X_L, mcl-1) with those of "death" factors (e.g., bax, bcl-X_S, bad, bak,

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bik-1) play a key role in regulating the physiologic cell death of B lymphocytes (10). The expression of Bcl-2 is biphasic in B-cell development. Bcl-2 is expressed at high levels only in progenitor and mature B-cell stages, and it is low during all intermediate stages (Fig. 1) (29,35). During B lymphoid development, levels of Bcl-2 protein follow those of *bcl-2* mRNA, suggesting that regulation occurs at the level of transcription (19,29). The mechanism by which transcriptional repression occurs between the pro- and pre-B cell stage is not known.

NF-κB family members form homo- or heterodimers with each other and remain bound in an inactive cytoplasmic complex with inhibitory proteins, called IκBs. Upon stimulation by a wide variety of agonists, including cytokines and growth factors, IκB is phosphorylated, ubiquitinated, and degraded, exposing the nuclear localization sequence of NF-κB members, thereby promoting their nuclear translocation (46). The subunit composition of NF-κB changes during B-cell development (Fig. 1). In precursor B cells the predominant species is p50/RelA while in immature B cells it is p50/cRel (18,30,36). This differential expression underscores the hypothesis that different NF-κB members may have different functions during B-cell development.

NF-κB is known to regulate numerous genes whose products are critical in the development and function of the immune system. Such genes are involved in response to viral infections, inflammatory and acute phase reactions, processes in which PCD is tightly controlled. NF-κB factors have been implicated as both activators and repressors of PCD, depending on the stimulus and cell type examined. For example, NF-κB p50/RelA is protective in the tumor necrosis factor-α (TNF-α) model of PCD (4,31, 45,47). On the other hand, there are established indications that NF-κB may be involved in promoting PCD. v-rel is cytopathic in murine fibroblasts (43). The same protein, if expressed in avian cells, causes a transforming phenotype. In addition, cRel expression in the avian embryo is correlated with cells undergoing PCD (1). Finally, the anti-inflammatory drug aspirin (sodium salicylate) protects neuronal cells by downregulation of NF-κB, thereby implicating this family of factors in the promotion of cell death during inflammation (17). Taken together, these observations indicate that NF-κB members can have dramatically different effect during PCD in different cell systems.

In complement to this work, we have shown that stably expressing a transdominant inhibitor of NF-κB activity, termed IκB-αΔN (7,22) in FL5.12 cells, significantly delayed death following cytokine withdrawal. NF-κB member RelA is constitutively present in the nucleus of these cells. Between 2 to 8 h after cytokine withdrawal, the major NF-κB inhibitor, IκB-α, is degraded and NF-κB member cRel is translocated to the nucleus. In addition, transient overexpression of: (a) IκB-αΔN delays, (b) RelA has no effect, and (c) cRel precipitates PCD in FL5.12 cells after cytokine withdrawal. Finally, bone marrow derived B cells from transgenic mice expressing IκB-αΔN die more slowly than nontransgenic cells when cultured in the absence of survival factors. This role of NF-κB in cytokine-mediated PCD is specific because when these factors are exogenously provided, the differential death is abolished (Sohur et al., in press). In summary, these data propose that in cytokine-mediated PCD in early lineage B cells: (i) NF-κB is apoptogenic, (ii) RelA has no apparent function, and (ii) cRel may mediate proapoptotic role of NF-κB.

In this report, we advance a mechanistic model in which NF-κB induces PCD by repression of *bcl-2* transcription in the FL5.12 model of progenitor B lymphocytes, upon cytokine withdrawal. Our results show that in FL5.12 cells, Bcl-2 protein decreases

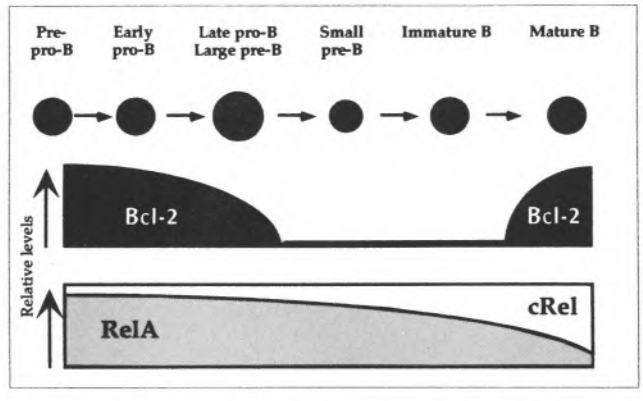

FIG.1. Differential expression of Bcl-2 and NF-κB during B cell development (18,29,30,35,36).

postcytokine withdrawal due, in part, to transcriptional repression of its gene. We further demonstrate that the human *bcl-2* promoter contains three putative NF-κB enhancer elements that associate with FL5.12 extracts in vitro. Assays of expression show that the *bcl-2* promoter is repressed at early time points after cytokine withdrawal. This repression is alleviated when the κB sites are mutated. These results support the hypothesis that cytokine withdrawal-mediated NF-κB activity directly represses *bcl-2* transcription, thereby promoting PCD in early lineage B cells.

MATERIALS AND METHODS

Cell Culture

The murine FL5.12 pro-B lymphocyte line (6,33,39) was maintained in 5% CO_2 in Iscove's modified medium (Mediatech), supplemented with 10% heat-inactivated fetal bovine serum, 10% WEHI-3B conditioned medium (IL-3 source), 1× penicillin/streptomycin, and 50 μM β-mercaptoethanol. *Drosophila* Schneider (S2) cells were cultured as previously described (25).

Mutageneses and Transfections

Site-directed mutageneses of the κB sites in the *bcl-2* promoter were carried out as per manufacturer's directions (Biorad T7 mutagenesis kit). The primers for κB1, κB2, and κB3 used were 5′-ACA CTT GAT TCT GAT CTT GAA CTC TTG GCA TGA-3′, 5′-TAT AGC TGA TTT TAG CCT TAA CAA TGA ATC AGG A-3′, 5′-AAT GTC AAT CCG CAG CAA TAA CAA CCG GAG ATC T-3′, respectively. At least two independent clones of each mutant construct were isolated and the *bcl-2* promoter region was completely sequenced (Vanderbilt-Ingram Cancer Center Sequencing Core). FL5.12 cells were transiently transfected with either bcl-2/luc or mut-bcl-2/luc (10 μl/mg) together with SV40-βgal (2 μg) as an internal transfection control, via DEAE-Trypsin protocol. Forty-eight hours posttransfection, multiple independent transfectants were pooled and pelleted by centrifugation, washed once in serum-free medium, and resuspended in complete medium without IL-3. At the indicated time points, 1×10^6 cells were removed, processed into cell lysates, and analyzed for luciferase activity according to manufacturer's instructions (Analytical Bioluminescence). Schneider S2 cells were cotransfected with 2 μg reporter construct, 1 μg β-galactosidase control plasmid, and 4 μg κB expression plasmid as indicated. All transfectants were equalized to 12 μg total DNA using the empty Δp expression vector (25). Cells were transfected by calcium phosphate precipitation and, 48 h posttransfection, cell lysates were processed for luciferase activity and normalized to β-galactosidase activity.

Protein Isolation and Western Analysis

FL5.12 cells were washed three times with phosphate-buffered saline (PBS) and resuspended in complete medium without IL-3. The time $t = 0$ was taken as the midpoint between the time when IL-3+ medium was withdrawn and IL-3– medium was added. At indicated times, cells were washed once with ice-cold PBS (all centrifugations done at $800 \times g$ for 5 min) and cell pellets stored at –80°C until further use. Protein isolation was carried out at 4°C. Pellets were thawed on ice in 1 ml of 1× Wu buffer (42) [2× Wu buffer stock: 20 mM HEPES, pH 7.40, 3 mM $MgCl_2$, 0.2 mM EGTA, 10% glycerol, 100 mM β-glycerophosphate, 1 mM dithiotreitol (DTT), 2 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF)], pipetted vigorously, allowed to stand for 10 min, and pipetted again. The samples were centrifuged at $11{,}000 \times g$ for 30 min. Supernatants were collected as cytoplasmic fractions. Nuclei were washed in 1 ml of 1× Wu buffer centrifuged for 15 min, resuspended by shearing through an 18-gauge needle in 1× Wu buffer, supplemented with 450 mM KCl, and centrifuged for 30 min. These nuclear fractions and the cytoplasmic fractions were dialyzed overnight with buffer D (20 mM HEPES, pH 7.90, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 50 mM β-glycerophosphate, 1 μM pepstatin A). Dialysates were centrifuged for 30 min and Bradford analysis (BioRad) was conducted to determine concentration.

Equal amounts of cytoplasmic (100–150 μg) or nuclear fractions (50–75 μg) were fractionated by SDS-polyacrylamide gel electrophoresis prior to electroblot transfer to polyvinylidene fluoride (PVDF) Immobilon membrane (Millipore). Membranes were immunoblotted with the following antisera: Bax (sc-493) (Santa Cruz Biotechnology); Bcl-2 (Pharmingen 15616 E); Bad (B31420) (Signal Transduction Laboratories). Immune complexes were visualized via chemiluminescent detection according to the manufacturer's protocols (Renaissance, NEN Life Science Products).

Northern Analysis

RNA from FL5.12 cells deprived of IL-3 for the indicated times was isolated by Tri Reagent (Molecular Research Center, Inc.). For *bcl-2* Northern blots, mRNA was enriched for poly(A) species (Oligotex, Qiagen). Either 2.5 μg poly(A) mRNA or 12–15 μg

of total RNA were fractionated by formaldehyde-agarose gel electrophoresis prior to transfer to nitrocellulose (HyBond-N, Amersham). ^{32}P-labeled sequence-specific cDNA probes for *bcl-2*, *bax*, and glyceraldehyde phosphate dedehydrogenase (GAPDH) were generated by random priming (Prime It II, Stratagene) and hybridized to the membranes. Membranes were washed twice (5 min followed by 30 min) with solution I [1× SSPE (20× SSPE/NaOH, pH 7.4, 3 M NaCl, 200 mM $NaH_2PO_4 \cdot H_2O$, 25 mM EDTA), 0.5% SDS] and solution II [0.2× SSPE, 1.0% SDS] once (30 min), both at room temperature. Finally, membranes were washed twice for 30 min at 60°C with solution III [0.1% SSPE, 0.5% SDS] before exposure to film at −80°C and development.

Nuclear Run-On Analysis

The assay was a modification of (16,34). FL5.12 cells (5×10^7) were washed three times with ice-cold PBS (unless otherwise noted, cells and nuclei were spun at $800 \times g$ for 5 min). Pellets were suspended in 2.0 ml of NP40 lysis buffer [1 mM Tris-HCl, pH 7.4, 1 mM NaCl, 3 mM $MgCl_2$, 0.8% Nonidet P40 (NP40) (v/v)], incubated on ice for 5 min, centrifuged, washed with 2.0 ml of NP40 lysis buffer. Pellets were resuspended in 100 µl of nuclear storage buffer [50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 40% (v/v) glycerol] and stored at −80°C. Nuclei were thawed, centrifuged, and pellets were resuspended in reaction buffer [50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM of each ATP, CTP, and GTP, and 333 µCi of 800 Ci mmol^{-1} [α-^{32}P]UTP (NEN)] and incubated at 30°C for 30 min. Each reaction mixture was added to 1 ml of ice-cold PBS (supplemented with 1 mM $MgCl_2$) and centrifuged. To nuclei, 2 µl of 1 mg/ml yeast tRNA, 348 µl of THC buffer [20 mM Tris-HCl (pH 7.4) 1 mM $CaCl_2$], and 25 µl of 1 U/µl RQ1 DNase (Promega) were added and incubated at 37°C for 30 min. Incubation was continued for another 15 min after addition of 50 µl of 0.2 M EDTA and 50 µl of 10% sodium dodecyl sulfate (SDS). A phenol-chloroform extraction was followed by precipitation in 20% trichloroacetic acid (TCA) for 30 min on ice and centrifugation at $11,000 \times g$ for 30 min. RNA pellets were rinsed four times with 5% TCA, resuspended in water, and precipitated overnight with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2. RNA samples were pelleted, washed with 70% ethanol, and resuspended in water. For each experiment set, counts were normalized between samples. The 5′ terminal regions unique between the *bcl-2* family members of the murine *bcl-2* and murine bax cDNAs (gift of Dr. S. Korsmeyer) were amplified by polymerase chain reaction (PCR). A *BglI*/*Bam*HI fragment from Bluescript II SK-(Stratagene), spanning the multiple cloning site, was used as a negative control. For each slot, 2 µg of DNA was denatured in 0.3 M NaOH by incubation at 65°C for 30 min. The DNA solution was neutralized with ammonium acetate and applied to nitrocellulose presoaked in 1 M ammonium acetate. Filters were washed with 5× SSC [20× SSC, pH 7.0, 3 M NaCl, 0.3 M sodium citrate], dried and baked for 2 h under vacuum at 80°C before use. Filters were soaked in 5× SSC and incubated at 65°C for at least 6 h in a prehybridization buffer [5 ml, 4× TES (0.04 M Tris, pH 7.3, 1.2 M NaCl, 0.02 M EDTA), 2.0 ml 0.1% Ficoll/polyvinylpyrrolidone (PVP), 2.0 ml 5% sodium pyruvate, 0.9 ml of 1 mg/ml of tRNA, and 0.1 ml of 2 mg/ml polycytidine]. Normalized counts were loaded in plastic bags with strips and volume was equalized to 2.0 ml with water, bags sealed, and incubated in a hybridization oven at 65°C for 4 days. Strips were washed twice with 2× SSC at 65°C and incubated twice with 2× SSC at 65°C for 40 min each. Filters were further incubated with 1:4000 dilutions of RNase A and 20,000 U/ml RNase T1 (Boehringer Mannheim) in 2× SSC at 37°C for 30 min. Filters were washed twice with 2× SSC at 37°C and incubated twice with 2× SSC 1 min each. Filters were dried and exposed overnight and bands quantified in a Fujix phosphoimager.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed as previously detailed (24). Probes for κB1, κB2, and κB3 within the *bcl-2* promoter (+ strands only) were 5′-AAC TCG AGC CAA GAG GGA AAC ACC AGA ATC AAC TCG AGA A-3′, 5′-AAC TCG AGT TCA TTG GGA AGT TTC AAA TCA GCC TCG AGA A-3′, and 5′-AAC TCG AGC CGG TTG GGA TTC CTG CGG ATT CTC GAG-3′, respectively.

RESULTS

It has been shown that endogenous levels of Bcl-2 decrease after cytokine withdrawal in FL5.12 cells (38) and forced expression of bcl-2 significantly delays FL5.12 PCD (6,20,39). We sought to characterize the expression patterns of bcl-2 family members after induction of PCD. Immunoblots using cytoplasmic extracts isolated from FL5.12 cells in a time course after cytokine withdrawal were performed (Fig. 2). As previously noted (38), levels of Bcl-2 protein begin to decrease 8 h postcytokine removal and become undetectable by 23 h. In our clones, no alteration in the cytoplasmic levels of death inducers

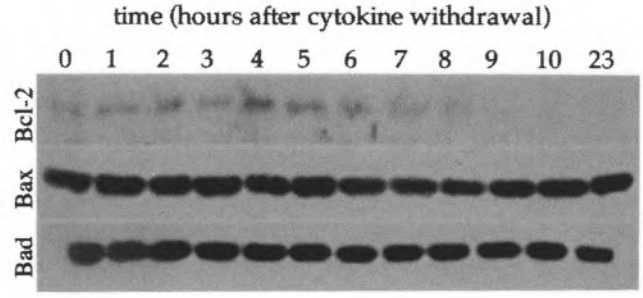

FIG. 2. Levels of Bcl-2 decrease following IL-3 withdrawal in FL5.12 pro-B cells. Bcl-2 protein levels within hours following IL-3 withdrawal. Equal amount of fractionated cytoplasmic protein extracts (100–150 μg) were analyzed for bcl-2 family members, Bax and Bad. Panels shown are representative of a minimum of two independent Western analyses from at least two separate time point collections.

bax or bad was detected (Fig. 2). These results support previous models of B lineage apoptosis, which propose that specific stimuli create an imbalance in the bcl-2:bax ratio in favor of the death protein (39).

To address the mechanism by which bcl-2 levels are decreased, we first analyzed the transcriptional regulation of the *bcl-2* gene. Northern analysis revealed that *bcl-2* mRNA levels decreased in a temporal pattern consistent with the disappearance of the Bcl-2 protein (Fig. 3A). *Bax* and *bad* mRNA level remain constant (Fig. 3A). To assay directly the transcriptional activity of the *bcl-2* gene, nuclear run-on analyses were performed and quantified. Figure 3B shows the summary of two separate run-on experiments demonstrating a reduction in the rate of *bcl-2* transcription at two time points following cytokine withdrawal. As might be predicted from the analysis of *bax* mRNA by Northern blot, the rate of *bax* transcription remained constant.

Examination of the *bcl-2* promoter sequence reveals three putative NF-κB enhancer motifs in the region located between the P1 and P2 start sites of transcription (Fig. 4A). All three motifs can form specific complexes as demonstrated by EMSA (Fig. 4B) with nuclear extracts from cytokine withdrawn (6 h post) FL5.12 cells; however, the κB1 appears to bind with highest affinity. Bacterially produced NF-κB1 (p50), NF-κB2 (p52), cRel, and RelA can associate with all three κB enhancer motifs in vitro (data not shown). In order to address whether the putative NF-κB enhancer elements are critical for *bcl-2* expression, site-directed oligonucleotide mutagenesis was performed to introduce nonfunctional mutations in all of the κB elements within the *bcl-2* promoter. Wild-type and mutant promoter constructs driving the expression of a luciferase reporter were introduced transiently in FL5.12 cells. Both plasmids show relatively high basal activity, consistent with Western and

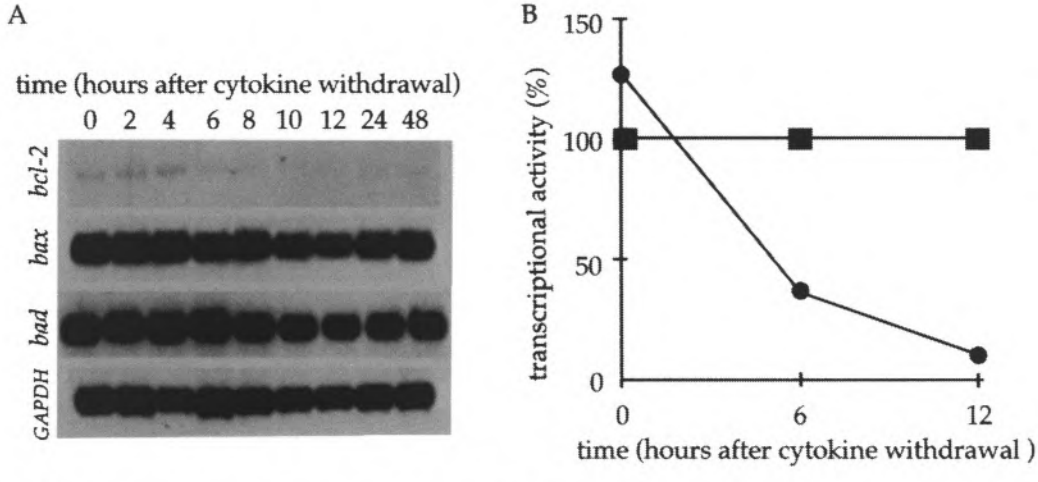

FIG 3. (A) Bcl-2 transcripts decrease following IL-3 withdrawal. Northern blot analysis of mRNA isolated at the indicated times and probed with radiolabeled cDNAs specific for murine *bcl-2* (top panel), bax (second panel), bad (third panel), and glyceraldehyde-phosphate-dehydrogenase (GAPDH, bottom panel). (B) Bcl-2 transcription rates (●) decrease following IL-3 withdrawal. Summary of two independent nuclear run-on transcriptions quantified by phosphoimager analysis of *bcl-2* transcripts with respect to bax transcripts. No alteration in bax transcription (■) was observed in any experiment. Individual lanes were adjusted based on background plasmid control (murine GAPDH).

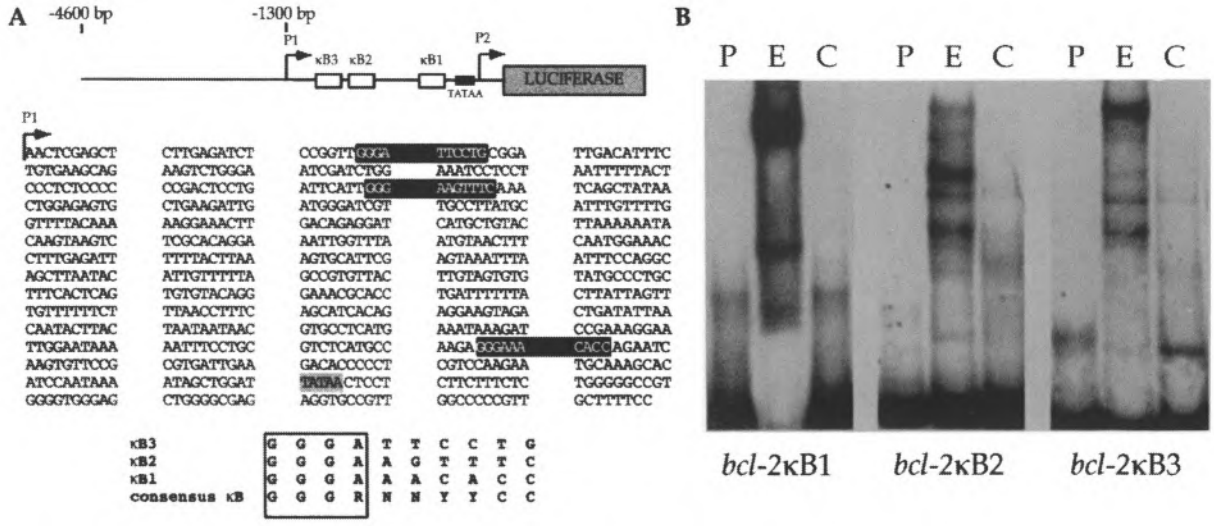

FIG. 4. The *bcl-2* promoter contains three putative κB recognition sites. (A) Top panel is a schematic of the parental *bcl-2* promoter luciferase reporter construct used in transfection experiments. The sites are designated as κB1, κB2, and κB3 with the site nearest to the second start site (P2) referred to as κB1. The middle panel details the sequence and positioning of the three NF-κB recognition sites. The lower panel compares each putative κB with the canonical consensus site. (B) EMSAs of individual putative κB elements from the *bcl-2* promoter using nuclear extracts from FL5.12 cells (6 h postcytokine withdrawal). The inclusion of 50× cold oligonucleotide as a competitor DNA to the indicated probe effectively competes for binding. P = probe, E = FL5.12 extract (6 h postcytokine withdrawal), C = 50× competition with cold DNA.

Northern results. However, upon cytokine withdrawal, the activity of the wild-type promoter decreases and reaches a minimum point at 6 h into the time course. In contrast, transfections using the triple κB mutant promoter/luc displayed a slightly attenuated basal activity, unaffected by cytokine withdrawal (Fig. 5). These data demonstrate that the κB motifs within the *bcl-2* promoter function during cytokine withdrawal-mediated PCD.

To dissect the interaction of specific NF-κB members with individual κB sites in the *bcl-2* promoter, we chose to perform our studies in *Drosophila* Schneider S2 cells (S2). The S2 line is characterized by a lack of endogenous NF-κB factors able to transactivate mammalian κB enhancer motifs and thus represents a null cell system in which to explore individual NF-κB member function (25). The NF-κB/cRel proto-oncoprotein is specifically translocated to the nucleus after cytokine withdrawal in the same time frame that *bcl-2* promoter activity is repressed (Sohur et al., in press). Therefore, we first chose to analyze the effect of cRel on the *bcl-2* promoter. Cotransfection of a *c-rel* expression vector with the wild-type *bcl-2* promoter resulted in 2.5-fold repression of *bcl-2*-driven luciferase activity (Fig. 6A). Mutation of either κB1 or κB3 relieves the repression mediated by cRel. Mutation of κB2 alone has no effect (Fig. 6A). Additional experiments sought to characterize the role of reconstituted NF-κB p50/RelA. The wild-type or triple-mutant *bcl-2* promoter/

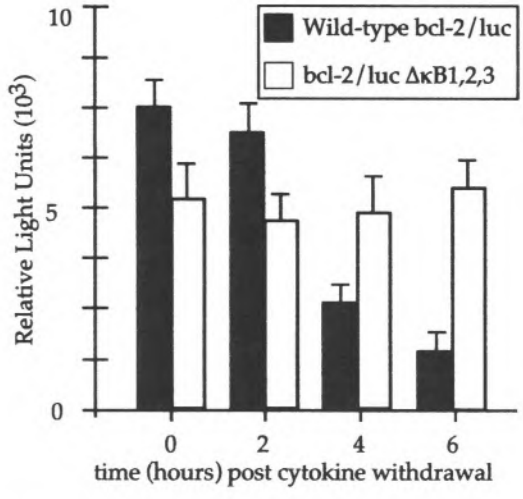

FIG. 5. Repression of the *bcl-2* promoter is alleviated in FL5.12 cells post-IL-3 withdrawal by mutation of the three putative κB elements. Cells were transfected with either bcl-2/luc (black bars) or mut-bcl-2/luc (gray bars) together with SV40-βgal as an internal transfection control. Forty-eight hours posttransfection, multiple independent transfectants were consolidated and replaced in IL-3 medium, after washes. At the indicated time points, 1×10^6 cells were processed for luciferase activity. Assays are the average of five individual transfection experiments. SEM is indicated.

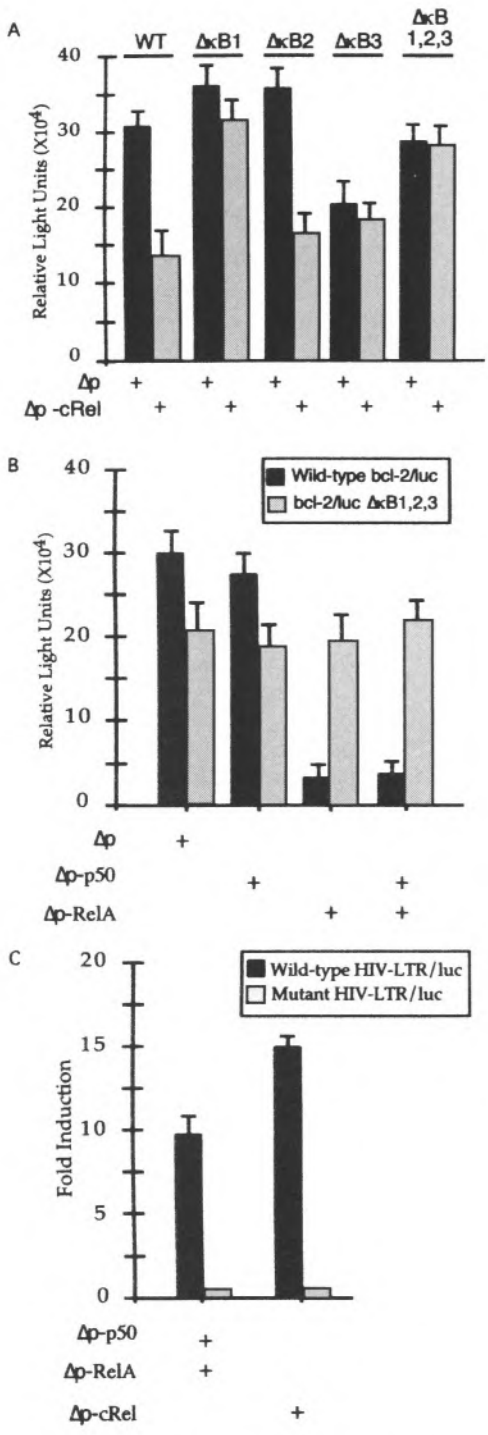

FIG. 6. NF-κB factors specifically repress the *bcl-2* promoter in *Drosophila* S2 cells. (A) S2 cells were cotransfected with β-galactosidase control plasmid, a bcl-2/luc construct and with either empty expression vector (Δp) shown as black bars or with vector expressing murine c-rel (Δp-cRel) shown as gray bars. Wild-type (WT) and ΔκB1, ΔκB2, and ΔκB3 represent the individual mutations in each of the three putative κB sites and the triple mutant is designated ΔκB1, 2, 3. (B) S2 cells were cotransfected with β-galactosidase control plasmid, wild-type *bcl-2* promoter (solid bars), or the triple κB mutant (empty bars) and with either empty vector (Δp) or vector containing either NF-κB1 p50 (Δp-p50) or RelA (Δp-RelA) singly or in combination. Black bars represent the wild-type *bcl-2* reporter construct; gray bars represent the triple-mutant reporter construct. (C) S2 cells were cotransfected with β-galactosidase control plasmid reporter, HIV-LTR/luc construct and with either vectors expressing *c-rel* (Δp-cRel) or RelA (Δp-RelA) and NF-κB1 p50 (Δp-p50). Results are shown as fold induction over cells cotransfected with empty expression vector (Δp). Black bars represent the wild-type HIV-LTR reporter construct; gray bars represent the HIV-LTR reporter construct with both its sites κB mutated. All luciferase reporter assays represent the average of at least six individual transfection experiments. SEM is indicated.

luc constructs were transfected together with expression vectors encoding NF-κB/p50, NF-κB/RelA, or both. The results showed that RelA alone or the p50/RelA combination also have an inhibitory effect on the wild-type *bcl-2* promoter, while showing no effect on the mutant promoter (Fig. 6B).

Activation of a human immunodeficiency virus long terminal repeat/luciferase (HIV-LTR/luc) construct shown to be specifically NF-κB responsive (27) was observed under conditions identical to those that demonstrate repression of the *bcl-2* promoter. HIV-LTR/luc was stimulated 15.2-fold over empty expression vector at the same concentration of transfected c-rel expression plasmid (4 μg) (Fig. 6C). Furthermore, the HIV-LTR/luc was stimulated 9.8-fold over expression vector if p50/RelA was co-transfected. These controls confirm that the repression observed by NF-κB on the *bcl-2* promoter is not the result of general squelching, but rather a specific repression of sequence κB motifs within the *bcl-2* gene 5′ region.

DISCUSSION

In many models of PCD in immunology, cytokine withdrawal promotes downregulation of the Bcl-2, thereby triggering apoptosis, for example, IL-2-dependent CTLL-2, and IL-3-dependent BAF3 cells and 32D cells (2,11,32). Treatment with ionizing radiation, TNF-α, and ceramide, designed to induce apoptosis in HL-60 and U-937 human leukemia cells, also decreases *bcl-2* expression (8). These agonists are known to activate NF-κB [(46) and ref. within]. Given that transcriptional regulation of *bcl-2* is important for B-cell development, that the pattern of NF-κB expression changes during B-cell development, and that both Bcl-2 and NF-κB are implicated in the regulation of apoptosis, we set out to examine the cross-talk between these families. Using the FL5.12 model, our data demonstrate that *bcl-2* is downregulated in response to cytokine withdrawal by

transcriptional repression mediated through κB elements within the *bcl-2* promoter. In addition to transcriptional regulation of *bcl-2*, we have noted that Bcl-2 protein rapidly disappears after cytokine withdrawal, with an approximate half-life of 4.5 h. Half-life of Bcl-2 protein can change dramatically: in the OCI/AML-2 leukemia cell line has a half-life of 20 h but it changes to 43 h with hydrocortisone treatment and 12 h with all-*trans* retinoic acid treatment (5,21). Previously, the half-life for Bcl-2 in resting lymphocytes was reported to be 10.5 h (35). These data suggest that Bcl-2 may be actively degraded, once an apoptotic cascade is triggered. We also note that Bcl-2 levels in FL5.12 are relatively low compared to Bax and Bad. We appreciate that early B cells are a 'hair-trigger" away from death during the developmental scheme, considering that approximately 75% of these cells die by apoptosis (13,15,40). Thus, presence of Bcl-2 at a low level fits in well with our hypothesis in the sense that subtle decreases in the level of Bcl-2 would prime the B cell to die, making this mechanism a sensitive and efficient rheostat. In summary, progenitor B cells may be poised to die in response to the lack of survival factors and the relatively low levels of Bcl-2 may allow for a rapid shifting of cells towards PCD.

Analysis of the ability of NF-κB members to bind to the three putative κB elements within the *bcl-2* promoter in the mammalian NF-κB null S2 *Drosophila* cell line yielded interesting results. Both cRel (Fig. 6A) and p50/RelA (Fig. 6B), two physiologically significant components of the NF-κB because of their strong transactivation domains, can mediate repression in these cells. The HIV-LTR/luc, an NF-κB responsive reporter, is strongly induced under these same conditions (Fig. 6C). These results confirm that the repression of the *bcl-2* promoter by NF-κB members is specific and not a result of general squelching. That RelA mediates repression in S2 cells is in contrast with our data in the FL5.12 system, where overexpression of RelA has no effect (Sohur et al., in press). One explanation for this anomaly is that the S2 cell line is an NF-κB null system and therefore cannot accurately mimic the dynamic interactions between NF-κB members present in the FL5.12 nucleus. These data highlight the contextual action of NF-κB members on the *bcl-2* promoter.

There is precedent for NF-κB behaving as both a transcriptional activator and repressor in the same cell. The *Drosophila* protein Dorsal, a homolog of the Rel/NF-κB family, has demonstrated the ability to activate the *twist* and *snail* genes while repressing the *decapentaplegia* (*dpp*) and *zerknullt* (*zen*) in the midline of the developing embryo. Ptashne and coworkers (28) have described a negative regulatory element (NRE) found adjacent to Dorsal binding sites in the *zen* promoter, able to bind a Dorsal/Dorsal switch protein (DSP1), the *Drosophila* homolog of HMG1. This DSP1 interaction converts the regulatory function of Dorsal into a transcriptional repressor. A similar transcriptional activator/repressor switch has been evoked for regulation of the human interferon-beta (IFN-β) enhancer by NF-κB (28). Our observations have not yet revealed the presence of a repressor NRE adjacent to the κB motifs in the *bcl-2* gene.

A negative response element between P1 and P2 (5′ UTR), which was p53 responsive, was shown to be responsible for downregulation of *bcl-2* (37). It is interesting to note that the κB1 site, described in this report, is located within the previously identified p53 response element. The significance of this observation in early B cells is at present unclear. Transfection experiments with the *bcl-2* promoter, using p53 –/– cells may be considered, to elucidate this issue.

Future studies will be geared towards understanding the nature of the NF-κB complexes at the *bcl-2* promoter during cytokine withdrawal apoptosis. We have been unable to show differential DNA binding of NF-κB members, particularly cRel, at the highest point of NF-κB activity (6 h after cytokine withdrawal) in FL5.12 cells as demonstrated by Western analysis and luciferase reporter assays (Figs. 1 and 5). There are several explanations for the lack of success in this endeavor. First, the formal possibility exists that NF-κB does not modulate *bcl-2* transcription after cytokine withdrawal in FL5.12 cells. However, we have shown that mutating the NF-κB sites in the human *bcl-2* promoter alleviates its repression after cytokine withdrawal (Fig. 5). We have also extensively analyzed this promoter in an NF-κB null line (S2 *Drosophila* cells) and a panel of other cell lines to confirm that NF-κB indeed represses the human *bcl-2* promoter (Fig. 6). This combined evidence strongly suggests that NF-κB and the human *bcl-2* promoter postcytokine withdrawal are coupled and that our inability to demonstrate this by supershift assay in EMSA is due to technical limitations of the cell type and available commercial antibodies.

It is entirely possible that NF-κB/cRel acts on the *bcl-2* promoter as part of a multiprotein complex in the same way that p300 and HMG(I)Y have been shown to participate in NF-κB's action on genes (14,41). Thus, the 30-mer *bcl-2* κB1 we have used in our in vitro experiments may not be sufficient to foster binding. Alternatively, NF-κB/cRel may be part of a complex in FL5.12 cells postcytokine withdrawal such that recognition with available antibodies is not possible because its epitopes are masked. Note that there are several specific bands in our EMSAs

that do not supershift with any antibodies we have used in this project.

Several approaches may be useful for future experiments to determine the identity of the NF-κB member(s) binding to the *bcl-2* promoter postcytokine withdrawal. Ultimately, techniques such as in vivo footprinting will be required to demonstrate that the NF-κB site(s) on the *bcl-2* promoter are occupied postcytokine withdrawal. As other antibodies become available, they may prove successful in supershift experiments. Two other possibilities include using immunohistochemistry to detect nuclear cRel in FL5.12 cells postcytokine withdrawal and UV cross-linking (23) to determine the identity of NF-κB member(s) in these experiments.

In summary, these results suggest that the *bcl-2* gene is one downstream target for the transcriptional regulatory activity of NF-κB transcription complex during early B lineage apoptosis. In our model, progenitor B cells are poised to die by PCD unless continually provided with survival signals. Withdrawal of these signals in the bone marrow microenvironment activates NF-κB, which in turn represses the *bcl-2* promoter. Thus, a subtle decrease in the level of Bcl-2 via both transcriptional repression and active degradation of Bcl-2 protein results in a rapid and efficient shift in favor of death-promoting complexes and apoptosis ensues.

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