

Overexpression of RelA Causes G₁ Arrest and Apoptosis in a Pro-B Cell Line*

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NF- κ B/Rel family proteins form a network of post-translationally regulated transcription factors that respond to a variety of extracellular stimuli and mediate distinct cellular responses. These responses include cytokine gene expression, regulated cell cycle activation, and both the protection from and induction of the cell death program. To examine the function of individual Rel family proteins in B cell development and resolve their role in the signaling of apoptosis, we used a tetracycline-regulated gene expression system to overexpress either c-Rel or RelA in the transformed pro-B cell line 220-8. Elevated levels of RelA, but not c-Rel, induced a G₁ cell cycle arrest followed by apoptosis. Both the DNA binding and transactivation domains of RelA were required for this effect. When RelA was overexpressed in the immature B cell line WEHI 231 or the mature B cell line M12, neither cell cycle arrest nor apoptosis was evident. The differential effects of elevated RelA levels in these cell lines suggests that susceptibility to NF- κ B-induced apoptosis may reflect a relevant selection event during B cell development.

Classical NF- κ B¹ is a heterodimer composed of two protein subunits, p50 and p65, that are members of the Rel family of transcription factors (1). This family consists of five known members, c-Rel, RelA (p65), RelB, p50, and p52 (2–6), and is identified by a characteristic N-terminal 300 amino acid Rel homology domain. This region contains sequences for DNA binding, dimerization, and nuclear localization. Association of Rel family dimers with a second family of proteins, the I κ Bs, is responsible for the cytoplasmic sequestration of inactive NF- κ B in unstimulated cells (7).

NF- κ B was originally identified as a B cell-specific transcription factor that bound to a decameric sequence within the intronic enhancer of the immunoglobulin (Ig) κ light chain gene (8). It had been shown previously that the κ B site within this

enhancer is critical for enhancer activity in reporter assays (9) and that NF- κ B activation controlled the ability of this enhancer to activate transcription (10–12). During early B cell development, Ig κ loci undergoing gene rearrangement were also transcriptionally active (13–15), and this activation correlated with the presence of active nuclear NF- κ B (8, 10). These observations led to the hypothesis that NF- κ B played a critical role in the activation of Ig κ locus transcription and rearrangement during early B cell development (9–11, 14, 16). It was later appreciated that NF- κ B was in fact a ubiquitously expressed, inducible factor (17), responsible for the activation of a diverse array of genes (1), but its role in B cell development continues to be of significant interest.

The role of NF- κ B in the developmental regulation of gene expression has been studied by generating mice deficient in individual Rel family members (18–23). Whereas none of the single knock-out mice showed any obvious defect in early B cell development, a recent report of mice deficient in both p50 and p52 revealed an essential role for NF- κ B in the generation of mature B cells (24). Interestingly, mice deficient in either p50 or c-Rel have normal numbers of mature B cells, but these cells fail to activate appropriately in response to antigen receptor stimulation leading to humoral immunodeficiency (18, 20). The role of RelA in B cell development could not be assessed since deficiency in this factor proved lethal by day 15 of embryogenesis, although adoptive transfer experiments suggested that B cell expression of RelA was not required for normal development (21, 25). Evaluation of RelA-deficient pre-morbid fetuses attributed death to massive liver cell apoptosis. Thus, whereas their precise role in early B cell development remains uncertain, genetic studies implicate Rel family members in the regulation of both cell activation and cell death.

A variety of other studies has demonstrated a role for NF- κ B in both protection from and induction of apoptosis (26–32). These reports encompass a range of both cell types and apoptosis-inducing stimuli. Additional observations have suggested a role for NF- κ B in growth arrest and differentiation (24, 25, 33–37), and recently NF- κ B transcription has been correlated with cell cycle progression (38). These observations lead to the conclusion that the cell type and context of an NF- κ B-inducing stimulus are critical determinants in the outcome of a signal that can lead to proliferation, differentiation, or death.

In an effort to clarify the effects of NF- κ B on cell proliferation and viability during B cell development, we utilized the tetracycline-regulated expression system (39, 40) to examine the individual effects of either RelA or c-Rel overexpression in a pro-B cell line. Whereas elevated levels of RelA resulted in a G₁ cell cycle arrest followed by the induction of apoptosis, the overexpression of c-Rel did not affect cell growth or viability. Both the transactivating potential and the DNA binding specificity of RelA were required for these effects. To investigate cell

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¹ The abbreviations used are: NF- κ B, nuclear factor- κ B; HA, hemagglutinin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; tTA, tetracycline-regulated transactivator protein; 7-AAD, 7-amino actinomycin D; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor α .

type specificity, RelA was also overexpressed in immature and mature B lymphoma cell lines. Interestingly, elevated levels of RelA in these lymphoma cell lines did not result in apoptosis. From these observations, we conclude that RelA expression can result in cell growth arrest, leading to the induction of apoptosis, and that this apoptotic potential may be developmentally stage-specific.

EXPERIMENTAL PROCEDURES

Cell Culture—The pro-B cell line, 220-8, the immature B cell lymphoma, WEHI 231, and the mature B cell lymphoma M12 were cultured in RPMI 1640 with glutamine, supplemented with 10% fetal bovine serum (Gemini Biological Products), 50 μ g/ml penicillin/streptomycin, and 10^{-4} M β -mercaptoethanol.

Cells were transfected by electroporation, and stable transfectants were selected in either mycophenolic acid or G418 (Life Technologies, Inc.). The transfectant pools were single cell-cloned by limiting dilution into antibiotic-containing media to establish clonal populations. All selections were performed and cultures maintained in the presence of 1 μ g/ml tetracycline (Sigma) to repress tTA expression. Expression was induced by harvesting cells by centrifugation and then replating them into media lacking tetracycline.

Expression Constructs—The *gpt* expression cassette from pSV2-*gpt* was cloned into the pTet-tTA plasmid ((41) a gift from Dr. David Schatz) (pTet-tTA*gpt*) to allow establishment of stable transfectants. Correspondingly, the neomycin drug resistance cassette from pGK-neo was cloned into the target plasmid of the inducible system, pTet-splice (41), to create pTetsplice-neo (pTSN). Finally, a hemagglutinin epitope tag (HA-tag) consisting of three tandem HA epitopes (a generous gift from Dr. Susan Michaelis) was cloned into the *EcoRV* site of the pTSN polylinker to generate pTSN.flu. When *HindIII* was used as a cloning site, an in-frame C-terminal HA epitope tag was generated. A custom-designed linker (Life Technologies, Inc.), containing a stop codon, was added at the end of the HA epitope. All cDNA fragments were then cloned into the *HindIII* site of pTSN.flu. In each case, translational reading frame was confirmed by DNA sequencing.

The murine *relA* cDNA (a gift from Dr. Sankar Ghosh) was digested with *Bsu36I* and blunted with mung bean nuclease (Boehringer Mannheim), truncating the gene at the 3' end of the open reading frame. Custom-made oligonucleotide linkers (Life Technologies, Inc.) were designed to maintain the reading frame and ligated to the 3' end. A fragment containing the *relA* open reading frame was then cloned into pTSN.flu. The RelA TADt construct, containing a deletion of the C-terminal transactivation domains, was cloned using a polymerase chain reaction strategy, resulting in a gene fragment missing the 79 C-terminal amino acids. The *c-rel* cDNA (a gift from Sankar Ghosh) was excised by *DraI* digestion (eliminating the last two amino acids of the reported open reading frame), and then cloned into pTSN.flu using oligonucleotide adapters. The mutated *relA* cDNA (RRPA) obtained from Dr. Sankar Ghosh was similarly cloned into pTSN.flu. The *relA-c-rel* chimeric genes were created using a polymerase chain reaction protocol. The 79 C-terminal amino acids (amino acid 471–549) of RelA were fused to the N terminus (433 amino acids) of c-Rel to generate c-Rel.TAD, and the C-terminal 135 amino acids of the c-Rel protein (amino acids 434–568) were fused to the N terminus (470 amino acids) of the RelA cDNA. These gene fusions were then cloned into pTSN.flu.

Electrophoretic Gel Mobility Shift Assays—Nuclear extracts were prepared using a Nonidet P-40 lysis method (42). Briefly, cells were spun down and washed once in 1 \times PBS, resuspended in hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μ l/ml aprotinin (Sigma)), and incubated for 15 min on ice. The cells were then lysed in 0.5% Nonidet P-40 and the nuclei pelleted. The nuclei were salt-extracted (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μ l/ml aprotinin) and incubated with vigorous shaking for 15 min at 4°C. Nuclear remnants were removed by centrifugation, and the supernatant containing extracted protein was stored in aliquots at –80°C. Protein concentration was determined via a BCA colorimetric assay (Pierce).

5–10 μ g of nuclear extract was added to 2 μ l of 10 \times binding buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 50% glycerol), 20 μ g of bovine serum albumin, 6 μ g of poly(dI-dC), 3 mM GTP, and 1% Nonidet P-40 (included only in NF- κ B DNA binding analysis), and 10^5 cpm polynucleotide kinase (New England Biolabs) end-labeled oligonucleotide probe. A final salt concentration of 10 mM was established by adding the appropriate amount of 1 M NaCl to the volume of the protein extract. The

NF- κ B probe used in these experiments was 5' TAACAGAGGG-GACBBBCCGAGAGCCA (B indicates BrdUrd nucleotides). The quality of all extracts was monitored by gel mobility shift assay using an octamer binding site oligonucleotide: 5' GCCTCATTTGCATGGACT-TAGCTTGTCATGCAAATGAGG. The 20- μ l binding reactions were incubated 10 min at room temperature, loaded onto a 4% polyacrylamide gel (that had been pre-run for 60 min at 150 V with buffer recirculation) in 0.25 \times TBE, and electrophoresed at 150 V for 3–4 h. The gel was dried under vacuum and exposed to a PhosphorImager screen (Molecular Dynamics) for 12–36 h.

Where indicated, supershift analysis was performed by incubating anti-RelA antibody (Santa Cruz Biotechnology) or anti-c-Rel antibody (Santa Cruz Biotechnology) with the protein extract for 60 min at 4°C. The binding buffer was then added, and the reaction was incubated for an additional 10 min at room temperature before gel loading.

Western Blotting—Whole cell extracts were prepared by harvesting 5×10^5 – 10^6 cells, washing once in 1 \times PBS, and then lysing in sample buffer (10% glycerol, 3% SDS, 62.5 mM Tris pH 6.8). The samples were then mixed with an equal volume of bromophenol blue loading dye (containing 1 M β -mercaptoethanol), boiled, and then electrophoresed on a 7.5% SDS-polyacrylamide gel. The gel was blotted onto a 0.45- μ m nitrocellulose membrane (Protran, Schleicher & Schuell) by electroelution. The blots were stained with Ponceau S to assess protein transfer and then blocked 30 min to overnight in 5% powdered milk/PBS-T (1 \times PBS, 0.1% Tween 20). Primary antibodies included anti-c-Rel and anti-RelA (listed above), used at 1:1000 dilutions in 5% powdered milk/PBS-T and anti-HA-epitope (Boehringer Mannheim, clone 12CA5) used at a 1:400 dilution in 5% milk/PBS-T. Incubations with the primary antibodies were 1–3 h at room temperature, and subsequent washes were done in PBS-T. Secondary antibody incubations, either anti-mouse Ig or anti-rabbit Ig, were performed at 1:3000 dilution in 5% milk/PBS-T for 30–60 min. The blots were imaged using chemiluminescence (ECL; Amersham Pharmacia Biotech).

Cell Viability Assay—Cell viability was assessed via the trypan blue exclusion properties of a cell culture. Cultures were analyzed in duplicate as follows: 4×10^5 cells were harvested by centrifugation (to remove tetracycline) and resuspended in 20 ml of complete media lacking tetracycline. Control cultures were similarly prepared with the addition of tetracycline to a final concentration of 1 μ g/ml. 1.5-ml aliquots were removed from cultures at the indicated time points. Each aliquot of cells was harvested by centrifugation and resuspended in a small volume (varying with expected cell numbers) of PBS, diluted 1:1 with a trypan blue solution (Life Technologies, Inc.), and counted using a hemacytometer. The total number of cells in 1.5 ml was calculated and recorded as the cell number for the appropriate time point.

Annexin V Staining— 10^6 cells were pelleted, washed in wash buffer (1 \times PBS, 3% fetal bovine serum, and 10 mM Hepes, pH 7.4), resuspended in 100 μ l of wash buffer, and split into duplicate tubes. Each sample was pelleted and resuspended in wash buffer supplemented with either 2 mM CaCl_2 or 2 mM EDTA. The FITC-Annexin V reagent (CLONTECH) was added at a 1:20 dilution, and samples were incubated for 30 min on ice. The cells were then washed once with 1.5 ml of wash buffer with or without CaCl_2 and resuspended in 500 μ l of the appropriate wash buffer. 7-AAD was added at a concentration of 1 μ g/ml, and cells were incubated 10 min at room temperature before analysis on a FACScan (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson).

Cell Cycle Analysis—Cell cycle analysis was performed as reported previously (43). Cell cultures were pulsed with 30 μ M bromodeoxyuridine (BrdUrd; Sigma B5002) for 60 min. 3×10^6 cells were pelleted and washed in PBS, 5 mM EDTA. The cells were fixed in methanol, pelleted, resuspended in 1 ml of 2 N HCl, 0.2 mg/ml pepsin, and incubated at room temperature for 30 min. 3 ml of 0.1 M sodium tetraborate, pH 8.5 (Sigma), was added to neutralize the HCl, the cells were pelleted, washed once in IFA (0.009 M Hepes/0.15 M NaCl, pH 7.7, 4% heat-inactivated newborn calf serum (Life Technologies, Inc.), 0.1% sodium azide), washed once in IFA + 0.5% Tween 20, resuspended in anti-BrdUrd antibody (PharMingen Clone 3D4) diluted 1:1000 in IFA + 0.5% Tween 20, and incubated for 30 min at room temperature. The cells were then washed in IFA, 0.5% Tween 20 and resuspended in 100 μ l of anti-mIgG₁ (PharMingen) diluted 1:200 in IFA, 0.5% Tween 20 and incubated for 30 min at room temperature. A final wash was done in IFA, 0.5% Tween 20, and the cells were resuspended in PBS, 20 μ g/ml RNase and incubated for 15 min at room temperature. 7-AAD was added at a final concentration of 1 μ g/ml, and the samples were analyzed using the FACScan and CellQuest software.

RESULTS

Tetracycline-regulated Expression of RelA and c-Rel—An inducible expression system was used to individually overexpress the RelA and c-Rel proteins in the Abelson virus-transformed pro-B cell line 220-8 (14). In this system, the DNA binding domain of the Tet repressor protein (TetR), whose DNA binding is inhibited in the presence of tetracycline, is fused to the transactivation domain of the herpesvirus VP16 protein, resulting in a tetracycline-regulated transactivator protein (tTA) (39). When Tet operator sequences are positioned upstream of a minimal promoter, the binding of tTA results in transcriptional activation of a downstream target gene. In the presence of tetracycline, tTA protein cannot bind its operator sequences, and the target gene is not transcribed. By placing the tTA gene itself under control of the tTA-inducible promoter, an autoregulatory loop is established resulting in tight control of target gene expression (41).

A vector containing tTA expression and mycophenolic acid resistance cassettes was used to generate stable 220-8 transfectants. Single cell clones were screened for inducible expression of the tTA fusion protein and a clone expressing high levels of tTA10 was identified for use in subsequent experiments (data not shown). A second vector, in which either the *relA* or *c-rel* cDNA (fused in-frame with an HA epitope tag) was placed under the regulatory control of tet operator sequences, was transfected into tTA10, and stable transfectants were selected for G418 resistance. Single cell clones were analyzed by Western blot for inducible expression of either RelA or c-Rel protein. In the presence of tetracycline, no detectable protein was made, but upon removal of tetracycline, protein expression was seen as early as 5 h after antibiotic removal, and expression was stable for up to 5 days (Fig. 1, A and B). Levels of the induced RelA and c-Rel proteins were similar to the levels of the corresponding endogenous proteins (Fig. 1 and data not shown).

The appearance of NF- κ B binding activity in nuclear extracts paralleled the accumulation of induced protein (Fig. 1C), with maximal binding detected after 72 h of induction. Super-shift analysis of these complexes using specific antibodies identified the presence of both RelA and c-Rel in these induced κ B site-binding complexes (Fig. 1C and data not shown). Thus, induction of either RelA or c-Rel protein resulted in both activation and nuclear translocation of NF- κ B in the absence of an additional stimulus. NF- κ B can also be induced in untransfected cells by culturing them in the presence of bacterial lipopolysaccharide (LPS). LPS induces nuclear NF- κ B complexes containing predominantly RelA (Fig. 1C) and p50 (35, 36, 44).

Overexpression of RelA, but Not c-Rel, Leads to Cell Death—During our initial analysis of induced RelA or c-Rel protein expression, we noted a significant amount of cell death in pro-B cell clones expressing the RelA construct, but not in those cultures expressing either the c-Rel construct or the parental tTA construct itself. Untransfected LPS-treated 220-8 cultures failed to show increased cell death, however (data not shown). A cell viability analysis, utilizing trypan blue exclusion, revealed a striking loss of cells upon induction of the RelA protein (Fig. 2). In contrast, cells expressing either the tTA regulatory protein or the c-Rel protein show only a modest decrease in cell growth compared with control 220-8 clones.

A kinetic analysis of the effect of RelA expression on cell number revealed a time lag between protein expression and cell death. Although we could detect RelA as soon as 5 h after the removal of tetracycline, with its expression peaking at 36 h (Fig. 1, A and B), significant cell loss was not seen until 72 h. Interestingly, this onset of cell loss coincided with the maximal induction of nuclear κ B site binding activity (Fig. 1C and data

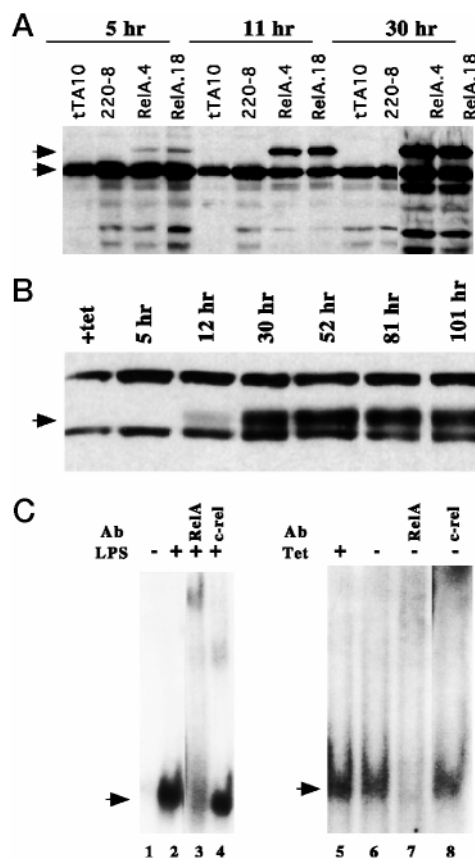


FIG. 1. Time course of induced Rel protein expression. A, two RelA-transfected pro-B cell clones, RelA.4 and RelA.18, were induced to express the RelA protein by removal of tetracycline. At the indicated time points, 10^6 cells were removed and whole cell lysates made. Half of each sample was analyzed by Western blotting, using an anti-p65 antibody. RelA-transfected clones were compared with both the original 220-8 cell line and with the parental tTA-expressing cell line, tTA10. The upper arrow indicates transfected RelA protein, and the lower arrow indicates the endogenous RelA protein. Transfected RelA is larger due to its C-terminal epitope tag. B, c-Rel clone.19 was analyzed as representative of c-Rel-overexpressing clones, in a protein expression time course, similar to the protocol outlined above. The Western blot was developed with an anti-HA antibody (Boehringer Mannheim), and the arrow indicates the c-Rel-HA fusion protein. C, antibody supershift analysis of induced NF- κ B site binding complexes. Left panel, nuclear extracts were prepared from 220-8 cells cultured in the absence (lane 1) or presence (lanes 2–4) of LPS. These extracts were analyzed by electrophoretic mobility shift without (lanes 1 and 2) or with (lanes 3 and 4) preincubation with anti-RelA or anti-c-Rel antisera as indicated. Right panel, nuclear extracts were prepared from a RelA-transfected clone cultured in the presence (lane 5) or absence (lanes 6–8) of tetracycline. These extracts were analyzed by electrophoretic mobility shift without (lanes 5 and 6) or with (lanes 7 and 8) preincubation with anti-RelA or anti-c-Rel antisera as indicated. The specific NF- κ B DNA binding complex is indicated by the arrow in each panel.

not shown). This implies that the observed cell death might depend upon the nuclear translocation of RelA and subsequent activation of cellular gene expression.

RelA-induced Cell Death Is Apoptotic—To characterize cell death in this system, we stained cells with FITC-conjugated recombinant Annexin V (Fig. 3). Annexin V is a protein that binds phosphatidylserine. During the early stages of apoptosis, phosphatidylserine, which is primarily localized to the inner leaflet of plasma membranes in healthy cells, is exposed on the outer leaflet where it can be detected by Annexin V binding (45, 46). Cells were also stained with 7-AAD, a DNA-binding dye that stains cells that have lost their membrane integrity (a late event in both apoptotic and non-apoptotic cell death). Induction of tTA expression in control cultures resulted in a moderate increase in cell death. When cultures expressing elevated levels

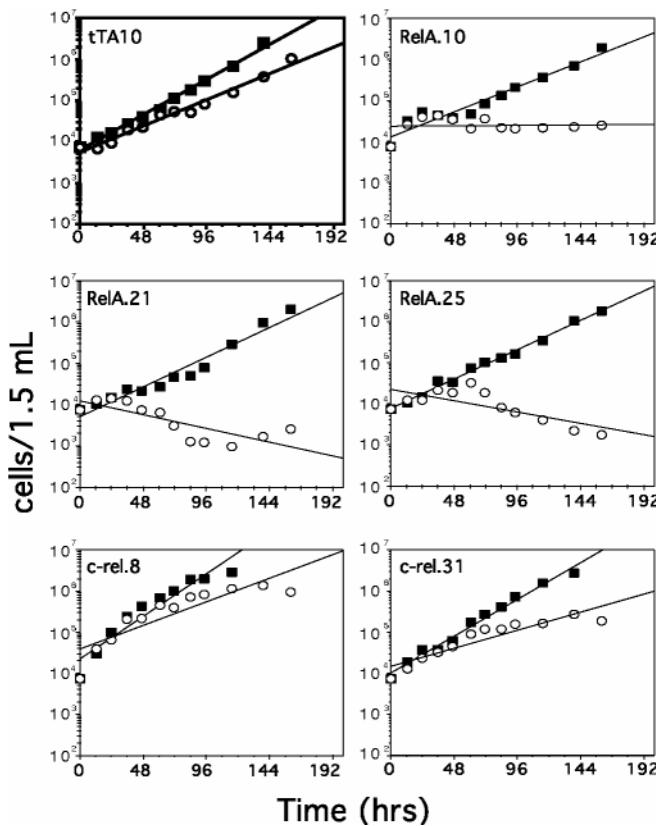
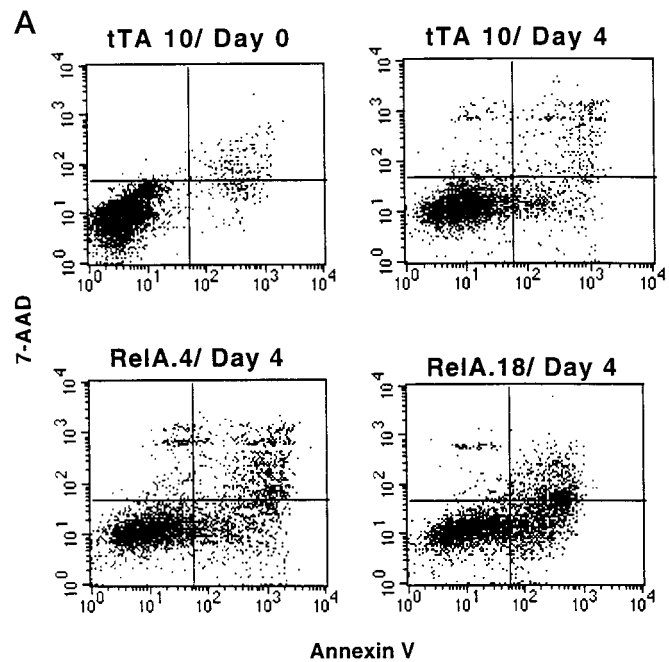


FIG. 2. RelA induction is associated with cell death. The viability of parental, RelA-expressing and c-Rel-expressing clones was assessed by a cell counting analysis. Duplicate cultures were seeded at a starting cell concentration of 1×10^4 cells/ml, either in presence or absence of tetracycline. Aliquots were removed and counted every 12 h. The total number of trypan blue-excluding cells within the 1.5-ml aliquot was calculated. Duplicate culture calculations were averaged at each time point and are represented on the graphs as single points. The profile of cultures growing in the presence of tetracycline is indicated by the filled squares, and the profile of cultures growing under inducing conditions (the absence of tetracycline) is indicated by the open circles. tTA10, the parental clone is shown in the upper left hand panel. Three independent RelA-overexpressing clones (RelA.10, RelA.21, and RelA.25) and two independent c-Rel-overexpressing clones (c-Rel.8 and c-Rel.31) were examined.

of RelA were compared with parental cultures, we found that clones overexpressing RelA had 2–3-fold more Annexin V binding 7-AAD excluding cells, indicative of the induction of apoptosis (Fig. 3). We detected twice the number of dead cells in the RelA-expressing clones as we did in control cultures. In addition, we performed a gel electrophoretic analysis of DNA purified from these cultures. RelA expression was associated with internucleosomal cleavage of DNA characteristic of apoptosis (data not shown).

A G_1 Cell Cycle Arrest Precedes Induction of Apoptosis—The lag between induction of RelA expression and the apparent onset of apoptosis led us to investigate whether cell cycle progression is affected by elevated levels of RelA protein. Parental (tTA10) and RelA-transfected clones were induced by removal of tetracycline. After various lengths of time, bromodeoxyuridine (BrdUrd) was added to the media, and incubation was continued for another hour. Cells continuing to cycle will incorporate BrdUrd into their DNA during S phase. At each time point, cells were permeabilized, stained with 7-AAD and FITC-conjugated anti-BrdUrd antibody, and analyzed by flow cytometry (Fig. 4A). Whereas the parental and RelA overexpressing clones showed indistinguishable cell cycle distributions in the presence of tetracycline, the induction of RelA overexpression led to a dramatic G_1 cell cycle arrest (Fig. 4A). The fraction of



B

Sample	Time (Days)	Live cells	Apoptotic cells	Dead cells
tTA10	0	94 %	3 %	3 %
tTA10	4	80	10	8
RelA.4	4	61	18	17
RelA.18	4	54	29	15

FIG. 3. Annexin V staining of RelA-overexpressing cells. A, parental tTA10 and two RelA-transfected clones were induced to express protein by removal of tetracycline. After 4 days of growth under inducing conditions, 5×10^6 cells were double-stained with Annexin V-FITC and 7-AAD and analyzed by flow cytometry. Induced cultures were compared with the corresponding cultures growing in the presence of tetracycline (only tTA10 is shown; the other uninduced cultures were identical to tTA10). Annexin V stains apoptotic cells, and 7-AAD stains dead cells regardless of their mechanism of death. B, the table was generated using CellQuest software to quantify the fraction of Annexin V-FITC⁺/7-AAD⁻ (live), Annexin V-FITC⁺/7-AAD⁺ (apoptotic), and Annexin V-FITC⁻/7-AAD⁺ (dead) cells from A, above.

RelA-expressing cells in G_1 increased from 45 to 75%, whereas the parental clone remained essentially unchanged (Fig. 4B). Furthermore, apoptotic cells, characterized by their sub- G_1 DNA content, accumulated in the RelA-expressing culture and not in the control culture (apoptotic cells were not included in quantitative analyses of cell cycle distribution). In RelA overexpressing cultures, as shown in Fig. 4B, the fraction of cells in S phase decreased much more rapidly than the fraction of apoptotic cells (as defined by sub- G_1 DNA content) increased, confirming the impression that cell cycle arrest precedes apoptosis in this system.

Induction of Apoptosis Requires the Transactivating Potential of RelA—Since Rel family members heterodimerize, it was possible that RelA overexpression induced cell cycle arrest and apoptosis indirectly, by altering the distribution of various Rel family dimers. Alternatively, RelA might provoke these phenomena by directly altering target gene expression. To delineate the importance of transcriptional transactivation by RelA for the induction of G_1 cell cycle arrest and apoptosis, we generated 220-8 clones inducibly expressing a truncation mu-

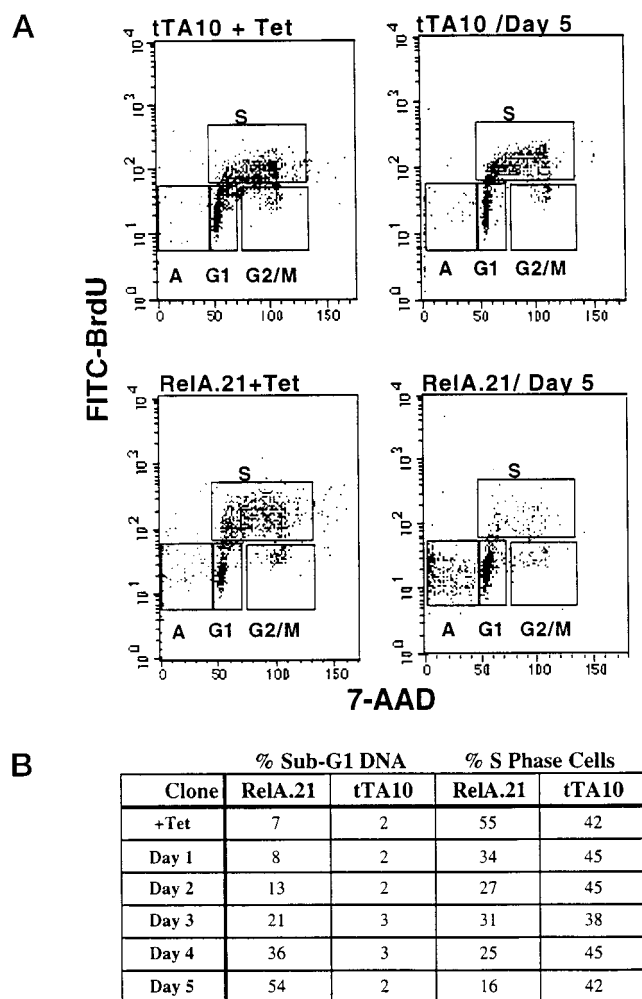


FIG. 4. Cell cycle analysis of RelA-overexpressing cells. **A**, a parental tTA10 culture and a representative RelA-overexpressing culture (RelA.21) were induced by removal of tetracycline and cultured for up to 5 days. Each day aliquots of cells were pulsed with BrdUrd for 60 min, harvested, permeabilized, and stained sequentially with a murine anti-BrdUrd antibody, an anti-mouse IgG1 FITC-conjugated secondary reagent and 7-AAD. The staining profiles of the uninduced and 5-day-induced cultures are shown. The boxed regions indicate the various cell cycle stages (G₁, S, and G₂/M) and cells with sub-G₁ DNA content (A). **B**, the subpopulations of cells in the tTA10 and RelA.21 cultures in each stage of the cell cycle before induction and on each of 5 days after induction were quantified using CellQuest software and the gates indicated in **A**, above. Cells with sub-G₁ DNA content are generally considered apoptotic. The percentage of cells in S phase was calculated exclusive of the fraction of dead cells (box A) in each culture.

tant of RelA that lacks the previously characterized C-terminal transactivation domain (amino acids 471–549) (47, 48). When the growth of clones expressing this truncated protein was examined, no obvious defects were noted beyond the mild growth retardation observed with the parental tTA clone (Fig. 5).

To corroborate this observation, we studied a previously characterized RelA transactivation mutant, RRPA (49). This point mutation alters a protein kinase A phosphorylation site that is essential for the transactivation potential of RelA. We verified inducible expression of the RRPA mutant RelA by Western blot and its ability to bind DNA by mobility shift analysis (data not shown). In contrast to the phenotype associated with overexpression of wild-type RelA, overexpression of the RRPA mutant protein did not result in any proliferative defects (Fig. 5). Cell growth, in both the presence and absence of tetracycline, was similar to the parental control. These re-

sults strongly suggest that the transactivating potential of the RelA protein is necessary for the apoptotic phenotype seen in clones overexpressing RelA.

Both the DNA Binding and Transactivation Domains of RelA Are Specifically Required for Induction of Apoptosis—Whereas the preceding experiments showed that transactivation potential was necessary for RelA to induce growth arrest and apoptosis, it remained uncertain whether the requirement was specific for the RelA transactivation domain. The amino acid sequences of the c-Rel and RelA transactivation domains are quite distinct from one another, suggesting that differences in transactivation may underlie the effects of distinct Rel family dimers. To test this idea, we generated two RelA/c-Rel chimeric transcription factors. In c-Rel/TAD, the transactivation domain of RelA was replaced by the analogous domain of c-Rel (the C-terminal 156 amino acids (50)), and in the chimera RelA-TAD, the transactivation domain of c-Rel was replaced by the analogous domain of RelA (the 79 C-terminal amino acids). We transfected these chimeric proteins into tTA10 cells under tetracycline regulation and analyzed cell viability following induction (Fig. 6). Control experiments confirmed that both chimeric proteins were able to specifically bind DNA *in vitro* and to transactivate an NF- κ B-dependent reporter construct *in vivo* (data not shown).

Surprisingly, neither chimeric protein reproduced the striking growth arrest and apoptotic phenotype observed with wild-type RelA overexpression. These results support the conclusion that both the RelA transactivation domain and its DNA binding and dimerization domain are critical for this phenotype.

Overexpression of RelA in B Cell Lymphoma Cell Lines Does Not Cause Apoptosis—Recent reports have suggested a role for NF- κ B in the protection of fibroblasts from TNF- α -induced apoptosis and immature B cells from anti-IgM-induced apoptosis. To explore the possibility that the effect of RelA on cell cycle progression and apoptosis differed at different stages of development, we established the tetracycline-regulated expression system in two B cell lymphoma lines, WEHI 231 and M12. Whereas the 220-8 cell line is representative of the pro-B cell stage of development, WEHI 231 has been used as a model for the immature B cell stage and M12 for the mature B cell stage of development. We found that overexpression of RelA in WEHI 231 or in M12 does not alter cell growth (Fig. 7). Expression levels and kinetics of RelA protein induction in the WEHI and M12 cell clones were similar to those in the 220-8 transfectants (data not shown) and thus cannot account for the difference in phenotypes. Multiple attempts to generate additional distinct pro-B cell lines expressing RelA were unsuccessful. We believe that this was due to the extreme toxicity of even modest amounts of excess RelA to these cells, despite tightly regulated expression. This discrepancy in the effects of RelA overexpression in pro-B and immature B cells suggests that there may be a developmental basis for the susceptibility of pro-B cells to the induction of apoptosis by RelA.

DISCUSSION

NF- κ B was initially discovered because of its ability to bind a site in the Ig κ locus intronic enhancer. Subsequently, NF- κ B-binding sites were found in transcriptional regulatory elements associated with a broad array of genes involved in immune or inflammatory responses (1). A number of recent experiments perturbing the composition of NF- κ B/Rel family dimers have revealed previously unsuspected roles for this family of transcription factors in regulating both cell division and apoptosis (21, 29, 32, 51).

The levels of the various Rel family members and their contributions to NF- κ B DNA binding activity are normally controlled by both transcriptional and post-translational mech-

FIG. 5. Mutant RelA proteins do not affect pro-B cell growth. The cell viability assay was performed as in Fig. 2. Growth in the presence of tetracycline is indicated by the *filled squares*, and growth under inducing conditions is indicated by the *open circles*. TADT 19 and TADT 31 are clones expressing the transactivation domain truncation mutant RelA protein, and RRPA 6 and RRPA 12 are clones expressing the protein kinase A site mutant RelA protein.

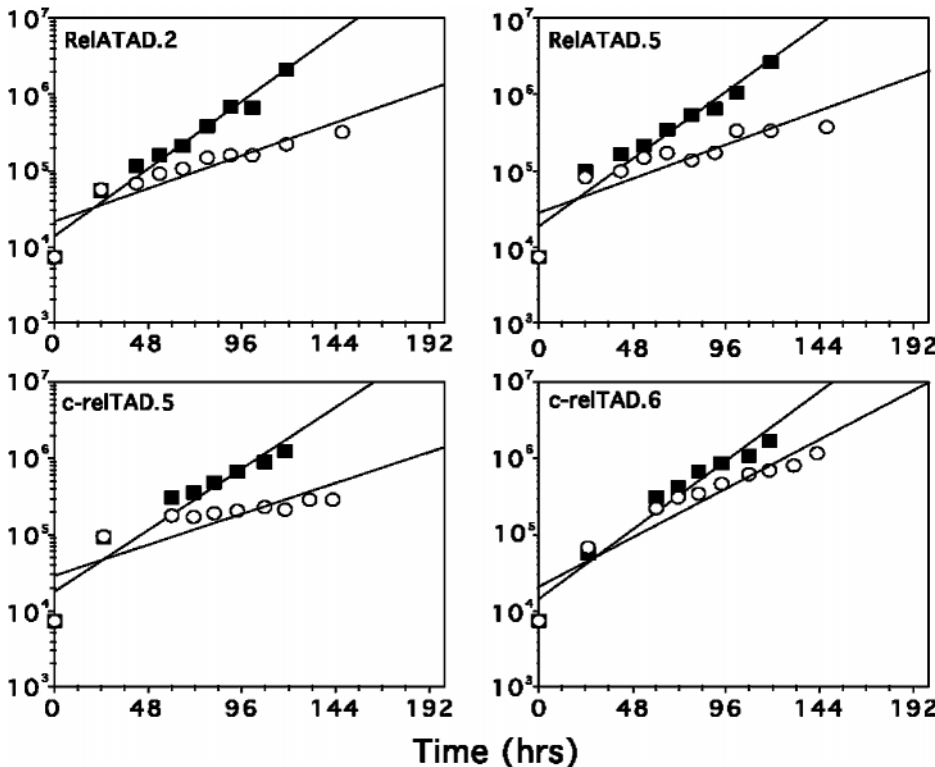
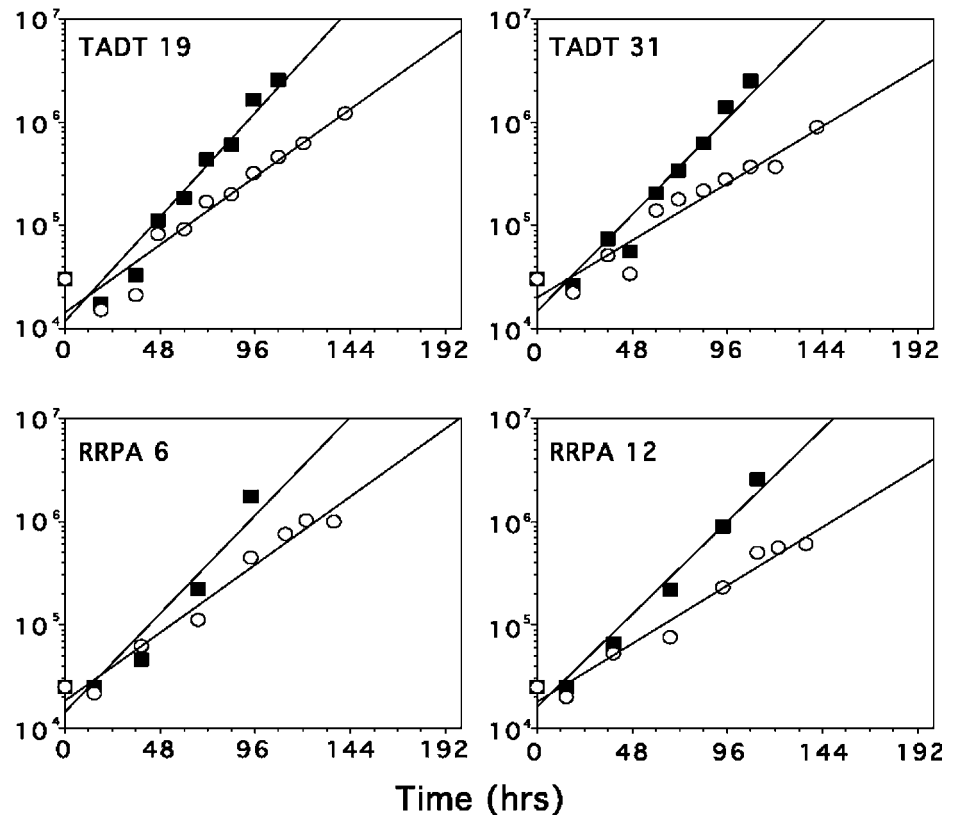


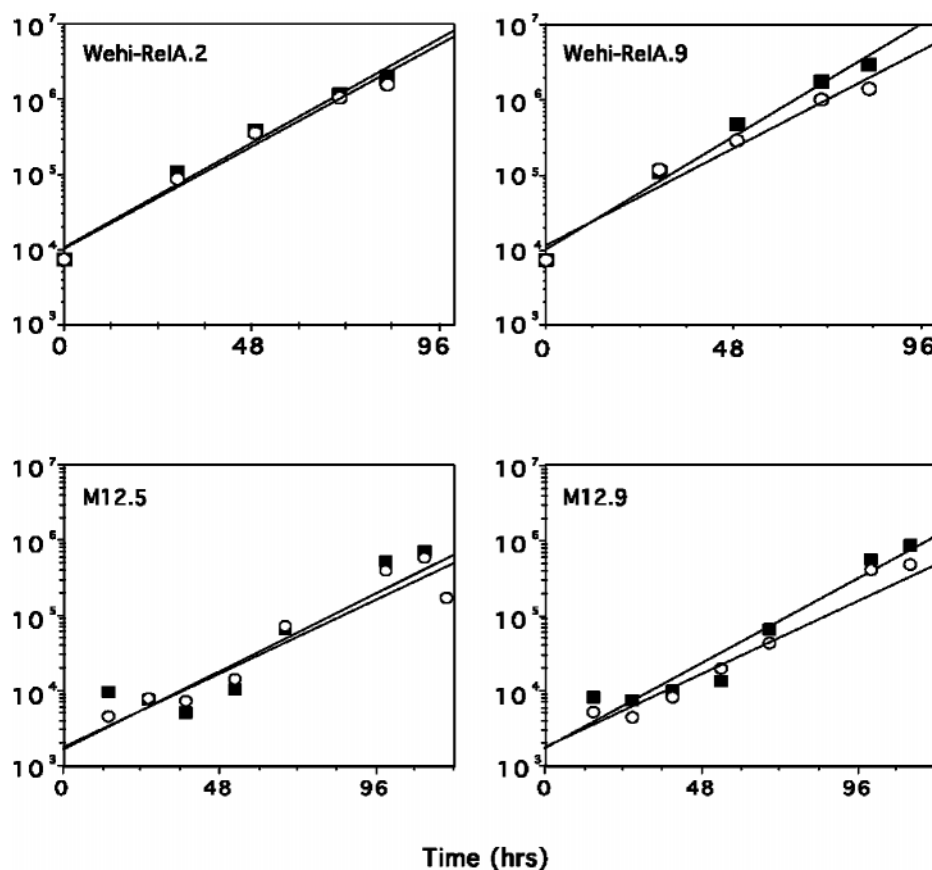
FIG. 6. RelA-c-Rel chimeric proteins do not affect pro-B cell growth. This cell viability assay was performed as in Fig. 2. Growth in the presence of tetracycline is indicated by the *filled squares*, and growth under conditions of protein induction is indicated by the *open circles*. The *upper panels* represent the growth profiles of cells expressing a chimeric protein composed of the RelA DNA binding domain and the c-Rel transactivation domain (RelATAD.2 and RelATAD.5). The *lower panels* represent the growth profile of cells expressing a chimeric protein composed of the c-Rel DNA binding domain and the RelA transactivation domain (c-RelTAD.5 and c-RelTAD.6).

anisms. For example, NF- κ B activation can lead to increased synthesis of I κ B α (but not I κ B β) (52–54), p50 (55), and c-Rel (56–58). This web of interactions among the Rel family members and their inhibitors has contributed to difficulty in identifying the unique functions of each family member. We used an inducible gene expression system to disrupt normal cellular regulation of the Rel family, leading to the overexpression of either RelA or the c-Rel protein in a pro-B cell line. The inap-

propriate regulation of these proteins resulted in stimulation of nuclear NF- κ B DNA binding activity. Whereas elevated levels of RelA led to the induction of a G₁ cell cycle arrest followed by apoptosis, overexpression of c-Rel had little effect on cellular proliferation or cell death.

Since RelA is known to interact with several I κ B family members (7, 59–61), it is possible that its overexpression induced apoptosis indirectly by altering the cytoplasmic localiza-

FIG. 7. RelA overexpression does not affect the growth of WEHI 231 B lymphoma cells. This cell viability assay was performed as in Fig. 2. The tTA-regulated expression system was established in the immature B cell lymphoma WEHI 231 and the mature B cell lymphoma M12. Two RelA-expressing clones from each cell line were analyzed. Growth in the presence of tetracycline is indicated by the filled squares, and growth under conditions of protein induction is indicated by the open circles.



tion of various NF- κ B complexes imposed by the I κ B proteins. The recently cloned I κ B ϵ (60, 62, 63) has been reported to selectively interact with RelA homodimers, and both I κ B α and I κ B β are known to interact with RelA-containing heterodimers. The sequestration of various I κ Bs through interaction with inappropriately regulated RelA protein might lead to nuclear translocation and target gene activation. The overexpression of c-Rel, however, did not cause apoptosis although it might be expected that a similar redistribution phenomenon would also result from elevated levels of this protein subunit. The specificity of the RelA-induced cell cycle arrest suggests that the initiation of this signaling pathway is a direct result of RelA-specific target gene activation.

Our failure to observe cell cycle arrest or apoptosis in cells expressing the RelA transactivation domain truncation mutant protein, TADt, or the protein kinase A phosphorylation site point mutant protein, RRPA, confirm the conclusion that the effects of RelA overexpression were due to its transcriptional transactivation potential. It is important to realize that the kinetics of the cell cycle arrest differ significantly from the induction of apoptosis. Whereas the cell cycle arrest was detectable after less than 24 h of protein induction, apoptosis was not observed until after 72–96 h, suggesting that the cells might be dying as a result of their inability to continue normal cell cycle transit. This interpretation suggests a role for RelA in the regulation of cell cycle proteins. The involvement of c-Rel in the regulation of p53 and p21 transcript stability and E2-F DNA binding activity has been reported (51). We examined each of these cell cycle regulators in our system, as well as the transcriptional regulation of the *c-myc* gene, whose role in cell division and apoptosis has been extensively investigated (64–68), and we were unable to identify any significant perturbations (data not shown).

Alternatively, it is possible that RelA contributes directly to the transcriptional activation of proteins involved in the regu-

lation of apoptosis. A recent report showed that RelA was required for the induction of FasL expression in a human T cell line, for example (69). The time lag we observed in the onset of cell death after RelA induction might reflect the time necessary to activate the expression of FasL and for FasL to interact with Fas and activate the apoptosis pathway. Identification of the downstream target gene(s) whose altered expression results in the induction of this G₁ cell cycle arrest and apoptosis awaits further investigation.

Several lines of investigation into the relationship between NF- κ B and apoptosis have supported distinct functions in both the protection from and the induction of programmed cell death. Deletion of the *relA* gene in mice results in embryonic lethality. Massive hepatocyte apoptosis was observed in pre-morbid fetuses, implicating RelA in the prevention of cell death. Experiments with fibroblasts from RelA knock-out mice showed that these cells were sensitized to TNF- α -induced apoptosis (29). Although the viability of wild-type fibroblasts was unaffected by TNF- α stimulation, up to 80% of TNF- α -stimulated *relA*^{-/-} fibroblasts were killed. Apoptosis in this system could be inhibited by the expression of exogenous RelA, suggesting that TNF- α stimulation of NF- κ B activity was involved in the protection of fibroblasts from apoptosis during TNF- α signaling. Similar experiments examining this TNF- α signal in macrophage, T cell, and fibrosarcoma lines corroborated the observations in fibroblasts (30, 32). Additional work done with the immature B cell line, WEHI 231, which expresses surface immunoglobulin (sIgM), also defined a role for NF- κ B in the protection from apoptosis induced by surface receptor cross-linking (31). The mechanism of NF- κ B protection from these apoptotic stimuli remains unclear.

A role for NF- κ B in the induction of apoptosis has also been observed. A report examining the expression of c-Rel in the developing avian embryo found that cells undergoing apoptosis expressed elevated levels of c-Rel protein. When c-Rel overex-

pression was induced by viral transduction into primary avian hematopoietic cells, the cells underwent an uncharacterized growth arrest that was followed by the induction of programmed cell death (26). Transduction of *c-rel* into the corresponding primary fibroblasts appeared to extend the lifespan of these cells, leading to the speculation that the biological effects of c-Rel overexpression were cell type-specific. NF- κ B activation was also required in the induction of apoptosis following the infection of AT-3 cells (a prostate carcinoma line) with Sindbis virus. Blocking NF- κ B DNA binding activity inhibited the induction of apoptosis accompanying this viral infection (27). Interestingly, this requirement for NF- κ B was not seen in Sindbis-virus induced apoptosis of N18 (neuroblastoma) cells, again emphasizing the differential effects of NF- κ B activation depending on cell type. The activation of nuclear NF- κ B DNA binding activity is also observed in serum-starved 293 cells (a human embryonic kidney cell line), concomitant with apoptosis of these cells (28). Transfection of a dominant-negative mutant of the RelA subunit, truncated at its C-terminal transactivation domains, attenuated the observed cell death, suggesting the importance of transcriptional activation by NF- κ B in this model of apoptosis. These reports encompass a range of cell types and apoptosis-inducing stimuli and suggest that both the cell type and context of an NF- κ B-activating signal are critical in determining whether cells are protected from or induced to undergo apoptosis.

We addressed the paradoxical roles of NF- κ B in apoptosis by examining both the cell type specificity and the signaling context of its pro-apoptotic activity in transfected B lineage cells. LPS stimulation of transformed precursor B cells (including those used in our studies) results in the nuclear translocation of NF- κ B, composed primarily of the p50/RelA heterodimer, and does not result in any obvious alteration in proliferation or induction of apoptosis (9, 17). When we induced transfected pro-B cells to overexpress RelA, we also observed the activation of nuclear NF- κ B DNA binding complexes similar to those seen with LPS stimulation. However, under these conditions, cells experience growth arrest and subsequently undergo apoptosis. The explanation for these disparate results may lie in compensatory signaling pathways induced by LPS treatment that might counteract the induction of growth arrest by nuclear NF- κ B. Stimulating RelA-overexpressing clones with LPS concomitant with RelA induction does not rescue these cells, however (data not shown), suggesting that the RelA-induced NF- κ B signal is dominant in the induction of cell death under these conditions.

We approached the cell type specificity issue by overexpressing RelA in the immature B cell line WEHI 231 and the mature B cell line M12. The overexpression of RelA in these cells did not result in either cell cycle arrest or induction of apoptosis. In addition to representing distinct stages of B cell development, 220-8, WEHI 231, and M12 cells represent different states of cellular NF- κ B. In most cells, NF- κ B is present in an inactive cytoplasmic form, but in several subsets of cells, including mature B cells and macrophages (17, 33), nuclear NF- κ B DNA binding activity is constitutive. It is at the immature stage of B cell development, represented by WEHI 231, that this constitutive activity is acquired. The 220-8 line represents an earlier stage in B cell development that precedes this constitutive activation. During the analogous stage of development in the bone marrow, B cells that do not successfully rearrange the Ig heavy chain locus and synthesize heavy chain protein are eliminated via an apoptotic mechanism (70). NF- κ B may be the factor responsible for initiation of this apoptotic pathway. Similarly, specific populations of developing B cells within the avian bursa of Fabricius (71) are highly susceptible to the

induction of cell death. 220-8 cells might represent a stage of development that is the mammalian equivalent of avian B cell populations that exploit NF- κ B-induced apoptosis to perform cellular selection. The WEHI 231 and M12 cell lines, having progressed beyond this susceptible stage, would not be expected to be sensitive to the apoptosis-inducing function of NF- κ B.

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