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Sequential Induction of NF-κB/Rel Family Proteins during B-Cell Terminal Differentiation

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The NF-κB/Rel family of at least five transcription factor polypeptides is thought to function both as a developmental regulator in B cells and as a rapid response system in all cells. To examine this notion in more detail, we determined the protein contents of both the inducible and constitutive NF-κB/Rel activities in a pre-B-cell line, 70Z/3, and a mature B-cell line, WEHI 231. NF-κB p50/p65 is the major inducible nuclear complex after lipopolysaccharide or phorbol myristate acetate treatment of 70Z/3 cells. The constitutive and inducible complexes in WEHI 231 cells are mainly composed of p50 and Rel. The constitutive or induced activities are all sensitive to IκB-α, but this inhibitor is very short-lived in WEHI 231 cells, suggesting that the balance between synthesis and degradation of IκB-α determines whether a particular cell lineage has constitutive activity. A patterned expression of the NF-κB/Rel activator proteins emerges from an analysis of other B-lineage cell lines and splenic B cells: mainly p50 and p65 in pre-B (and non-B) cells, a predominance of Rel and p50 in mature B cells, and expression of p52 and RelB in plasmacytoma lines. This ordered pattern of regulators may reflect the requirement for expression of different genes during terminal B-cell differentiation because different combinations of NF-κB/Rel family members preferentially activate distinct κB sites in reporter constructs.

B-cell differentiation is an ordered process culminating in expression of rearranged immunoglobulin (Ig) heavy (H)- and light (L)-chain gene products (7). Pre-B cells express μ IgH protein in their cytoplasm, while the transition from the pre-B to B stage is accompanied by the rearrangement and expression of the κ L-chain gene. Thus, the regulation of Igκ gene expression is considered a crucial element in B-cell differentiation. The κ L-chain intronic enhancer contains several transcription factor binding sites, including a κB site and three E boxes, that contribute to enhancer activity both in vitro and in vivo (42). Integrity of the κB site is necessary for any enhancer activity to be manifest: it is bound by the NF-κB protein (24, 41). Among B-lineage cell lines, the presence of nuclear NF-κB activity correlates with κ gene expression (42). One of the most useful cell lines has been 70Z/3, which contains a functionally rearranged but transcriptionally inactive Igκ locus (35). Stimulation of 70Z/3 cells with the B-cell mitogen lipopolysaccharide (LPS) causes nuclear translocation of NF-κB and concomitant κ L-chain transcription, followed by surface expression of an assembled IgM (33). By analogy, the constitutive NF-κB activity in mature B cells has been thought to play a role in the regulation of Igκ gene expression and B-cell differentiation.

NF-κB is actually present in the cytoplasm of most cells as an inactive complex with an inhibitor protein, IκB (3). From the inactive NF-κB/IκB complex, DNA binding NF-κB can be released by stimulating cells with various exogenous agents (15). For example, NF-κB is activated in B cells by LPS, phorbol myristate acetate (PMA), or cross-linking of surface IgM. In T cells, NF-κB responds to a variety of activating stimuli, such as PMA, phytohemagglutinin, interleukin-2 (IL-2), and cross-linking of surface CD3, CD28, and T-cell receptors. The signal transduction pathways leading to the activation of NF-κB by these stimuli are yet to be identified. Approximately 10-bp κB sites for NF-κB have been found in the promoters/enhancers of many cellular and viral genes. These putative target genes of NF-κB, including Igκ, cytokines, cytokine receptors, and Myc, are involved in the regulation of the acute-phase response, inflammation, lymphocyte activation, and cell growth or differentiation. Deregulation or mutation of some NF-κB/Rel and IκB family members has been associated with tumorigenesis (14).

Both NF-κB and IκB are multigene families (4, 26). The mammalian NF-κB/Rel family members identified so far include NFKB-1 (p50 and its precursor, p105), NFKB-2 (p52 and its precursor, p100), p65 (RelA), c-Rel (Rel), and RelB. The approximately 300-amino-acid Rel homology domain shared by this family mediates DNA binding, dimerization, and interaction with IκB. With few exceptions, members of the NF-κB/Rel family can form heterodimers with other members. The IκB family of proteins includes IκB-α (MAD-3, pp40), IκB-β, IκB-γ, and Bcl-3. Prominent within these IκB-related proteins are multiple repeated sequences of 33 amino acids, termed ankyrin repeats, which appear to be responsible for interaction with the Rel domain.

Although NF-κB p50/p65 was identified as a nearly ubiquitous cytoplasmic factor, many members of the NF-κB/Rel and IκB families are inducible genes. In many cell types, NFKB-1, NFKB-2, c-Rel, RelB, IκB-α, and Bcl-3 are induced in response to those stimuli that activate NF-κB/Rel activity (6, 16, 17, 30, 32, 38). Rel is constitutively made in lymphoid cells; its expression is also inducible (16). Overexpression of Rel leads to tumorigenesis or programmed cell death (1). Certain members of the NF-κB/Rel and IκB families, such as p105 and c-Rel, are under autoregulation presumably due to the presence of κB sites in their promoters. The autoregulation of IκB-α by p65 implies that it too is under NF-κB regulation (40, 44).

Except for mature B cells, most uninduced cells lack nuclear

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NF-κB/Rel binding activity. Nuclear KBF-1 (a p50 homodimer) and p52 homodimers have been detected in unstimulated T cells and macrophages by using oligonucleotide probes containing the IL-2 or IL-2 receptor (IL-2R) κB sites to which the homodimers bind especially well (21, 22). It was recently reported that crude spleen B-cell extracts express p50 and RelB in constitutive complexes (25).

As a step toward understanding the physiological role of NF-κB in Igκ gene regulation, B-cell proliferation, and differentiation, we have characterized the nature and regulation of the constitutive NF-κB activity in B-lineage cells. Our results demonstrate that the various NF-κB/Rel complexes are differentially expressed in B-lineage cell types. Furthermore, different NF-κB/Rel complexes have distinct target gene specificities.

MATERIALS AND METHODS

Cell lines and tissue culture conditions. All B-lineage cells were maintained in RPMI 1640 containing 10% defined fetal calf serum (HyClone), penicillin-streptomycin, and 50 mM β-mercaptoethanol. Cells were split regularly at a dilution of 1:10 or 1:15. 3T3 cells were cultured in Dulbecco modified Eagle medium with 10% bovine serum.

Stimulation conditions for cells were the following: cycloheximide (10 μg/ml) added 30 min before the addition of other stimuli; LPS (Sigma L2880; 10 μg/ml) for the indicated time; PMA (100 nM) for 4 h; and tumor necrosis factor alpha (TNF-α; Genzyme; 20 ng/ml) for 2 h. For cross-linking surface IgM on B cells, goat anti-mouse IgM $F(ab')_2$ (Jackson ImmunoResearch) was added at a concentration of 30 μg/ml for 4 h. For phosphopeptide analysis, cells were treated with PMA for 10 min.

Purification of resting splenic B cells. Erythrocytes were removed from single-cell splenocyte populations by ammonium chloride lysis. Crude spleen extracts were prepared from these cells. Resting B cells were purified from these crude spleen populations by one round of complement lysis with a Thy 1.2 antibody to remove T cells. The remaining cells were then fractionated through Percoll gradients (8) to isolate small resting B cells, contained between 66 and 70% Percoll. These cells were collected, washed extensively, and incubated in medium for stimulation with anti-IgM or LPS.

Electrophoretic mobility shift assay (EMSA) and antibody inhibition. The nuclear and cytosolic fractionation procedure was slightly modified from the basic protocol of Dignam et al. (9). Cells were harvested, washed once with phosphate-buffered saline and resuspended into buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-sulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) plus 0.1% Nonidet P-40 (NP-40). Cells were lysed on ice for 2 min and checked for complete lysis under a microscope. Nuclei were spun down, and the supernatant was saved as the cytosolic fraction. The nuclear pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), sonicated for a few seconds to decrease viscosity, and rotated in the cold room for 30 min. Ultracentrifugation was performed to remove insoluble debris. The supernatant was nuclear extract.

The EMSA was performed as described previously (41). For antibody inhibition experiments, antibody was purified by ammonium sulfate precipitation of crude immune serum. The optimal amount of antibody used in EMSA inhibitions was determined by using purified proteins. For a 20-μl DNA binding reaction, 5 μg of purified antibody was added per reaction. Antibody was incubated with a mixture of nuclear extract and poly (dI-dC) in 1× DNA binding buffer (41) on ice for 30 min before the addition of ^{32}P-labeled κB probe. The reaction was continued for 15 min at room temperature, and the mixture was loaded onto a 5% native polyacrylamide gel. For high-resolution electrophoresis, gels were prerun at 160 V for 2.5 h, continuing for 3 to 3.5 h after the samples were loaded.

Immunoprecipitation and pulse-chase experiments. Cells were harvested, washed, and incubated in RPMI medium without either methionine or phosphate for 40 min prior to labeling for 2 h in the presence of 0.5 to 2 mCi of [^{35}S]methionine or $^{32}P_i$ per ml at a cell density of 10×10^6/ml. For pulse-chase experiments, aliquots of [^{35}S]methionine-labeled cells were chased with cold medium and harvested at 0, 10, 20, 40, 60, 120, 300, and 960 min. Cells were lysed in 1 ml of NP-40 buffer (0.1% NP-40, 250 mM NaCl, 50 mM Tris, 1 mM EDTA) for coimmunoprecipitation experiments or in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris [pH 7.4], 75 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate [DOC], 0.01% sodium dodecyl sulfate [SDS], 2.5 mM EDTA) for phosphopeptide analyses. These buffers contained mixtures of protease and phosphatase inhibitors (100 μM $NaVO_4$, 50 mM NaF, 10 mM sodium pyrophosphate, and 1 mM aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). Total lysates (500 to 1,000 μl) were precleared with 5 μl of normal rabbit serum for 30 min on ice, and then 80 μl of 50% protein A-plus-protein G Sepharose beads (Oncogene Sciences) was added. After removal of nonspecific immune complexes, either 4 to 6 μl of specific serum (1:100) or 5 μg of affinity-purified antibody was added, and the mixture was incubated for 2 h at 4°C. Immune complexes were precipitated with 30 μl of protein A-plus-protein G beads. After extensive washing, the beads were resuspended in 30 μl of protein loading buffer, boiled, and loaded onto SDS-polyacrylamide gels.

Western blot (immunoblot) analysis. Equal amounts of protein lysates (5 μg of nuclear extract and 20 μg of cytosol, equivalent to 10^6 cells per sample) from 70Z/3 and WEHI 231 cells were loaded onto each lane of several identical protein gels. After transferral to nitrocellulose filters, the blots were stained with Ponceau S to confirm the uniformity of protein loading in each lane. Filters were then blocked with 2% nonfat milk–TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 1 h and incubated with specific antibody at a dilution of 1:1,000 in 2% nonfat milk–TBST for 2 h at room temperature. After three washes with TBST, filters were incubated with the secondary antibody (goat anti-rabbit IgG–horseradish peroxidase conjugate) for 1 h, and antibody-reactive bands were revealed by chemiluminescent detection (ECL Western detection kit; Amersham International).

Transfection and luciferase assay. p50, p65, and Rel were subcloned into a retroviral vectro, pGD (40). None or 0.2, 0.4, or 0.6 μg of the p50, p65, and Rel expression constructs alone or in combination was cotransfected with 1 μg of different luciferase reporter constructs into L929 cells by the calcium phosphate transfection method. The total amount of transfected DNA was normalized to 0.6 μg in all transfections using empty vector DNA. Both expression and luciferase constructs were added to 100 μl of transfection solution (75 μl of 2× HBS [8 g of NaCl, 0.105 g of Na_2HPO_4, and 6.5 g of HEPES in 500 ml], 9.3 μl of 2 M $CaCl_2$), and the mixture was immediately applied to each well of a 24-well plate. L929 cells were grown to 50% confluency at the time of transfection. Transfections were performed in triplicate. Two days posttransfection, the cells were lysed and luciferase activity was measured by luminometer (Promega luciferase assay system).

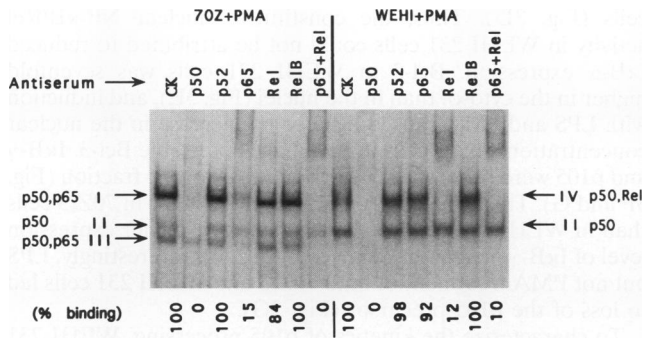

FIG. 1. Components of nuclear NF-κB/Rel complexes and sensitivities to IκB. Shown is EMSA of 70Z/3 and WEHI 231 nuclear extracts with antibody inhibition. Purified specific antibodies (5 μg) to each NF-κB/Rel component were preincubated with nuclear extracts prior to addition of a loop oligonucleotide probe containing an MHC class I κB site (10). Nuclear extracts were prepared from cells treated with PMA for 4 h. Equal amounts of nuclear extract (2.5 μg) were applied to each lane. CK represents control normal rabbit serum. Positions of specific NF-κB/Rel complexes (I through IV) are indicated by arrows. Each complex was quantitated by a PhosphorImager (Molecular Dynamics), and the percentage of residual binding was determined (indicated below the lanes).

RESULTS

NF-κB/Rel complexes in two model cell lines. Sen and Baltimore (42) originally observed constitutive NF-κB binding activity in nuclear extracts of mature B-cell lines but saw no such activity in extracts of pre-B-cell lines. NF-κB activity in pre-B-cell lines, however, can be induced by LPS or PMA treatment (41). To characterize the proteins involved in the NF-κB activity of B-lineage cells, we examined the constitutive and inducible NF-κB complexes in two cell lines representative of pre-B and mature B stages, 70Z/3 and WEHI 231. Equal amounts of nuclear extract protein prepared from uninduced or induced 70Z or WEHI 231 cells were subjected to EMSA. Pretreatment with purified antibody specific for individual NF-κB/Rel members was used to identify the protein constituents of the shifted complexes. Antibody specificities were tested by using purified p50, p65, or Rel protein. Under our experimental conditions, there was no cross-reactivity between a given antibody and noncognate members of the NF-κB/Rel protein family (not shown).

Since NF-κB/Rel heterodimers bind equally well to both Igκ and major histocompatibility complex (MHC) class I κB sites, but p50 and p52 homodimers bind with higher affinity to the class I site than to the Ig κB site (10, 31), we used the class I κB site as the probe in most EMSAs described in this report. As reported previously (41), uninduced 70Z/3 cells contained little or no detectable nuclear NF-κB activity by EMSA (data not shown), but a 4-h treatment with LPS or PMA induced the nuclear appearance of NF-κB/Rel activity (Fig. 1). The PMA-induced complex I was specifically inhibited by anti-p50 and anti-p65 antibodies (decreased 100 and 85%, respectively) but very little by other antibodies. Complex II was a p50 homodimer because it was blocked by anti-p50 antibody only (Fig. 1). Complex III was probably a degradation product of p50/p65 because its properties were like that of complex I. Therefore, p50/p65 is the inducible complex in 70Z/3 cells. By contrast and as expected (42), WEHI 231 expressed constitutive nuclear NF-κB/Rel which was further stimulated by PMA or LPS. The constitutive and inducible NF-κB/Rel complex IV in WEHI 231 cells was inhibited by both anti-p50 and anti-Rel antibodies (decreased 100 and 88%, respectively) but not by other antibodies (Fig. 1), indicating that a p50/Rel dimer is the predominant NF-κB/Rel complex in WEHI 231 cells, although some p50/p65 was present. Complex II had the properties of a p50 homodimer. Thus, 70Z/3 and WEHI 231 cells contain different NF-κB/Rel complexes: p50/p65 is the major inducible complex in 70Z/3 cells, and p50/Rel is the major constitutive and inducible complex in WEHI 231 cells. Both cell types had a basal level of $p50_2$ that was not inducible upon stimulation (data not shown).

The nuclear NF-κB/Rel complexes were then tested for their susceptibility to inhibition by IκB-α. For this purpose, IκB-α was purified as a fusion protein with glutathione *S*-transferase (27). The inducible nuclear complex in 70Z/3 cells and the constitutive complex in WEHI 231 cells were equally sensitive to inhibition by IκB-α–glutathione *S*-transferase (data not shown). Thus, there is no major qualitative difference in the sensitivity to IκB-α inhibition between the constitutive and the inducible nuclear NF-κB/Rel complexes in these two cell types.

Most cellular NF-κB/Rel in unactivated cells is stored in the cytoplasm as a non-DNA-binding complex with IκB or an IκB-containing precursor molecule like p105 (19, 36, 37). To examine the inactive complexes in 70Z/3 and WEHI 231 cells, we used immunoprecipitation analysis of ^{35}S-radiolabeled lysates with antibody against p65, Rel, or IκB-α. Under conditions that allowed coimmunoprecipitation of associated proteins, in 70Z/3 cells, IκB-α was coprecipitated with serum to either p65 or Rel (Fig. 2A, lanes 2 and 3) (36). Both p65 and Rel in 70Z/3 cells were also associated with the p105 precursor (lanes 2 and 3). Interestingly, 70Z/3 cells contained not only p65 but also a reasonable amount of cytoplasmic Rel (lane 3). However, the Rel complexes in 70Z/3 cells seem to be less responsive to stimulation by LPS or PMA in that they do not appear in the nucleus (Fig. 1). A small portion of Rel may also be associated with p65 (lane 3), as has been suggested previously (36).

There is a high level of expression of Rel protein in WEHI 231 cells. IκB-α, p105, and p65 were associated with Rel (Fig. 2A, lane 7) (36). There is much less p65 protein than Rel protein in the cytoplasm of WEHI 231 cells, and it is mainly associated with IκB-α rather than p105 (lanes 6 and 7). The IκB-α antibody coimmunoprecipitated IκB-α and Rel protein (lane 8) but not with any of the precursor, p105 (lane 4), in WEHI 231 cells. Therefore, IκB-α and p105 are mutually exclusive, perhaps indicating that only one IκB-like molecule is in complex with a dimer of NF-κB/Rel protein. In 70Z/3 cells, IκB-α antibody failed to detect IκB-α protein, possibly because of the low level of IκB-α in 70Z/3 cells. Alternatively, the antibody may have low affinity for IκB-α protein in a complex form.

Because p50 protein comigrates with the precipitating antibody, it has been difficult to quantitate the p50 protein by immunoprecipitation. Therefore, to resolve p50-containing complexes, we used DOC treatment followed by EMSA to examine the inactive NF-κB/Rel complexes in cytosol. The DOC-released NF-κB/Rel complex was mainly p50/Rel in WEHI 231 cells and p50/p65 in 70Z/3 cells (Fig. 2B). Thus, there is a p50/p65/IκB complex in the cytoplasm of 70Z/3 cells and a p50/Rel/IκB complex in WEHI 231 cells, from which IκBs are readily removed by detergent or by physiological stimulation.

Expression level of NF-κB/Rel and IκB proteins in 70Z/3 and WEHI 231 cells. In addition to EMSA and immunoprecipitation, we performed Western blot analysis to investigate the relative amounts of NF-κB/Rel and IκB proteins in these two cell lines. The NF-κB/Rel components that translocated to

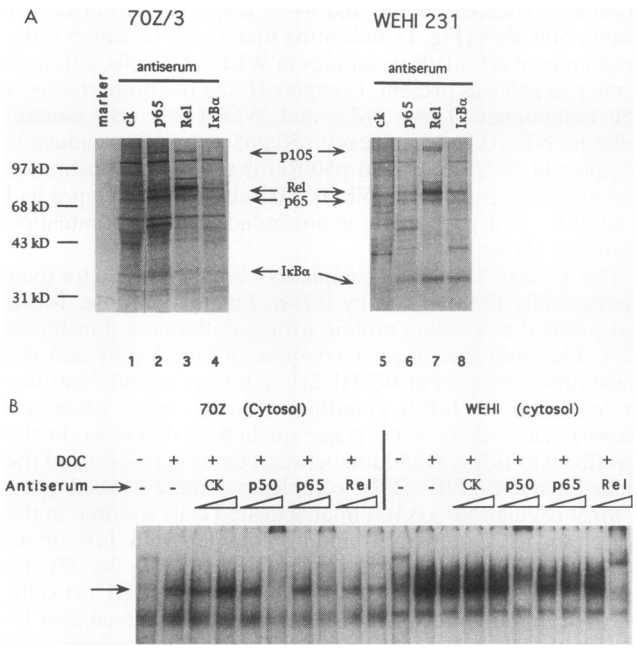

FIG. 2. NF-κB/Rel components in the cytosolic fractions of 70Z/3 and WEHI 231 cells. (A) Coimmunoprecipitation of NF-κB/Rel and IκB-α proteins in 70Z/3 and WEHI 231 cells. Cytosolic lysates from ^{35}S-labeled 70Z/3 and WEHI 231 cells were immunoprecipitated with normal rabbit serum (CK) or serum against p65, Rel, or IκB-α in an NP-40-based buffer (see Materials and Methods) that allows coimmunoprecipitation of associated protein complexes. Specific protein bands are marked with arrows. (B) DOC-released cytoplasmic complex is p50/p65 in 70Z and p50/Rel in WEHI 231 cells. Ten micrograms of cytosolic proteins was treated with 0.8% DOC to release NF-κB/Rel complexes from IκB-α, after which 1% NP-40 and specific antibody were added to the EMSA reaction. The arrow indicates the NF-κB/Rel complexes.

the nucleus after a 4-h LPS or PMA induction were also examined. To limit the study to immediate responses to stimulation, de novo protein synthesis was blocked with cycloheximide. Equal amounts of lysate protein were analyzed in each lane (5 μg from nuclei and 20 μg from cytosol, equivalent to 10^6 cells per lane).

From several gels probed with antibody specific to NF-κB and IκB proteins, it was evident that WEHI 231 cells had 3- to 10-fold-higher levels of p50, Rel, IκB-α, Bcl-3, and p105 than did 70Z/3 cells (Fig. 3). p65 protein was twofold less in WEHI 231 than in 70Z/3 cells (Fig. 3B). There was a continuous degradation (and therefore resynthesis) of p65, Rel, IκB-α, and p105 in WEHI 231 cells because a 4-h cycloheximide treatment reduced the levels of these proteins (Fig. 3B, C, D, and G). About 30% of total cellular p50 and 20% of the Rel proteins was in the nuclear extract of uninduced WEHI 231 cells, but none was found in 70Z/3 nuclei (Fig. 3A and C). The nuclear p50 and Rel made up the preponderance of the constitutive NF-κB/Rel activity, as detected by EMSA in WEHI 231 nuclei (Fig. 1A). Upon induction, 50 to 60% of total p50 and Rel proteins were found in WEHI 231 nuclei (Fig. 3A and C), demonstrating induced translocation of these proteins. The same stimuli also caused nuclear translocation of 30 to 40% of total p50 and 35 to 55% of p65 protein in 70Z/3 cells (Fig. 3A and B).

With respect to the NF-κB-bound proteins, there was a discernible level of IκB-α in WEHI 231 cells but little in 70Z/3 cells (Fig. 3D). Thus, the constitutive nuclear NF-κB/Rel activity in WEHI 231 cells could not be attributed to reduced IκB-α expression. Bcl-3 in WEHI 231 cells was sevenfold higher in the cytosol than in the nuclei (Fig. 3E), and induction with LPS and PMA caused little or no increase in the nuclear concentration. 70Z/3 cells did not have detectable Bcl-3. IκB-γ and p105 were present exclusively in the cytosolic fraction (Fig. 3F and G). The level of IκB-γ was slightly higher in 70Z/3 cells than in WEHI 231 cells, consistent with the higher expression level of IκB-γ mRNA in 70Z/3 cells (20, 26). Interestingly, LPS but not PMA treatment of both 70Z/3 and WEHI 231 cells led to loss of the p105 precursor (Fig. 3G).

To characterize the kinetics of p105 processing, WEHI 231 cells were induced at various time periods by either LPS or PMA. Equal amounts of lysate were resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using anti-p50 antibody. LPS induction causes increased processing of p105, whereas PMA did not (Fig. 3H). The induced processing became obvious after 1 h of LPS treatment (decreased 45%) and further enhanced after 4 h of stimulation (decreased 65%). Other stimuli, such as anti-IgM and TNF-α, like PMA, did not cause p105 processing in WEHI 231 cells (data not shown). These results suggest that various stimuli trigger different regulatory pathways for the activation of NF-κB/Rel protein.

IκB-α protein turns over rapidly in WEHI 231 cells. IκB-α is rapidly degraded following stimulation with a variety of agents (5, 18). Free IκB-α is degraded within about 30 min, whereas it is stable for longer than 6 h when complexed with p65 (40). Since there is continuous synthesis of IκB-α in WEHI 231 cells (Fig. 3D), there must be a continuous turnover maintaining a dynamic balance of IκB-α in these cells. To test this interpretation, we measured the turnover of IκB-α in both 70Z/3 and WEHI 231 cells by pulse-chase experiments (Fig. 4). Cells were labeled with [^{35}S]methionine for 2 h and chased with cold medium for various times. Lysates were immunoprecipitated with anti-IκB-α antibody, and the ^{35}S-labeled protein bands corresponding to IκB-α from each time point were quantitated. IκB-α in WEHI 231 cells had a much faster and more extensive degradation than in 70Z/3 cells (Fig. 4). The fast turnover of IκB-α protein may be partly responsible for the constitutive nuclear p50/Rel and p50/p65 in WEHI 231 cells.

Modification by phosphorylation has been suggested as one of the regulatory mechanisms of NF-κB activation in vitro (12). We therefore tested if phosphorylation of endogenous NF-κB/Rel or IκB proteins in 70Z/3 and WEHI 231 cells might account for the constitutive nuclear NF-κB/Rel activity in WEHI 231 cells. Results from phosphopeptide analyses indicated that both the basal and inducible phosphorylation patterns of p65, Rel, and IκB-α were comparable in these two cells (data not shown). Therefore, the constitutive nuclear component of NF-κB/Rel in WEHI 231 cells is not a consequence of a readily discernible difference in basal protein phosphorylation between these cells and 70Z cells.

Sequential expression of various NF-κB/Rel complexes in B-lineage cells. The presence of two different predominant complexes in two B-lineage cell lines prompted us to examine other B-cell lines of various differentiation stages. M12 is a B-cell lymphoma derived from BALB/c mice that was adapted to in vitro culture. These cells synthesize IgG2a in both a secreted and a membrane form. P3X63Ag (termed XAg here) is derived from the MOPC-21 myeloma and secretes IgG1. S194, S107, and J558L were derived from IgA-producing plasmacytomas and thus represent the terminal differentiation stage of B cells.

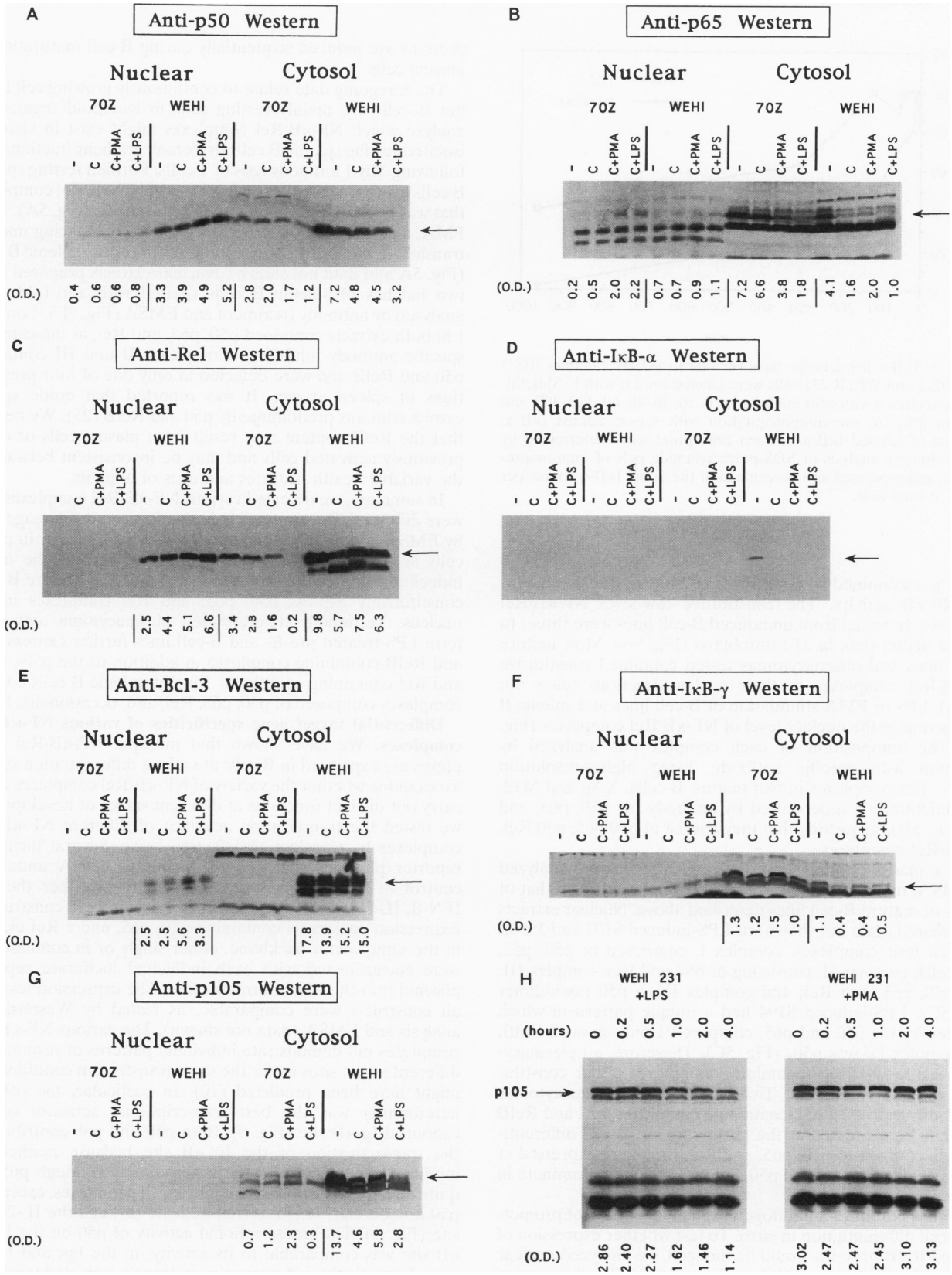

FIG. 3. Western blot analyses of lysates prepared from 70Z/3 and WEHI 231 cells induced with LPS or PMA. 70Z/3 and WEHI 231 cells were untreated (−) or treated for 4 h with cycloheximide (C; 10 μg/ml), LPS (10 μg/ml), or PMA (100 nM). Each protein blot was incubated with serum specific to p50 (A, F, G, and H), p65 (B), Rel (C), IκB-α (D), or Bcl-3 (E). For panel H, WEHI 231 cells were treated with either LPS (10 μg/ml) or PMA (100 nM) and analyzed by Western blot analysis with the anti-p50 serum. Arrows indicate specific p105 protein bands. The intensity of each protein bands was quantitated by densitometer and represented as relative optical density (O.D.) value.

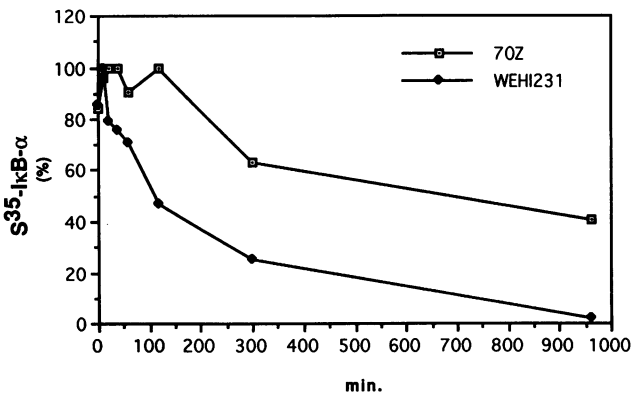

FIG. 4. IκB-α has a faster turnover rate in WEHI 231 than 70Z/3 cells. 70Z/3 and WEHI 231 cells were labeled for 2 h with [^{35}S]methionine and chased with cold medium for 0, 10, 20, 40, 60, 120, 300, and 960 min prior to immunoprecipitation with serum against IκB-α. Amounts of labeled IκB-α at each time point were determined by PhosphoImager analysis of SDS-polyacrylamide gels of immunoprecipitates and expressed as a percentage of the initial IκB-α counts per minute at time zero.

We first examined whether these cell lines express constitutive NF-κB activity. The constitutive low-level NF-κB/Rel complexes in nuclei from uninduced B-cell lines were three- to fivefold higher than in 3T3 fibroblasts (Fig. 5A). Most mature B-cell lines and plasmacytomas tested contained constitutive NF-κB/Rel complexes in their nuclear fractions (data not shown). LPS or PMA stimulation of B-cell lines and splenic B cells increased the nuclear level of NF-κB/Rel complexes (Fig. 5A). The composition of each complex was analyzed by inhibition with specific antibody, using higher-resolution EMSA. The complexes in two mature B cells, XAg and M12, were inhibited or supershifted by antibody for p50, p65, and Rel (Fig. 5B), suggesting that they consist of p50/p65, p50/Rel, or p65/Rel complexes.

When plasmacytomas S194, S107, and J558L were analyzed by EMSA, the complex pattern was very different from that of pre-B- or mature B-cell lines described above. Nuclear extracts of untreated (data not shown) or LPS-induced S107 and J558L revealed four complexes: complex I, composed of p50, p52, and RelB; complex II, consisting of p52 and Rel; complex III, with p50, p65, and Rel; and complex IV, a p50 homodimer (Fig. 5C). LPS-induced S194 had a unique pattern in which complex V was p50 and p65, complex VI only showed RelB, and complex IV was $p50_2$ (Fig. 5C). Therefore, all plasmacytomas expressed RelB-containing complexes either constitutively or upon induction. Two of the three plasmacytomas tested also expressed p52-containing complexes. p52 and RelB may thus be expressed at the latest stage of B-cell differentiation, in contrast to p50, p65, and Rel, which are expressed at an intermediate stage, and p50 and p65, which predominate in the pre-B-cell stage.

LPS is a potent B-cell mitogen capable over days of promoting B-cell differentiation in vitro. To test whether expression of p52 and RelB proteins would be induced, we analyzed nuclear extracts obtained from 70Z/3 pre-B- and M12 B-cell lines that were treated with LPS for 3 days. LPS treatment caused the cells to differentiate into lymphoblasts with a dramatic increase in cell volume. The binding pattern of their nuclear extracts consisted of the same four complexes found in plasmacytomas (Fig. 5C and D). These results suggest that the NF-κB/Rel proteins are induced sequentially during B-cell maturation to plasma cells.

The foregoing data relate to continuously growing cell lines, but B cells are mainly resting cells in lymphoid organs. To analyze which NF-κB/Rel complexes might exist in vivo, we isolated resting splenic B cells by Percoll gradient fractionation following Thy 1 antibody lysis of T cells. Purified resting splenic B cells express a constitutive low level of NF-κB/Rel complexes that was fivefold higher than in 3T3 fibroblasts (Fig. 5A). LPS, PMA, and anti-IgM were capable of further inducing nuclear translocation of NF-κB/Rel complexes in resting splenic B cells (Fig. 5A and data not shown). Nuclear extracts prepared from two batches of splenic B cells treated with anti-IgM were analyzed by antibody treatment and EMSA (Fig. 5E). Complex I in both extracts contained p50, p65, and Rel, as indicated by specific antibody inhibition. Complexes II and III contained p50 and RelB and were detected in only one of four preparations of spleen extract. It was reported that crude spleen extract contains predominantly p50 and RelB (25). We believe that the RelB content may result from plasma cells or other previously activated cells and may be inconsistent because of the variable health histories and ages of animals.

In summary, we detected various NF-κB/Rel complexes that were differentially expressed in both splenic and B-lineage cells by EMSA and antibody supershift analysis (Table 1). In pre-B cells as in nonlymphoid cells, NF-κB p50/p65 is the major inducible complex that appears in the nucleus. Mature B cells constitutively express p50, p65, and Rel complexes in the nucleus. Terminally differentiated plasmacytoma and long-term LPS-treated pre-B- and B-cell lines further express p52- and RelB-containing complexes in addition to the p50-, p65-, and Rel-containing complexes. Finally, splenic B cells contain complexes composed of p50, p65, Rel, and, occasionally, RelB.

Differential target gene specificities of various NF-κB/Rel complexes. We have shown that multiple NF-κB/Rel complexes are expressed in B cells at various differentiation stages. To examine whether the variety of NF-κB/Rel complexes could carry out distinct functions at different stages of development, we tested the transcription activities of different NF-κB/Rel complexes by transient transfection assay. Several luciferase reporter plasmids that contain luciferase cDNA under the control of divergent κB sites derived from either the Igκ, IFN-β, IL-2R, or Myc regulatory sequences were constructed. Expression constructs containing p50, p65, and c-Rel cDNAs in the same vector backbone, either singly or in combination, were cotransfected with each individual luciferase reporter plasmid into the L929 fibroblast line. The expression levels of all constructs were comparable, as tested by Western blot analysis and EMSA (data not shown). The various NF-κB/Rel complexes did demonstrate individual patterns of response on different target sites under the same transfection conditions, as might have been predicted (10). In particular, the p50/p65 heterodimer was the best transcriptional activator of the canonical Ig κB site (Fig. 6). Both p50 and p65 contribute in the transactivation of the Ig κB site because neither homodimer was as good as the heterodimer, although p65 was quite effective on its own. In contrast, all complexes, except the p50 homodimer, failed to transactivate through the IL-2R κB site (Fig. 6). The transcriptional activity of p50 on the IL-2R κB site was comparable to its activity on the Igκ and IFN-β sites. Intriguingly, p65 homodimer strongly activated transcription through both the IFN-β and Myc κB sites but not the IL-2R site. Rel synergized with p65 in the activation of the IFN-β κB site. Overall, Rel itself was a weak transcription activator on the selected κB sites studied. The optimal κB sites for Rel are yet to be identified. The results suggest that various

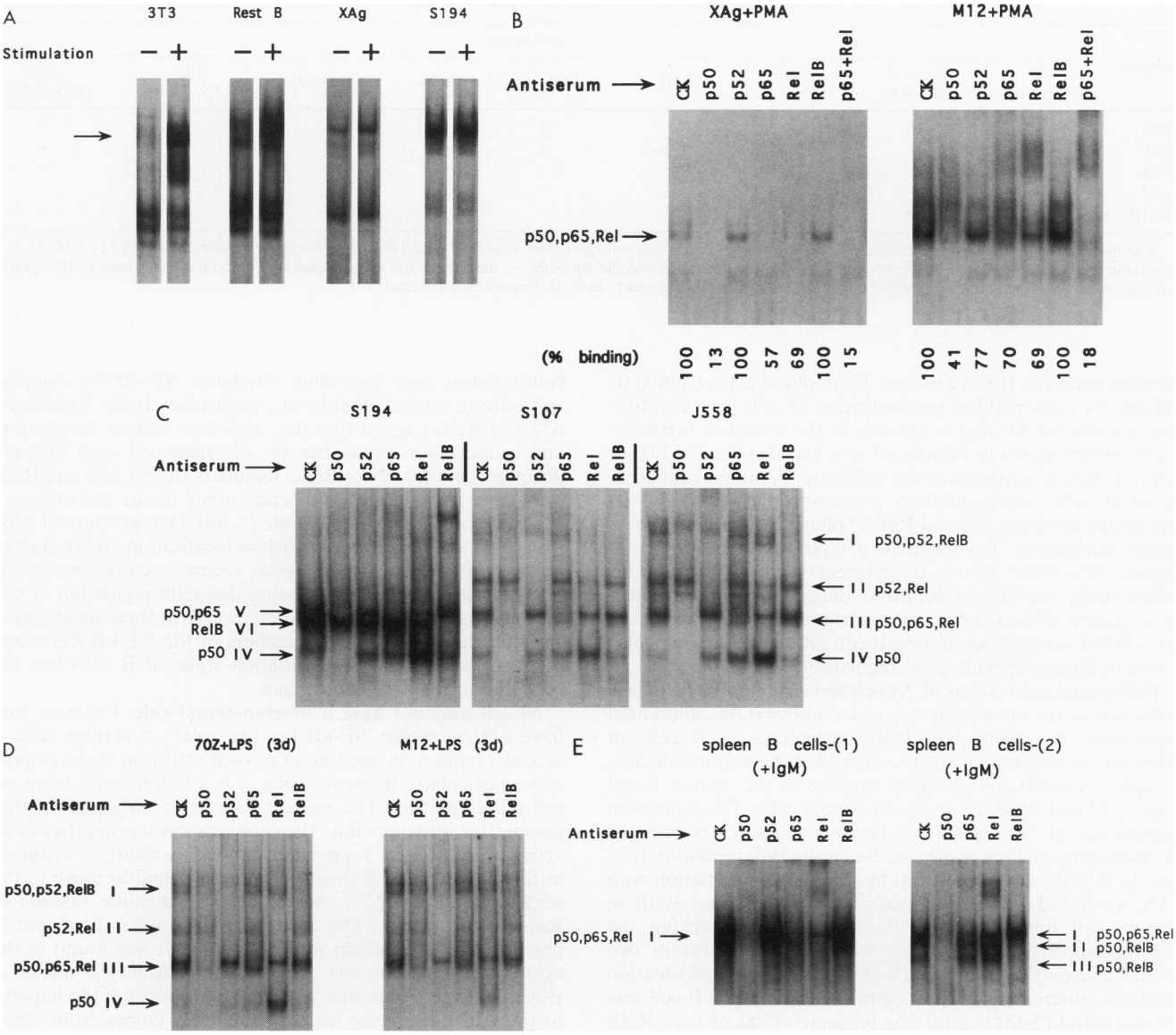

FIG. 5. NF-κB/Rel components are differentially expressed in B cells of various stages of differentiation. (A) Constitutive NF-κB/Rel activity in B-cell lines and splenic B cells. Shown is an EMSA of 5 μg of nuclear extracts from various cells; + indicates treatment of cells with TNF-α (3T3 cells), anti-IgM (resting B cells), or PMA (XAg and S194 cells). (B) Mature B cells express p50, p65, and Rel in the nucleus. Shown is an EMSA of nuclear extracts from XAg and M12 cell lines that were induced with PMA for 4 h. The NF-κB/Rel components in each complex were assessed by antibody inhibition as described for Fig. 1A. Percentage of residual binding was quantitated by a PhosphoImager. (C) S194, S107, and J558L plasmacytoma cells contain additional p52 and RelB complexes. Shown is an EMSA of nuclear extracts from S194, S107, and J558L plasmacytoma cells treated with PMA for 4 h. The S107 line used in this study is a different subline from that described by Atchison and Perry (2), which lacks NF-κB. (D) Long-term LPS stimulation of 70Z/3 and M12 cells leads to the induction of p52 and RelB complexes. Shown is an EMSA of nuclear extracts from 70Z/3 and M12 cells cultured in the presence of LPS (10 μg/ml) for 3 days (3d). (E) NF-κB/Rel components in two preparations of resting splenic B cells. Shown is an EMSA of nuclear extracts from two preparations of resting splenic B cells stimulated with anti-IgM for 4 h.

NF-κB/Rel components can regulate distinct sets of target genes during B-cell activation, although the particular responses seen in this experiment were a consequence both of the products of the transfected genes and of endogenous complexes that they may induce.

DISCUSSION

Originally, NF-κB was defined as an activity that shifted the mobility of a DNA probe containing a κB site. Later, the subunit composition of latent NF-κB in unactivated cells was found to be p50 and p65. When these subunits were shown to be related to Rel, other members of the NF-κB/Rel family were identified, and the composition of NF-κB in various cell types became an issue. Here we have examined the composition of NF-κB in cells of the B-lymphocyte lineage and have found that it changes in a developmentally ordered fashion. In pre-B cells, typified by 70Z/3 cells, cytoplasmic, IκB-bound p50/p65 is the predominant form, with a minor fraction of

TABLE 1. Summary of nuclear NF-κB/Rel components

Complex	EMSA result[a]								
	3T3 (TNF-α)	Spleen B (IgM, 4 h)	70Z/3 (LPS, 4 h)	WEHI 231	M12, XAg	S194	S107, J558L	70Z (LPS, 3 days)	M12 (LPS, 3 days)
p50	+	+	+	+	+	+	+	+	+
p65	+	+	+	Low	+	+	+	+	+
Rel	−	+	Low	+	+	−	+	+	+
p52	−	−	−	−	−	−	+	+	+
RelB	−	Low	−	−	−	+	+	+	+

[a] The nuclear DNA-binding complexes of NF-κB/Rel proteins in various cell types were assessed by inhibition with a specific antiserum followed by EMSA. +, a detectable level of the tested NF-κB/Rel component was found with the specific antibody; −, the component was undetectable in the nuclear extract by the specific antibody under the described experimental conditions; low, a barely detectable level of the protein was found.

p50/Rel bound to IκB. In mature B-lymphoid cells, typified by WEHI 231 cells, p50/Rel predominates. B cells have constitutive, nuclear NF-κB that is sensitive to the inhibitor, IκB-α; its constitutivity seems to be related to a fast turnover of IκB-α, not to a lack of synthesis of the inhibitor. Terminally differentiated B cells, represented by plasmacytomas, have NF-κB complexes involving p52 and RelB. Splenic B cells, which are a mixed population, have largely p50/p65 and p50/Rel complexes. NF-κB/Rel factors of different subunit compositions differentially activate transcription, depending on the particular sequence of the κB site in a reporter construct. The shift in NF-κB/Rel composition during B-cell differentiation may play a role in lineage-specific gene regulation.

Differential expression of NF-κB/Rel proteins in B-lineage cells. One of the most intriguing observations is the differential expression of various NF-κB/Rel complexes in B cells of different developmental stages. p50/p65 is the major inducible complex in pre-B cells; p50/Rel appears at the mature B-cell stage; p52 and RelB occur late in plasma cells. The sequential appearance of NF-κB/Rel components suggests a sequential programming of their synthesis. Since the differentiation process in B cells can be induced by long-term stimulation with LPS, we tested whether the expression of p52 and RelB in plasma cell lines could be reproduced by mimicking the differentiation process in vitro with LPS treatment of two earlier-lineage B-cell lines derived by different transformation methods. Interestingly, both a pre-B- and mature B-cell line treated with LPS for several days began to utilize p52 and RelB in induced complexes. The induction of p52 and RelB complexes in these cell lines by LPS treatment is unlikely to result solely from the nuclear translocation of NF-κB activity, since mature B cells already have constitutive nuclear p50 and Rel proteins yet do not usually express high levels of p52 and RelB.

The differential target gene specificity of each NF-κB/Rel component suggests that the ordered expression of each member of the NF-κB/Rel protein family during B-cell development may be to help regulate genes that are involved in stage-specific function. c-Rel is made primarily in lymphoid tissues, and oncogenic v-Rel is associated with B-cell lymphoma, suggesting that Rel protein may participate in cell cycle control in mature B cells. p52 and RelB are expressed predominantly in plasma cells. Different NF-κB/Rel complexes bind with variable affinity to different κB target sequences. Also, although p50 homodimer binds equally well to the Igκ, MHC class I, and IFN-β κB sites, it transactivates expression only from the MHC class I site (10). This is due to a conformational change in p50 when binding to the MHC class I site. p52/p65 had the capacity to bind to various κB sites, but it failed to activate reporter constructs with either H2 or IL-2R κB sites (6, 34, 39). Therefore, both binding affinity and conformation may determine whether a NF-κB/Rel complex can activate transcription from a particular κB site. Studies on p52 and RelB suggest that they also have unique recognition and transcription properties for divergent κB sites that are distinct from other NF-κB/Rel members (6, 38). p52 and RelB were able to synergize with each other in the activation of reporter construct with Ig κB site (6, 38). Deregulation of p100 that leads to changes in the nuclear localization and transcriptional properties of the p52 gene seems to correlate with its oncogenicity in B cells, suggesting that tight regulation of p52 may also be important to maintain B cells at their steady state. The full expression of five members of the NF-κB/Rel members at the terminal differentiation stage of B cells has the potential to regulate many genes.

NF-κB may not have a developmental role. Previous data have suggested that NF-κB has two roles: in mature cells, it acts as a rapid response transcriptional activator; in developing cells, particularly B-lineage cells, it is a determinant of pre-B cell differentiation. The present data bring an aspect of that perspective into question. The rapid response properties of its activity are strongly supported: p50/p65 is clearly a cytoplasmically stored gene activator awaiting a signal to move to the nucleus, bind to DNA, and initiate an activation cascade of Rel-related proteins. The evidence for NF-κB involvement in pre-B-cell differentiation was largely that it was found in the cytoplasm of pre-B cells and constitutively in the nuclei of B-lymphoid cell lines, like WEHI 231. However, if the impetus to grow in these cells, which presumably comes from oncogenes that are active in them, is equivalent to an activation stimulus, then their properties may not relate to their differentiation state but only to their activation state. Very recently, we have created a mouse with a null mutation in the NFKB-1(p50) gene, and it can make surface-Igμκ-positive B cells in normal amounts (43). This finding suggests that p50 at least may not play a role in differentiation events that lead to mature B cells.

One way to determine whether the constitutive NF-κB in B-cell lines is an indicator of their differentiation state or their activation state would seem to be to examine resting splenic B cells. The literature, however, suggests that B cells either have (28) or do not have (29) endogenously active NF-κB. We reexamined this question by purifying resting B cells by using Percoll gradients (8). They show a low level of nuclear NF-κB, more than 3T3 cells and less than B-cell lines. They also can be induced to translocate more NF-κB to their nuclei; this is a mixture of p50/p65 and p50/Rel. The cells purified from Percoll gradients, though, should be a mixture of memory B cells and virgin B cells, with perhaps other cell types included. Therefore, the definitive answer to the question of whether resting virgin splenic B cells express constitutive NF-κB activity

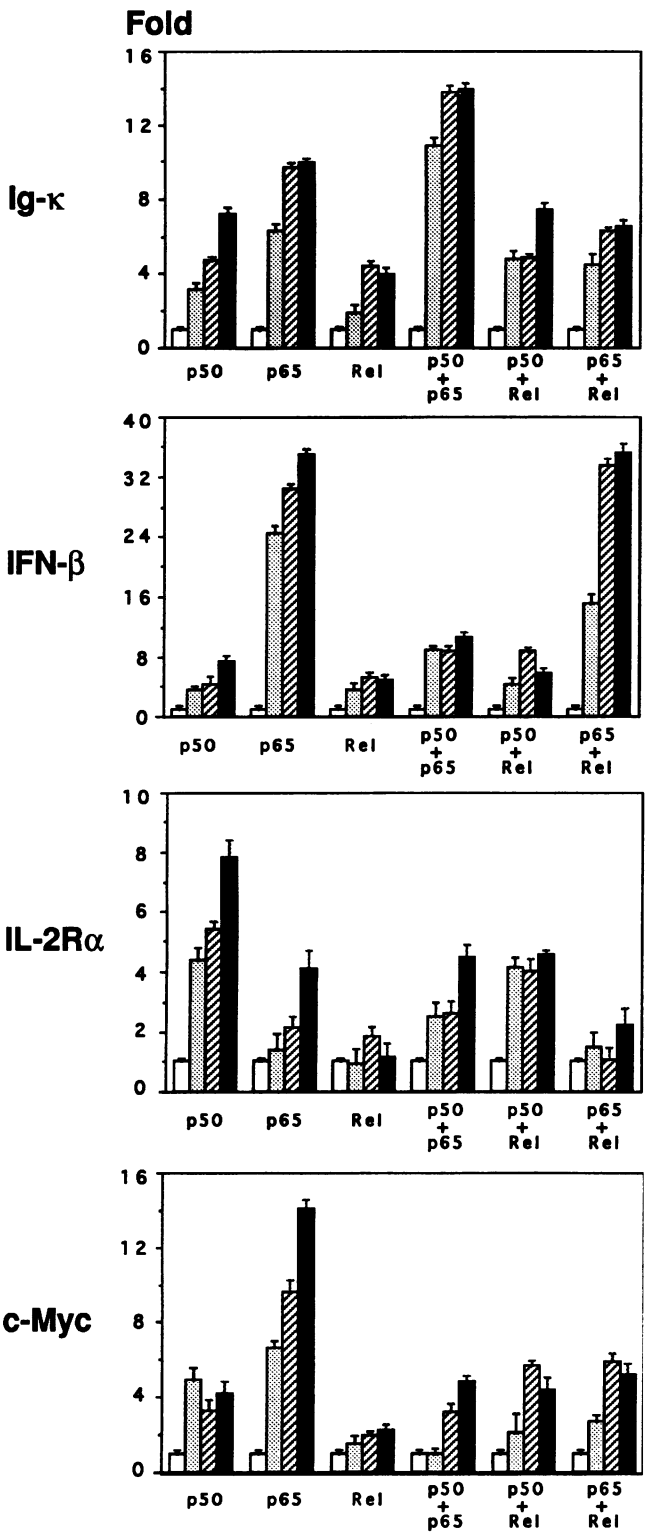

FIG. 6. Various combinations of NF-κB/Rel components demonstrate different transcriptional activities on divergent κB sites. Abilities of different combinations of p50, p65, and Rel expression constructs to differentially activate luciferase reporter constructs containing dimerized κB sites corresponding to Ig κ (GGGACTTTCC), IL-2R (GG GAATCTCC), Myc (GGGTTTTCCC), and IFN-β (GGGAAAT TCC) sites were determined. Luciferase activity is shown as fold induction over transfections with vector alone. All transfections were done in triplicate for several dosages of expression constructs. (□, 0 μg; ▨, 0.2 μg; ▨, 0.4 μg; and ▩, 0.6 μg).

awaits a more detailed study of defined subpopulations of cells, which was beyond the scope of this investigation. Klug et al. (23) recently reported the detection of a constitutive NF-κB activity in IL-7-expanded pre-B cells from the Whitlock-Witte bone marrow culture system. The constitutive activation of NF-κB in the nucleus of pre-B cells may reflect a natural differentiation process through interaction with stromal cells in bone marrow or result from an activation process due to the presence of IL-7 in the in vitro differentiation system.

In our studies, both crude and purified resting spleen B-cell extracts are mainly composed of p50, p65, and Rel, the same components also observed in mature B-cell lines. One of four tested splenic B-cell preparations also expressed RelB (Fig. 5E). This is in contrast to the consistent observation by Lernbecher et al. (25) of p50/RelB in crude spleen B cells. Not only might the health status of their mice cause this result, as suggested by our occasional similar observation, but also their inclusion of activated splenic cells in their preparations might be a factor because we excluded such cells by use of Percoll gradient purification.

The activation process. The cytoplasmic NF-κB complexes with IκB can be dissociated and NF-κB can rapidly be translocated to the nucleus by an activation stimulus. Activation is caused by cell-specific mediators such as TNF-α, antigens, LPS, PMA, and IL-1. The mechanism of release of NF-κB from IκB is controversial and may involve multiple pathways; both phosphorylation of IκB and its degradation appear to play roles in activation. Whatever the events that ensue from an activation stimulus, it leads to loss of detectable IκB which is then resynthesized (5). If the activation signal is transient, the new IκB can capture the existing NF-κB and return it to the cytoplasm (5, 36, 40, 44). If the activation process continues, a steady state of synthesis and degradation of IκB is established in an activated cell (Fig. 3) (36). The movement of NF-κB to the nucleus in an activated cell leads to increased transcription of genes with κB regulatory sites. Among such genes are IκB-α, Rel, and p105, all of which are transcribed more extensively after activation (16, 17, 30). Thus, even though IκB-α is degraded very rapidly in activated cells, it is made at a higher rate than in unactivated cells.

The properties of WEHI 231 cells are therefore those of chronically activated cells. They have a high level of IκB-α that turns over rapidly and a high expression of Rel. The oncogenes active in WEHI 231 cells are not known, but they could give rise to a chronically activated state. What causes the constitutive NF-κB activity in WEHI 231 B cells? Since WEHI 231 cells express high levels of IκB-α, IκB-γ, and Bcl-3, the possibility that IκB proteins are not expressed in WEHI 231 cells is ruled out. There is no major qualitative difference between the inducible complex in 70Z/3 cells and the constitutive complex in WEHI 231 cells because both are sensitive to inhibition by IκB-α, although it remains possible that p50/p65 and p50/Rel complexes have different affinities for IκB. The basal and stimulated phosphorylation statuses of IκB-α, p65, and Rel proteins are comparable in these two cell types, suggesting that there is no major difference in the activities of protein kinases. Several parameters may be related to the constitutive p50 and Rel activities in WEHI 231 cells. One of the striking results is that IκB-α turns over rapidly in WEHI 231 cells. However, the mechanism responsible for IκB-α degradation remains to be identified. WEHI 231 cells express

a lower level of IκB-γ protein than 70Z/3 cells do. The 2.6-kb spliced mRNA variant corresponding to IκB-γ is expressed at a higher level in pre-B-cell lines than in mature B cells (12, 27). Such a differentiation-mediated down-regulation of the IκB-γ mRNA may be relevant to the nuclear translocation of p50 homodimer. In addition, the high expression of Bcl-3 protein in WEHI 231 cells may facilitate nuclear transport of p50; Bcl-3 as a coactivator of p50 may in turn enhance the transcription of target genes through its interaction with p50 (11).

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