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Identification of a Novel NF-κB p50-Related Protein in B Lymphocytes

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In most cell types other than mature B lymphocytes and macrophages, the transcription factor NF-κB remains in an inactive form in the cytosol by being bound to the inhibitory proteins IκBα and IκBβ. To investigate the regulation of constitutively active NF-κB in B lymphocytes, we have examined the composition of Rel protein complexes in different mouse B-cell lines. As reported previously, the constitutively active complex in mature B cells was predominantly p50:c-Rel. However, the κB binding complex in the plasmacytomas that were examined lacked c-Rel and instead contained only a p50-related protein. This p50-related protein (p55) cross-reacts with three different p50 antisera, exists in both the cytosol and the nucleus, and is the protein that binds to κB sites in plasma cells. Transfection of reporter constructs into plasma cells indicates that the p55 complex is also transcriptionally active. The p55 protein can be detected in splenocytes from mice lacking the p105/p50 gene, and therefore it appears to be the product of a distinct gene. The implications of the existence of a NF-κB p50-related protein in plasma cells that is capable of binding to κB sites and activating transcription are discussed.

The transcription factor NF-κB generally exists in most cells as an inactive cytosolic protein that is bound to inhibitory proteins known as IκBs (2). Treatment of cells with a wide variety of agents such as lipopolysaccharide (LPS), interleukin-1, tumor necrosis factor, and oxygen free radicals causes the dissociation of the inactive cytosolic complex, due to the degradation of the inhibitors, IκBα and IκBβ (6, 32, 33). Thus, NF-κB acts like a second messenger, transducing signals from the cell surface to the nucleus. In keeping with such a role, induction of NF-κB is also a key element in the inducible expression of a wide variety of genes, including those encoding interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor, beta interferon, and adhesion molecules (6, 32, 33). The genes whose expression is influenced by NF-κB all play important roles in the body's response to situations of infection, stress, and injury (16). Thus, it appears that NF-κB plays an important role in coordinating the activation of many immune response genes and therefore plays a critical role in innate immunity.

NF-κB subunits belong to a family of proteins known as the *rel* family (6, 32, 33). The NF-κB p50 subunit is produced as a longer precursor of 105 kDa (p105) and is proteolytically processed to its final size of 50 kDa (5, 8, 12, 19, 25). The other members of this family include p100, which is processed to p52, p65, c-Rel, and RelB (3, 21, 26, 27). Different members of this family of proteins can form both hetero- and homodimers and bind to DNA (6, 33). Although heterodimers where one of the subunits is p65, c-Rel, or RelB are transcriptionally active, homodimers of p50 or p52 are thought to be transcriptionally inactive since these proteins lack transcriptional activation domains (6, 28, 33). It has also been demonstrated that different Rel proteins play distinct roles in vivo. These differences have recently been highlighted in the phenotypes of mutant mice generated by gene-targeting techniques (4, 15, 31, 34). For example, mice lacking p50 or c-Rel exhibit defects in immune responses, whereas the absence of p65 leads to neonatal lethality due to massive apoptosis of hepatocytes in the liver. Although these mutants have helped to demonstrate the importance of members of this transcription factor family in normal development and physiology, they have failed to provide significantly greater insight about their regulation in vivo. We have approached the regulation of Rel transcription factors by focusing on B lymphocytes and on the regulation of stage-specific expression of the immunoglobulin κ light-chain gene. By determining which Rel complexes are present at different developmental stages, we hope to learn more about their function and also to begin to understand the mechanism by which NF-κB activity is regulated in vivo.

NF-κB was first discovered as a nuclear transcription factor that bound specifically to the intronic enhancer of the immunoglobulin κ light-chain gene (30). Subsequent studies carried out with Abelson virus-transformed pre-B-cell lines indicated that NF-κB was inactive in these cells but was constitutively activated in mature B cells and plasma cells (17, 29). Therefore, the activation of NF-κB appeared to be a developmentally regulated event, and active NF-κB was believed to be responsible for developmental stage-specific expression of the κ light-chain gene (1, 17, 29, 30). However, this simple and attractive model of NF-κB as a critical regulator of immunoglobulin κ light-chain expression has been challenged recently by a number of observations. In particular, it was found that primary pre-B cells from the bone marrow contain active nuclear NF-κB, a result that is in contrast to previous observations for Abelson virus-transformed pre-B-cell lines (14), and it has been suggested that the lack of nuclear NF-κB in pre-B-cell lines might be a consequence of transformation by the Abelson virus. Also, the disruption of the genes encoding three members of this family, p50, c-Rel, and RelB, resulted in mice that did not exhibit any apparent defect in B-lymphocyte differentiation (4, 15, 31, 34). Despite these observations, it remains true that B lymphocytes are one of only a few cell types where this normally inactive transcription factor is constitutively active. We have therefore continued to examine the

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regulation of this NF-κB in B cells in an attempt to understand the mechanism responsible for constitutive activation of NF-κB in these cells.

In the course of carrying out these studies, we found that the constitutively active κB binding complex in plasmacytoma cell lines was composed of a novel p50-related protein, which we term p55. We have shown that p55 is present in p50 knockout mice and therefore is the product of a distinct gene. We have demonstrated cross-reactivity of this protein with three different p50 antisera and have shown that this protein forms a κB-DNA binding complex in plasma cells. Significant κB-dependent reporter gene activity was also observed in these plasma cells, suggesting that the p55 protein was transcriptionally active. The presence of a transcriptionally active, p50-related protein, therefore, has implications for the expression of different genes where p50 dimers have been previously suggested to play important roles.

MATERIALS AND METHODS

Antibodies. (i) p50. Three different p50 antibodies were used. One, α-p50-1, was a polyclonal antibody raised against a 15-amino-acid peptide (amino acids [aa] 351 to 365), and then affinity purified against the same peptide, from Santa Cruz Biotechnology. It was used for both Western blotting (immunoblotting) and supershift assays. Another, α-p50-2, was an affinity-purified antibody raised against a bacterially overexpressed fragment of p50 NF-κB (aa 1 to 387) that was purified to homogeneity (as determined by silver staining of overloaded gels). It was also used for Western blotting and supershift assays. A third, α-p50-3, was raised against a glutathione *S*-transferase fusion of a portion of p50 from aa 152 to 250 that includes the unique sequence that interrupts the Rel homology domain of p50. This antibody was used only for Western blotting.

(ii) p65. Three different antibodies were used. α-p65-1, polyclonal, affinity-purified antibody from Santa Cruz Biotechnology raised against a unique p65 peptide (aa 3 to 19), was used for Western blotting and supershift assays. Another polyclonal antibody, α-p65-2, was from Rodrigo Bravo and was used for Western blotting. The third antibody, α-p65-3, was from Garry Nolan and was also used for Western blotting.

(iii) c-Rel. Two affinity-purified antibodies, one raised against an N-terminal peptide (aa 152 to 176) (α-cRel-N) and the other raised against a C-terminal peptide (aa 498 to 517) (α-cRel-C), obtained from Santa Cruz Biotechnology, were used for both Western blotting and supershift assays.

(iv) Other antibodies. A polyclonal IκBγ antibody raised against aa 785 to 971 of p105 as a glutathione *S*-transferase fusion was a gift of Hsiou-chi Liou. A polyclonal p50B antibody (serum) and a polyclonal RelB antibody (serum) were from Rodrigo Bravo.

Supershift assays. Ten-microgram aliquots of nuclear extracts were taken and made up to a final volume of 10 μl with 100 mM KCl, 25 mM Tris (pH 7.5), 1 mM dithiothreitol, 0.5 mM EDTA, and 5% glycerol. Then the labeled probe was added along with 2 μg of dI-dC, 3 mM GTP, and the binding buffer, and the mixture was incubated at room temperature for 15 min. Then 1 μl of the appropriate antibody, or an immunoglobulin G (IgG) as a control, was added to each sample, and the mixture was incubated for 8 h at 4°C. The samples (20 μl) were then electrophoresed under standard conditions (7).

Western analysis. Following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the proteins on the gel were electrophoretically transferred onto Immobilon-P membranes (Millipore) at 100 V for 1 h at 4°C (or at 30 V, overnight at room temperature), using a Hoefer Mighty Small Transphor apparatus. The membranes were then blocked with BLOTTO and incubated with the primary antibody at various dilutions for 2 to 12 h. The membranes were washed and then incubated with the horseradish peroxidase-conjugated secondary antibody at a 1:2,000 dilution. The bound antibodies were detected by using the enhanced chemiluminescence (ECL) protocol (Amersham).

Fractionation of cytoplasmic extracts. Large-volume extracts were prepared from $\sim 10^9$ cells of each type as described by Dignam et al. (4a). The cytoplasmic S100 fraction was used for chromatography of the Mono Q column. The column was equilibrated at 50 mM KCl, 25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.5 mM sodium EDTA, 0.05% Nonidet P-40 (NP-40), and 5% glycerol. After loading of the cytoplasmic extract, the column was washed with the same buffer and finally the bound proteins were eluted with a gradient from 50 to 600 mM KCl at 0.5 ml/min. A total of 35 1-ml fractions were collected.

Isolation of primary B cells. Mature C57 B10 mice were sacrificed, and spleens were removed and placed in RPMI medium containing 10% fetal calf serum. The spleens were crushed, and the T cells were depleted by treatment with an anti-Thy-1 monoclonal antibody plus complement. The cells were then layered on a discontinuous gradient of Percoll and centrifuged at 1,200 × *g* for 20 min. Cells banding at the 60 to 70% interface were recovered and washed with RPMI. The cells were then cultured overnight at 10^6 cells per ml in RPMI–5% fetal calf serum. B cells prepared this way were >95% pure as judged by fluorescence-activated cell sorting. Nuclear and cytoplasmic fractionations were then done as described above.

Transfection by the DEAE-dextran protocol. Approximately 10^7 cells were used for each transfection. The DNA (~5 μg) was taken up in $MgCl_2$-$CaCl_2$-containing buffer. Then DEAE-dextran was added to a final concentration of 0.5 μg/ml in a total volume of 1 ml. The cells were taken up in this solution and incubated for 20 min. Then medium with chloroquine was added, and the mixture was incubated for a further 30 min. The cells were spun down, washed, and then plated in fresh medium for 48 h. The cells were then harvested, washed with phosphate-buffered saline, and lysed in an NP-40-containing buffer, and the extract was assayed for luciferase activity as recommended by Promega.

RESULTS

Plasmacytoma cells contain nuclear κB binding complexes lacking c-Rel. Nuclear κB binding activity can be detected only in mature B cells and plasma cells and can be induced in pre-B cells by treatment with agents such as LPS or phorbol myristate acetate. We noticed that the mobilities of κB binding complexes from the nuclei of pre-B cells treated with LPS and mature B cells were different from the mobility of the complex seen in plasma cells, suggesting a difference in their composition (Fig. 1A). While both the pre-B-cell and mature B-cell complexes migrated at positions for heterodimers, the complex from plasma cells migrated at a position similar to that for p50 homodimers. Also, while the deoxycholate-induced cytoplasmic complex from S194 cells migrated at the same position as the WEHI 231 nuclear complex, the nuclear complex from plasma cells was clearly different (Fig. 1B). To determine the subunit composition of these complexes, we carried out supershift experiments using specific antibodies to p50, p65, and c-Rel (Fig. 1C). The different cell lines that we tested can be arranged according to their stages of development as follows: PD31 (pre-B, μ^+) > WEHI 231 (early mature B, surface IgM positive [$sIgM^+$]) > BCL1 (mature B, $sIgM^+$ $sIgD^+$) > 2PK-3 (activated B, $sIgG^+$, can secrete IgG2a) > S194 (plasmacytoma, IgG secreting) > P1-17 (plasmacytoma, IgA secreting).

The p50 antibody supershifted the complex from the nuclear extracts of all cells tested, but the only complex that supershifted with the p65 antibody was from the LPS-induced pre-B cells (PD31). By contrast, the complexes from the mature B-cell lines (WEHI 231, BCL1, and 2PK-3) were shifted completely with the c-Rel antibody but were unaffected by the p65 antibody, and these results are similar to those reported previously (20, 23). The complexes from the plasma cell lines (S194 and P1-17) were unaffected by either p65 or c-Rel antibodies. Therefore, treatment of pre-B cells (PD31) with agents such as LPS resulted in the activation and nuclear translocation of p50:p65, while the complex detected in the nuclei of mature B cells (WEHI 231, BCL1, and 2PK-3) was p50:c-Rel. Surprisingly, further differentiation to plasma cells (S194 and P1-17) resulted in the disappearance of c-Rel from the cells; instead, a complex containing only p50 (or a related protein) was detected. However, in contrast to some previously published reports (18, 20), we did not detect significant amounts of RelB in any of the nuclear κB binding complexes (data not shown).

To ensure that the identification of p50:c-Rel in mature B cells was not limited to transformed cell lines, we extended our analysis to primary B cells. Although it is very difficult to obtain sufficient numbers of primary pre-B cells that belong to distinct developmental stages, mature B cells from adult spleens can be isolated as a relatively homogeneous population of small, resting B cells. We isolated significant quantities of such cells (approximately 95% pure), made nuclear extracts, and used them in supershift experiments. The purification protocol avoided the use of positive selection, as we were concerned that cross-linking cell surface markers might result in transmission

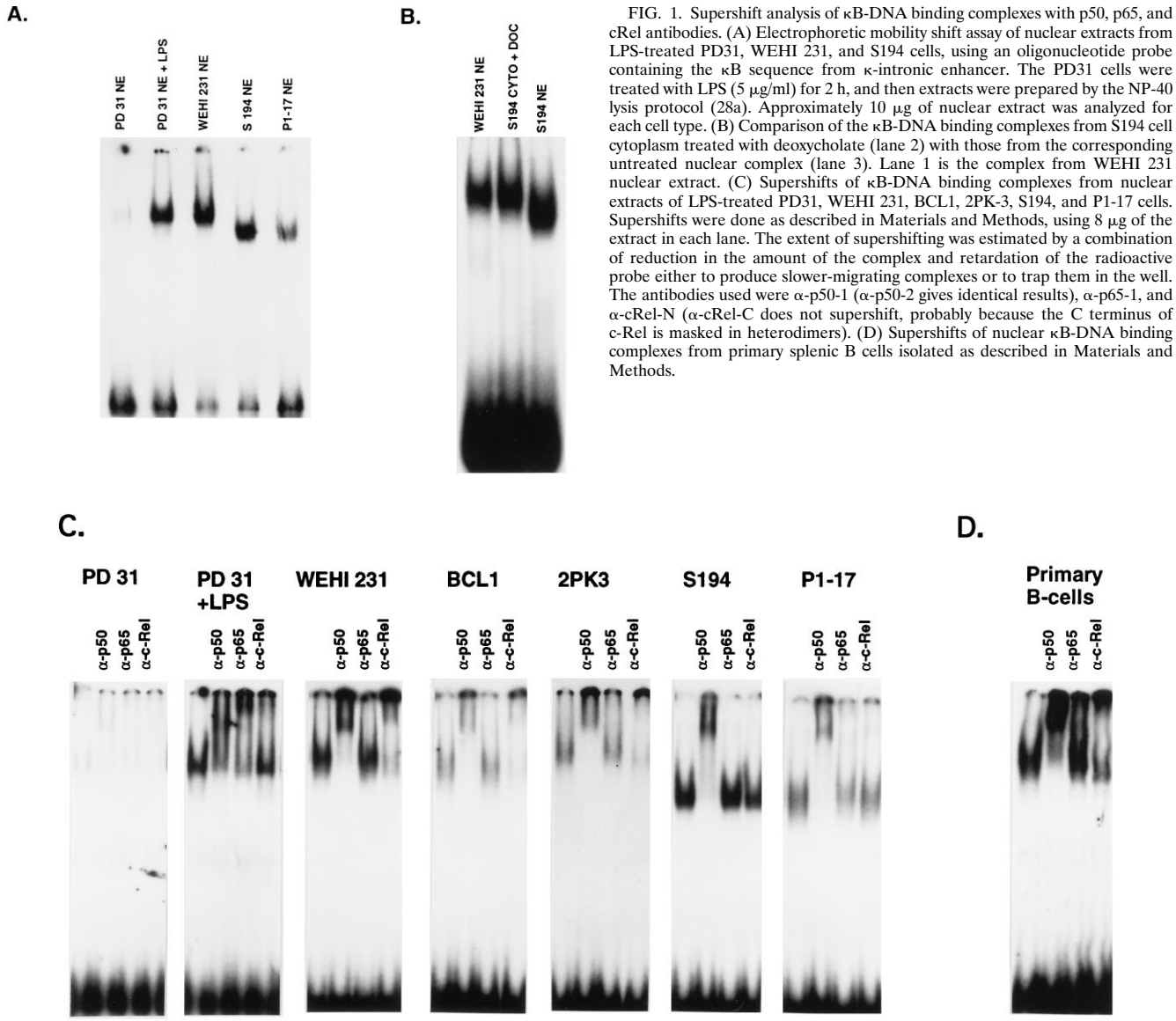


FIG. 1. Supershift analysis of κB-DNA binding complexes with p50, p65, and cRel antibodies. (A) Electrophoretic mobility shift assay of nuclear extracts from LPS-treated PD31, WEHI 231, and S194 cells, using an oligonucleotide probe containing the κB sequence from κ-intronic enhancer. The PD31 cells were treated with LPS (5 μg/ml) for 2 h, and then extracts were prepared by the NP-40 lysis protocol (28a). Approximately 10 μg of nuclear extract was analyzed for each cell type. (B) Comparison of the κB-DNA binding complexes from S194 cell cytoplasm treated with deoxycholate (lane 2) with those from the corresponding untreated nuclear complex (lane 3). Lane 1 is the complex from WEHI 231 nuclear extract. (C) Supershifts of κB-DNA binding complexes from nuclear extracts of LPS-treated PD31, WEHI 231, BCL1, 2PK-3, S194, and P1-17 cells. Supershifts were done as described in Materials and Methods, using 8 μg of the extract in each lane. The extent of supershifting was estimated by a combination of reduction in the amount of the complex and retardation of the radioactive probe either to produce slower-migrating complexes or to trap them in the well. The antibodies used were α-p50-1 (α-p50-2 gives identical results), α-p65-1, and α-cRel-N (α-cRel-C does not supershift, probably because the C terminus of c-Rel is masked in heterodimers). (D) Supershifts of nuclear κB-DNA binding complexes from primary splenic B cells isolated as described in Materials and Methods.

of an activation signal. The supershift experiments on the nuclear complexes using anti-p50, anti-p65, and anti-c-Rel antibodies indicated the presence of p50 and c-Rel in the nuclear complex with some p65 (Fig. 1D). Thus, the presence of c-Rel in the nuclei of these splenic B cells was similar to the results obtained with WEHI 231 and BCL1 cells.

Western analysis reveals two forms of p50 in B cells. The results obtained in supershift experiments were further verified by immunoblot analysis of nuclear and cytoplasmic fractions (Fig. 2A and E). These immunoblots indicated that while the pre-B-cell cytoplasm contained p50, a novel p50-reactive protein that we call p55 (discussed below), p65, and c-Rel, short-term induction with LPS or phorbol myristate acetate caused the nuclear uptake of mostly p50 and p65. The cytoplasm of the mature B-cell line WEHI 231 had increased levels of the proteins p50, p55, and c-Rel (the level of p65 had not increased), while the nucleus had primarily p50 and c-Rel. The plasma cell lines did not have any detectable c-Rel protein in either the nucleus or the cytoplasm, and only low levels of p50, p55, and p65 were present in the cytoplasm. The nuclear fractions of plasma cells had only the p55 protein.

As mentioned above, immunoblot analysis with the p50 antibody detected two strongly cross-reactive bands, one at ~50 kDa and the other at ~55 kDa (Fig. 2A). We could also detect the precursor for p50, the p105 protein, in the cytoplasmic fractions. The 50-kDa band correlated in size to the purified p50 from rabbit lungs, and hence we refer to it as p50. The larger p55 band was unlikely to be an unrelated cross-reacting species because it also appeared to undergo cytosol-to-nucleus translocation. Also, the only anti-p50-reactive protein detected in the nuclei of S194 plasma cells was larger than the p50 protein (Fig. 2A, lane 8). We suspected that this intermediate-size protein in S194 cells was a degradation product of the p55 form, as we detected a few smaller proteins, and therefore we tested the cytoplasmic and nuclear extracts of another plasma cell line, P1-17. The immunoblot analysis of fractions from this cell line showed that the two p50-related forms could be detected in the cytoplasm (although the amount of the smaller

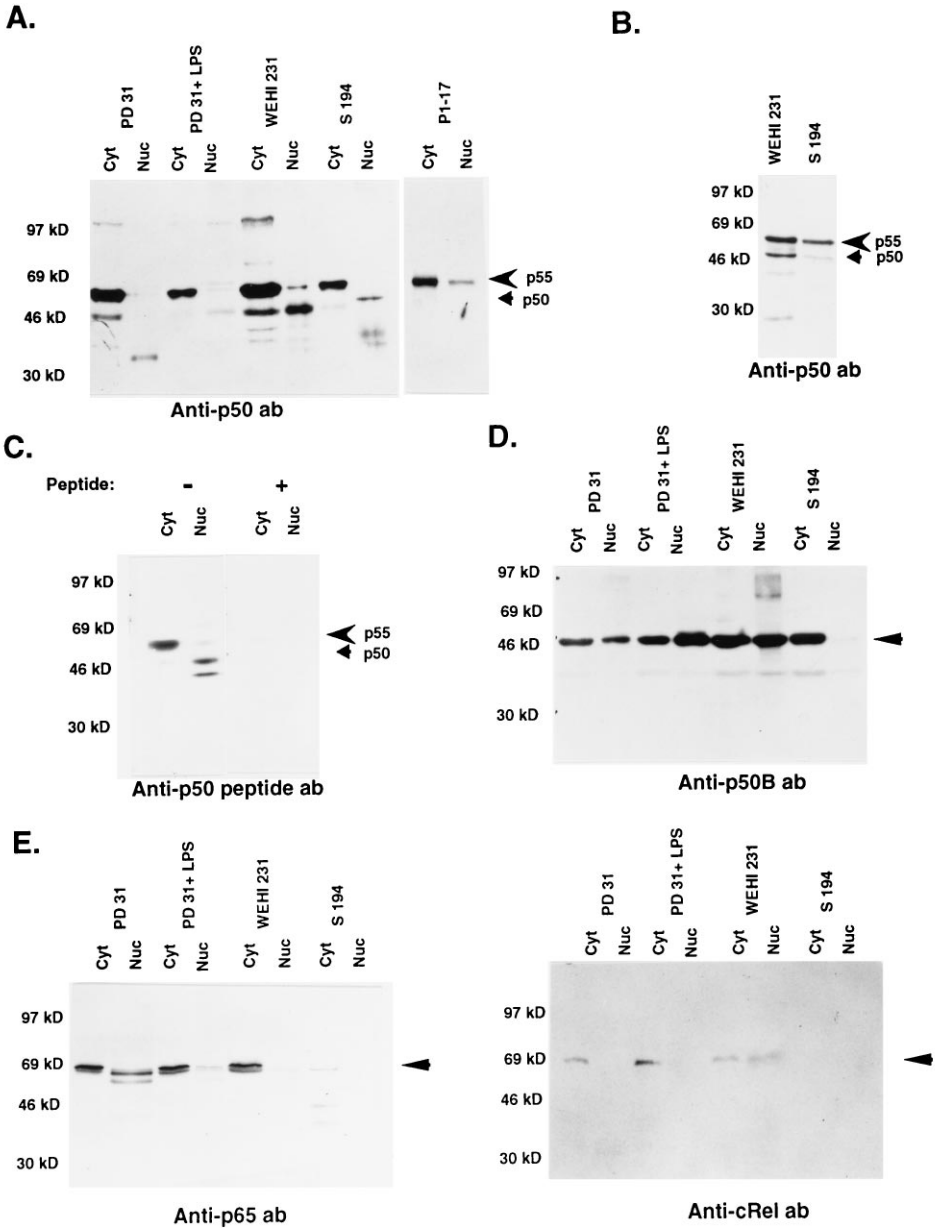


FIG. 2. Western analysis of cytoplasmic and nuclear fractions with antibodies against different Rel proteins. (A) Western analysis of cytoplasmic (Cyt) and nuclear (Nuc) fractions of PD31, LPS-treated PD31, WEHI 231, S194, and P1-17 cells, using the α-p50-2 antibody (Ab) (α-p50-1 and α-p50-3 give similar results). Each lane contained 25 μg of extract. The specifically bound antibodies were detected by using an ECL kit from Amersham. (B) Western analysis of WEHI 231 and S194 cells directly lysed in SDS sample buffer and heated at 90°C for 15 min. The boiled lysates were spun in a tabletop ultracentrifuge at 100,000 × *g* for 45 min. Equal amounts of the supernatant were then analyzed in an SDS–10% polyacrylamide gel. Western blotting and detection of p50 protein with the α-p50-2 antibody were carried out as described in Materials and Methods. (C) Specific blocking of the α-p50-1 antibody by the immunogenic peptide. The left half shows that both the p55 and p50 proteins are specifically detected in cytoplasmic and nuclear fractions (as in panel A) of WEHI 231 cells, using an antibody raised against a unique peptide from p50. If, however, the antibody is pretreated with 1 μg of the immunogenic peptide per μl, all of the binding is specifically blocked (right half). (D) Western analysis of the cytoplasmic and nuclear extracts, as for panel A, with an antibody to p50B. (E) Western analysis of the cytoplasmic and nuclear extracts, as for panels A and D, with α-p65-2 and α-cRel-N antibodies. For the p65 blot, the upper, darker band is that of p65.

form was less and was visible only on longer exposures of the ECL blots), but the only form present in the nucleus was the p55 form. To determine if the intermediate p50-reactive band in S194 nuclear extracts was generated during the process of making the extracts, we lysed some S194 cells directly in SDS sample buffer and analyzed the whole-cell extract on a Western blot (Fig. 2B). Only two p50-reactive bands, p50 and p55, that migrated at the same positions as in the other cells were now seen, and the intermediate band was no longer detected. Reprobing the Western blots with a different p50 antibody raised against a 15-aa peptide yielded an identical pattern, suggesting that this other form was either a novel processing product of p105 or a very closely related protein (Fig. 2C). We could also specifically prevent this antibody from recognizing both forms if the immunogenic peptide was used to pretreat the antibody (Fig. 2C). Moreover, antibodies against p65, c-Rel (Fig. 2E), p52 (p50B) (Fig. 2D) (3, 27), and RelB (not shown) (26) do not cross-react against this 55 kDa form, strengthening our argu-

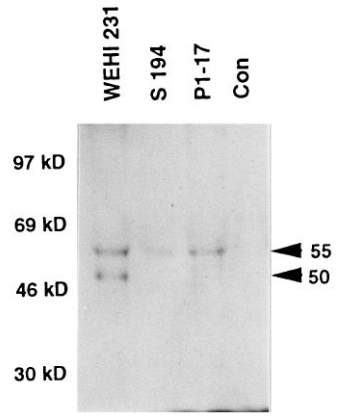


FIG. 3. Identification of p50 and p55 polypeptides bound to the κB-DNA sequence in complexes formed with nuclear extracts from WEHI 231, S194, and P1-17 cells. Approximately 95 μg of WEHI 231, 50 μg of S194, and 100 μg of P1-17 nuclear extracts were incubated with 0.5 μg of unlabeled κB probe mixed with 20,000 cpm of the regular labeled probe of the same oligonucleotide. The electrophoretic mobility shift assay gel was then exposed to film to identify the retarded complexes, and the bands were excised. The proteins were eluted in a SDS-containing buffer, precipitated with acetone to concentrate them, run on an SDS-gel, and analyzed by Western blotting with the α-p50-1 antibody.

ment that they are not nonspecific cross-reacting proteins. The pattern of expression of p52 in these B-cell lines, however, was quite interesting (Fig. 2D). It was present in both the nucleus and the cytoplasm of uninduced pre-B cells and mature B cells but was present only in the cytoplasm of plasma cells (Fig. 2D). Because the pre-B-cell nuclear extract did not exhibit any κB-DNA binding activity, the p52 in the pre-B-cell nucleus was probably bound to an inhibitor. The nuclear form of p52 in LPS-induced PD31 cells or WEHI 231 cells was also not a major component of the κB complexes, since p52 antibodies did not supershift these complexes (data not shown).

p55 from plasmacytoma nuclear extracts binds to κB sites. To determine whether the p55 form actually bound to the κB sequence, we carried out the following experiment. We ran an electrophoretic mobility shift assay gel with large amounts of nuclear extracts from WEHI 231, S194, and P1-17 plasma cells. The characteristic retarded complexes were excised, and the proteins were eluted and analyzed by immunoblotting with an anti-p50 antibody. The results clearly showed that while the WEHI 231 complex had both the p55 and p50 proteins, the plasma cell complexes had only the p55 form (Fig. 3). If the protein was run without the probe and the same region of the gel was excised, no p50 or p55 bands could be detected. Therefore, the p55 form is the protein that is bound to the κB sequence in plasma cells and is capable of activating transcription from reporters driven by κB sites (see Fig. 7).

The identification of the p50 and p55 forms raises the question of how they are produced. One possibility is that they are generated through differential proteolysis of the p105 protein. Alternatively, the p55 form could arise through posttranslational modification of the p50 form. To test these possibilities, we transfected a mouse p105 cDNA tagged at its N terminus with the 7-aa epitope from influenza virus (flu epitope) into WEHI 231 cells. Upon analyzing the extracts from the transfected cells with an anti-flu epitope monoclonal antibody, we observed only a single flu epitope-tagged p50-like protein (Fig. 4). Although it is possible that generation of p55 involves N-terminal processing resulting in the removal of the flu epitope tag, we feel it is more likely that p55 is the product of

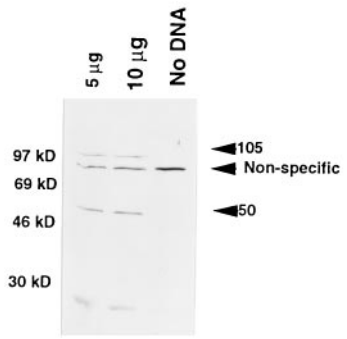


FIG. 4. Transfection of flu epitope-tagged p105 into WEHI 231 cells. The flu epitope was fused to the mouse p105 coding sequence by using PCR. The flu-tagged cDNA was then transfected into WEHI 231 cells by the DEAE-dextran procedure (see Materials and Methods). The cells were harvested after 48 h and lysed, and Western analysis was performed. Antibody bound to target proteins was detected by ECL. Con, control.

a different but related gene and that similarities in their sequences result in antigenic cross-reactivity.

p55 is present in mice lacking the p105/p50 gene. To further establish that p55 was the product of a distinct but related gene, we analyzed extracts from splenocytes of mice lacking the p105/p50 gene and compared them to extracts from wild-type littermates. For comparative purposes, we also analyzed extracts from WEHI 231 and P1-17 cell lines. The results indicated clearly that p55 could be detected in all samples, but p50 was absent in the extracts from $p50^{-/-}$ mice and from the P1-17 cells (Fig. 5). The detection of the p55 protein in the extracts from splenic cells of $p50^{-/-}$ mice therefore suggests that p55 is the product of a gene distinct from but related to the p50 gene.

The p50 and p55 forms exist in distinct cytoplasmic pools. The identification of p55 raised the question of whether p55 and p50 exist in distinct pools in the cytoplasm of pre-B cells. To address this question, we fractionated the cytoplasmic extracts of cells from different developmental stages by using anion-exchange chromatography on a Mono Q fast protein liquid chromatography (FPLC) column. We had previously seen that under the mild conditions of anion-exchange chromatography, the different Rel protein complexes (with or without IκB proteins) were not disrupted and hence allowed for the analysis of complexes that exist in the cytosol. Cytosolic extracts from PD31, WEHI 231, and S194 cells were therefore chromatographed separately under identical conditions (Fig.

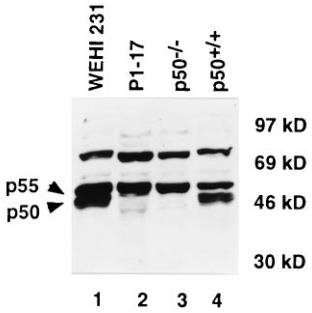


FIG. 5. p55 can be detected in mice lacking the p105/p50 gene. Splenocytes from wild-type and knockout mice were isolated, and extracts were prepared. These extracts, along with extracts from WEHI 231 and P1-17 cells, were fractionated by SDS-PAGE and analyzed by immunoblotting with the p50 antibody. The immunoblots were then visualized by ECL.

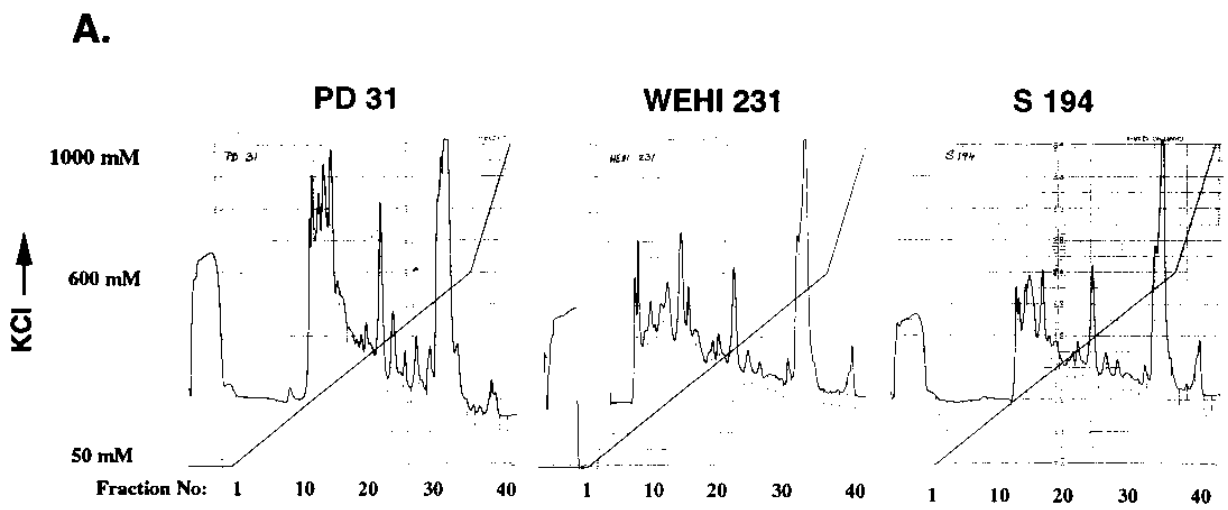


FIG. 6. Fractionation of cytoplasmic extracts of PD31, WEHI 231, and S194 cells. (A) A_{280} profiles of the fractions obtained by chromatographing 15 mg of cytoplasmic extracts from PD31, WEHI 231, and S194 cells on a 1-ml Mono Q FPLC column. The proteins bound to the column were eluted with a 35-ml gradient from 50 to 600 mM KCl, and 35 1-ml fractions were collected. (B) Western analysis of Mono Q fractions 8 to 31 with antibodies to p50 (α-p50-2), c-Rel, (α-cRel-N), p65 (α-p65-3), IκBα, and IκBγ. Thirty microliters of each fraction was run on an SDS-gel, blotted, and analyzed with different antibodies as described in Materials and Methods. The bound antibodies were detected by ECL (Amersham). The fractions were analyzed on three different gels (Hoefer minigels), and all blots for one antibody were processed together and exposed on one film to ensure that the levels of detected proteins in different fractions of the different cell types were comparable. No c-Rel protein was detected in S194 fractions, and therefore the results are not shown.

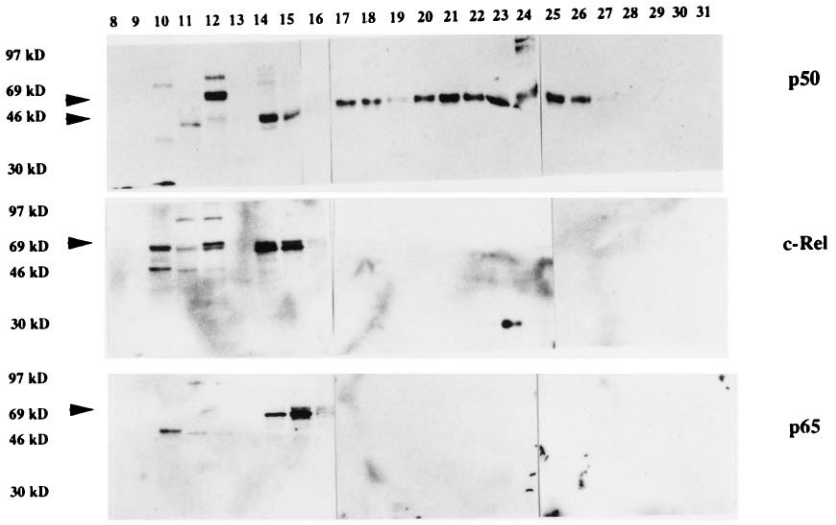
6A). The fractions obtained from these runs were then assayed by immunoblot analysis with specific antibodies for the presence of the different proteins (Fig. 6B). Using such an approach, we found that fractions containing p50 also contained p65 and c-Rel for PD31 and WEHI 231 cells. In S194 cells, which do not have any c-Rel protein, p50-containing fractions had only p65. The additional immunoreactive protein of ~55 kDa (p55) eluted over the later portion of the gradient away from p50, p65, or c-Rel. It is possible that the p55 form detected in the cytoplasm was associated with an IκB-like molecule. However, fractions containing p55 do not contain significant amounts of either IκBα or IκBβ (data not shown). We have also been unable to recover κB-DNA binding activity from fractions containing p55 (fractions 17 to 24), using the simple deoxycholate–NP-40 treatment (data not shown). However, carrying out a denaturation-renaturation protocol on the protein in these fractions reveals weak κB sequence binding activity at ~55 kDa (data not shown), suggesting that cytosolic p55 might be present in complexes containing novel IκB-like proteins. Of the different IκB forms described, one candidate, IκBγ, might be specific for p55 because it has a greater affinity for p50 than other Rel family proteins. We therefore used an antibody raised against IκBγ protein to test the Mono Q fractions and found that the 70-kDa IκBγ protein eluted in fractions 22 to 24 (Fig. 6B), fractions that contain p55 but not IκBα or IκBβ (not shown). Therefore, it is possible that p55 in the cytoplasm is in a complex with IκBγ. It is interesting that on Northern (RNA) analysis, the level of IκBγ message is sharply reduced in plasma cells in relation to earlier developmental stages, and hence the regulation of nuclear translocation of p55 may in part be due to reduced synthesis of IκBγ (Fig. 1A) (8, 10).

Transcriptional activation of the intronic enhancer. To determine the ability of p55 to regulate transcription from a reporter driven by the κ-intronic enhancer, transient transfections were done in S107 plasma cells, which do not have any nuclear κB-DNA binding complexes (Fig. 7). The reporter construct contained two κB sites cloned upstream of a luciferase gene. There was minimal expression from this reporter in S107 cells and moderate expression in both WEHI 231 cells (presumably due to nuclear p50:c-Rel) and S194 cells (presumably through p55:p55). Cotransfection of p50 into the S107 cells did not increase transcription much beyond the basal levels, while transfection of either p65 and c-Rel increased transcription moderately. Therefore, these results suggested that unlike p50, endogenous p55 is capable of directing transcription from κB sites. However, the possibility that p55 associates with as yet uncharacterized partners in forming the transcriptionally active complex is yet to be ruled out.

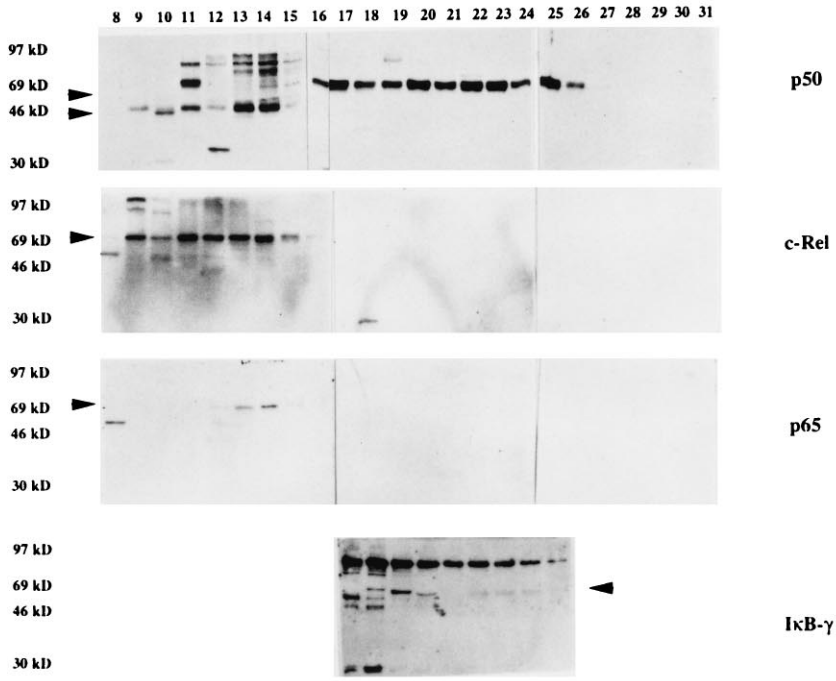
DISCUSSION

The major finding in this report is the identification of a novel 55-kDa protein, antigenically related to NF-κB p50, that is detected in the κB-DNA binding complexes from the nuclei of mature B and plasma cells. The identification of the p55 homodimers in plasma cells (where they can activate transcription) may help to explain the enigma of the transcription factor KBF-1. KBF-1 was characterized as a transcription factor that is partly responsible for the expression of major histocompatibility complex class I genes from an *H-2* site (11, 13). The cloning of its gene based on peptide sequences from an *H-2*-binding protein in HeLa cells resulted in the isolation of the NF-κB p105 gene (12). But surprisingly, this p50 KBF-1 has been repeatedly shown to be unable to activate reporter genes in cells from the *H-2* site in vivo, and subsequent studies have demonstrated a dominant inhibitory effect of p50 on NF-κB-mediated transcription (28). However, if, as our results suggest, there is an alternate form of p50 that contains different and/or additional sequences and is capable of activating transcription as a homodimer (as seen in plasma cells), then it is possible that KBF-1 is actually the p55 form and not the p50 form. Although we were initially surprised that two forms of p50 had not been reported earlier, an examination of the literature revealed that in two earlier reports, UV cross-linking of κB-DNA binding complexes in activated T cells revealed four complexed proteins, p75 (p65), p85 (c-Rel), and two p50-like proteins, p55 and p50 (the two proteins were shown to be

B **PD 31**



WEHI 231



S 194

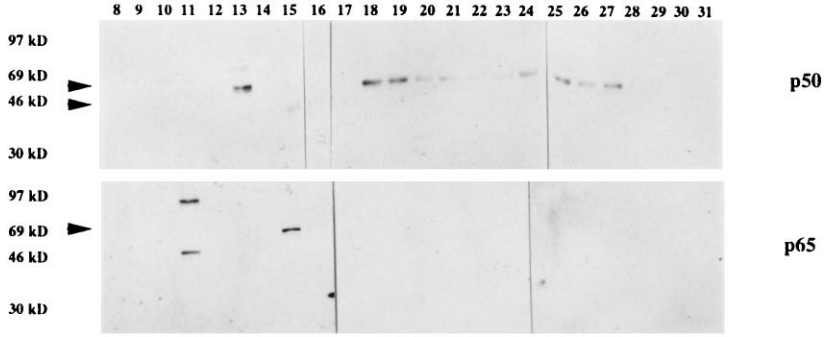


FIG. 6—*Continued.*

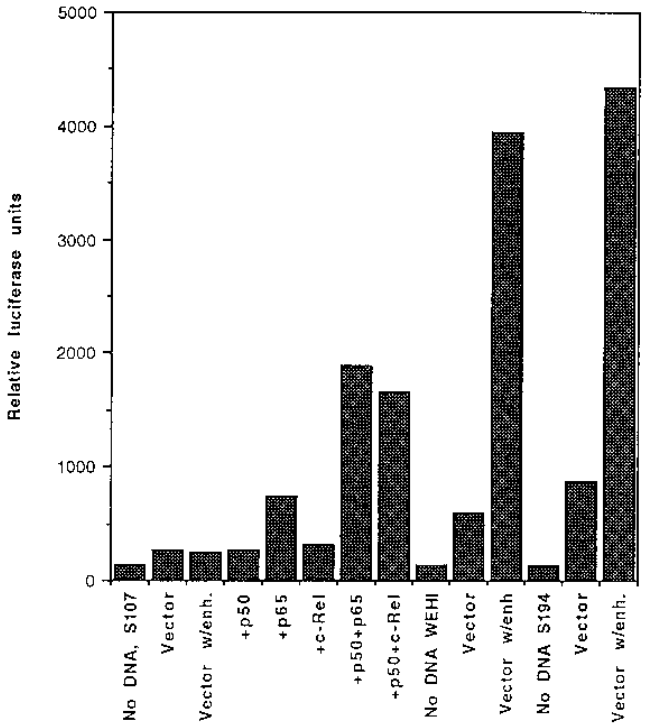


FIG. 7. Transcriptional competence of p50:p65 and p50:c-Rel. Transcription was measured by determining the activity of the luciferase enzyme produced by the reporter constructs, using the standard assay conditions recommended by Promega. The emitted light was measured with a luminometer. The p50, c-Rel, and p65 cDNAs were cloned in pRC/CMV vectors, and equal amounts (~5 μg) were transfected into cells by the DEAE-dextran method (see Materials and Methods). Following transfection, cells were harvested after 48 h and the extracts were assayed for luciferase activity. enh, enhancer.

related by peptide mapping experiments) (24). It is possible that these two p50 forms in T cells are the same forms that we observe in B cells. Also, in another study of WEHI 231 cells, the published immunoblots indicate a p55-like protein very similar to the one described in this report (22).

The question naturally arises about the nature of p55. We initially thought that p55 was an alternatively spliced form of p50 that was expressed in a tissue-specific manner. In particular, the recent identification of two alternatively spliced variants of the murine NF-κB p105 gene, p98 and p84, raised the possibility that they serve as precursors of the p55 isoform (9). Alternatively, p55 could be a posttranslationally modified form of p50, although our failure to observe two p50-like products upon transfecting an epitope-tagged p105 into mature B cells made this possibility less likely (Fig. 4). Finally, p55 could be the product of a distinct but p50-related gene. The definitive answer to this question can come only from the cloning and sequencing of the cDNA encoding this protein. However the presence of p55 in p105/p50 knockout animals strongly suggests that p55 is in fact the product of a distinct gene. In any case, the existence of such a p50-like protein may help to explain many instances where p50 dimers were implicated in the expression of different genes. Also, if p55 is the product of a different gene, one that can bind to κB sites to activate transcription, then gene disruption studies on individual Rel family members would give incomplete answers.

In the case of B-cell lines, our results allow us to propose the following outline for the regulation of Rel complexes. In a mature B-cell line (and presumably in pre-B cells from the bone marrow), NF-κB is activated, probably because a developmental signal targets the NF-κB:IκB complex. The active NF-κB (mostly p50:p65) leads to the transcriptional up-regulation of p50 and c-Rel, thereby gradually switching the composition of the complex to p50:c-Rel (20, 23). Subsequently, in plasma cells, as a result of a mechanism yet to be understood, the majority of the c-Rel protein disappears although the levels of c-Rel mRNA are relatively unaltered. Instead, the κB-DNA binding complex that is detected in the nuclei of these cells is composed of only p55. The transcriptional shutoff of IκBγ in plasma cells might explain the appearance of p55 in the nuclei of these cells, since chromatographic experiments on the cytosol from earlier developmental stages suggest that p55 and IκBγ coelute. These nuclear p55 homodimers appear to be capable of driving expression from immunoglobulin κB sites but may also be used for the expression of other targets such as major histocompatibility complex class I genes, which are known to depend on activation from the *H-2* site.

The presence of an NF-κB p50-related protein in B lymphocytes that can bind to κB sites and direct transcription might also help explain the relatively mild developmental phenotype of mice lacking certain Rel subunits (4, 15, 31, 34). Published results for lymphocytes of different knockout mice indicate that low levels of κB-specific complexes can be detected in the nuclear extracts, and it is therefore possible this complex contains p55. Also, the description of p55, along with other studies that have reported the existence of multiple splice variants of the NF-κB p105 gene (9), points out a level of complexity that has not been appreciated previously. We are also particularly struck by the relative abundance of the p55 form in certain cell lines, suggesting that in these cells the p50 and p55 forms might compete for binding to DNA. Although the presence of p55 raises a number of questions, definitive answers to these questions will come only after the cloning and characterization of the gene encoding p55.

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