Enhanced IκBα Degradation Is Responsible for Constitutive NF-κB Activity in Mature Murine B-Cell Lines

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Nuclear factor κB (NF-κB) is a ubiquitous transcription factor which binds to decameric DNA sequences (κB sites) and regulates transcription of multiple genes. The activity of NF-κB is regulated by an inhibitor protein, IκB, which sequesters NF-κB in the cytoplasm. Release of IκB and subsequent nuclear translocation of NF-κB generally require activating signals. However, in mature murine B cells, the DNA-binding activity of NF-κB is constitutively nuclear and activates the Igk gene, a marker for mature B cells. To understand the basis for the constitutive NF-κB activation, we examined the properties of NF-κB and IκB in both pre-B and mature B cells, the regulated and constitutive states, respectively. We found that expression of IκBα and p105, members of the IκB family, and Rel, a member of the NF-κB family, is augmented in mature B cells. Both IκBα and p105 are associated with NF-κB proteins and sequester most of these proteins in the cytoplasm of mature B cells. However, rapid IκBα dissociation and degradation lead to continuous nuclear translocation of a small fraction of NF-κB proteins, which represent the constitutively active NF-κB in mature B cells. We estimate that the protease activity is at least 35-fold greater in mature B cells than in pre-B cells. Rapid degradation of IκBα is directly involved in constitutive NF-κB activation, because stabilization of IκBα by a protease inhibitor causes loss of NF-κB activity in mature B cells. These results provide evidence that continuous and rapid degradation of IκBα in the absence of external stimuli is causally involved in the constitutive activation of NF-κB in mature murine B cells.

Nuclear factor κB (NF-κB) was originally identified as a tissue-specific transcription factor which is involved in the activation of immunoglobulin κ light-chain gene transcription in mature B cells (39). Subsequent studies, however, revealed that NF-κB activity is quite ubiquitous (for reviews, see references 1 and 15). NF-κB is a dimeric protein complex whose subunits may be any members of the following related family of proteins: p50 (NFκB1), p52 (NFκB2), p65 (RelA), v-Rel, Rel, RelB, or Rel/ΔΔ (p52Δ) (4, 6, 14, 22, 26, 30, 32, 36, 37). This family also includes a Drosophila morphogen, Dorsal, required for the establishment of dorsal-ventral polarity of the organism (40). These proteins share extensive sequence homology at their ~300-amino-acid N-terminal region, which contains both the dimerization and DNA-binding domains (14a). NF-κB proteins regulate transcription of a wide variety of target genes, including genes that encode immunomodulators, cytokines, lymphokines, viral proteins, immunoglobulin, and neuronal proteins through the κB sites located in their promoter and/or enhancer elements (for reviews, see references 1 and 15). Additionally, NF-κB autoregulates its subunit proteins (p50-p105 and Rel), as well as its inhibitor, IκBα (8, 11, 17, 42).

The activity of NF-κB is regulated at the level of subcellular localization. Typically, NF-κB is sequestered in the cytoplasm by an inhibitor protein, IκBα (2, 3). IκBα also belongs to a family of related proteins which includes IκBα, IκBγ, IκBε, IκB-3, p105, and p100 (10, 13, 18, 21, 24, 25, 28, 29, 31, 33, 35, 43, 44). All IκB proteins contain characteristic ankyrin repeat motifs which are involved in association with NF-κB proteins (19, 23, 27). A major IκBα in most cell types appears to be IκBα, as its rapid degradation following various stimuli is directly related to the activation of inactive cytoplasmic NF-κB complexes (7, 20, 38, 41).

In contrast to that of most cell types, the DNA-binding activity of NF-κB is constitutively nuclear in mature murine B cells (39). However, the molecular basis for the constitutive NF-κB activation remains obscure. The present study attempted to identify an event directly involved in constitutive NF-κB activation in mature B cells by comparing it to the regulated system of pre-B cells. We demonstrated that in mature B cells (i) IκBα and p105, two of the IκBα proteins, and Rel, an NF-κB subunit protein, are overexpressed; (ii) IκBα and p105 are associated with NF-κB subunit proteins; (iii) most of the NF-κB proteins are indeed sequestered in the cytoplasm by IκBα proteins; (iv) only a small pool of NF-κB appears in the nucleus; (v) NF-κB-IκBα interaction is unstable, whereas NF-κB-p105 interaction is not affected; (vi) the half-life of IκBα is much shorter than that of pre-B cells, whereas that of Rel is the same; and (vii) inhibition of IκBα degradation inhibits constitutive NF-κB activity. These results support a model in which rapid and continuous degradation of IκBα leads to release of a small pool of NF-κB into the nucleus, resulting in constitutive activation of NF-κB in mature B cells.

MATERIALS AND METHODS

Cell culture. Murine pre-B-cell lines (702Z/3, AKA1.G1, RAW253, NFS25.C3, and ABE8L2) and B-cell lines (WEHI231, WEHI279, CH33, CH31, and CH1) were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 50 μM β-mercaptoethanol, 1,250 U of penicillin G, and 0.5 mg of streptomycin sulfate per ml. Pre-B-cell lines NFS25.C3 and ABE8L2 and B-cell lines WEHI279 and CH1 were purchased from the American Type Culture Collection. AKA1.G1, RAW253, CH33, and CH31 were a generous gift from R. Hymen. Tosylsulfonl-phenylala-
nly chloromethyl ketone (TPCK) was purchased from Sigma. Treatment of WEHI231 and 70Z/3 cells with TPCK at 25 μM for 24 h had very little effect on their survivability.

**Northern (RNA) blot analysis.** RNA was isolated from each cell line as previously described (9). Five micrograms of total RNA was electrophoresed in a 1% formaldehyde agarose gel and transferred to Hybond-N (Amersham). The RNA was fixed on the membrane by UV irradiation, and the membrane was prehybridized at 65°C in 7% sodium dodecyl sulfate (SDS)-0.2 M sodium phosphate (pH 7.2)-1% bovine serum albumin-1 mM EDTA. Hybridization was carried out in the same buffer at 65°C. The probes were murine IκBa (1.2-kb EcoRI fragment), murine NFκB1 (0.9-kb HindIII fragment), murine Rel (1.8-kb BamHI-EcoRI fragment), the murine κ light chain (0.5-kb BglII fragment of pKConSt (a generous gift from R. Grosschedl), and murine glyceraldehyde-3-phosphate dehydrogenase (280-bp HindIII-PstI fragment of pMGAP).

**Electrophoretic mobility shift assays.** Cell extracts were prepared as described by Dignam et al. (12). Electrophoretic mobility shift assays were performed as described previously (22). Briefly, 5 μg of either a cytoplasmic or a nuclear extract was incubated with 1 μg of poly(dl-dC) (Pharmacia) for 20 min on ice, and a 32P-labeled double-stranded oligonucleotide containing the κB site from the immunoglobulin κ light-chain gene (5'-TCAACAGAGGGGACTTCCGAGAGCC-3') was added. Binding of the probe was carried out for 20 min at room temperature. The resulting complexes were resolved by 4% non-denaturing polyacrylamide gels. The mutant κB competitor was a double-stranded oligonucleotide, 5'-TCAACA GAGCTCACATTAGAGAGCC-3'. The AP-1 oligonucleotide was 5'-GCGCTGCTACACCAGCCCTGC-3' (the underlined sequence is the binding site). A mutation, TCACTCA, was introduced in the AP-1-binding site for the mutant AP-1 oligonucleotide. The bacterial glutathione S-transferase (GST)-IκB proteins used were described previously (21).

**Metabolic labeling and immunoprecipitation.** Cells (10^7) were grown for 1 h in 1 ml of methionine-cysteine-free Dulbecco modified Eagle medium supplemented with 10% dialyzed fetal calf serum, 50 μM β-mercaptoethanol, nonessential amino acids (Gibco), and 2 mM glutamine. [35S]methionine-cysteine was then added at 1 μCi/ml, and the mixture was further incubated for appropriate periods. Cells were then washed twice with phosphate-buffered saline and lysed in IP buffer (20 mM Tris HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, 0.1% Triton X-100). The specific proteins were immunoprecipitated with affinity-purified antisera and protein A-Sepharose (Pharmacia). Antiserum to Rel was as previously described (22). Antiserum to p50 was raised against a 22-mer peptide (SDLAYLQCAGGDGRQDTREKE, amino acids 171 to 193 of murine p50 [14]). Anti-p65 serum was raised against the N-terminal 19-amino-acid peptide (MDDL FPLIPSPESAPASGP) of murine p65 (32). IκBa antisemur was raised against the GST-fused N-terminal region of the murine IκBa protein. The subcellular fractionation procedure used was that of Dignam et al. (12), with some modifications. Following labeling of the cells, cytoplasmic extracts were prepared by gently pipetng the cells in hypotonic buffer (12) and pelleting the nuclei. The nuclear pellets were rinsed once with hypotonic buffer (the supernatants were added to the cytoplasmic fraction) and dissolved in IP buffer containing 0.5% SDS and disrupted by 30 gentle strokes with a 21-gauge needle. Both the cytoplasmic (NaCl and SDS were added to final concentrations of 100 mM and 0.5%, respectively) and the nuclear fractions were boiled for 5 min and diluted fivefold with IP buffer without SDS.

**RESULTS**

*Expression of IκBa, p105, and Rel is augmented in mature B cells.* One possible explanation for constitutive NF-κB activity is that transcription of IκB in mature B cells is inhibited. To test this notion, RNAs obtained from pre-B-cell and mature B-cell lines were analyzed by Northern blot analysis. Surprisingly, IκBa was overexpressed 12-fold in mature B cells (Fig. 1A, lanes 6 to 10), as measured by densitometry, compared with pre-B cells (lanes 1 to 5). The expression level of another IκB family member, p105, the precursor of p50, was also enhanced 2.2-fold in mature B cells (Fig. 1A). The level of Rel mRNA was also increased 4.4-fold, in agreement with previous observations (16). As can be seen in the lower panel, expression of the κ light-chain gene was observed in all of the B-cell lines (lanes 6 to 10) but not in pre-B-cell lines (lanes 1 to 5). These data tentatively rule out inhibition of IκB transcription as a possible explanation for constitutive NF-κB activation in mature B cells. Instead, genes encoding two of the IκB proteins, IκBa and p105, are increased in mature B cells. Since the promoters of the genes encoding proteins IκBa, p105, and Rel contain κB sites (8, 11, 17, 42), it is perhaps not surprising to see enhanced expression of these genes in cells in which NF-κB activity is constitutive.

**NF-κB in WEHI231 cells is sensitive to inhibition by IκB.** An alternate possible mechanism for constitutive NF-κB activation is its insensitivity to inhibition by IκB proteins. If NF-κB complexes are insensitive to inhibition by IκB, overproduction of IκB seen in mature B cells would not inhibit NF-κB activity. To determine whether NF-κB activity is still sensitive to inhibition by IκB, bacterially expressed murine IκBa and IκBy, as GST fusion proteins (21), were added to nuclear extracts

**FIG. 1.** (A) Northern blot analysis of IκBa, p105, and Rel in pre-B-cell and mature B-cell lines. Total RNAs (5 μg of each) isolated from the various cells were analyzed as described in Materials and Methods. Lanes: 1 to 5, murine pre-B-cell lines; 6 to 10, mature murine B-cell lines. Blots were sequentially hybridized, exposed, stripped, and rehybridized with murine IκBa, p105, Rel, Iκγ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. (B) Inhibition of DNA-binding activity of constitutive NF-κB complexes in WEHI231 cells by bacterially expressed IκB proteins. Nuclear extracts isolated from WEHI231 cells were incubated with 200 ng each of GST-IκBa (lane 2), GST-IκBy (lane 3), or GST (lane 4) and analyzed by electrophoretic mobility shift assay. The band at the bottom of the gel is the free probe. There are two major NF-κB complexes in WEHI231 cells, the upper p50-Rel heterodimer and the lower p50 homodimer (29, 35).
prepared from WEHI231 cells and analyzed by gel shift assays. As shown in Fig. 1B (lane 1), two major NF-κB complexes were observed in mature B cells, a more slowly migrating p50-Rel heterodimer and a faster-migrating p50 homodimer (29a). When fusion protein GST-IκBa was added, the DNA-binding activity of the upper p50-Rel heterodimer was primarily inhibited (lane 2). The faster-migrating p50 homodimer was affected to a much lesser extent (lane 2). GST-IκBγ inhibited both of the complexes (lane 3). GST alone had no inhibitory effect (lane 4). These results are in agreement with previously published observations which showed that IκBγ is able to inhibit the DNA-binding activity of p50 homodimers, whereas IκBa has little effect (21, 25). Partially purified IκBa proteins from 70Z/3 cellular extracts have also been shown to inhibit constitutive NF-κB activity (3). Since exogenously added IκBa effectively inhibited NF-κB DNA-binding activity, insensitivity of NF-κB to inhibition by IκB is unlikely to account for its constitutive activity in mature B cells, as suggested previously.

**IκBa associates with NF-κB proteins in WEHI231 cells.** In the WEHI231 mature B-cell line, IκB is overexpressed (Fig. 1A), NF-κB complexes are sensitive to inhibition by exogenously added IκBa (Fig. 1B), and yet NF-κB is constitutively active in the nucleus. If IκB is posttranslationally modified such that it cannot associate with NF-κB subunits, then NF-κB would be constitutively active despite IκB overproduction. To test this notion directly, coimmunoprecipitation assays were performed with an affinity-purified antisera specific to Rel protein, a major subunit of the NF-κB complex in mature B cells (see below). As shown in Fig. 2, Rel is associated with multiple proteins (lane 1). The specificity of immunoprecipitation was determined by competition with excess Rel epitope peptides (compare lanes 1 and 2). To determine whether IκB proteins are present in the Rel complex, the Rel-associated proteins were released by boiling in SDS and further immunoprecipitated with antisera specific to IκBa and p105. As demonstrated in lanes 3 and 4, p105 and IκBa, respectively, are directly associated with Rel. p65, another member of the NF-κB family, is also associated with p105 (lane 7) and IκBa (lane 8), although the amount of p105 associated with p65 is minimal. Competition with the epitope peptide shows the specificity of immunoprecipitation (compare lanes 5 and 6). The upper band, which coimmunoprecipitated with p65, was the Rel protein, as demonstrated by secondary immunoprecipitation (data not shown). The antisera to p65 did not cross-react with Rel (see Fig. 3). These results demonstrate that whatever posttranslational modification IκBa or p105 may have undergone, it can still associate with NF-κB proteins in mature B cells.

**Approximately 10% of NF-κB proteins are nuclear in WEHI231 cells.** In mature B cells, expression of IκBa, p105, and Rel is increased; the NF-κB and IκBa proteins are associated; and yet NF-κB is constitutively active. A possible explanation for these observations is that NF-κB proteins are not sequestered in the cytoplasm, even though they are associated with IκBa proteins (Fig. 2). It has been shown that antisera to chicken IκBa (pp40) affects the mobility of the constitutive NF-κB complexes in WEHI231 cells (25), suggesting that the NF-κB-IκBa complex is nuclear and capable of DNA binding. To measure the amounts of the NF-κB and IκBa proteins in the nuclei versus the cytoplasm of mature B cells, WEHI231 cells were metabolically labeled and fractionated into nuclear and cytoplasmic pools and immunoprecipitated with antisera specific for each of the NF-κB and IκBa proteins. Surprisingly, only ~10% of the total Rel and p50 proteins was detected in the nucleus (Fig. 3, lanes 7 to 12). Most of the NF-κB proteins remained sequestered in the cytoplasm (Fig. 3, lanes 1 to 6), in association with either IκBa or p105 protein (Fig. 2), which were only cytoplasmic (compare lanes 13 to 16 and 17 to 20). The specificity of immunoprecipitation was shown by competition with corresponding excess epitope peptides (lanes 2, 4, 6, 8, 10, 12, and 14) or bacterially expressed IκBa protein (lanes 16 and 20). It is worth noting that although only a fraction of the p50 and Rel proteins is present in the nucleus, they represent all of the DNA-binding activity in WEHI231 cells (29a). Thus, IκB proteins (IκBa, p105, and perhaps other minor IκB proteins) sequester ~90% of NF-κB proteins in the cytoplasm, demonstrating that IκB proteins are functional in inhibiting NF-κB and that NF-κB-IκBa complexes are indeed cytoplasmic in mature B cells.

**Rapid dissociation and degradation of IκBa in WEHI231 cells.** The data so far demonstrate that IκB proteins are functional and sequester most of the NF-κB proteins in mature B cells. Only ~10% of NF-κB proteins escape inhibition by IκB and translocate to the nucleus. What is the molecular basis for nuclear translocation of small amounts of NF-κB proteins? One possible explanation is unstable interactions between NF-κB and IκBa proteins. If the interaction between NF-κB and IκBa proteins is unstable, then there would be some free NF-κB which is not associated with IκBa proteins and translocates into the nucleus before IκBa can block it. To test the stability of NF-κB and IκBa interactions in WEHI231 cells, the
disassociation of Rel-associated IkB proteins synthesized during a pulse period with [35S]Met-Cys was measured by chasing the proteins for various time periods with cold media and compared to that in 70Z/3 cells. In pre-B-cell line 70Z/3, more than 50% of IkB was still bound to Rel during the 5.5-h chase period (Fig. 4A, lanes 1 to 3, and 4B). In contrast, in mature B cells nearly all of the IkB was dissociated from Rel protein during the 5.5-h chase period (Fig. 4A, lanes 4 to 7, and 4B). In comparison, the stability of Rel and Rel-associated p105 was not drastically affected between these cell types. Similar results were obtained with p65-associated IkB proteins (data not shown), demonstrating that the subunit composition of NF-кB does not affect the stability of IkBα association. The data in Fig. 4A and B demonstrate that even though IkBα is initially bound to Rel, it is quickly lost from Rel protein complexes only in mature B cells. Thus, the interaction between IkBα and Rel is indeed much more unstable in WEHI231 than in 70Z/3 cells.

To examine the stability of IkBα in mature B cells further, its half-life was directly measured in 70Z/3 and WEHI231 cells. Figure 4C and D shows that the half-life of IkBα in 70Z/3 cells is 1-145 min (Fig. 4C, lanes 1 to 5, and 4D, dotted line), consistent with the observation of Henkel et al. (20), whereas that in WEHI231 cells is 3-40 min (Fig. 4C, lanes 6 to 10, and 4D, solid line). Thus, IkBα degrades 3.5-fold more rapidly in mature B cells than in pre-B cells. Although the overall difference between the half-lives of IkBα in 70Z/3 and WEHI231 cells is only 3.5-fold, the activity of the protease(s) involved must be increased at least 35-fold, because the amount of IkBα synthesized in WEHI231 cells is at least 10-fold greater than that in 70Z/3 cells (Fig. 1A and 4C). Thus, in mature B cells, IkBα continuously and rapidly degrades in the absence of external signals because of an apparent increase in protease activity.

Inhibition of IkBα degradation causes loss of the constitutive NF-кB activity in a variety of mature B cell lines. The data so far point toward rapid degradation of IkBα as the primary cause of constitutive NF-кB activation in WEHI231 cells. Therefore, we attempted to inhibit its degradation by a serine protease inhibitor, TPCK, as described recently by Henkel et al. (20). The half-life of IkBα was determined by a pulse-chase experiment in the presence and absence of a nontoxic dose of TPCK (Fig. 5A). Addition of TPCK to WEHI231 cells increased the half-life of IkBα twofold (~35 versus ~65 min; Fig. 5A and B). This dose was sufficient to block lipopolysaccharide-induced IkBα degradation in 70Z/3 cells (8), as demonstrated previously (40). Since TPCK was added during a part of the pulse period and the entire chase period, the amount of IkBα at time 0 min (Fig. 5A, lane 6) was greater than that at time 0 min without TPCK (Fig. 5A, lane 1) because of the
accumulation during the pulse with TPCK (15 min). However, TPCK treatment had no effect on the level or the half-lives of the Rel and p65 proteins (data not shown).

If degradation of IκBa is directly involved in constitutive NF-κB activation, inhibition of IκBa degradation is expected to result in either loss or reduction of the constitutive NF-κB activity in these cells. To test this hypothesis directly, gel shift assays were performed with nuclear extracts isolated from WEHI231 cells treated with or without TPCK. As shown in Fig. 6A, TPCK caused loss of constitutive NF-κB activity (compare lanes 1 and 4). The binding activity of the p50-Rel heterodimer was inhibited more efficiently than that of the p50 homodimer. This was not due to direct inhibition by residual TPCK possibly left in the extracts, because addition of extracts isolated from TPCK-treated cells had no effect on the DNA-binding activity of the p50-Rel complex (data not shown). However, TPCK treatment had no effect on the binding activity of the AP-1 complex (compare lanes 6 and 9), demonstrating the specificity of the inhibition of the DNA-binding activity of NF-κB by TPCK. The specificity of the binding activities was shown by competition with excess wild-type (lanes 2 and 7) and mutant (lanes 3 and 8) oligonucleotide. Probes alone showed no binding complexes (lanes 5 and 10).

To determine if the inhibition of constitutive NF-κB activity by TPCK is a general phenomenon of mature B cells, a panel of murine B-cell lines was also tested. The nuclear extracts prepared from TPCK-treated mature B-cell lines showed loss of constitutive NF-κB activity (Fig. 6B, compare alternate lanes). The p50 homodimer was also significantly inhibited. This was most likely due to sequestration of p50 by overproduced IκBa, as previously shown by cotransfection assays (5). Although the effect of TPCK may not be limited to inhibition of the protease(s) involved in IκBa degradation, these observations support the notion that enhanced degradation of IκBa and constant translocation of a small pool of NF-κB proteins are directly involved in the constitutive NF-κB activation in mature murine B cells.

**FIG. 5.** (A) TPCK inhibits IκBa degradation in WEHI231 cells. WEHI231 cells were pulse-labeled with [35S]Met-Cys for 2 h. TPCK (25 μM) was added to half of the sample for 15 min during the pulse period. The samples were washed with phosphate-buffered saline and returned to growth media without (lanes 1 to 6) and with (lanes 7 to 12) 25 μM TPCK for the periods shown. IκBa was immunoprecipitated as described in the legend to Fig. 2. (B) Half-lives of IκBa in WEHI231 cells in the absence and presence of TPCK. The density of IκBa bands in panel A was measured and plotted against time. Molecular sizes are shown on the right in kilodaltons.

**FIG. 6.** (A) TPCK inhibits constitutive NF-κB DNA-binding activity in WEHI231 cells. Nuclear extracts were prepared from WEHI231 cells either exposed (lanes 4 and 8) or not exposed (lanes 1 to 3 and 6 to 8) to 25 μM TPCK for 30 min, and the DNA-binding activities of NF-κB (lanes 1 to 4) and AP-1 (lane 6 to 9) complexes were measured by electrophoretic mobility shift assay using the immunoglobulin κ light chain κB or AP-1 site. Lanes: 2 and 7, competition with a 50-fold excess of cold wild-type competitor oligonucleotides for the κB and AP-1 sites, respectively; 3 and 8, competition with a 50-fold excess of mutant oligonucleotides for the κB and AP-1 sites, respectively; 5 and 10, probes for the κB and AP-1 sites, respectively. Mu, mutant; WT, wild type. (B) TPCK inhibits NF-κB DNA-binding activity in various mature B-cell lines. Mature B-cell lines were treated with 25 μM TPCK for 30 min and analyzed as for panel A. Two major NF-κB complexes (p50-Rel and p50-p50) were found in each cell line without TPCK treatment (odd-numbered lanes). The identities of the cell lines are shown above the lanes.

**DISCUSSION**

We have demonstrated that constitutive activation of NF-κB activity in mature murine B-cell lines is regulated by IκBa. Inhibition of IκBa degradation leads to loss of constitutive NF-κB activity. We propose that in mature B cells, there is enhanced degradation of IκBa leading to constitutive NF-κB activation.

**Upregulation of IκBa in mature B cells—a paradox.** Since IκB is the primary regulator of NF-κB activity, intuitively, one would have expected it to be decreased in activity or level in mature B cells, thereby leading to constitutive NF-κB activation. On the contrary, transcription of the genes encoding IκBa and p105 was increased 12- and 2.2-fold, respectively, over that in pre-B cells (Fig. 1A). The level of Rel, a member of the NF-κB family, was also increased in mature B cells compared with pre-B cells (Fig. 1B). Upregulation of IκBa, p105, and Rel is probably due to the presence of κB sites in the promoters of the genes encoding these proteins (8, 11, 17, 42), whose activity is enhanced by constitutive NF-κB in mature B cells. Because the level of IκB transcription is increased in mature B cells, we tested four hypotheses which may account for the constitutive activation of NF-κB activity.

(i) **NF-κB DNA-binding activity is refractory to IκB inhibition.** Our rationale was that in B cells, the NF-κB proteins may have undergone a modification(s) which makes them refractory to inhibition by IκB proteins. Therefore, overproduction of IκB proteins is not detrimental to NF-κB activity. The data shown in Fig. 1A and previously published observations (3, 25), however, rule out this possibility because addition of exoge-
nous IκBα or IκBγ inhibits the DNA-binding activity of NF-κB proteins present in mature B cells.

(ii) IκBα is unable to associate with NF-κB proteins. Since NF-κB proteins form a complex in the cytoplasm with IκB proteins, it was formally possible that either NF-κB or IκB proteins have undergone modifications which do not allow their in vivo association. This could result in continuous translocation of NF-κB proteins to the nucleus. The data in Fig. 2 and recent studies by Rice and Ernst (34), however, show that in WEHI231 cells, IκBα and p105 are associated with NF-κB subunit proteins. Thus, in mature B cells, NF-κB is complexed with IκB proteins.

(iii) Nuclear translocation of NF-κB–IκB proteins. It was formally possible that the NF-κB–IκB complex is not sequestered in the cytoplasm in mature B cells, as suggested by the mobility shifts of the constitutive NF-κB complexes in WEHI231 cells with an antiseraum to chicken pp40-IκBα (25). We tested this possibility by immunoprecipitating NF-κB and IκB proteins from the nuclear and cytoplasmic fractions of WEHI231 cells. Our data show that almost all of the NF-κB proteins were indeed cytoplasmic in association with IκB proteins (Fig. 3). No detectable amount of IκBα proteins was seen in the nucleus. In addition, only ~10% of NF-κB was nuclear, and this represents the constitutive NF-κB activity in mature B cells. By using an affinity-purified antisera to murine IκBα, we did not detect any supershift of constitutive NF-κB complexes in WEHI231 nuclear extracts (data not shown). Thus, the NF-κB–IκB complex is sequestered in the cytoplasm and nuclear NF-κB is free of associated IκB proteins within the sensitivity of the assays utilized.

(iv) Unstable NF-κB and IκB interactions. Another possibility was that the association of NF-κB proteins with IκBα in mature B cells was unstable because of a rapid exchange with newly synthesized IκBα. Thus, a small amount of free NF-κB proteins is constitutively released in the cytoplasm and translocated to the nucleus before newly synthesized IκBα can block it. We tested this hypothesis by pulse-chase experiments followed by coimmunoprecipitation studies (Fig. 4). These studies demonstrated that IκBα is rapidly lost from the Rel complex in WEHI231 cells compared with 70Z/3 cells (Fig. 4A and B). Similar results were also obtained with p65 antiseraum (data not shown). Furthermore, this unstable interaction was associated with rapid degradation of IκBα proteins (Fig. 4C and D).

Because the amount of IκBα produced in WEHI231 cells was more than 10 times that synthesized in 70Z/3 cells (Fig. 1A and 4C), the 3.5-fold difference in the half-lives of these two cell types means that the apparent activity of protease is at least 35-fold higher in WEHI231 cells. The increase in protease activity in mature B cells may be due to an increase in the activity or level of the protease(s) itself. It is equally likely that IκBα is modified such that digestion by the protease is enhanced without directly affecting its level or activity. Enhanced proteolysis is, however, specific to IκBα because the half-lives of Rel or p105 were similar in the two cell types. These data strongly indicate that IκBα associated with Rel or p65 has a short half-life in mature B cells compared with that in pre-B cells and that this is due to the apparent increase in protease activity.

It is perhaps noteworthy that during induction of NF-κB in pre-B cells, IκBα is completely degraded and rapidly synthesized (8, 19, 38, 41). In contrast, in uninduced pre-B or mature B cells, there is a steady state between the rates of degradation and synthesis of IκBα proteins (34). The present study showed that both the synthesis and degradation of IκBα are greatly enhanced in mature B cells versus pre-B cells.

Stabilization of IκBα inhibits constitutive NF-κB activity in mature B-cell lines. The data on the half-life of IκBα proteins suggested that if the degradation of IκBα can be slowed or inhibited, there should be little or no constitutive NF-κB activity in mature B cells. This prediction was borne out because addition of TPCK, a protease inhibitor shown to inhibit NF-κB induction in 70Z/3 cells by inhibiting IκBα degradation (20), also inhibited the activity of NF-κB complexes in mature B cells (Fig. 6). This effect was specific because another transcription factor, AP-1 activity, was not affected.

Interestingly enough, the rates of degradation of IκBα differed only twofold between TPCK-treated and untreated WEHI231 cells (Fig. 5). How can a twofold difference in IκBα protein stability cause inhibition of all of the NF-κB activity? A possible solution was provided by the data in Fig. 3, which show that only about 10% of the NF-κB proteins translocated to the nucleus at steady state in mature B cells. Since the amount of IκBα present in unstimulated cells is enough to retain 90% of NF-κB proteins in the cytoplasm if the concentration of IκBα, a major IκB protein in mature B cells, is increased twofold, it is not surprising that the remaining 10% of NF-κB is completely inhibited.

Constitutive NF-κB activity seems to require constant translocation of NF-κB from the cytoplasm, because nuclear NF-κB activity can be inhibited in as little as 15 min if TPCK is added (Fig. 6). Alternatively, overproduced IκBα may enter the nucleus and inhibit the DNA-binding activity of the constitutive NF-κB complex (45).

The apparent activity of a protease(s) specific to IκBα is greatly enhanced in mature B cells. However, what causes this enhanced activity of IκBα proteolysis is not known. The underlying mechanism may involve either modification(s) of IκBα (such as phosphorylation) or activation of an IκBα-specific protease(s). Signals required for enhanced IκBα degradation in mature B cells, as well as during differentiation of pre-B to mature B cells, should be identified.

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