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Distinct Functional Properties of IκBα and IκBβ

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The biological activity of the transcription factor NF-κB is controlled mainly by the IκBα and IκBβ proteins, which restrict NF-κB to the cytoplasm and inhibit its DNA binding activity. Here, we carried out experiments to determine and compare the mechanisms by which IκBα and IκBβ inhibit NF-κB-dependent transcriptional activation. First, we found that in vivo IκBα is a stronger inhibitor of NF-κB than is IκBβ. This difference is directly correlated with their abilities to inhibit NF-κB binding to DNA in vitro and in vivo. Moreover, IκBα, but not IκBβ, can remove NF-κB from functional preinitiation complexes in in vitro transcription experiments. Second, we showed that both IκBs function in vivo not only in the cytoplasm but also in the nucleus, where they inhibit NF-κB binding to DNA. Third, the inhibitory activity of IκBβ, but not that of IκBα, is facilitated by phosphorylation of the C-terminal PEST sequence by casein kinase II and/or by the interaction of NF-κB with high-mobility group protein I (HMG I) on selected promoters. The unphosphorylated form of IκBβ forms stable ternary complexes with NF-κB on the DNA either in vitro or in vivo. These experiments suggest that IκBα works as a postinduction repressor of NF-κB independently of HMG I, whereas IκBβ functions preferentially in promoters regulated by the NF-κB/HMG I complexes.

Regulation of gene expression by members of the NF-κB/Rel family of transcription factors illustrates a complex mechanism for sensing the immediate cellular environment and responding to these perceptions in an appropriate fashion. The NF-κB/Rel signal transduction pathway operates at the center of a variety of different signaling events in the living cell (reviewed in references 3, 4, 6, 21, 25, 54, 60, and 63). For example, NF-κB is an essential regulator for the expression of numerous genes involved in the function and development of the immune system, recruitment of leukocytes from the circulation into extra vascular space, inflammatory and acute responses, and viral genes, etc. In mammals there are five Rel proteins, p50, p52, p65 (RelA), Rel (c-Rel), and RelB, all of which contain a so-called Rel homology region (RHR) that includes DNA binding and dimerization domains and a nuclear localization signal (NLS). Members of the Rel family form homo- or heterodimers (NF-κB is the p50/p65 heterodimer) which bind to a specific decameric DNA sequence called the κB motif (3, 4, 6, 21, 25, 54, 60, 63). Unlike most transcriptional activators, NF-κB resides in the cytoplasm and must translocate to the nucleus to function. The cellular partitioning of NF-κB is tightly regulated by the IκB proteins, which are complexed with NF-κB in the cytoplasm (2). Two different IκB proteins (IκBα and IκBβ), sharing a characteristic ankyrin repeat motif required for their interaction with the RHR, have been identified (26, 61). These interactions have two functional consequences. First, NF-κB/IκB complexes are sequestered in the cytoplasm, because the IκBs mask the NLS through direct protein-protein interactions, and second, IκBs can prevent NF-κB from binding to the DNA. After exposure of the cells to a large variety of stimuli, IκB proteins are degraded by the ubiquitin/proteasome pathway, and free NF-κB translocates to the nucleus, where it activates gene transcription (3–6, 9–11, 14, 16, 21, 29, 39, 51, 54, 60, 62, 63).

IκBα and IκBβ share common properties but also exhibit significant differences. For example, both IκBα and IκBβ interact with the same spectrum of Rel proteins, inhibit their DNA binding, and restrict their distribution to the cytoplasm. Degradation of IκBα occurs with all of the NF-κB inducers tested (reviewed in references 6, 21, 60, and 63), while IκBβ responds to only a subset (24, 43, 61). For example, treatment of cells with tumor necrosis factor alpha (TNF-α) causes a transient activation of NF-κB and a transient loss of IκBα with no effect on IκBβ (11). In contrast, induction with interleukin-1, lipopolysaccharide, or Tax I results in degradation of both IκBα and IκBβ, and the activity of NF-κB persists for several hours following stimulation despite the presence of newly synthesized IκBα.

An important component of the NF-κB system is the positive regulation of the IκBα gene by NF-κB. The rapid reappearance of IκBα following its destruction is the result of the activation of the IκBα gene by NF-κB (15, 17, 19, 30, 35, 53, 56). Moreover, this reaccumulation correlates with the inhibition of NF-κB activity in the nucleus, because IκBα can enter the nucleus (1, 47, 65). This idea is also supported by the observation that in cells derived from mice lacking IκBα, NF-κB activation is prolonged after TNF-α treatment (12, 32). However, the NF-κB activity persists for several hours after stimulation of cells with lipopolysaccharide or interleukin-1, which degrade both IκBα and IκBβ despite the resynthesis of IκBα (24, 43, 61). It appears that the newly synthesized and unphosphorylated IκBβ, although it associates with NF-κB, cannot mask the NLS, resulting in the nuclear translocation of the entire complex, which is refractory to IκBα inhibition (57).

The NF-κB transcriptional activity is subject to further levels of regulation after its release from cytoplasmic IκBs. The best-characterized example is virus induction of the human beta interferon (IFN-β) gene. In this case, the high-mobility group protein I(Y) [HMG I(Y)] binds cooperatively with NF-κB to the PRDII element (the IFN-β κB site) and stimulates its transcriptional activity (58–60). Thus, HMG I(Y) functions as a gene-specific coactivator of NF-κB.

In this study we have determined and compared the mechanisms by which IκBα and IκBβ function to inhibit NF-κB-dependent transcription. First, we showed that in cotransfection experiments IκBα is a stronger inhibitor of NF-κB than is IκBβ. Second, we established that both IκBs can function in

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the nucleus, where they directly inhibit binding of NF-κB to promoters in vivo. Third, the different inhibitory activities of IκBα and IκBβ in vivo correlate with their differential abilities to inhibit NF-κB DNA binding in vitro. We showed that IκBβ but not IκBα can be converted to a strong inhibitor of NF-κB DNA binding by in vitro phosphorylation of the PEST sequence by casein kinase II (CKII) or by the interaction of NF-κB with HMG I on selected promoter elements. Unphosphorylated IκBβ forms ternary complexes with NF-κB on the DNA which can be detected either in vivo or in vitro. Finally, we demonstrated that these properties of IκBα and IκBβ correlate with their ability to differentially inhibit NF-κB-dependent transcription in vitro.

MATERIALS AND METHODS

Plasmid constructions. Mammalian expression vectors encoding human IκBα and mouse IκBβ and IκBβΔC were constructed by subcloning the respective cDNAs in the PCDNA3 vector (Invitrogen) at the *Eco*RI, *Not*I, and *Not*I-*Hin*dIII sites, respectively. IκBβΔC contains a deletion of the last 50 amino acids (aa), including the PEST and CKII sites. The corresponding bacterial expression vectors were constructed after subcloning to the pET25b expression vector (Novagen) and in frame with the six-His moiety. To generate a bacterial expression vector for p65, we subcloned a PCR fragment bearing the entire open reading frame (ORF) of human p65 between the *Bam*HI and *Xho*I sites of the pET25b vector. The GAL4-p65FL protein was constructed by subcloning the p65 ORF (*Bam*HI fragment) in the M1 plasmid (50) and in frame with the GAL4 DNA binding domain (aa 1 to 147). Similarly, the GAL4-p65AD protein was generated by subcloning the *Eco*RI-*Bam*HI fragment (aa 298 to 551) of human p65 to M1. The PMVN constructs were generated by subcloning the ORFs of IκBα, IκBβ, and IκBβΔC in the PMVN vectors (provided by I. Sadowski) and in frame with VP16 and the simian virus 40 (SV40) NLS. The $PRDII_4LACZ$ plasmid was generated by replacing the chloramphenicol acetyltransferase (CAT) gene with the *lacZ* gene taken from CMVβGAL (42).

Transfection and cell culture. HeLa, P19, or COS cells were transfected by the calcium phosphate method as previously described (58, 59). Transfections were carried out with the amounts of plasmids indicated in the figure legends, and in every case vector DNA was added as necessary to achieve a constant amount of transfected DNA. Virus or TNF-α inductions were carried out as previously described (58, 59).

Expression and purification of proteins in *Escherichia coli.* Bacterial PACYC BL21(DE3) cells were transformed with plasmids expressing the desired His-tagged proteins, expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) during mid-logarithmic growth phase, and the cells were grown for 3 h at 30°C. His-tagged proteins were purified and dialyzed as previously described (58). Briefly, the cells were lysed by sonication, and the His-tagged proteins were purified by incubation with Ni-nitrilotriacetic acid-agarose beads in a buffer containing 8 M urea, 10 mM Tris, 100 mM Na_2PO_4, 10 mM immidazole, and 10 mM β-mercaptoethanol. Bound proteins were eluted with 25, 50, 150, and 500 mM and 1 M immidazole. Purified proteins were renatured by dialysis in a buffer containing 6 M urea, 500 mM NaCl, 0.1% Nonidet P-40 (NP-40), 10% glycerol, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The urea concentration was gradually decreased to 0 M by the addition of urea-free buffer, and a final dialysis was made in buffer containing 100 mM NaCl, 0.1% NP-40, 10% glycerol, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 2 mM DTT, and 0.5 mM PMSF. The term recombinant NF-κB refers to the in vitro-reconstituted p50/p65 heterodimer prepared by corenaturation of equal amounts of p50 and p65 polypeptides.

Preparation of whole-cell extracts. Monolayer COS cell cultures were washed three times in phosphate-buffered saline (PBS) and harvested by scraping in PBS. The cells were pelleted at 10,000 × *g* for 15 s and resuspended in extract buffer containing 20 mM HEPES, 300 mM KCl, 0.2 mM EDTA, 0.5% NP-40, 1 mM DTT, 7% glycerol, 0.5 mM PMSF, 5 μg of pepstatin A per ml, and 10 μg of aprotinin per ml. The resuspended cells were subjected to three rounds of freeze-thawing on dry ice and ice, respectively. After the final thaw, the mixture was spun down at 14,000 rpm at 4°C for 5 min, and the supernatant was retrieved, frozen in dry ice, and stored at −80°C.

Preparation of nuclear and cytoplasmic extracts. Monolayer HeLa cell cultures from 10 plates (150 mm diameter) were washed and harvested as before. Cells were centrifuged for 10 min at 1,850 × *g* and the packed cell volume (pcv) was measured. The cells were washed with 5 pcv of PBS and centrifuged for 10 min at 1,850 × *g*. The cells were resuspended in 5 pcv of hypotonic buffer containing 10 mM HEPES (pH 7.9 at 4°C), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT and centrifuged for 5 min at 1,850 × *g*. The cells were resuspended to a final volume of 3 pcv in hypotonic buffer and allowed to swell on ice for 10 min. The swollen cells were homogenized in a Dounce homogenizer with 12 strokes. The nuclei were pelleted for 15 min at 3,300 × *g*. The cytoplasmic extract was removed, and 0.11 volume of 10× cytoplasmic buffer containing 0.3 M HEPES (pH 7.9 at 4°C), 1.4 M KCl, and 0.03 M $MgCl_2$ was added. The packed nucleus volume (pnv) was measured, and the nuclei were resuspended in 1/2 pnv low-salt buffer containing 20 mM HEPES (pH 7.9 at 4°C), 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT. One-half pnv of high-salt buffer containing the same reagents as the low-salt buffer except with 1.2 M KCl instead of 0.02 M KCl was added dropwise to the mixture. The nuclei were allowed to extract for 30 min with gentle stirring at 4°C and then were centrifuged for 15 min at 8,000 rpm.

EMSAs. Electrophoretic mobility shift assays (EMSAs) with recombinant proteins were performed as follows. Proteins and DNA probes were incubated for 15 min at room temperature in 10 mM Tris (pH 7.9)–50 mM NaCl–1 mM EDTA–5% glycerol–2 mM DTT–20 μg of bovine serum albumin–0.3% NP-40. Protein-DNA complexes were resolved by electrophoresis through 5% acrylamide-bisacrylamide (29:1) gels in 0.5× TBE (40 mM Tris, 45 mM boric acid, and 1 mM EDTA) at 200 V for 4 h. EMSAs with whole-cell extracts were carried out by preincubating the extract in a mixture containing 10 mM Tris (pH 7.9), 50 mM NaCl, 1 mM EDTA, 5% glycerol, 2 mM DTT, 2 μg of poly(dI · dC), and 0.1% NP-40 for 15 min on ice. The DNA probes were added and incubated for an additional 15 min on ice. The protein-DNA complexes were resolved as described above.

In vitro phosphorylation of proteins. Recombinant proteins (5 μg) were incubated with 20 mM Tris-HCl–50 mM KCl–10 mM $MgCl_2$–1 mM ATP–10 U of CKII (New England Biolabs) for 30 min at 30°C. Phosphorylation of proteins was confirmed by using [γ-^{32}P]ATP (100 μCi) in place of cold ATP in the kinase reaction mix.

Western blotting. Cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol for 3 h at 80 V and 4°C. The blots were blocked for one hour in TBST (20 mM Tris [pH 7.6], 137 mM NaCl, and 0.1% Tween 20) containing 5% dry milk. The blots were washed in TBST and incubated with primary antibody (Santa Cruz) for 1 h at room temperature with shaking. The blots were washed in TBST and incubated with secondary antibody (Santa Cruz) under the same conditions. The blots were washed, and bound antibody was visualized by chemiluminescence (Amersham).

Affinity purification of DNA binding complexes. Cell extracts (500 μl) were incubated with 1 μg of biotinylated κB oligonucleotide, 60 μl of streptavidin agarose beads, and 20 μg of poly(dI · dC) for 1 h at 4°C with gentle mixing. Bound complexes were pelleted at 10,000 × *g* for 1 min, and the pellet was washed with buffer containing 30 mM HEPES, 140 mM KCl, and 3 mM $MgCl_2$. An additional 0.5 μg of biotinylated DNA and 30 μl of strepavidin beads were added to the wash. The wash mixture was incubated for 30 min at 4°C with gentle mixing. Bound complexes were pelleted at 14,000 rpm for 1 min, and the supernatant was removed. The pellet was washed extensively and resuspended in 2× SDS loading buffer containing 130 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 0.2 mM bromophenol blue, and 284 mM β-mercaptoethanol, and the bound complexes were resolved by SDS-PAGE. Western blotting was performed as described above.

In vitro transcription. Recombinant proteins were incubated with 200 ng of template DNA for 10 min at room temperature. Thirty-eight microliters of a premixed batch containing 9 μl of HeLa cell nuclear extract (HNE), 0.3 μl of 1 M $MgCl_2$, 0.8 μl of 25 mM nucleoside triphosphates (NTPs), and 16 μl of DB buffer (25 mM HEPES [pH 7.9], 40 mM KCl, 10% glycerol, and 1 mM DTT) was added to the protein-DNA mix and allowed to incubate for 1 h at 30°C. Alternatively, recombinant proteins were incubated with 200 ng of template for 10 min at room temperature. Thirty-seven microliters of a premixed batch containing 9 μl of HNE, 0.3 μl of 1 M $MgCl_2$, and 16 μl of DB buffer was added to the protein-DNA mix and allowed to incubate for 20 min at 30°C. Next, 1 μl of 25 mM ribonucleoside triphosphates (rNTPs) was added to each reaction mixture, followed by 1 h of incubation at 30°C. IκBs were either added after the formation of the NF-κB/DNA complex and incubated for an additional 10 min or added after preincubation of the template with HNE. For the single-round transcription experiments, Sarkosyl was added to a final concentration of 0.05% followed by rNTPs. Transcription was terminated by adding 100 μl of stop mix (0.4 M sodium acetate, 0.2% SDS, 10 mM EDTA, 50 μg of tRNA per ml, and 200 μg of proteinase K per ml) followed by phenol extraction and ethanol precipitation. The RNA pellet was washed with cold 70% ethanol, dried, resuspended in 5 μl of H_2O, and annealed with the E4 primer in 1× PE buffer containing 50 mM Tris (pH 8.3 at 42°C), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP, and 0.5 mM spermidine at 55°C for 20 min. The reaction mix was allowed to cool at room temperature for 10 min, and 1 U of avian myeloblastosis virus reverse transcriptase (Life Sciences) contained in 10 μl of 1× PE buffer with 7 mM sodium pyrophosphate was added to the mix. The reverse transcription reaction mixture was incubated for 30 min at 42°C, and the reaction was terminated. An equal volume of loading dye (98% formamide, 10 mM EDTA, 0.01% xylene cyanol, and 0.01% bromophenol blue) was added to the reaction mixture. The transcripts were resolved on an 8% acrylamide-bisacrylamide (19:1)–5 M urea gel in 1× TBE (80 mM Tris, 90 mM boric acid, and 2 mM EDTA) run at 200 V for 1.5 h.

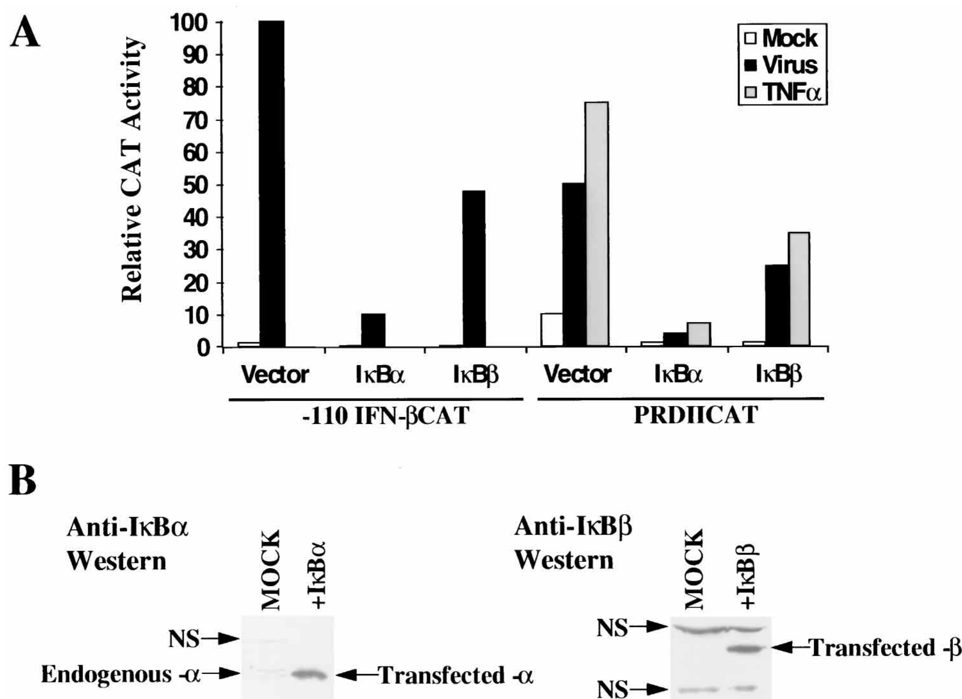


FIG. 1. (A) Human HeLa cells were cotransfected with 4 μg of −110 IFN-β CAT or 8 μg of PRDII CAT reporter along with 16 μg of mammalian expression vector encoding IκBα or IκBβ. At 24 h posttransfection the cells were either mock, virus, or TNF-α induced for 16 h, and the CAT activities were determined. Shown are averages from eight independent experiments. The variability from experiment to experiment was less than 15%. (B) IκBα and IκBβ are expressed at comparable levels following transfection. Shown is a Western blot probed with the IκBα (left) and IκBβ (right) antibodies. Whole-cell extracts prepared from cells transfected with the IκBα and IκBβ expression vectors were fractionated on by SDS–10% PAGE and subjected to immunoblot analysis. NS, nonspecific.

RESULTS

IκBα is a stronger inhibitor of NF-κB than IκBβ. In a previous study, Thompson et al. (61) established that IκBα and IκBβ interact with the same spectrum of Rel proteins in vivo or in vitro. To investigate whether these interactions lead to comparable inhibition of NF-κB-dependent transcriptional activation, we carried out cotransfection experiments with human HeLa cells and ectopically expressed IκBα and IκBβ. Reporter constructs bearing the CAT gene fused to the intact IFN-β promoter or to four copies of the PRDII element (the NF-κB site of the IFN-β promoter) were cotransfected along with expression vectors encoding IκBα or IκBβ. The endogenous NF-κB DNA binding activity, consisting of p50/p65 heterodimers and p65 homodimers (59), was induced either by virus infection or by treatment with TNF-α, and the CAT activities were determined. Figure 1A shows that the expression of either IκBα or IκBβ decreased the levels of virus induction from both reporters, as well as the TNF-α-induced transcription from the PRDII element. However, IκBα appears to be a fivefold-stronger inhibitor of NF-κB compared to IκBβ. Figure 1B shows that this difference is not due to their different levels of expression, since IκBα and IκBβ are expressed in similar amounts as determined by Western blot analysis of the transfected cells. As the IκBβ antibody is less sensitive than the IκBα antibody, we believe that the actual levels of expressed IκBβ are higher than those of IκBα (data not shown).

To examine in detail the different inhibitory properties of IκBα and IκBβ, we performed cotransfection experiments with mouse P19 cells, which are devoid of NF-κB (59), using the PRDII reporter activated by transfection of a small, constant amount of the NF-κB p65 subunit along with increasing amounts of each of the IκB-expressing plasmids. As shown in Fig. 2A, increasing amounts of transfected IκBα or IκBβ expression plasmids inhibited NF-κB-dependent transcription in a dose-dependent manner. Again, IκBα was a five-times-stronger inhibitor of NF-κB than IκBβ. Interestingly, an IκBβ derivative that lacks the carboxyl-terminal 50 amino acids (IκBβΔC), including the PEST sequence and two putative CKII sites (18, 34), did not efficiently inhibit NF-κB-dependent transcription (Fig. 2A). This is not a peculiarity of the cell type, since identical results were obtained with both HeLa and COS cells (data not shown). Again, the relative levels of the expressed proteins were determined by Western blotting, with whole-cell extracts prepared from transfected cells and immunoblotted with either p65-, IκBα-, or IκBβ-specific antibodies. As shown in Fig. 2B, transfection of the p65 expression vector leads to the accumulation of the p65 protein (lane 2). In agreement with previous studies (56), we found that p65 stimulates the synthesis of IκBα protein but not IκBβ protein (Fig. 2B, lane 2) (61). Cotransfection of p65 plus IκBα, p65 plus IκBβ, or p65 plus IκBβΔC led to the synthesis of equivalent amounts of all IκB proteins compared to IκBα (compare lanes 3, 4, and 5 of Fig. 2B). Actually, IκBβΔC appears to accumulate in larger amounts than IκBβ even though it displays a weaker inhibitory activity. Thus, the different inhibitory properties of IκBα and IκBβ are not due to their different levels of expression. Finally, we found that the carboxyl terminus of IκBβ appears to be necessary for NF-κB inhibition in vivo, as is true for IκBα (8, 20, 49, 62a).

IκB proteins inhibit NF-κB DNA binding in the nucleus. Two common properties are shared by the IκB proteins. First, they mask the NLS of NF-κB and therefore inhibit its nuclear translocation in vivo, and second, they inhibit binding of

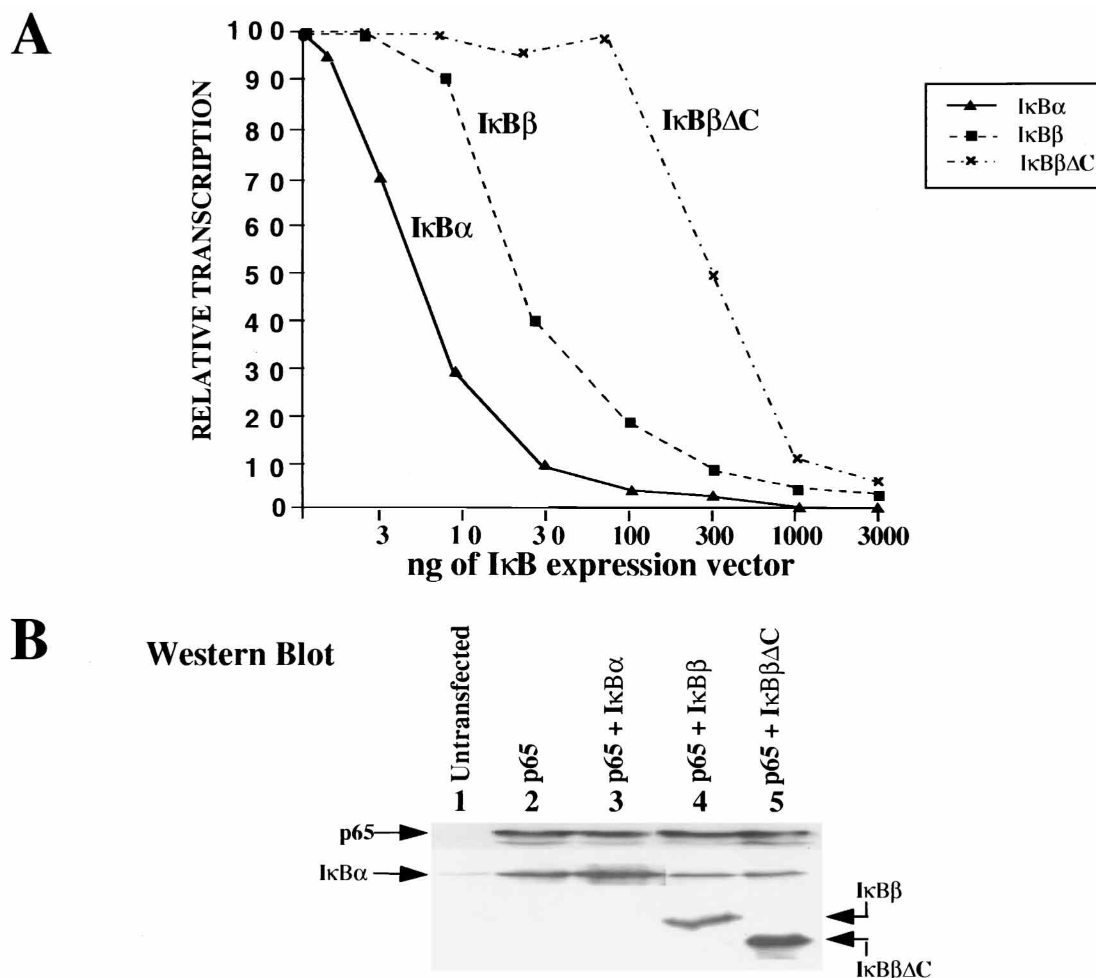
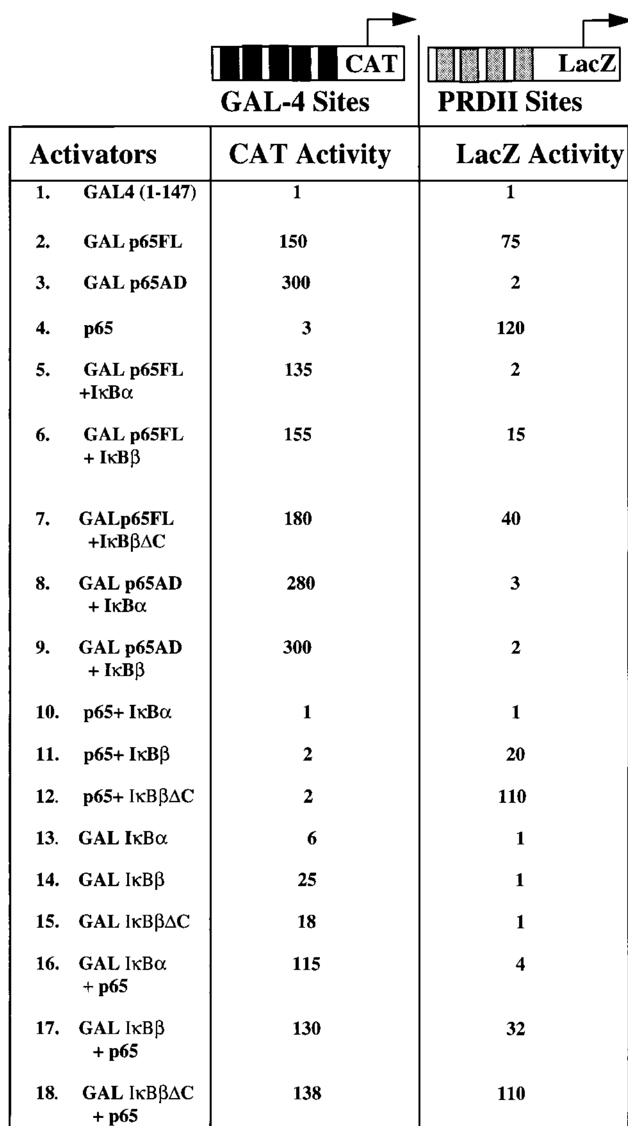


FIG. 2. (A) Mouse P19 cells were cotransfected with the PRDII reporter (1 μg) along with the p65 expression vector (200 ng) with the indicated amounts of the IκB-expressing plasmids. The fold activation of transcription by p65 alone was 89, and this was taken as 100%. Shown are results from one of four independent experiments. The variability between experiments was less than 20%. (B) Whole-cell extracts, prepared from cells transfected with the indicated expression plasmids, were immunoblotted with the p65 (top), IκBα (middle), and IκBβ (bottom) antibodies.

NF-κB to the DNA in vitro (1, 47, 65). On the other hand, previous studies have provided compelling biochemical evidence that IκB proteins can be found in the nucleus, but they did not provide a direct functional role for the nuclear IκB proteins. Therefore, it is possible that the observed inhibitory activity of IκB proteins on NF-κB-dependent transcription may be entirely due to the cytoplasmic sequestration of NF-κB by IκBs and not due to their ability to inhibit NF-κB binding in the nucleus. To examine this possibility, we took advantage of a previous observation that addition of a second NLS (derived from the SV40 T antigen) to the p65 subunit generates a protein which is not sequestered in the cytoplasm by IκB proteins (10). We fused the DNA binding domain of GAL4 (aa 1 to 147), which contains a functional NLS (55), to the full-length p65 protein (GALp65FL) or to the activation domain (GALp65AD). Thus, the GALp65FL protein contains two functional NLSs, one in GAL4 and the other in the RHR of p65, and IκB proteins interact only with the p65 NLS and not with that of GAL4 (see below). Mouse P19 cells were transfected simultaneously with two different reporters bearing either five GAL4 sites upstream of the CAT gene or four PRDII sites upstream of the β-galactosidase gene along with various p65 derivatives in either the presence or the absence of the IκB-expressing plasmids. Figure 3 (line 2) shows that in the same cells GALp65FL activates transcription from both reporters, because it binds to both sites. In contrast, GALp65AD (containing the activation domain of p65 fused to GAL4) activates transcription from only the GAL4 reporter (Fig. 3, line 3), whereas wild-type p65 activates from only the PRDII reporter (line 4). Interestingly, cotransfection of GALp65FL with either IκBα or IκBβ did not affect activation of transcription from the GAL4 reporter (compare line 2 with lines 5 and 6 in Fig. 3), indicating that the NLS of GAL4 suffices to drive the GALp65FL/IκB complex into the nucleus. Moreover, IκBs complexed with the RHR of p65 do not inhibit activation of transcription by p65AD if the complex is tethered to a promoter via the heterologous DNA binding domain of GAL4 (Fig. 3, lines 5 and 6). However, in the same cells the same complex did not activate transcription from the PRDII sites (Fig. 3, lines 5 and 6), suggesting that the nuclear NF-κB/IκB is transcriptionally inactive because IκBs inhibit binding to the PRDII sites. Again, IκBα appears to be a stronger inhibitor of nuclear NF-κB than IκBβ (compare lines 5 and 6 of Fig. 3), and IκBβΔC only marginally inhibited DNA binding of NF-κB



	GAL-4 Sites (CAT)	PRDII Sites (LacZ)
Activators	**CAT Activity**	**LacZ Activity**
1. GAL4 (1-147)	1	1
2. GAL p65FL	150	75
3. GAL p65AD	300	2
4. p65	3	120
5. GAL p65FL +IκBα	135	2
6. GAL p65FL + IκBβ	155	15
7. GALp65FL +IκBβΔC	180	40
8. GAL p65AD + IκBα	280	3
9. GAL p65AD + IκBβ	300	2
10. p65+ IκBα	1	1
11. p65+ IκBβ	2	20
12. p65+ IκBβΔC	2	110
13. GAL IκBα	6	1
14. GAL IκBβ	25	1
15. GAL IκBβΔC	18	1
16. GAL IκBα + p65	115	4
17. GAL IκBβ + p65	130	32
18. GAL IκBβΔC + p65	138	110

FIG. 3. IκBα and IκBβ inhibit NF-κB-dependent transcription in the nucleus. Lines 1 to 12, mouse P19 cells were transfected simultaneously with the GAL4-CAT and PRDII-LacZ reporters (0.5 μg each) along with the indicated activators (2μg) in the presence or absence of IκB proteins (2 μg). Lines 13 to 18, the transfection cocktail contained the same amount of reporters as above, 2 μg of GAL4-IκB fusion, and 2 μg of p65 expression vectors. CAT and β-galactosidase activities were determined 48 h after transfection. Shown are results from one of five independent experiments, and the variability was less than 10% between experiments.

(Fig. 3, line 8). As a control, we showed that the GALp65AD protein is not affected by the IκBs (compare line 3 with lines 8 and 9 in Fig. 3). Moreover, both IκBs inhibited activation by wild-type p65, albeit to different extents (compare line 4 with lines 10, 11, and 12 in Fig. 3), in agreement with the data presented in Fig. 2.

In a reciprocal set of experiments, we fused the GAL4 DNA binding moiety to either IκBα, IκBβ, or IκBβΔC. As shown in Fig. 3, all three GAL-IκB fusions, especially the two GAL-IκBβ derivatives, can stimulate transcription on their own (compare line 1 with lines 13, 14, and 15) when tethered to a promoter, a result consistent with previous studies with avian and yeast cells (44). Coexpression of p65 plus the GAL-IκB fusions led to a dramatic increase in transcription from the GAL4 sites but to inhibition of transcription from the PRDII sites (compare lines 4, 13, 14, and 15 with lines 16, 17, and 18 in Fig. 3). Thus, GAL-IκBs recruit p65 in the nucleus at the GAL4 sites but in the same cells inhibit binding of p65 at the PRDII sites and decrease transcription. These experiments strongly suggest that IκBα and IκBβ function in vivo by inhibiting not only nuclear translocation but also the DNA binding function of NF-κB in the nucleus. Furthermore, it appears that the differences in the inhibitory properties of IκBα, IκBβ, and IκBβΔC with regard to NF-κB-dependent transcription directly correlate with their abilities to inhibit NF-κB binding in the nucleus (see below).

IκBα but not IκBβ efficiently removes NF-κB from the DNA. The transfection experiments described above suggested that the different inhibitory properties of IκBα and IκBβ are due, at least in part, to their differential abilities to prevent NF-κB DNA binding in vivo. To further examine this possibility, we performed EMSAs with recombinant purified NF-κB and IκB proteins. The coding sequences for IκBα, IκBβ, and IκBβΔC were cloned in the bacterial expression vector pET25b, bearing six histidine residues at the 3′ end of the ORF. Figure 4A shows an SDS-PAGE gel, stained with Coomassie blue, containing the purified IκBα, IκBβ, and IκBβΔC proteins. A constant amount of NF-κB (p50/p65 heterodimer) was allowed to interact with the PRDII oligonucleotide and then challenged with increasing amounts of IκB proteins. The complexes were analyzed by electrophoresis, and results of a representative experiment are shown in Fig. 4B. Consistent with previous studies, IκBα efficiently removes NF-κB from the DNA (Fig. 4B, lanes 2 to 7) (64). Quantitation of results from several similar experiments revealed that 50% inhibition of NF-κB binding occurs at a 1:1 molar ratio between NF-κB and IκBα. However, recombinant IκBβ displayed a different effect on NF-κB binding. Surprisingly, IκBβ forms a ternary complex with NF-κB on the DNA and inhibited DNA binding only at very high molar ratios (Fig. 4B, lanes 8 to 13) (50% inhibition of NF-κB binding requires a 30:1 molar ratio of IκBβ over NF-κB). Interestingly, IκBβΔC also forms a ternary complex with NF-κB on the DNA, but in contrast to the case for full-length IκBβ, it does not inhibit DNA binding even at high molar ratios (Fig. 4B, lanes 14 to 19). The presence of IκBβ in ternary complexes with NF-κB on the DNA was verified by antibody supershift experiments (data not shown). Identical results were obtained when NF-κB and IκBs were preincubated in the absence of DNA, followed by the addition of the probe (data not shown). Thus, it appears that IκBα and IκBβ have differential abilities to inhibit binding of NF-κB in vitro. Qualitatively similar results were obtained when EMSA experiments were carried out with proteins expressed in COS cells. As shown in Fig. 4C, mock-transfected COS cells do not contain detectable amounts of NF-κB binding activity (lane 1). Transfection of the p65 expression vector results in synthesis of p65, which binds to the PRDII oligonucleotide (Fig. 4C, lane 2). However, coexpression of IκBα inhibited p65 binding (Fig. 4C, lane 5). In contrast, IκBβ only partially inhibited binding (Fig. 4C, lane 3), whereas IκBβΔC formed a stable ternary complex with p65 on the DNA (lane 4). Taken together, these results strongly suggest that IκBα and IκBβ differ in their inhibitory NF-κB DNA binding activities. In addition, the carboxyl-terminal 50 aa of IκBβ is required for the inhibition of NF-κB DNA binding in vivo and in vitro. Deletion of this region results in a protein that forms stable complexes with NF-κB on the DNA.

Recombinant IκBβ phosphorylated at the C terminus by CKII efficiently inhibits NF-κB binding. We have shown that

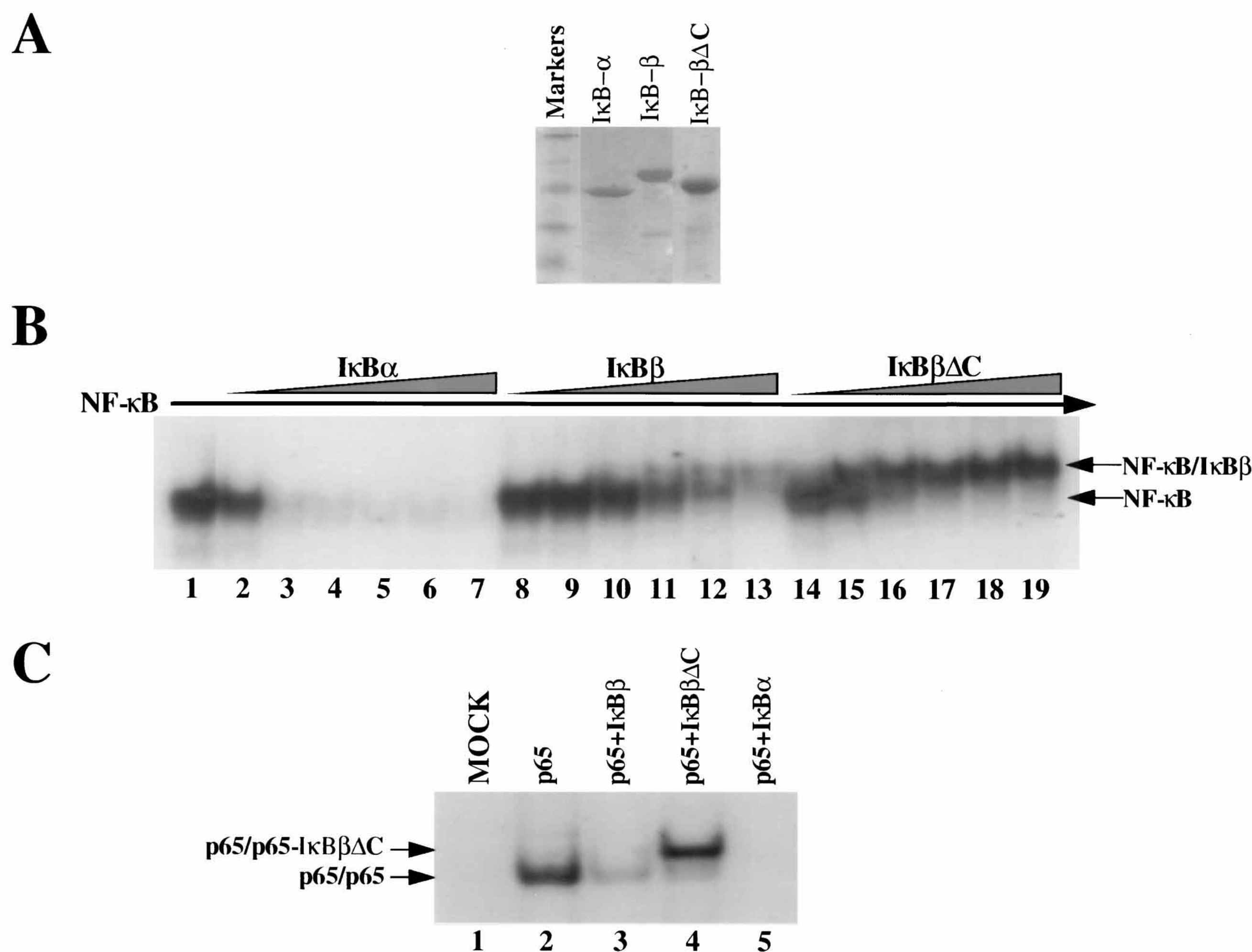


FIG. 4. Recombinant IκBβ is a weak inhibitor of NF-κB binding. (A) Coomassie blue-stained SDS-PAGE gel containing the bacterially expressed and purified His-tagged IκBα, IκBβ, and IκBβΔC. (B) Results of a representative EMSA experiment using the PRDII element as a probe along with recombinant NF-κB and IκB proteins. Lane 1, NF-κB (p50/p65 heterodimer) alone; lanes 2 to 7, increasing amounts of IκBα; lanes 8 to 13, increasing amounts of IκBβ; lanes 14 to 19, increasing amounts of IκBβDC. The molar ratios between NF-κB and IκBs were 1:1 (lanes 2, 8, and 14), 1:3 (lanes 3, 9, and 15), 1:10 (lanes 4, 10, and 16), 1:30 (lanes 5, 11, and 17), 1:100 (lanes 6, 12, and 18), and 1:300 (lanes 7, 13, and 19). The arrows at the right indicate the NF-κB and NF-κB/IκBβ/DNA complexes. (C) Results of an EMSA experiment using the PRDII element as a probe along with whole-cell extracts prepared from COS cells transfected with the expression vectors indicated on the top.

full-length IκBβ inhibits NF-κB binding to DNA only at high concentrations and that deletion of its carboxyl terminus eliminates this inhibitory activity. Therefore, the carboxyl terminus of IκBβ modulates the ability of the protein to inhibit NF-κB binding. Inspection of this sequence revealed the presence of a consensus site for CKII (34). Multiple CKII sites have also been identified at the C terminus of IκBα (7, 38, 49, 52, 62a). To examine whether phosphorylation of the IκBβ C terminus by CKII alters its ability to inhibit NF-κB binding, we incubated recombinant IκB proteins with CKII and examined their ability to inhibit NF-κB binding. Figure 5A shows a Coomassie blue-stained SDS-PAGE gel containing the recombinant proteins used as substrates in the in vitro kinase assays with CKII. Figure 5B shows the autoradiogram of the equivalent SDS-PAGE gel after phosphorylation with [γ-^{32}P]ATP. As shown in Fig. 5B (lanes 1 and 2), neither p50 nor p65 is a substrate for CKII in vitro. However, both IκBα and IκBβ are phosphorylated by CKII (Fig. 5B, lanes 3 and 4). The phosphorylation site in IκBβ appears to be at the C terminus, because the IκBβΔC protein is not phosphorylated (Fig. 5B, lane 5). The specificity of these reactions was also demonstrated by showing that CKII phosphorylates the intact HMG I protein (Fig. 5B, lane 6) but not an HMG I derivative lacking the HMG I CKII site (lane 7) (46). Figure 5C shows the results of an EMSA experiment where we compared the abilities of mock- and CKII-phosphorylated recombinant IκB proteins to inhibit NF-κB binding. Remarkably, IκBβ phosphorylated in vitro by CKII inhibits NF-κB binding more efficiently (eightfold) than the unphosphorylated protein and does not form a ternary complex with NF-κB (compare lanes 26 to 31 with lanes 32 to 37 in Fig. 5C). By contrast, CKII-phosphorylated IκBα is only a twofold-better inhibitor than the unphosphorylated form (compare lanes 2 to 7 with lanes 8 to 13) (8). These effects are specific to the phosphorylation of IκBα and IκBβ, because CKII has no effect on the ability of IκBβΔC to influence NF-κB DNA binding (compare lanes 14 to 19 with lanes 20 to 25 in Fig. 5C). These results strongly suggest that the carboxyl terminus of IκBβ regulates its inhibitory activity presumably by phosphorylation, consistent with a previous report (18) (see Discussion).

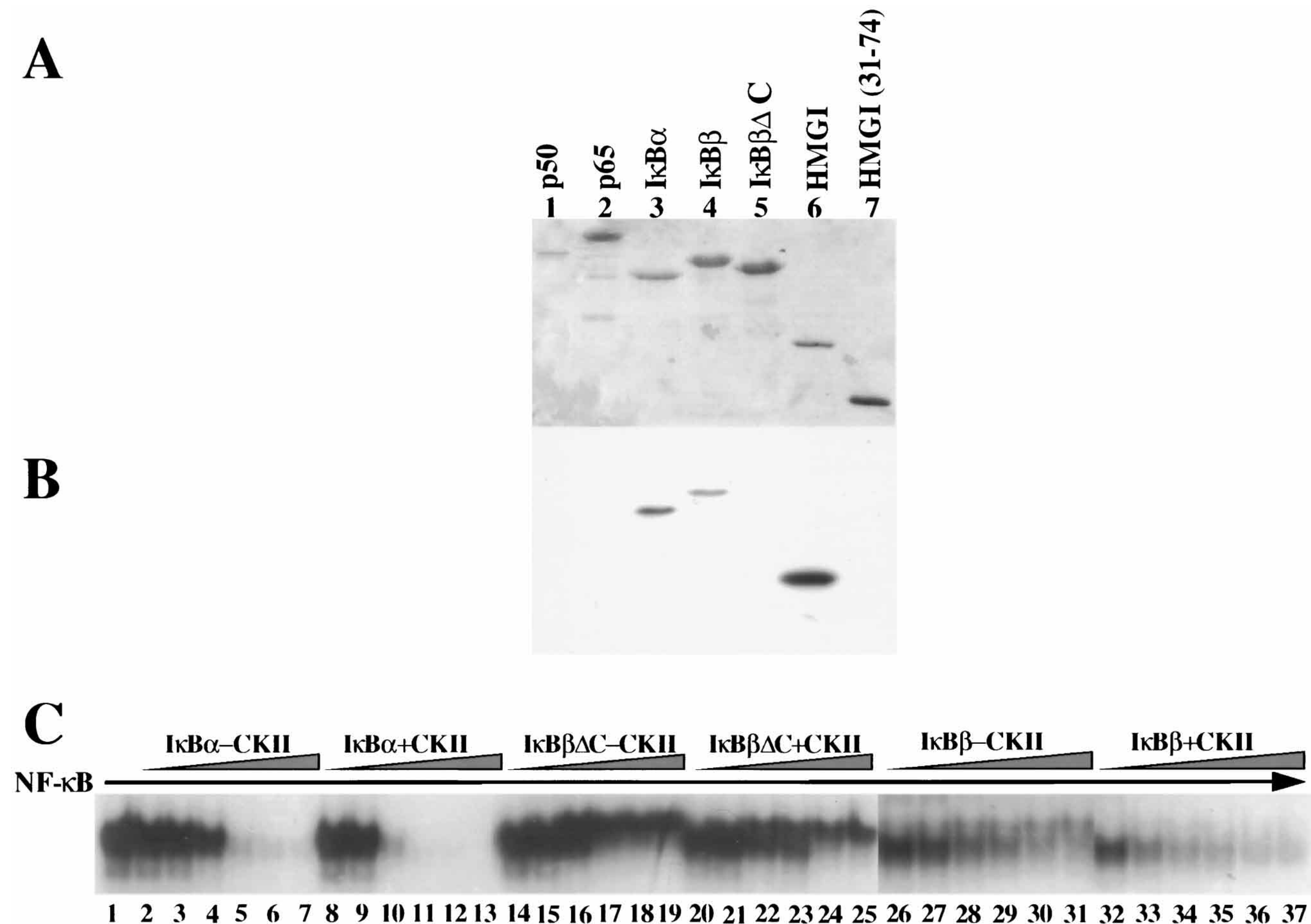


FIG. 5. Phosphorylation of the C-terminal PEST domain of IκBβ by CKII potentiates its inhibitory activity. (A) Coomassie blue-stained SDS-PAGE gel containing recombinant p50 (lane 1), p65 (lane 2), IκBα (lane 3), IκBβ (lane 4), IκBβΔC (lane 5), HMG I (lane 6), and a truncated HMG I protein (lane 7). (B) The amounts of proteins shown in panel A were incubated with CKII plus [γ-^{32}P]ATP, analyzed by SDS-PAGE, and autoradiographed. (C) Results of an EMSA experiment using the PRDII element as a probe and recombinant NF-κB either in the presence or in the absence of increasing concentrations of mock- or CKII-treated IκB proteins. Lane 1, NF-κB (p50/p65 heterodimer) alone; lanes 2 to 7, NF-κB plus increasing amounts of mock-treated IκBα; lanes 8 to 13, NF-κB plus increasing amounts of CKII IκBα. The molar ratios between NF-κB and IκBα were 30:1, 10:1, 3:1, 1:1, 1:3, and 1:10 for lanes 2 to 7, respectively, and for lanes 8 to 13, respectively. Lanes 14 to 19, NF-κB plus increasing amounts of mock-treated IκBβΔC; lanes 20 to 25, NF-κB plus increasing amounts of CKII-treated IκBβΔC. The molar ratios between NF-κB and IκBβΔC were 10:1, 3:1, 1:1, 1:3, 1:10, and 1:30 for lanes 14 to 19, respectively, and for lanes 20 to 25, respectively. Lanes 26 to 31, NF-κB plus increasing amounts of mock-treated IκBβ; lanes 32 to 37, NF-κB plus increasing amounts of CKII-treated IκBβ. The molar ratios between NF-κB and IκBβ were 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 for lanes 26 to 31, respectively, and for lanes 32 to 37, respectively.

Unphosphorylated IκBβ efficiently removes NF-κB from NF-κB/HMG I ternary complexes. Previous studies have established that NF-κB and the high-mobility group protein HMG I(Y) bind simultaneously to the PRDII elements from opposite sides of the DNA helix, forming a cooperative ternary complex (58). To examine the role of HMG I in the ability of IκBs to remove NF-κB from the PRDII element, we carried out EMSA experiments with the results shown in Fig. 6. In agreement with our previous results, incubation of HMG I and NF-κB results in the formation of a ternary complex on the PRDII element (Fig. 6A, lanes 1 and 2) but not on the IgκB element, which binds NF-κB but does not bind HMG I (Fig. 6B, lanes 1 and 2) (58). Addition of increasing amounts of IκBα to the preformed complexes removes NF-κB equally efficiently in the absence or presence of HMG I (Fig. 6A and B, compare lanes 3 to 7 with lanes 8 to 12). In contrast, IκBβ removed NF-κB 10 to 20 times more efficiently in the presence of HMG I than in its absence (compare lanes 13 to 17 with lanes 18 to 22). This effect appears to depend on the formation of the HMG I/NF-κB ternary complex on DNA, because HMG I did not have any effect when the IgκB site was used as the probe (Fig. 6B). Thus, HMG I may alter the structure of NF-κB on the DNA in such a way that IκBβ can efficiently recognize and remove NF-κB from the DNA. The biological significance of this observation was demonstrated in transfection experiments where we compared the abilities of IκBβ to inhibit transcription from the PRDII and IgκB elements. It has been previously shown that HMG I binding at PRDII but not at IgκB is required in vivo for NF-κB-dependent transcription (58). As shown in Fig. 6C, transfected IκBβ inhibits NF-κB-dependent transcription from the PRDII reporter five times more efficiently than that from the IgκB reporter. In contrast, IκBα is equally effective with both reporters (Fig. 6D). Thus, IκBβ more efficiently removes NF-κB from promoters activated by the HMG I/NF-κB complex, such as PRDII, than from the IgκB element, which does not require HMG I binding.

NF-κB/IκBβ/DNA ternary complexes are formed on promoters in vivo. We have shown that unphosphorylated IκBβ or

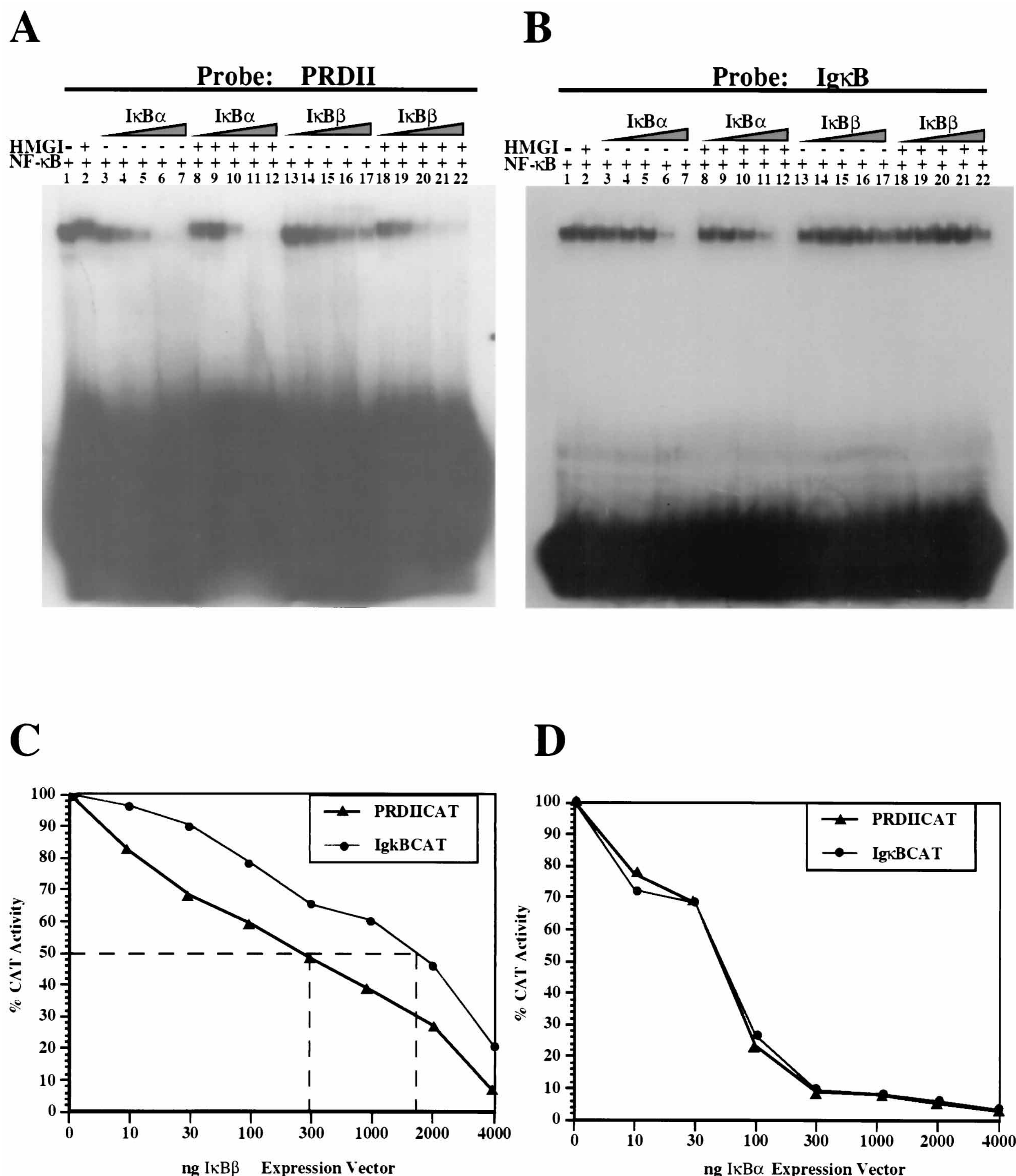


FIG. 6. IκBβ efficiently removes NF-κB from the HMG I/NF-κB/DNA complexes. (A) Results of an EMSA experiment using the PRDII as a probe and recombinant NF-κB (p50/p65 heterodimer), IκBα, IκBβ, and HMG I. Lane 1, NF-κB alone; lane 2, NF-κB plus HMG I; lanes 3 to 7, NF-κB plus increasing amounts of IκBα; lanes 8 to 12, NF-κB/HMG I plus increasing amounts of IκBα; lanes 13 to 17, NF-κB plus increasing amounts of IκBβ; lanes 18 to 22, NF-κB/HMG I plus increasing amounts of IκBβ. (B) Same as in panel A, but the probe was the IgκB element. The amount of HMG I used was 20 ng. In both panels, the molar ratios between NF-κB and IκBα were 1:0.1, 1:0.3, 1:1, 1:3, and 1:10 for lanes 3 to 7, respectively, and for lanes 8 to 12, respectively. The molar ratios between NF-κB and IκBβ were 1:1, 1:3, 1:10, 1:30, and 1:100 for lanes 13 to 17, respectively, and for lanes 18 to 22, respectively. (C) Mouse P19 cells were cotransfected with the PRDII reporter (1 μg) plus a constant amount of GAL-p65 FL (3 μg) along with the indicated amounts of either IκBα- or IκBβ-expressing vector. Shown are results from one of three independent experiments. Dotted lines indicate 50% inhibition. (D) Same as in panel C, but the reporter was the IgκBCAT plasmid.

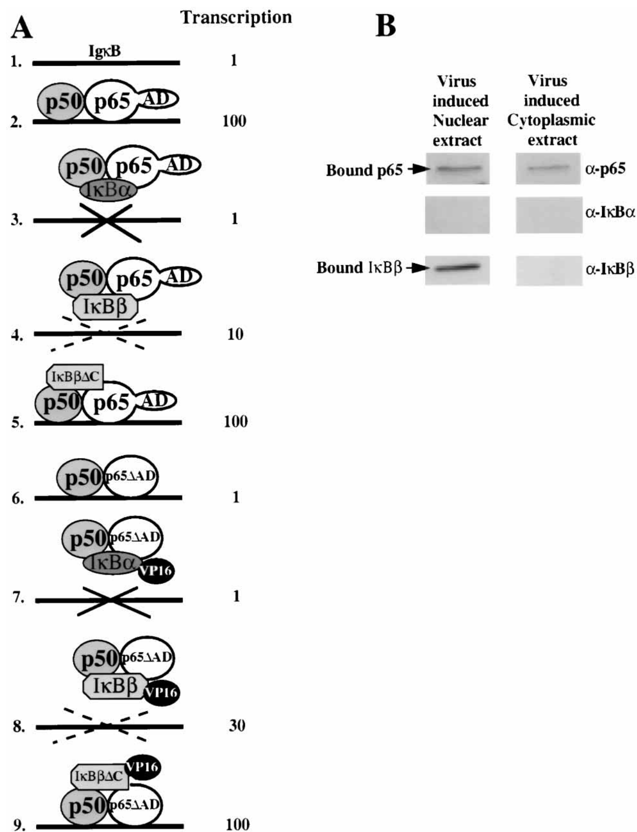


FIG. 7. In vivo identification of the NF-κB/IκBβ/promoter complexes. (A) Mouse P19 cells were cotransfected with the IgκBCAT reporter plasmid (1 μg) and the indicated activators in the presence or absence of different IκB derivatives. Line 1, reporter alone; line 2, full-length NF-κB (1 μg); line 3, NF-κB plus IκBα (3 μg); line 4, NF-κB plus IκBβ (3 μg); line 5, NF-κB plus IκBβΔC (3 μg); line 6, NF-κB lacking activation domain (NF-κBΔAD) (1 μg); line 7, NF-κBΔAD plus PMVN1-IκBα (3 μg); line 8, NF-κBΔAD plus PMVNI-IκBβ (3 μg); line 9, NF-κBΔAD plus PMVN1-IκBβΔC (3 μg). (B) A biotinylated κB oligonucleotide was incubated with nuclear or cytoplasmic extract prepared from virus-infected HeLa cells and washed, and the DNA-bound proteins were immunoblotted with the p65 (top), IκBα (middle), and IκBβ (bottom) antibodies.

IκBβΔC forms stable ternary complexes with NF-κB on the DNA. As there are two forms of IκBβ in the cells, the phosphorylated and the nonphosphorylated forms (57), we hypothesized that nonphosphorylated nuclear IκBβ may be complexed with NF-κB on promoters because we showed that it cannot inhibit DNA binding. To examine this possibility, we performed transient-transfection experiments with P19 cells and the NF-κB and IκB derivatives, as shown in Fig. 7A. We deleted the activation domain of NF-κB (NF-κBΔAD) and fused the SV40 NLS and the VP16 activation domain to IκBα and IκBβ. Thus, a ternary complex formed in vivo on the promoters between NF-κBΔAD and IκB-VP16 must be transcriptionally active. As shown in Fig. 7, transfection of the IgκB reporter along with NF-κB leads to 100-fold stimulation of transcription. Consistent with our previous data, coexpression of IκBα, IκBβ, or IκBβΔC completely abolished, decreased, or did not affect this activation, respectively (Fig. 7A, lines 3, 4, and 5). Deletion of the p65 activation domain (NF-κBΔAD) abolished transcriptional activation (Fig. 7A, line 6). Cotransfection of NF-κBΔAD and IκBα-VP16 did not stimulate transcription (Fig. 7A, line 7). However, coexpression of NF-κBΔAD with IκBβ-VP16 or IκBβΔC-VP16 significantly stimulated transcription, 30- and 100-fold, respectively (Fig. 7A, lines 8 and 9). Thus, it appears that the NF-κB/IκBβ-VP16 complex binds to the promoter and that the VP16 activation domain is responsible for the activated transcription. Moreover, the difference in the abilities of the two IκBβ derivatives as well as of IκBα to stimulate transcription after being recruited by NF-κB parallels their abilities to inhibit NF-κB binding.

The experiments described above, taken together with the results shown in Fig. 4 and 5 as well as with those of other studies (57), strongly suggest that unphosphorylated IκBβ is complexed with nuclear NF-κB on the DNA. To provide additional evidence for this, we incubated a biotinylated κB oligonucleotide with nuclear or cytoplasmic extracts derived from virus-infected HeLa cells and precipitated the κB-bound proteins with strepavidin agarose. The precipitated proteins were extensively washed, analyzed by SDS-PAGE, and then immunoblotted with p65, IκBα, and IκBβ antibodies. As shown in Fig. 7B, p65 is detected in the nuclei as well as in the cytoplasm of virus-infected cells. Remarkably, IκBβ, but not IκBα, is detected as part of the virus-inducible NF-κB/DNA complex in the nucleus. Thus, IκBβ, presumably the unphosphorylated form, is part of the virus-inducible NF-κB complex assembled on κB sites.

IκB proteins compete with the basal transcriptional apparatus for NF-κB. The experiments presented in the previous sections strongly suggested that IκB proteins can terminate NF-κB-dependent transcription in the nucleus. Moreover, if the function of IκB proteins in the nucleus is to inhibit NF-κB binding, then it is expected that they would compete with the basal transcriptional machinery for NF-κB interactions (33, 37). To address this possibility, we performed in vitro transcription experiments using recombinant NF-κB and IκB proteins. The test reporter plasmid bears six copies of the PRDII element cloned immediately upstream of the E4 TATA box and coding region. As a control, we used a reporter bearing five GAL4 sites upstream of the E4 cassette (40). Correctly initiated transcripts were detected and quantitated by primer extension with a primer from the E4 coding sequence. Recombinant NF-κB or GAL4-AH was incubated with the appropriate reporter plasmid, followed by the addition of increasing concentrations of IκB proteins and HNE. Figure 8 shows that recombinant NF-κB (p50/p65 heterodimer) or GAL4-AH stimulates transcription from the respective reporter plasmids (compare lane 1 with lane 2). However, increasing the concentration of IκBα drastically inhibits transcription from PRDII but not from the GAL4 reporter (Fig. 8, lanes 3 to 5). In contrast, IκBβ inhibits transcription only at the highest concentration (Fig. 8, lanes 6 to 8), whereas IκBβΔC does not affect NF-κB-dependent transcriptional activation (lanes 9 to 11). Thus, there is a perfect correlation between the abilities of the three IκBs to inhibit DNA binding in vitro and their abilities to terminate NF-κB-activated transcription either in vivo or in vitro. Another important implication from these experiments is that the NF-κB/IκBβ or NF-κB/IκBβΔC ternary complexes formed on a promoter in vitro are transcriptionally active.

IκBα but not IκBβ disrupts preformed NF-κB/functional PIC interactions. The experiment described above taken together with previous studies (33, 37) established that IκB proteins can directly inhibit NF-κB-dependent transcription in vitro because they inhibit DNA binding. However, we did not know in what stage of the transcription cycle IκB proteins can remove NF-κB from the promoter. Since activators can influence transcription at numerous stages during the transcription cycle (reviewed in reference 48), we investigated first at which stage NF-κB acts by carrying out single-round in vitro tran-

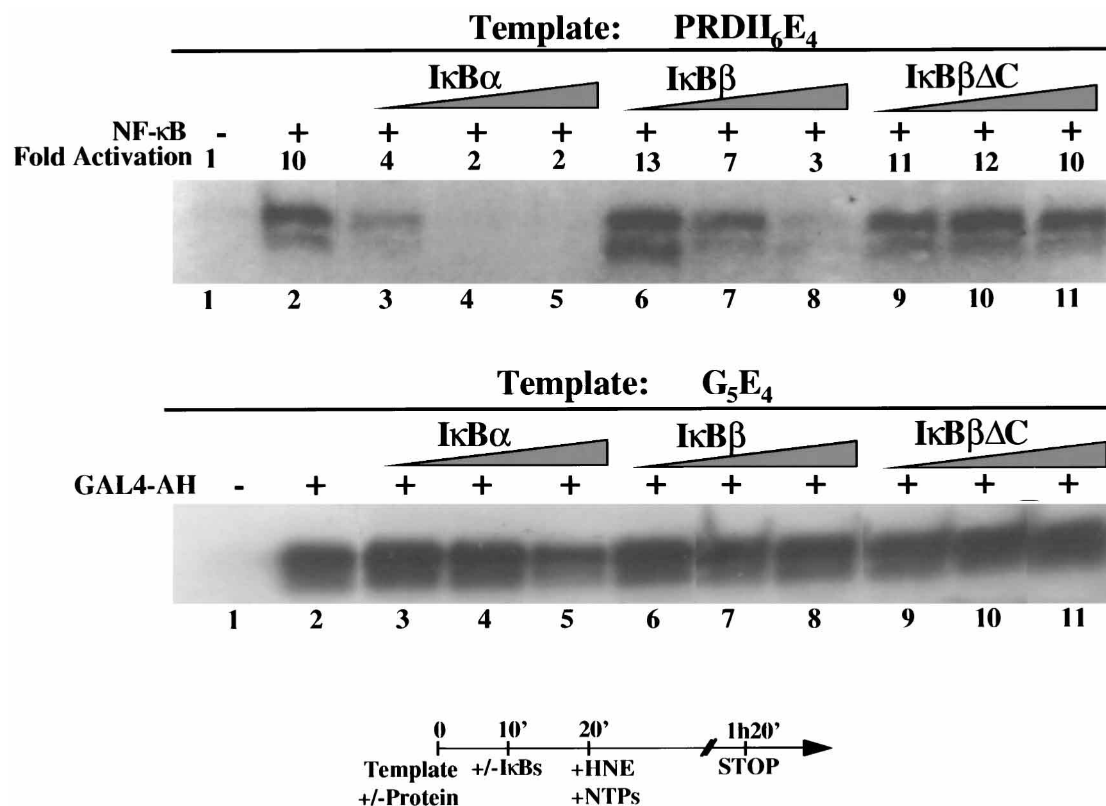
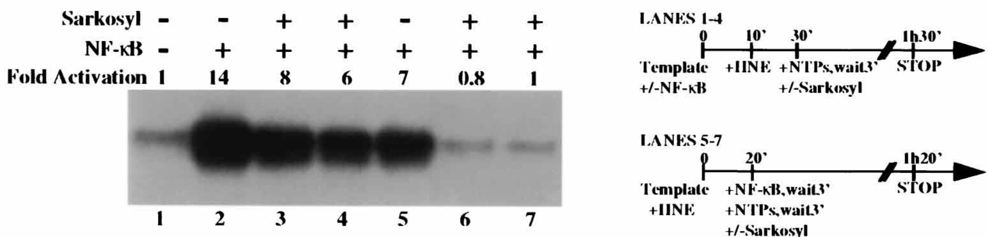
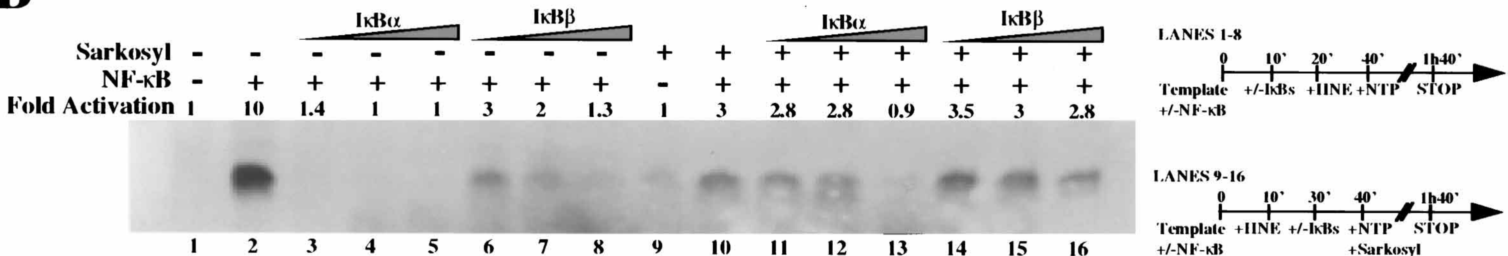


FIG. 8. IκBα and IκBβ inhibit NF-κB-dependent transcription in vitro. Shown are results from a representative in vitro transcription experiment using the PRDII$_6$E4 (top) and G$_5$E4 (bottom) templates and a constant amount of NF-κB or GAL4-AH in the absence of IκB (lanes 2) or in the presence of recombinant IκBα (lanes 3 to 5), IκBβ (lanes 6 to 8), or IκBβΔC (lanes 9 to 11). The order of addition is indicated in the kinetic scheme shown at the bottom. The molar ratios of activators and IκBα, IκBβ, and IκBβΔC were 1:1, 1:3, and 1:10 for lanes 3 to 5, respectively, for lanes 6 to 8, respectively, and for lanes 9 to 11, respectively. The amount of NF-κB and GAL4-AH was 300 ng.

scription experiments. This was accomplished by using low concentrations of the detergent Sarkosyl, which does not disrupt fully formed preinitiation complexes (PICs) but does prevent formation of new PICs and therefore reinitiation of transcription by RNA polymerase II (27, 28). Thus, the amount of RNA produced is proportional to the number of PICs formed before the addition of the detergent. The template bearing the six PRDII sites upstream of the minimal E4 promoter was incubated with recombinant NF-κB for 10 min to allow DNA binding, followed by treatment with HNE for 20 min. During that period, functional PICs were assembled (27, 28), transcription was initiated by the addition of NTPs, and transcript elongation was allowed by incubation for an additional 1 h. As shown in Fig. 9A, lane 1, in the absence of exogenously added NF-κB, the level of transcription is low, similar to that obtained by using a reporter containing only the E4 TATA box (not shown). However, addition of NF-κB stimulated transcription 14-fold (Fig. 9A, lane 2). Addition of Sarkosyl immediately after the NTPs at 0.04% (Fig. 9A, lane 3) and 0.06% (lane 4) decreased the level of transcription about twofold. This result strongly suggests that in our system NF-κB supports at least two rounds of activated transcription per template and that Sarkosyl inhibited the second round. A different result was obtained when we carried out the reciprocal experiment. In this case the templates were preincubated with the nuclear extract alone for 20 min, followed by the addition of NF-κB. Three minutes later we added NTPs and Sarkosyl, and the reaction mixture was incubated for an additional 1 h. As shown in Fig. 9A, lane 5, NF-κB can stimulate transcription when added late in the reaction. Interestingly, NF-κB did not stimulate transcription when it was added late followed by Sarkosyl (Fig. 9A, lanes 6 and 7). In EMSA experiments we have shown that these amounts of Sarkosyl do not affect NF-κB DNA binding (data not shown). The results obtained from this experiment are consistent with the notion that NF-κB does not activate transcription from PICs assembled in its absence but is required to be present during their assembly. That is, NF-κB stimulates transcription by promoting the assembly of functional PICs, because PICs that are allowed to be formed in the absence of NF-κB do not respond to added NF-κB.

Next, we investigated whether IκBα and IκBβ can remove NF-κB from committed functional PICs in single-round transcription experiments. NF-κB/template complexes were incubated with saturated amounts of IκBα and IκBβ for 10 min, followed by treatment with HNE for 20 min to allow formation of PICs. After that time, NTPs were added, and the transcripts were quantitated by primer extension. As shown in Fig. 9B, both IκBα (lanes 3 to 5) and IκBβ (lanes 6 to 9) can remove NF-κB from the DNA, consistent with the experiment of Fig. 8. However, as indicated in lanes 11 to 13 and 14 to 16 of Fig. 9B, IκBα but not IκBβ can remove NF-κB from a committed PIC in single-round transcription experiments. Interestingly, much larger amounts of IκBα are required to remove NF-κB from a committed functional PIC than before its assembly (compare lanes 3 to 5 with lanes 11 to 13 in Fig. 9B), implying





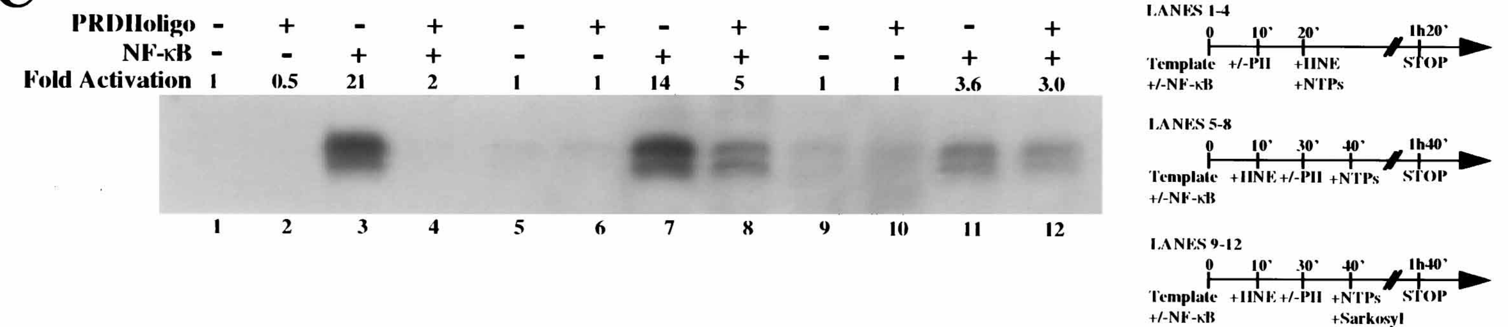


FIG. 9. IκBα but not IκBβ removes NF-κB from functional PICs. (A) NF-κB stimulates the formation of functional PICs. Shown are results of an in vitro transcription experiment using recombinant NF-κB in the presence or the absence of 0.04% (lanes 3 and 6) or 0.06% (lanes 4 and 7) Sarkosyl. The kinetic schemes are shown on the right of the autoradiogram. (B) IκBα but not IκBβ inhibits single-round in vitro transcription from NF-κB. The kinetic schemes are indicated on the right of the gel. The molar ratios between NF-κB and IκBα were 1:3, 1:10, and 1:30, whereas those between NF-κB and IκBβ were 1:10, 1:30, and 1:100. (C) Results of a representative in vitro transcription experiment showing that NF-κB is stably bound to the promoter during formation of PICs. The kinetic schemes are shown on the right of the gel.

that the interactions between NF-κB and components of the basal machinery stabilize its association with the promoter. To examine this possibility, we carried out in vitro transcription experiments in the presence of a 125-fold excess of PRDII oligonucleotide added at different times during the in vitro transcription experiment. As shown in Fig. 9C, addition of the PRDII competitor after the incubation of NF-κB with the template completely inhibited activation of transcription (compare lane 3 with lane 4). However, when the competitor oligonucleotide was added after the formation of a functional PIC, it did not inhibit single-round transcription, indicating that NF-κB is stably bound to the promoter (Fig. 9C, compare lane 11 with lane 12). As expected, in the absence of Sarkosyl the oligonucleotide competitor decreased transcription threefold, indicating that NF-κB dissociates from the promoter after each round of transcription (Fig. 9C, compare lane 7 with lane 8). In conclusion, our experiments strongly suggest that IκBα but not IκBβ can remove NF-κB during the process of activation of transcription.

DISCUSSION

Members of the IκB family of proteins interact with NF-κB/Rel proteins and inhibit their transcriptional activity by cytoplasmic sequestration (6, 9, 60, 63). In this paper we showed that in addition to cytoplasmic sequestration, IκBs function in the nucleus to inhibit NF-κB DNA binding and therefore terminate the NF-κB-dependent transcriptional activation. In principle, the NF-κB/IκB complexes are restricted in the cytoplasm because IκBs mask the NLSs of the NF-κB proteins. However, IκBs can also be detected in the nucleus, especially after their reaccumulation (1, 47), where we showed that they function by removing NF-κB bound on promoters. Inhibition of IκBα resynthesis or inactivation of the IκBα gene results in prolonged activation of NF-κB (1, 12, 32, 47) and persistent transcription of its target genes. Thus, IκBs function as postinduction repressors of NF-κB.

We carried out a comparative analysis of the mechanisms by which IκBα and IκBβ function to inhibit NF-κB-dependent transcriptional activation either in vivo or in vitro. The most striking difference between IκBα and IκBβ is that recombinant unphosphorylated IκBβ is a very weak inhibitor of NF-κB binding and that it forms ternary complexes with NF-κB on the DNA. Moreover, deletion of the IκBβ PEST sequence gives rise to a protein that is stably associated with NF-κB on the DNA without inhibiting DNA binding. In sharp contrast, recombinant IκBα inhibits NF-κB binding very efficiently, and removal of its PEST sequence decreases its ability to associate with NF-κB in vitro and in vivo in either the presence or the absence of DNA (references 8, 20, 49, and 62a and our unpublished observations). Thus, in both cases the PEST sequence is required for inhibition of DNA binding, but in IκBα it is also required for strengthening the protein-protein interactions with NF-κB. On the other hand, the ankyrin repeats in IκBβ, but not those in IκBα, suffice for stable interaction with NF-κB on the DNA. This functional distinction may be due either to the number of ankyrin repeats per se, six in IκBβ versus five in IκBα, or to specific amino acid sequences in the repeats.

Our results taken together with previous studies (reviewed in reference 63) suggest that although the ankyrin repeats of IκBs are required for association with NF-κB, they are not sufficient for inhibition of DNA binding in the absence of the C-terminal acidic region. How does the acidic region of the IκBs function to assist in inhibition of NF-κB DNA binding? We showed that phosphorylation of IκBβ by CKII at the acidic region converts IκBβ from a weak to a strong inhibitor of NF-κB binding. Similar results have been reported for the ability of IκBβ to inhibit c-Rel DNA binding (18). By contrast, the ability of IκBα to inhibit NF-κB binding is not significantly increased by CKII phosphorylation (8). Thus, the acidic C-terminal regions of IκBα and IκBβ have distinct requirements for phosphate groups in order to assist in inhibition of NF-κB binding. Comparison of IκBα and IκBβ C-terminal sequences revealed that there are 14 and 9 negative amino acids in IκBα and IκBβ, respectively. Moreover, there are two putative CKII phosphorylation sites at the C terminus of IκBβ (18), and if these sites are fully phosphorylated, then the total charge of -9 prior to phosphorylation would be -13 after CKII phosphorylation. Thus, the weak inhibitory activity of the unphosphorylated IκBβ maybe due to the low abundance of acidic amino acids at its carboxyl terminus. This idea is also supported by previous studies on IκBα, where a decrease of the negative charges at the carboxyl terminus adversely decreased its inhibitory activity on NF-κB binding (20). The acidic domains of IκBα and IκBβ may directly interact with the positively charged DNA binding surface of NF-κB (23, 45), thus competing with the DNA for these interactions. Alternatively, a strong acidic motif may simply be used by IκBs to dissociate the NF-κB from the DNA by repulsive electrostatic forces with the negatively charged DNA. In summary, our experiments taken together with other in vivo and in vitro studies (18, 31, 41, 57) establish that phosphorylation of IκBβ by CKII is required for inhibition of NF-κB DNA binding. It is important to mention here that an interaction between IκBβ and NF-κB, even on the DNA, does not suffice for dissociation of NF-κB, as revealed by the property of IκBβΔC to form stable complexes with NF-κB on the DNA.

We showed that recombinant and unphosphorylated IκBβ removes NF-κB very efficiently from ternary complexes containing HMG I at PRDII. However, HMG I does not alter the efficiency with which IκBα inhibits NF-κB binding at this site. Since HMG I and NF-κB directly contact each other (58, 63a), we believe that these interactions induce a conformational change in NF-κB in such a way that allows IκBβ, but not IκBα, to interact in a qualitatively different manner with the RHR. These putative conformational changes induced by HMG I on NF-κB require DNA binding of both proteins, since HMG I does not alter IκBβ function when NF-κB binding sites that do not bind HMG I (57), such as the IgκB element, are used. The in vivo significance of these observations was demonstrated in transfection experiments where we showed that the reporter construct containing the PRDII element is inhibited more efficiently by IκBβ than the IgκB reporter which does not bind HMG I.

An intriguing finding of this study is that IκBα and IκBβ function as inhibitors of NF-κB not only in the cytoplasm but also in the nucleus. Previous studies have shown that both IκBs interact with the same spectrum of Rel proteins and restrict them in the cytoplasm. In this paper we provided additional evidence that IκBs can also work in the nucleus to inhibit NF-κB from binding to promoters and/or remove NF-κB from actively transcribed promoters. Similar results have been reported for the *Drosophila* proteins cactus (IκB homolog) and dorsal (NF-κB homolog) in yeast cells (36). Thus, IκBs function as postinduction inhibitors of NF-κB-dependent gene activation. Following IκBα degradation and subsequent NF-κB activation, the IκBα protein is rapidly accumulated, resets the NF-κB switch in the cytoplasm, and enters the nucleus, where as we showed, it represses NF-κB. Several observations are consistent with this model. First, IκBα has been detected in the nucleus complexed with NF-κB even in unstimulated cells (47).

Second, in IκBα−/− cells but not in wild-type cells, NF-κB binding persists for several hours following TNF-α induction (12, 32). Third, we showed that once in the nucleus, IκBα inhibits NF-κB binding to promoters, resulting in repression of NF-κB-dependent transcription. Finally, in in vitro transcription experiments we established that IκBα can remove NF-κB from functional PICs. The last observation has important implications for the mechanics of IκBα action. That is, IκBα not only interacts with nuclear NF-κB off the DNA but can also remove NF-κB from a promoter during the process of transcriptional activation. Interestingly, at this step NF-κB is so tightly bound to the promoter that it cannot be removed by cold oligonucleotide competitors. In contrast, the NF-κB/promoter interactions become weaker between sequential rounds of transcription, as revealed by oligonucleotide competitions in in vitro transcription experiments. Taken together, these experiments strongly suggest that IκBα works as a postinduction repressor of NF-κB.

In the case of IκBβ, we have shown that its ability to remove NF-κB from the DNA critically depends on phosphorylation of the C-terminal PEST sequence by CKII and on whether NF-κB is complexed with HMG I. Thus, in contrast to IκBα, IκBβ can efficiently inhibit NF-κB only when one or both of these requirements are fulfilled in the living cell. Interestingly, it is only the nonphosphorylated form of IκBβ that can be detected in the nucleus shortly after IκBβ resynthesis following degradation (57). Unphosphorylated IκBβ interacts with NF-κB bound to promoter elements, and we showed in both in vivo and in vitro experiments that the association of IκBβ with NF-κB does not interfere with the ability of NF-κB to stimulate transcription. Thus, it is possible that in some genes regulated by NF-κB, nuclear unphosphorylated IκBβ is part of the enhancer complex and may influence transcription. Interestingly, BCL-3, which is another IκB-like protein, forms complexes with the p50 or p52 subunit of NF-κB on promoters in vivo and in vitro (13, 22). Consistent with this possibility is our observation that IκBβ directly interacts with a component of the basal machinery (unpublished results). However, in cases like that of the IFN-β gene, where HMG I is required for NF-κB activation (58), unphosphorylated IκBβ efficiently inhibits NF-κB binding to the promoter. Thus, nuclear IκBβ may function as a gene-specific inhibitor of NF-κB.

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