Volume 3 · Number 2 · 1993
GENE EXPRESSION

Diverse molecular mechanisms of inhibition of NF-κB/DNA binding complexes by IκB proteins

Thierry Léveillard and Inder M. Verma

Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California

The activity of the rel/NF-κB/dorsal family of κB site binding proteins is regulated by IκB proteins. The ankyrin repeat motif identified in IκB family members, which include IκBα (pp40/MAD-3), IκBγ, and bcl3, directly associates with κB site binding proteins, resulting in specific DNA-binding inhibition of rel, p50, or p65 dimers. We report that IκBγ, containing eight ankyrin repeats, mediates a reversible inhibition of $(p50)_2$-DNA complex but cannot displace preformed DNA–protein complexes. IκBα and bcl3, on the other hand, can displace preformed DNA–protein complexes. IκBα specifically displaces $(p65)_2$ or p50/p65-DNA complexes but requires the C-terminal 37 amino acids in addition to the ankyrin repeat domain. Human bcl3 protein specifically displaces $(p50)_2$-DNA complexes. Because IκBα and bcl3 can displace preformed $(p65)_2$ or $(p50)_2$-DNA complexes, respectively, we propose that they can act as repressors or antirepressors of NF-κB-induced gene expression.

NF-κB was first identified as a lymphoid-specific transcription factor that bound to a decameric sequence (GGGACTTTCC)—the κB site—present in the enhancer region of the κ light chain (Sen and Baltimore, 1986a,b). Since then, the family of NF-κB proteins has been extended to include many lymphoid and non-lymphoid κB site binding proteins, the product of protooncogene rel and the morphogen dorsal in Drosophila (Steward, 1987; Kieran et al., 1990; Ghosh et al., 1990). Myriad genes implicated in the immune and acute phase responses, as well as replication of HIV, appear to be regulated by κB proteins (Baeuerle, 1991). In most unstimulated cells, NF-κB is sequestered in the cytoplasm, complexed with a family of inhibitor proteins referred to as IκB (Baeuerle and Baltimore, 1988a,b; Zabel and Baeuerle, 1990). Stimulation by a wide variety of agents such as antigens for lymphocytes, lipopolysaccharide (LPS), cytokines like tumor necrosis factor α and IL-1, the tumor promoter phorbol myristate acetate (PMA), UV light, oxygen radicals, double-strand RNA, and viral infection leads to the release of NF-κB from IκB, which then translocates to the nucleus and activates target genes (Baeuerle and Baltimore, 1989; Baldwin et al., 1991; Lenardo and Baltimore, 1989; Schreck et al., 1991; Stein et al., 1989). Dissociation of the NF-κB/IκB complex is thought to be regulated by phosphorylation of the inhibitor protein (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Kerr et al., 1991).

Biochemical analysis reveals that NF-κB is composed of 50 kDa (p50) and 65 kDa (p65) proteins that share extensive amino acid sequence homology with v-rel, the transforming protein of reticuloendotheliosis virus strain T,

Received February 2, 1993; revision accepted March 24, 1993.

Correspondence: Inder M. Verma, Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92186-5800 Tel (619) 453-4100, ext. 462 Fax (619) 558-7454

 1052-2166/93/0302/135-16$2.00

its cellular homologue c-rel, and the Drosophila morphogen dorsal (Theilen et al., 1966; Steward, 1987; Kawakami et al., 1988; Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Gilmore, 1990). The homology domain extends over 300 amino acids at the N-terminus, which includes the DNA-binding, dimerization, and IκB binding regions, and has been referred to as the rel homology domain (Gilmore, 1990). The p50 subunit of NF-κB is processed from a 105 kDa (p105) precursor in an ATP-dependent reaction (Fan and Maniatis, 1991). However, no cellular protease(s) involved in this processing event has been characterized to date. The precursor (p105) does not efficiently bind to DNA and is found in the cytoplasm (Blank et al., 1991), or in the nucleus associated with the transcriptional activator *tax* in HTLV-1-infected cells (Hirai et al., 1992). The sequences in the C-terminal half of p105 that inhibit DNA binding contain eight ankyrin repeats of 30–34 amino acids each. These repeats were first described in cdc10 of Schizosaccharomyces pombe and SW16 and SW14 of Saccharomyce cerevisiae proteins involved in cell-cycle control (Aves et al., 1985; Breeden and Nasmyth, 1987; Andrew and Hershkowitz, 1989). This motif, thought to mediate protein–protein interaction, has now been found in a number of unrelated proteins involved in cell growth and differentiation (Blank et al., 1992). More interestingly, the ankyrin repeat motifs have been identified in the inhibitors of DNA-binding activity of NF-κB/rel-related proteins (Kerr et al., 1991, 1992). The amino acid sequence of chicken phosphoprotein pp40 and MAD-3, an intermediate-early gene induced in adherent monocytes (both of them are referred here as IκBα) reveals five ankyrin repeats (Davis et al., 1991; Kerr et al., 1991; Haskill et al., 1991). The proto-oncogene bcl3 found on chromosome 19 adjacent to the breakpoint in the translocation t(14;19), which occurs in some cases of chronic lymphocytic leukemia, codes for a protein containing seven tandem copies of the ankyrin motif (Ohno et al., 1990). IκBγ, a 70 kDa protein generated from a subgenomic messenger RNA in a variety of lymphoid cell lines, is identical to the C-terminal half of p105 and includes eight ankyrin repeats (Inoue et al., 1992a). A p105-related protein, p100, also contains eight ankyrin repeats in the C-terminus (Schmid et al., 1991; Neri et al., 1991), but no corresponding C-terminal protein has yet been identified. Cactus, an inhibitor of dorsal binding to DNA, has recently been molecularly cloned and contains six complete ankyrin repeats (Geisler et al., 1992; Kidd, 1992). Functional analysis of ankryin-containing IκB inhibitors has led to the emergence of two classes of activities. The first class, represented by IκBα (pp40/MAD-3), inhibits DNA binding of the heterodimeric p50/p65 NF-κB complex and rel complexes, but not p50 homodimers (Zabel and Baeuerle, 1990; Kerr et al., 1991; Haskill et al., 1991). The second class includes IκBγ and bcl-3, which inhibit DNA binding of p50/p65 and the $(p50)_2$ homodimer (Inoue et al., 1992a; Hatada et al., 1992; Liou et al., 1992; Kerr et al., 1992). The bcl3 protein, however, does not inhibit DNA binding of c-rel (Kerr et al., 1992). The finding that different classes of inhibitors distinguish between different classes of NF-κB/rel-related complexes raises the very interesting question of how cells regulate the binding at κB sites.

We report that ankyrin repeats in IκB proteins directly interact with the members of the rel/NF-κB family, resulting in specific DNA-binding inhibition of p50 or p65 homodimers. IκBγ mediates a reversible inhibition of $(p50)_2$-DNA binding complex but is unable to displace preformed DNA–protein complex. In contrast, IκBα and bcl3 can displace preformed DNA–protein complexes. IκBα (pp40/MAD-3) specifically displaces $(p65)_2$ or p50/65 complexes, while human bcl-3 protein specifically displaces $(p50)_2$ complex. The ankyrin repeat domain of IκBα is necessary, but not sufficient, for displacing $(p65)_2$ preformed complexes. The C-terminal domain of IκBα is additionally required for displacement activity. We propose that both IκBα and bcl3 can potentially act as repressors or antirepressors of NF-κB-induced transcriptional activation.

Materials and methods

Plasmid construction

Plasmids for overproducing glutathione-S-transferase (GST)-fused proteins were constructed using pT7GT (L. J. Ransone, unpublished data), in which GST is under the control of a T7 promoter. Plasmid pGST-IκBγΔN (construct A) is a slight modification of pGST70ΔGS (Inoue et al., 1992a). A 1892 bp Hind III fragment from pBS70HN (constructed from mouse

p70 cDNA cloned in Bluescript SK− (Inoue et al., 1992a) using site-directed mutagenesis) was put in-frame of a GST peptide leader to generate a 704 amino acid GST-fused protein, 471 of which at the C-terminal end correspond to IκBγ sequence from amino acid position 137 to 607. Briefly, two oligonucleotides were designed to introduce Hind III and Nhe I sites at positions 592 and 1493, respectively, of the IκBγ nucleotide sequence. Introduction of the Hind III site changes amino residues AL at position 137, 138 to PM, and the Nhe I site changes residues PQ at 436, 437 to AS. Construction of pGST-IκBΔN (construct E) and pGST-ANK-IκB (construct H) have been described previously (Inoue et al., 1992b).

In order to make the chimeric construct pGST-ANK5-C70 (construct F), a Bluescript construct pBS-ANK5-C70 was created by cleaving pBS IκB* (Inoue et al., 1992b) with Xba I in the Bluescript sequence downstream of the chicken pp40 cDNA (IκBα) sequence, filling in with Klenow fragment DNA polymerase I, then redigesting with Spe I at position 2289 to isolate a 3788 bp fragment. pBS70HN was cleaved with Nhe I (position 1493) and EcoR 1 (Bluescript sequence downstream of the IκBγ sequence) to isolate a 997 bp fragment. Both fragments were then religated. A 1542 bp Nhe I-EcoR I fragment from pBS-ANK5-C70 was isolated, filled in with Klenow fragment DNA polymerase I, and ligated with a filled-in BspD I site of pT7GT to overproduce a 591 amino acid GST-fused protein.

pGST-ANK8-C40 (construct B) was generated by digesting pBS70HN with Nhe I and EcoR V as above, and a 4450 bp fragment was isolated. pBS IκB* was cleaved by Spe I (position 2289) and Sma I downstream of pp40 cDNA (IκBα) sequence to isolate a 911 bp fragment. Both fragments were religated to create pBS-ANK8-C40. A 1814 bp Hind III fragment (position 592 of IκBγ nucleotide sequence to position downstream in the Bluescript sequence) was isolated and cloned in-frame in Hind III site of pT7GT to overproduce a 598 amino acid GST-fused protein.

pGST-ANK8 (construct C) was created by digesting plasmid pGST-IκBγΔN (construct A) with Nhe I at position 1493, filled in with Klenow fragment DNA polymerase I, digested with Dra III at position 2033. After 3′ overhang ends were removed using T4 DNA polymerase, a 4590 bp fragment was isolated and religated. Peptide sequence CLF was added at the C-terminal end of the protein. pGST-ANK8 (construct C) encodes a 534 amino acid GST-fused protein.

pGST-IκBγΔN was cleaved with Kpn I at positions 1400 and 1685; 3′ overhang ends were removed with T4 DNA polymerase and religated to generate pGST-ANK7γ (construct D), with peptide sequence ASKS added at the C-terminal end of the protein to produce a 504 amino acid GST-fused protein.

To generate pGST-ANK6α (construct G), pGST-IκBΔN (construct E) was digested with Alwn I (position 2388) and BstE II (position 2988), 3′ overhang Alwn I ends were removed, 5′ BstE II overhang ends were filled in with T4 DNA polymerase, and the products were religated. Peptide sequence VTWLLGGR was added at the C-terminal end of the 459 amino acid GST-fused protein.

pGST-ANK10-C40 (construct I) was created by cleaving pBSIκB* with Nhe I and Spe I (positions 1746 and 2289 respectively). A 543 bp fragment was isolated, then ligated in-frame in the Spe I site at position 2289 in pGST-IκBΔN (construct E) to overproduce a 671 amino acid protein.

pGST-IκB-ΔANK$_1$ (construct J) was generated by cleaving pBS-IκB-ANK1 (Inoue et al., 1992b) with EcoR I at positions 1469 and downstream of the coding sequence to isolate a 1718 bp fragment that was filled in with Klenow DNA polymerase I and ligated in-frame in the filled-in BspD I site of pT7GT to overproduce a 577 amino acid protein.

pGM-GST-p65 (L. D. Kerr, unpublished data) was created by inserting with site-directed mutagenesis an EcoR I site in-frame of the initiating ATG of human p65 cDNA (Nolan et al., 1991). The 1668 bp EcoR I fragment was then ligated in the EcoR I site of T7 vector pGM-GST (L. J. Ransone, unpublished data) to overproduce a GST-fused full-length p65 protein.

pETp50-361ΔC was constructed by polymerase chain reaction, using oligonucleotides GGG ATCCATCTTCACCATGGA and GGCGAATTC TCACCTTTGCACTTC and modified mouse p105 cDNA (pBS-F-NF-κB-p105), which contains flu epitope (J.-I. Inoue, unpublished), as a template (Ghosh et al., 1990). The 1093 BamH I-EcoR I fragment was ligated in pBluescript SK− to create pBSp50-361, which was then cleaved with Nco I (position 292) and EcoR I (downstream

coding sequence). The 1080 bp fragment was ligated into pET11d (Novagen) between the Nco I and EcoR I sites. A 361 amino acid protein was overproduced using the p105 initiating codon.

pBSIκB*TS (mutant M) was created by site-directed mutagenesis using pBSIκB* and the oligonucleotide GTCTTCCTCCAATACTCGAG TCATCATACATAAG in order to replace two cyteines at positions 309–310 of pp40 with two serine residues.

pBScLK2 encodes human bcl-3 cDNA (Ohno et al., 1990) inserted in the EcoR I site of Bluescript SKII+ (Stratagene) using Not I-EcoR I linker adaptators (T. W. McKeithan, unpublished data).

All recombinant constructs were checked by either di-deoxy sequencing or restriction mapping or GST-fused protein expression and/or Western blotting.

Bacterial protein overproduction and purification

Overproduction of bacterial GST-fused proteins was done as previously described (Inoue et al., 1992a) with minor modifications. Briefly, E. coli strain BL21 was transformed with each plasmid and then induced for overproduction by adding isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hours at 37°C. GST-fusion proteins were then purified using glutathione-sepharose (Pharmacia). After elution with reduced glutathione, purified proteins were dialyzed against one liter of buffer B [15 mM Tris, pH 7.5, 75 mM NaCl, 7.5% (v/v) glycerol, 1.5 mM dithiotreitol (DTT), 1.5 mM EDTA, 0.5 mM phenylmethylsulfonyl-fluoride (PMSF), and 2.5 μg/μl pepstatin A and leupeptin].

Bacterial p50-361ΔC was overproduced by induction by IPTG then purified as follows. Cells were harvested, resuspended in MTPBS [1% Triton X-100, 1% Tween 20, 10 mM DTT in phosphate-buffered saline (PBS; pH 7.3)] and disrupted by sonication. Cell debris was pelleted by centrifugation, and the supernatant was dialyzed against buffer B. p50-361ΔC protein was precipitated with 32% saturated amonium sulfate (Pognonec et al., 1991), resuspended in buffer B, then purified on a κB site DNA affinity column (Kadanoga and Tjian, 1986). Protein fractions eluted in a 1 M NaCl step were pooled and dialyzed against buffer B. Bacterial p50 was a generous gift of C. S. Duckett and was produced from a pET11-p50 construct using the parental initiating ATG and encoding mouse p50, including the glycine-rich region to position 412. Bacterial bcl3 was a generous gift of T. W. McKeithan and was overproduced using a pET11-bcl3 clone encoding the full-length human bcl3. GST-p65 was purified on glutathione sepharose.

Gel shift assay

Electrophoretic mobility shift assays were done as follows. Bacterially expressed DNA-binding protein was mixed with 10–200 ng of bacterial inhibitor protein for 30 minutes on ice in binding buffer [15 mM Tris; pH 7.5, 75 mM NaCl, 7.5% (v/v) glycerol, 1.5 mM DTT, and 100 μg/μl bovine serum albumin (BSA)], followed by the addition of 4 fmoles of polynucleotide kinase ^{32}P-labeled HIV κB oligonucleotide (AGCTTC AGAGGGGACTTTCCGAGAGG/AGTCTCCCC TGAAAGGCTCTCCAGCT). After incubation for the indicated time at 24°C, protein–DNA complexes were resolved by electrophoresis on 4% polyacrylamide gel (37.5:1 acrylamide:bis) as described previously (Léveillard et al., 1991). In some cases, bacterial DNA-binding proteins were preincubated with 4 fmoles of kinased κB oligonucleotide for 15 minutes at 24°C in binding buffer. Simultaneously, bacterial inhibitor proteins or in vitro translated proteins were incubated with 400 fmoles of unlabeled κB oligonucleotide. Both pre-incubated reactions were mixed together then analyzed after a 10 to 20 minute incubation at 24°C.

Protein–protein association assay

^{35}S-labeled proteins were translated in vitro in the presence of L-[^{35}S]methionine by using wheat germ extract (Fig. 3) or rabbit reticulocyte lysate. ^{35}S-labeled proteins were mixed with GST-fused inhibitor proteins for one hour at 4°C in gel shift binding buffer; glutathione-sepharose (Pharmacia) was then added, followed by incubation for 30 minutes at the same temperature with agitation. Protein complex attached to sepharose was washed twice with a hundred volume of 40 mM Tris, pH 7.5; 150 mM NaCl; 0.02% Triton-X100 and analyzed by 10% SDS/polyacrylamide gel electrophoresis (SDS/PAGE).

Results

Ankyrin repeats of IκBγ can mediate $(p50)_2$-DNA binding inhibition

Our first goal was to investigate the contribution of the ankyrin domains of IκB inhibitors. We designed GST-fusion plasmid constructs encoding various regions of the inhibitors, mouse IκBγ and chicken pp40 (MAD-3/IκBα). The recombinant constructs (A through K) are schematically drawn in Figure 1. A Coomassie blue-stained pattern of GST-fused proteins expressed in bacteria following purification on glutathione beads is shown in Figure 2A. All the chimeric constructs are able to synthesize the expected full-length proteins.

The bacterially expressed, purified chimeric proteins were assayed for their ability to inhibit the DNA binding activity of p50. Results in Figure 2B show that, as expected, construct A (GST-IκBγΔN) is able to prevent DNA binding of p50 homodimer $(p50)_2$ (lane 2), whereas construct E (GST-IκBαΔN) has little or no inhibitory effect (lane 5; Inoue et al., 1992a,b). A fused protein corresponding to the ankyrin repeat region of IκBγ (construct C) is able to inhibit DNA binding (lane 4), suggesting that the ankyrin repeat domain of IκBγ is sufficient to mediate DNA-binding inhibition. Construct B contains the ankyrin repeat domain of IκBγ, as well as the C-terminal region of IκBα, and—like construct C (lane 4)—this chimeric protein is capable of inhibiting DNA-binding activity (lane 3). These results indicate that the ankyrin repeat domain of IκBγ is sufficient to mediate DNA-binding inhibition of $(p50)_2$.

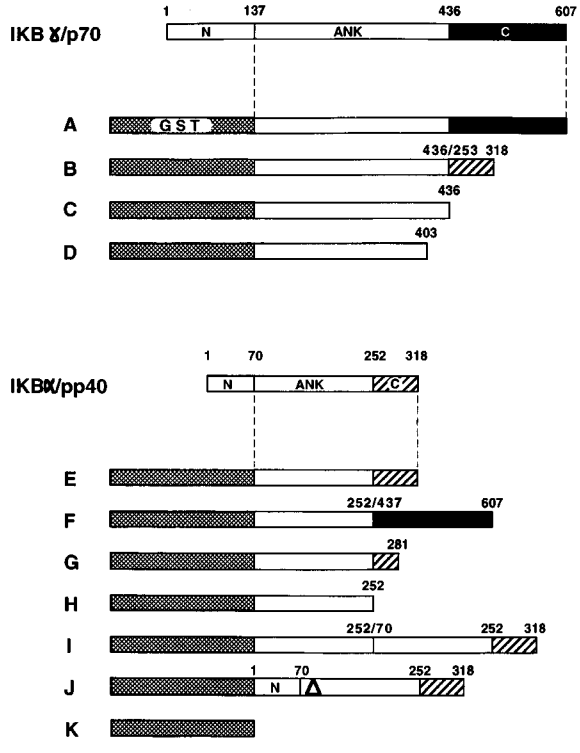

Figure 1. Schematic representation of the GST-fusion proteins. Constructs are denoted alphabetically on the left. Cross-hatched portions represent the GST sequence; open boxes represent ankyrin repeats; filled boxes represent the C-terminal region of IκBγ; striped boxes represent the C-terminus of pp40 (IκBα); the open triangle represents mutated ankyrin repeat one in pp40 (IκBα). Amino acid positions where fusions or truncations were carried out are indicated.

We have previously shown that IκBα (pp40/MAD-3) inhibits the DNA-binding activity of NF-κB complex but has no effect on the DNA-binding activity of $(p50)_2$ (Kerr et al., 1991; Inoue et al., 1992b; construct E, lane 5). Similarly, constructs F and H, containing either the five ankyrin repeats of IκBα linked to the C-terminus of IκBγ or the five ankyrin repeats alone, do not inhibit DNA binding (lanes 6 and 7). To analyze further whether all eight ankyrin repeats of IκBγ are needed for its inhibitory activity, we deleted the C-terminal (eighth) ankyrin repeat to generate construct D, which is unable to inhibit DNA-binding activity of $(p50)_2$ (lane 11). We therefore conclude that the inhibition of DNA-binding activity of the $(p50)_2$ homodimer by IκBγ requires an intact ankyrin repeat domain. Furthermore, the ankyrin repeat domain alone is sufficient to mediate DNA-binding inhibition.

In an analogous experiment, we tested the ability of the same set of GST-fusion proteins to prevent DNA binding of the p65 homodimer, $(p65)_2$ (Fig. 2C). Construct A, containing eight IκBγ ankyrin repeats, inhibited binding of $(p65)_2$ to the κB site (compare lanes 3 and 4). Construct B also fully inhibited $(p65)_2$-DNA complex formation (lane 5), whereas construct D, lacking the eighth ankyrin repeat, was only capable of partial inhibition (lane 6). As reported before, IκBα (pp40/MAD-3; construct E) is also able to prevent $(p65)_2$ binding (lane 7; Inoue et al., 1992b). Construct F, lacking the C-terminal domain of IκBα but containing the

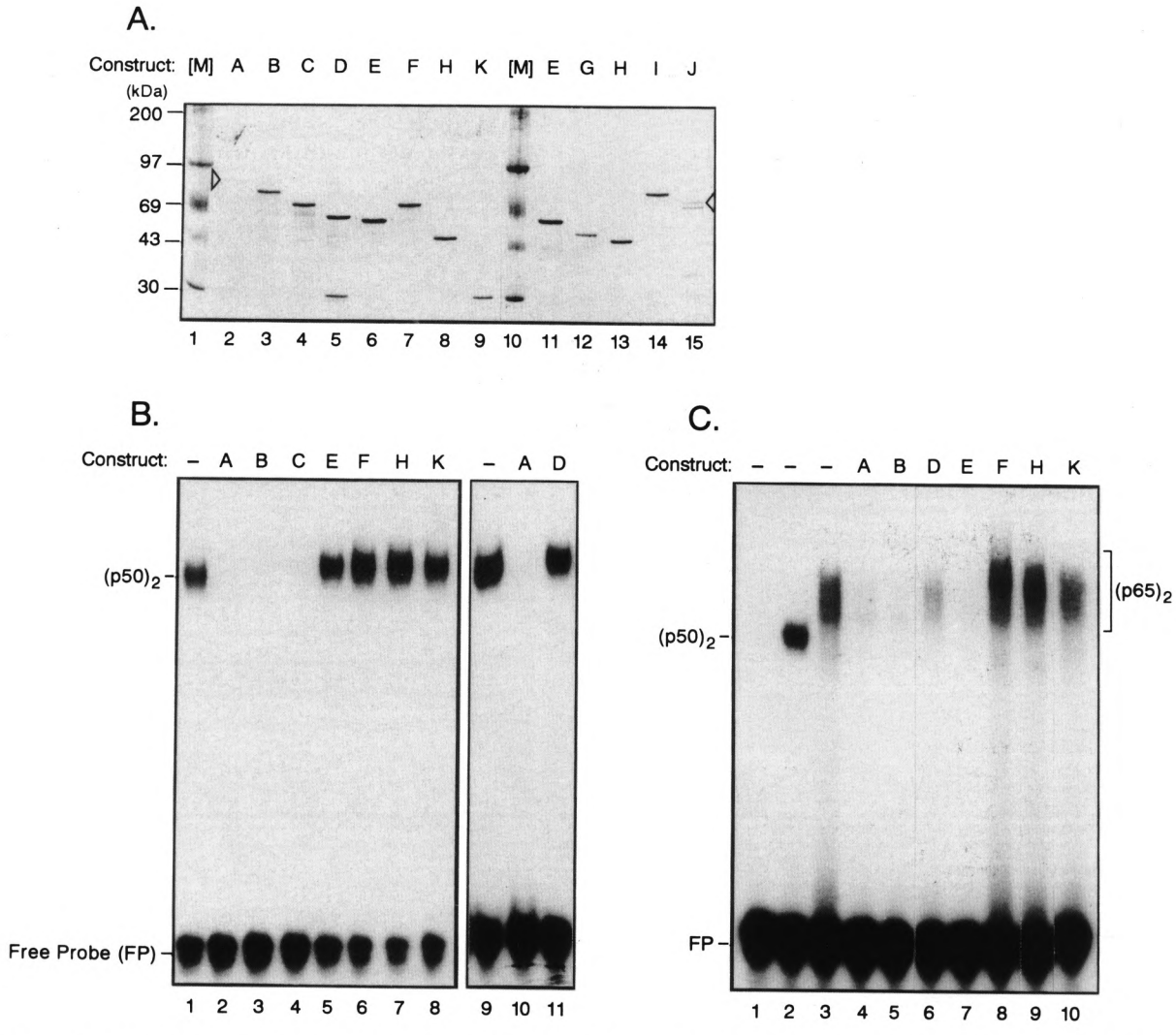

Figure 2. DNA-binding activity of chimeric constructs. **A.** Size analysis. Coomassie-stained, 10% SDS/PAGE of the GST-fused inhibitor proteins. Lane 1 [M], molecular weight markers; lane 2, construct A (GST-IκBγΔN); lane 3, construct B (GST-ANK8-C40); lane 4, construct C (GST-ANK8); lane 5, construct D (GST-ANK7γ); lane 6, construct E (GST-IκBΔN); lane 7, construct F (GST-ANK5-C70); lane 8, construct H (GST-ANK-IκB); lane 9, construct K (GST); lane 10, markers (M); lane 11, construct E; lane 12, construct G (GST-ANK6α); lane 13, construct H; lane 14, construct I (GST-ANK10-C40); lane 15, construct J (GST-ΔANK1). **B.** DNA-binding inhibition of $(p50)_2$. GST-fusion proteins incubated with bacterially expressed p50 (5 ng) and labeled κB oligonucleotide were resolved on 4% nondenaturing gel. Lane 1, bacterially expressed p50 (5 ng); lane 2, construct A (50 ng); lane 3, construct B (100 ng); lane 4, construct C (100 ng); lane 5, construct E (100 ng); lane 6, construct F (100 ng); lane 7, construct H (100 ng); lane 8, construct K (100 ng); lane 9, p50 (5 ng); lane 10, construct A (50 ng); lane 11, construct D (100 ng). Lanes 1–8 and 9–11 correspond to two separate experiments. Positions of the $(p50)_2$-DNA complex and the free probe (FP) are indicated. **C.** DNA-binding inhibition of $(p65)_2$. GST-fusion proteins were incubated with bacterially expressed GST-p65 (5 ng) and analyzed as described in Materials and Methods. Lane 1, DNA alone; lane 2, p50 (5 ng); lane 3, GST-p65 (5 ng); lane 4, construct A (100 ng); lane 5, construct B (100 ng); lane 6, construct D (100 ng); lane 7, construct E (50 ng); lane 8, construct F (50 ng); lane 9, construct H (50 ng); lane 10, GST control (100 ng). The overproduction and purification of GST-p65 result in multiple bands on SDS polyacrylamide gel; they are thought to be proteolytic products following induction. This might explain the apparent heterogeneity of the $(GST\text{-}p65)_2$-DNA complex.

C-terminal of IκBγ as substitute, was unable to inhibit the formation of $(p65)_2$-DNA complex. Similarly, construct H, containing the five IκBα ankyrin repeats but missing the entire C-terminus, showed no inhibitory activity (lane 9), emphasizing that inhibition by IκBα requires both the intact ankyrin repeat domain and the C-terminus. The enhanced binding in the presence of constructs F and H is likely due to stabilization of $(p65)_2$-DNA complex, because both of these proteins can associate with p65 (see Fig. 3A). In contrast, IκBγ can mediate DNA-binding inhibition solely by the ankyrin repeat domain. Similar results were obtained with a number of constructs using purified NF-κB complex (p50/p65) from human placenta (data not shown). In the experiments carried out here, we chose p65 instead of p50/p65 complex because of the lack of sufficient amounts of purified p50/p65 complex.

Physical association of IκB with p50 and p65

To gain insight into the mechanism of inhibition, we tested the association of various inhibitor constructs with in vitro translated p50 and p65. Briefly, GST-fused chimeric proteins (Fig. 1) were mixed with ^{35}S-labeled in vitro translated p50 or p65 proteins in buffer conditions under which gel shift assays shown in Figure 2 were carried out. Figure 3A shows that both the IκBγ- and IκBα-based chimeric proteins associated with p65, whereas the control construct, generating only GST protein (construct K, lane 2), shows no association. Although most of the association is with full-length p65, shorter proteins, probably representing premature termination or internal initiation, can also be observed. Association of IκB inhibitors with p65 is not sufficient, however, to cause DNA-binding inhibition. Constructs F and H are unable to inhibit DNA binding of p65 (Fig. 2C, lanes 8 and 9), even though they associate with p65—as does construct E, which *is* capable of DNA-binding inhibition (Fig. 2C, lane 6). Construct G is also able to associate with p65 (data not shown).

We also tested the association of various IκB chimeric constructs with p50 (Fig. 3B). The IκBγ constructs containing eight intact ankyrin repeats associate with p50 (Fig. 3B, lanes 11–13). Construct E, containing five intact ankyrin repeats of IκBα and the C-terminus, also associates (lane 14), but mutants either lacking the C-terminus (lane 16) or containing the C-terminus of IκBγ (lane 15) show no association with p50 (Inoue et al., 1992b). Therefore, it appears that IκBα constructs require an intact C-terminus for association with p50 (Fig. 3B). Once again, association of the inhibitor with κB protein is not sufficient for inhibitory activity, be-

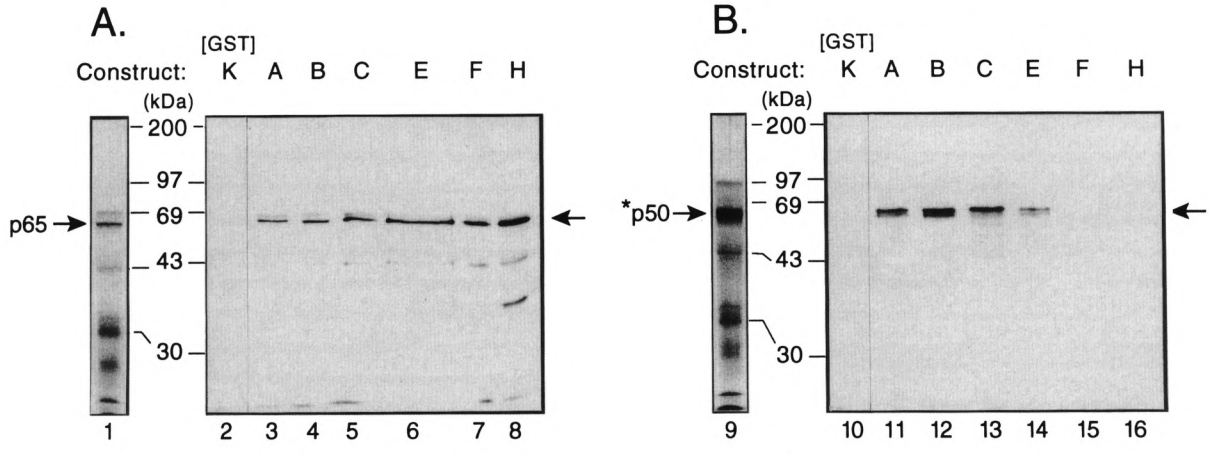

Figure 3. Association assay between GST-fused inhibitor constructs and in vitro translated p65 (**A**) and p50 (**B**). p65 (lanes 1–8) or *p50 (flu-epitope-tagged; lanes 9–16) translated in vitro, in wheat germ extract (Promega) were incubated with GST-fused protein inhibitors as described and were analyzed by 10% SDS/PAGE. Lanes 1 and 9, in vitro translated p65 and p50 respectively; lanes 2 and 10, GST control; lanes 3 and 11, construct A; lanes 4 and 12, construct B; lanes 5 and 13, construct C; lanes 6 and 14, construct E; lanes 7 and 15, construct F; lanes 8 and 16, construct H. Molecular weight standards are indicated.

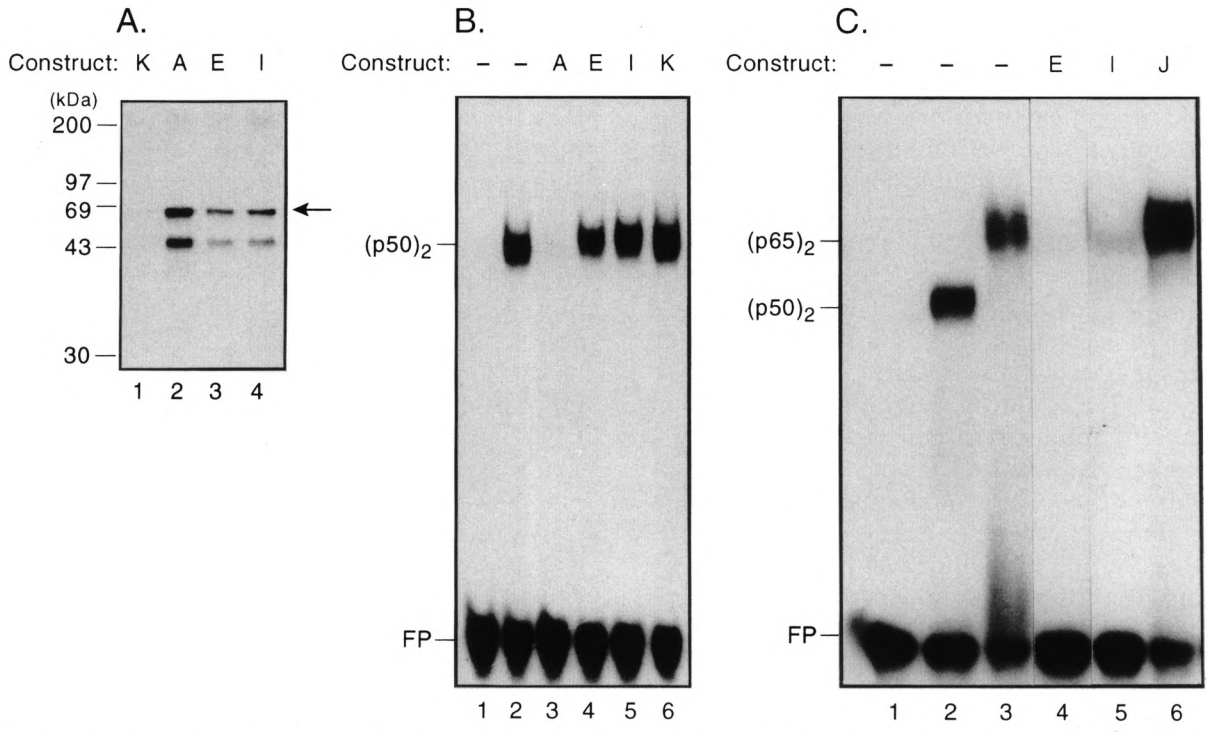

Figure 4. Analysis of constructs containing ten ankyrin repeats (construct I). **A.** Association assays. In vitro translated flu-tagged *p50 RNA using rabbit reticulocyte lysate (Promega) was used as source of labeled p50. Constructs are denoted alphabetically at the top of each lane. The full-length epitope-tagged p50 product is indicated by an arrow. A Coomassie blue-stained 10% polyacrylamide/SDS gel of construct I is shown in Figure 2A, lane 14. **B.** Ten ankyrin repeats of pp40 (IκBα) sequence do not prevent $(p50)_2$-DNA binding. Bacterially expressed p50 (5 ng) protein was incubated with labeled κB oligonucleotide and chimeric IκB proteins; lane 1, probe alone; lane 2, p50 (5 ng); lane 3, construct A (100 ng); lane 4, construct E (50 ng); lane 5, construct I (50 ng); lane 6, control construct K (100 ng). $(p50)_2$-DNA complex and free probe (FP) are indicated. **C.** DNA-binding inhibition of $(p65)_2$ by construct I. Bacterially expressed p50 (5 ng; lane 2) or GST-fused p65 (5 ng; lanes 3–6) was preincubated with protein buffer (lanes 2, 3), construct E (50 ng; lane 4), construct I (50 ng; lane 5), and construct J (50 ng; lane 6) for 30 minutes at 4°C before the addition of labeled κB oligonucleotide and analyzed as described. Positions of the $(p50)_2$- and $(p65)_2$-DNA complexes and free probe (FP) are indicated.

cause construct E associates with p50 (Fig. 3B, lane 14) but is unable to inhibit DNA binding of $(p50)_2$ (Fig. 2B, lane 5). Based on the data in Figure 3, we conclude that association between IκB and NF-κB subunits is not sufficient to mediate DNA-binding inhibition but is a prerequisite to prevent binding to the κB site.

Spatial arrangement rather than absolute number of ankyrin repeats is required for inhibitory activity

Because IκBγ contains eight ankyrin repeats compared to five in IκBα, and is capable of inhibiting both $(p50)_2$ and $(p65)_2$, whereas IκBα inhibits only $(p65)_2$ or p50/p65 heterocomplex, we asked whether increasing the number of ankyrin repeats in IκBα could alter its specificity. Accordingly, we generated construct I (Fig. 1), which contains ten IκBα ankyrin repeat motifs in tandem. Like its parent molecule, construct E, it associates with p50 (Fig. 4A, lane 4) but is unable to inhibit its DNA-binding activity (Fig. 4B, lane 5). Construct I is able to inhibit the DNA-binding activity of $(p65)_2$ (Fig. 4C, lane 5), indicating that the additional five ankyrin repeats do not render it inactive. Construct J, containing mutant ankyrin repeat 1, was unable to prevent DNA binding (Inoue et al., 1992b). We suggest that the specificity of inhibitory activity of IκB proteins lies in the amino acid sequences and spatial arrangement of ankyrin repeats.

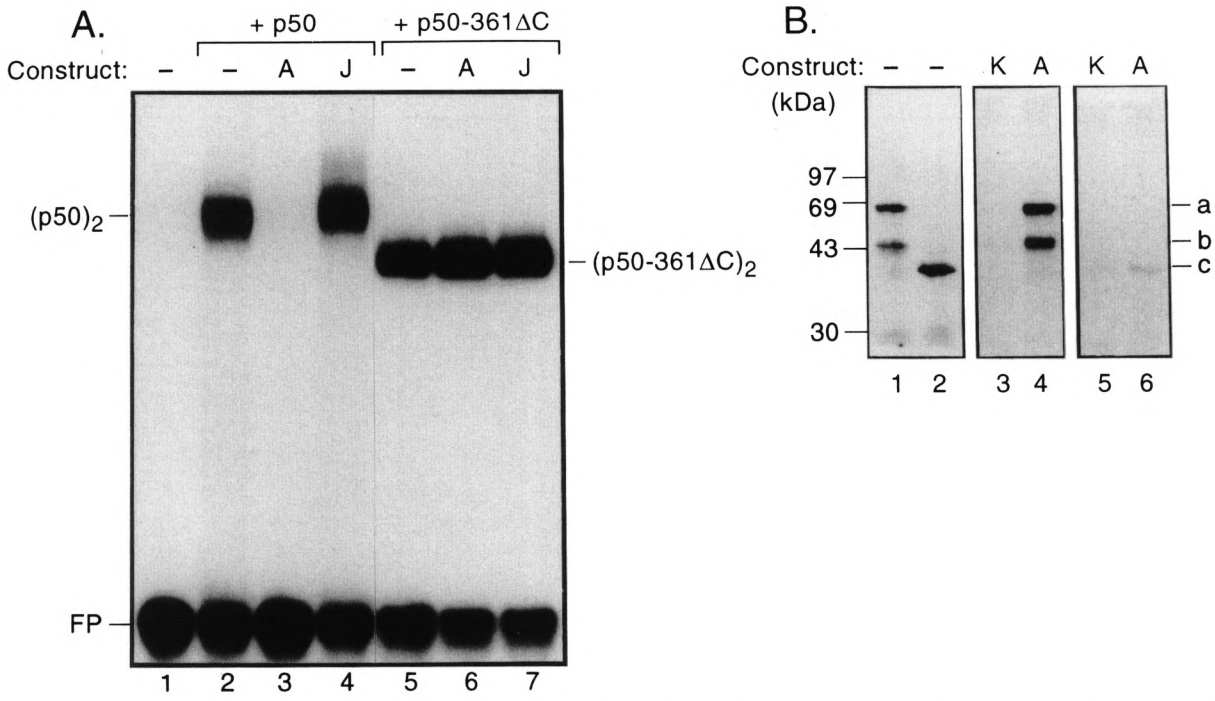

Figure 5. DNA binding and protein association characterization of p50 and p50-361ΔC. **A.** $(p50)_2$ DNA-binding inhibition by IκBγ requires the presence of the nuclear localization sequence and C-terminal sequence of p50. Bacterially expressed p50 (5 ng; lanes 2–4) or p50-361ΔC (5 ng; lanes 5–7) was incubated with binding buffer (lanes 2, 5), construct A (100 ng; lanes 3, 6), or construct J (50 ng; lanes 4, 7) for 30 minutes on ice. Labeled κB oligonucleotide was then added, and samples were loaded on a 4% non-denaturing gel after a 5 minute incubation at 24°C. $(p50)_2$- and $(p50\text{-}361\Delta C)_2$-DNA complexes are indicated. **B.** Association between IκBγ and p50 protein involves the nuclear localization sequence of p50 and a C-terminal domain. In vitro translated epitope-tagged p50 (lanes 1, 3, and 4) or p50-361ΔC (lanes 2, 5, and 6) were incubated with GST protein (lanes 3 and 5) or construct A (lanes 4 and 6). Letters a and b represent full-length and shorter product, respectively, of p50 and p50-361ΔC RNA translation using the rabbit reticulocyte lysate instead of wheat germ extract used in Figure 3, while c indicates the product of the p50-361ΔC.

Inhibition by IκBγ requires the C-terminal sequence of p50, including the nuclear localization signal

We were interested in investigating which regions of p50 outside the rel homology domain may be required for association and DNA-binding inhibition by IκBγ. We generated a p50 construct wherein C-terminal amino acids 362 to 412 were deleted. The resulting construct p50-361ΔC also lacks the putative nuclear localization sequence (KRQK) at position 362–365. Results in Figure 5A show that, like $(p50)_2$, the mutant p50-361ΔC protein is able to form a complex with the κB site (compare lanes 2 and 5). However, construct A, containing the eight ankyrin repeats of IκBγ, is able to inhibit the DNA-binding activity of $(p50)_2$ (lane 3) but has no effect on the DNA-binding activity of the mutant protein (lane 6) lacking the nuclear localization signal and the C-terminal sequences. Since DNA-binding inhibition requires direct association between p50 and IκBγ, we performed association assays as described before. The association of C-terminal truncated p50 protein to IκBγ is greatly diminished (Fig. 5B, lane 6) compared to the full-length p50 (lane 4). This result is consistent with the data shown in Figure 3, that association between IκB and NF-κB proteins is obligatory for inhibition of DNA-binding activity.

IκBγ mediates a reversible inhibition of $(p50)_2$-DNA binding

The mechanism by which IκBγ inhibits DNA binding of $(p50)_2$ was investigated further by performing time course experiments. Briefly,

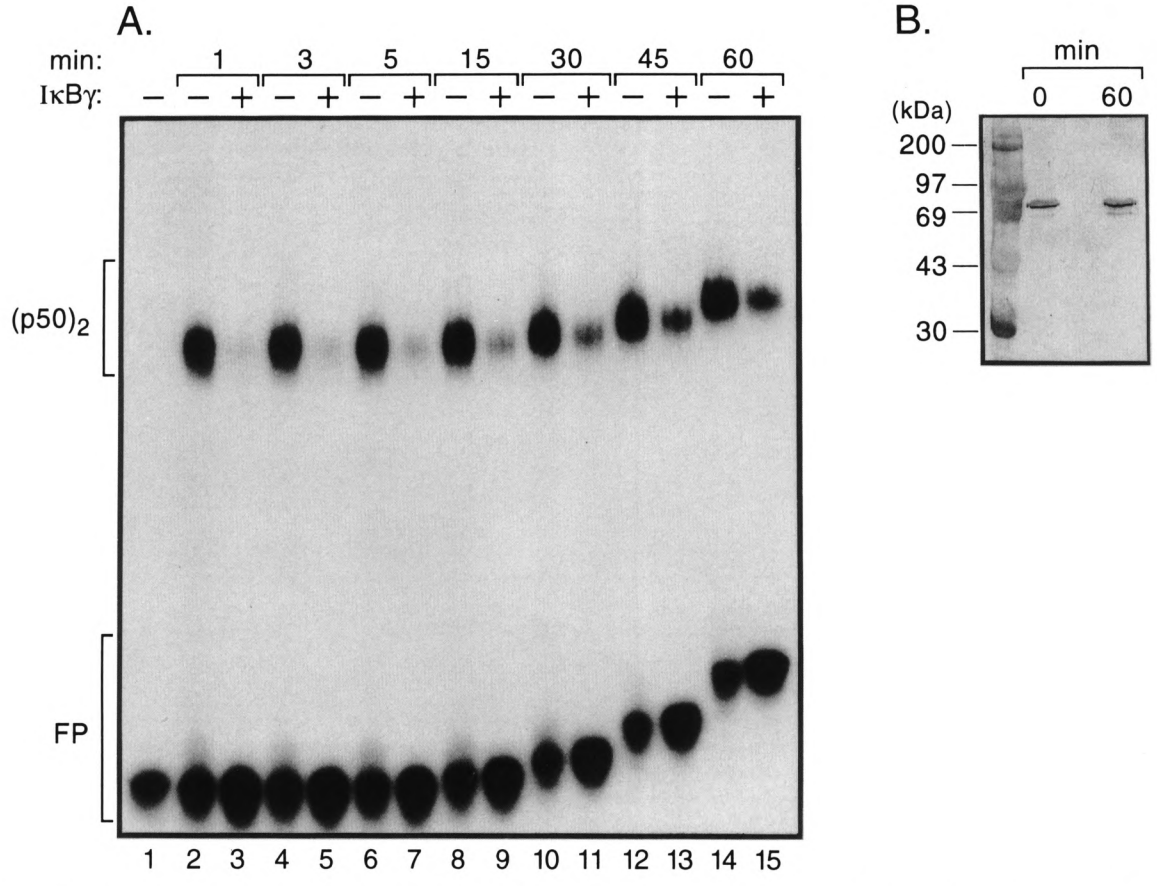

Figure 6. Reversible binding of IκBγ to $(p50)_2$-DNA complex. **A.** Time course of DNA-binding inhibition of $(p50)_2$ by IκBγ. Bacterially expressed and purified p50 protein was incubated in standard gel shift assay buffer with or without ~50 ng of construct A for 30 minutes at 4°C, followed by addition of 4 fmoles of labeled κB oligonucleotide. Aliquots of the reaction mixture were loaded at different times on a gel run at room temperature. Lane 1, labeled κB oligonucleotide; time points are indicated on the top of the figure; – and + are for with or without added construct A at the beginning of the experiment. The position of the $(p50)_2$-DNA complex and free probe (FP) are shown by brackets. The setup of the experiment was such that samples loaded at 60 minutes have a 59 minute shorter gel running time than samples loaded at 1 minute. **B.** Western blot analysis of construct A protein shows the absence of degradation of the IκBγ inhibitor. An experiment identical to the protocol used in **A** was repeated using unlabeled κB oligonucleotide. In the 0 minute lane, the binding buffer was added with the κB oligonucleotide (time 0 minutes in **A**). In the 60 minute lane, the same binding buffer was added after 60 minutes incubation time at 24°C (time 60 minutes in **A**). Samples were loaded on a 10% SDS/polyacrylamide gel, then transferred to Immobilon-P (Millipore) under standard conditions. Construct A protein was detected using rabbit 5177C antibody (Inoue et al., 1992a) and mouse anti-rabbit antibody coupled to alkaline phosphatase (Promega).

bacterially expressed p50 protein was incubated with or without construct A (Fig. 1) for 30 minutes at 4°C, followed by addition of labeled κB probe, which was counted as the 0 minute time point. Aliquots were withdrawn at various times following incubation at room temperature and loaded on a non-denaturing gel. The setup of the experiment was such that the 60 minute time point was electrophoresed for 59 minutes less (Fig. 6A, lanes 14 and 15) than the one minute time point (lanes 2 and 3). Therefore, the mobility of the gel-shift complex and the free probe is skewed. Results indicate that the inhibition mediated by IκBγ changes dramatically during the time course of the experiment. One minute after the addition of the probe, $(p50)_2$-DNA complex is completely inhibited by construct A (Fig. 6A, lanes 3 versus 2); however, by 5 minutes, complexes begin to appear (lane 7) and gradually but quantitatively increase by 60

minutes (lane 15). In comparison, in the absence of the inhibitor, $(p50)_2$-DNA complex is instantly formed (lane 2) and is essentially unaffected during the entire time course. The association of IκBγ with $(p50)_2$ is therefore dynamic and reversible.

The possibility that the protein encoded by construct A was degraded during the experiment was tested. Briefly, a reaction identical to that described above (Fig. 6A) was set up with unlabeled κB oligonucleotide probe. Samples were analyzed by Western blotting with the antibody 5177C raised against IκBγ (Inoue et al., 1992a) at 0 and 60 minutes (Fig. 6B). No product degradation or decrease in the quantity of expected construct A protein was observed after 60 minutes, ruling out the possibility of degradation of the protein as a mechanism for the release of DNA-binding inhibition observed in Figure 6A.

IκBα and bcl3 displace preformed protein–DNA complexes

We next asked whether IκB proteins are able to displace preformed DNA complexes. Briefly, preformed $(p50)_2$ or $(p65)_2$ complexes were assembled by incubating with labeled probe, and 100-fold excess unlabeled κB probe was added prior to addition of IκB proteins. If the labeled probe is released following association of IκB proteins, it will not form a new complex but will be diluted by the excess unlabeled probe. Results in Figure 7 show that 100 ng IκBγ (construct A, Fig. 1), as well as full-length IκBγ (data not shown), was unable to displace the preformed $(p50)_2$ (lane 3) or $(p65)_2$ (lane 7) DNA complex. On the other hand, 100 ng bcl3 protein was able to displace completely the preformed $(p50)_2$-DNA complex (lane 4) but not $(p65)_2$-DNA complex (lane 8). In contrast, 100 ng IκBα (construct E, Fig. 1), as expected from data in Figure 2, is unable to displace binding of $(p50)_2$ (lane 2) but completely displaces the $(p65)_2$-DNA complex (lane 6). IκBα is also able to displace the binding of NF-κB complex to the κB site (Zabel and Baeuerle, 1990; data not shown), presumably by displacing the p50/p65 heterocomplex. Thus, it appears that both IκBα and bcl3 proteins have a stronger association with p65 or p50 DNA-binding subunits, respec-

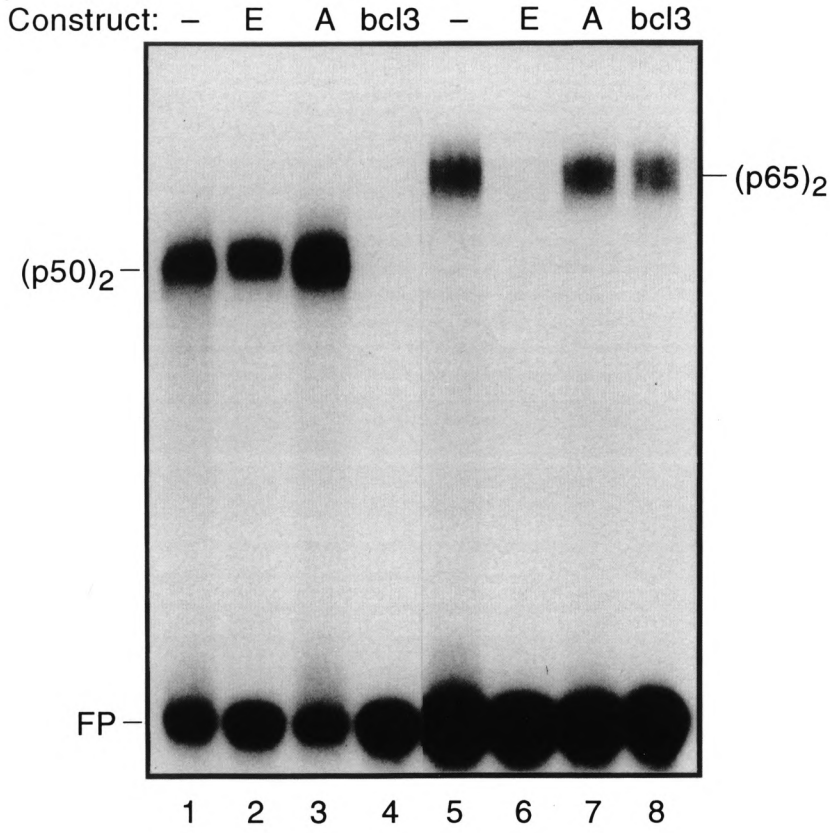

Figure 7. IκBα and bcl3 displace preformed $(p50)_2$-DNA complex. Bacterially expressed p50 (5 ng; lanes 1–4) or GST-p65 (5 ng; lanes 5–7) were incubated in binding buffer with labeled κB oligonucleotide, followed by the addition of 100 molar excess of cold κB oligonucleotide and GST protein buffer (lanes 1 and 5), approximately 100 ng of construct E (lanes 2 and 6), construct A (lanes 3 and 7), or full-length bcl3 protein (lanes 4 and 8). After 15 minutes at 24°C, samples were loaded on a 4% non-denaturing acrylamide gel. $(p50)_2$- and $(p65)_2$-DNA complexes and free probe (FP) are indicated.

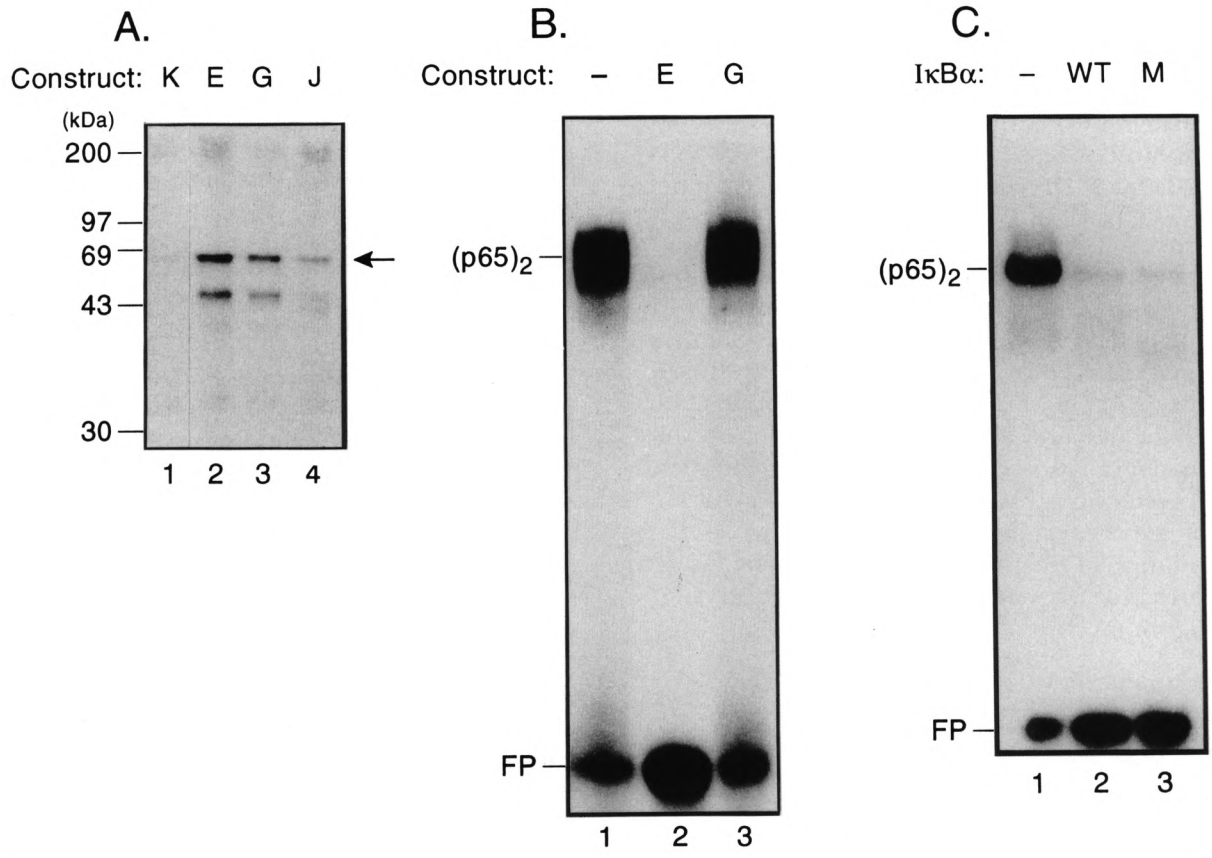

Figure 8. C-terminal region of IκBα (pp40) is required for displacement of preformed $(p65)_2$-DNA complex. **A.** Association assay between GST-fused inhibitor proteins and in vitro translated p50. p50 RNAs were translated using rabbit reticulocyte lysate and association assays performed as described. Lane 1, GST; lane 2, construct E; lane 3, construct G; lane 4, construct J. Arrow indicates position of full-length flu-epitope tagged p50. **B.** Construct G does not displace preformed $(p65)_2$-DNA complex. Bacterial GST-fused p65 (5 ng) was preincubated with labeled κB oligonucleotide for 15 minutes at 24°C, before the addition of 100-fold excess of unlabeled κB oligonucleotide in the presence of protein buffer (lane 1) or an equal amount of construct E (50 ng) or construct G (50 ng), lanes 2 and 3, respectively. DNA-protein complexes were resolved on a non-denaturing gel after a 15 minute incubation at 24°C. $(p65)_2$-DNA complex and free probe (FP) are indicated. **C.** Displacement of preformed $(p65)_2$-DNA complex by in vitro translated wild-type and two serine mutants of IκBα/pp40. Wild-type and pp40 (IκBα) cysteine mutants (309, 310 Cys, Cys→Ser, Ser, mutant, M) were translated in vitro using rabbit reticulocyte lysate (Promega) under standard conditions. Bacterially expressed GST-p65 was preincubated with labeled κB for 15 minutes at 24°C in binding buffer. Twenty μl of reticulocyte reaction mixture (lane 1), WT or M mutant translation reaction (lanes 2 and 3, respectively) were added in the presence of 100-fold excess of unlabeled κB oligonucleotide. Samples were loaded on a 4% non-denaturing gel after a further 20 minute incubation at 24°C. $(p65)_2$-DNA complex and free probe (FP) are indicated.

tively, and can displace them from preformed DNA complexes. In contrast, IκBγ is unable to dissociate preformed κB DNA complexes.

Displacement of $(p65)_2$-DNA preformed complex requires a domain at the C-terminus of IκBα

We have previously shown that the C-terminus of IκBα (pp40) was required to inhibit DNA binding of the p50/p65 heterocomplex (Inoue et al., 1992b). Additionally, we have shown that construct H, which lacks the C-terminal 66 aa, is unable to inhibit $(p65)_2$-DNA complex (Fig. 2C, lane 9), even though it is able to associate with p65 (Fig. 3A, lane 8). It therefore appears that the ankyrin repeat motifs in IκBα are sufficient to associate with p65 but require a domain in the C-terminal region to effect DNA displacement. Furthermore, the C-terminal domain of IκBα is specific, because replace-

ment with the C-terminal domain of IκBγ (construct F) does not inhibit the DNA-binding activity of p65 (Fig. 2C, lane 8). To further delineate the C-terminal domain required for DNA displacement, we generated construct G, lacking 37 aa at the C-terminus of IκBα. Since construct H, containing only the 5 ankyrin repeats of IκBα, associates with p65 (Fig. 3A, lane 8), we would expect that construct G also associates with p65. Interestingly, unlike construct H (3B, lane 16), construct G can now associate with p50 (Fig. 8A, lane 3). Nevertheless, construct G, lacking the C-terminal 37 aa, is still unable to inhibit DNA-binding activity of $(p65)_2$ (Fig. 8B, lane 3). Thus the region representing the extreme C-terminal 37 aa may be directly involved in DNA displacement. Detailed sequence analysis of this region revealed an α-helix and a pair of cysteine residues (at position 309 and 310) that may play a role in redox regulation (Matthews et al., 1992). Mutation of the cysteine residues to serine, however, did not influence the DNA binding inhibition (Fig. 8C). It should be noted that both the wild-type (IκBα/pp40, Fig. 1) and the cysteine 309/310→Ser mutants were translated in vitro (compared to bacterially expressed IκB proteins in all other experiments shown here) and were capable of mediating inhibition of $(p65)_2$-DNA complex (Fig. 8C, lanes 2 and 3). Therefore, DNA-binding inhibition at least by IκBα is carried out at molar ratios and does not require an excess of inhibitory protein.

Discussion

IκB activity was first described as an NF-κB-associated protein whose dissociation, induced by various stimuli, led to nuclear translocation of the transactivator NF-κB (Baeuerle and Baltimore, 1988a,b). IκB proteins do not bind to the κB site (Kerr et al., 1991, 1992), although weak binding has been observed with p105 (A. Israël, personal communication). Furthermore, IκBα has been shown to act as a transcriptional activator when linked to heterologous DNA-binding protein (Morin and Gilmore, 1992). The bcl3 protein has also been shown to cause transactivation in some cases (U. Siebenlist and D. Baltimore, personal communication). All IκB proteins identified to date contain an ankyrin repeat domain (Davis et al., 1991; Haskill et al., 1991; Ohno et al., 1990; Inoue et al., 1992a; Tewari et al., 1992), which is essential for their inhibitory activity (Inoue et al., 1992b; Hatada et al., 1992).

Ankyrin repeats provide specificity for DNA-binding inhibition

Both the specific sequences and number of ankyrin repeat motifs provide specificity for DNA-binding inhibition by IκB proteins. IκBα containing five ankyrin repeats is capable of inhibiting the $(p65)_2$-DNA binding complex (Fig. 2C), as well as the p50/p65-DNA-binding complex (data not shown), but has no effect on the DNA-binding activity of $(p50)_2$ (Fig. 2C; Inoue et al., 1992b; Kerr et al., 1992). IκBγ and bcl3, which contain eight and seven ankyrin repeat motifs, respectively, compared to five in IκBα, inhibit the DNA-binding activity of $(p50)_2$ (Fig. 7; Kerr et al., 1992). Lack of inhibition of $(p50)_2$-DNA complex by IκBα is not simply due to fewer number of ankyrin repeats, because construct I, containing ten IκBα ankyrin repeats in tandem, does not inhibit formation of $(p50)_2$-DNA complex (Fig. 4B, lane 5). From the data presented here and that reported before (Inoue et al., 1992b), it is clear that association of IκB proteins with κB proteins is necessary but not sufficient to mediate DNA-binding inhibition. Although the precise sites of association between IκB and κB proteins have not yet been mapped, it appears that the putative nuclear transport signal is involved (Beg et al., 1992; Fig. 5A). We have previously shown that association of IκBα with c-rel requires the N-terminal 51 aa of c-rel (Kerr et al., 1991). It would appear, therefore, that the association of IκBα and perhaps IκBγ with κB proteins encompasses the DNA-binding and dimerization domains, as well as the nuclear transport signal, thereby precluding accessibility to DNA. One way to explain the inability of IκBα to inhibit DNA binding by $(p50)_2$ homodimers but not by p50/p65 heterodimers may be that $(p50)_2$ binding to κB sites introduces a bend in DNA different from the one induced by p50/p65, which consequently does not allow a stable association with IκBα (Schreck et al., 1990). Regarding the stability of association between ankyrin repeat domain and NF-κB subunits, it is interesting to note that although construct E can associate with p50 and p65, the gel shift complex does not show any supershift (Fig. 2B and C), suggesting that the

association of IκB to κB proteins has a rapid on/off rate in the presence of DNA.

Mechanism of DNA-binding inhibition by IκB

In the experiments described here and those reported before, a molar excess of IκB proteins was used to inhibit κB binding to DNA. This is partly due to the use of IκB proteins generated in bacteria, which may be only partially active. Since in vitro translated IκBα can inhibit $(p65)_2$-DNA binding activity (Fig. 8C), at least for IκBα, inhibition may be stochiometric. Some IκBα and bcl3 have been found in the nucleus (Davis et al., 1990; Kerr et al., 1991, 1992; Morin and Gilmore, 1992), but IκBγ has only been localized in the cytoplasm (Inoue et al., 1992a). Therefore, IκBγ must interact with NF-κB proteins in the cytoplasm, in the absence of DNA. Results in Figure 5 confirm this notion, because removal of the nuclear localization signal from p50 abolishes the inhibitory activity of IκBγ, even though the DNA-binding activity is not compromised. The C-terminal domain of p105, related to IκBγ (p70), has been shown to regulate the nuclear uptake of p50 and other members of the rel family (Henkel et al., 1992; Rice et al., 1992). IκBγ may also play a role in regulating the processing of p105, the p50 precursor.

IκBα can displace the preformed $(p65)_2$ or p50/p65-DNA complex (Fig. 7; Kerr et al., 1991), but this displacement requires the presence of C-terminal 37 aa (compare construct G with construct E in Fig. 8B). In the absence of the C-terminal 37 aa, IκBα (construct G) can associate with both p65 and p50; however, in the absence of the entire 66 aa at the C-terminal (construct H), there is no association with p50 (Fig. 3B). Thus the IκBα can be functionally divided into at least three domains: (1) an N-terminal domain whose removal has little effect on its biochemical activity; (2) an ankyrin repeat domain that is sufficient to bind to p65 but has little influence on its DNA-binding activity; and (3) the 66 aa C-terminal domain, which can be further subdivided into two regions. One region, comprising the first 31 aa (construct G), provides association with p50 yet cannot displace preformed $(p65)_2$-DNA complex; the second region, comprising the terminal 37 aa, is essential for DNA displacement activity (Fig. 8). Inspection of the C-terminal 37 aa reveals an α helical region which is highly acidic (13/37 residues) and several likely phosphorylation sites. It is worth pointing out that the C-terminal domain functions in cis with the association domain of the same IκB molecules, because linking it to IκBγ (construct B) does not allow DNA displacement of preformed $(p50)_2$ complexes (data not shown). Similarly, the C-terminal domain of IκBγ cannot substitute for the C-terminal domain of IκBα (Fig. 2B).

Inhibitor, repressor, and antirepressor

Because IκB can dissociate from the NF-κB-DNA complex, it was proposed that free IκB could enter the nucleus and cause rapid release of NF-κB from high-affinity binding sites in enhancers and promoter elements to terminate NF-κB-dependent activation of gene expression (Zabel and Baeuerle, 1990). The idea that IκB may play an active role in transcriptional regulation within the nucleus is also supported by the fact that even after preincubation of NF-κB in vitro with all purified components of a transcriptional system (including basal transcription factors and co-activator) and template DNA, it completely inhibited stimulation of transcription by NF-κB (Kretzschmar et al., 1992). We have shown that IκBα and human bcl3 specifically dissociate preformed $(p65)_2$ and $(p50)_2$-DNA complexes (Fig. 7). The DNA-binding affinity of κB proteins has been calculated to be high, in the range of $1\text{–}5 \times 10^{-11}$ M (Baeuerle, 1991); yet addition of IκBα and bcl3 displaces this complex.

Co-expression of p50 suppressed transactivation by p65, presumably by competitive DNA binding of transcriptionally inactive p50 dimer (Schmitz and Baeuerle, 1991). Since bcl3 can displace a preformed $(p50)_2$-DNA complex (Fig. 7), we propose a model whereby bcl3 acts as an antirepressor. It displaces $(p50)_2$ from the DNA-binding site, thereby allowing binding of appropriate NF-κB proteins to initiate transcription of the κB site-containing genes. Thus bcl3 and IκBα proteins can function both as transcription inhibitors by preventing DNA binding and as transcriptional activators by displacing inactive or inappropriate NF-κB complexes from DNA. This idea has been borne out by recent experiments showing that bcl3 inhibits transcription by endogenous κB proteins but leads to transcriptional activation at higher concentrations, presumably by displacing inactive $(p50)_2$ (Franzoso et al., 1992). Recently, it has been suggested that bcl3 also activates transcription by p52 (also referred to as p49 or p50B;

U. Siebenlist, personal communication). Thus the modulation of IκB proteins may dictate whether they will function as inhibitors or activators.

Based on their cellular localization, IκB proteins can be divided into two categories. The first category comprises p105 and IκBγ, which remain in the cytoplasm (Inoue et al., 1992a; Rice et al., 1992), although p105 has been found in the nucleus in association with the *tax* protein of HTLV-1 (Hirai et al., 1992; Neumann et al., 1992). IκBγ, generated from subgenomic mRNA and identical to the C-terminal portion of p105, is able to inhibit DNA binding by rel, p50, p65, and p50/p65 heterodimer (Inoue et al., 1992a). However, it is unable to displace a preformed $(p50)_2$ or $(p65)_2$ or p50/p65 DNA complex (Fig. 7). Since IκBγ is not transported to the nucleus, it is unlikely to participate in gene expression by displacing productive or nonproductive NF-κB complexes.

The second category comprises IκBα and bcl3, which can be translocated to the nucleus either by passive diffusion or by active transport or in association with κB-binding proteins (Davis et al., 1990; Kerr et al., 1991; Morin and Gilmore, 1992; P. Baeuerle and D. Baltimore, personal communication). Since both IκBα and bcl3 can displace preformed complexes (Fig. 7), they are likely to participate directly in gene expression by κB proteins, presumably by acting as repressors or anti-repressors of κB binding proteins. Finally, it is worth pointing out that the mode of action of IκB inhibitors is quite distinct from other inhibitors of DNA-binding activity, such as Id and *twin* of IPOU (Benzera et al., 1990; Treacy et al., 1992). In these cases, the proteins lack the basic region, which results in the formation of a nonproductive homo- or heterodimer, and thus function as dominant negative mutants. Similarly, transcriptional repression mediated by histone H1 is distinct because it inhibits by nonspecific DNA binding (Croston et al., 1991).

Acknowledgments

We are grateful to Drs. T. Gilmore, S. Miyamoto, and L. Kerr for critical reading of the manuscript. We thank all members of the Verma laboratory for various reagents and encouragement. We thank Pat McClintock for help in preparing the manuscript.

This work was supported by funds from the National Institutes of Health, the American Cancer Society, the H. N. and Frances C. Berger Foundation, and the Council for Tobacco Research-USA, Inc., to I. M. Verma. I. M. Verma is an American Cancer Society Professor of Molecular Biology.

The costs of publishing this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC Section 1734 solely to indicate this fact.

Thierry Léveillard is currently at CNRS-LGME, 11, rue Humann, 67085 Strasbourg, France.

References

B. J. Andrews and I. Herskowitz (1989), Nature 342, 830–833.

S. J. Aves, B. W. Durkacz, A. Carr, and P. Nurse (1985), EMBO J 4, 457–463.

A. S. Baldwin, J. C. Azizkhan, D. E. Jensen, A. A. Beg, and L. R. Coodly (1991), Mol Cell Biol 11, 4943–4951.

P. A. Baeuerle (1991), Biochim Biophys Acta 1072, 63–80.

P. A. Baeuerle and D. Baltimore (1988a), Cell 53, 211–217.

P. A. Baeuerle and D. Baltimore (1988b), Science 242, 540–546.

P. A. Baeuerle and D. Baltimore (1989), Genes Dev 3, 1689–1698.

A. A. Beg, S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr. (1992), Genes Dev 6, 1899–1913.

R. Benezra, R. L. Davis, D. Lockshou, D. L. Turner, and H. Weintraub (1990), Cell 61, 49–59.

V. Blank, P. Kourilsky, and A. Israël (1991), EMBO J 10, 4159–4167.

V. Blank, P. Kourilsky, and A. Israël (1992), Trends Biochem Sci 17, 135–140.

L. Breeden and K. Nasmyth (1987), Nature 329, 651–654.

G. E. Croston, L. A. Kerrigan, L. M. Lira, D. R. Marshak, and J. T. Kadonaga (1991), Science 251, 643–649.

J. N. Davis, W. Bargmann, M.-Y. Lim, and H. R. Bose, Jr. (1990), J Virol 64, 584–591.

N. Davis, S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr. (1991), Science 253, 1268–1271.

C.-M. Fan and T. Maniatis (1991), Nature 354, 395–398.

G. Franzoso, V. Bours, S. Park, M. Tomita-Yamaguchi, K. Kelly, and U. Siebenlist (1992), Nature 359, 339–342.

R. Geisler, A. Bergmann, Y. Hiromi, and C. Nüsslein-Volhard (1992), Cell 71, 613–621.

T. D. Gilmore (1990), Cell 62, 841–843.

S. Ghosh and D. Baltimore (1990), Nature 344, 678–682.

S. Ghosh, A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore (1990), Cell 62, 1019–1029.

S. Haskill, A. A. Beg, S. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr. (1991), Cell 65, 1281–1289.

E. N. Hatada, A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T. W. McKeithan, and C. Scheidereit (1992), Proc Natl Acad Sci USA 89, 2489–2493.

T. Henkel, U. Zabel, K. Van Zee, J. M. Müller, E. Fanning, and P. A. Baeuerle (1992), Cell 68, 1121–1133.

H. Hirai, J. Fujisawa, T. Suzuki, K. Ueda, M. Muramatsu, A. Tsuboi, N. Arai, and M. Yoshida (1992), Oncogene 7, 1737–1742.

J.-I. Inoue, L. D. Kerr, A. Kakizuka, and I. M. Verma (1992a), Cell 68, 1109–1120.

J.-I. Inoue, L. D. Kerr, D. Rashid, N. Davis, H. R. Bose, Jr., and I. M. Verma (1992b), Proc Natl Acad Sci USA 89, 4333–4337.

J. T. Kadonaga and R. Tjian (1986), Proc Natl Acad Sci USA 83, 5889–5893.

K. Kawakami, C. Scheidereit, and R. G. Roeder (1988), Proc Natl Acad Sci USA 85, 4700–4704.

L. D. Kerr, J.-I. Inoue, N. Davis, E. Link, P. A. Baeuerle, H. R. Bose, Jr., and I. M. Verma (1991), Genes Dev 5, 1464–1476.

L. D. Kerr, C. S. Duckett, P. Wamsley, Q. Zhang, P. Chiao, G. Nabel, T. McKeithan, P. A. Baeuerle, and I. M. Verma (1992), Genes Dev 6, 2352–2363.

S. Kidd (1992), Cell 71, 623–635.

M. Kieran, V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël (1990), Cell 62, 1007–1018.

M. Kretzschmar, M. Meisterernst, C. Scheidereit, G. Li, and R. G. Roeder (1992), Genes Dev 6, 761–774.

M. J. Lenardo and D. Baltimore (1989), Cell 58, 227–229.

T. Léveillard, G. A. Kassavetis, and E. P. Geiduschek (1991), J Biol Chem 266, 5162–5168.

E. Link, L. D. Kerr, R. Schreck, U. Zabel, I. M. Verma, and P. A. Baeuerle (1992), J Biol Chem 267, 239–246.

H.-C. Liou, G. P. Nolan, S. Ghosh, T. Fujita, and D. Baltimore (1992), EMBO J 11, 3003–3009.

J. R. Matthews, N. Wakasugi, J.-L. Virelizier, J. Yodoi, and T. Hay (1992), Nucleic Acids Res 20, 3821–3830.

P. J. Morin and T. D. Gilmore (1992), Nucleic Acids Res 20, 2453–2458.

A. Neri, C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. K. Chaganti, and R. Dalla-Favera (1991), Cell 67, 1075–1087.

M. Neumann, K. Tsapos, J. A. Scheppler, J. Ross, and B. R. Franza, Jr. (1992), Oncogene 7, 2095–3014.

G. P. Nolan, S. Ghosh, H.-C. Liou, P. Tempst, and D. Baltimore (1991), Cell 64, 961–969.

H. Ohno, G. Takimoto, and T. W. McKeithan (1990), Cell 60, 991–997.

P. Pognonec, H. Kato, H. Sumimoto, M. Kretzschmar, and R. G. Roeder (1991), Nucleic Acids Res 19, 6650.

N. R. Rice, M. L. MacKichan, and A. Israël (1992), Cell 71, 243–253.

R. M. Schmid, N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel (1991), Nature 352, 733–736.

M. L. Schmitz and P. A. Baeuerle (1991), EMBO J 10, 3805–3817.

R. Schreck, H. Zorbas, E.-L. Winnacker, and P. A. Baeuerle (1990), Nucleic Acids Res 18, 6497–6502.

R. Schreck, P. Rieber, and P. A. Baeuerle (1991), EMBO J 10, 2247–2258.

R. Sen and D. Baltimore (1986a), Cell 46, 705–716.

R. Sen and D. Baltimore (1986b), Cell 47, 921–928.

F. Shirakawa and S. Mizel (1989), Mol Cell Biol 9, 2424–2430.

B. Stein, H. J. Rahmsdorf, A. Steffen, M. Litfin, and P. Herrlich (1989), Mol Cell Biol 9, 5169–5181.

R. Steward (1987), Science 238, 692–694.

M. Tewari, P. Dobranski, K. L. Mohu, D. E. Cressman, J.-C. Hsu, R. Bravo, and R. Taub (1992), Mol Cell Biol 12, 2898–2908.

G. Theilen, R. Zeigel, and M. Tweihaus (1966), J Natl Cancer Inst 37, 747–749.

M. N. Treacy, L. I. Neilson, E. E. Turner, X. He, and M. G. Rosenfeld (1992), Cell 68, 491–505.

M. B. Urban and P. A. Baeuerle (1990), Genes Dev 4, 1975–1984.

U. Zabel and P. A. Baeuerle (1990), Cell 61, 255–265.