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The PEST-Like Sequence of IκBα Is Responsible for Inhibition of DNA Binding but Not for Cytoplasmic Retention of c-Rel or RelA Homodimers

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In most cells, proteins belonging to the Rel/NF-κB family of transcription factors are held in inactive form in the cytoplasm by an inhibitor protein, IκBα. Stimulation of the cells leads to degradation of the inhibitor and transit of active DNA-binding Rel/NF-κB dimers to the nucleus. IκBα is also able to inhibit DNA binding by Rel/NF-κB dimers in vitro, suggesting that it may perform the same function in cells when the activating signal is no longer present. Structurally, the human IκBα molecule can be divided into three sections: a 70-amino-acid N terminus with no known function, a 205-residue midsection composed of six ankyrin-like repeats, and a very acidic 42-amino-acid C terminus that resembles a PEST sequence. In this study we examined how the structural elements of the IκBα protein correlate with its functional capabilities both in vitro and in vivo. Using a battery of IκBα mutants, we show that (i) a dimer binds a single IκBα molecule, (ii) the acidic C-terminal region of IκBα is not required for protein-protein binding and does not mask the nuclear localization signal of the dimer, (iii) the same C-terminal region is required for inhibition of DNA binding, and (iv) this inhibition may be accomplished by direct interaction between the PEST-like region and the DNA-binding region of one of the subunits of the dimer.

The Rel/NF-κB family of transcription factors is expressed in all human cell types examined to date and influences the regulation of many genes, including many of those involved in the immune or inflammatory response (see reference 16 for a review). Members of the family include c-Rel, p65 (RelA), RelB, p50 (NF-κB1), and p52 (NF-κB2), and, among these, nearly all possible DNA-binding homodimers and heterodimers have been described. In most cells these proteins are found not in the nucleus but in the cytoplasm, where they are complexed with an inhibitor (IκB) (1). Stimulation of the cell with any of a variety of agents, e.g., lipopolysaccharide (LPS), tumor necrosis factor, phorbol myristyl acetate, etc., results in the rapid transit of some of the cytoplasmic NF-κB to the nucleus and in the appearance of sequence-specific DNA-binding activity (see reference 16 for a review).

Several IκB's have been characterized. Two of them are the high-molecular-weight precursors of p50 and p52 (34, 36, 40, 43, 47). Each of these (p105 and p100, respectively) is able to dimerize with c-Rel, p65, p50, or p52, and such dimers are strictly cytoplasmic and unable to bind DNA. In these dimers, the long C-terminal region of the precursor apparently covers the nuclear localization signal (NLS) of each subunit (6, 19, 33). Only when the precursor is proteolytically processed to yield the N-terminal p50 or p52 region is the resulting dimer able to enter the nucleus and bind DNA. Processing of the precursor occurs at a slow rate, which is not markedly affected by treatment of the cells with activating agents (12, 34). Thus, these precursor-containing dimers are not a major source of rapidly induced DNA-binding activity.

In contrast, a third inhibitor, IκBα, responds very quickly to activating signals. Within minutes after addition of tumor necrosis factor alpha to the culture medium, nearly all of the IκBα is released from cytoplasmic dimers. Treatment of cells with LPS, phorbol myristyl acetate, or interleukin-1 evokes a similar response (3, 9, 11, 13, 39, 46). Thus, complexes containing IκBα are a major source of the rapidly induced NF-κB DNA-binding activity.

IκBα performs several critical functions. First, by binding to a dimer and masking the NLS of each of the subunits, it is able to retain most Rel family dimers in the cytoplasm. The NLS is required for IκBα binding (4, 15, 25), suggesting that IκBα directly contacts the subunits at those points. Second, in response to activating signals, IκBα releases the dimer. Phosphorylation of IκBα often accompanies activation (3, 9, 11), but it is not yet known whether phosphorylation is required. It is clear, however, that IκBα becomes very labile when its complex receives an activating signal (3, 9, 11, 13, 39, 46). Thus, the protein presumably contains sequences that, when recognized, target it for rapid degradation. Third, IκBα not only binds to dimers but also prevents c-Rel- and p65-containing dimers from binding DNA (2, 23). Indeed, when added to dimers that are already bound to DNA, it terminates such binding (52). Thus, removing nuclear Rel–NF-κB complexes when the activating signal is no longer present may be yet another role for IκBα in cells (53).

How does IκBα perform these various functions? The human protein consists of 317 amino acids, about 200 of which are arranged in six ankyrin-like repeats (17). Ankyrin repeats have a rather loose consensus sequence of 33 residues and appear to function, in groups of six repeats, in protein-protein interaction (31, 35). Upstream of the ankyrin repeats are 70 amino acids, to which no function has been assigned to date. Downstream of the repeats is a very acidic 42-amino-acid stretch, part of which resembles the PEST motif that, in other proteins, is highly correlated with rapid protein turnover (41).

What sequences in IκBα are required for binding to a Rel- or p65-containing dimer? The N-terminal 70 amino acids are

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not required, as their deletion does not affect binding (18, 21). Sequences within the ankyrin repeats are required, as mutations within this region generally eliminate binding (18, 21). The region(s) of c-Rel or p65 contacted by the ankyrin repeats, however, is not known. Regarding the C terminus, there are conflicting reports in the literature. In one set of experiments performed with bacterially expressed and renatured proteins, deletion of 38 C-terminal amino acids (in a mutant which also lacked the 70 nonessential N-terminal residues) rendered IκBα unable to bind p65 (18). In a second study, performed with a bacterial glutathione *S*-transferase–IκBα fusion protein and p65 translated in vitro in a wheat germ lysate, essentially the same IκBα mutations did not affect binding to p65 (27). A third study found that deletion of the C terminus eliminated binding of in vitro-translated IκBα to a bacterial glutathione *S*-transferase–c-Rel fusion protein (21). However, since the deletion removed not only the acidic region but also part of the sixth ankyrin repeat, it is impossible to conclude which region(s) was the important one. Thus, the extent to which the C-terminal region of IκBα contributes to binding is an unsettled question. Which region of IκBα contacts the NLS is also unresolved.

In this study we examined how the structural elements of the IκBα protein correlate with its functional capabilities. Specifically, we tested the effects of various mutations in the acidic C-terminal region of IκBα on its ability to interact with c-Rel and p65 both in vitro and in vivo. The following questions were asked. (i) How many IκBα molecules bind to a c-Rel or p65 homodimer? (ii) Which region of IκBα masks the NLS? (iii) Which region of IκBα is required for inhibition of DNA binding? (iv) What is the mechanism of this inhibition? Using a battery of IκBα mutants, we show that (i) a dimer binds a single IκBα molecule both in vitro and in vivo, (ii) the acidic C-terminal region of IκBα is not required for protein-protein binding and does not mask the NLS, (iii) the same C-terminal region is required for inhibition of DNA binding, and (iv) this inhibition may be accomplished by direct interaction between the PEST-like region and the DNA-binding region of one of the subunits of the dimer. We also show that, at least in the case of c-Rel, assays using in vitro-translated protein do not always reflect the situation in vivo.

MATERIALS AND METHODS

Plasmids. Human IκBα (17) in a cytomegalovirus-based expression vector was a gift of Al Baldwin. The insert was excised with *Hin*dIII and *Xba*I and cloned into pRc/CMV (Invitrogen). This construct was used for in vitro translation, transfection into 293 cells, and making the template for mutagenesis. Human p65 in a cytomegalovirus-based expression vector (42) (a gift of Craig Rosen) was used for transfection. Murine p65 (38) in Bluescript SK (a gift of Garry Nolan) was used for in vitro translation. The human c-Rel expression vector pRSV c-rel and the pRel-NA vector expressing C-terminally truncated c-Rel (Δ-Rel) have been described previously (48). For in vitro translation, the *Eco*RI insert from pUC8-c-Rel (10) was cloned into pRc/CMV.

Mutagenesis. All mutants were generated by using Bio-Rad's Muta-Gene phagemid in vitro mutagenesis system, version 2. Mutations were confirmed by sequence analysis with Sequenase 2.0 (U.S. Biochemical).

Oligonucleotides. Oligonucleotides were synthesized by an Applied Biosystems synthesizer. Those used as sequencing primers or in DNA binding assays were used without purification. For the electrophoretic mobility shift assay (EMSA), we used the interleukin-6 promoter κB site (underlined) with its natural flanking sequences, 5′-TCAAATGTGGGATTTTCCCATGAGTCT-3′ (28). Complementary strands were renatured and labeled with [γ-^{32}P]ATP as previously described (39).

For mutagenesis, oligonucleotides in which the wild-type sequence extended for 15 to 28 bases on either side of the desired mutation(s) were used. To make deletions encompassing the C terminus of IκBα (mutants A, B, and D), we retained the natural termination codon and downstream untranslated sequences. Mutants containing multiple point mutations were generated with a single long oligonucleotide. Mutagenic oligonucleotides were synthesized as the 5′-triphosphate or were phosphorylated by using T4 polynucleotide kinase and, in most cases, were purified from urea-containing polyacrylamide gels. The sequences of all the mutagenic oligonucleotides are available upon request.

In vitro translation. Transcription-translation reactions were performed according to the manufacturer's directions by using the wheat germ or reticulocyte TNT coupled system from Promega.

Transfection. Human 293 cells were seeded at 10^6/6-cm-diameter dish. Twenty-four hours later they were transfected by the calcium phosphate method.

Metabolic labeling and immunoprecipitation. Twenty-four to forty-eight hours after transfection, cells were grown in media containing [^{35}S]methionine and [^{35}S]cysteine (each at 50 μCi/ml; 1,000 Ci/mmol; Amersham) for 1 h. Cells were lysed in ELB buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) (15) and immunoprecipitated in buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl, and 1% Triton X-100. Precipitates were collected on protein A-Sepharose (Pharmacia) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Usually, 5% of the total lysate (from a 6-cm-diameter dish) was used per lane, and this gave a strong signal on X-ray film after an overnight exposure.

EMSA. Twenty-four hours after transfection, cells were lysed in ice-cold ELB buffer supplemented with protease inhibitors and 0.5 mM dithiothreitol, by using 0.2 ml of ELB per 6-cm-diameter dish. Lysates were clarified by centrifuging at 10,000 × *g* for 10 min. Lysates containing Δ-Rel were stored at 4°C and were used within 24 h. The binding reaction mixture was 10 mM HEPES (pH 7.5)–80 mM KCl–1 mM EDTA–1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–6% glycerol–0.5 μg of poly(dI-dC)–0.5 μg of sonicated double-stranded salmon sperm DNA–^{32}P-labeled oligonucleotide (1 ng; ~0.5 × 10^5 to 3 × 10^5 cpm)–cellular lysate (0.5 to 6 μl) in a total volume of 10 to 20 μl. The mixture was incubated at room temperature for 30 min. In the experiments in Fig. 6B, the reaction mixture minus ^{32}P-DNA was preincubated with 1 μl of antiserum for 10 min at room temperature. Lysate (5 μl) from cells expressing IκBα was then added. After 10 min of incubation, the ^{32}P-DNA was added, and incubation continued for 30 min more. Products were analyzed on 6.6% acrylamide gels made up in 50 mM Tris (pH 8.4)–45 mM boric acid–0.5 mM EDTA–0.001% Nonidet P-40.

Immunoblotting. Lysates prepared for EMSA were also analyzed by immunoblotting with the ECL system of Amersham. Two to twenty microliters of the lysate was used per lane, and samples were transferred and processed as previously described (39). Primary antisera were used at a 1:1,000 dilution.

Immunofluorescence. Our procedure has been described previously (5). Briefly, transfected 293 cells were seeded onto collagen-coated glass coverslips 24 h after transfection. After an additional 16 h the cells were fixed in 2% paraformaldelhyde, rinsed, and permeabilized in phosphate-buffered saline (PBS) containing 0.1% Nonidet P-40, blocked with goat serum, and incubated with primary antibody (anti-c-Rel 1136 or anti-p65 1226) at 1:80 for 1 h at room temperature. Secondary antibody was anti-rabbit immunoglobulin G, conjugated with fluorescein isothiocyanate (Kirkegaard and Perry Laboratories, Inc.); it was used at 1:20. After being washed in PBS, coverslips were mounted on slides by using Vectashield (Vector Laboratories), and cells were examined with a Leitz Ortholux-II microscope.

Antisera. All antisera were raised in rabbits against synthetic peptides coupled to keyhole limpet hemocyanin. Anti-IκBα serum 1258 (raised against the C terminus of IκBα), anti-Rel serum 1136 (raised against an internal peptide in human c-Rel), and anti-p65 serum 1226 (raised against the C terminus of p65) have been described previously (40, 48). Peptide 1309 is NH_2-Phe-Gln-Ala-Ala-Glu-Arg-Pro-Gln-Glu-Trp-Ala-Met-Glu-Gly-Cys (residues 2 to 15 of human IκBα) (17). Peptide 1206 is NH_2-Lys-Ala-Gly-Ile-Asn-Pro-Phe-Asn-Val-Pro-Glu-Lys-Gln-Leu-Asn-Asp-Ile-Glu-Cys (residues 124 to 141 of human c-Rel), peptide 1506 is NH_2-Ile-Glu-Ile-Ile-Glu-Gln-Pro-Arg-Gln-Arg-Gly-Met-Arg-Phe-Arg (residues 10 to 24 of human c-Rel), and peptide 1507 is NH_2-Asp-Glu-Lys-Asp-Thr-Tyr-Gly-Asn-Lys-Ala-Lys-Lys-Gln-Lys-Thr-Thr-Leu-Leu-Phe-Gln-Lys-Leu-Cys (residues 282 to 304 of human c-Rel) (10). A cysteine residue at the C termini of 1309 and 1206 was added to facilitate coupling to hemocyanin, as described by Liu et al. (29). Peptide 1506 was coupled through its N terminus by using dinitrodifluorobenzene. Peptides were purchased from Multiple Peptide Systems (San Diego, Calif.). Below, each antiserum is referred to by a descriptive name (e.g., h-rel-I for human c-Rel internal and MAD-3-C for MAD-3 [IκBα] C-terminal, etc.) and by its number (e.g., 1136 and 1258, etc.).

RESULTS

Stoichiometry of binding. To understand the interaction of IκBα with Rel family proteins, it is essential to know the stoichiometry of binding. It has been shown that Rel proteins in solution exist as dimers, with few or no monomers, and that DNA binding is accomplished by dimers (7, 14, 24, 45, 50). Our first question was how many IκBα molecules bind to a dimer? In a previous study, Hatada et al. (18) used bacterially expressed and renatured proteins, coupled with glutaraldehyde cross-linking, to suggest that a p65 dimer binds a single IκBα.

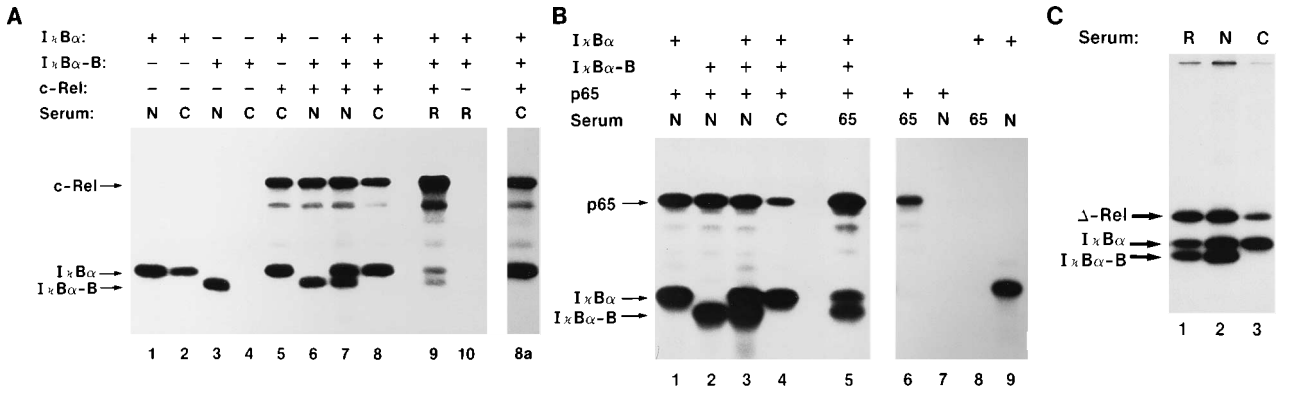

FIG. 1. A single IκBα binds to a c-Rel or p65 dimer. (A) c-Rel, IκBα, and IκBα-B were translated in vitro in a wheat germ lysate, combined as indicated in the figure, and immunoprecipitated with anti-c-Rel (antibody 1206 [h-rel-I]) or anti-IκBα (antibody 1309 [MAD-3-N] or 1258 [MAD-3-C]). Precipitates were analyzed by SDS-PAGE. Lane 8a is a longer exposure of lane 8. (B) p65, IκBα, and IκBα-B were translated in vitro in a reticulocyte lysate, combined as indicated, and immunoprecipitated with anti-p65 (antibody 1226 [65-C]) or anti-IκBα (antibody 1309 or 1258). Precipitates were analyzed by SDS-PAGE. (C) Human 293 cells were cotransfected with plasmids expressing a C-terminal deletion mutant of c-Rel (Δ-Rel), IκBα, or IκBα-B. Forty-eight hours later, the cells were grown in the presence of [^{35}S]methionine and [^{35}S]cysteine for 1 h. The lysate was immunoprecipitated with anti-c-Rel (antibody 1136 [h-rel-I]) or anti-IκBα (antibody 1309 or 1258), and precipitates were analyzed by SDS-PAGE. Antisera: N, 1309; C, 1258; R, 1206 (A) or 1136 (C); 65, 1226.

Because such cross-linking experiments rest on certain untestable assumptions (see Discussion), we wanted to examine the stoichiometry of binding by a completely independent method. Equally important, we wanted to determine whether the answer obtained in vivo is the same as that in vitro.

To analyze the binding of IκBα to c-Rel homodimers, we employed both wild-type IκBα and a deletion mutant of IκBα (mutant IκBα-B) lacking the 14 C-terminal amino acids. If more than one IκBα binds to a c-Rel dimer, then complexes containing both full-length IκBα and mutant IκBα should exist. These complexes should be precipitable by an IκBα peptide antibody (MAD-3-C) directed towards the C terminus of the protein; that is, MAD-3-C should precipitate the complexed mutant IκBα-B even though this antibody cannot recognize the mutant protein. If, on the other hand, a c-Rel dimer binds only one IκBα molecule, no complexes containing both IκBα forms will be found.

c-Rel, IκBα, and IκBα-B were each translated in vitro, mixed in various combinations, and immunoprecipitated (Fig. 1A). As expected, IκBα was recognized by both MAD-3-C (lane 2) and MAD-3-N, an antibody directed towards the N terminus of the protein (lane 1). IκBα-B was recognized by MAD-3-N (lane 3) but not by MAD-3-C (lane 4). When IκBα was mixed with c-Rel, the resulting complex was precipitable with MAD-3-C (lane 5). As expected, the c-Rel–IκBα-B complex was precipitable by MAD-3-N (lane 6). When all three proteins—c-Rel, IκBα, and IκBα-B—were mixed, both wild-type and mutant IκBα were still able to bind c-Rel, since an anti-Rel serum coprecipitated both of them (lane 9). However, MAD-3-C precipitated only full-length IκBα (as well as c-Rel) and not the deletion mutant (lane 8). Thus, there were no complexes precipitable by MAD-3-C that contained c-Rel, IκBα, and IκBα-B. This result strongly suggests that the complexes consist of a c-Rel homodimer plus a single IκBα molecule. The same experiment was performed with in vitro-translated p65, IκBα, and IκBα-B, and the same result was obtained (Fig. 1B).

To verify that this stoichiometry applies to proteins expressed in vivo, a c-Rel expression vector was cotransfected with vectors encoding IκBα and IκBα-B. To avoid inducing synthesis of endogenous IκBα, we used a mutant of c-Rel (Δ-Rel) from which most of the C-terminal transactivating domain has been deleted. It has been shown previously for c-Rel, v-Rel, and p65 that this domain is not required for IκBα binding (4, 15, 23, 25). As with the in vitro-translated proteins, the mutant and wild-type IκBα proteins were each able to bind the Δ-Rel homodimer (Fig. 1C, lane 1), but no complexes containing the homodimer, IκBα, and IκBα-B were detectable (lane 3). We conclude that in vivo, as well as in vitro, a Rel protein dimer binds a single IκBα molecule.

What binds to the NLS? It has been shown that binding of IκBα to Rel protein dimers requires the intact NLS of the dimer. Deletion or mutagenesis of the NLS leaves dimerization and DNA binding unaffected, but ability to bind IκBα is abolished both in vitro and in vivo (4, 15, 25). The simplest interpretation of this result is that IκBα contacts the NLS directly. We therefore designed experiments to test whether the highly acidic C-terminal region of IκBα might bind to the positively charged NLS. Deletions and point mutations were introduced into IκBα (Fig. 2), and the effects of these changes on IκBα binding to c-Rel and p65 were determined.

As detailed below, these experiments demonstrated that the acidic C-terminal region of IκBα is not involved in contacting the NLS of c-Rel or p65 NLS in vivo. However, the initial in vitro experiments did suggest possible involvement. c-Rel, IκBα, and each of the mutants were translated in vitro and assayed for their ability to coimmunoprecipitate. As shown in Fig. 3A, most of the IκBα mutants retained the ability to bind c-Rel, but four of the most drastic mutations did not. In IκBα mutant C, residues 285 to 302 were deleted, while in mutant G, 8 of the 10 acidic residues in the same region were converted to uncharged amino acids. Both of these mutants showed a much reduced ability to bind c-Rel in vitro (lanes 5 and 8). No binding at all was observed between c-Rel and mutant A, in which the entire C-terminal region was deleted (lane 4), and very little binding was seen between c-Rel and mutant J (deletion of residues 279 to 284) (lane 11). These results indicate that the region between residues 279 and 290 is important for binding of IκBα to c-Rel in vitro.

When the proteins were coexpressed in vivo, however, the result was different. Contrary to the in vitro results, c-Rel could associate in vivo with mutants J and C when assayed by coimmunoprecipitation (Fig. 3B, lanes 10 and 11). Very limited binding was also observed between c-Rel and mutant A (lane 12). The same results were obtained when Δ-Rel replaced full-length c-Rel (lanes 6 to 8). These data show that, while

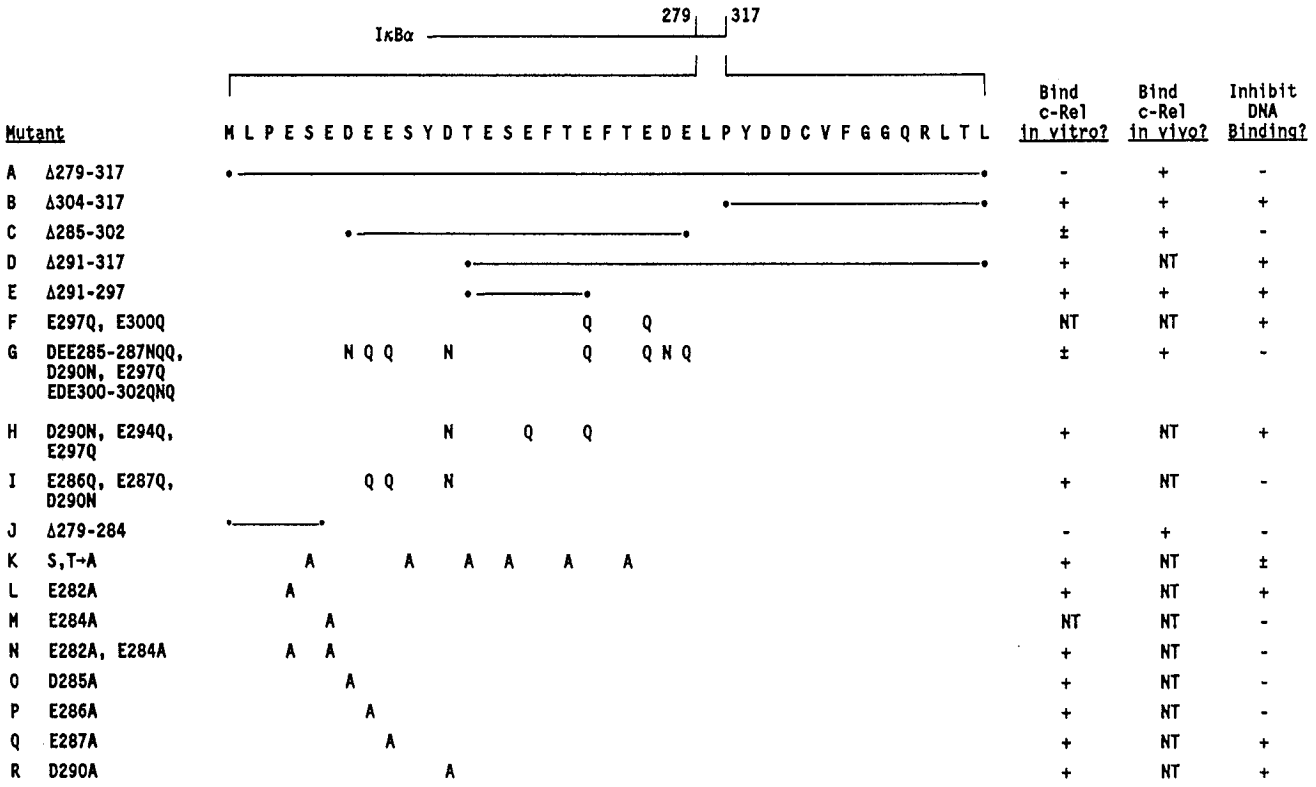

FIG. 2. IκBα mutants. The amino acid sequence of the C-terminal region of IκBα is shown from residue 279 to residue 317, which is the C terminus of the protein. Deletions are indicated (•—•). For point mutations, the mutant amino acids are indicated. On the left, point mutations are described in the one-letter code as the wild-type amino acid, its residue number, and the mutant amino acid, e.g., E282A for E-282→A. Δ, deletion. Results presented in the text are summarized on the right. ±, reduced binding or inhibitory activity relative to wild-type IκBα; NT, not tested.

important for binding to c-Rel in vitro, the J and C mutations are tolerated in vivo. This suggests that c-Rel and/or IκBα expressed in vivo differs from that translated in vitro.

In contrast, in vitro-translated p65 was able to bind to all of the IκBα mutants, including mutant A (data not shown). Similarly, cotransfection experiments demonstrated binding of p65 and IκBα mutants J, C, and A in vivo (Fig. 3B, lanes 2 to 4). Thus, deletion of the entire C-terminal region of IκBα does not prevent binding to p65 in vitro or in vivo, and deletion of most of that region does not prevent binding to c-Rel in vivo.

Since binding to c-Rel and p65 occurs in the absence of the IκBα C-terminal region, it is unlikely that this region contacts the NLS. We tested this directly with an immunofluorescence assay, asking whether various IκBα mutants were able to retain cotransfected Δ-Rel or p65 in the cytoplasm. When Δ-Rel was transfected alone, it was localized to the nucleus (Fig. 4a), and

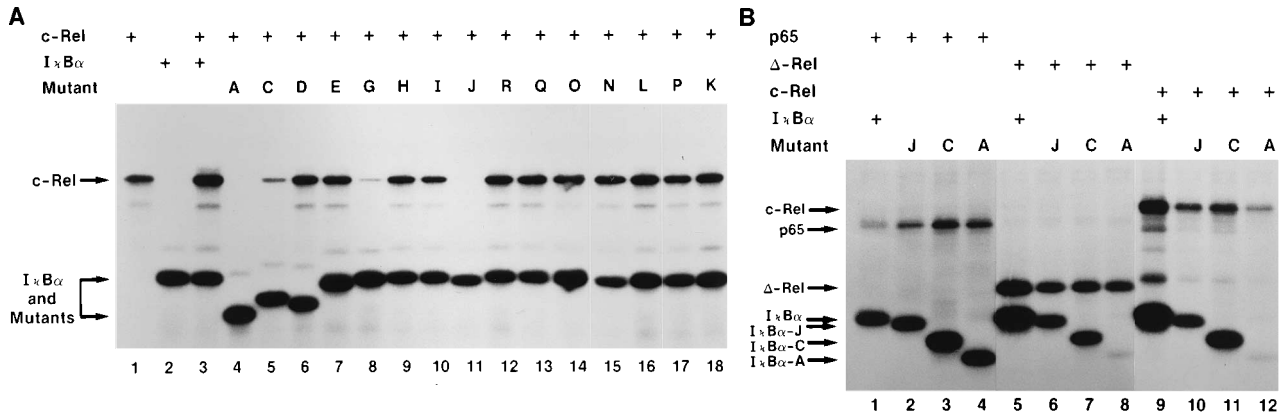

FIG. 3. Binding of IκBα mutants to c-Rel and p65. (A) c-Rel, IκBα, and the IκBα mutants were each individually in vitro translated in a wheat germ lysate containing [^{35}S]methionine and were then combined as indicated. Coimmunoprecipitation was assayed by using anti-IκBα (antibody 1309 [MAD-3-N]; lanes 2 to 17) or anti-c-Rel (antibody 1206 [h-rel-I]; lane 1) and SDS-PAGE. (B) Cotransfections were performed with plasmids expressing a Rel family protein (c-Rel, Δ-Rel, or p65) and an IκBα protein (wild type or mutant IκBα-J, IκBα-C, or IκBα-A). Cells were grown in the presence of [^{35}S]methionine and [^{35}S]cysteine, and lysates were immunoprecipitated with anti-c-Rel (antibody 1136 [h-rel-I]; lanes 5 to 12) or anti-p65 (antibody 1226 [65-C]; lanes 1 to 4). Precipitates were analyzed by SDS-PAGE.

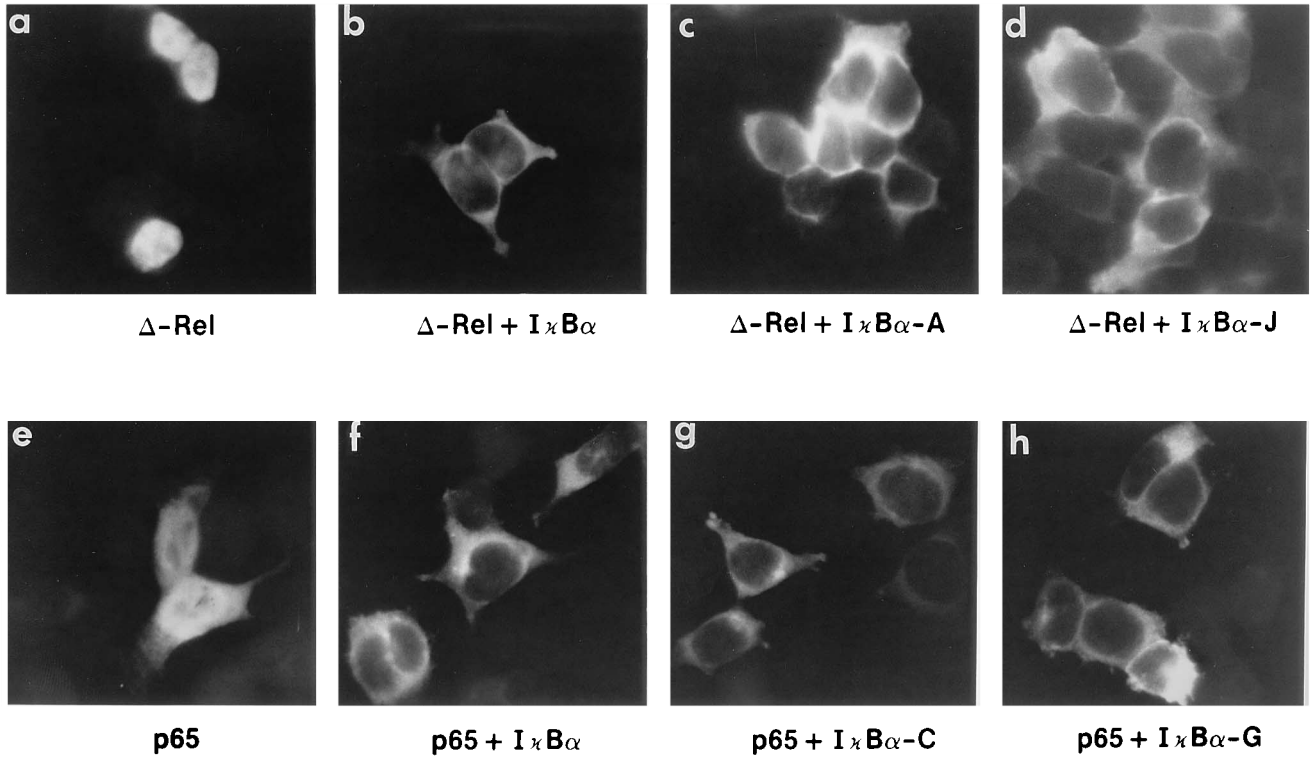

FIG. 4. IκBα mutants retain Δ-Rel and p65 in the cytoplasm. Human 293 cells were transfected with plasmids expressing either Δ-Rel (a) or p65 (e) alone or were cotransfected with plasmids expressing Δ-Rel and an IκBα (b to d) or with p65 and an IκBα (f to h). Cells were analyzed by immunofluorescence using anti-c-Rel (antibody 1136 [h-rel-I]) (a to d) or anti-p65 (antibody 1226 [65-C]) (e to h).

when cotransfected with wild-type IκBα it was cytoplasmic (Fig. 4b). Similarly, cotransfection with any of the IκBα mutants, including those unable to bind to c-Rel in vitro (mutants A, C, G, and J), resulted in cytoplasmic Δ-Rel (Fig. 4c and d and data not shown). (The fact that mutant A bound Δ-Rel in vivo [Fig. 4c] but could not be coprecipitated with c-Rel or Δ-Rel [Fig. 3B] suggests that its binding is very unstable.) When p65 replaced Δ-Rel in the immunofluorescence experiments, similar results were obtained. When transfected alone, p65 was found in nuclei and the cytoplasm (Fig. 4e). Some cytoplasmic p65 is expected, since it efficiently induces synthesis of endogenous IκBα (44, 46). When cotransfected with IκBα mutants C or G, however, p65 was wholly cytoplasmic (Fig. 4g and h). Thus, the highly acidic C-terminal region of IκBα is not involved in binding to the NLS of Rel protein dimers. Since both the N terminus and the C terminus of IκBα are dispensable for binding, the role of the NLS must be mediated by the portion of the molecule containing the six ankyrin repeats.

IκBα C-terminal region and DNA binding. IκBα not only binds to c-Rel or p65; it also prevents their binding to DNA. It is possible that these two functions are separable, i.e., are derived from different regions of the IκBα molecule, since IκBα can bind to p50 (and retain it in the cytoplasm) but cannot inhibit its DNA binding (4, 15). We therefore tested the ability of the IκBα mutants with mutations in the C terminus to inhibit DNA binding by Δ-Rel or p65.

p65 was transfected into human 293 cells, or it was cotransfected with wild-type IκBα or a deletion mutant. Twenty-four hours later, whole-cell extracts were made and tested for DNA binding activity. As expected, cells expressing p65 alone had considerable DNA binding activity (Fig. 5A, lane 1), and this activity was absent in cells expressing both p65 and wild-type IκBα (lane 2). Several of the IκBα mutants were able to completely suppress DNA binding by p65. These included IκBα-B (lane 4), IκBα-D (lane 6), IκBα-E (lane 7), and IκBα-F (lane 8). Suppression by IκBα-H (lane 10) was nearly complete. The region of IκBα affected in these mutants extends from residue 290 to the C terminus (residue 317). In mutant D, residues 291 to 317 are deleted, and in mutant H, residue 290 is changed from Asp to Asn. Since mutants D and H both inhibit DNA binding by p65, residues 290 to 317 are not required for this function.

In contrast, most mutations N-terminal to IκBα residue 290 had a marked effect on the ability to inhibit DNA binding. Deletion of the entire C-terminal region in mutant A (residues 279 to 317) abolished inhibitory activity against p65 (Fig. 5A, lane 3). The smaller deletion in mutant J (residues 279 to 284) also destroyed activity (lane 12), as did deleting or mutating residues 285 to 287 in mutants C (lane 5), G (lane 9), and I (lane 11). This result is not due to a failure to express these mutants, since immunoblot analysis showed that wild-type and mutant IκBα's were all expressed at comparable levels (Fig. 5A, lower panels). Similarly, the results cannot be attributed to instability of the complexes formed between p65 and mutant IκBα's. To demonstrate this, complexes were isolated from cotransfected cells grown in the presence of ^{35}S-amino acids. Cell extracts were prepared and incubated exactly as for the DNA-binding assays, except that, instead of adding DNA, the complexes were immunoprecipitated with anti-p65. SDS-PAGE revealed coprecipitation of p65 with wild-type IκBα, as expected, but also p65 with the IκBα mutants, including mutant A (data not shown). Thus, mutating the IκBα region between

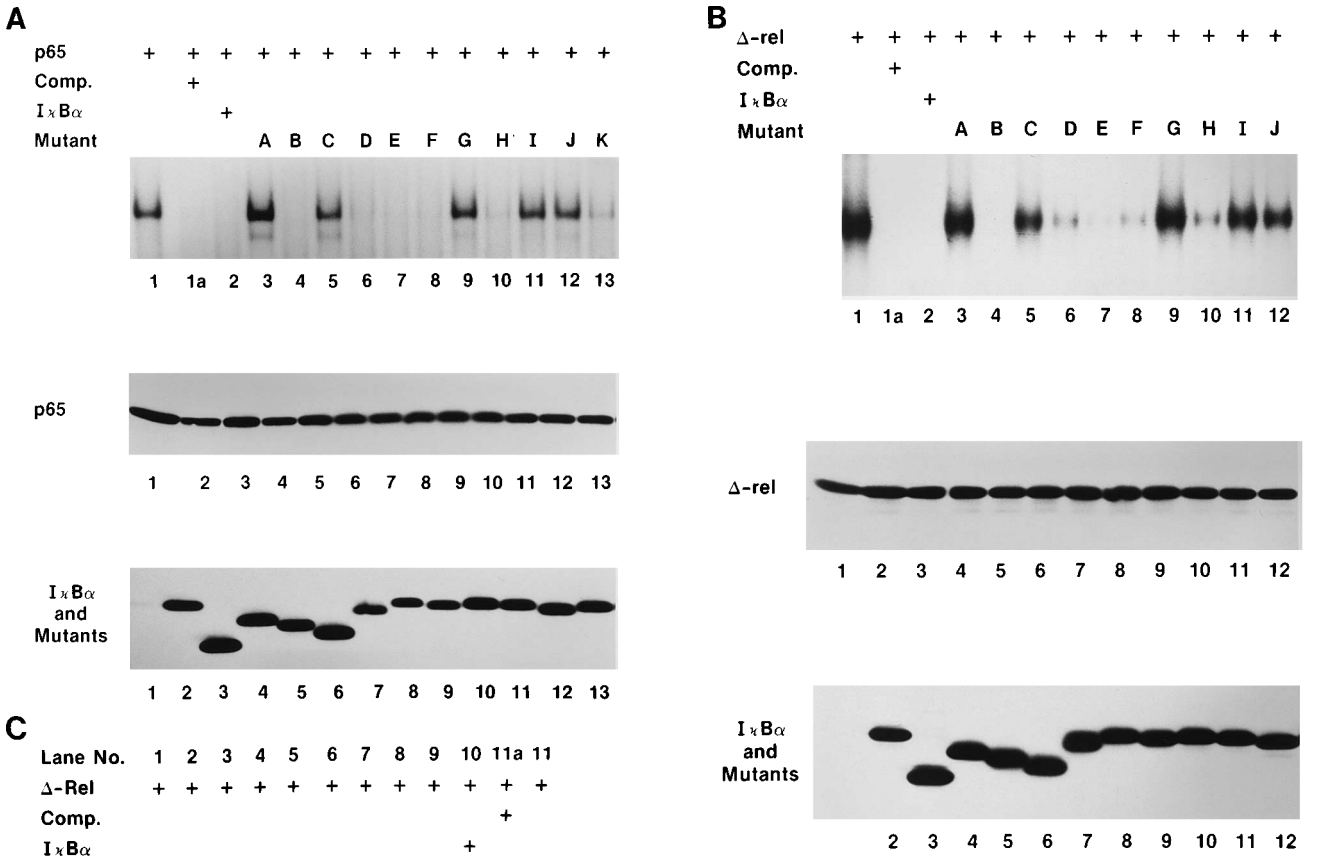

FIG. 5. Some IκBα C-terminal mutants fail to inhibit DNA binding by p65. (A) (Upper panel) Human 293 cells were transfected with a plasmid expressing p65 alone (lanes 1 and 1a), or they were cotransfected with plasmids expressing p65 and IκBα (lane 2) or p65 and an IκBα mutant (lanes 3 to 13). Whole-cell extracts were prepared 24 h later, and aliquots of these were assayed for ability to bind a ^{32}P-oligonucleotide containing the κB binding site from the interleukin-6 gene. Comp., a 40-fold excess of an unlabeled-oligonucleotide competitor. (Lower panels) The transfected cells expressed p65 and an IκBα. Equal aliquots of the whole-cell extracts were analyzed by immunoblotting using anti-p65 (antibody 1226 [65-C]) or anti-IκBα (antibody 1309 [MAD-3-N]). The lanes correspond to those in the upper panel. (B) Same as panel A, except that Δ-Rel was substituted for p65 throughout. Δ-Rel was monitored with h-rel-I (antibody 1136). (C) Same as panel B, with a different panel of IκBα mutants.

residues 279 and 287 leaves the protein able to bind p65 but unable to inhibit DNA binding by p65.

DNA binding experiments were also performed with Δ-Rel and the IκBα mutants, and the results were qualitatively the same (Fig. 5B). The only difference observed was that mutation of IκBα residues 290 to 303 decreased inhibitory activity slightly (lanes 6 to 8 and 10) whereas it had little or no noticeable effect when the mutants were assayed with p65. As with p65, the major effect on inhibitory activity came from mutating various residues between positions 279 and 287 (lanes 5, 9, 11, and 12). IκBα's with these mutations (mutants C, G, I, and J) were expressed (Fig. 5B, lower panel) and were able to coprecipitate with Δ-Rel under conditions of the DNA-binding assay (data not shown). Only mutant A, in which the entire C-terminal region is deleted, failed to remain associated with Δ-Rel under these conditions (just as in Fig. 3B). The inhibitory activity of mutant A on Δ-Rel cannot be measured, therefore, since the complex dissociates prior to the assay.

We next tried to locate the critical residues within the region spanning residues 279 to 287 by testing six point mutants, each of which had a single acidic residue changed to alanine. Mutating Glu-282 (mutant L) had little or no effect on ability to inhibit DNA binding by Δ-Rel (Fig. 5C, lane 1), while mutating Glu-287 (mutant Q) or Asp-290 (mutant R) had a slight effect (lanes 6 and 7). (In two other experiments the inhibitory activity of mutant R was the same as that of mutant Q, unlike the

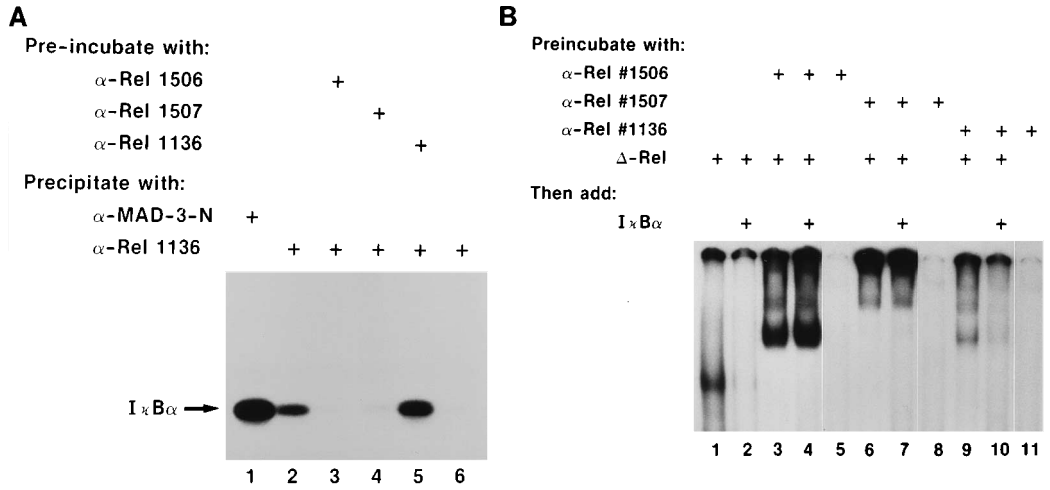

FIG. 6. Blocking the DNA-binding region prevents stable association of Δ-Rel and IκBα. (A) Immunoprecipitation assay. A nonradioactive lysate from 293 cells transfected with Δ-Rel was incubated for 1 h with anti-Rel DNA-binding region (antiserum 1506) (lane 3), anti-rel-NLS (antiserum 1507) (lane 4), anti-rel-I (antiserum 1136) (lane 5), or no antiserum (lanes 1 and 2). A lysate from ^{35}S-labeled cells transfected with IκBα was then added, and the mixture was incubated for an additional hour. Anti-rel-I (antiserum 1136) (lanes 2 to 5) or anti-IκBα (antiserum 1309 [MAD-3N]) (lane 1) was then added, and complexes were collected on protein A-Sepharose and analyzed by SDS-PAGE. Lane 6, ^{35}S-lysate with no Δ-Rel lysate, precipitated with anti-rel-I (antiserum 1136). (B) EMSA. 293 cells were transfected with a plasmid expressing Δ-Rel or IκBα, and whole-cell lysates were prepared 24 h later. These were combined as indicated and assayed for ability to bind a ^{32}P-oligonucleotide containing the κB binding site from the IL-6 gene.

result shown in Fig. 5C.) However changing Glu-284 (mutant M), Asp-285 (mutant O), or Glu-286 (mutant P) had a much greater effect and resulted in a significant loss of activity (lanes 2, 4, and 5). As above, controls showed that the mutants were expressed (Fig. 5C, lower panel) and were able to remain associated with Δ-Rel under the assay conditions (data not shown). Results were similar with p65, except that none of the point mutants was as poor an inhibitor as mutant J or I (data not shown). These results demonstrate that the three consecutive acidic residues at positions 284 to 286 are crucial for inhibitory activity.

Residues 284 to 286 lie within a consensus casein kinase II phosphorylation site

S E
X XX XX
T D

where the X's are amino acids enriched in acidic residues. We therefore wondered whether the three acidic residues at positions 284 to 286 are themselves involved in binding to p65 and Δ-Rel or whether they simply generate a phosphorylation site and it is the phosphoserine that is required for binding. To test this, we used an IκBα mutant (K) in which the six serine and threonine residues between positions 283 and 299 were changed to alanine. It was shown above that mutations C-terminal to residue 290 do not affect inhibitory activity, and four of the six residues changed in mutant K are downstream of 290. Mutant K, therefore, should reveal the effect on inhibitory activity of eliminating Ser-283 (the potential casein kinase II site) and Ser-288 (not a casein kinase II site). The result was that mutant K was less effective than wild-type IκBα in inhibiting p65 but it was more effective than mutants A, C, G, I, and J (Fig. 5A, lane 13). The same result was obtained when Ser-283 was individually changed to Ala (data not shown). Thus, neither Ser-283 nor its phosphorylated form is absolutely required for inhibition of DNA binding, and the acidic residues at positions 284 to 286 play a role independent of their creating a casein kinase II site. There is one indication, however, that a phosphoserine at position 283, while not necessary for inhibiting activity, might contribute to it. When this residue was individually mutated to aspartic acid in order to mimic the effect of a negatively charged phosphoserine, the resulting protein showed no loss in inhibitory activity (data not shown). That is, while the Ala mutant was slightly impaired in inhibiting activity, the Asp mutant was not. Thus, a negative charge at position 283 is helpful, suggesting that this may be supplied in vivo by phosphorylation of the serine residue.

Results presented above suggest that there are at least two distinct interactions between a c-Rel or p65 homodimer and IκBα. One interaction requires the NLS of Rel/p65 and the ankyrin domain of IκBα; this interaction is necessary for binding of a dimer to IκBα. The second interaction involves a portion of the C-terminal region of IκBα; this interaction is dispensable for protein-protein binding but is required for inhibition of DNA binding. It should be noted, however, that the C-terminal region by itself apparently cannot inhibit DNA binding. We tested a synthetic peptide corresponding to IκBα residues 280 to 303 for inhibitory activity and found that it had none.

What c-Rel sequences interact with the IκBα C-terminal region? An attractive hypothesis is that the acidic C terminus of IκBα binds directly to the positively charged DNA-binding sequences of a Rel family dimer, thus preventing their contact with DNA. In fact, both Kerr et al. (23) and Kumar and Gelinas (25) have suggested interaction between chicken pp40 and the v-Rel protein in this region. The amino acid sequence between residues 10 and 30 of human c-Rel is shown below. Residues critical to DNA binding have not been determined for c-Rel, but in v-Rel,

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10              **** **30
IEIIEQPRQRGMRFRYKCEGR
      * *  *    *
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simultaneous mutation of the arginines at positions 17, 19, and 22 (asterisks below the sequence) abolished DNA binding. Mutation of the cysteine at position 27 also affected binding (26). There are somewhat more data available for the highly

related DNA binding site in p50: each of the residues with asterisks above them is critical (8, 30, 32, 49). To test the hypothesis that IκBα contacts c-Rel in this region, we used an antibody (antiserum 1506) raised against residues 10 to 24.

Results presented above showed that all of the IκBα mutants, including mutant A, were able to bind c-Rel in transfected cells. Under immunoprecipitation conditions all of the mutants remained bound to c-Rel, except for mutant A. That is, when all of the contacts normally made by the IκBα C terminus are prevented, as they are in mutant A, the c-Rel–IκBα-A complex that was formed in vivo is unstable in vitro under our conditions. If those contacts involve the c-Rel DNA-binding region, then blocking that or nearby regions with antibody should prevent the association of c-Rel with wild-type IκBα in vitro, just as removing the IκBα contact sequences prevented in vitro binding of mutant A.

Lysate from cells transfected with Δ-Rel was incubated for an hour with the antiserum raised against residues 10 to 24 of c-Rel (antiserum 1506), then for an additional hour with ^{35}S-labeled IκBα (also from transfected cells), and finally with a second anti-Rel serum (antiserum 1136) capable of precipitating c-Rel and any IκBα associated with it. The result was that the 1506 antibody prevented association between Δ-Rel and IκBα (Fig. 6A, lane 3). Controls showed that ^{35}S-IκBα was present (lane 1), that it associated with c-Rel in the absence of antiserum 1506 (lane 2), and that preincubation with the 1136 antibody did not block association (lane 5). As an additional control we used an antibody raised against a 23-amino-acid peptide that includes the c-Rel NLS. This antibody (antiserum 1507) was expected to block association of IκBα with Δ-Rel, and it did (lane 4). Thus, association of Δ-Rel and IκBα was prevented not only by an NLS antibody but by an antibody directed against a portion of the c-Rel DNA-binding sequence and/or nearby upstream sequences.

The same result was obtained when association was monitored by EMSA rather than by immunoprecipitation (Fig. 6B). Surprisingly, antibody 1506 did not block DNA binding by c-Rel but, rather, supershifted the c-Rel–DNA complex (lane 3). This suggests that the major antigenic determinant in peptide 1506 is upstream of the residues directly involved in DNA binding. That being true, we were able to test whether preincubation of Δ-Rel with the antibody would block the inhibitory action of IκBα. We found that it did (lane 4). As expected, the NLS antibody also prevented IκBα inhibition (lane 7), whereas preincubation with h-rel-I antibody (antibody 1136) had no effect (lane 10). Thus, blocking sequences near the DNA-binding region of Δ-Rel with antibody prevented inhibition by IκBα. This is consistent with the possibility that IκBα contacts c-Rel directly at this region.

DISCUSSION

The IκBα molecule can be divided structurally into three major segments: an N-terminal region (amino acids 1 to 70 in human IκBα) with no known function, a middle region (residues 71 to 275) containing six ankyrin repeats, and a C-terminal region (residues 276 to 317) highly enriched in acidic residues. Previous studies have shown that IκBα's ability to inhibit DNA binding is not affected by deletion of its N-terminal region but that most mutations within the ankyrin-containing segment abolish this activity (18, 21). It has also been shown that binding between IκBα and a Rel family dimer requires the NLS of the latter: deletion or mutation of the NLS has no effect on dimerization or DNA binding of the dimer, but it completely eliminates binding by IκBα (4, 15, 25). In this report we show that it must be the ankyrin-like region of IκBα that mediates binding to a c-Rel or p65 homodimer and that the NLS of the dimer is masked as a result of this binding. While the acidic C terminus is dispensable for protein-protein binding in vivo, it is required for inhibition of DNA binding. Further, these tasks are accomplished by a single IκBα molecule per dimer. Each of these points is discussed below.

Stoichiometry of binding. In a previous study, Hatada et al. (18) used glutaraldehyde cross-linking followed by SDS-PAGE to establish the number of IκBα molecules per dimer. The size of the cross-linked product indicated the presence of a single IκBα. Recently Isoda and Nüsslein-Volhard (22) cross-linked the *Drosophila* Cactus and Dorsal proteins through disulfide bonds and obtained the same result. However, such experiments rely on the assumptions that all members of a complex will be covalently joined by the cross-linking agent and, furthermore, that the size of the cross-linked complex can be accurately deduced from its SDS-PAGE mobility. We therefore decided to approach the question of the stoichiometry of binding by a completely independent method.

We used two distinguishable forms of IκBα—wild type and a mutant with a 14-amino-acid C-terminal deletion—and asked whether both forms can bind to the same c-Rel or p65 homodimer. To assay binding, an antibody reactive against the C terminus of IκBα was used; it recognizes wild-type IκBα but not the deletion mutant. The result was very clear: the antibody precipitated complexes containing the dimer and wild-type IκBα, but the deletion mutant was never seen in these complexes. This strongly suggests that a dimer binds a single IκBα molecule.

How could this result be misleading? The experiment assumes that the antibody can recognize a complex with two IκBα's if any such complexes exist. If for some reason the IκBα C terminus becomes inaccessible when two molecules are bound, the experimental result would be meaningless. We do not regard this as a serious possibility, however, since the same antibody is able to precipitate essentially all bound IκBα from a cellular lysate. The experimental result would also be meaningless if the antibody dislodged the mutant IκBα from complexes containing two molecules. While this possibility cannot be ruled out, the binding between the dimer and mutant IκBα does not appear to be particularly unstable. For example, the binding experiments in Fig. 1A and B were performed under conditions of excess IκBα (and mutant) versus c-Rel or p65. If wild-type IκBα binds significantly more stably than the mutant to c-Rel or p65, we might expect to see more wild type than mutant in the bound products. Yet their levels of binding appear roughly comparable (Fig. 1A, lane 9, and B, lane 5). Thus, it seems reasonable to conclude that a dimer binds a single IκBα molecule.

Binding of IκBα to the NLS. It has been well documented that binding of IκBα to a Rel family dimer requires the dimer NLS (4, 15, 25). The simplest interpretation of this result is that IκBα contacts the NLS directly. If this is so, which region of IκBα would be involved? It is unlikely to be the N-terminal region, since deletion of amino acids 1 to 77 of chicken pp40 or amino acids 1 to 70 of human IκBα did not affect binding to Rel protein dimers (18, 21). Similarly, we show in this report that deletion of the C-terminal region (residues 279 to 317 of human IκBα) does not affect binding of IκBα to c-Rel or p65 in vivo: mutant A not only bound c-Rel and p65 but retained them in the cytoplasm. Thus, if IκBα binds directly to the NLS, that binding must be mediated by the ankyrin-like region of IκBα. Furthermore, since each subunit of the dimer has an NLS, the ankyrin region would have to mask both of them, either by binding directly to both or by binding to one and sterically hindering the other. Since each NLS has at least

three positively charged residues, binding might result at least in part from contact between these basic residues and acidic ones located in the IκBα ankyrin region. However, it is not obvious from inspection of the IκBα sequence which acidic residues might be involved. This suggests either that proper positioning of the negative charge results from the three-dimensional structure of the molecule or that the simple idea of NLS-ankyrin contact is mistaken. The crystal structures of these proteins should help to clarify this point.

There are other ankyrin-repeat-containing inhibitors of Rel family dimers, and their binding specificities vary. Like IκBα, IκBγ retains dimers in the cytoplasm (20) and may accomplish this by binding directly to the NLS. Similarly, dimers containing p105 or p100 are cytoplasmic because the C-terminal region of the precursor masks the NLS (6, 19, 33). Bcl-3, however, acts differently. As with IκBα, the ankyrin region of Bcl-3 is required for binding, but the NLS of the p50 dimer is not (37, 51, 54). Further, the p50 NLS does not appear even to be masked when Bcl-3 is bound (54). It will be interesting to learn whether Bcl-3 binds to other (non-NLS) positively charged residues in p50 or whether its binding specificity is actually fundamentally different from that of IκBα.

The fact that neither the N-terminal region nor the C-terminal region of IκBα is necessary for binding to dimers has implications for the mechanism of activation. One current hypothesis is that stimulation of the cell results in transduction of a signal to IκBα, which, perhaps because of a phosphorylation event(s), is released, then recognized, and degraded. If this is true, the critical modifying event must affect the ankyrin region, since both the upstream and the downstream regions are irrelevant to binding. If, on the other hand, the critical modifying event does not cause release of IκBα, but rather targets it for degradation while it is still bound, then that event could theoretically affect any region of the molecule. If it were to affect the N- and/or C-terminal region and not the ankyrin region, then complexes containing the corresponding N- or C-terminal deletion mutant(s) might not be activatable.

Inhibition of DNA binding. While the ankyrin region of IκBα appears to be necessary and sufficient for binding to c-Rel or p65, it is not sufficient to inhibit DNA binding. Inhibitory activity requires the C-terminal region and, more specifically, the short negatively charged segment (SEDE) between residues 283 and 286. Point mutation or deletion of sequences downstream from these residues had relatively little effect on activity, while mutation or deletion of some or all of these residues drastically reduced inhibitory activity.

The critical region has several interesting aspects. First, the serine residue is a potential casein kinase II phosphorylation site. Changing that serine to alanine reduced inhibitory activity somewhat, suggesting that phosphorylation of the serine may be required for maximal activity. To test whether a negative charge at residue 283 contributes to inhibitory activity, the serine was also individually changed to aspartic acid. That mutant inhibited DNA binding as well as wild-type IκBα, suggesting that Ser-283 is phosphorylated in vivo and that the negatively charged phosphate helps in the inhibition of DNA binding.

The critical region is also of interest because it contains at least three negatively charged residues, suggesting that it interacts with a positively charged region in c-Rel or p65. The available evidence is consistent with the possibility that the positive charge resides in the DNA-binding region itself. Kumar and Gelinas (25) have shown that mutation of three such residues in the v-Rel protein markedly affected pp40 binding in vitro. Similarly, we showed that an antibody raised against these and upstream residues in the human c-Rel protein was able to block binding of IκBα in vitro. One might argue that the v-Rel mutations or the antibody binding could disrupt association indirectly by disturbing the conformation of v-Rel or c-Rel. In our case, however, the protein must be very close to native conformation since it can still dimerize and bind DNA. Thus, the results suggest that some sequence at or near the DNA-binding region of c-Rel contacts IκBα and that this is the mechanism by which IκBα inhibits DNA binding. The positively charged residues required for DNA binding and the negatively charged residues in the IκBα C terminus are attractive candidates for this contact. One consequence of such binding is that the PEST-like sequence, which may play a role in the rapid degradation of IκBα, would be shielded. Nevertheless, other less interesting explanations of the data cannot be ruled out. For example, if the DNA-binding region of c-Rel is close to the NLS, antibody 1506 might block the latter and interfere with IκBα binding in that way.

The third interesting feature of the IκBα C-terminal critical region is that it is so small—only four residues which, according to secondary-structure predictions, are part of an extended alpha helix. This small region would presumably be able to contact only one member of the dimer, suggesting that blocking DNA binding by one member is sufficient to reduce total binding activity greatly. This is not to rule out the possibility that in wild-type IκBα the downstream acidic residues also contribute to inhibitory activity, perhaps by contacting the second member of the dimer. Yet mutants lacking the downstream region still inhibit very well (mutants B, D, and E, for example), so the role of those acidic residues, if any, is clearly secondary to the role of the upstream residues at positions 283 to 286.

While IκBα is a potent inhibitor of DNA binding in vitro, it is not known whether it also plays this role in vivo. It is clear that in transfected cells overexpressed IκBα can enter the nucleus (53), but whether it does so in normal cells has not been established. Work is in progress in this and other laboratories to try to resolve this issue.

Association in vivo versus in vitro. Results presented above showed that the ability of c-Rel to associate with various IκBα mutants depended on conditions of the assay. When tested in vivo, by immunofluorescence, all of the C-terminal mutants were able to bind c-Rel. When tested by immunoprecipitation following transfection, all mutants but one were able to bind. But when tested by immunoprecipitation following in vitro translation, two mutants (C and G) bound weakly and two more (mutants A and J) failed to bind at all. Each of these four had a mutation in the region that is required for inhibition of DNA binding.

The reason for the difference in results is not known. One possibility is that cellular c-Rel and/or IκBα differs somehow from that translated in vitro. For example, one or more posttranslational modifications might contribute to the stability of the c-Rel–IκBα complex. Their contribution would not be crucial for binding of wild-type IκBα to c-Rel when both are translated in vitro. When the contact between c-Rel and the IκBα C-terminal region is eliminated, however, the importance of the hypothetical modifications could be magnified. Alternatively, there may be no crucial posttranslational modifications. The difference in results may stem solely from the difference in stability of the wild-type versus the mutant IκBα complexes. If this is so, then a higher protein concentration, longer incubation time, and/or different ionic or detergent conditions might produce more complexes containing mutants A, C, G, and J in vitro.

Whatever the basis for the difference in results, it may not be limited to c-Rel and IκBα C-terminal mutants. For example,

p50 binds IκBα poorly when both are translated in vitro, but binding is readily detected in cells (4, 11a). The C-terminal region of p105 did not bind to c-Rel in vitro but was able to bind in vivo (40). A C-terminal deletion mutant of IκBα that was expressed in *Escherichia coli* failed to bind p65, which had also been isolated from bacteria (18). This IκBα mutant had both N-terminal deletions and C-terminal deletions (residues 1 to 70 and 280 to 317), and its lack of binding to p65 might reflect its origin and/or a possible stabilizing influence of the N-terminal region. Finally, Kumar and Gelinas (25) mutated three arginine residues in the DNA-binding region of v-Rel and found that they lost not only the DNA binding capability but also the ability to bind pp40 (chicken IκBα). They concluded that pp40 contacts v-Rel at the DNA-binding region and that this contact is essential for stability of the complex. In their experiments, both v-Rel and pp40 were translated in vitro. On the basis of the results presented here, it is likely that the missing contact is between the v-Rel DNA-binding region and the pp40 acidic C-terminal region, just as in c-Rel and our IκBα mutant A. This complex is unstable in vitro but stable in vivo, and we predict that the v-Rel–pp40 complex will behave the same way.

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REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA binding activity in an apparently cytoplasmic precursor of the NFκB transcription factor. Cell **53:**211–217.
2. **Baeuerle, P. A., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3:**1689–1698.
3. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
4. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6:**1899–1913.
5. **Beisel, C. E., J. F. Edwards, L. L. Dunn, and N. R. Rice.** 1993. Analysis of multiple mRNAs from pathogenic equine infectious anemia virus (EIAV) in an acutely infected horse reveals a novel protein, Ttm, derived from the carboxy terminus of the EIAV transmembrane protein. J. Virol. **67:**832–842.
6. **Blank, V., P. Kourilsky, and A. Israël.** 1991. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J. **10:**4159–4167.
7. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R.-P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. Mol. Cell. Biol. **12:**685–695.
8. **Bressler, P., K. Brown, W. Timmer, V. Bours, U. Siebenlist, and A. S. Fauci.** 1993. Mutational analysis of the p50 subunit of NF-κB and inhibition of NF-κB activity by *trans*-dominant p50 mutants. J. Virol. **67:**288–293.
9. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
10. **Brownell, E., N. Mittereder, and N. R. Rice.** 1989. A human rel proto-oncogene cDNA containing an Alu fragment as a potential coding exon. Oncogene **4:**935–942.
11. **Cordle, S. R., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-Rel and related NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. **268:**11803–11810.

11a. **Ernst, M. K., et al.** Unpublished data.

12. **Fan, C.-M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature (London) **354:**395–398.
13. **Frantz, B., E. C. Nordby, G. Bren, N. Steffan, C. V. Paya, R. L. Kincaid, M. J. Tocci, S. J. O'Keefe, and E. A. O'Neill.** 1994. Calcineurin acts in synergy with PMA to inactivate IκB/MAD-3, an inhibitor of NF-κB. EMBO J. **13:**861–870.
14. **Fujita, T., G. P. Nolan, S. Ghosh, and D. Baltimore.** 1992. Independent mode of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev. **6:**775–787.
15. **Ganchi, P. A., S.-C. Sun, W. C. Greene, and D. W. Ballard.** 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. Mol. Biol. Cell **3:**1339–1352.
16. **Grilli, M., J. J.-S. Chiu, and M. J. Lenardo.** 1993. NF-κB and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–62.
17. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281–1289.
18. **Hatada, E. N., M. Naumann, and C. Scheidereit.** 1993. Common structural constituents confer IκB activity to NF-κB p105 and IκB/MAD-3. EMBO J. **12:**2781–2788.
19. **Henkel, T., U. Zabel, K. van Zee, J. M. Müller, E. Fanning, and P. A. Baeuerle.** 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell **68:**1121–1133.
20. **Inoue, J.-I., L. D. Kerr, A. Kakizuka, and I. Verma.** 1992. IκBγ, a 70 kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68:**1109–1120.
21. **Inoue, J.-I., L. D. Kerr, D. Rashid, N. Davis, H. R. Bose, Jr., and I. M. Verma.** 1992. Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA **89:**4333–4337.
22. **Isoda, K., and C. Nüsslein-Volhard.** 1994. Disulfide cross-linking in crude embryonic lysates reveals three complexes of the *Drosophila* morphogen dorsal and its inhibitor cactus. Proc. Natl. Acad. Sci. USA **91:**5350–5354.
23. **Kerr, L. D., J.-I. Inoue, N. Davis, E. Link, P. A. Baeuerle, H. R. Bose, Jr., and I. M. Verma.** 1991. The Rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev. **5:**1464–1476.
24. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le-Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
25. **Kumar, S., and C. Gelinas.** 1993. IκBα-mediated inhibition of v-Rel DNA binding requires direct interaction with the RxxRxRxxC Rel/κB DNA-binding motif. Proc. Natl. Acad. Sci. USA **90:**8962–8966.
26. **Kumar, S., A. B. Rabson, and C. Gelinas.** 1992. The RxxRxRxxC motif conserved in all Rel/κB proteins is essential for the DNA-binding activity and redox regulation of the v-Rel oncoprotein. Mol. Cell. Biol. **12:**3094–3106.
27. **Leveillard, T., and I. M. Verma.** 1993. Diverse molecular mechanisms of inhibition of NF-κB/DNA binding complexes by κB proteins. Gene Exp. **3:**135–150.
28. **Libermann, T. A., and D. Baltimore.** 1990. Activation of interleukin-6 gene expression through the NF-κB transcription factor. Mol. Cell. Biol. **10:**2327–2334.
29. **Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18:**690–693.
30. **Liu, J., M. Sodeoka, W. S. Lane, and G. L. Verdine.** 1994. Evidence for a non-α-helical DNA-binding motif in the Rel homology region. Proc. Natl. Acad. Sci. USA **91:**908–912.
31. **Lux, S. E., K. M. John, and V. Bennett.** 1990. Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. Nature (London) **344:**36–42.
32. **Matthews, J. R., W. Kaszubska, G. Turcatti, T. N. C. Wells, and R. T. Hay.** 1993. Role of cysteine62 in DNA recognition by the p50 subunit of NF-κB. Nucleic Acids Res. **21:**1727–1734.
33. **Matthews, J. R., E. Watson, S. Buckley, and R. T. Hay.** 1993. Interaction of the C-terminal region of p105 with the nuclear localisation signal of p50 is required for inhibition of NF-κB DNA binding activity. Nucleic Acids Res. **21:**4516–4523.
34. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB mediated signal transduction. Genes Dev. **7:**705–718.
35. **Michaely, P., and V. Bennett.** 1993. The membrane-binding domain of ankyrin contains four independently folded subdomains, each comprised of six ankyrin repeats. J. Biol. Chem. **268:**22703–22709.
36. **Naumann, M., F. G. Wulczyn, and C. Scheidereit.** 1993. The NF-κB precursor p105 and the proto-oncogene product Bcl-3 are IκB molecules and control nuclear translocation. EMBO J. **12:**213–222.
37. **Nolan, G. P., T. Fujita, K. Bhatia, C. Huppi, H.-C. Liou, M. L. Scott, and D. Baltimore.** 1993. The bcl-3 proto-oncogene encodes a nuclear IκB-like mol-

ecule that preferentially interacts with NF-κB p50 and p52 in a phosphorylation-dependent manner. Mol. Cell. Biol. **13:**3557–3566.
38. **Nolan, G. P., S. Ghosh, H.-C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. Cell **64:**961–969.
39. **Rice, N. R., and M. K. Ernst.** 1993. In vivo control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
40. **Rice, N. R., M. L. MacKichan, and A. Israël.** 1992. The precursor of NF-κB has IκB-like functions. Cell **71:**243–253.
41. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:** 364–368.
42. **Ruben, S. M., R. Narayanan, J. F. Klement, C.-H. Chen, and C. A. Rosen.** 1992. Functional characterization of the NF-κB p65 transcriptional activator and an alternatively spliced derivative. Mol. Cell. Biol. **12:**444–454.
43. **Scheinman, R. I., A. A. Beg, and A. S. Baldwin.** 1993. NF-κB p100 (Lyt-10) is a component of H2TF1 and can function as an IκB-like molecule. Mol. Cell. Biol. **13:**6089–6101.
44. **Scott, M. L., T. Fujita, H.-C. Liou, G. P. Nolan, and D. Baltimore.** 1993. The p65 subunit of NF-κB regulates IκB by two distinct mechanisms. Genes Dev. **7:**1266–1276.
45. **Simek, S., and N. R. Rice.** 1988. $p59^{v\text{-}rel}$, the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed chicken lymphoid cells. J. Virol. **62:**4730–4736.
46. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
47. **Sun, S.-C., P. A. Ganchi, C. Beraud, D. W. Ballard, and W. C. Greene.** 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA **91:**1346–1350.
48. **Tan, T.-H., G. P. Huang, A. Sica, P. Ghosh, H. A. Young, D. L. Longo, and N. R. Rice.** 1992. κB site-dependent activation of the interleukin-2 receptor α-chain gene promoter by human c-Rel. Mol. Cell. Biol. **12:**4067–4075.
49. **Toledano, M. B., D. Ghosh, F. Trinh, and W. J. Leonard.** 1993. N-terminal DNA-binding domains contribute to differential DNA-binding specificities of NF-κB p50 and p65. Mol. Cell. Biol. **13:**852–860.
50. **Urban, M. B., R. Schreck, and P. A. Baeuerle.** 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. **10:**1817–1825.
51. **Wulczyn, F. G., M. Naumann, and C. Scheidereit.** 1992. Candidate proto-oncogene *bcl*-3 encodes a subunit-specific inhibitor of transcription factor NF-κB. Nature (London) **358:**597–599.
52. **Zabel, U., and P. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61:**255–265.
53. **Zabel, U., T. Henkel, M. dos Santos Silva, and P. Baeuerle.** 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J. **12:**201–211.
54. **Zhang, Q., J. A. Didonato, M. Karin, and T. W. McKeithen.** 1994. BCL3 encodes a nuclear protein which can alter the subcellular location of NF-κB proteins. Mol. Cell. Biol. **14:**3915–3926.