MOLECULAR AND CELLULAR BIOLOGY, Apr. 1995, p. 2166–2172 Vol. 15, No. 4
0270-7306/95/$04.00+0
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Domain Organization of IκBα and Sites of Interaction with NF-κB p65

ELLIS JAFFRAY,[1] KATRINA M. WOOD,[2] AND RONALD T. HAY[1]*

School of Biological and Medical Sciences, University of St. Andrews, Fife KY16 9AL, Scotland,[1] *and Department of Cellular Science, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, England*[2]

Received 10 October 1994/Returned for modification 30 December 1994/Accepted 10 January 1995

The DNA-binding activity and cellular distribution of the transcription factor NF-κB are regulated by the inhibitor protein IκBα. IκBα belongs to a family of proteins that contain multiple repeats of a 30- to 35-amino-acid sequence that was initially recognized in the erythrocyte protein ankyrin. Partial proteolysis has been used to study the domain structure of IκBα and to determine the sites at which it interacts with NF-κB. The data reveal a tripartite structure for IκBα in which a central, protease-resistant domain composed of five ankyrin repeats is flanked by an unstructured N-terminal extension and a compact, highly acidic C-terminal domain that is connected to the core of the protein by a flexible linker. Functional analysis of V8 cleavage products indicates that IκBα molecules lacking the N-terminal region can interact with and inhibit the DNA-binding activity of the p65 subunit of NF-κB, whereas IκBα molecules which lack both the N- and C-terminal regions are incapable of doing so. Protease cleavage of the N terminus of IκBα was unaffected by the presence of the p65 subunit of NF-κB, whereas bound p65 blocked cleavage of the flexible linker connecting the C-terminal domain to the ankyrin repeat-containing core of the protein. This linker region is highly conserved within the human, rat, pig, and chicken homologs of IκBα, and while it has been suggested that it represents a sixth ankyrin repeat, it is also likely that this is a flexible region of the protein that interacts with NF-κB.

To a large extent, gene expression is controlled by the frequency of transcriptional initiation at the promoter. Initiation of transcription is preceded by the formation of a large nucleoprotein complex containing promoter DNA, basal transcription factors, upstream activator proteins, and a variety of other proteins. In many cases, the rate at which transcription initiates is limited by the availability or activity of the DNA-binding upstream activators. One such upstream activator is the transcription factor NF-κB, whose DNA-binding activity and nuclear/cytoplasmic distribution are controlled by the IκB inhibitor proteins. In unstimulated cells, NF-κB is held in the cytoplasm, in a form that is unable to bind DNA, by the inhibitory IκB proteins. Exposure of cells to a wide variety of stimuli results in release of the transcription factor from the IκB proteins, allowing the active DNA-binding form of the transcription factor to translocate to the nucleus, where it binds to its recognition sites in the upstream regions of a wide variety of genes that respond to immune and inflammatory signals, including human immunodeficiency virus type 1 (reviewed in references 20 and 28). NF-κB is a dimer of proteins that share a highly conserved region known as the Rel homology domain which contains the sequences responsible for DNA binding, dimerization, and nuclear localization. The 11-bp binding site of NF-κB is recognized by the protein in an unusual way involving base and backbone contacts with the DNA over one complete helical turn (9, 10). In humans, the family of proteins consists of p50 (15, 26), p52 (6, 35, 42), p65 (36, 40), c-Rel (47), and RelB (41). Although it appears that almost all combinations of homo- and heterodimers can exist, the typical form of NF-κB that is activated in response to extracellular signals is composed of a heterodimer of p50 and p65. p50 represents the N-terminal region of a p105 precursor from which it is processed, by a pathway thought to involve ubiquitinylation of the protein (37). The C-terminal region removed from p105 contains multiple repeats of a 30- to 35-amino-acid sequence present in the erythrocyte protein ankyrin (30) and found in all proteins with IκB activity (16). In lymphoid cells, the C-terminal region of p105 has been identified as an independent entity known as IκBγ (23, 29) that preferentially inhibits the DNA-binding activity of p50 homodimers. In the p105 precursor molecule, the C-terminal region is thought to function as a *cis*-acting inhibitor of p105 DNA-binding activity (22). Although p50 does not possess a transcriptional activation domain, its p65 partner does have an acidic activation domain that accounts for the transcriptional activity of the NF-κB heterodimer (14, 43). NF-κB activity is regulated by its association with the inhibitor protein IκBα or MAD3 (2, 18), which like the C-terminal region of p105 and the proto-oncoprotein Bcl-3 contains multiple ankyrin repeats. When cells are exposed to stimuli which activate NF-κB, the transcription factor is freed of the inhibitory activity of IκBα. Examination of the metabolism of IκBα after activation reveals that the protein is rapidly degraded (3, 7, 11, 21, 33, 38, 45). Prior to degradation, IκBα is modified to a more slowly migrating form that results from phosphorylation (3, 7, 11, 21, 33, 45), suggesting that it is a substrate for kinases that are components of signal transduction pathways. Inhibition of protein degradation via the 26S proteosome results in accumulation of the hyperphosphorylated form of IκBα and a failure to activate NF-κB, indicating that IκBα proteolysis is a necessary step in NF-κB activation (37). Degradation of IκBα is rapidly followed by induction of IκBα mRNA in a mechanism that is regulated by interaction of NF-κB with DNA recognition sites located in the promoter of the IκBα gene (8, 27, 45). Resynthesized IκBα protein appears transiently in the nucleus,

* Corresponding author. Mailing address: School of Biological and Medical Sciences, Irvine Building, University of St. Andrews, Fife KY16 9AL, Scotland. Phone: 0334 463396. Fax: 0334 463400. Electronic mail address: rth@st-and.ac.uk.

where it negatively regulates NF-κB-dependent transcription (1). The precise mechanism by which IκBα proteins inhibit both the DNA-binding activity and nuclear translocation of NF-κB proteins has not been determined, but deletion analysis, antibody accessibility experiments, and protease sensitivity studies have indicated that the nuclear localization signals of p50 and p65 are occluded by bound IκBγ and IκBα, respectively (4, 22, 32). Mutational analyses of IκBα, IκBγ, and pp40 have indicated that the integrity of the ankyrin repeats and C-terminal acidic domains is required for interaction with the corresponding NF-κB proteins (5, 19, 24). In the absence of structural information on the IκB proteins, partial proteolysis has been used to define the domain organization of IκBα and probe its interaction with the p65 subunit of NF-κB. IκBα displays a tripartite organization with a central protease-resistant domain containing five ankyrin repeats, an unstructured N-terminal extension that is susceptible to proteolysis, and a small, highly acidic C-terminal domain connected to the core of the protein by a protease-sensitive linker. Functional analysis of the V8 cleavage products indicates that while the N-terminal region is dispensable for IκB activity, IκBα molecules which lack both the N- and C-terminal regions are incapable of interacting with and inhibiting the DNA-binding activity of the p65 subunit of NF-κB. Protease cleavage of the N terminus of IκBα was unaffected by the presence of p65, whereas bound p65 inhibited cleavage of the flexible linker connecting the C-terminal domain to the ankyrin repeat-containing core of the protein. Although it has been suggested that this highly conserved region of the protein represents a sixth ankyrin repeat, it is also likely that this surface-exposed region of IκBα participates in the interaction with NF-κB.

MATERIALS AND METHODS

Expression and purification of proteins. IκBα was expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* JM101, and the fusion protein was purified by affinity chromatography on glutathione-Sepharose as described previously (32). The fusion protein was eluted in a buffer containing 50 mM Tris HCl (pH 8.0), 0.5 M NaCl, and 10 mM reduced glutathione and cleaved with thrombin (1 U/mg of protein). The digest was terminated by addition of phenylmethylsulfonyl fluoride (PMSF) to 1 mM, and the concentration of NaCl was reduced to 0.25 M by dilution with 50 mM Tris HCl (pH 8.0) prior to loading onto a column of Q-Sepharose equilibrated with 50 mM Tris HCl (pH 8.0)–0.25 M NaCl. Bound protein was eluted with an increasing gradient of NaCl from 0.25 to 0.6 M. Fractions containing IκBα were quickly frozen and stored at −70°C. Some preparations of protein contained a minor contaminant resulting from thrombin cleavage of IκBα after R-61. A modified version of the protein containing the epitope tag GKPIPNPLLGLDST (17) fused to the C terminus (called IκB ctag) was expressed and purified in an identical fashion (39). From a 10-liter culture of bacteria, 80 mg of purified IκBα could be recovered. The p65 subunit of NF-κB [amino acids 12 to 317; designated p65(12–317)] was expressed as a fusion with GST, purified on glutathione-Sepharose, and cleaved with thrombin as described above. Before further purification by DNA affinity chromatography, the NaCl concentration was reduced to 0.08 M by dilution with 20 mM sodium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. After extensive washing, bound protein was eluted from the DNA-Sepharose column with 20 mM sodium phosphate buffer (pH 7.5)–0.5 M NaCl–2 mM dithiothreitol. Approximately 60 mg of purified protein was recovered from a 10-liter culture of bacteria. p65 protein was stored as described above.

Proteolytic digestion and identification of the fragments. IκBα, 25 μM in 50 mM Tris HCl (pH 8.0)–0.45 M NaCl, was digested with a range of concentrations (indicated in the figure legends) of either chymotrypsin (*N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK] treated; Sigma) or V8 protease (Sigma) at 20°C for 60 min. Reactions were terminated by the addition of PMSF to a final concentration of 1 mM. Cleavage products were heated to 100°C for 2 min in the presence of sodium dodecyl sulfate (SDS) and mercaptoethanol prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. To determine the point at which cleavage occurred, proteins were electrophoretically transferred to Hyperbond (Beckman) and the amino-terminal sequence was determined in a Beckman microsequencer with on-line phenylthiohydantoin analysis by Graham Kemp, University of St. Andrews. The masses of the cleaved products, and thus the deduced carboxy termini, were determined on a matrix-assisted laser desorption time-of-flight mass spectrometer (Vestek) by Ian Davidson, University of Aberdeen.

Gel electrophoresis DNA binding and protein association assays. The ability of proteolytic fragments of IκBα to inhibit the DNA-binding activity of p65 homodimers was determined in a gel electrophoresis DNA binding assay essentially as described previously (31). Binding reaction mixtures contained 2 nM ^{32}P-labelled double-stranded oligonucleotide 5′-CTGGGGACTTTCCAGG-3′ (top strand) derived from the human immunodeficiency virus type 1 enhancer, 2 nM p65(12-317) (described above), and 1.5 nM IκBα or proteolytic digestion product. After incubation at 20°C for 30 min, free DNA and DNA-protein complexes were separated on a native polyacrylamide gel. In the protein association experiments, GST-p65(12-317), GST-p65Δ(1-317), and GST were bound to glutathione-Sepharose (0.5 mg of fusion protein or 0.25 mg of GST per packed ml of beads). Each assay mixture, in a total volume of 200 μl, contained 20 μl (packed volume) of GST- or fusion protein-bound beads, 250 nM IκBα or V8 digestion product in 10 mM Tris HCl (pH 8.0), 100 mM NaCl, 1 mg of bovine serum albumin (BSA) per ml, and 0.1% Nonidet P-40 (binding buffer). Proteins were allowed to interact for 60 min at 20°C, after which the beads were collected by centrifugation and washed four times with binding buffer. Beads were resuspended in gel loading buffer containing SDS and mercaptoethanol and heated at 100°C for 2 min prior to analysis by Western blotting (immunoblotting).

Western blotting. Proteins were separated in SDS-polyacrylamide minigels prior to electrophoretic transfer onto polyvinylidene difluoride membranes (Sigma). Membranes were incubated in blocking buffer (phosphate-buffered saline [PBS] containing 5% nonfat milk and 0.05% Tween 20) followed by primary antibody diluted in the same buffer and then washed twice with PBS containing 0.05% Tween 20. Detection of immobilized antigen-antibody complexes was performed by using horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse immunoglobulins with an enhanced chemiluminescence detection system (Amersham). Primary antibodies used were affinity-purified immunoglobulins obtained from a rabbit immunized with recombinant IκBα, monoclonal antibody MAD 10B, which recognizes an epitope located between amino acids 21 and 48 of IκBα (data not shown), and monoclonal antibody SV5-Pk (18).

RESULTS

IκBα contains a structured central domain with flexible N- and C-terminal extensions. Large proteins are often composed of a limited number of independently folded domains connected by flexible interdomain linkers. Limited proteolysis is an excellent tool with which to probe this structural organization, as the compact, highly structured domains are resistant to proteolysis whereas the unstructured connecting regions are susceptible to cleavage. IκBα expressed in bacteria and purified by a combination of affinity and ion-exchange chromatography was incubated with a variety of proteases, and the digestion products were fractionated by SDS-PAGE. On the basis of these observations, the domain structure of IκBα was explored by using chymotrypsin, which cleaves after bulky hydrophobic residues, and V8 protease, which cleaves after amino acids with acidic side chains. In each case, the ratio of IκBα to protease was varied over a wide range to identify regions of the protein most and least susceptible to proteolytic cleavage. Cleavage sites were identified by N-terminal sequencing and laser desorption time-of-flight mass spectrometry. At low concentrations of chymotrypsin, cleavage takes place at the N terminus of the protein, after residues W-11 and L-20. Higher concentrations of the enzyme lead to cleavage in the C terminus of IκBα after Y-251 and W-258. At the highest concentration of chymotrypsin used, a small, highly resistant domain comprising residues 67 to 201 was detected (Fig. 1). A similar situation was apparent when IκBα was exposed to V8 protease. Initial cleavage took place in the N-terminal region of the protein after residues E-48 and E-51, with further cleavage taking place after residue E-275. At high concentrations of V8 protease, a stable domain comprising residues 52 to 275 accumulates (Fig. 2). These data suggest that IκBα contains a highly structured central domain that is resistant to proteolysis with flexible N- and C-terminal extensions that are sensitive to proteolytic digestion. IκBα is a member of a growing family of proteins that are characterized by the possession of a number of repeats of a 35-amino-acid sequence that was first recognized in the erythrocyte protein ankyrin (18, 30). These ankyrin repeats are

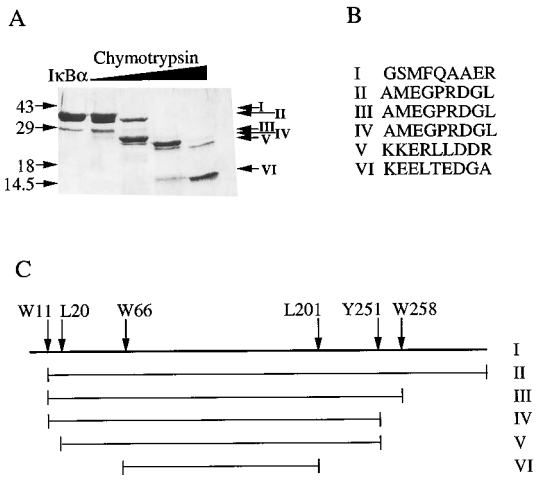

FIG. 1. Digestion of IκBα with chymotrypsin. (A) IκBα (25 μM, 16 μg) was incubated either in the absence or in the presence of 9.4, 38, 113, or 300 ng of TLCK-treated chymotrypsin. Products of digestion were separated in an SDS–12.5% polyacrylamide gel and stained with Coomassie blue. Molecular mass markers are indicated in kilodaltons on the left. (B) Digestion products were electrophoretically transferred to Hyperbond and stained with amido black, and the N-terminal sequences of fragments I to VI were determined in a Beckman microsequencer. (C) Molecular masses of fragments were determined by matrix-assisted laser desorption time-of-flight mass spectrometry (Vestek). The deduced structure for each of the fragments is shown. The indicated amino acids are N terminal to the cleavage site.

located in the central domain of IκBα and therefore appear to fold into a compact, protease-resistant structure.

Functional properties of domains. Digestion of IκBα with low concentrations of V8 protease generates two species (II and III) which lack the N terminus of the protein but are otherwise intact (Fig. 2). At higher concentrations of V8 protease, the C-terminal region of the protein is also removed, leaving the central domain of the protein which contains the

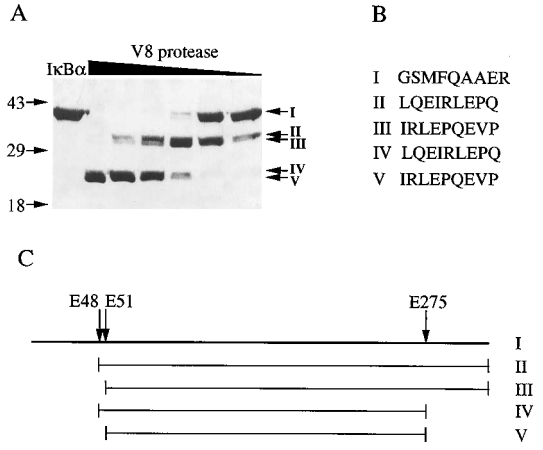

FIG. 2. Digestion of IκBα with V8 protease. (A) IκBα (25 μM, 16 μg) was incubated either in the absence or in the presence of 150, 75, 38, 19, 9.5, or 4.7 ng of V8 protease. Products of digestion were separated in an SDS–10% polyacrylamide gel and stained with Coomassie blue. Molecular mass markers are indicated in kilodaltons on the left. (B) Digestion products were electrophoretically transferred to Hyperbond and stained with amido black, and the N-terminal sequences of fragments I to VI were determined in a Beckman microsequencer. (C) Molecular masses of fragments were determined by matrix-assisted laser desorption time-of-flight mass spectrometry (Vestek). The deduced structure for each of the fragments is shown. The indicated amino acids are N terminal to the cleavage site.

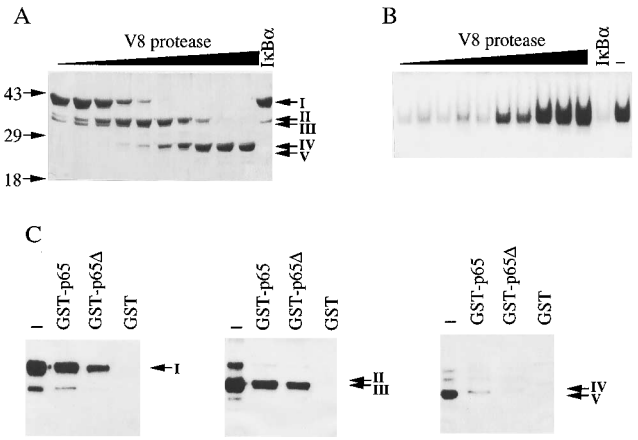

FIG. 3. Functional properties of IκBα fragments generated by V8 protease cleavage. (A) IκBα (25 μM, 16 μg) was incubated either in the absence or in the presence of a range of concentrations of V8 protease as described in the legend to Fig. 2. Reactions were terminated with PMSF, and a fraction of the products was analyzed in an SDS–10% SDS polyacrylamide gel and stained with Coomassie blue. Molecular mass markers are indicated in kilodaltons on the left. (B) Ability of V8 digestion products of IκBα to inhibit the DNA-binding activity of NF-κB p65 homodimers. The DNA-binding activity of recombinant p65 was determined in a gel electrophoresis DNA binding assay. Binding reaction mixtures contained 2 nM ^{32}P-labelled double-stranded oligonucleotide (CTGGG GACTTTCCAGG), 2 nM p65(12-317), and 1.5 nM IκBα or proteolytic digestion product. DNA-protein complexes were resolved on a native 6% polyacrylamide gel and visualized by autoradiography of the dried gel. (C) Ability of V8 digestion products of IκBα to associate with NF-κB p65 homodimers. Unfractionated V8 digestion products of IκBα (lane −) or digestion products bound to either GST or GST-p65 fusion proteins immobilized on glutathione-agarose were separated in an SDS-polyacrylamide gel and analyzed by Western blotting with IκBα-specific, affinity-purified immunoglobulins.

ankyrin repeats. One of the characteristics of IκBα is its ability to inhibit the DNA-binding activity of the p65 subunit of NF-κB. The products of digestion of IκBα with V8 protease were therefore tested for the ability to interact with and inhibit the DNA-binding activity of homodimeric p65. IκBα was incubated with 10 different amounts of V8 protease, and digestion was terminated by the addition of PMSF. The extent of digestion was determined by analysis of the products in an SDS-polyacrylamide gel followed by staining with Coomassie blue (Fig. 3A). Each digested product was added to a reaction mixture containing the bacterially expressed DNA-binding domain of p65 (amino acids 12 to 317) and a ^{32}P-labelled double-stranded oligonucleotide containing its recognition site. Residual DNA-binding activity was determined in a gel electrophoresis DNA binding assay. In the absence of IκBα, p65 forms a stable complex with its DNA recognition site but is unable to do so in the presence of IκBα. Comparison of the extent of digestion, as monitored by SDS-PAGE, with the ability to inhibit the DNA-binding activity of p65 reveals that even when most of the IκBα has been converted into forms II and III, the truncated protein is still capable of inhibiting p65 DNA binding (Fig. 3A and B). However, further digestion of IκBα to generate forms IV and V is accompanied by loss of its inhibitory activity (Fig. 3A and B). Thus, it appears that whereas the N-terminal region of IκBα is dispensable, the C-terminal region is required for its ability to inhibit the DNA binding of NF-κB p65. As a prerequisite for inhibition of the DNA-binding activity of p65, IκBα must physically interact with p65. The ability of the truncated forms of IκBα to interact with p65 was tested directly when two different p65-GST fusion proteins, bound to glutathione-agarose, were incubated with V8-digested IκBα and proteins remaining bound to the beads after

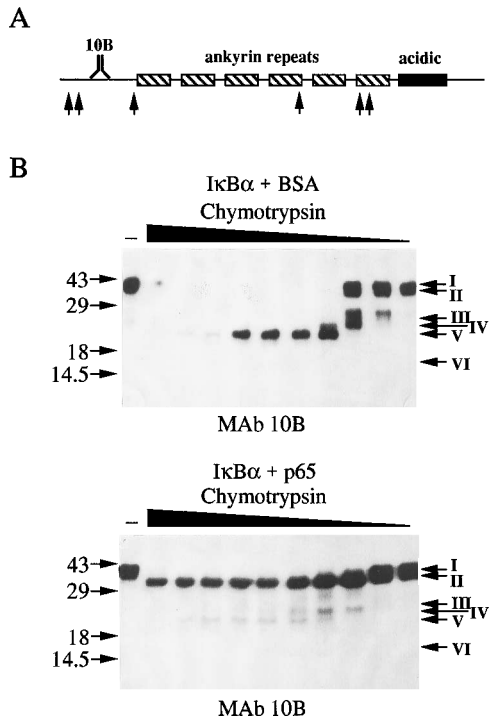

FIG. 4. Chymotrypsin cleavage of IκBα in the presence of NF-κB p65. (A) Proposed domain structure, chymotrypsin cleavage sites, and binding site of monoclonal antibody MAD 10B in IκBα. (B) IκBα (4.8 μM, 2.4 μg) was incubated with either BSA (12 μM, 12 μg) or NF-κB p65 (24 μM, 12 μg) and then digested with either 0, 100, 87.5, 75, 62.5, 50, 37.5, 25, 12.5, 6.2, or 3.1 ng of TLCK-treated chymotrypsin for 60 min at 20°C. A small fraction of the digestion products was separated by electrophoresis in an SDS–12.5% polyacrylamide gel and detected by Western blotting using monoclonal antibody MAD 10B (MAb 10B). Molecular mass markers are indicated in kilodaltons on the left. Chymotryptic cleavage products I to VI are those described in detail in Fig. 1.

extensive washing were resolved by SDS-PAGE. IκBα protein fragments were detected by Western blotting using an affinity-purified rabbit antiserum. Both forms of p65 physically interact with IκBα forms I, II, and III but fail to bind to IκBα forms IV and V (Fig. 3C). The regions of IκBα that are required for physical interaction with p65 and inhibition of its DNA-binding activity therefore appear to coincide and demonstrate that the N-terminal 51 amino acids are dispensable for this purpose.

Chymotrypsin cleavage of IκBα in the presence of NF-κB p65. Changes in the protease susceptibility of specific regions of a protein that accompany binding of a ligand can be used to infer which regions of the protein are in contact with the ligand. The experimental strategy adopted was recently used to demonstrate that bound IκBγ protected the nuclear localization signal of NF-κB p50 from digestion with trypsin (32). IκBα was mixed and allowed to interact with either p65 or an equivalent amount of BSA as a control. The protease susceptibility of IκBα under these conditions was determined by incubation with a range of concentrations of protease and detection of the cleaved IκBα products by Western blotting using antibodies specific for IκBα. In the absence of p65, IκBα is cleaved by chymotrypsin at six surface-exposed sites to generate forms I to VI, which can be detected by PAGE (Fig. 1 and 4). When IκBα is incubated with chymotrypsin in the presence of BSA and the digestion products are detected by Western blotting with monoclonal antibody MAD 10B, fragments I to V are clearly visible. At low concentrations of chymotrypsin, cleavage takes place after W-11 to generate fragment II, but at higher concentrations, IκBα is cleaved in the C-terminal region of the protein after residues W-258 and Y-251 to generate fragments III and IV, respectively (Fig. 4B, upper panel). Further cleavage after L-20 generates fragment V. The epitope recognized by monoclonal antibody MAD 10B is located between K-21 and E-48 (data not shown), and although fragment V becomes undetectable, fragment VI does not appear on the Western blot, as cleavage after W-66 removes the epitope bound by the antibody (Fig. 4B). In contrast to the progressive chymotryptic cleavage of IκBα observed in the presence of BSA, IκBα bound to p65 is extremely resistant to degradation by chymotrypsin. Only fragment II is generated, and it is not further processed to fragment III, IV, or V, although the decrease in the amount of MAD 10B-reactive material suggests that cleavage after W-66 is still taking place (Fig. 4B, lower panel). It is also likely that cleavage after L-20 is taking place although the fragment generated is not resolved from fragment II, as all of the C-terminal cleavages appear to be blocked by bound p65. Thus, it appears that chymotrypsin cleavage of IκBα after residues W-11, L-20, and W-66 is unaffected by the presence of bound p65, whereas residues L-201, Y-251, and W-258 are protected from cleavage by bound p65.

V8 protease cleavage of IκBα bound to p65. To obtain further information on sites of interaction between IκBα and p65, the accessibility of acidic residues in IκBα to V8 protease was determined, both in the free protein and in the presence of bound p65. By using the strategy outlined above, IκBα was digested with V8 protease in the presence of BSA, and the products of digestion were detected by Western blotting using monoclonal antibody MAD 10B (Fig. 5B, right panels) or an affinity-purified immunoglobulin G preparation (Fig. 5B, left panels). At low V8 concentrations, cleavage takes place after residues E-48 and E-51 to generate the previously recognized fragments II and III (Fig. 2) and a rapidly migrating fragment that corresponds to the N terminus of the protein, as it is recognized by MAD 10B. Although a species that corresponds to fragments II and III is detected by MAD 10B (epitope within residues 21 to 48), it is clear that this fragment is not generated by cleavage at E-48 or E-51 but extends from the N terminus of the protein to the cleavage site after E-275. Higher concentrations of V8 lead to cleavage of fragments II and III after E-275 to generate the previously characterized fragments IV and V (Fig. 2), which are not detected by MAD 10B but are detected, albeit weakly, by the polyclonal antibodies (Fig. 5B, left panels). In the presence of p65, cleavage after E-48 and E-51 is unaffected, as the rapidly migrating fragment that represents the N terminus of the protein is generated at the same concentrations of V8 as is the fragment observed when IκBα is incubated with BSA (Fig. 5B). However, bound p65 completely blocks cleavage after E-275, as fragments II and III accumulate and fragments IV and V are not detected with the polyclonal antibodies (Fig. 5B, left panels). Likewise, the fragment from residues 1 to 275 detected with MAD 10B in the presence of BSA is not detected in the presence of bound p65 (Fig. 5B, right panels).

Although it is clear from the experiments described above that V8 cleavage releases an intact N terminus from IκBα, it was not possible to determine the fate of the C-terminal fragment. To address this problem, we used IκB ctag (Fig. 6A), in which the C terminus is fused to a 14-amino-acid sequence derived from P protein of simian virus type 5 (1). This C-terminal epitope tag is recognized by monoclonal antibody SV5-Pk (17). IκB ctag was incubated with either BSA or p65 and exposed to a range of concentrations of V8 protease, and the digestion products were detected by Western blotting with the anti-tag monoclonal antibody. In the presence of BSA, we

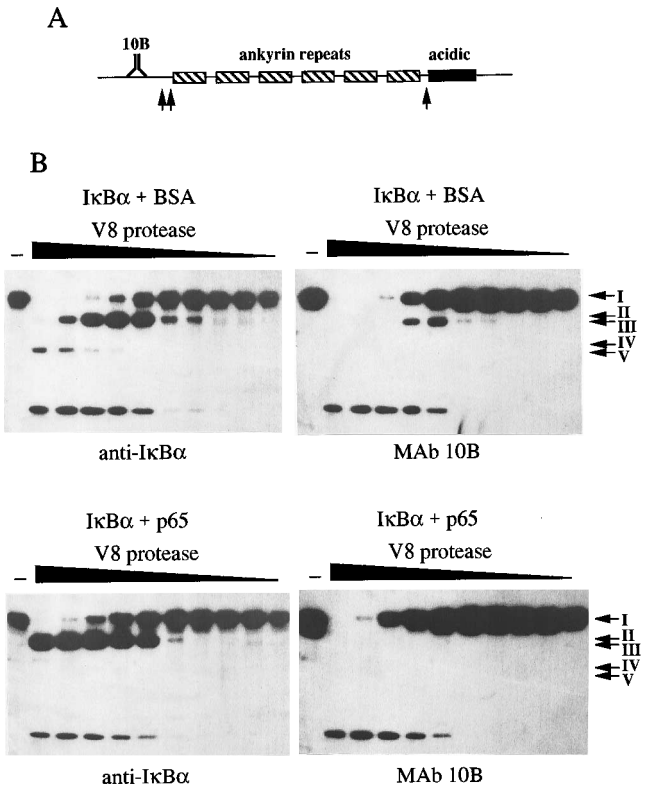

FIG. 5. Protease V8 cleavage of IκBα in the presence of NF-κB p65. (A) Proposed domain structure, V8 protease cleavage sites, and binding site of MAb 10B in IκBα. (B) IκBα (4.8 μM, 2.4 μg) was incubated with either BSA (10 μM, 10 μg) or NF-κB p65 (20 μM, 10 μg) and then digested with either 0, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, or 0.19 ng of protease V8 for 60 min at 20°C. A small fraction of the digestion products was separated by electrophoresis in an SDS–10% polyacrylamide gel and detected by Western blotting using either affinity-purified immunoglobulins specific for IκBα (anti-IκBα; left panels) or monoclonal antibody MAD 10B (MAb 10B; right panels). Protease V8 cleavage products I to V are those described in detail in Fig. 2.

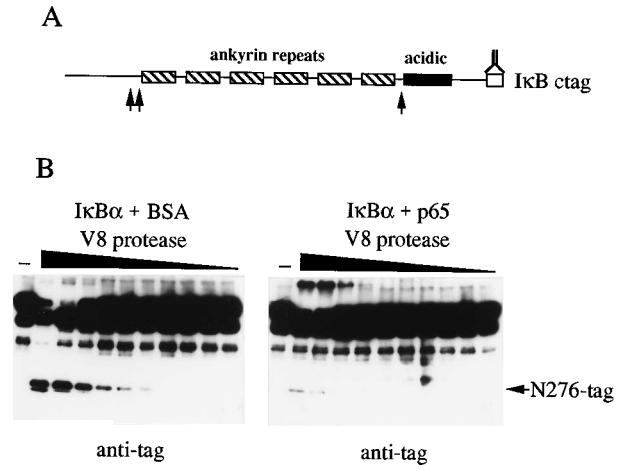

FIG. 6. Protease V8 cleavage of IκB ctag alone or in the presence of NF-κB p65. (A) Proposed domain structure, V8 protease cleavage sites, and binding site of MAb SV5-Pk in IκB ctag. (B) IκB ctag (4.8 μM, 2.4 μg) was incubated with either BSA (10 μM, 10 μg) or NF-κB p65 (20 μM, 10 μg) and then digested with either 0, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, or 0.19 ng of protease V8 for 60 min at 20°C. A small fraction of the digestion products was separated by electrophoresis in an SDS–12.5% polyacrylamide gel and detected by Western blotting using monoclonal antibody SV5-Pk. The position of the fragment which results from the previously characterized cleavage after E-275 (Fig. 2) and extends from N-276 to the C terminus of the IκB ctag protein is indicated (N276-tag).

detected a rapidly migrating species that, from molecular weight estimates, is likely to represent a fragment that results from the previously characterized cleavage after E-275 (Fig. 2) and extends from N-276 to the end of the epitope tag (Fig. 6B, left panel). As expected, this cleavage is not detected in the presence of bound p65 (Fig. 6B, right panel). The resistance of this small C-terminal fragment to cleavage with V8 protease is surprising, as this region of the protein is rich in the acidic amino acid residues (14 of 41 amino acids are aspartic or glutamic acid), none of which are recognized by the protease. It is therefore possible that the C terminus of IκBα represents a small, independently folded domain that is resistant to proteolysis.

DISCUSSION

Partial proteolysis experiments reported here and summarized in Fig. 7 suggest a tripartite structure for the IκBα protein in which a conserved central domain containing the ankyrin repeats is flanked by a relatively unstructured N-terminal domain and a small C-terminal region that may form an independently folded domain. The N-terminal domain of IκBα is extremely susceptible to protease digestion, with both chymotrypsin and V8 protease cleaving at multiple sites. This region also contains a thrombin cleavage site that is cleaved in the native protein when high concentrations of thrombin are used to release IκBα from its GST fusion partner. These sites are equally accessible when p65 is bound to IκBα (Fig. 4 and 5), indicating that they are unlikely to be directly involved in the IκBα-p65 interaction. IκBα molecules in which the N-terminal region had been removed by cleavage with V8 protease were still capable of interacting with and inhibiting the DNA-binding activity of NF-κB p65 (Fig. 3). Analysis of deleted versions of IκBα (19) and chicken pp40 (24) also reached the conclusion that the N-terminal region was dispensable for interaction with NF-κB. The flexibility and surface exposure of the N-terminal region are also suggested by the vigorous antibody response that this region elicits when the intact protein is used as an immunogen. A significant fraction of the polyclonal antibody response is directed against this region (Fig. 5), and the monoclonal antibodies obtained were directed against this part of the protein. The central region of IκBα is a compact, independently folded domain that is relatively resistant to proteolysis. This domain appears to be constructed from ankyrin repeats (30), each of which is composed of 30 to 35 amino acids. Comparisons of the sequences of a number of proteins with IκB-like activities such as human MAD3 (IκBα), porcine ECI-6, rat RL/IF-1, and chicken pp40 (12, 13, 18, 46) have revealed that the proteins contain five ankyrin repeats, although on the basis of alignments with IκBγ and Bcl-3, it has been suggested that six repeats are present (19, 25). Examination of the sequences in the area of the potential sixth repeat reveals that while this region in IκBγ and Bcl-3 conforms to the

FIG. 7. Summary of proposed domain structure and protease accessibility of IκBα in the presence or absence of bound NF-κB p65. Cleavage sites for chymotrypsin (c) and V8 protease (v) are indicated, as are the proposed ankyrin repeats (■). ▨, low-homology repeat; □, acidic domain; ▼, cleavage blocked by bound p65; ▽, cleavage unaffected by bound p65.

ankyrin repeat consensus, only vestiges of a repeat are recognizable in IκBα, ECI-6, RL/IF-1, and pp40. Repeats 1 to 5 appear to pack into a single folded unit that is largely resistant to cleavage with proteases, although at high concentrations of chymotrypsin, cleavage within the unit, at the end of repeat 4, is evident (Fig. 7). In fact, the region corresponding to a sixth repeat is highly susceptible to cleavage with chymotrypsin, indicating that it is rather unstructured. In the presence of p65, cleavage within this region by chymotrypsin and at the boundary of this region by V8 protease is blocked, suggesting either that this region of the protein directly interfaces with p65 and is thus occluded in the IκBα-p65 complex or that p65 induces a conformational change in IκBα that renders it resistant to proteolysis (Fig. 4 to 7). It is also worth noting that this region is highly conserved among the IκBα homologs MAD3, ECI-6, RL/IF-1, and pp40, which all bind NF-κB but release the transcription factor after undergoing rapid, signal-induced proteolytic degradation. Immediately C terminal to the V8 cleavage site at E-275 is a highly acidic region of the protein that appears to form a small, independently folded domain. This conclusion is based on the observation that although this region of the protein contains a high proportion of aspartates and glutamates, these potential cleavage sites are not recognized by the V8 protease. Functional analysis of the V8-cleaved products indicates that the integrity of this region of the protein is essential for interaction with and inhibition of NF-κB p65 (Fig. 3), a conclusion that was also reached by deletion analysis (19, 24). This domain of the protein is again highly conserved in the IκBα homologs but is also present at a similar position in the C-terminal region of p105 or IκBγ, where it is also required for inhibitory activity (19). Fusion of the acidic domain of IκBγ to the DNA-binding domain of GAL4 demonstrates that this region of the protein has the potential to act as an acidic activator of transcription (34), although the importance of this observation for NF-κB dependent transcription has yet to be established.

ACKNOWLEDGMENTS

We thank Graham Kemp and Paul Talbot (University of St. Andrews) for protein sequencing, Ian Davidson (University of Aberdeen) for mass analysis, and Bill Blyth for photography. The help of Rick Randall and Tomas Hanke in the construction and detection of epitope-tagged protein is gratefully appreciated.

This work was supported by BBSRC (United Kingdom) and benefited from collaboration funded by the European Communities Concerted Action (project ROCIO). K.M.W. was supported by the Wellcome Trust.

REFERENCES

1. **Arenzana-Seisdedos, F., J. Thompson, M. S. Rodriguez, F. Bachelerie, D. Thomas, and R. T. Hay.** Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activities of NF-κB. Mol. Cell. Biol., in press.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:**540–546.
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 IκB-α: 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. **Blank, V., P. Kourilsky, and A. Israel.** 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.
6. **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.
7. **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.
8. **Chiao, P. J., S. Miyamoto, and I. M. Verma.** 1994. Autoregulation of IκBα activity. Proc. Natl. Acad. Sci. USA **91:**28–32.
9. **Clark, L., and R. T. Hay.** 1989. Sequence requirement for specific interaction of an enhancer binding protein (EBP1) with DNA. Nucleic Acids Res. **17:**499–515.
10. **Clark, L., J. Nicholson, and R. T. Hay.** 1989. Enhancer binding protein (EBP1) makes base and backbone contacts over one complete turn of the DNA double helix. J. Mol. Biol. **206:**615–626.
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.
12. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science **253:**1268–1271.
13. **de Martin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F. H. Bach.** 1993. Cytokine inducible expression in endothelial cells of an IκBα-like gene is regulated by NF-κB. EMBO J. **12:**2773–2779.
14. **Fujita, T., G. P. Nolan, S. Ghosh, and D. Baltimore.** 1992. Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev. **6:**775–787.
15. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62:**1019–1029.
16. **Gilmore, T. D., and P. J. Morin.** 1993. The IκB proteins: members of a multifunctional family. Trends Genet. **9:**427–433.
17. **Hanke, T., P. Szawlowski, and R. E. Randall.** 1992. Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. J. Gen. Virol. **73:**653–660.
18. **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.
19. **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.
20. **Hay, R. T.** 1993. Control of nuclear factor-κB DNA binding activity by inhibitor proteins containing ankyrin repeats. Biochem. Soc. Trans. **21:**926–930.
21. **Henkel, T., T. Machleidt, I. Alkalay, M. Krönke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
22. **Henkel, T., U. Zabel, K. van Zee, J. M. Muller, 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.
23. **Inoue, J.-I., L. D. Kerr, A. Kakizuka, and I. M. Verma.** 1992. IκBγ, a 70kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68:**1109–1120.
24. **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.
25. **Kidd, S.** 1992. Characterization of the Drosophila *cactus* locus and analysis of interactions between cactus and dorsal proteins. Cell **71:**623–635.
26. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le-Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
27. **LeBail, O., R. Schmidt-Ullrich, and A. Israël.** 1993. Promoter analysis of the gene encoding the IκB-α/MAD-3 inhibitor of NF-κB: positive regulation by members of the rel/NF-κB family. EMBO J. **12:**5043–5049.
28. **Liou, H.-C., and D. Baltimore.** 1993. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. Curr. Opin. Cell Biol. **5:**477–487.
29. **Liou, H.-C., G. P. Nolan, S. Ghosh, T. Fujita, and D. Baltimore.** 1992. The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50. EMBO J. **11:**3003–3009.
30. **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.
31. **Matthews, J. R., W. Kaszubska, G. Turcatti, T. N. C. Wells, and R. T. Hay.** 1993. Role of cysteine 62 in DNA recognition by the p50 subunit of NF-κB. Nucleic Acids Res. **21:**1727–1734.
32. **Matthews, J. R., E. A. Watson, S. L. 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.
33. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD-3 (IκBα) and enhanced processing of the NF-κB precursor p105 are

obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
34. **Morin, P. J., G. S. Subramanian, and T. D. Gilmore.** 1993. Gal4-IκBα and Gal4-IIκBγ activate transcription by different mechanisms. Nucleic Acids Res. **21:**2157–2163.
35. **Neri, A. C., L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, and R. S. K. Dalla-Favera.** 1992. B cell lymphoma associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell **67:** 1075–1087.
36. **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.
37. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteosome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell **78:**773–785.
38. **Rice, N. R., and M. K. Ernst.** 1993. In vivo control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
39. **Rodriguez, M. S., I. Michalopoulos, F. Arenzana-Seisdedos, and R. T. Hay.** Inducible degradation of IκBα in vitro and in vivo requires the acidic C-terminal domain of the protein. Mol. Cell. Biol., in press.
40. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C.-H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a *rel*-related human cDNA that potentially encodes the 65 kD subunit of NF-κB. Science **251:**1490–1493.
41. **Ryseck, R.-P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12:**674–684.
42. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.
43. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
44. **Schreck, R., P. Rieber, and P. A. Baeuerle.** 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. **10:**2247–2258.
45. **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.
46. **Tewari, M., P. Dobrzanski, K. L. Mohn, D. E. Cressman, J.-C. Hsu, R. Bravo, and R. Taub.** 1992. Rapid induction in regenerating liver of RL/IF-1 (an IκB that inhibits NF-κB, RelB-p50, and c-Rel-p50) and PHF, a novel κB site-binding complex. Mol. Cell. Biol. **12:**2898–2908.
47. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. J. Virol. **52:**172–182.