A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB

Patrick A. Baeuerle[1] and David Baltimore

Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142 USA, and Massachusetts Institute of Technology, Department of Biology, Cambridge, Massachusetts 02139 USA

The NF-κB transcription factor was affinity-purified from deoxycholate (DOC)-treated cytosol of HeLa cells and shown to contain both a 50-kD polypeptide (p50) with a DNA-binding specificity identical to that of nuclear NF-κB and a 65-kD protein (p65) lacking DNA binding activity. Electrophoretically purified p50, after renaturation, gave rise to a protein–DNA complex that migrated faster than that made by native NF-κB. Reconstitution of p50 and p65 together produced a protein that combined with DNA to form a complex with electrophoretic mobility indistinguishable from that of the complex formed by nuclear extracts and DOC–treated cytosolic fractions. Sedimentation and gel filtration analyses indicate thats alone, the p50 protein exists as a dimer; two molecules of p65 bind to it to form a heterotetramer. Unlike IκB, the specific inhibitor of NF-κB, p65 displayed no inhibitor activity and was not released from NF-κB by DOC. p65 did not change the DNA binding specificity or the stimulatory effect of GTP on the p50 homodimer. Surprisingly, NF-κB could only be inactivated by IκB when p65 was bound. It would appear that one function of p65 is to make NF-κB susceptible to inhibition by IκB.

[*Key Words*: Purification of NF-κB; homodimers of NF-κB]

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The rate of synthesis of messenger RNA from most genes of a eukaryotic cell is regulated to meet the needs of a cell for certain individual polypeptides. Short DNA sequence elements within promoters and enhancers have been recognized as motifs that, by their interaction with proteins, determine the specific regulatory program of the genes they control (for review, see Yamamoto 1985; Hatzopoulos et al. 1988). These regulatory DNA sequences, which are located in the proximity of the genes they control, contain binding sites for various sequence-specific DNA-binding proteins. In many instances it has been shown that the occupation of the binding sites by specific proteins is essential for the activity of the elements and, subsequently, for the transcriptional activity of the gene (for review, see Johnson and McKnight 1989).

A well-studied example of a polypeptide that induces the synthesis of mRNA from genes by binding to enhancer and promoter elements is the nuclear factor κB (NF-κB) (Sen and Baltimore 1986a,b; for review, see Lenardo and Baltimore 1989). This DNA-binding protein recognizes a motif with the sequence 5′-GGGACTTTCC-3′ (Sen and Baltimore 1986a) and some variations of it in enhancers and promoters of a variety of tissue-specifically expressed genes (for review, see Lenardo and Baltimore 1989). Deletion and mutational analysis of NF-κB binding sites and oligonucleotide insertion experiments have shown that the binding of this protein to regulatory DNA sequence is responsible for the transcriptional activation of genes in response to stimulation of cells by active phorbol ester and bacterial lipopolysaccharide (Lenardo et al. 1987; Nelson et al. 1988; Pierce et al. 1988), agents that mimic T-cell activation (Nabel and Baltimore 1987; Boehnlein et al. 1988), the tax protein of HTLV-I (Ballard et al. 1988; Leung and Nabel 1988), interleukin-1 (Lowenthal et al. 1989; Osborn et al. 1989), tumor necrosis factor α (Osborn et al. 1989), and double-stranded RNA (Lenardo et al. 1989; Visvanathan and Goodburn 1989). In vitro transcription assays using purified protein support the view that NF-κB is a transcriptional activator (Kawikami et al. 1988). Stimulation of cells, [e.g., by 12-*O*-tetradecanoylphorbol 13-acetate (TPA)] activates the DNA binding activity of NF-κB by a post-translational mechanism (Sen and Baltimore 1986b). The activated NF-κB then can bind to an enhancer or promoter element and, by a yet unknown mechanism, trigger a dramatic increase in mRNA synthesis.

Recently we found that, in unstimulated cells, NF-κB is present in the cytoplasm in a sequestered form that can be activated in vitro by treating cytosol with the dissociating agent sodium deoxycholate (DOC) (Baeuerle and Baltimore 1988a). DOC was shown to release IκB, an inhibitory protein with a native molecular size of 60–70 kD, from NF-κB (Baeuerle and Baltimore 1988b). This inhibitor of DNA binding can inactivate NF-κB in a reversible and specific manner. Recent studies have indi-

[1]Present address: Genzentrum der Ludwig-Maximilians-Universitaet Muenchen, D-8033 Martinsried, FRG.

cated that a variety of other transcription factors are also associated with proteins that regulate their activity. Examples are the AP-1/*jun* transcription factor, which is only a high-affinity DNA-binding protein in a heterodimeric complex with the *fos* protein (Halazonetis et al. 1988; Nakabeppu et al. 1988; Rauscher III et al. 1988); GAL4, which is inhibited in its transcriptional activation function, but not in its DNA binding, by GAL80 (Ma and Ptashne 1987); the serum response factor, which requires a bound 62-kD protein to be serum responsive (Shaw et al. 1989); and the helix–loop–helix proteins that associate together as heterodimers to form complexes with high DNA affinity (Murre et al. 1989). Here we report that the activated form of NF-κB contains two polypeptides with disparate functions. A 65-kD protein (p65) copurified with the DNA-binding 50-kD subunit of NF-κB (p50). It is a non-DNA-binding subunit present in NF-κB complexes from nuclear extracts and DOC-treated cytosol. The p50 component appears to form a homodimer that has two p65 molecules attached. Unlike IκB, which has a similar native molecular size, p65 does not inhibit DNA binding and is not released from NF-κB by DOC. The p65 polypeptide was required to render NF-κB susceptible to inhibition by IκB.

Results

Purification of NF-κB from cytosol of HeLa cells

The DNA binding activity of NF-κB in the cytosol from unstimulated HeLa cells was activated by the addition of DOC (Baeuerle and Baltimore 1988a). The NF-κB released from its inhibitory protein IκB (Baeuerle and Baltimore 1988b) then was adsorbed to an affinity resin that had covalently attached multimerized oligonucleotides (Kadonaga and Tjian 1986) containing the NF-κB binding site found in the mouse κ light-chain enhancer (Sen and Baltimore 1986a) and the HIV enhancer (Nabel and Baltimore 1987). About 10% of the total NF-κB in the cytosol was recovered in the 0.4 M KCl eluate from the specific DNA column as active NF-κB. The efficiency of activation could be increased by subsequent passes of the DOC-treated cytosol over the regenerated resin. NF-κB was purified further by three cycles of sequence-specific DNA affinity chromatography in the presence of the nonspecific competitor poly[d(I-C)]. The specific activity of the NF-κB in the final 0.4 M KCl eluate was >100,000-fold increased over that in the cytosol. The yield was ~3% with respect to the first affinity eluate.

The high-salt eluate obtained after the fourth cycle of affinity chromatography (referred to as affinity-purified NF-κB) was seen to contain two prominent polypeptides of 50 (p50) and 65 kD (p65) on reducing SDS–polyacrylamide gels after silver staining (Fig. 1A). Also, a minor band of 45 kD was seen. To assess whether the DNA binding activity of NF-κB could be attributed to the polypeptides detected in silver-stained SDS-gels, fractions covering a complete lane of an SDS gel were assayed for DNA binding activity by an electrophoretic mobility-shift assay (EMSA) after elution and renaturation of proteins (Hager and Burgess 1980; Baeuerle and Baltimore 1988a). Only the molecular weight region containing p50 gave rise to a specific DNA binding activity that complexed with a wild-type κ enhancer fragment but not with one mutated in the NF-κB binding site (Lenardo et al. 1987) (Fig. 1B, lane 8). p65 did not exhibit any DNA binding activity after renaturation (Fig. 1B, lane 5), whereas p45 showed a nonspecific DNA binding activity (Fig. 1B, lane 9).

Figure 1. Purification and characterization of NF-κB from DOC-treated cytosol. (*A*) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of purified NF-κB. The protein in 200 μl of the affinity eluate after the fourth cycle of DNA affinity chromatography was precipitated by acetone and analyzed on a reducing SDS—10% polyacrylamide gel (Laemmli 1970) followed by silver staining (Wray et al. 1981). The positions and apparent molecular weights of the two major polypeptide species are indicated by arrows. The small arrow indicates the position of a 45-kD polypeptide. The fuzzy bands between the 50- to 65-kD bands were also seen in lanes that were not loaded with protein and are presumably finger proteins. Molecular weight standards were: myosin (220 kD), phosphorylase (97 kD), BSA (67 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD), and soybean trypsin inhibitor (20 kD). (*B*) Sizing of the DNA binding activity of purified NF-κB. A duplicate of the gel lane shown in *A* was not fixed and stained but cut into 12 different molecular size fractions as guided by rainbow markers (Amersham). Proteins were eluted and renatured (Hager and Burgess 1980; Baeuerle and Baltimore 1988a) and fractions assayed for DNA binding activity in an EMSA using a wild-type (wt) and mutant (mu) κ enhancer fragment as described earlier (Baeuerle and Baltimore 1988a,b). The numbering of lanes corresponds to the numbering of the molecular size fractions in *A*. Positions of size markers are indicated on the top. The position of κB-specific protein–DNA complex in lane *8* (filled arrowhead) and the position of unbound DNA (open arrowhead) are shown. The position of a nonspecific protein–DNA complex is shown in lane *9* (small arrow). A fluorogram of a native gel is shown. (*C*) Methylation interference analysis of NF-κB complexes I and II. A ^{32}P-labeled *Hin*dIII–*Sal*I 50-bp fragment (the plasmid was a kind gift of Dr. Michael Lenardo) was partially methylated and used in EMSAs. The bound and free DNA was eluted from a DEAE filter (see Experimental procedures), cleaved with piperidine, and analyzed on denaturing 12.5% polyacrylamide gels. Fluorograms are shown. (*Left*) coding strand; (*right*) noncoding strand. (Lanes *1* and *5*) Purines (lane *5*, Pu > Py); (lanes *2* and *6*) unbound DNA; (lanes *3* and *7*) DNA bound to affinity-purified NF-κB (complex I); (lanes *4* and *8*) DNA bound to electrophoretically purified 50-kD NF-κB (complex II). The arrows indicate guanosine residues whose methylation inhibited complex formation. The sequence on the bottom summarizes the results. Dots indicate guanosine residues that are essential for protein binding. (*D*) Titration of affinity-purified NF-κB with DNA. Various amounts of ^{32}P-end-labeled κ enhancer fragment of known specific activity were incubated with a constant amount of NF-κB in the absence of poly[d(I-C)]. The DNA binding reactions were performed as described (Baeuerle and Baltimore 1988a) in the presence of 20 μg of BSA. The specific activity of the ^{32}P-labeled DNA probe was calculated from both the known amount of DNA used for kinasing and from the specific activity of the [^{32}P]ATP, assuming complete kinasing and was 1780 and 2000 Ci/mmole, respectively. The position of the specific NF-κB–DNA complex (filled arrowhead) and position of unbound DNA (open arrowhead) are shown. A fluorogram of a native gel is shown.

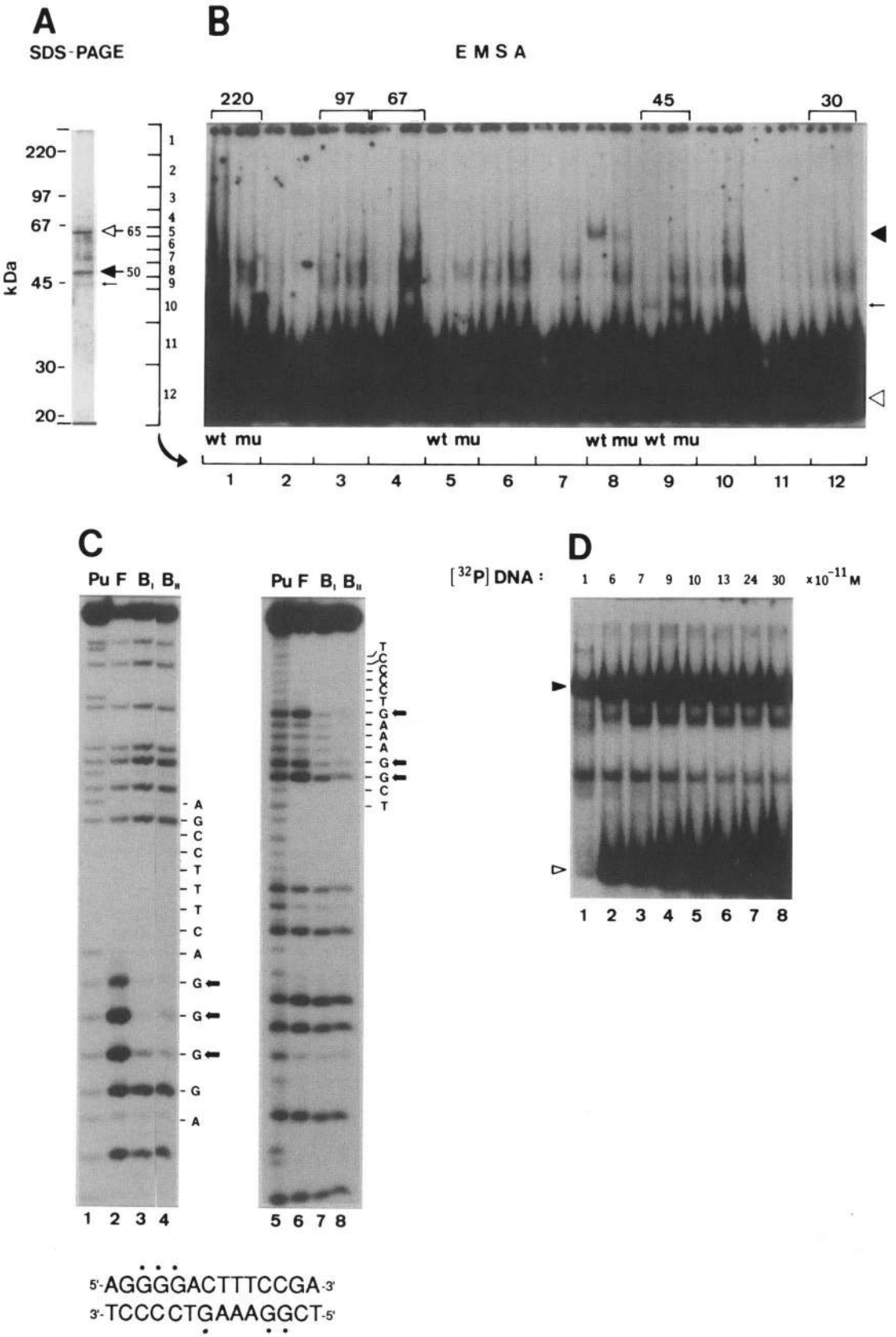

Figure 1. (*See facing page for legend.*)

For analysis by methylation interference, larger amounts of p50 were electrophoretically purified and renatured. The separated p50 gave a methylation interference pattern identical to that of affinity-purified NF-κB, defining the cognate sequence as 5′-GGGACTTTCC-3′ (Fig. 1C). Thus, p50 purified from DOC-treated cytosol has the same DNA binding specificity as NF-κB purified from nuclear extracts of a human Burkitt's lymphoma (Kawakami et al. 1988) and calf spleen (Lenardo et al. 1988). Moreover, its molecular weight matches that reported for human NF-κB (Kawakami et al. 1988). These data suggest that the DNA-binding subunits of cytosolic and nuclear NF-κB are identical.

As expected for a highly purified fraction, the NF-κB could be assayed by EMSA in the absence of a nonspecific competitor (Fig. 1D). A titration showed that poly[d(I-C)] was only a very weak competitor for the specific DNA binding of NF-κB: At a concentration of 0.1 mg/ml of poly[d(I-C)], binding of the radioactive DNA probe was reduced by only 50% (data not shown). When NF-κB was titrated with increasing known concentrations of specific DNA probe in the absence of nonspecific competitor, the binding of DNA to NF-κB saturated at $\sim 7 \times 10^{-11}$ M DNA as determined by liquid scintillation counting of the protein–DNA complexes (Fig. 1D). This concentration of DNA should be equivalent to the number of DNA-binding sites in the preparation. The number of NF-κB DNA-binding sites per HeLa cell that can be activated by DOC was calculated to be ~200 on the assumption that NF-κB binds as a homodimer. At a concentration of DNA of 10^{-11} M, almost all DNA probe was found in complex with NF-κB (Fig. 1D, lane 1). This suggests that the dissociation constant of the specific DNA–NF-κB complex was in the range of 10^{-12} M or even lower, reflecting an extremely high affinity of NF-κB for its cognate DNA.

A 65-kD protein is associated with the 50-kD DNA-binding subunit of NF-κB

Affinity-purified cytosolic NF-κB gave a protein–DNA complex in native gels that was identical in mobility with that formed by the NF-κB contained in crude cytosol or a nuclear extract from TPA-treated HeLa cells (data not shown) suggesting that purification did not change the protein composition of NF-κB. We refer to this protein–DNA complex as complex I (in this paper we will refer to a given DNA–protein complex or to its protein component by the same roman numeral). The

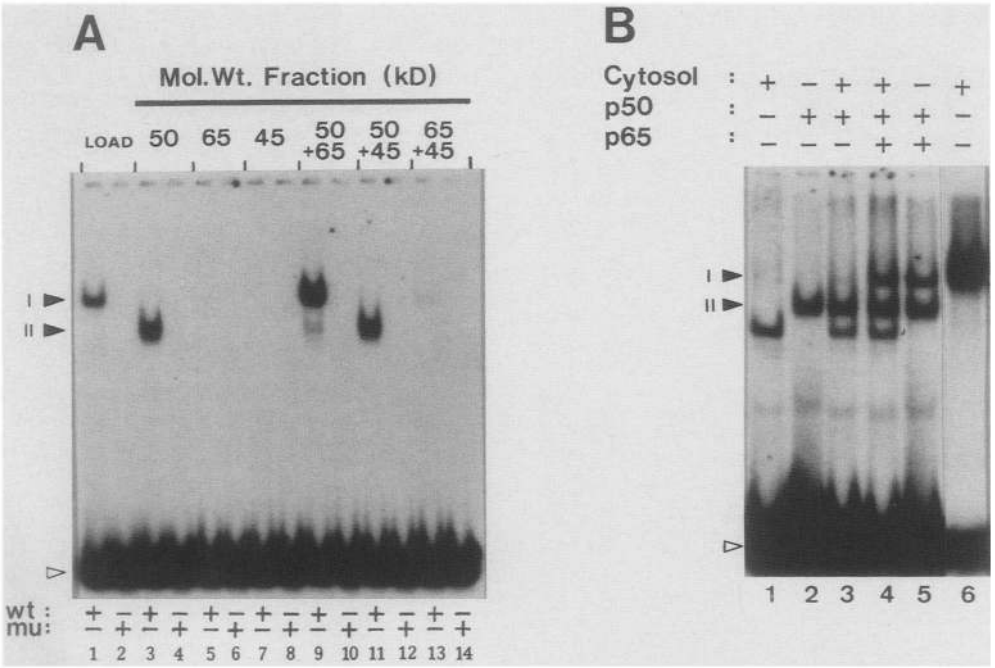

Figure 2. Identification and assay of a 65-kD protein associated with the 50-kD DNA-binding subunit of NF-κB. (*A*) Reconstitution of a native NF-κB complex with the 50- to 65-kD polypeptides. Affinity-purified NF-κB (see Fig. 1A) was subjected to SDS–PAGE. The 65-, 50-, and 45-kD molecular weight fractions were excised from gels as guided by rainbow markers (Amersham) and the proteins were eluted. Proteins were renatured separately (lanes 3–8), and allowed to renature in the combinations indicated on the top (lanes *9–14*). Renatured fractions were analyzed for DNA binding activity in an EMSA with wild-type (wt) and mutant (mu) κ enhancer fragments. A fluorogram of a native gel is shown. Positions of complexes I and II (filled arrowheads) and of unbound DNA probe (open arrowhead) are shown. (*B*) Assay of a cytosolic fraction for p65 activity. HeLa cell cytosol (2 μg of protein) (lane *1*) and gel-purified p50 (lane *2*) were reacted for 15 min at room temperature either alone (lane *3*) or in the presence of gel-purified p65 (lane *4*). (Lane *5*) Reaction of equivalent amounts of p50 and p65 in the absence of cytosol; (lane *6*) an equivalent amount of cytosol after treatment with DOC (Baeuerle and Baltimore 1988a,b). After the binding reactions, samples were analyzed in an EMSA. A fluorogram of a native gel is shown.

mobility of complex I was, however, different from that obtained with electrophoretically purified p50 (referred to as complex II), which migrated significantly faster (Fig. 2A, cf. lanes 1 and 3). This finding suggests that complex I contained additional components that were removed during electrophoretic purification of p50. To test whether p65 or p45, which are also abundant in highly purified fractions (Fig. 1A), can restore complex I, p50 was renatured together with protein eluted from either the 65- or the 45-kD range. As shown in Figure 2A (lane 9), renaturation of p50 in the presence of p65 led to an almost quantitative transformation of complex II into complex I. This shows that complex I can be reconstituted in vitro using two distinct polypeptide species. Because p65 did not show any DNA binding activity of its own (Fig. 2A, lanes 5 and 6), it probably gave rise to complex I by protein–protein interaction with p50. This notion is supported by the observation that the absence of p65 from complex II had no significant influence on the methylation interference pattern produced by NF-κB (Fig. 1C). The p45 protein did not change the mobility of complex II (Fig. 2A, lane 11). Most likely, this protein copurified during DNA affinity chromatography because it is a DNA-binding protein on its own (see Fig. 1B, lane 9) that recognizes a sequence within or overlapping the NF-κB binding site that is not affected in the mutant NF-κB probe.

Specific bands with the mobility of complex II are rarely seen when fractions from different cell lines (Sen and Baltimore 1986a,b; Baeuerle and Baltimore 1988a) and tissues (data not shown) are analyzed by EMSA. The predominance of complex I in those fractions suggests that p65 is a ubiquitous subunit of NF-κB and is available when NF-κB becomes activated by DOC in vitro or by TPA treatment in vivo. Therefore, we tested whether there is any free p65 detectable in the cytosol where activation of NF-κB is thought to occur. As shown in Figure 2B (lane 5), p65 can be assayed after addition of electrophoretically purified p50 by the formation of complex I. [Because the amount of p50 was not reduced significantly in this mixing experiment (see Fig. 2B, lane 2), p65 may have reacted preferentially with denatured p50 in the fraction, thereby restoring its activity.] When HeLa cell cytosol was incubated with p50, hardly any complex I was formed (Fig. 2B, lane 3). However, when electrophoretically purified p65 was added, the same amount of complex was obtained as in the absence of cytosol (Fig. 2B, cf. lanes 4 and 5). Because almost no active p65 was detectable in HeLa cell cytosol, most of the p65 contained in the complex I seen after DOC activation (Fig. 2B, lane 6) must have been present before activation, presumably in the NF-κB–IκB complex.

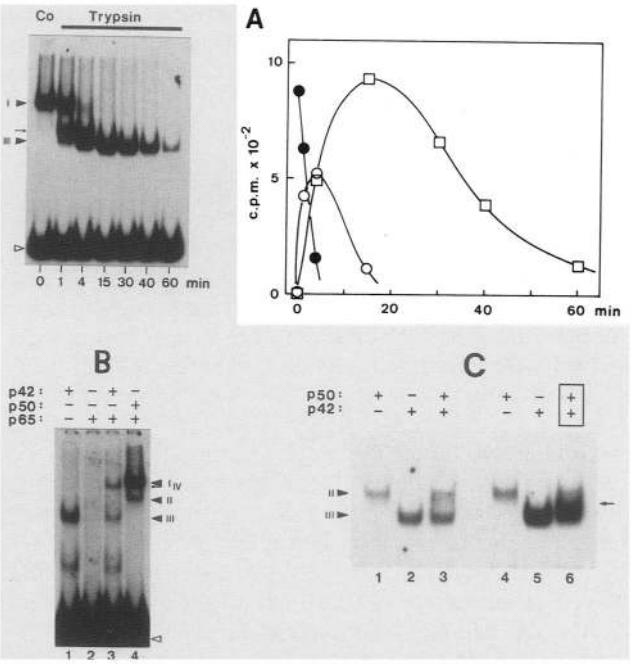

Figure 3. Analysis of protein complexes of NF-κB using a proteolyzed form of the 50-kD subunit. (*A*) Trypsin treatment of complex I. Equal amounts of affinity-purified NF-κB were incubated with trypsin followed by the addition of soybean trypsin inhibitor after the indicated periods of time. (*Left*) Analysis of digests by EMSA. A fluorogram of a native gel is shown. Positions of complex I and a novel complex designated III (filled arrowheads) are shown. The position of an intermediate complex (small arrow) is shown. (*Right*) Quantitation of the ^{32}P-radioactivity in the three protein–DNA complexes by Cerenkov counting. (●) Complex I; (○) intermediate complex; (□) complex III. (*B*) Combined renaturation of p42 and p65. Affinity-purified NF-κB was treated for 15 min with trypsin followed by the addition of trypsin inhibitor. SDS–sample buffer (Laemmli 1970) was added, and the solution was boiled immediately and subjected to SDS–PAGE. Proteins renatured from molecular size fractions of SDS gels were analyzed by an EMSA. A fluorogram of a native gel is shown. (Lane *1*) Protein renatured from the 42-kD fraction of trypsinized complex I; (lane *2*) protein renatured from the 65-kD fraction of untreated affinity-purified NF-κB; (lane *3*) combined renaturation of proteins; (lane *4*) combined renaturation of equivalent amounts of p50 and p65. The position of free DNA probe (filled arrowhead) is shown. The position of a novel complex designated IV (small arrow) is shown. (*C*) Combined renaturation of p42 and p50. Renatured proteins were analyzed by an EMSA. A section of a fluorogram of a native gel is shown. (Lanes *1* and *4*) Renatured p50; (lanes *2* and *5*) renatured p42; (lane *3*) mix of equivalent amounts of renatured p42 and p50; (lane *6*) combined renaturation of p50 with p42. The position of a novel intermediate complex (small arrow) not observed in lane *3* is shown. The renaturation efficiency of p42 was higher than that of p50 (cf. lanes *3* and *5*). Therefore, not equal amounts of active p50 and p42 were available for the formation of a heterodimer. The positions of complexes II and III (filled arrowheads) are shown.

The composition of active NF-κB complexes

Complex I was analyzed by protease treatment of affinity-purified NF-κB. Trypsin rapidly degraded complex I and, along with an intermediate form, gave rise to a novel relatively trypsin-resistant NF-κB complex (referred to as complex III) with a considerably increased mobility in native gels (Fig. 3A, left panel). Prolonged digestion produced no faster migrating complexes but reduced the amount of complex III. From quantitation of

the forms in Figure 3A (left), it appears that trypsin degrades complex I rapidly, leading first to the intermediate form which then is degraded to complex III with no apparent loss of DNA binding activity. The high mobility of complex III resulted from both truncation of p50 and, to a greater extent, from the loss of p65. This was shown by sizing the DNA binding activity that gave rise to complex III in an SDS gel (data not shown) and by a reconstitution experiment with electrophoretically purified p65 (Fig. 3B). In reducing SDS gels, the DNA binding activity of complex III was confined to the 42-kD fraction (data not shown) indicating that trypsin removed only 8 kD from p50. The electrophoretically purified 42-kD NF-κB (p42) formed a protein–DNA complex comigrating with that of complex III (Fig. 3B, lane 1). When renatured together with electrophoretically purified p65, p42 gave rise to a novel slower-migrating complex (Fig. 3B, lane 3). This indicated that the removed 8-kD domain was not required for binding of p65. The protein–DNA complex formed by p42 and p65 (referred to as complex IV) migrated in native gels almost as slowly as complex I (Fig. 3B, cf. lanes 3 and 4).

The DNA binding activity of the renatured 50-kD NF-κB (complex II) migrated with an apparent molecular size of 100 kD after both gel filtration and glycerol gradient centrifugation (Table 1), indicating that p50 forms a homodimer in solution. The same appeared to be true for the 42-kD NF-κB, which had a peak molecular size of 80 to 90 kD (Table 1). When p42 and p50 were allowed to renature together, a novel complex of intermediate mobility was observed (Fig. 3C, lane 6) that had the same mobility in native gels as the intermediate seen during tryptic digestion of complex I (Fig. 3A, left panel). Most likely this complex contained a p50/p42 heterodimer, implying that p50 and p42 also bound as homodimers to DNA. The intermediate was not observed when p50 and p42 were mixed after renaturation (Fig. 3C, lane 3) even after treatment with elevated temperature and dissociating agents (data not shown) suggesting that the homodimers formed by renatured p50 and p42 were very stable.

The DNA binding activity of protein complex I gave a peak upon gel filtration implying a size of ~250 kD (Table 1). Because complexes I and II showed an identical DNA binding specificity (Fig. 1C), we assume that the core of complex I is a p50 homodimer as found in complex II. The size of 250 kD of complex I then is explained most simply by the binding of two p65 molecules to the p50 homodimer, which would add up to a size of 230 kD. Surprisingly, the protein complex I sedimented in glycerol gradients at a rate consistent with a size of 100 kD (Table 1), but nevertheless gave rise to protein–DNA complex I in EMSAs (data not shown). Because only the larger size of complex I, seen after gel filtration, would explain the different mobilities of the protein–DNA complexes I and II in native gels (Fig. 2A), we assume that complex I dissociates during glycerol gradient centrifugation—either into complex II and free p65, or into a p50/p65 heterodimer—and could reassociate before or during the DNA binding reactions.

When very minute amounts of activity were assayed, the DNA binding activity of protein complexes I, II, and III of NF-κB were increased dramatically in the presence of millimolar concentrations of GTP (Fig. 4). This effect of GTP was shown earlier to be specific for NF-κB (Lenardo et al. 1988). GTP stimulation of DNA binding of

Table 1. *Approximate molecular size of NF-κB-related protein complexes*[a]

	Glycerol gradient centrifugation	Gel filtration	Proposed composition
Complex I	~100	~250	$(50)_2(65)_2$
Complex II	~100	~100	$(50)_2$
Complex III	~90	~90	$(42)_2$

[a] Molecular sizes estimated in kilodaltons.
Glycerol gradient centrifugation was performed as described earlier (Baeuerle and Baltimore 1988b). Complexes I and III were run as a mix, and complex II was run separately. Gel filtration was performed on Sephadex G-200 (Pharmacia) with a mix of complexes I and III and on Sephacryl S-200 (Pharmacia) with a mix of all three forms. Fractions were assayed by EMSA for the DNA binding activity of protein complexes. Molecular size standards were as described (Baeuerle and Baltimore 1988b) except that in some experiments α-amylase from sweet potato (Sigma; 200 kD) was used instead of immunoglobulin G (158 kD). All of the molecular size estimates were made by comparison to standards under the assumption that the NF-κB-related proteins adopt a reasonably globular configuration.

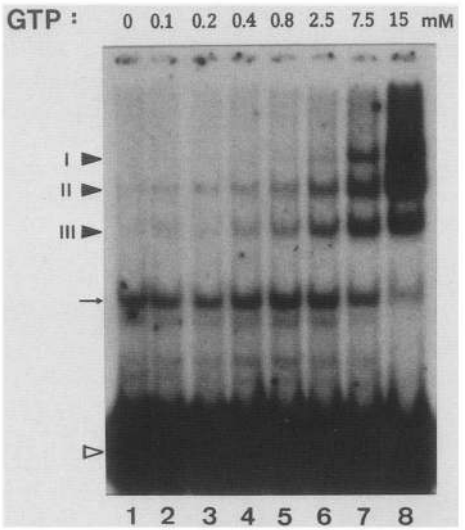

Figure 4. The effect of GTP on the DNA binding activity of the various NF-κB complexes. Fractions containing similar amounts of activity of electrophoretically purified p50, affinity-purified NF-κB, and trypsinized NF-κB were mixed and diluted in buffer H containing 0.4 M KCl so that hardly any DNA binding activity was detectable (lane *1*). Increasing concentrations of GTP in 20 mM Tris-HCl (pH 7.5) were added along with the DNA binding assay mix and samples analyzed by an EMSA. A fluorogram of a native gel is shown. The positions of NF-κB complexes I, II, and III (filled arrowheads) and the position of unbound DNA probe (open arrowhead) are shown. The position of a non-specific DNA binding activity (small arrow) is shown, which is not influenced by GTP.

NF-κB was obviously not dependent on the presence of p65 or the 8-kD domain removed from p50 by trypsin treatment.

IκB can inactivate NF-κB only in the presence of p65

To further examine the role of the non-DNA-binding 65-kD subunit of NF-κB, we tested the physiological NF-κB and the artificial complexes II and III for inactivation by IκB. Unlike p65, IκB is released after DOC treatment from NF-κB and can be recovered in the flowthrough fraction from the first affinity chromatography step (Baeuerle and Baltimore 1988b). The DNA binding activity of complexes II and III (mixed p50 and p42) was virtually unaffected in the presence of increasing amounts of a gel filtration fraction containing IκB (Fig. 5A, lanes 1–5). IκB was active under the assay conditions used because the DNA binding activity of affinity-purified NF-κB was almost completely inhibited by even the smallest amount of IκB used (Fig. 5A, lanes 6–10). Also, the complex I NF-κB obtained by the combined renaturation of gel-purified p50 and p65 was strongly inhibited (Fig. 5B, lanes 3 and 4). These results show that IκB could not inactivate the p50 or p42 homodimers. Inhibition of DNA binding by IκB is only seen with the NF-κB complex containing p65, suggesting that p65 modifies the p50 homodimer such that it is susceptible to inhibition by IκB. It is unlikely that IκB bound to the p50 or p42 homodimers without subsequent inhibition because no novel slower migrating complexes were formed (Fig. 5A, lanes 2–5).

To investigate whether the 8-kD domain that was removed from p50 by tryptic cleavage was required for the inhibition by IκB, we incubated the in vitro reconstituted complex of p42 and p65 with active IκB. As shown in Figure 5B (lane 2), IκB could inhibit its DNA binding activity as well as that of complex I under identical conditions (Fig. 5B, lane 4). This suggests that inhibition of NF-κB by IκB did not depend on the 8-kD domain removed from p50 but solely on the bound p65.

We have summarized our findings in diagrammatic form (Fig. 6).

Discussion

The active form of the human NF-κB transcription factor appears from data presented here to be composed of two distinct polypeptide subunits. One is a 50-kD protein that forms a homodimer in solution and binds as a homodimer to the κB motif in DNA. The other subunit is a hitherto undescribed 65-kD protein that does not exhibit DNA binding activity but copurifies with p50. From the size of the NF-κB determined by gel filtration, we believe that two molecules of p65 are bound to the p50 homodimer. In complex with p50, p65 did not significantly affect the DNA binding specificity and activity of p50, which suggests that it does not bind in close proximity to its DNA-binding domain. Therefore, p65 could be positioned on a surface of the p50 homodimer away from the surface that contacts the double helix. In this position, p65 could mediate the communication between the 50-kD DNA-binding subunit and the transcriptional machinery. Recent in vitro transcription assays that used purified human nuclear NF-κB containing both subunits are consistent with this notion (Kawakami et al. 1988). Although a 51-kD polypeptide was identified by UV-crosslinking as the specific DNA-binding protein in the purest fraction, a 68-kD protein was evident.

The NF-κB reconstituted in vitro from the p50 and p65 polypeptides gives rise to a protein–DNA complex that displays an electrophoretic mobility in native gels very similar or identical to that of the complex obtained with NF-κB in nuclear extracts or DOC-treated cytosolic frac-

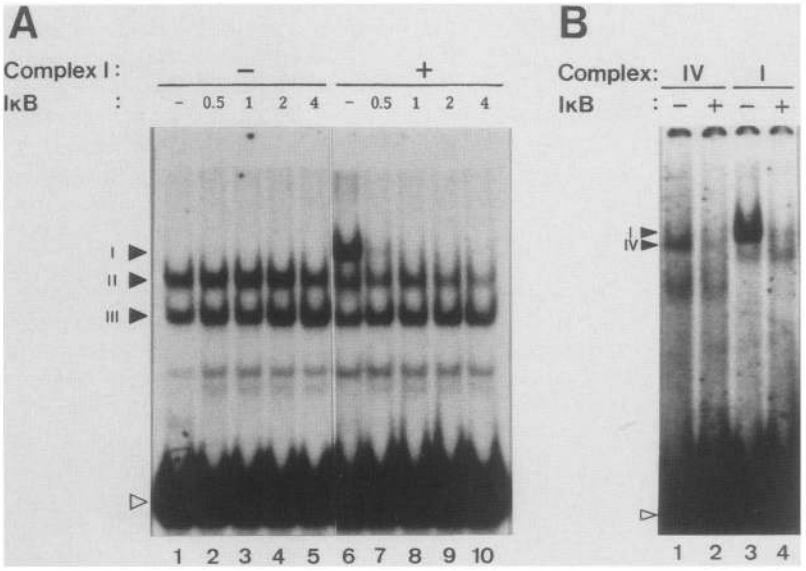

Figure 5. The effect of IκB on the DNA binding activity of the various NF-κB complexes. (*A*) The effect of IκB on complexes I, II, and III. (Lanes *1–5*) Equal amounts of binding activity of electrophoretically purified p50 and trypsinized affinity-purified NF-κB were reacted with increasing amounts of a gel filtration fraction containing active IκB as described (Baeuerle and Baltimore 1988b). (Lanes *6–10*) Same as lanes *1–5* but in the presence of affinity-purified NF-κB. Samples were analyzed for DNA binding activity in an EMSA. Fluorograms of native gels are shown. The positions of complexes I, II, and III (filled arrowheads) and the position of unbound DNA probe (open arrowhead) are shown. (*B*) The effect of IκB on the complex of p42 and p65. A fraction containing p42 that was renatured together with electrophoretically purified p65 (lanes *1* and *2*) and a fraction containing p50 that was renatured together with electrophoretically purified p65 (lanes *3* and *4*) were reacted with a gel filtration fraction containing active IκB. Samples were analyzed by an EMSA. A fluorogram of a native gel is shown. The positions of complexes I and IV (filled arrowheads) and of free DNA probe (open arrowhead) are shown.

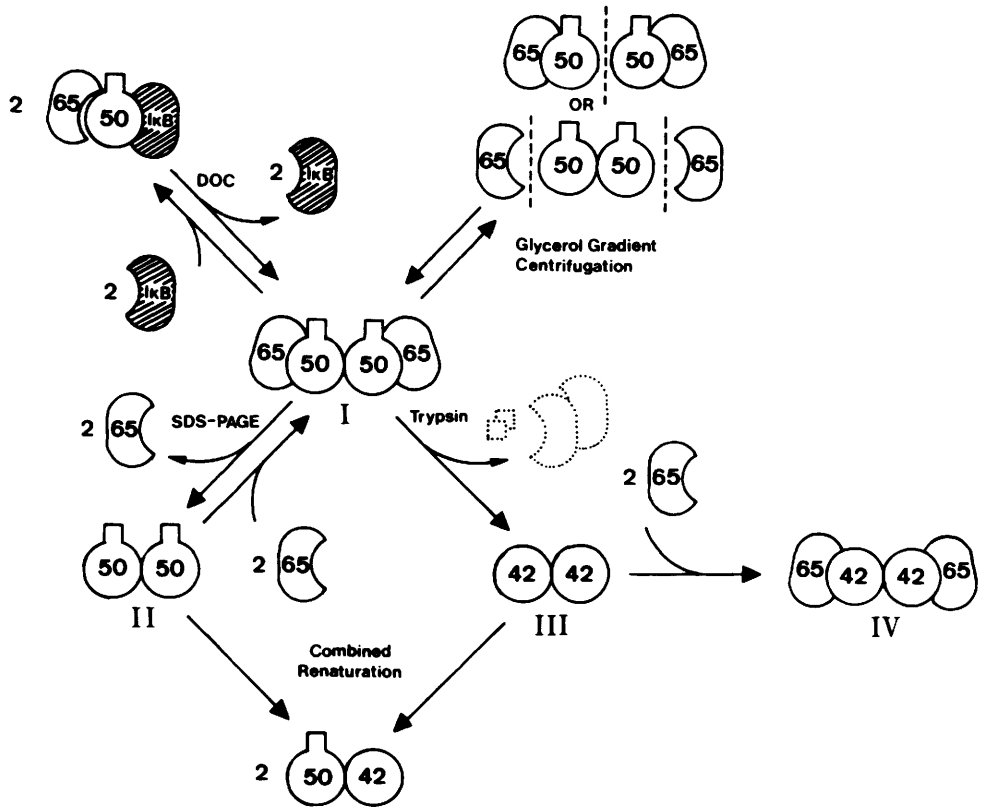

Figure 6. A model showing the relationships between the various complexes of NF-κB described in this study. The position of IκB and p65 on the p50 molecule is arbitrary. It is also conceivable that IκB binds via p65 to p50.

tions from a variety of human cell lines, including the T cell lines H-9 and Jurkat (data not shown). This suggests that p50 and p65 are ubiquitous components of NF-κB, although we cannot exclude at present that there are cell type-specific isoforms of either proteins. The two subunits of NF-κB may serve as binding sites for yet other polypeptides. A possible candidate is HIVEN86, a protein identified by DNA-affinity precipitation assays (Franza et al. 1987) that used the NF-κB binding sites in the HIV enhancer and the promoter of the interleukin-2 receptor α-chain gene (Boehnlein et al. 1988). HIVEN86 is induced in its synthesis or binding activity by conditions that mimic T-cell activation.

Recently we identified a protein, IκB, that is associated with the cytosolic inactive form of NF-κB (Baeuerle and Baltimore 1988b). When bound to NF-κB, IκB inhibits the dimerization, DNA binding activity, and the nuclear translocation of p50. These are activities that clearly distinguish IκB from p65. Moreover, treatment with DOC releases the IκB activity from the inactive cytoplasmic NF-κB and the activated form contains p65.

Is p65 contained in the inactive complex of p50 and IκB? This remains a confusing issue. Sedimentation in glycerol gradients and gel filtration of the inactive mouse NF-κB suggested that two rather than three components, each 55–70 kD, were present (Baeuerle and Baltimore 1988b). Also, the human complex sedimented in glycerol gradients with a size that was consistent with a heterodimeric complex of p50 and a single 60–70 kD IκB molecule (data not shown). Treatment of the glycerol-gradient fraction with DOC, however, gave rise to complex I, indicating the presence of p65. Recently, a faster migrating complex similar to that of human complex II was obtained after glycerol gradient centrifugation and DOC activation of a partially purified mouse NF-κB–IκB complex (S. Ghosh and D. Baltimore, unpubl.). It is possible that in the presence of IκB, p65 is loosely associated with p50 and can be separated under certain conditions. The possibility that p65 is an abundant protein that requires multiple purification steps to be separated completely from p50-IκB is not supported by our finding that hardly any free p65 is detectable in a cytosolic fraction.

IκB specifically inhibits the DNA binding activity of NF-κB from nuclear extracts of mature B cells and various TPA-induced cells as well as that of NF-κB from DOC-treated cytosol (Baeuerle and Baltimore 1988b). Here we report that a form of active NF-κB that is devoid of the p65 subunit does not become inhibited by IκB, which suggests that p65 has an important role in the process of inactivation of NF-κB by IκB. We assume that NF-κB can bind only as a homodimer with high affinity to DNA by analogy to other transcription factors, for example, the estrogen receptor (Kumar and Chambon 1988), GCN4 (Hope and Struhl 1986), and CREB (Yama-

moto et al. 1988). This notion is supported by the finding that mutational alteration of one half-site of the NF-κB binding site completely prevents factor binding (Lenardo et al. 1987; Nabel and Baltimore 1987). A simple mechanism by which IκB could then keep NF-κB in an inactive state is to prevent the formation of a homodimer by stabilizing the p50 monomer. When NF-κB is inactivated in vitro by the addition of active IκB (Baeuerle and Baltimore 1988b), or in vivo after prolonged treatment of cells with TPA (Baeuerle et al. 1989), the p50 homodimer has to be dissociated again to form an inactive complex that apparently contains only one p50 molecule. It appears that this cannot happen if p65 is not bound to p50, which suggests that P65 assists IκB to dissociate the homodimer. For example, p65 could serve as a receptor for IκB (but that would require the presence of p65 in the inactive complex of NF-κB). Another possibility is that IκB has no proper access to its binding site on p50, unless p50 is modified by bound p65. Only when the relevant genes are cloned, providing access to large amounts of proteins and allowing the application of genetic methods, will it be possible to settle these issues.

Experimental procedures

Preparation of the DNA-affinity resin

A synthetic, double-stranded oligonucleotide with the sequence:

5′-GATCCAGAGGGGACTTTCCGAGA-3′
3′-GTCTCCCCTGAAAGGCTCTCTAG-5′

was phosphorylated and ligated as described (Kadonaga and Tjian 1986). About 600 μg of ^{32}P-trace-labeled DNA [referred to as (κB)*n*] with an average size of 500 bp was incubated with 5 ml of cyanogen bromide-activated Sepharose 4B (Sigma) for 4 hr at 37°C in 10 mM KH_2PO_4, adjusted to pH 3.5 with acetic acid. After extensive washes with 2 M KCl, H_2O, 1% SDS, and 2% (vol/vol) NP-40 the ^{32}P radioactivity that was bound to a portion of the resin was determined by Cerenkov counting. About 50 μg of DNA were bound per milliliter of wet resin, corresponding to ~3.3 nmoles of binding sites of NF-κB.

Purification of NF-κB from cytosol

Cytosol was prepared as described (Baeuerle and Baltimore 1988a) from ~5 × 10^{10} HeLa cells grown as a spinner culture in 50 liters of modified Eagle's medium supplemented with 10% horse serum. Cytosolic protein (10 mg/ml) in buffer D (Dignam et al. 1983) containing 1% (vol/vol) NP-40 was mixed with 4 ml of (κB)*n*-Sepharose followed by the addition of solid DOC (sodium salt) to a final concentration of 1%. After a brief incubation on ice, a 10% (vol/vol) solution of NP-40 was added to give a total concentration of 2% nonionic detergent. The mix was incubated end-over-end for 2 hr at 4°C and then transferred into a wide column. The resin was washed with 30 ml of buffer H [20 mM HEPES (pH 7.9), 1 mM DTT, 0.5 mM EDTA, 0.25 mM EGTA, 0.1 mM PMSF, 0.2% (vol/vol) NP-40, 0.1% (wt/vol) DOC, 20% (vol/vol) glycerol] containing 0.15 M KCl. Bound protein was eluted step-wise with 8 ml of 0.2 M KCl, 4 ml of 0.3 M KCl, and 7.5 ml of 0.4 M, all in buffer H. The bulk of active NF-κB was contained in the 0.4 M KCl fraction. Hardly any activity was found in fractions eluted with higher salt. To the 0.4 M KCl eluate, 2 ml of (κB)*n*-Sepharose, 2.5 mg of poly[d(I-C)] (Pharmacia), and fresh PMSF were added and the volume adjusted to 30 ml with a final concentration of 0.1 M KCl by the addition of buffer H. After a 2-hr batch-wise incubation at 4°C, the resin was washed and eluted as described above using half of the buffer volumes. Two more cycles of DNA affinity chromatography in the presence of 0.2 mg/ml poly[d(I-C)] were performed. Protein concentrations were determined by a bicin choninic acid microassay (Pierce), and in the most highly purified fractions estimated from silver-stained SDS–polyacrylamide gels using a bovine serum albumin (BSA) standard dilution series. The resin was regenerated by washes with 2 M KCl and 1% SDS. The amount of NF-κB activity in the fractions was determined by EMSA as described (Sen and Baltimore 1986a; Baeuerle and Baltimore 1988a) in the presence of 20 μg of BSA and 2 μg of poly[d(I-C)] per assay.

Renaturation of proteins from SDS–polyacrylamide gels

Proteins were eluted from reducing SDS–polyacrylamide gels and renatured according to Hager and Burgess (1980) with the modifications described earlier (Baeuerle and Baltimore 1988a). Here, a novel renaturation buffer was used that consisted of twofold concentrated EMSA-binding buffer (Sen and Baltimore 1986a), 10% buffer D (Dignam et al. 1983), and 0.1% (vol/vol) NP-40. For EMSA, one volume of the renatured fraction was added to one volume of an assay mix that contained 20 μg of BSA, 0.8 μg of poly[d(I-C)], and the ^{32}P-labeled DNA probe.

Tryptic digestion of NF-κB

Digestion was performed with 10 μg/ml TPCK-trypsin (Worthington) at room temperature in buffer H containing 0.4 M KCl. The reaction was stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor (Sigma).

Methylation interference analysis

The following modifications to the protocol of Sen and Baltimore (1986a) were made. Protein–DNA complexes were separated on 1% low-melt agarose gels in EMSA running buffer (Sen and Baltimore 1986a) then transferred onto DEAE paper by Southern blotting. The bands of free and bound DNA were identified by autoradiography of the filter, then excised and the DNA eluted with 1 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA at 68°C for 2 hr followed by cleavage with piperidine (Maxam and Gilbert 1980).

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