IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention

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NF-κB is an inducible transcription factor comprised of a 50-kD (p50) and a 65-kD (p65) subunit. Induction of NF-κB activity, which is a critical event in many signal transduction pathways, involves release from a cytoplasmic inhibitory protein, IκB, followed by translocation of the active transcription factor complex into the nucleus. Earlier studies suggested that IκB targets the p65 subunit of NF-κB. However, we demonstrate by in vitro and in vivo methods that the recently cloned IκB/MAD-3 interacts with both the p50 and p65 subunits of NF-κB, as well as c-Rel. Furthermore, an alternatively spliced, dimerization-deficient transforming variant of p65 (p65Δ) interacts extremely weakly with IκB/MAD-3, suggesting that dimerization is important for interaction. We demonstrate that the conserved nuclear localization sequences (NLSs) of NF-κB and c-Rel are the targets for IκB/MAD-3 interaction. Indirect immunofluorescence experiments demonstrate that IκB/MAD-3 expression retains both p65 and p50 in the cytoplasm. Furthermore, and most important, a p65 that contains an SV40 large T antigen NLS in addition to its own NLS is no longer retained in the cytoplasm in the presence of IκB/MAD-3. We propose that IκB/MAD-3 masks the NLSs of NF-κB and c-Rel and that this constitutes the mechanism for cytoplasmic retention of these proteins.

[*Key Words*: IκB; NF-κB; c-Rel; dimerization; NLS]

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The transcription factor NF-κB was initially described as a DNA-binding protein involved in the regulation of expression of the immunoglobulin κ light-chain gene (Sen and Baltimore 1986). Subsequent studies have established its role as an important regulator of expression of a number of immune function and inflammation response genes as well as a regulator of human immunodeficiency virus (HIV) gene expression (for review, see Baeuerle 1991; Baeuerle and Baltimore 1991 and references therein). NF-κB is sequestered in the cytoplasm of many cells where it is bound to an inhibitor protein called IκB (Baeuerle and Baltimore 1988b). Various reagents and stimuli, including the tumor promoter PMA, B- and T-cell mitogens, cytokines such as tumor necrosis factor α and interleukin-1 (IL-1), reactive oxygen intermediates, UV light, serum growth factors, double-stranded RNA, as well as viral infection, can lead to the release of NF-κB from IκB (Lenardo and Baltimore 1989; Stein et al. 1989; Baeuerle and Baltimore 1991; Baldwin et al. 1991; Schreck et al. 1991). Free NF-κB can then move into the nucleus and transcriptionally regulate target genes through interaction with κB-like DNA sequences (for review, see Baeuerle 1991 and references therein). In vitro studies have suggested that the mechanism of release of NF-κB is through phosphorylation of IκB (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990), although other mechanisms may be involved as well (Schreck et al. 1991).

NF-κB is typically characterized as a heterodimer of a 50-kD protein (p50), which is derived from a 105-kD precursor (p105) and a 65-kD (p65) protein (Baeuerle and Baltimore 1989; Ghosh et al. 1990; Kieran et al. 1990). The recent cloning of the p50 and p65 subunits has revealed homologies with the proto-oncogene c-*rel* and the *dorsal* maternal effect gene of *Drosophila* (Bours et al. 1990; Ghosh et al. 1990; Kieran et al. 1990; Meyer et al. 1991; Nolan et al. 1991; Ruben et al. 1991). Both homodimers and heterodimers of p50 and p65 can bind to

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κB sequences (Nolan et al. 1991; Ruben et al. 1991; Urban et al. 1991). However, only p50/p65 heterodimer and p65 homodimer DNA binding can be inhibited by IκB, suggesting that the p65 subunit is the target for IκB (Baeuerle and Baltimore 1989; Nolan et al. 1991; Ruben et al. 1991; Urban et al. 1991). Two forms of IκB (IκB-α and IκB-β) have been purified from cellular extracts (Zabel and Baeuerle 1990). The relationships of these two forms to the recently cloned IκB/MAD-3 and to the c-Rel-associated protein pp40 are not completely clear (Davis et al. 1991; Haskill et al. 1991).

The mechanism by which NF-κB is retained in the cytoplasm by IκB is largely unknown. However, the predicted protein sequences of IκB/MAD-3 and pp40 have revealed the presence of ankyrin repeats, 30- to 34-amino-acid structures that are present in some proteins associated with the cytoskeleton (Lux et al. 1990; Davis et al. 1991; Haskill et al. 1991). Thus, one possibility is that IκB can bind to both NF-κB and cytoskeletal elements in the cytoplasm, thereby preventing NF-κB from entering the nucleus. However, it has been reported recently that ankyrin repeats found in a transcription factor can also function as a dimerization domain (Thompson et al. 1991). In fact, deletion of two ankyrin repeats prevents IκB/MAD-3 from associating with NF-κB (Haskill et al. 1991). Therefore, it is also possible that the ankyrin repeats serve as a dimerization "interface" between IκB and NF-κB. In this case, the uptake of NF-κB into the nucleus might be prevented through the masking of the nuclear localization sequence (NLS) of NF-κB by IκB. Furthermore, NLSs have been shown to be the targets for receptor proteins that are involved in nuclear transport (Adam and Gerace 1991). Interestingly, c-Rel, Dorsal, both of the subunits of NF-κB, as well as the recently identified p49/lyt-10 and RelB/I-Rel proteins, have highly conserved NLSs (Steward 1987; Brownell et al. 1989; Bours et al. 1990, 1992; Ghosh et al. 1990; Kieran et al. 1990; Meyer et al. 1991; Neri et al. 1991; Nolan et al. 1991; Ruben et al. 1991, 1992a; Schmid et al. 1991; Ryseck et al. 1992), some of which have been shown to be functional in nuclear transport (Gilmore and Temin 1988; Blank et al. 1991).

In this study we show that IκB/MAD-3 can interact with c-Rel and both the p50 and the p65 subunits of NF-κB in vitro and can functionally inhibit the activity of the p50 and p65 subunits in vivo. Our analysis demonstrates that the NLSs of p50, p65, and c-Rel are essential targets for IκB/MAD-3 binding, thereby suggesting a mechanism for the cytoplasmic retention of these proteins through the masking of these sequences. Furthermore, indirect immunofluorescence experiments demonstrate that IκB/MAD-3 retains both p65 and p50 in the cytoplasm and that the addition of a heterologous NLS to p65 overrides the cytoplasmic retention mediated by IκB/MAD-3. We also show a direct correlation between the ability of p65 to heterodimerize with p50 and to bind IκB/MAD-3. These studies have been extended to include the analysis of an alternatively spliced form of p65, which is highly transforming (Narayanan et al. 1992) and which we show has almost no affinity for IκB/MAD-3.

Results

IκB/MAD-3 binds to both the p50 and p65 subunits of NF-κB

Both homodimers and heterodimers of the p50 and p65 subunits of NF-κB are capable of binding κB-like sequences (Nolan et al. 1991; Ruben et al. 1991; Urban et al. 1991). To test the effect of IκB/MAD-3 on the DNA binding of in vitro-translated p50 and p65, we carried out a gel-shift assay using a radiolabeled major histocompatibility complex (MHC) class I enhancer-binding site probe, shown previously to bind NF-κB. Either a truncation of p105, which corresponds approximately to wild-type p50 (Fig. 1B, construct 2), or full-length wild-type p65 (Fig. 1A, construct 1) was used in the gel-shift assay. No effect of IκB/MAD-3 addition was seen on the ability of p50 to interact with the κB probe (Fig. 2A, lanes 4,5) while p65 DNA binding was inhibited (Fig. 2A, lanes 2,3), in agreement with previous findings (Haskill et al. 1991; Nolan et al. 1991; Ruben et al. 1991).

To study direct interactions between IκB/MAD-3 and p50 or p65, we carried out coimmunoprecipitation experiments. An antibody (Ab 9) generated against an amino-terminal peptide of IκB/MAD-3 that could specifically immunoprecipitate ^{35}S-labeled in vitro-translated IκB/MAD-3 was used for these experiments. Gel-shift assays showed that Ab 9 did not affect the ability of IκB/MAD-3 to inhibit NF-κB DNA binding (data not shown), which suggested that Ab 9 could recognize IκB/MAD-3 when it is bound to NF-κB and was therefore suitable for use in coimmunoprecipitation studies. Surprisingly, coimmunoprecipitation assays using ^{35}S-labeled in vitro-translated p50 or p65 with bacterially expressed IκB/MAD-3 revealed that both proteins bound to IκB/MAD-3, although less p50 was precipitated as compared with p65 (Fig. 2B, cf. lanes 3 and 6). The low levels of background evident in the absence of IκB/MAD-3 show that neither the antibody nor the immune serum has any significant affinity for p50 or p65 (Fig. 2B, lanes 2,5). We believe that the low background levels are likely the result of the presence of an endogenous IκB-like activity in the lysates that is recognized by Ab 9. In addition, no coimmunoprecipitation of p50 or p65 with IκB/MAD-3 was observed using preimmune serum (data not shown). To exclude the possibility that p50 was being immunoprecipitated from rabbit reticulocyte lysates through association with endogenous p65-like activities, we have carried out immunoprecipitations with proteins translated in wheat germ lysates, which do not have any detectable NF-κB activity, and have obtained identical results (data not shown). More importantly, a truncated form of p50 that was capable of dimerizing and binding DNA is not capable of binding IκB/MAD-3 (see below and also Fig. 3B, lanes 7–9). These results strongly suggest that the p50 and IκB/MAD-3 interaction is taking place through direct binding and is not mediated through interaction with p65. Further evidence for the interaction between p50 and IκB/MAD-3 is presented later in the text.

There are at least two possible explanations why p50

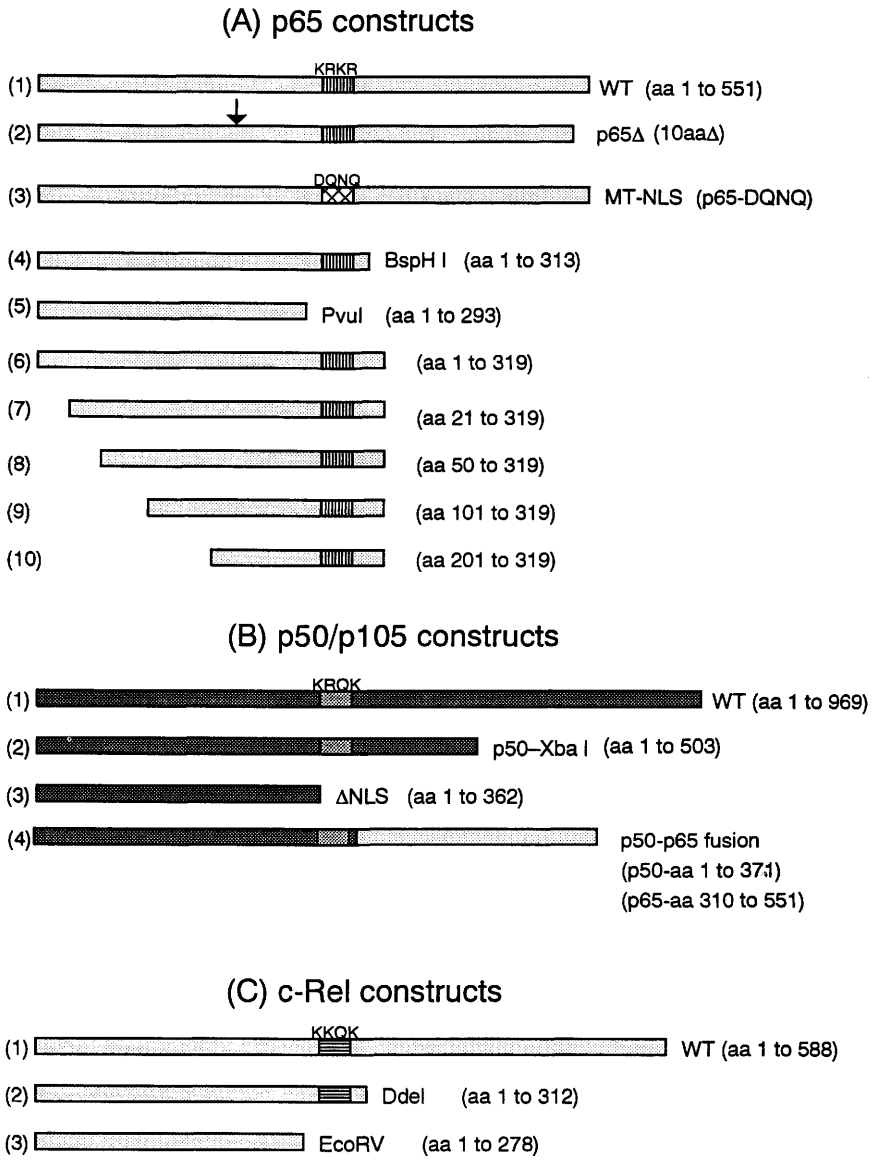

Figure 1. Schematic drawing of p65, p50/p105, and c-Rel constructs. (*A*) p65 constructs used for different experiments. (*1*) The full-length wild-type p65. Vertical lines indicate the NLS; the sequence of the four highly conserved basic residues (KRKR) is shown above it. All other p65 constructs have the same four residues and are also indicated by vertical lines, except for *3*, which was made from full-length p65 by mutation of these residues to DQNQ and is indicated by crosshatching. (*2*) An alternatively spliced form of p65 (p65Δ), which has a deletion of residues 222–231, the approximate position of which is shown by the arrow. (*4,5*) Carboxy-terminal deletions. The restriction enzymes used for cutting the p65 cDNA and the lengths of the proteins produced are shown at the *right* of the constructs. (*6–10*) Amino-terminal deletions of the p65 cDNA made by using a PCR-mediated approach. The lengths of the proteins are shown at *right*. (*B*) (*1*) The full-length p50/p105 protein with its NLS, indicated by light stippling. (*2*) A protein made by cutting the p105 gene with *XbaI*, approximately corresponding to the wild-type p50. (*3*) Made by a PCR-mediated approach with the NLS deleted. The lengths of *2* and *3* are shown at *right*. (*4*) A fusion protein that contains amino acids 1–371 of p50 and amino acids 310–551 of p65. (*C*) (*1*) The wild-type c-Rel protein with the NLS indicated by horizontal lines. (*2,3*) Carboxy-terminal deletions that contain or do not contain the c-Rel NLS, respectively, generated by cutting the c-Rel cDNA with the enzymes shown at *right*. The lengths of the proteins are shown at *right*. Abbreviations/symbol: (WT) Wild-type; (NLS) nuclear localization sequence; (aa) amino acids; (MT) mutant; (Δ) deletion.

DNA binding is not inhibited by IκB/MAD-3 in a gel-shift assay although association is observed in the coimmunoprecipitation assay. The simplest explanation is that because of its lower affinity for p50, considerably more IκB/MAD-3 is required to inhibit p50 DNA binding. However, even 50 times more IκB/MAD-3 than that used to inhibit p65 DNA binding did not inhibit p50 DNA binding (data not shown). The second possibility is that p50 bound to IκB/MAD-3 is still capable of contacting a κB-binding site DNA probe. In this manner, binding site probe could lead to the dissociation of p50 from IκB/MAD-3. To determine whether this model is true, we carried out a coimmunoprecipitation experiment in which NF-κB-binding site DNA was added to preformed p50-IκB/MAD-3 or p65-IκB/MAD-3 complexes (Fig. 2C). Addition of this DNA reduced the amount of coimmunoprecipitated p50 to background levels, whereas no noticeable effect of DNA was seen on coimmunoprecipitation of p65 with IκB/MAD-3 (Fig. 2C, cf. lanes 3 and 4 with lanes 7 and 8). Furthermore, a 200-fold excess of nonspecific DNA had no effect on coimmunoprecipitation of either p50 or p65 (data not shown). This demonstrates that high-affinity binding site DNA causes the dissociation of p50–IκB/MAD-3 complexes and explains the inability of IκB/MAD-3 to inhibit p50 DNA binding in a gel-shift assay.

The NLSs of p50, p65, and c-Rel are targets for IκB/MAD-3

To identify the regions of p50, p65, and c-Rel that are involved in IκB/MAD-3 binding, carboxy-terminal deletions of the p50/p105, p65, and c-Rel cDNAs were constructed using either restriction enzyme sites or a polymerase chain reaction (PCR)-mediated approach (see Fig. 1). We were aware of the highly conserved NLSs present

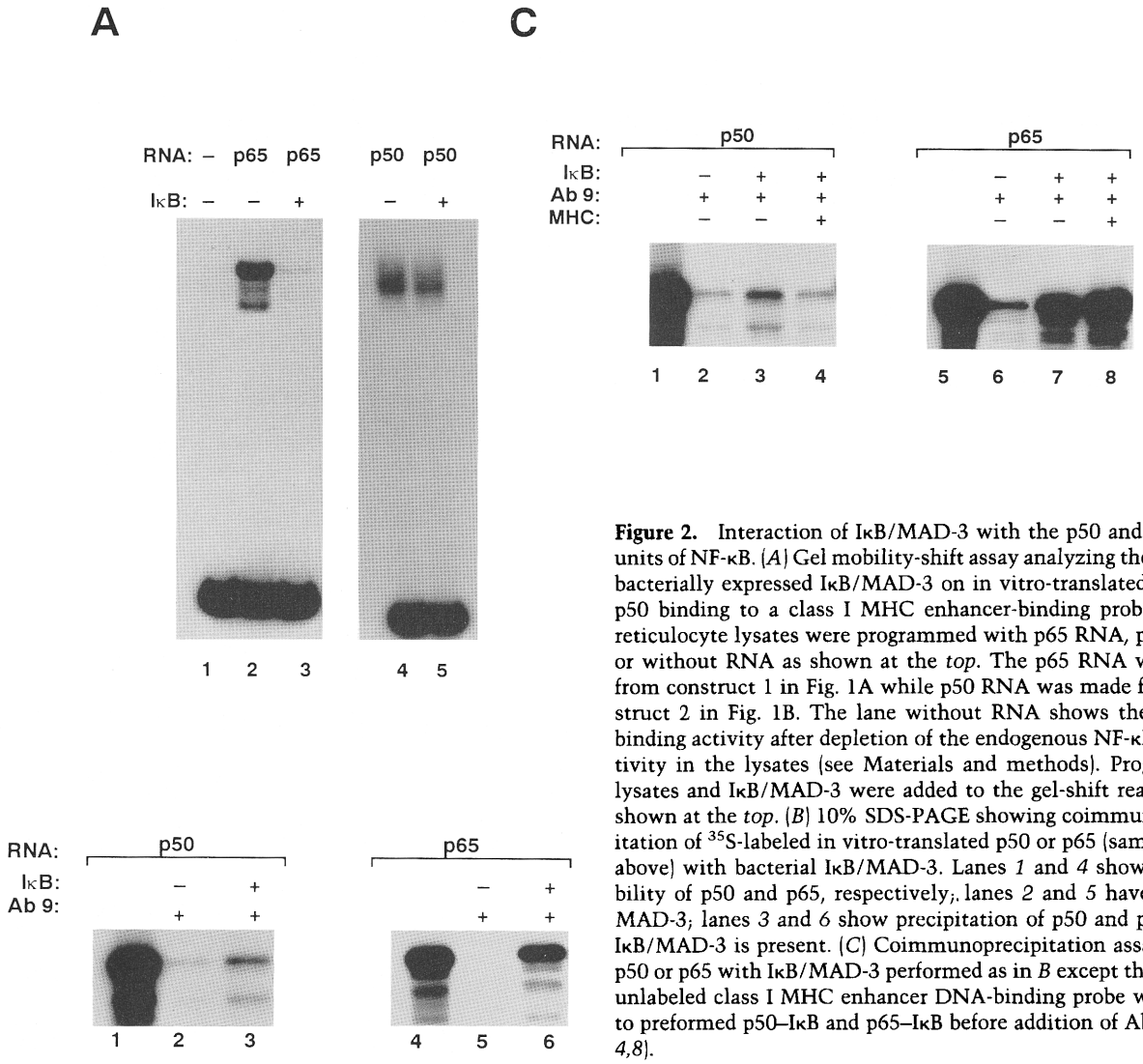

Figure 2. Interaction of IκB/MAD-3 with the p50 and p65 subunits of NF-κB. (*A*) Gel mobility-shift assay analyzing the effect of bacterially expressed IκB/MAD-3 on in vitro-translated p65 and p50 binding to a class I MHC enhancer-binding probe. Rabbit reticulocyte lysates were programmed with p65 RNA, p50 RNA, or without RNA as shown at the *top*. The p65 RNA was made from construct 1 in Fig. 1A while p50 RNA was made from construct 2 in Fig. 1B. The lane without RNA shows the level of binding activity after depletion of the endogenous NF-κB-like activity in the lysates (see Materials and methods). Programmed lysates and IκB/MAD-3 were added to the gel-shift reactions as shown at the *top*. (*B*) 10% SDS-PAGE showing coimmunoprecipitation of ^{35}S-labeled in vitro-translated p50 or p65 (same as in *A* above) with bacterial IκB/MAD-3. Lanes *1* and *4* show the mobility of p50 and p65, respectively;. lanes *2* and *5* have no IκB/MAD-3; lanes *3* and *6* show precipitation of p50 and p65 when IκB/MAD-3 is present. (*C*) Coimmunoprecipitation assays using p50 or p65 with IκB/MAD-3 performed as in *B* except that 2 ng of unlabeled class I MHC enhancer DNA-binding probe was added to preformed p50–IκB and p65–IκB before addition of Ab 9 (lanes *4,8*).

in these transcription factors and their possible involvement in IκB/MAD-3 binding. Therefore, two different carboxy-terminal deletion constructs were made for each protein: one that contained the NLS and another that had the NLS deleted. A gel-shift assay showed that the DNA-binding activity of an NLS-containing form of p65 (Fig. 1A, construct 4, amino acids 314–551 deleted) was inhibited in the presence of IκB/MAD-3 (Fig. 3A, lanes 1,2). These results show that amino acids 1–313 of p65 are sufficient for IκB/MAD-3 binding. However, removal of an additional 20 amino acids, including the NLS, produced a p65 protein (Fig. 1A, construct 5) that was no longer inhibited by IκB/MAD-3 (Fig. 3A, lanes 3,4). Coimmunoprecipitation experiments using these two p65 proteins with IκB/MAD-3 confirmed that the region containing the NLS is essential for IκB/MAD-3 binding (Fig. 3B, lanes 1–6). Using a similar approach, we tested the inhibition of DNA binding of two carboxy-terminal truncations of c-Rel (Fig. 1C, constructs 2 and 3) by IκB/MAD-3. Our results demonstrated that the presence of the NLS was necessary for IκB/MAD-3 to inhibit c-Rel DNA binding (Fig. 3A, lanes 5–8). We have shown above that p50 (which contains a NLS) can bind IκB/MAD-3 (Fig. 2B). We then tested the ability of a p50 protein that does not contain an NLS to bind IκB/MAD-3 (Fig. 1B, construct 3). An IκB/MAD-3 coimmunoprecipitation experiment showed that this protein was no longer capable of binding IκB/MAD-3 (Fig. 3B, lanes 7–9). Therefore, we conclude that the regions containing the highly conserved NLSs of p50, p65, and c-Rel are critical for IκB/MAD-3 binding, whereas the regions carboxy-terminal to the NLSs are expendable. Moreover, each of the proteins with the deleted NLS was able to bind DNA, demonstrating that the deletions were not causing conformational changes that dramatically alter the folding of these proteins.

We then tested whether mutations of the highly conserved basic residues in the NLS of p65 would affect its interaction with IκB/MAD-3 (Fig. 3C). Amino acid substitutions in the NLS of p65, KRKR to DQNQ (Fig. 1A,

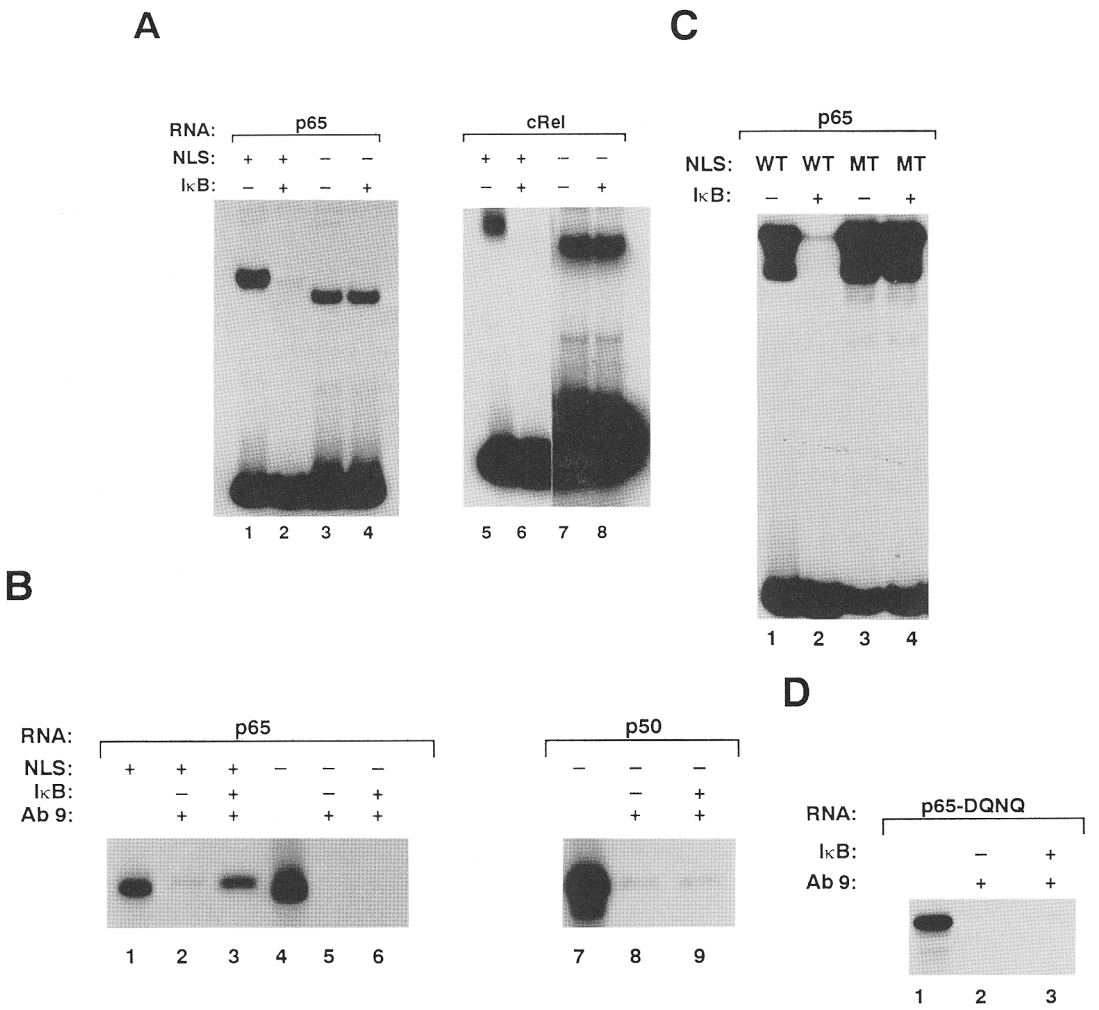

Figure 3. IκB/MAD-3 targets the NLSs of c-Rel and of the p50 and p65 subunits of NF-κB. (*A*) Gel-shift assay showing the effect of deleting the NLS of p65 (lanes *1–4*) and of c-Rel (lanes *5–8*) on their abilities to interact with IκB/MAD-3. Lanes *1* and *2* have in vitro-translated p65 derived from construct 4 shown in Fig. 1A; lanes *3* and *4* have p65 derived from construct 5 in Fig. 1A. The c-Rel protein used in lanes *5* and *6* was made from construct 2 in Fig. 1C; the c-Rel protein used in lanes *7* and *8* was made from construct 3 in Fig. 1C. The presence or absence of the NLS in the various proteins used and the addition of IκB/MAD-3 are indicated at the *top*. (*B*) 10% SDS-PAGE showing the effect of deleting the NLS of p65 and p50 on their ability to coimmunoprecipitate with IκB/MAD-3. Lanes *1–3* have the same p65 protein as in lanes *1* and *2* in *A*; lanes *4–6* have the same p65 as in lanes *3* and *4* in *A*; lanes *7–9* have a p50 protein with no NLS, derived from construct 3 in Fig. 1B. The addition of IκB/MAD-3 and the presence or absence of a NLS is shown at the *top*. (*C*) Gel-shift assay showing the effect of amino acid substitutions in the NLS of in vitro-translated p65 (KRKR to DQNQ) on its ability to interact with IκB/MAD-3. Lanes *1* and *2* show wild-type p65 (Fig. 1A, construct 1); lanes *3* and *4* show p65-DQNQ (Fig. 1A, construct 3). IκB/MAD-3 was added as shown at the *top*. (*D*) 10% SDS-PAGE demonstrating the inability of a p65 with amino acid substitutions in the NLS (KRKR to DQNQ) to coimmunoprecipitate with IκB/MAD-3. The addition of IκB/MAD-3 is indicated at the *top*.

construct 3), prevented inhibition by IκB/MAD-3 in a gel-shift assay as opposed to wild-type p65, which was almost completely inhibited (Fig. 3C, cf. lanes 2 and 4). The same result was obtained in a coimmunoprecipitation assay using this p65–DQNQ mutant with IκB/MAD-3, demonstrating that the mutation dramatically reduced the ability of p65 to bind IκB/MAD-3 (Fig. 3D). Deletion of these four NLS amino acids (KRKR) also resulted in a protein that was no longer capable of binding IκB/MAD-3 (data not shown). These results suggest that a direct contact is made between IκB/MAD-3 and the NLS of p65.

Heterodimerization of p65 and p50 correlates with the ability to bind IκB/MAD-3

We have shown above that the NLSs of p50, p65, and c-Rel are required for IκB/MAD-3 binding. To determine whether additional regions are also necessary for IκB/MAD-3 binding, we generated amino-terminal deletions

of p65 using a PCR-mediated approach (see Fig. 1A, constructs 6–10, and Materials and methods) and then carried out a series of IκB/MAD-3 coimmunoprecipitation assays (Fig. 4A). p65 was used for these experiments because it is a high-affinity target for IκB/MAD-3 and because carboxy-terminal deletions had revealed that p50, p65, and c-Rel contain virtually identical IκB/MAD-3 target regions (Fig. 3A,B). All p65 proteins contained the NLS that was necessary for IκB/MAD-3 binding while the carboxy-terminal region containing amino acids 320–551 had been deleted. (Fig. 1A, constructs 6–10). When the complete amino-terminal region was present (Fig. 1A, construct 6), the resulting protein bound IκB/MAD-3 with high affinity (Fig. 4A, lanes 1–3), as expected. A 20-amino-acid amino-terminal deletion (Fig. 1A, construct 7) produced a protein that was capable of binding IκB/MAD-3 (Fig. 4A, lanes 4–6). However, a 49-amino-acid deletion (Fig. 1A, construct 8) yielded a protein with dramatically reduced affinity for IκB/MAD-3 (Fig. 4A, lanes 7–9). A p65 protein with a 100-amino-acid deletion (Fig. 1A, construct 9) also had very low affinity for IκB/MAD-3 (Fig. 4B, lanes 10–12). A 200-amino-acid deletion of p65 (Fig. 1A, construct 10) resulted in a protein with no affinity for IκB/MAD-3 (lanes 13–15). Interestingly, only the proteins generated from constructs 6 and 7 above, which bound IκB/MAD-3 with high affinity, were also capable of binding DNA (data not shown) and of heterodimerization with p50 (Fig. 4B, lanes 1–4). This demonstrates that the 20 amino-terminal amino acids are expendable for IκB/MAD-3 binding, DNA binding, and heterodimerization with p50. Moreover, proteins derived from constructs 8 and 9 have a dramatically reduced affinity for IκB/MAD-3 (Fig. 4A, lanes 7–12) and have little or no affinity for p50 (Fig. 4B, lanes 5–8). We believe that the low affinity of these proteins for IκB/MAD-3 is the result of their inability to form dimers.

A dimerization-deficient, transforming variant of p65 does not bind IκB/MAD-3

An alternatively spliced form of p65 mRNA, encoding a protein with a 10-amino-acid deletion from residues 222 to 231 (see Fig. 1A, construct 2), which severely inhibits its ability to dimerize and hence to bind DNA, has been reported recently (Ruben et al. 1992b). This form of p65 (p65Δ) is highly transforming when transfected into Rat-1 cells, as determined by foci formation, growth in soft agar, and tumor formation in athymic nude mice (Narayanan et al. 1992). To potentially understand p65Δ function, we tested whether IκB/MAD-3 could associate with this protein. Interestingly, only very low-affinity interaction was observed between IκB/MAD-3 and p65Δ in a coimmunoprecipitation assay (Fig. 4C). This result demonstrates that the internal 10-amino-acid deletion of p65Δ that blocks dimerization also blocks interaction with IκB/MAD-3. This is consistent with the results shown above that only a p65 protein with the ability to form dimers can interact with IκB/MAD-3.

IκB/MAD-3 can block trans*-activation by p65 and by a p50/65 chimera*

To determine whether a functional interaction could be shown in vivo between IκB/MAD-3 and the p50 and p65 subunits of NF-κB, a series of transient transfection experiments were carried out. Expression plasmids encoding NF-κB subunits were introduced into Jurkat T cells along with a CAT reporter plasmid containing a minimal *fos* promoter and either three copies of the H-2K^b class I MHC NF-κB site or three copies of a mutant site incapable of binding NF-κB. *Trans*-activation was measured as chloramphenicol acetyltransferase (CAT) activity by thin layer chromatography (TLC) separation of acetylated [^{14}C]chloramphenicol. As shown in Figure 5 (lane 1), Jurkat cells exhibit some basal activity mediated by the MHC NF-κB sites. This basal activity is inhibited by IκB/MAD-3 (Fig. 5, lanes 2,4) and is abolished by replacing the functional NF-κB sites in the reporter construct with mutated sites incapable of binding NF-κB (Fig. 5, lane 9), suggesting that NF-κB-like activities are mediating this expression. The ability of IκB/MAD-3 to block this basal activity suggests that either these cells are rapidly turning over NF-κB such that IκB/MAD-3 can sequester newly synthesized NF-κB in the cytoplasm or that IκB/MAD-3 can enter the nucleus and block this activity. Expression of p50 does not activate transcription through the NF-κB-binding sites and may inhibit basal activity (Fig. 5, lane 3). Expression of p65 in these cells caused a marked activation of this reporter, as evidenced by the acetylation of 85% of the chloramphenicol present in this assay (Fig. 5, lane 5). Furthermore, *trans*-activation by p65 was strongly inhibited by IκB/MAD-3 expression (Fig. 5, lane 6). IκB/MAD-3 did not inhibit gene expression mediated by the adenovirus major late

Figure 4. Effect of amino-terminal deletions of p65 on its ability to bind IκB/MAD-3. (*A*) 15% SDS-PAGE showing the effect of amino-terminal deletions on the ability of in vitro-translated p65 to coimmunoprecipitate with IκB/MAD-3. The lengths of the various proteins are shown at the *top*, and the constructs from which they were derived are shown in Fig. 1A (constructs 6–10). (Lanes *1,4,7,10,13*) The mobility of the different proteins. The addition of IκB/MAD-3 is shown above the lanes. The positions of migration of molecular mass markers are shown at the *left* (in kD). (*B*) 15% SDS-PAGE showing the effect of amino-terminal deletions on the ability of in vitro-translated p65 to coimmunoprecipitate with in vitro cotranslated p50 using antibodies against p50 (Ab 2). The lengths of the various p65 proteins are shown at the *top*; the constructs from which they were derived are shown in Fig. 1A (constructs 6–10). (Lanes *1,3,5,7,9*) The mobility of various proteins. p50 is shown with the large arrowhead; the various p65 proteins are shown with small arrowheads. (Lanes *2,4,6,8,10*) The results of the coimmunoprecipitation experiment. (*C*) 10% SDS-PAGE showing the result of a coimmunoprecipitation experiment using in vitro-translated p65Δ, an alternatively spliced form of p65, with IκB/MAD-3. The p65Δ construct used is shown in Fig. 1A, construct 2. The addition of IκB/MAD-3 is shown at the *top*.

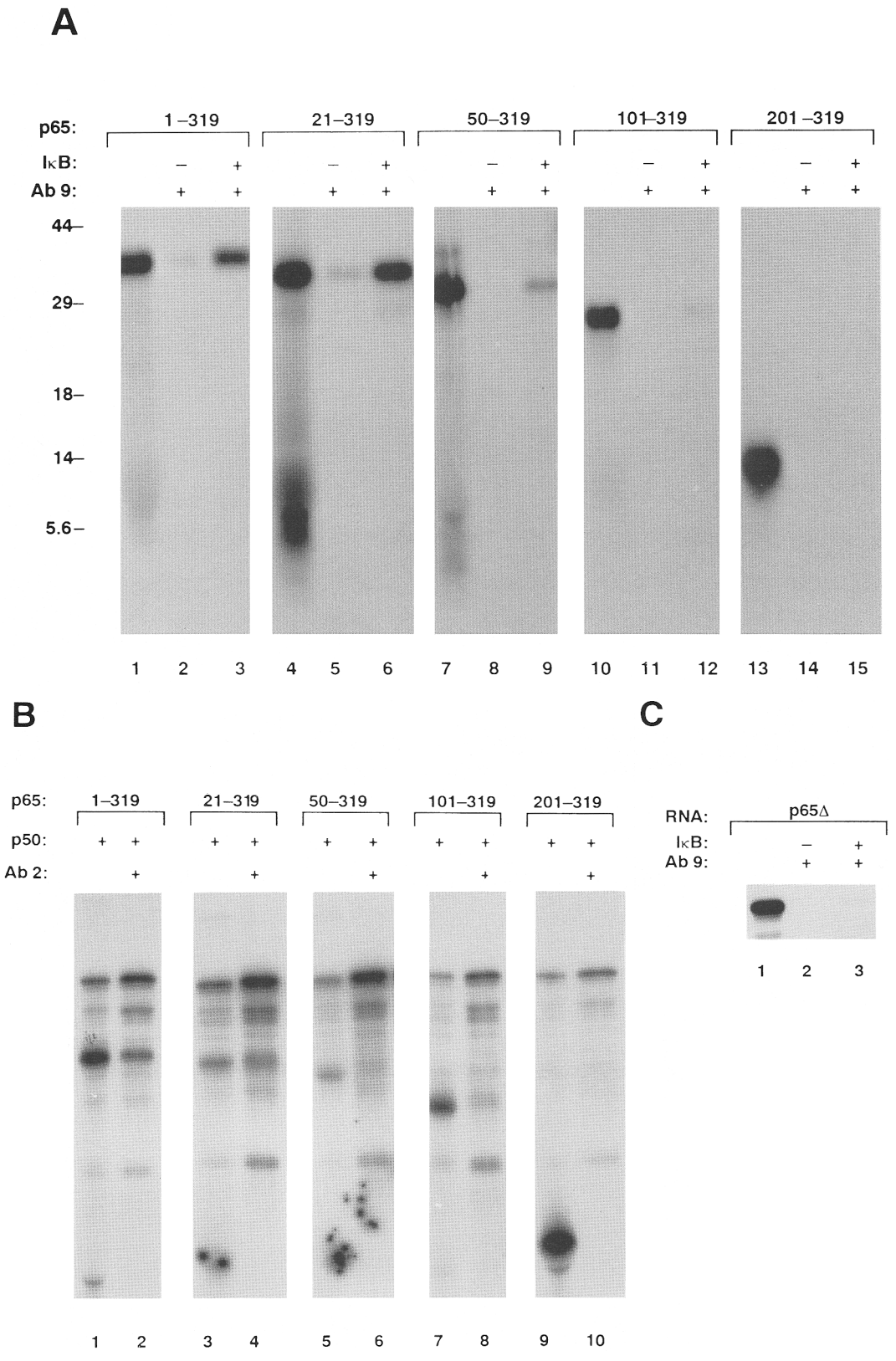

Figure 4. (*See facing page for legend.*)

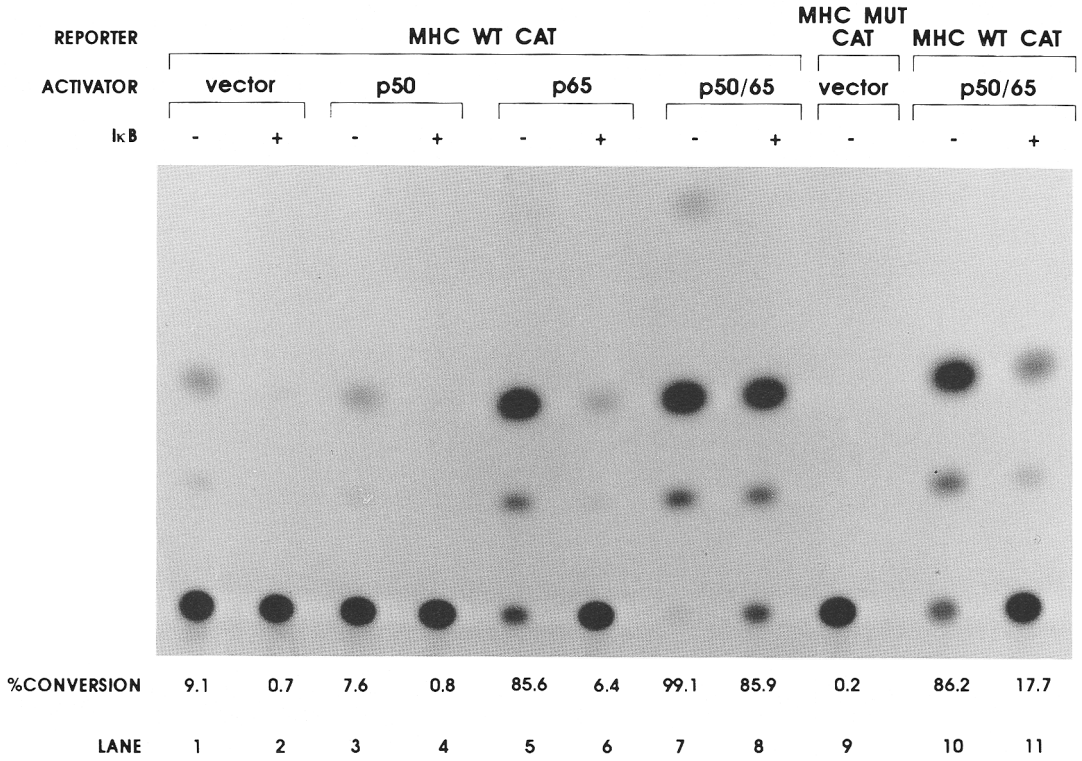

Figure 5. IκB/MAD-3 can block *trans*-activation by p65 and by a p50/65 chimera. Jurkat T cells were transfected by electroporation, and extracts were prepared as described in Materials and methods. Each transfection contains 5 μg of reporter plasmid (MHC WT CAT or MHC MUT CAT, as shown). Lanes *1*, *2*, and *9* contain no activators; lanes *3–8*, *10*, and *11* contain 2.5 μg of p50, p65, or p50/p65 fusion expression plasmids as indicated at the *top*. Lanes *2*, *4*, *6*, *8*, and *11* contain 2.5 μg of IκB/MAD-3 expression plasmid as indicated. All lanes have the same amount of DNA (10 μg) by including empty vector as indicated. Lanes *1–9* were generated from extracts corresponding to 5×10^5 transfected Jurkat cells. In contrast, lanes *10* and *11* were generated from 1×10^5 cells. The amount of radioactivity present in acetylated and unacetylated forms of chloramphenicol was measured by scanning in an Ambis Beta camera and is shown below each lane as % conversion. Each of these experiments has been repeated at least three times.

promoter (data not shown), demonstrating specificity of the inhibitor for the NF-κB subunits.

We have shown above that p50 can interact with IκB/MAD-3 (Fig. 2B, lanes 1–3). To test whether this interaction occurs in vivo, we made use of a p50/65 chimera consisting of amino acids 1–371 of p50 followed by amino acids 310–551 of p65 (Ruben et al. 1992b). The chimera contains the DNA-binding sequence and NLS of p50 connected to the transcriptional activation domain of p65. The chimera was shown to be a stronger *trans*-activator than p65 (Fig. 5, cf. lanes 5 and 7), making it difficult to observe any possible inhibition by IκB/MAD-3 under the nonlinear conditions used for the CAT assay (Fig. 5, lane 8). However, when fivefold less protein was used in the CAT assay, the percent conversion of chloramphenicol was equal to that of p65 (Fig. 5, cf. lanes 5 and 10). The cotransfection of IκB/MAD-3 with the p50/65 chimera blocked CAT expression mediated by this *trans*-activator, although not to the same extent as p65, suggesting an in vivo interaction between p50 and IκB/MAD-3 (Fig. 5, lane 11). This is consistent with the observation that IκB/MAD-3 has lower affinity for p50 than for p65 (Fig. 2B). The p65 portion of the chimera is unable to interact with IκB/MAD-3 by immunoprecipitation and can be deleted without any effect on IκB/MAD-3-binding ability (Fig. 3A, lanes 1,2). Thus, the ability of the chimera to interact with IκB/MAD-3 must be the result of the presence of amino acid sequences from p50. Furthermore, the inhibition by IκB/MAD-3 is not likely to be taking place through the association of the p50 portion of the chimera with endogenous p65 in Jurkat cells, as transfection of the p50 plasmid alone would have led to elevated transcription through the activation domain of the endogenous p65 molecule. However, no such activation by p50 alone was noticed (Fig. 5, lane 3). On the basis of these results and those shown above, we conclude that IκB/MAD-3 can bind p50, p65, and c-Rel in vitro and p50 and p65 in vivo.

IκB/MAD-3 retains both the p50 and p65 subunits of NF-κB in the cytoplasm

The in vitro analysis of p65-IκB/MAD-3 interaction indicated that the amino acid sequence KRKR, which corresponds to the NLS identified in the related c-Rel and p50 proteins (Gilmore and Temin 1988; Blank et al.

1991; Henkel et al. 1992), is required for IκB/MAD-3 interaction. To establish that these amino acids comprise the bona fide NLS of p65, the subcellular distribution of this subunit and the NLS mutant was examined by indirect immunofluorescence (Fig. 6). To distinguish endogenous NF-κB from that produced by transfection, expression vectors where the amino-terminal sequences of the NF-κB proteins were fused in-frame with a sequence encoding a 10-amino-acid epitope of the influenza hemagglutinin antigen (HA) (Kolodziej and Young 1991) were constructed. Forty-eight hours after transfection, the subcellular localization of the recombinant proteins was examined by indirect immunofluorescence using a monoclonal anti-HA-antibody, followed by a rhodamine-conjugated anti-mouse antibody. COS cells transfected with the plasmid HAp65, which encodes the epitope-tagged wild-type p65, demonstrated strong nuclear immunofluorescence, whereas those cells transfected with the HAp65–DQNQ expression vector, which encodes the NLS mutant, showed strong cytoplasmic staining (Fig. 6). We therefore conclude that amino acids KRKR are essential for nuclear targeting of p65.

Previous studies have established that in unstimulated cells, a large proportion of NF-κB is complexed with IκB in the cytoplasm (Baeuerle and Baltimore 1988a). To examine whether IκB/MAD-3 can mediate intracellular distribution of NF-κB, the plasmids encoding the epitope-tagged p50 and p65 were cotransfected with or without an IκB/MAD-3 expression plasmid and their subcellular localization was examined. Whereas in the absence of IκB/MAD-3 both proteins showed strong nuclear staining, a redistribution to the cytoplasm was observed in cells that were cotransfected with IκB/MAD-3 (Fig. 6). These findings provide formal proof that expression of IκB/MAD-3 alone is sufficient to alter the subcellular localization of both p50 and p65 NF-κB.

A heterologous NLS overrides cytoplasmic retention of p65 by IκB/MAD-3

Having established that IκB/MAD-3 can affect the distribution of the p50 and p65 subunits of NF-κB, the importance of the p65 NLS in this process was examined. A new series of expression vectors were constructed where, in addition to the HA epitope tag, the amino-terminal sequences of HAp65 and HAp65–DQNQ were fused in-frame with a sequence encoding the NLS of the large T antigen (Kalderon et al. 1984). The SV40 NLS was func-

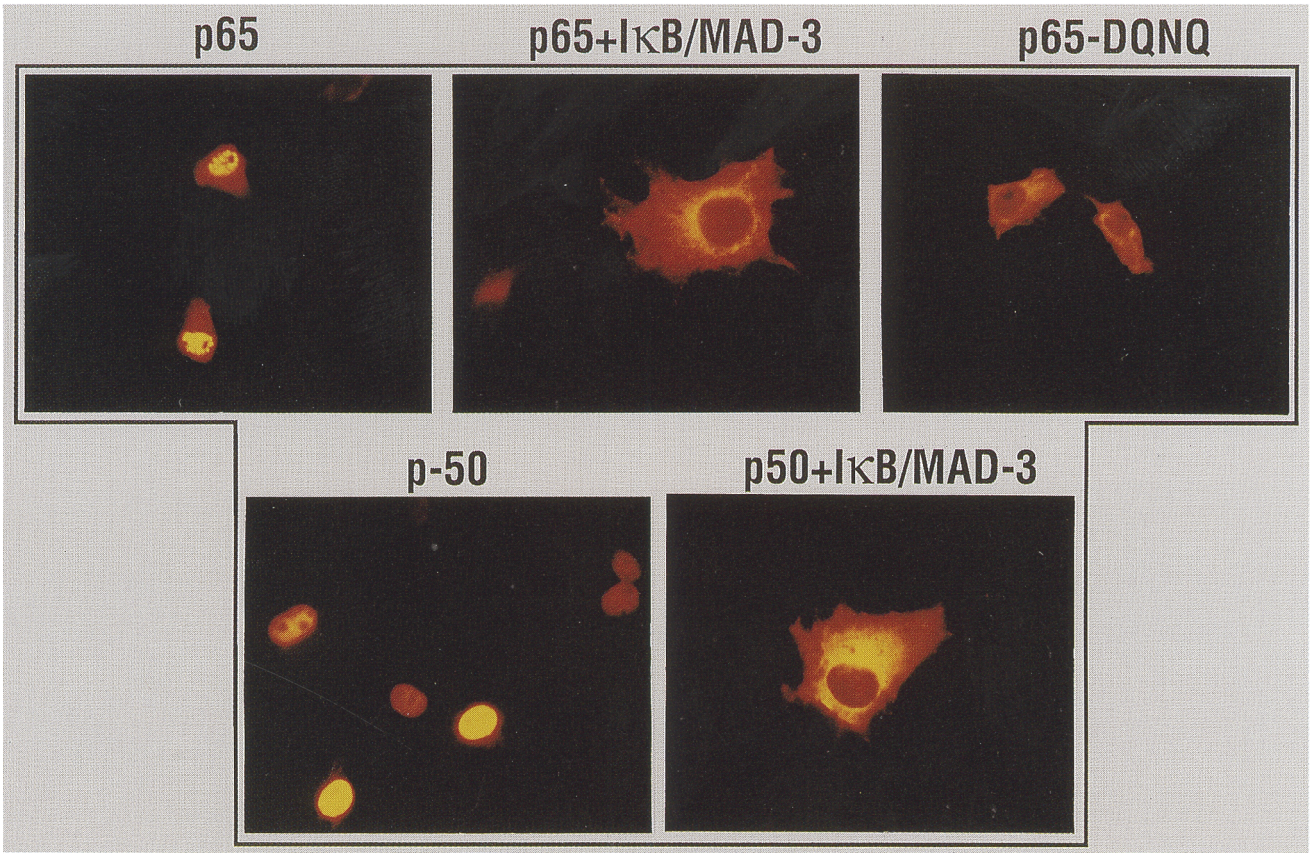

Figure 6. Subcellular distribution of NF-κB p65 and p50 in the presence and absence of IκB/MAD-3. COS-7 cells were transfected with expression vectors encoding the HA-tagged p65, p65–DQNQ, and p50 proteins in the presence or absence of IκB/MAD-3 as indicated. Indirect immunofluorescence was performed 48 hr post-transfection. Antibody staining was accomplished using an anti-HA antibody followed by a rhodamine-conjugated secondary antibody.

tional as SV–p65–DQNQ protein localized to the nucleus (Fig. 7). We also note that the SV40 NLS affected the p65 localization pattern as a distinct clustered appearance of HAp65 in the nucleus gave way to a more uniformly intense nuclear fluorescence with SVp65. (Fig. 7). More importantly, the SV40 NLS-tagged authentic p65 remained nuclear in the presence of IκB/MAD-3 (Fig. 7). These findings indicate that IκB/MAD-3 targets p65 through the NLS sequence and further suggests that the presence of a heterologous NLS can override IκB/MAD-3 function.

Discussion

A mechanism for the cytoplasmic retention of NF-κB and c-Rel

Short stretches of mostly basic amino acids have been characterized as signals necessary for the nuclear transport of proteins and have been called NLSs (Kalderon et al. 1984; Silver 1991). Several proteins have been identified that bind NLSs in vitro and in vivo (for review, see Adam and Gerace 1991; Nigg et al. 1991 and references therein). These proteins are thought to function as receptors that first bind and then translocate NLS-bearing proteins into the nucleus through the nuclear pores, although the exact mechanism of this process is not completely understood (Nigg et al. 1991; Silver 1991). Other studies have suggested that the location of the NLS in the protein is also critical (Roberts et al. 1987). NLSs that lie in less accessible regions of the proteins do not function well, which suggests that NLSs need to be exposed to be recognized by NLS receptor proteins (Roberts et al. 1987). All members of the NF-κB/c-Rel family have highly conserved NLSs. In addition, previous studies have shown that the integrity of the v-Rel and p50 NLSs is necessary for nuclear transport (Gilmore and Temin 1988; Blank et al. 1991; Henkel et al. 1992). It has also been proposed that the mechanism for the cytoplasmic retention of NF-κB could be through the masking of its NLS by IκB (Baeuerle 1991; Silver 1991). Here, we demonstrate that the NLSs of c-Rel, p50, and p65 are required for IκB/MAD-3 binding (Fig. 3). Our results suggest that IκB/MAD-3 makes direct contact with the NLS residues of the p65 subunit of NF-κB (Fig. 3C,D). In addition, we demonstrate that IκB/MAD-3 can retain both the p50 and p65 subunits of NF-κB in the cytoplasm (Fig. 6). Consistent with our immunofluorescence experiments, Western blot analysis of IκB/MAD-3 has revealed that it is predominantly cytoplasmic in HeLa cells (data not

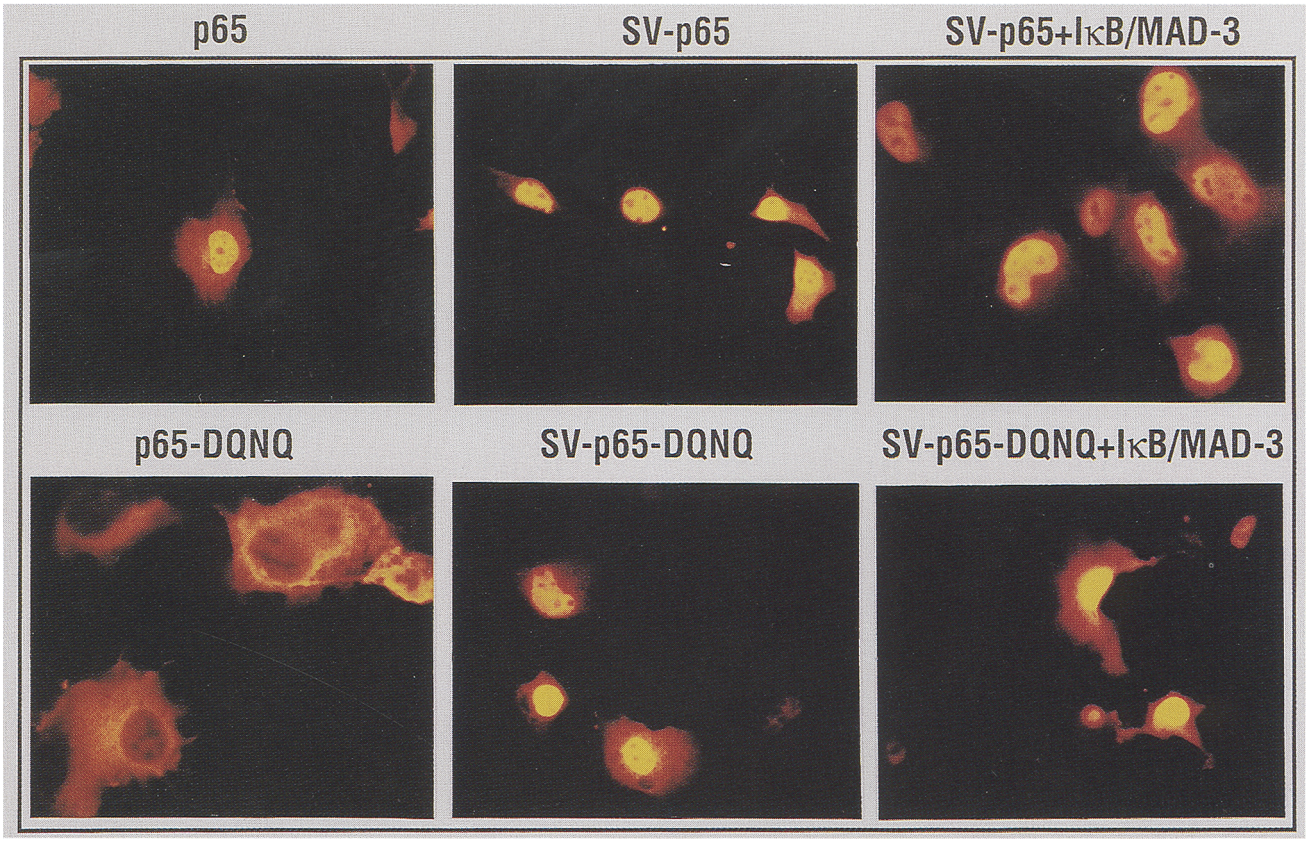

Figure 7. Subcellular distribution of SV40 NLS-tagged p65 and p65–DQNQ in the presence and absence of IκB/MAD-3. COS-7 cells were transfected with expression vectors encoding the HA-tagged p65 or p65–DQNQ or vectors expressing the HA-tagged proteins with the SV40 NLS (SV prefix) in the presence or absence of IκB/MAD-3 expression vector. Indirect immunofluorescence was performed 48 hr post-transfection using the anti-HA antibody followed by a rhodamine-conjugated secondary antibody.

shown). Furthermore, we demonstrate that the protein SV–p65, which contains a second NLS derived from the SV40 large T antigen, remains nuclear in the presence of IκB/MAD-3 (Fig. 7). Thus, the presence of an NLS, which cannot interact with IκB, is sufficient to override the cytoplasmic retention by this inhibitory protein.

We propose a model that involves the masking of the NLSs of c-Rel and of NF-κB by IκB/MAD-3, thereby preventing NLS receptors from recognizing these sequences and transporting these proteins into the nucleus. Dissociation of NF-κB-IκB/MAD-3 complexes caused by the different inducers of NF-κB would result in the unmasking of these sequences and would allow NLS receptors to bind and translocate NF-κB into the nucleus. It has been demonstrated recently that the carboxy-terminal domain of the cytoplasmic 105-kD precursor of p50 masks its own NLS by an intramolecular mechanism and thereby prevents transport into the nucleus (Blank et al. 1991; Henkel et al. 1992). This is consistent with our data that intermolecular masking of the NLSs of these proteins by IκB/MAD-3 is responsible for their cytoplasmic retention. Another possible (and mutually nonexclusive) mechanism for retention of NF-κB in the cytoplasm is through the binding of NF-κB-IκB/MAD-3 complexes to some nondiffusible cytoplasmic structure. However, the results presented in Figure 7 argue against this type of mechanism because the addition of a heterologous NLS to p65 confers resistance to cytoplasmic retention by IκB/MAD-3.

In the NF-κB subunits, the NLSs and residues immediately amino-terminal to them have a high number of basic and acidic amino acids (e.g., 12 of 14 residues in this region are charged in p65). This arrangement, along with the requirement for basic residues in the NLS of p65 (KRKR) for IκB/MAD-3 binding, suggests that charged interactions constitute an important part of NF-κB-IκB/MAD-3 complex formation. This may explain the sensitivity of NF-κB-IκB/MAD-3 complexes to treatment with the anionic detergent deoxycholate (Baeuerle and Baltimore 1988a; Haskill et al. 1991).

Binding of IκB/MAD-3 to p50

Previous studies have indicated that the p65 subunit of NF-κB is required for the inhibition of the DNA binding of NF-κB (p50/p65) by IκB (Baeuerle and Baltimore 1989). These results were based on the observation that p50/p65 heterodimers were inhibited while p50/p50 homodimers were not inhibited (Baeuerle and Baltimore 1989; Haskill et al. 1991; Nolan et al. 1991). In this study we have shown that IκB/MAD-3 binds to both the p50 and the p65 subunit of NF-κB in vitro, although with different affinities (Fig. 2B), and that IκB/MAD-3 can block the *trans*-activation properties of both proteins in a transient expression experiment (Fig. 5). Furthermore, coexpression of IκB/MAD-3 with p50 results in the cytoplasmic retention of p50 (Fig. 6).

Consistent with previous results, we have shown that p50 homodimer DNA binding is not inhibited by IκB/MAD-3 (Fig. 2A). However, we have found that p50 binds IκB/MAD-3 in a coimmunoprecipitation assay and that high-affinity binding site DNA can dissociate p50–IκB/MAD-3 complexes but not p65–IκB/MAD-3 complexes (Fig. 2C). One possible explanation for these results is that certain regions of p50 that are important in DNA binding are not contacted by IκB/MAD-3. These regions would be free to contact DNA, thus leading to dissociation of p50–IκB/MAD-3 complexes by an allosteric mechanism. Consistent with this proposal is the recent observation that p50 undergoes a conformational change upon binding certain κB sites (Fujita et al. 1992). Furthermore, our data may explain why IκB inhibits the ability of p65 to stimulate transcription in vitro but does not inhibit the ability of p50 to do so (Kretzschmar et al. 1992). Our results demonstrate that the lack of inhibition of DNA binding by an inhibitory protein is not a definitive assay for protein–protein interaction.

The interaction between p50 and IκB/MAD-3 has several important biological implications. First, it suggests that IκB/MAD-3 has the potential to interact with both the p50 and p65 subunits in an NF-κB complex. In this way, both the p50 and p65 proteins could interact with IκB/MAD-3, thus preventing a NLS receptor from interacting with either protein. Second, it has implications for the regulation of p50 homodimers. Almost all cell lines that have p50/p65 NF-κB in the nucleus also have p50/p50 homodimers (called KBF1) in the nucleus. Similarly, cell lines that have inactive NF-κB in the cytoplasm may also have p50 homodimers in the cytoplasm. Interestingly, certain agents that induce NF-κB DNA-binding activity, presumably through its dissociation from IκB/MAD-3, also induce p50 homodimer DNA-binding activity (A.A. Beg and A.S. Baldwin, unpubl.). These observations suggest that some amounts of p50/p50 KBF1 may also be kept in the cytoplasm by a mechanism similar to p50/p65 NF-κB. Third, this suggests a mechanism for the assembly of the inactive pool of cytoplasmic NF-κB complexes through the ability of p50, p65, and IκB/MAD-3 to bind each other. Fourth, our data suggest that if IκB/MAD-3 were to enter the nucleus, it may dissociate p50/p65 heterodimers (Zabel and Baeuerle 1990), but not p50 homodimers, from DNA.

Dimerization of p65 is correlated with IκB/MAD-3 binding

The members of the NF-κB/c-Rel family can exist both as homodimers and as heterodimers in various combinations (Ballard et al. 1990; Kieran et al. 1990; Hansen et al. 1992). We demonstrate here that IκB/MAD-3 can interact with high affinity only with those p65 molecules that are also capable of heterodimerization with p50 (Fig. 4). We interpret these results to mean that IκB/MAD-3 would only associate with a homo- or heterodimerized p50 or p65 molecule but not with their monomers, presumably because the conformational changes associated with dimerization generate the high-affinity binding site for IκB/MAD-3. Alternatively, IκB/MAD-3 may require simultaneous interaction with both subunits; hence, the

need for dimerization. This may form the basis of a mechanism that would ensure that IκB/MAD-3 would only associate with dimeric, and thus biologically functional, NF-κB molecules. Release of these NF-κB molecules would lead to a rapid transcriptional response as opposed to the release of nonfunctional monomeric ones, which would first have to dimerize to carry out their function. An alternate and mutually nonexclusive interpretation for our results is that the region of p65 involved in dimerization is also involved in direct contacts with IκB/MAD-3.

Consistent with the first model is that an alternatively spliced form of p65 (p65Δ) that has little dimerization potential has an extremely weak affinity for IκB/MAD-3 (Fig. 4C). p65Δ, which is expressed at high levels in some erythroid and lymphoid colonies derived from the bone marrow, is highly transforming when transfected into Rat-1 cells (Narayanan et al. 1992). One possible model for transformation could be through the ability of p65Δ to bind a cofactor required for NF-κB-mediated transcriptional regulation (Narayanan et al. 1992). The transcriptional activation domain of p65Δ is not disrupted by the deletion, and within this activation domain is a leucine zipper-like motif critical for transcriptional activity (Ruben et al. 1992b). A mutation within this region of p65 severely decreases its transcriptional activity (Ruben et al. 1992b) and, in the context of p65Δ, abolishes its ability to transform Rat-1 cells (Narayanan et al. 1992). We speculate that the high transformation potential of p65Δ could be the result of its ability to move rapidly into the nucleus because of its low affinity for IκB/MAD-3. Indirect immunofluorescence experiments demonstrate that p65Δ is nuclear even in the presence of IκB/MAD-3 (S.M. Ruben and C.A. Rosen, unpubl.).

Relationships between different IκB-like activities

Two forms of IκB (IκB-α and IκB-β) have been purified and partially characterized (Zabel and Baeuerle 1990). The specificity of the two forms for different members of the NF-κB family has, however, been somewhat contradictory. According to one report, IκB-α is specific for NF-κB (p50/p65), whereas IκB-β interacts with both NF-κB and c-Rel (Kerr et al. 1991). However, others have shown that IκB-α can inhibit the DNA-binding activity of both NF-κB and c-Rel (Davis et al. 1991). We have shown here that IκB/MAD-3 can inhibit DNA binding of both NF-κB and c-Rel (Fig. 3A). On the basis of this information, it is difficult to assign IκB/MAD-3 to one of the two forms of IκB. However, peptide sequences of IκB-α are identical to the predicted protein sequence of IκB/MAD-3 (Davis et al. 1991), suggesting that IκB/MAD-3 encodes IκB-α. The other IκB-like activity that has recently been cloned is the c-Rel-associated protein pp40, which can also interact with both NF-κB and c-Rel (Davis et al. 1991). Antibodies against pp40 react with IκB-β (Kerr et al. 1991). In addition, phosphopeptide maps of pp40 and IκB-β are very similar, suggesting that pp40 encodes IκB-β (Kerr et al. 1991). However, only the cloning or the generation of peptide sequences of authentic IκB-α and IκB-β can unequivocally resolve this matter. In addition, the proto-oncogene *bcl 3* has also been shown to encode IκB-like activity (Hatada et al. 1992). Interestingly, Bcl 3 protein seems to inhibit DNA binding of p50 homodimers, as well as that of p50/p65 heterodimers (Hatada et al. 1992), and therefore is not likely to be either IκB-α or IκB-β. A recent report indicates that a separate mRNA derived from the p50/p105 gene encodes a 70-kD protein (IκB-γ) that corresponds to the carboxyl terminus of p105 and that functions as an IκB (Inoue et al. 1992).

The presence of different IκB-like activities suggests that the regulation of NF-κB/Rel family members is quite complex. However, these observations are not surprising considering that these transcription factors play critical roles in numerous signal transduction pathways and that inappropriate expression of these proteins can lead to neoplastic transformation.

Materials and methods

Plasmids used for in vitro and in vivo expression of p50, p65, c-Rel, and IκB/MAD-3

Expression plasmids for the in vitro synthesis of human p50 and p65 proteins were the generous gift of B. Stein (University of North Carolina, Chapel Hill). Both cDNA inserts had deletions in their 5′ untranslated regions, resulting in at least a 10-fold increase in the in vitro translation product synthesis. For the p50 expression plasmid, a *Tha*I–*Xba*I fragment from a p105 expression plasmid (Kieran et al. 1990) was cloned into the *Eco*RI–*Xba*I sites in pGEM3 (Promega). p50 protein was synthesized in rabbit reticulocyte lysates by linearizing the plasmid with *Xba*I and using T7 RNA polymerase for transcription. The entire coding region of p65 was amplified by PCR using primers containing *Hin*dIII and *Xba*I sites followed by cloning into pGEM4 (Promega). RNA synthesis was carried out using T7 RNA polymerase after linearizing the plasmid with *Xba*I. The human c-Rel expression plasmid was the generous gift of W. Greene (Gladstone Institute, San Francisco, CA). In vitro transcription was carried out from the SP6 promoter. The cytomegalovirus (CMV) promoter-containing plasmid soCMIN was used for eukaryotic expression of full-length p65, p50, and the p50–p65 fusion protein (amino acids 1–371 from p50 and 310–551 from p65) as described previously (Ruben et al. 1992b). IκB/MAD-3 cDNA was cloned into the soCMIN vector as well. Epitope-tagged p65 and p65–DQNQ were constructed by PCR using a 5′ primer encoding the amino acid sequence MYPYDVPDYA corresponding to the influenza HA protein (Kolodziej and Young 1991), followed by cloning the amplified product into the plasmid soCMIN. For attachment of the SV40 NLS, a 5′ primer encoding the amino acid sequence MGPKKKRKVGGG, followed by a sequence corresponding to the HA eptitope tag, was used to amplify HA-tagged p65 and HA-tagged p65–DQNQ. The amplified product was cloned into the soCMIN vector.

Mutagenesis of p50, p65, and c-Rel

The p50 protein carboxy-terminal truncation lacking the NLS was made by a PCR-assisted approach using one primer complementary to the T7 promoter and another with complementarity extending to amino acid 362 and by using p50 expression plasmid as template. The PCR product was gel purified and used for in vitro transcription and translation. The p65 product containing the NLS was made by restriction digestion of the plasmid with *Bsp*HI, and the one lacking it was made by digestion

with *Pvu*I. Similarly, the c-Rel expression plasmid was digested with *Dde*I or *Eco*RV to make constructs containing or not containing the NLS, respectively. A schematic drawing of the different proteins is shown in Figure 1.

Mutagenesis of the NLS of p65 (KRKR to DQNQ) using PCR was carried out as described (Higuchi 1990). Amino-terminal deletions were made by using the PCR-based method described by Kain et al. (1991). First, a DNA fragment [universal promoter (UP)] containing the T7 promoter, an upstream untranslated region, and the translation start site was made by PCR as described (Kain et al. 1991). Second, p65 gene fragments were amplified using a carboxy-terminal primer with 5′-end complementarity extending to amino acid 319 of the p65 cDNA and by one of five different amino-terminal primers. The 5′ ends of these amplified p65 PCR products had 9 bp homologous to the 3′ end of the UP. Third, each of the p65 products and the UP were mixed in equimolar amounts and taken through 15 cycles of a PCR reaction without any primers, using the conditions specified in Kain et al. (1991). After the 15 cycles, a primer homologous to the T7 promoter, the carboxy-terminal p65 primer, and additional *Taq* polymerase were added, and PCR was carried out for another 30 cycles under conditions described previously (Kain et al. 1991). The PCR products were then gel purified and used for in vitro transcription and translation. The sizes of the protein products made from the different PCR products are shown in Figure 1A, constructs 6–10.

IκB/MAD-3 antibody (Ab 9)

A peptide containing IκB/MAD-3 amino acids 6–20 was injected into rabbits and serum prepared by standard procedures. Specificity of the IκB/MAD-3 antiserum was characterized by immunoprecipitation of ^{35}S-labeled IκB/MAD-3 translated in rabbit reticulocyte lysates and by Western blotting against purified IκB/MAD-3.

Protein expression

Expression of IκB/MAD-3 in *Escherichia coli* was carried out by cloning the IκB/MAD-3 cDNA into the pDS expression plasmid (Gentz et al. 1989). Expression and purification of IκB/MAD-3 were carried out by a protocol described previously (Ruben et al. 1992b). In vitro translations were carried out in rabbit reticulocyte lysates that were depleted of their endogenous NF-κB-like activity as described previously (Haskill et al. 1991).

Gel-shift assays

Gel-shift DNA-binding reactions were carried out as described previously (Haskill et al. 1991). Reactions to determine the effect of IκB/MAD-3 on the ability of different proteins to bind DNA were carried out by adding 50 ng of bacterially expressed IκB/MAD-3 to the reactions. After 10 min at room temperature, poly[d(I-C)]/[d(I-C)] and binding site DNA probe were added, and the reactions were allowed to proceed for 15 min, followed by gel electrophoresis.

Immunoprecipitation assays

IκB/MAD-3 coimmunoprecipitations were carried out as follows: To 5 μl of ^{35}S-labeled in vitro-translated proteins, either 1 μl of bacterially expressed and purified IκB/MAD-3 (10 ng) or 1 μl of buffer without IκB/MAD-3 was added, and the binding reactions were allowed to proceed for 5 min at room temperature. One microliter of IκB/MAD-3 antiserum (Ab 9) was then added to all reactions and allowed to proceed for another 10 min. The reactions were then transferred to tubes containing 20 μl of protein A–Sepharose beads (Sigma) (1 : 1 mixture) equilibrated previously in dilution buffer [10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 0.025% sodium azide, 0.1% Triton X-100] and incubated for 10 min. The beads were then washed twice with 500 μl of dilution buffer, once with 500 μl of TSA solution (dilution buffer without Triton X-100), and once with 500 μl of 50 mM Tris-HCl (pH 6.8) as described (Springer 1989). The samples were then subjected to SDS-PAGE followed by fluorography at −70°C for 12 hr to 7 days.

Coimmunoprecipitations shown in Figure 2C were carried out as described above except that 2 ng of cold NF-κB-binding site was added (as explained in the legend to Fig. 2), following addition of IκB/MAD-3 or buffer in the immunoprecipitation reactions.

Coimmunoprecipitations of p65 mutants with p50 (Fig. 4B) were carried out by cotranslating these proteins in rabbit reticulocyte lysates and then carrying out coimmunoprecipitations, as described above, using antiserum (Ab 2) against human p50 (Kieran et al. 1990). Ab 2 was a kind gift from A. Israël (Pasteur Institute, Paris, France).

Cell culture, transfections, and CAT assays

Jurkat T cells were grown to a density of 10^6 cells/ml in RPMI 1640 supplemented with 10% bovine calf serum. Approximately 2.5×10^6 cells in a volume of 0.5 μl were transfected with 10 μg of DNA using a Bio-Rad Gene Pulser at a setting of 300 V and 960 μF. The cuvette width was 0.4 cm. Expression plasmids encoding activators and IκB/MAD-3 are described above in the section on plasmids. The reporter plasmid, MHC WT CAT, consists of three tandem repeats of the class I enhancer element NF-κB site, TGGGGATTCCCCA, inserted into the *Sal*I site of Δ56CAT as described (Baldwin et al. 1991). The mutant reporter (MHC MUT CAT) had three tandem repeats of TGCGGATTCCCGA in place of the enhancer sequence (Baldwin et al. 1991). CAT activities of cell lysates were assayed by acetylation of [^{14}C]chloramphenicol (New England Nuclear) for 2 hr at 37°C, followed by extraction in ethyl acetate and TLC in 95 : 5 chloroform/methanol. Conversion of chloramphenicol to acetylated forms was measured both by autoradiography and by real-time radiation imaging using an Ambis quantitation system according to the manufacturer's instructions.

Indirect immunofluorescence of COS cells expressing NF-κB proteins

COS-7 cells were plated at a density of 1×10^5 cells per 35-mm-diam. dish 24 hr before transfection. Cells were transfected with 1 μg of plasmid DNA by the DEAE–dextran transfection procedure as described previously (Cullen 1988). At 48 hr post-transfection, cells were washed with phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde in PBS (pH 7.8) for 30 min at room temperature, washed for 5 min in PBS plus 10 mM glycine, incubated for 5 min with PBS–1% Triton, incubated with PBS plus 25 mM glycine for 30 min, washed again with PBS–glycine and, finally, blocked with 5% goat serum (Vector Laboratories) in antibody binding buffer [1% bovine serum albumin in PBS (pH 7.8), with 0.5 M NaCl and 0.05% Tween 20] for at least 1–5 hr at room temperature. After being blocked, HA monoclonal antibody was diluted 1 : 20 in antibody binding buffer and added to the cells. Cells were incubated at 4°C overnight. The following day, cells were washed twice with PBS containing 10 mM glycine for 10 min each and then incubated in the dark with rhodamine isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (diluted 1 : 100 in antibody binding buffer plus 2% goat serum) with gentle shaking at room temperature for 1

hr. Cells were subsequently washed three times with PBS plus 10 mM glycine. Coverslips were added, and the cells were viewed with a light fluorescence microscope.

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