Molecular Biology of the Cell
Vol. 3, 1339–1352, December 1992

IκB/MAD-3 Masks the Nuclear Localization Signal of NF-κB p65 and Requires the Transactivation Domain to Inhibit NF-κB p65 DNA Binding

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Submitted August 14, 1992; Accepted September 23, 1992

The active nuclear form of the NF-κB transcription factor complex is composed of two DNA binding subunits, NF-κB p65 and NF-κB p50, both of which share extensive N-terminal sequence homology with the v-*rel* oncogene product. The NF-κB p65 subunit provides the transactivation activity in this complex and serves as an intracellular receptor for a cytoplasmic inhibitor of NF-κB, termed IκB. In contrast, NF-κB p50 alone fails to stimulate κB-directed transcription, and based on prior in vitro studies, is not directly regulated by IκB. To investigate the molecular basis for the critical regulatory interaction between NF-κB and IκB/MAD-3, a series of human NF-κB p65 mutants was identified that functionally segregated DNA binding, IκB-mediated inhibition, and IκB-induced nuclear exclusion of this transcription factor. Results from in vivo expression studies performed with these NF-κB p65 mutants revealed the following: 1) IκB/MAD-3 completely inhibits NF-κB p65-dependent transcriptional activation mediated through the human immunodeficiency virus type 1 κB enhancer in human T lymphocytes, 2) the binding of IκB/MAD-3 to NF-κB p65 is sufficient to retarget NF-κB p65 from the nucleus to the cytoplasm, 3) selective deletion of the functional nuclear localization signal present in the Rel homology domain of NF-κB p65 disrupts its ability to engage IκB/MAD-3, and 4) the unique C-terminus of NF-κB p65 attenuates its own nuclear localization and contains sequences that are required for IκB-mediated inhibition of NF-κB p65 DNA binding activity. Together, these findings suggest that the nuclear localization signal and transactivation domain of NF-κB p65 constitute a bipartite system that is critically involved in the inhibitory function of IκB/MAD-3. Unexpectedly, our in vivo studies also demonstrate that IκB/MAD-3 binds directly to NF-κB p50. This interaction is functional as it leads to retargeting of NF-κB p50 from the nucleus to the cytoplasm. However, no loss of DNA binding activity is observed, presumably reflecting the unique C-terminal domain that is distinct from that present in NF-κB p65.

INTRODUCTION

During the development of a normal immune response, the clonal expansion of activated $CD4^+$ T lymphocytes is tightly controlled by the transient transcriptional induction of specific cellular genes, including those encoding the growth factor interleukin-2 and the alpha subunit of its high-affinity membrane receptor complex (Crabtree, 1989; Greene *et al.*, 1989). Both of these growth-related genes contain promoter-proximal regulatory sequences that serve as functional binding sites for NF-κB (Böhnlein *et al.*, 1988; Hoyos *et al.*, 1989), a heterodimeric transcription factor that is rapidly translocated from the cytoplasm to the nucleus during T-cell

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activation (Sen and Baltimore, 1986a,b; Lenardo and Baltimore, 1989). Like these inducible cellular transcription units, the 5′ long terminal repeat (LTR)[1] of the type 1 human immunodeficiency virus (HIV-1) contains two tandem NF-κB binding sites that have been implicated in the capacity of this pathogenic retrovirus to respond to a variety of immunomodulatory signals that normally lead to T-cell activation (Kaufman *et al.*, 1987; Muesing *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1987). Thus, NF-κB represents one critical host transcription factor involved not only in normal T-cell activation and growth but also in governing the initial activation of HIV-1 gene expression in virally-infected $CD4^+$ T lymphocytes (Greene *et al.*, 1989).

Biochemical (Baeuerle and Baltimore, 1989) and molecular cloning (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991; Ballard *et al.*, 1992) studies indicate that the heterodimeric NF-κB complex found in the nucleus of activated T cells is comprised of at least two DNA binding subunits, termed NF-κB p65 and NF-κB p50. Both of these subunits share significant N-terminal amino acid sequence homology (~300 amino acids) with the v-Rel oncoprotein, its cellular counterpart (c-Rel), and the *dorsal* gene product, a maternal morphogen in *Drosophila* (Gilmore, 1990, and references therein). Notably, the NF-κB p65 subunit exhibits two unique biologic activities that are integral to the function and regulation of the NF-κB complex. First, NF-κB p65, unlike NF-κB p50, contains a strong C-terminal transactivation domain that is essential for efficient transcription directed from the HIV-1 κB enhancer (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992; Ruben *et al.*, 1992b). Second, this subunit acts as an intracellular receptor for IκB, a specific cytoplasmic inhibitor that appears critically involved in the regulation of NF-κB shuttling from the cytoplasm to the nucleus during T-cell activation (Baeuerle and Baltimore, 1988a,b; Molitor *et al.*, 1990; Urban and Baeuerle, 1990). In contrast to the positive transcriptional effects exerted by NF-κB p65, comparative studies with NF-κB p50 have suggested that it primarily serves to augment the DNA binding affinity of the heterodimeric NF-κB p65/p50 complex (Baeuerle and Baltimore, 1989; Nolan *et al.*, 1991). It also has been reported that the homodimeric form of NF-κB p50 acts as a physiologic repressor of κB-dependent transcription (Schmitz and Baeuerle, 1991; Kang *et al.*, 1992). Furthermore, despite its extensive N-terminal sequence homology with NF-κB p65, the DNA binding activity of NF-κB p50 is not subject to IκB-mediated inhibition in vitro (Baeuerle and Baltimore, 1989; Zabel and Baeuerle, 1990; Nolan *et al.*, 1991; Inoue *et al.*, 1992b). Instead, it appears that NF-κB p50 is expressed as an inactive 110-kDa precursor polypeptide whose DNA binding potential is regulated via proteolytic processing to remove an inhibitory C-terminal domain (Ghosh *et al.*, 1990; Kieran *et al.*, 1990).

Although the transactivation and IκB binding properties of NF-κB p65 are clearly distinct from those of NF-κB p50, the molecular mechanism underlying IκB-mediated inhibition of NF-κB function specifically through the NF-κB p65 subunit (Urban and Baeuerle, 1990) remains unknown. In this regard, long-range deletion mapping studies with in vitro synthesized proteins suggest that the N-terminal Rel homology domain of NF-κB p65 contains structural determinants essential for its regulation by IκB (Nolan *et al.*, 1991). To define precisely this critical regulatory interaction in a more physiologic setting, we have analyzed the biochemical properties of a series of in vivo synthesized NF-κB p65 variants after transfection of the corresponding cDNA expression vectors in monkey COS cells. These studies clearly demonstrate that NF-κB p65 contains two domains that function in concert to mediate assembly with IκB/MAD-3, resulting in the inhibition of NF-κB p65 DNA binding activity. Specifically, one major subregion of NF-κB p65 critically involved in IκB/MAD-3 binding corresponds to the functional nuclear localization signal (NLS) within the Rel homology domain. In addition, the C-terminal transactivation domain of NF-κB p65 is required for IκB-mediated inhibition of DNA binding but is completely dispensible for the assembly of NF-κB p65 with IκB/MAD-3. Surprisingly, IκB/MAD-3 also binds to and regulates the subcellular distribution of the structurally related NF-κB p50 subunit in the absence of NF-κB p65. Together, these findings suggest that IκB/MAD-3 prevents the nuclear translocation of NF-κB by directly masking the NLS of either of these integral Rel-related subunits. However, the selective inhibition of NF-κB p65 DNA binding occurs through a more complex interaction involving the unique C-terminus of this transactivator.

MATERIALS AND METHODS

Expression Vectors and Reporter Plasmids

All human NF-κB p65 (Ruben *et al.*, 1991; Ballard *et al.*, 1992), NF-κB p50 (amino acids 1-461; Kieran *et al.*, 1990), and IκB/MAD-3 (Haskill *et al.*, 1991) cDNAs were subcloned within the polylinker sequences of the pCMV4 expression vector (Andersson *et al.*, 1989) downstream of the cytomegalovirus immediate early promoter. Carboxy- and amino-terminal deletion mutants were amplified using the polymerase chain reaction from a template corresponding to the full-length human NF-κB p65 cDNA (Ruben *et al.*, 1991). Oligonucleotide primers used in these reactions were designed to incorporate a consensus translational initiation sequence (Kozak, 1987), an in-frame stop codon, and unique restriction sites to facilitate directional cloning into pCMV4. Specific site-directed mutations were introduced into the full-length NF-κB p65 cDNA using the phosphorothioate method and oligonu-

[1] Abbreviations used: CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; FCS, fetal calf serum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T cell leukemia virus type 1; LTR, long terminal repeat; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKA, protein kinase A; SDS, sodium dodecyl sulfate.

cleotide primers that introduced diagnostic restriction sites (Nakamaye and Eckstein, 1986). The Tax expression vector has been described previously (Rimsky *et al.*, 1988). Reporter plasmids contained either the full-length type 1 human T-cell leukemia virus (HTLV-1) LTR (Sodroski *et al.*, 1985) or the HIV-1 κB enhancer cassette linked to a heterologous TATA box (Stein *et al.*, 1989), cloned immediately upstream of the chloramphenicol acetyltransferase (CAT) reporter gene.

COS Cell Transfections and Extract Preparations

The COS monkey kidney cell line was maintained in Iscove's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics. One day before transfection, cells were seeded in 0.1% gelatin-treated six-well plates (3×10^5 cells/well). Plasmid DNA was transfected using the DEAE-dextran method (Holbrook *et al.*, 1987). Briefly, adherent cells were washed in phosphate-buffered saline (PBS), incubated with 0.5 ml of DNA cocktail (0.5 mg/ml DEAE-dextran and 2.5 μg/ml plasmid DNA in PBS) for 30 min at 37°C, followed by the addition of 4.5 ml of Iscoves medium containing 100 μM chloroquine. Cells were then incubated for an additional 2.5 h at 37°C. The medium was aspirated, and the cells were treated with 10% dimethyl sulfoxide for 2.5 min at room temperature. At 48–60 h after transfection, some cells were starved for 1 h in Dulbecco's modified Eagle medium lacking cysteine and methionine and then radiolabeled for 2 h with [^{35}S]cysteine and [^{35}S]methionine (150 μCi/ml each). Whole-cell extracts were prepared under low-stringency conditions by lysis with ELB buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM dithiothreitol [DTT], 1.0 mM phenylmethanesulfonyl fluoride) supplemented with a previously described protease inhibitor cocktail (Ballard *et al.*, 1990). After a 10-min incubation at 4°C, lysed cells were centrifuged at 37 000 × *g* for 15 min. Clarified supernatants were frozen in liquid nitrogen and stored at −70°C until used.

DNA Binding Studies

^{32}P-radiolabeled κB DNA probes were prepared by annealing a 27-base coding strand template (5'-CAACGGCAGGGGAATTCCCCTCTCCTT-3', κB-pd) to a complementary 10-base primer (5'-AAGGAGAGGG-3') and filling in the resultant overhang using the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP, [α-^{32}P]dCTP, dGTP, and dTTP (Ballard *et al.*, 1989). DNA binding reactions (20 μl) contained 4 μl of COS cell extract (~0.5 mg/ml) and 0.2 μg of double-stranded poly(dI-dC) buffered in 20 mM HEPES, pH 7.9, 5% glycerol, 1 mM EDTA, 0.5 mg/ml bovine serum albumin, 1% Nonidet P-40, and 5 mM DTT (Ballard *et al.*, 1989). Approximately 10 fmol of ^{32}P-labeled probe (200 000 cpm) was used per reaction. After incubating the reactions at room temperature for 25 min, the resultant nucleoprotein complexes were resolved on a native 5% polyacrylamide gel as previously described (Böhnlein *et al.*, 1988).

Immunoprecipitation Analysis

Rabbit antisera were prepared (Immunodynamics, La Jolla, CA) against carrier-conjugated peptides corresponding to unique terminal sequences present in NF-κB p65 (amino acids 1–21 and amino acids 535–551) (Ruben *et al.*, 1991), NF-κB p50 (amino acids 1–21) (Kieran *et al.*, 1990), and IκB/MAD-3 (amino acids 1–29) (Haskill *et al.*, 1991). For immunoprecipitation studies, 1 μl of the appropriate antipeptide antiserum was mixed with 100 μl of precleared ^{35}S-labeled COS extract and 400 μl of ELB buffer. Antibody binding reactions were incubated for 1 h at 4°C and then supplemented with 10 μl (packed volume) of protein A-agarose (Boehringer Mannheim, Indianapolis, IN). Solid-phase immune complexes were washed three times with 500 μl of ice-cold ELB buffer, boiled in the presence of 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 8 M urea, and fractionated on 10% SDS-polyacrylamide gels. Radiolabeled polypeptides were visualized by fluorography in the presence of Amplify (Amersham, Arlington Heights, IL).

Western Blot Analysis

Protein samples (~3.5 μg) were separated on 8.75% polyacrylamide gels followed by electrotransfer onto nitrocellulose membranes. Membranes were incubated in 5% dry milk in PBS/0.1% Tween 20 to inhibit nonspecific protein binding and then sequentially incubated with an appropriate dilution of specific rabbit antiserum (1 h) and horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham; 1:2500 dilution in PBS/0.1% Tween 20). Membranes were washed extensively with PBST after each of the incubation steps. Specific immune complexes were detected by enhanced chemiluminescence as specified by the manufacturer (ECL Western Blotting Kit; Amersham).

Indirect Immunofluorescence

COS cells were seeded on four-well chamber slides (Nunc, Naperville, IL) and transfected using the DEAE-dextran method. After 48 h, the cells were rinsed once with PBS and fixed for 20 min in 3% paraformaldehyde/10% FCS in PBS. After rinsing twice with PBS, the cells were incubated with the primary rabbit antiserum (diluted in PBS/10% FCS) for 1 h at room temperature, followed by three 5-min washes with PBS. The cells were then incubated for 1 h with the Texas red-conjugated donkey anti-rabbit IgG (Amersham; 1:500 dilution in PBS/10% FCS). After washing four times in PBS, the slides were mounted in Gel/Mount (Biomeda, Foster City, CA) and photographed using an immunofluorescent microscope (Diaplan; Leitz, Wetzlar, W. Germany).

Jurkat T-Cell Transfections and CAT Assays

The human T-cell line, Jurkat, was maintained in RPMI supplemented with 10% FCS, 2 mM glutamine, and antibiotics. Plasmid DNA was introduced into cells using DEAE-dextran (Holbrook *et al.*, 1987). Jurkat cells (5×10^6) were incubated in 250 μl of a 0.5 mg/ml DEAE-dextran solution containing 10 μg of plasmid DNA for 30 min at 37°C. Subsequently, 5 ml of RPMI containing 100 μM chloroquine was added, and the incubation continued for another 30 min at 37°C. Cell extracts were normalized for protein recovery (Bradford, 1976) and assayed for CAT activity 48 h posttransfection using the diffusion-based liquid scintillation counting method described by Neumann *et al.* (1987).

RESULTS

NF-κB p65-Mediated Activation of the HIV-1 κB Enhancer in Human T Lymphocytes is Inhibited by IκB/MAD-3

Biochemical reconstitution studies have suggested that the NF-κB p65/p50 heterodimer is functionally sequestered as a latent complex in the cytoplasm of resting T lymphocytes via specific protein–protein interactions between IκB and as yet undefined sequences within the N-terminal Rel homology domain of NF-κB p65 (Figure 1A) (Baeuerle and Baltimore, 1989; Molitor *et al.*, 1990; Urban and Baeuerle, 1990; Nolan *et al.*, 1991). Based on in vitro assays, the cytoplasmic form of NF-κB is likely released from IκB after phosphorylation of the inhibitor, thereby allowing the rapid translocation of NF-κB p65 to its nuclear site of action (Ghosh and Baltimore, 1990; Kerr *et al.*, 1991). Recently, Haskill *et al.* (1991) have isolated a human cDNA clone (IκB/MAD-3) encoding a 34- to 37-kDa polypeptide that inhibits

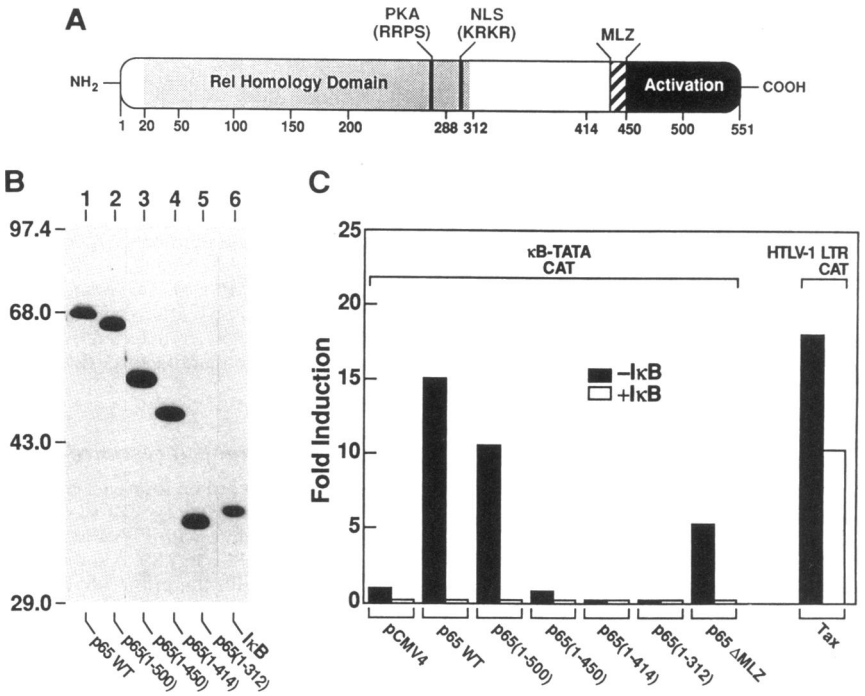

Figure 1. The transactivation function of NF-κB p65 is regulated by IκB/MAD-3 in vivo. (A) Primary domain structure of NF-κB p65. The N-terminal Rel homology domain (shaded) of NF-κB p65, which contains two highly conserved motifs, a basic tetrapeptide nuclear localization signal (NLS/KRKR), and a protein kinase A phosphorylation consensus sequence (PKA/RRPS), is shown in conjunction with the C-terminal transactivation domain (black) and the "mini-leucine zipper" motif (MLZ; hatched). The endpoints of specific N- and C-terminal NF-κB p65 truncation mutants used throughout this study are indicated. (B) Expression of C-terminally truncated NF-κB p65 mutants. Monkey COS cells were transfected using DEAE-dextran with cDNA expression vectors encoding either wild-type IκB/MAD-3 (lane 6), wild-type NF-κB p65 (lane 1), or C-terminal truncation mutants of NF-κB p65 containing the indicated N- and C-terminal endpoints (lanes 2–5). Recipient cells were metabolically labeled with [^{35}S]cysteine at 48 h posttransfection, and the corresponding extracts subjected to immunoprecipitation with polyclonal antipeptide antiserum specific for either NF-κB p65 (amino acids 1–21; lanes 1–5) or IκB/MAD-3 (amino acids 1–29; lane 6). (C) IκB/MAD-3 specifically inhibits NF-κB p65-dependent transactivation. Human Jurkat T cells were cotransfected with the indicated wild-type and truncated NF-κB p65 expression vectors, along with a CAT reporter plasmid containing the HIV-1 κB enhancer (κB-TATA-CAT) in the presence (□) or absence (■) of an IκB/MAD-3 expression vector. In control transfections (right), Jurkat cells were cotransfected with an HTLV-1 Tax expression vector and a CAT reporter plasmid linked to the full-length HTLV-1 LTR. CAT activities were measured after 48 h of culture and are presented as the relative fold-induction over basal levels obtained with unmodified pCMV4 expression vector alone.

the DNA binding activity of NF-κB in vitro. The predicted primary structure of this human inhibitor protein is also fully consistent with the partial amino acid sequence determined for purified rabbit IκB (Davis *et al.*, 1991). However, the intracellular biologic effects of IκB/MAD-3 on NF-κB-dependent transcription remain unexamined.

Transient transfection studies have revealed that NF-κB p65, but not NF-κB p50, contains unique C-terminal sequences (amino acids 451–551) (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992; Ruben *et al.*, 1992b) that are critical for activation of κB-directed transcription (Figure 1A). To investigate whether IκB/MAD-3 specifically regulates the transactivation function of NF-κB p65 in vivo, a series of C-terminally truncated cDNAs were constructed that encode stable NF-κB p65 variants lacking progressively larger portions of the C-terminal transactivation domain (Figure 1B). Jurkat human T cells were then cotransfected with recombinant expression vectors containing these truncated cDNAs and an HIV-1 κB-CAT reporter plasmid (κB-TATA-CAT) in the presence or absence of an expression vector encoding the 37-kDa human IκB/MAD-3 protein (Figure 1B). As shown in Figure 1C, coexpression of κB-TATA-CAT with full-length NF-κB p65 (p65 WT) potently stimulated κB-directed transcription (~15-fold) relative to the levels observed with unmodified pCMV4 expression vector. As expected (Ballard *et al.*, 1992), deletion of the C-terminal 101 amino acids (amino acids 451–551) of NF-κB p65 disrupted the transcriptional activity observed with the wild-type NF-κB p65 protein. More importantly, coexpression of IκB/MAD-3 with functionally active members of this deletion series completely abolished NF-κB p65-mediated transcription. In addition, specific deletion of a "mini-leucine zipper" motif located immediately adjacent to the primary transactivation domain (see Figure 1A), which is required for optimal NF-κB p65 activity (Ruben *et al.*, 1992b), did not sig-

nificantly affect the ability of IκB/MAD-3 to inhibit NF-κB p65-induced transcription (Figure 1C). In contrast, IκB/MAD-3 failed to significantly block Tax-mediated transcriptional activation of the HTLV-1 LTR, a response that proceeds independently of the NF-κB/Rel transcription factor pathway (Paskalis *et al.*, 1986; Shimotohno *et al.*, 1986; Brady *et al.*, 1987; Smith and Greene, 1990). These studies thus demonstrate that IκB/MAD-3 functions as a potent inhibitor of NF-κB p65 in vivo, presumably by a mechanism involving the proposed ability of IκB/MAD-3 to retain NF-κB in the cytoplasm (Baeuerle and Baltimore, 1988a,b).

IκB/MAD-3 Regulates the Nuclear Expression of Both NF-κB p65 and NF-κB p50 but Blocks the DNA Binding Activity of Only NF-κB p65

The previous finding that the Rel homology domain of NF-κB p65 is involved in its interaction with IκB (Nolan *et al.*, 1991) raised the possibility that homologous sequences present in the N-terminal half of NF-κB p50 might also interact with IκB. To determine the relative effects of IκB/MAD-3 on the DNA binding activity of NF-κB p65 and NF-κB p50, extracts from COS cells overexpressing each of these subunits, either in the absence or presence of IκB/MAD-3, were tested in gel retardation assays using a ^{32}P-labeled κB enhancer probe. As shown in Figure 2A, in vivo synthesized forms of both subunits bound to this κB enhancer probe in the absence of IκB/MAD-3, leading to the formation of distinct nucleoprotein complexes (lanes 1 and 3). Whereas coexpression of IκB/MAD-3 with NF-κB p65 completely blocked NF-κB p65 DNA binding activity (lane 2), coexpression of IκB/MAD-3 with NF-κB p50 failed to inhibit the formation of NF-κB p50/κB enhancer complexes (lane 4). These in vivo data are thus fully consistent with previous NF-κB DNA binding studies performed using in vitro synthesized proteins (Baeuerle and Baltimore, 1989; Zabel and Baeuerle, 1990; Nolan *et al.*, 1991; Inoue *et al.*, 1992b). These findings indicate that NF-κB p65 contains sequences required for IκB-mediated inhibition of DNA binding that are not found in NF-κB p50. However, these findings do not exclude the possibility that IκB/MAD-3 binds to NF-κB p50, even though it fails to block its DNA binding activity.

To directly compare the relative IκB/MAD-3 binding capacities of NF-κB p65 and NF-κB p50, coimmunoprecipitation studies were performed with subunit-specific antipeptide antisera using ^{35}S-labeled extracts from transfected COS cells. As shown in Figure 2B, the NF-κB p65-specific antiserum immunoprecipitated a major 65- to 72-kDa polypeptide in extracts from COS cells transfected with the wild-type NF-κB p65 expression vector (lane 1). Coexpression of IκB/MAD-3 with NF-κB p65 in these recipient cells resulted in the detection of a second major polypeptide of 35–40 kDa (lane 2), which is consistent with the molecular weight reported

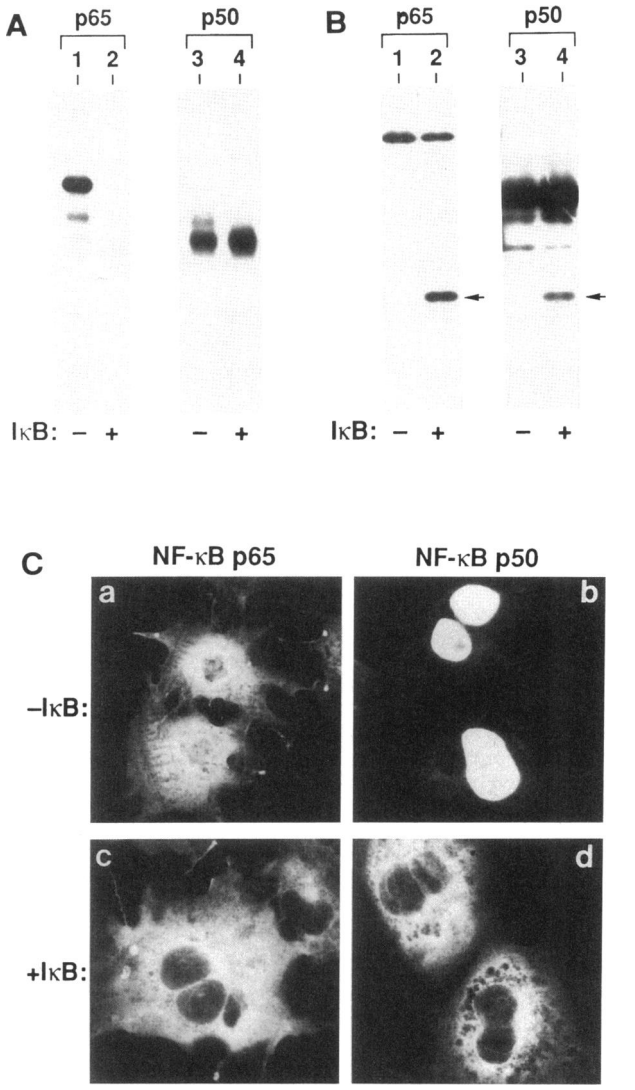

Figure 2. IκB/MAD-3 regulates both NF-κB p65 and NF-κB p50 in vivo. (A) IκB/MAD-3 selectively inhibits the DNA binding activity of NF-κB p65 but not that of NF-κB p50. COS cells were transfected with cDNA expression vectors encoding wild-type NF-κB p65 (lanes 1 and 2) or wild-type NF-κB p50 (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of an IκB/MAD-3 expression vector. The DNA binding activities of recipient cell extracts were analyzed by gel retardation assay using a ^{32}P-radiolabeled κB enhancer probe, and the resultant nucleoprotein complexes were visualized by autoradiography. (B) IκB/MAD-3 forms a stable complex with both NF-κB p65 and NF-κB p50. ^{35}S-labeled extracts from COS cells expressing NF-κB p65 (lanes 1 and 2) or NF-κB p50 (lanes 3 and 4) either alone (lanes 1 and 3) or in combination with IκB/MAD-3 (lanes 2 and 4) were subjected to immunoprecipitation analysis with subunit-specific antisera. Immunoreactive complexes were fractionated on a 10% SDS-polyacrylamide gel and detected by fluorography. The arrow indicates the position of IκB/MAD-3. (C) Assembly with IκB/MAD-3 prevents the nuclear import of both NF-κB p65 and NF-κB p50. COS cells were transfected with expression vectors encoding the indicated NF-κB subunit in the absence (a and b) or presence (c and d) of an IκB/MAD-3 expression vector. Transfected cells were fixed, treated with rabbit antiserum specific for each subunit, and subjected to indirect immunofluorescence staining using Texas red-conjugated anti-rabbit Ig.

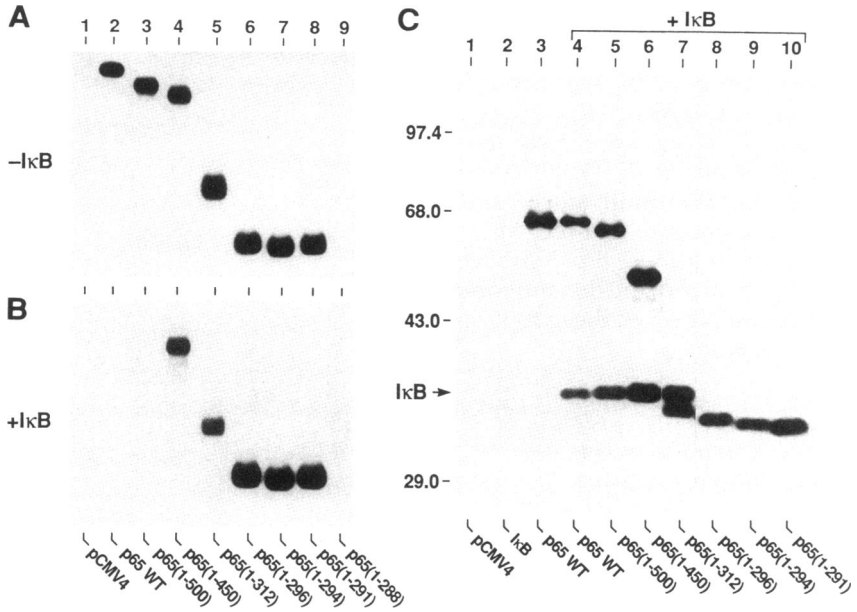

Figure 3. The N-terminal Rel homology domain and the unique C-terminus of NF-κB p65 act in concert to mediate a fully functional interaction with IκB/MAD-3. (A and B) The C-terminal transactivation domain of NF-κB p65 is required for IκB-mediated inhibition of DNA binding. Monkey COS cells were transfected with pCMV4 expression vectors encoding the indicated C-terminal truncation mutants (lanes 3–9) in the absence (A) or presence (B) of an IκB/MAD-3 expression vector. Control transfections performed with unmodified vector (pCMV4) and full-length NF-κB p65 (p65 WT) are shown in lanes 1 and 2, respectively. Extracts from recipient cells were incubated with a ^{32}P-labeled κB enhancer probe, and the resultant complexes were resolved on a 5% polyacrylamide gel and visualized by autoradiography. (C) The C-terminus of NF-κB p65 is dispensible for IκB/MAD-3 binding. ^{35}S-labeled extracts from COS cells expressing either unmodified pCMV4 (lane 1), IκB/MAD-3 (lane 2), wild-type NF-κB p65 (lane 3), or the indicated NF-κB p65 constructs in combination with IκB/MAD-3 (lanes 4–10) were subjected to immunoprecipitation analysis using an NF-κB p65-specific antipeptide antiserum (amino acids 1–21). Radiolabeled immune complexes were resolved by electrophoresis on a 10% SDS-polyacrylamide gel and detected by fluorography. The relative positions and sizes (in kilodaltons) of molecular weight markers are given on the left. The arrow identifies the position of IκB/MAD-3.

for human IκB/MAD-3 (Haskill *et al.*, 1991). This protein was also readily detected with anti-MAD-3 antiserum (Ganchi *et al.*, unpublished results). In reciprocal antibody binding studies with extracts from NF-κB p50-transfected COS cells, the NF-κB p50-specific antiserum immunoprecipitated a 48- to 55-kDa polypeptide (lane 3), consistent with the predicted size of this Rel-related subunit. Surprisingly, despite the inability of IκB/MAD-3 to block NF-κB p50 DNA binding activity (Figure 2A, lane 4), IκB/MAD-3 was readily detected in immunoprecipitates from COS cells coexpressing these polypeptides (Figure 2B, lane 4). These findings thus demonstrate the unexpected capacity of IκB/MAD-3 and NF-κB p50 to form stable complexes in vivo.

Studies were next performed to examine whether IκB/MAD-3 modulated the subcellular distribution of NF-κB p50 and NF-κB p65 (Figure 2C). Immunofluorescent staining of COS cells expressing NF-κB p65 with specific anti-NF-κB p65 antiserum revealed a diffuse, predominantly perinuclear, or whole-cell pattern of fluorescence (Figure 2C,a). In contrast, coexpression of IκB/MAD-3 with NF-κB p65 yielded a nuclear-excluded distribution of NF-κB p65 (Figure 2C,c). When NF-κB p50-expressing COS cells were probed with an NF-κB p50-specific antiserum, a primarily nuclear pattern of expression was observed (Figure 2C,b). However, consistent with the capacity of NF-κB p50 to engage IκB/MAD-3, this immunoreactive species was completely retargeted to the cytoplasm after coexpression with IκB/MAD-3 (Figure 2C,d). These results demonstrate that IκB/MAD-3 is capable of regulating the subcellular localization of the homodimeric forms of both NF-κB p65 and NF-κB p50.

The Transactivation Domain of NF-κB p65 is Selectively Required for IκB-Mediated Inhibition of DNA Binding but not for IκB/MAD-3 Binding

To determine the NF-κB p65 sequences required for IκB-mediated inhibition of DNA binding activity, the C-terminal deletion series of NF-κB p65 mutants were examined for DNA binding (Figure 3A), inhibition of binding by IκB/MAD-3 (Figure 3B), and stable association with IκB/MAD-3 (Figure 3C) after transfection in COS cells. In gel retardation assays, extracts from COS cells transfected with the wild-type NF-κB p65 cDNA supported the formation of a single nucleoprotein complex (Figure 3A, lane 2) that was absent in DNA-binding reactions performed using control extracts prepared from cells transfected with the parental pCMV4 expression vector (lane 1). Deletion of the unique C-terminal

non-Rel sequences of NF-κB p65, which encompass the principal transactivation domain (see Figure 1A) (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992), modestly enhanced the overall DNA binding activity of NF-κB p65 (Figure 3, lanes 5–8). These results are consistent with the earlier observations of Nolan *et al.* (1991), suggesting that these C-terminal sequences intrinsically attenuate the DNA binding activity of NF-κB p65. Deletion to amino acid 291, which removes a basic stretch of residues (KRKR; amino acids 301–304; see Figure 1A) resembling the functional nuclear localization signal present in NF-κB p50 (Henkel *et al.*, 1992), also had no negative effect on DNA binding (lane 8). However, removal of three additional amino acids to residue 288 completely disrupted DNA binding activity (lane 9), thus mapping the C-terminal boundary of the NF-κB p65 DNA binding domain to between amino acids 289 and 291 within the Rel homology domain.

In parallel experiments, this series of NF-κB p65 C-terminal deletion mutants was coexpressed in COS cells with an equivalent amount of an IκB/MAD-3 expression vector. Results from gel retardation studies performed with extracts from these cotransfected cells are shown in Figure 3B. Consistent with prior in vitro reconstitution studies (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991), coexpression of wild-type NF-κB p65 with IκB/MAD-3 in vivo completely blocked κB enhancer binding activity (lane 2). Similar inhibitory effects were observed with an NF-κB p65 mutant lacking the C-terminal 51 amino acids (lane 3). However, removal of all of the primary NF-κB p65 transactivation domain (amino acids 451–551; see Figure 1A) (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992) yielded an NF-κB p65 mutant that was entirely refractory to IκB-mediated inhibition (lane 4). Similarly, NF-κB p65 mutants terminated between amino acids 291 and 312 (lanes 5–8) retained DNA binding activity when coexpressed with an equivalent amount of the IκB/MAD-3 expression vector. Subsequent titration experiments performed with the latter NF-κB truncation mutant revealed significant inhibitory effects on DNA binding activity only at higher (at least 4:1 vector ratio) doses of IκB/MAD-3 (Ganchi *et al.*, unpublished results). These surprising results suggest that the C-terminal activation domain of NF-κB p65 is required for IκB-mediated inhibition of NF-κB p65 DNA binding.

The precise subregion(s) within NF-κB p65 required for IκB/MAD-3 binding in vivo remains unknown. To determine these requisite sequences, COS cells were cotransfected with expression vectors encoding IκB/MAD-3 and each of the NF-κB p65 C-terminal deletion mutants. These cell were then metabolically radiolabeled and subjected to immunoprecipitation analysis with specific antipeptide antiserum raised against the N-terminus of NF-κB p65. As shown in Figure 3C, this antiserum specifically immunoprecipitated a 65- to 72-kDa radiolabeled polypeptide from COS cells transfected with the NF-κB p65 expression vector (lane 3) but not from COS cells transfected with an expression vector encoding IκB/MAD-3 (lane 2) or lacking a cDNA insert (lane 1). When the IκB/MAD-3 cDNA was coexpressed with NF-κB p65, a 35- to 40-kDa radiolabeled polypeptide was readily coimmunoprecipitated from recipient cell extracts (lane 4), indicating the formation of stable NF-κB p65/IκB complexes in vivo. However, removal of the C-terminal activation domain sequences, which were required for IκB-mediated inhibition of DNA binding, had no effect on the interaction of IκB/MAD-3 with NF-κB p65 (lanes 5 and 6). Further deletion to residue 312 was also without effect (lane 7), but deletion to amino acid 296 or beyond, which disrupts the putative nuclear localization signal, yielded NF-κB p65 variants that failed to complex with IκB/MAD-3 (lanes 8–10). These coimmunoprecipitation studies thus establish that the C-terminal boundary required for IκB/MAD-3 binding lies between amino acids 297 and 312, which is different from the C-terminal boundary for DNA binding (amino acids 289–291). These results further demonstrate that the transactivation domain of NF-κB p65, which is required for IκB-mediated inhibition of DNA binding, is not itself an essential part of the IκB/MAD-3 binding site on NF-κB p65.

The Most N-terminal Residues of the Rel Homology Domain of NF-κB p65 are Required for DNA Binding but not for Association with IκB/MAD-3

To complement these C-terminal deletion mapping studies, reciprocal experiments were performed with a series of N-terminally truncated NF-κB p65 mutants. To optimize translation, each of these NF-κB p65 cDNA mutants was prepared using the polymerase chain reaction and matched 5′ oligonucleotide primers that incorporated a consensus translational initiation sequence (Kozak, 1987). Whole-cell extracts prepared from COS cells transfected with these mutant NF-κB p65 constructs were subsequently tested in gel retardation assays (Figure 4A). In control reactions, a single specific nucleoprotein complex was detected with extracts containing full-length NF-κB p65 (lane 2) but not with extracts from mock-transfected cells (lane 1). This DNA binding activity persisted after deletion of up to 20 N-terminal amino acids (lanes 3 and 4), which affects NF-κB p65 sequences that are divergent from NF-κB p50 (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991). However, removal of 10 or more additional residues, which involves the proximal boundary of the NF-κB p65 Rel homology domain, completely abolished this DNA binding activity (lanes 5–7). As shown in Figure 4B, the DNA binding activity of each of the active NF-κB p65 complexes was completely inhibited by coexpression with equivalent amounts of IκB/MAD-3 (Figure 4B; lanes 2–4). These findings indicate that, in

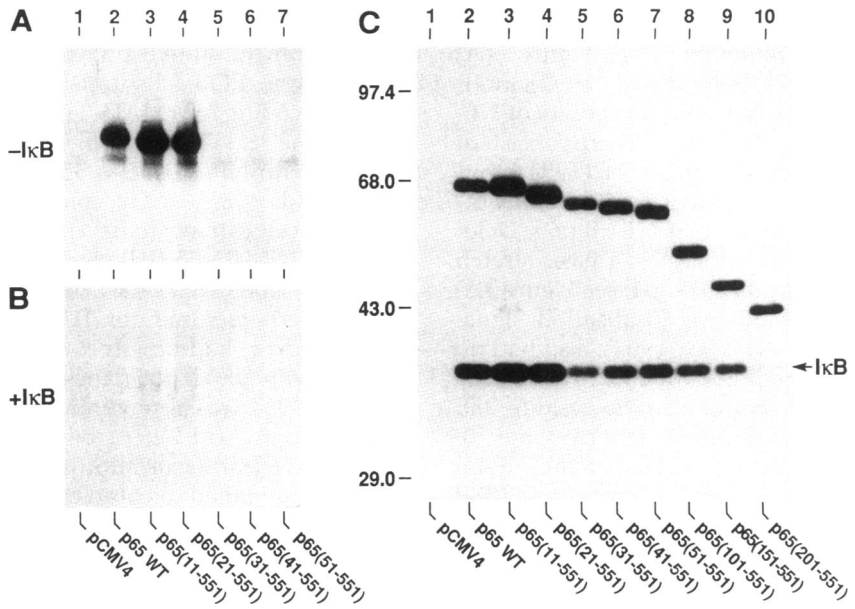

Figure 4. The N-terminal boundaries of both the NF-κB p65 DNA binding domain and IκB/MAD-3 binding domain are distinct. (A and B) The most N-terminal sequences of the Rel homology domain of NF-κB p65 are required for DNA binding. COS cells were transfected with the indicated NF-κB p65 expression vectors (lanes 2–7) in the absence (A) or presence (B) of an IκB/MAD-3 expression vector. Control transfections were performed with unmodified pCMV4 (lane 1) and full-length NF-κB p65 (p65 WT; lane 2). Recipient cell extracts were incubated with a ^{32}P-labeled palindromic κB enhancer probe, and the resultant nucleoprotein complexes were resolved on a 5% polyacrylamide gel. (C) The N-terminal half of the Rel homology domain of NF-κB p65 is dispensible for association with IκB/MAD-3. Extracts from COS cells coexpressing IκB/MAD-3 together with the parental pCMV4 expression vector (lane 1) or the indicated NF-κB p65 variants (lanes 2–10) were subjected to immunoprecipitation analysis using an antipeptide antiserum raised against the C-terminus of NF-κB p65 (amino acids 535–551). Immune complexes were subjected to reducing SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography. The relative positions and sizes (in kilodaltons) of molecular weight standards are as shown. The arrow indicates IκB/MAD-3.

contrast to the C-terminus of NF-κB p65, virtually the entire N-terminus of NF-κB p65 is required for DNA binding.

To delineate the N-terminal boundary of the IκB/MAD-3 binding domain present in NF-κB p65, a more extensive series of N-terminal truncation mutants was cotransfected into COS cells with equivalent amounts of the IκB/MAD-3 expression vector. Radiolabeled extracts from these cells were then prepared and subjected to immunoprecipitation analysis with an antiserum specific for the C-terminus of NF-κB p65. As shown in Figure 4C, IκB/MAD-3 was readily immunoprecipitated with this antiserum when coexpressed with the full-length NF-κB p65 protein (lane 2). Surprisingly, deletion of up to 150 N-terminal residues did not affect the formation of a stable NF-κB p65/IκB complex (lanes 3–9). However, deletion of 50 additional N-terminal amino acids completely abolished its ability to engage IκB/MAD-3 (lane 10). These deletion mapping results thus localize the N-terminal boundary of the NF-κB p65 receptor domain for IκB/MAD-3 to between amino acids 151 and 200. Therefore, in contrast to the DNA binding domain (amino acids 21–291), which spans virtually the entire Rel homology region, the IκB/MAD-3 binding domain (amino acids 151–312) encompasses only the distal half of this region.

The NLS of NF-κB p65 is a Primary Target for IκB/MAD-3 Binding

Inspection of the IκB/MAD-3 receptor domain within NF-κB p65 was remarkable for the presence of a basic subregion (KRKR; amino acids 301–304; see Figure 1A) within the Rel homology domain that is homologous to the functional NLS present at comparable positions in NF-κB p50 (RKRQK; Henkel *et al.*, 1992) and v-Rel (KRQR; Gilmore and Temin, 1988). To explore the potential role of this NF-κB p65 motif in IκB-mediated inhibition, IκB/MAD-3 binding, and nuclear translocation, site-directed mutagenesis was used to selectively delete these four codons from the wild-type NF-κB p65 cDNA. Monkey COS cells were transfected with the resultant cDNA expression vector (p65 ΔNLS) along with increasing doses of the human IκB/MAD-3 expression vector. Extracts from these COS cell transfectants were normalized for equivalent protein expression and subjected to gel retardation analysis using a ^{32}P-labeled κB enhancer probe (Figure 5A). In the absence of IκB/MAD-3, p65 ΔNLS (lane 6) appeared to bind DNA with increased affinity relative to the wild-type NF-κB p65 protein (lane 1). As expected, titration with increasing amounts of the IκB/MAD-3 expression vector sharply attenuated the DNA binding activity of

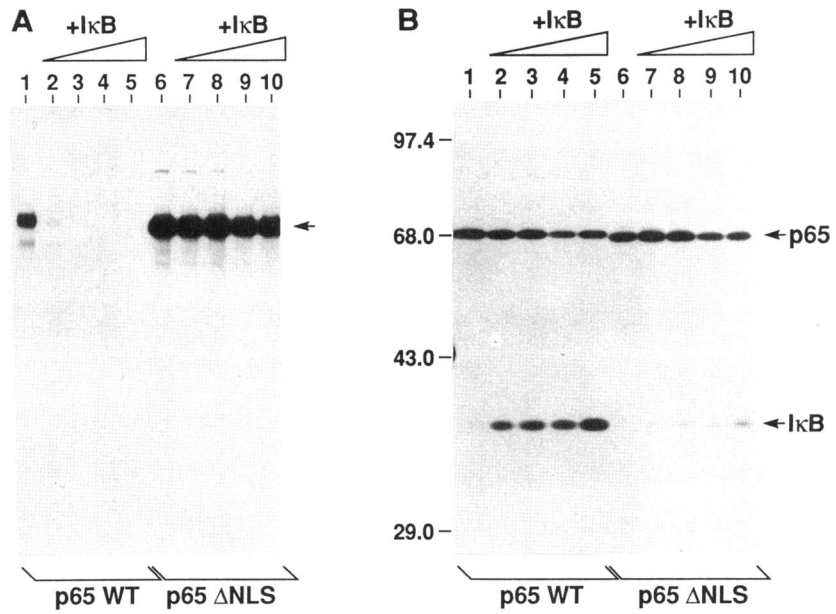

Figure 5. The NLS of NF-κB p65 is critically involved in IκB/MAD-3 function. (A) IκB/MAD-3 fails to inhibit the DNA binding activity of an NF-κB p65 variant lacking the highly conserved NLS. Monkey COS cell were transfected with expression vectors (0.5 μg) encoding either wild-type NF-κB p65 (lanes 1–5) or p65 ΔNLS (lanes 6–10), along with increasing doses (mock, lanes 1 and 6; 0.25 μg, lanes 2 and 7; 0.5 μg, lanes 3 and 8; 1.0 μg, lanes 4 and 9; 2.0 μg, lanes 5 and 10) of an IκB/MAD-3 expression vector. Recipient cell extracts were incubated with a ^{32}P-labeled κB enhancer probe, and the resultant nucleoprotein complexes were fractionated on a native 5% polyacrylamide gel. The arrow indicates NF-κB p65/κB enhancer complexes. (B) Deletion of the NLS of NF-κB p65 disrupts its ability to bind IκB/MAD-3. Whole-cell extracts described in A were subjected to immunoprecipitation analysis using an NF-κB p65-specific antipeptide antiserum (amino acids 535–551). Radiolabeled immunoprecipitates were fractionated by reducing SDS-PAGE and detected by autoradiography. The relative positions and sizes (in kilodaltons) of molecular weight standards are given on the left. The arrows indicate the positions of NF-κB p65 and IκB/MAD-3.

the wild-type protein (lanes 2–5). In contrast, similar titrations with IκB/MAD-3 in cells containing p65 ΔNLS failed to significantly block its DNA binding activity (lanes 7–10). As shown in Figure 5B, coimmunoprecipitation studies performed with an NF-κB p65-specific antiserum and ^{35}S-labeled extracts derived from these cells showed markedly diminished IκB/MAD-3 binding to the p65 ΔNLS protein (lanes 7–10) compared with the wild-type NF-κB p65 protein (lanes 2–5). Furthermore, as shown in Figure 6A, deletion of four amino acids immediately N-terminal (p65 Δ297–300; lane 1) or eight amino acids C-terminal (p65 Δ305–312; lane 3) to the KRKR motif (deleted in lane 2) did not significantly affect the IκB/MAD-3 binding capacity of these mutant proteins. Site-directed mutation of the nearby protein kinase A (PKA) phosphorylation consensus sequence (RRPS; amino acids 273–276) within the Rel homology domain of NF-κB p65 also had no effect on the interaction of NF-κB p65 with IκB/MAD-3. Together, these biochemical data indicate that the KRKR motif present in NF-κB p65 is selectively required for IκB-mediated inhibition and functions as a primary subregion mediating the stable assembly of NF-κB p65 with IκB/MAD-3 in vivo.

To confirm that this basic subregion within human NF-κB p65 is in fact a functional NLS, immunofluorescence studies were performed with NF-κB p65-specific antiserum and COS cell transfectants overexpressing either the Rel homology domain of NF-κB p65 (p65 Rel; amino acids 1–312) or a variant of this terminal deletion mutant lacking the KRKR motif, termed p65 Rel ΔKRKR. Of note, preliminary studies performed with the full-length form of NF-κB p65 revealed a predominantly perinuclear or whole-cell pattern of fluorescence (see Figure 2C), suggesting that the presence of the C-terminus intrinsically influences the subcellular disposition of NF-κB p65. However, as shown in Figure 6B, immunofluorescence staining of COS cells transfected with a C-terminal deletion mutant truncated downstream of the KRKR motif (p65 Rel) clearly demonstrated a nuclear pattern of expression. Selective deletion of the KRKR motif within this NF-κB p65 mutant profoundly altered the subcellular distribution, yielding a polypeptide (p65 Rel ΔKRKR) confined predominantly to the cytoplasmic compartment. Similar results were obtained with a mutant substituted within the KRKR domain (KRKR → AAAA; Ganchi *et al.*, unpublished results). Furthermore, deletion of a region adjacent to the KRKR motif (p65 Rel Δ293–296) failed to disrupt the nuclear targeting of this NF-κB p65 construct. These results confirm the NLS function of this motif in NF-κB p65.

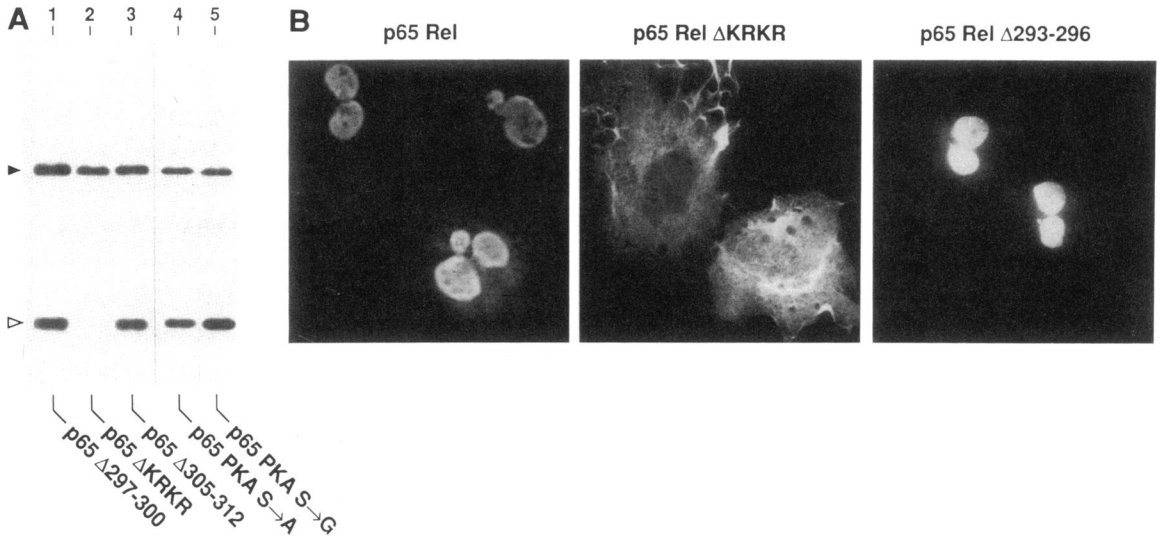

Figure 6. The functional NLS of NF-κB p65 is specifically targeted by IκB/MAD-3. (A) Neither the conserved PKA site nor the sequences flanking the NLS of NF-κB p65 are involved in the association with IκB/MAD-3. Extracts from COS cells expressing IκB/MAD-3 together with p65 Δ297–300 (lane 1), p65 ΔNLS (lane 2), p65 Δ305–312 (lane 3), p65 PKA S → A (lane 4), or p65 PKA S → G (lane 5) were subjected to immunoprecipitation analysis using an antipeptide antiserum raised against the C-terminus of NF-κB p65 (amino acids 535–551). Immune complexes were resolved on a 10% SDS-polyacrylamide gel and visualized by fluorography. The positions of NF-κB p65 (closed arrowhead) and IκB/MAD-3 (open arrowhead) are indicated. (B) The highly conserved KRKR motif of NF-κB p65 serves as a functional nuclear localization signal. COS cells were transfected with either p65 Rel, p65 Rel ΔKRKR, or p65 Rel Δ293–296, fixed 48 h posttransfection, and probed with an antipeptide antiserum raised against the N-terminus of NF-κB p65 (amino acids 1–21). Complexes were visualized using a Texas red-conjugated second antibody.

DISCUSSION

The NF-κB complex, a prominent member of the Rel family of transcription factors, is critically involved in both the physiologic activation of T cells in response to immunologic stimuli (Sen and Baltimore, 1986a,b; Lenardo and Baltimore, 1989) and the pathologic induction of HIV-1 gene expression in infected $CD4^+$ T lymphocytes (Muesing *et al.*, 1987; Nabel and Baltimore, 1987). The prototypic form of this heterodimeric complex is composed of two functionally distinct subunits, NF-κB p65 and NF-κB p50 (Baeuerle and Baltimore, 1989; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991), which share extensive N-terminal homology with the v-*rel* oncogene product (Figure 7) (Gilmore, 1990). In addition, other interactive Rel-related polypeptides recently have been identified that may also participate in the NF-κB signal transduction pathway through combinatorial associations involving either NF-κB p50 or NF-κB p65 (Neri *et al.*, 1991; Schmid *et al.*, 1991; Bours *et al.*, 1992; Ruben *et al.*, 1992a; Ryseck *et al.*, 1992). Although only NF-κB p50 was originally believed to possess DNA binding activity (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990), recent studies clearly have shown that NF-κB p65 is a κB-specific enhancer-binding protein in its own right (Nolan *et al.*, 1991; Ruben *et al.*, 1991; Ballard *et al.*, 1992). However, NF-κB p65 differs from NF-κB p50 with respect to several important functions. Specifically, NF-κB p65 contains a strong transactivation domain at its C-terminus that is conspicuously absent in NF-κB p50 (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992; Ruben *et al.*, 1992b). In addition, the in vitro DNA binding activity of NF-κB p65, but not of NF-κB p50, is selectively inhibited by IκB, a known cytosolic antagonist of NF-κB function (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991; Inoue *et al.*, 1992b). In this regard, several cDNAs encoding IκB-like inhibitors recently have been cloned, including human MAD-3 (Haskill *et al.*, 1991), chicken pp40 (Davis *et al.*, 1991), murine IκBγ (Inoue *et al.*, 1992a), and human Bcl-3 (Ohno *et al.*, 1990; Hatada *et al.*, 1992; Wulczyn *et al.*, 1992). The high degree of amino acid sequence homology shared among these various inhibitors, such as the presence of multiple ankyrin-like motifs (Davis *et al.*, 1991; Haskill *et al.*, 1991; Inoue *et al.*, 1992a), suggests a family of related proteins that may differentially regulate one or more members of the NF-κB/Rel transcription factor pathway. Furthermore, recent mutational analyses clearly have demonstrated that four of the ankyrin repeats present in pp40 are required to mediate its inhibitory effect on NF-κB p65 DNA binding activity (Inoue *et al.*, 1992b). However, the corresponding sequences of NF-κB p65 that interface with these IκB binding determinants and the effect of this interaction on NF-κB p65-dependent transcription in vivo have remained unknown. In the present study, we have

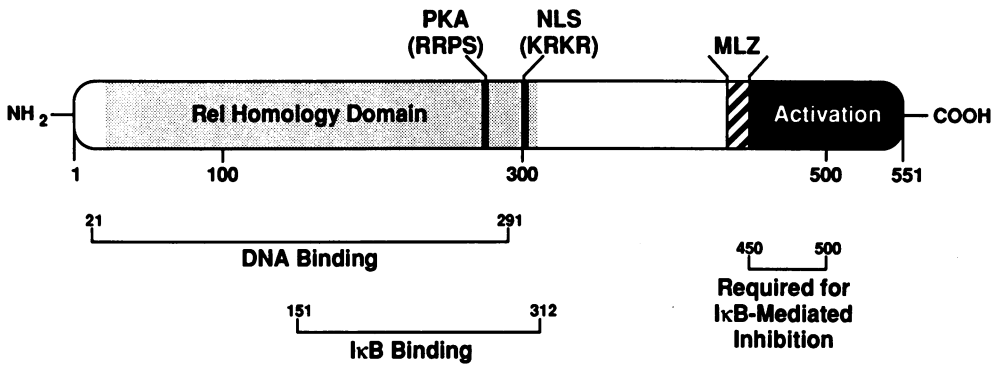

Figure 7. Defined organization of the NF-κB p65 DNA binding domain and functionally bipartite IκB/MAD-3 receptor sequences. Established amino acid endpoints for the maximal DNA binding (amino acids 21–291) and IκB/MAD-3 binding (amino acids 151–312) domains of NF-κB p65 are shown in relation to previously defined motifs (see Figure 1A). The region within the NF-κB p65 transactivation domain (amino acids 450–500) shown to be required for IκB-mediated inhibition of NF-κB p65 DNA binding is also bracketed.

explored in detail the molecular basis for the specific interaction between human NF-κB and IκB/MAD-3, focusing on defining the requisite interaction sequences within NF-κB p65 and the effects of IκB/MAD-3 binding on the various intrinsic biochemical properties of NF-κB p65 and NF-κB p50.

IκB/MAD-3 Inhibits NF-κB p65-Dependent Transcription In Vivo

Previous functional assays performed with Gal4/NF-κB p65 chimeric proteins identified a potent transactivation domain in the distal 101 amino acids of this 65-kDa subunit (Figure 7) (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992). Transient expression studies in human Jurkat T cells using the wild-type and C-terminally truncated forms of NF-κB p65 show that these activation sequences also function in the context of the native protein and that IκB/MAD-3 completely negates their stimulatory effects on transcription in vivo. Of note, IκB-mediated inhibition was unaffected by the selective deletion of a mini-leucine zipper motif that has been implicated as a part of the transactivation function of NF-κB p65 (Figure 7) (Ruben *et al.*, 1992b). These findings strongly suggest that MAD-3 represents at least one functional member of the proposed IκB family of polypeptides that has the capacity to regulate NF-κB p65-dependent transcription in vivo.

The NLS of NF-κB p65 is the Primary Functional Target of IκB/MAD-3

Phenotypic analysis of a series of N- and C-terminal NF-κB p65 truncation mutants has localized the maximal IκB/MAD-3 binding domain to a segment of the Rel homology region encompassing amino acids 151 through 312 (Figure 7). This region includes a basic tetrapeptide motif (KRKR; amino acids 301–304; Figure 7) (Ruben *et al.*, 1991) that is highly conserved among all members of the NF-κB/Rel polypeptide family (Gilmore, 1990). In this regard, it has been demonstrated that the corresponding motif within the 110-kDa precursor of NF-κB p50 (RKRQK) serves as a functional NLS whose expression is effectively masked *in cis* by inhibitory C-terminal sequences (Blank *et al.*, 1991; Henkel *et al.*, 1992). These inhibitory precursor sequences are ultimately removed by proteolytic processing, thereby generating the mature 50-kDa DNA binding protein (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). An alternatively spliced transcript selectively encoding this C-terminal inhibitory domain, termed IκBγ, recently has been cloned and shown to exhibit IκB-like activity *in trans* (Inoue *et al.*, 1992a). We demonstrate here that the basic KRKR motif present in NF-κB p65 is a functional NLS. Furthermore, specific deletion of this NLS markedly reduces the capacity of IκB/MAD-3 to stably associate with this transactivator protein. These findings suggest that IκB/MAD-3 directly binds to and masks the NLS of NF-κB p65, thereby preventing NF-κB p65 from entering the nuclear compartment. The highly conserved nature of this NLS among the various known Rel proteins underscores a potentially fundamental role for this basic motif in regulating the inducible nuclear import of multiple Rel family members via recognition by IκB.

In this regard, we now show that the subcellular localization of NF-κB p50 is also regulated by IκB/MAD-3, presumably by a similar mechanism that involves masking of its NLS (Henkel *et al.*, 1992). Recent studies suggest that the homodimeric form of this Rel-related subunit acts specifically as a repressor of NF-κB p65-dependent transcription from the interleukin-2 promoter (Kang *et al.*, 1992). It is not yet clear whether IκBγ, IκB/MAD-3, or another member of this growing family of repressors is the authentic physiologic inhibitor of NF-κB p50 function. Notwithstanding, the ability of the C-terminus of the NF-κB p50 precursor to mask its own NLS, coupled with its homology to both IκBγ and IκB/MAD-3, suggests that the NF-κB p50 NLS plays a

role similar to that of the NF-κB p65 NLS in IκB-dependent cytoplasmic retention.

The Unique C-terminal Half of NF-κB p65 Attenuates the Rel Domain-Encoded Functions of DNA Binding and Nuclear Localization Transactivation

In the present study, we have shown that deletion mutants of NF-κB p65 devoid of the unique C-terminal sequences are more efficiently localized within the nucleus when compared with the wild-type protein. Previous studies have suggested that these transcriptionally inactive NF-κB p65 mutants also bind DNA with increased affinity (Nolan *et al.*, 1991). The recently recognized capacity of NF-κB p65 to induce IκB/MAD-3 gene expression (Sun *et al.*, unpublished results) may contribute to these functional effects. Alternatively these effects could be a consequence of the disruption of noncovalent interactions that occur between the C-terminus of NF-κB p65 and the N-terminal Rel homology domain. In what may be a related finding, specific deletion of the NLS of NF-κB p65 also enhances its DNA binding activity, suggesting that this stretch of basic residues may serve as a primary contact point for the NF-κB p65 C-terminus.

This proposed type of intramolecular masking mechanism is not without precedent. For example, as noted above, the NLS within the Rel homology domain of the NF-κB p50 precursor is functionally sequestered by *cis*-acting C-terminal sequences (Blank *et al.*, 1991; Henkel *et al.*, 1992). In addition, C-terminally truncated variants of the Rel-related *dorsal* gene product, a maternal morphogen in *Drosophila*, have been shown to exhibit enhanced nuclear localization properties as compared with the wild-type protein (Rushlow *et al.*, 1989). Similarly, the cell cycle-dependent nuclear import of the yeast transcription factor, SWI5, is contingent on the regulated disruption of an intramolecular salt bridge involving its NLS (Moll *et al.*, 1991). By analogy, an interplay between the modular N- and C-terminal domains in NF-κB p65 may represent an alternative mechanism for regulating the potent transcriptional activity of NF-κB p65 in an IκB-independent manner. This inhibitory interaction could conceivably be relieved by dimerization with other Rel polypeptides (e.g., NF-κB p50) or by specific posttranslational modification (e.g., phosphorylation) that induces an "open" NF-κB p65 structural configuration.

The NF-κB p65 Transactivation Domain and NLS are Essential for a Fully Functional NF-κB p65/IκB Interaction

Although the N-terminal Rel homology domain of NF-κB p65 is fully capable of binding IκB/MAD-3, IκB-mediated inhibition of NF-κB p65 DNA binding activity requires additional sequences that map to the remote C-terminal transactivation domain. This relationship explains the paradoxical finding that IκB/MAD-3 efficiently engages both NF-κB p65 and NF-κB p50 but inhibits the DNA binding activity of only NF-κB p65 (see Figure 2, A and B). Specifically, these selective inhibitory effects of IκB/MAD-3 on NF-κB p65 do not derive from subtle differences between the Rel homology domains of NF-κB p65 and NF-κB p50 (Nolan *et al.*, 1991) but rather involve the unique C-terminal transactivation domain of NF-κB p65, which is missing in NF-κB p50. Coupled with the finding that the subcellular localization of NF-κB p50 can be regulated by IκB/MAD-3, the Rel homology domain itself can be considered an IκB-resistant DNA binding unit containing an IκB-sensitive NLS.

In this model, the ability of IκB/MAD-3 to block DNA binding activity depends critically on C-terminal sequences downstream of the Rel homology domain, which are highly divergent among the various NF-κB/Rel polypeptides (Gilmore, 1990). Given the ability of IκB/MAD-3 to control the inducible expression of NF-κB at the level of subcellular localization, this alternate inhibitory potential would seem redundant. However, should IκB/MAD-3 gain access to the nucleus, its capacity to inhibit DNA binding may serve a pivotal role in promoting disengagement of NF-κB p65 bound to its cognate enhancer (Zabel and Baeuerle, 1990). In this regard, an inhibitor of NF-κB DNA binding activity has been identified in nuclear extracts from T lymphocytes and further shown to be highly sensitive to treatment with deoxycholate (Kang *et al.*, 1992), a characteristic biochemical property of IκB (Baeuerle and Baltimore, 1988a,b). As such, through its dual capacity to block nuclear import as well as DNA binding, IκB could control both the rapid mobilization of NF-κB from the cytoplasm and the subsequent termination of NF-κB p65-dependent transcription in the nucleus.

ACKNOWLEDGMENTS

We thank Karen Koerber, Paul Morrow, Nancy Peffer, and Eric Dixon for excellent technical assistance; Dr. C. Rosen for the human NF-κB p65 clone; Dr. A. Baldwin for the human IκB/MAD-3 clone; Dr. B. Stein for the NF-κB p50 construct; and Diane Gearhart for manuscript preparation. P. Ganchi was supported in part by NIH training grant 5T32CA-09111. This work was funded by grants from the NIH (5RO-1AI-29117-05) and the J. David Gladstone Institutes.

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