Dimerization of NF-KB2 with RelA(p65) Regulates DNA Binding, Transcriptional Activation, and Inhibition by an IκB-α (MAD-3)

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Inducible expression of human immunodeficiency virus (HIV) is regulated by a cellular transcription factor, nuclear factor κB (NF-κB). NF-κB is composed of distinct subunits; five independent genes, NFKB1(p105), NFKB2(p100), RelA(p65), c-rel and relB, that encode related proteins that bind to κB DNA elements have been isolated. We have previously found that NFKB2(p49/p52) acts in concert with RelA(p65) to stimulate the HIV enhancer in Jurkat T-leukemia cells. Here we examine the biochemical basis for the transcriptional regulation of HIV by NFKB2. Using Scatchard analysis, we have determined the dissociation constants of homodimeric p49 and heterodimeric p49/p65 for binding to the HIV κB site. p49 has a ~18-fold-lower affinity for the HIV κB site ($K_D = 69.1$ pM) than does the ~50-kDa protein NFKB1(p50) derived from p105 ($K_D = 3.9$ pM). In contrast, the affinity of heterodimeric NFKB2(p49)/RelA(p65) for this site is ~-6-fold higher ($K_D = 11.8$ pM) than that of p49 alone. Consistent with these findings, in vitro transcription was stimulated 18-fold by the addition of preformed, heterodimeric NFKB2(p49)/RelA(p65) protein. Transcriptional activation of the HIV enhancer was also subject to regulation by recently cloned IκB-α(MAD-3). Recombinant IκB-α(MAD-3) inhibited the DNA binding activity of p65, p49/p65, and p50/p65 but stimulated the binding of NFKB2(p49) or NFKB1(p50). Functional activation of an HIV reporter plasmid by p49/p65 in transiently transfected Jurkat T-leukemia cells was also inhibited by coexpression of MAD-3. These data suggest that binding of the NFKB2 subunit to the HIV enhancer is facilitated by RelA(p65) and that this NFKB2(p49)/p65 heterodimeric complex mediates transcriptional activation which is subject to regulation by MAD-3.

Nuclear factor κB (NF-κB) is a family of sequence-specific DNA-binding proteins which regulate the transcription of a variety of cellular and viral genes (1, 26). These transcription factors can be detected in the cytoplasm of many cells (3) in association with IκB's, factors which inhibit their DNA-binding activity (2, 47). The DNA-binding activity of NF-κB can be stimulated by cellular activation with cytokines (37) or viral *trans*-activators (6, 28, 39). Cellular protein kinases can alter the phosphorylation of IκB's (13, 29), leading them to dissociate from NF-κB. This dissociation is likely to serve as an important regulatory step which makes NF-κB available for binding to κB elements in the promoters of genes involved in immune and inflammatory responses (26, 27). In addition to these cellular genes, NF-κB can also activate the transcription of genes encoded by eukaryotic viruses. Among these, the human immunodeficiency virus (HIV) enhancer contains two κB sites which play an important role in inducible activation of viral transcription (33).

Biochemical and molecular biological analysis of NF-κB has begun to reveal the complexity of this transcriptional regulatory pathway. Highly purified NF-κB is composed of two major subunits, with molecular masses of ~50 and ~65 kDa, respectively (4, 20). Isolation of cDNAs encoding the 50-kDa subunit (p50) suggested that the primary translation product of this subunit is a ~105-kDa protein which requires posttranslational modification to obtain the mature form (8, 14, 22, 32). Sequence comparison of p50 and the gene

encoding the 65-kDa subunit (p65) (36, 40) revealed a high homology to the c-rel proto-oncogene (9) and to the Drosophila morphogen dorsal (44). We have recently described another gene, p49/p100, which can generate an alternative ~50-kDa form of NF-κB (7, 34, 43). A ~50-kDa protein can be generated either by posttranslational processing of a ~100-kDa precursor or by the generation of an alternatively spliced form, p49; the N termini of p49 and p100 are identical, and p49 appears to be functionally indistinguishable from truncated forms of p100 (38). The gene encoding this product has been designated NFKB2, in contrast to the p105 subunit, which has been termed NFKB1 (30a).

A human cDNA clone that encodes a 35- to 37-kDa protein has been isolated. The protein, MAD-3, has been shown to have IκB-like properties (16). Purification and primary sequence analysis of rabbit IκB (10) has revealed its identity to MAD-3. The MAD-3/IκB protein contains five repeats of a sequence that was first identified in the erythroid protein ankyrin and is also present in a number of proteins involved in cell cycle control and tissue differentiation (35). The C termini of p100 and p105 also contain ankyrin/cell cycle repeats, and the C terminus of p105 has been shown to have IκB-like activity when expressed in *trans* (17, 30).

Cotransfection studies with p50 NFKB1 and p49 NFKB2 expression vectors have shown that these two genes, despite their homology, differ in the ability to activate κB-dependent transcription of the HIV enhancer in combination with RelA(p65) (38, 43). Here we characterize the biochemical properties of recombinant p49 as well as its interactions with p65 and with MAD-3 (16). The binding constants of p49

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homodimers and p49/p65 heterodimers to both the HIV κB site and the related H-2 site (5, 18) are determined, and the ability of NFKB2(p49)/RelA(p65) proteins to stimulate in vitro transcription of the HIV enhancer is characterized. We show that heterodimeric NFKB2(p49)/RelA(p65) has a higher affinity for κB than does NFKB2(p49), stimulates transcription of HIV in vitro and in vivo, and is subject to regulation by IκB-α/MAD-3 mediated through the RelA(p65) subunit.

MATERIALS AND METHODS

Plasmids. Human p50 NFKB2, a ~50-kDa N-terminal truncated form of p105, was generated from Rous sarcoma virus (RSV)-p105 by using site-directed mutagenesis to create a stop codon at the position corresponding to residue 453, followed by a BglII restriction site. Utilization of the previously created HindIII and NcoI sites 5' of the Kozak sequence (23) of RSV-p105 (38) allowed subcloning into the RSV and pET3D vectors as previously described (43).

Human p65 was isolated from a B-cell lymphoma cDNA library probed with a polymerase chain reaction-derived mouse p65 cDNA clone. The human clone appears to be identical to that described previously (40, 41). 3' untranslated sequence was removed by digestion with BsaHI, and the 5' overhang was filled in with Klenow enzyme. The NcoI site at the initiation ATG was used to subclone into the modified RSV β-globin vector (43), thus inserting a consensus Kozak sequence.

The MAD-3 cDNA (16) was kindly provided by Chiron Inc. and subcloned into the modified RSV vector (43) by ligation of an XbaI-Klenow-HindIII MAD-3 fragment into the modified RSV β-globin-derived backbone (43).

The HIV long terminal repeat (LTR) in vitro transcription template (pHIV380) was prepared by blunt-end ligation of a ~730-bp XhoI-HindIII Klenow-treated fragment from an HIV-chloramphenicol acetyltransferase (CAT) construct into the SacI site of the p(C₂AT)₁₉ G-less cassette vector (42), whose 3' overhangs had been removed by treatment with T4 polymerase. The adenovirus major late plasmid, pML200 (31), was a kind gift of U. Schibler, University of Geneva.

All other expression vectors and κB reporter plasmids have been described previously (33, 38).

Protein purification and formation of heterodimers. p49 and p50 proteins were expressed in *Escherichia coli* BL21(LysE) and partially purified by Sephacryl S-200 gel filtration fast protein liquid chromatography as described previously (38). Proteins were affinity purified by oligonucleotide chromatography (19), using the H-2 site as the target sequence (46). Proteins were eluted with a linear NaCl gradient in a buffer containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.9), 1 mM EDTA, 0.1 mM ZnCl₂, 0.1% Nonidet P-40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol, dialyzed against the same buffer with 50 mM NaCl, and stored in aliquots at −70°C. Proteins were judged to be at least 90% pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis. Heterodimers were formed by incubating concentrated proteins in a 3-μl volume for 15 to 40 min at 37°C and then diluting the mixture in the same dialysis buffer containing 0.1 mg of bovine serum albumin per ml.

Recombinant murine p65 protein, expressed in baculovirus (11), was a kind gift of T. Fujita, G. Nolan, and D. Baltimore.

Expression and purification of MAD-3 protein as a fusion protein with glutathione S-transferase (GST) will be described elsewhere (22a).

EMSA and chemical cross-linking. Proteins were incubated with radiolabeled DNA probes in a 20-μl reaction mixture containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, 3% glycerol, 50 μM MgCl₂, and 10 μM ATP. Nucleoprotein complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels in 0.25× Tris-borate-EDTA at 10 V/cm for 5 h at room temperature. Dried gels were exposed to Kodak XAR-5 film at −70°C with intensifying screens. The DNA probes used in this study were (i) a 182-bp ScaI-NheI restriction fragment from an HIV-1 LTR plasmid in which site-directed mutagenesis had been used to abolish the 3' κB site to eliminate the possibility of cooperative binding and double occupancy (Fig. 1A), (ii) a double-stranded oligonucleotide probe encoding a single HIV κB site (Fig. 1B),

5'-AGCTTGGGGACTTTCCACTAGTACG-3'
  3'-ACCCCTGAAAGGTGATCATGCTTAA-5'

and (iii), a double-stranded oligonucleotide probe encoding a single κB site and a single adjacent Sp1 site from the HIV enhancer (Fig. 5),

5'-GATCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGA-3'
  3'-GCGACCCCTGAAAGGTCCCTCCGCACCGGACTCTAG-5'

The κB motifs are shown in bold. Oligonucleotides were radiolabeled by using Klenow enzyme and [³²P]dCTP; ~0.5 ng was used in a standard electrophoretic mobility shift assay (EMSA) reaction.

For cross-linking, purified homodimeric and heterodimeric proteins (200 to 1,600 ng) were incubated with 4-ethyleneglycol bis(sulfosuccinimidyl succinate) (EGS; Pierce) for 15 min at room temperature in a 40-μl volume in the dilution buffer described above. The reaction was stopped by the addition of D-lysine (25 mM, final concentration), and one half of each sample was resolved by electrophoresis on a SDS-6% polyacrylamide gel and immunoblotting with an anti-p100 polyclonal antibody (38a) followed by a goat anti-rabbit horseradish peroxidase conjugate; the reaction was developed by using the ECL Western immunoblotting detection system (Amersham).

Determination of dissociation constants. Dissociation constants were determined essentially as described previously (11, 48). Gel-purified oligonucleotides encoding either the HIV or H-2 κB site, with T overhangs and C ends, were used as follows:

HIV κB probe,
5'-CCTTTTTTTTTTAGGGGACTTTCCGA-3'
            3'-TCCCCTGAAAGGCTTTTTTTTTTTCC-5'

H-2 probe,
5'-CCTTTTTTTTTTAGGGGATTCCCCGA-3'
            3'-TCCCCTAAGGGGCTTTTTTTTTTTCC-5'

The κB elements are shown in bold. Annealed oligonucleotides were radiolabeled with [α-³²P]dATP by using Klenow enzyme and chased with unlabeled dATP and dGTP. Unincorporated radionucleotides were removed by Sephadex G-50 gel filtration. Probes used were of a specific activity of at least 50,000 Ci/mmol. The concentration of active DNA-binding sites was calculated by EMSA analysis using a large excess of probe, and a constant amount of protein (~1 fmol)
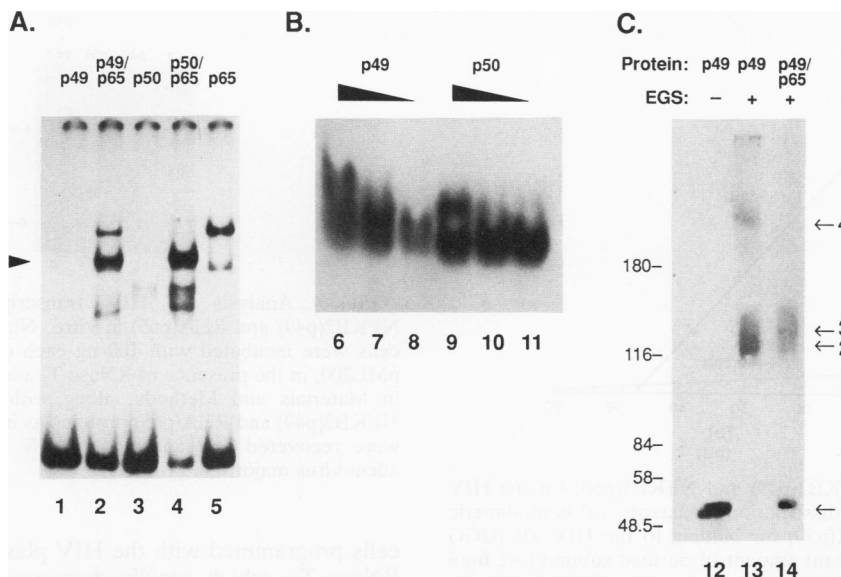
FIG. 1. Formation of NFKB2(p49) multimers in vitro. (A) NFKB2(p49), NFKB1(p50), and RelA(p65) proteins were incubated as NFKB2(p49) alone (lane 1), heterodimeric NFKB2(p49)/RelA(p65) (0.25 ng/2 ng; lane 2), NFKB1(p50) alone (0.12 ng; lane 3), heterodimeric NFKB1(p50)/RelA(p65) (0.12 ng/0.8 ng; lane 4), or an excess of RelA(p65) (4 ng) to compare its binding activity (lane 5) as described in Materials and Methods. A radiolabeled restriction fragment derived from the HIV-1 LTR was used under standard conditions, except that reaction mixtures contained 100 ng of poly(dI-dC) · poly(dI-dC). Nucleoprotein complexes were resolved by EMSA. (B) Formation of higher-order complexes at high protein concentration. Serial dilutions of NFKB2(p49) (50, 5, and 0.5 ng; lanes 6 to 8) and NFKB1(p50) (25, 2.5, and 0.25 ng; lanes 9 to 11) were incubated with a radiolabeled HIV κB probe and analyzed by EMSA under standard conditions. (C) Chemical cross-linking of NFKB2(p49) and RelA(p65). NFKB2(p49) and RelA(p65) proteins were incubated with EGS and analyzed by SDS-PAGE and immunoblotting with a polyclonal antibody specific to p49/p100 as described in Materials and Methods. Positions of native (arrow 1) and cross-linked (arrows 2, 3, and 4) species are indicated.

was incubated with a range of probe concentrations in a 10-μl reaction for 10 min under standard EMSA conditions for 1 h at room temperature. Dried gels were analyzed by using a Betascope analyzer (Betagen).

In vitro transcription. In vitro transcription experiments were performed with nuclear extracts from HeLa cells (HeLaScribe; Promega). Extracts (50 μg) were incubated at 30°C for 30 min in a 25-μl reaction volume containing ATP and CTP (all at 0.4 mM), 1 μl of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham), 7.5 mM MgCl$_2$, 100 ng each of plasmids pHIV380 and pML200, 10 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.2 mM dithiothreitol, and 8% glycerol. RNase T$_1$ (150 U; Bio-Rad) was added, and samples were incubated for a further 15 min. RNA was recovered by phenol-chloroform extraction followed by ethanol precipitation and resolved on 6% sequencing gels. Dried gels were exposed to Kodak XAR-5 film at −70°C with intensifying screens, and autoradiograms were analyzed by digital scanning, using hardware and software from BioImage Inc. (Ann Arbor, Mich.).

Transfections and CAT assays. Cell culture, transfections, and CAT assays were performed as described previously (43).

RESULTS

Dimerization and DNA binding of NFKB2(p49). The DNA-binding properties of recombinant NFKB2(p49) and NFKB1(p50) were compared by using the EMSA. When purified, recombinant p49 protein was combined with recombinant RelA(p65) and incubated with an HIV κB site probe, a novel complex with a mobility distinct from that of either subunit alone was detected (Fig. 1A, lane 2 versus 1 and 5).

A similar complex was also observed with NFKB1(p50) in combination with RelA(p65) (Fig. 1A, lane 4), consistent with previous findings that both NFKB2(p49) and NFKB1(p50) can form heterodimeric complexes with RelA(p65) (7, 36, 38). At high protein concentrations of p49 (Fig. 1B, lanes 6 to 8) and p50 (Fig. 1B, lanes 9 to 11), complexes with lower mobility were observed, suggesting that NFKB2(p49) and NFKB1(p50) are able to form higher-order oligomers. To investigate this observation further, the p49-containing complexes were incubated with the bifunctional chemical cross-linking agent EGS, resolved by SDS-PAGE, and detected by immunoblotting with an antiserum specific to p49. While native NFKB2(p49) migrated as predicted by its molecular weight (Fig. 1C, lane 12, arrow 1), cross-linked protein migrated predominantly as a ~100-kDa species, while a minor ~200-kDa band was also present (Fig. 1C, lane 13, arrows 2 and 4). These data suggest that NFKB2 exists predominantly in a dimeric form in solution. Cross-linked heterodimeric NFKB2/RelA migrated at a position consistent with the predicted size of the heterodimer (Fig. 1C, lane 14, arrow 3).

To examine the DNA-binding properties of NFKB2, the dissociation constants of binding to different κB sites were determined. Oligonucleotides were radiolabeled to high specific activity, and dilutions of probe were incubated with a constant concentration of protein. Bound and free DNA species were resolved by gel electrophoresis, and dissociation constants were determined by Scatchard analysis. The affinity of homodimeric NFKB2(p49) for the HIV κB site was approximately 18 times weaker than that of NFKB1(p50) (Fig. 2). Also, homodimeric p49 showed a higher affinity for the H-2 κB element than for the HIV κB site (Fig. 3), consistent with previous studies in which this
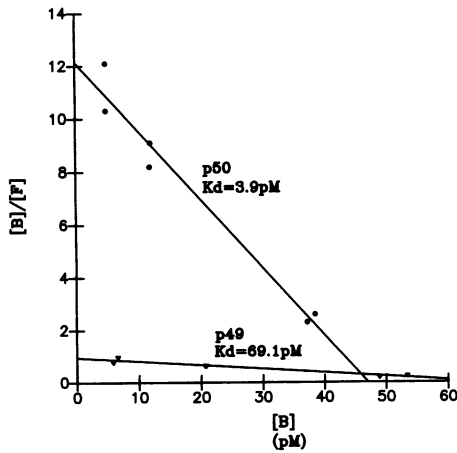
FIG. 2. Affinity of NFKB2(p49) and NFKB1(p50) for the HIV
κB site. To determine dissociation constants of homodimeric
NFKB2(p49) and NFKB1(p50) for binding to the HIV κB (GGG
GACTTTCC) site, a constant amount of purified subunit (~1 fmol
active protein) was incubated with a range of radiolabeled probe
concentrations, in a 10-μl reaction, and analyzed by EMSA as
described in Materials and Methods. Bound (B) and free (F) probe
species were quantitated by using a Betascope analyzer (Betagen),
and dissociation constants were determined by Scatchard analysis.
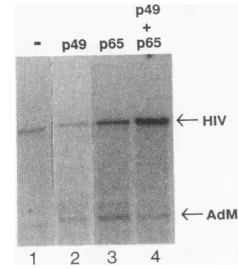Reactions were performed in triplicate.

FIG. 4. Analysis of HIV transcription by heterodimeric
NFKB2(p49) and RelA(p65) in vitro. Nuclear extracts from HeLa
cells were incubated with 100 ng each of plasmids pHIV380 and
pML200, in the presence of RNase T_1 and [α-^{32}P]UTP as described
in Materials and Methods, along with 100 ng of recombinant
NFKB2(p49) and RelA(p65) proteins as indicated. RNA transcripts
were recovered and analyzed on 6% sequencing gels. AdML,
adenovirus major late promoter.

difference had not been quantitated (7, 34, 38). However,
heterodimeric NFKB2(p49)/RelA(p65) showed a five- to
sixfold-higher affinity for the HIV site than did p49 alone
(Fig. 3A). The association of NFKB2(p49) with RelA(p65)
increased its affinity for the palindromic H-2 κB site less than
twofold under the same conditions (Fig. 3B).

**NFKB2(p49)/RelA(p65) heterodimers stimulate HIV tran-
scription in vitro.** The effect of heterodimeric NFKB2(p49)/
RelA(p65) protein on the transcriptional control of HIV was
examined in vitro by using a reporter plasmid in which the
HIV LTR was cloned upstream of the synthetic $(C_2AT)_{19}$
cassette (42) to allow the transcription of a ~380-nucleotide
RNA transcript lacking guanosine residues. LTR-directed
transcription was monitored in nuclear extracts of HeLa

cells programmed with the HIV plasmid in the presence of
RNase T_1, which rapidly degrades all G-containing tran-
scripts. Addition of preformed, heterodimeric NFKB2(p49)/
RelA(p65) stimulated transcription of HIV up to 18-fold (Fig.
4, lane 4), while addition of a similar quantity of
NFKB2(p49) or RelA(p65) alone stimulated HIV transcrip-
tion only 1.4-fold (lane 2) or 6.8-fold (lane 3), respectively, as
determined by digital scanning analysis of the autoradiogram
shown in Fig. 4. Transcription of a ~200-bp cassette driven
by the κB-independent adenovirus major late promoter was
not significantly affected by the addition of NFKB2(p49) and
RelA(p65) proteins.

**Binding of heterodimeric NFKB2(p49)/RelA(p65) to DNA is
blocked by MAD-3 protein.** The effect of the MAD-3 gene
product on the DNA binding of NFKB2(p49) was next
examined. Recombinant MAD-3 protein, expressed in E.
coli and purified as a fusion protein with GST, was incubated
with combinations of NF-κB subunits and analyzed by
EMSA. Addition of GST–MAD-3 protein inhibited the DNA
binding of RelA(p65) alone (Fig. 5, lanes 1 and 2). Recom-
binant MAD-3 protein had no inhibitory effect on
NFKB2(p49) or NFKB1(p50) binding alone and in fact
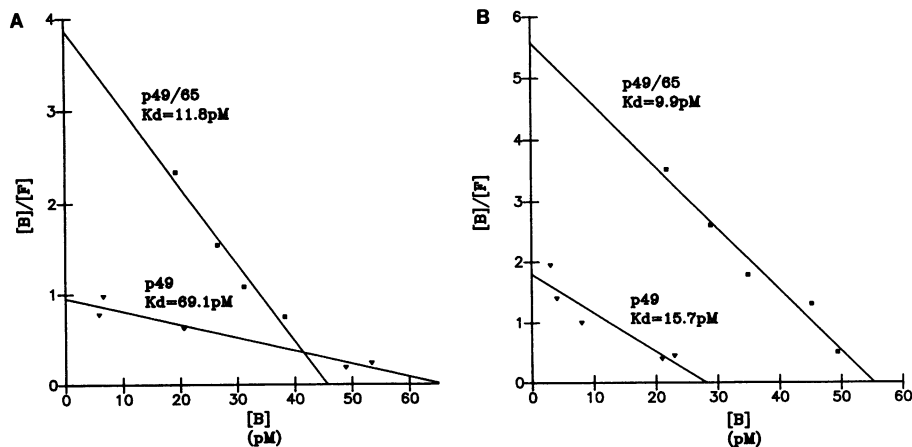stimulated the DNA binding of homodimeric p49 and p50

FIG. 3. Comparison of affinities of NFKB2(p49) and heterodimeric NFKB2(p49)/RelA(p65) for the HIV or class I major histocompatibility
complex κB sites by Scatchard analysis of binding to the HIV (GGGGACTTTCC) (A) and H-2 κB (GGGGAATCCCC) (B) sites.
NFKB2(p49)/RelA(p65) heterodimers were formed as described in Materials and Methods, and dissociation constants were determined as
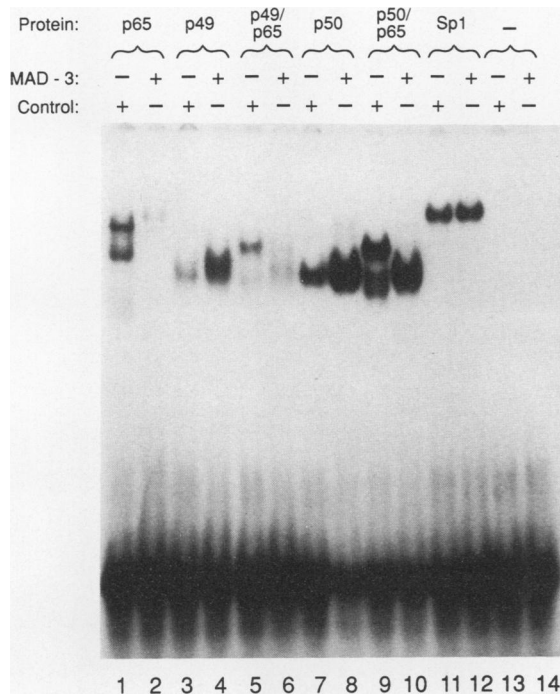described in the legend to Fig. 2. B, bound probe; F, free probe.

FIG. 5. Effects of recombinant MAD-3 on DNA binding of NF-κB proteins. MAD-3 protein expressed as a fusion protein with GST (100 ng) or a GST control was incubated under standard EMSA conditions with recombinant RelA(p65) (lanes 1 and 2), NFKB2(p49) (lanes 3 and 4), heterodimeric NFKB2(p49)/RelA(p65) (lanes 5 and 6), p50 (lanes 7 and 8), heterodimeric NFKB1(p50)/RelA(p65) (lanes 9 and 10), recombinant Sp1 (lanes 11 and 12), or alone (lanes 13 and 14). EMSA was performed as described in Materials and Methods.

(Fig. 5, lanes 3 versus 4 and 7 versus 8). When GST–MAD-3, in contrast to GST, was incubated with either NFKB2(p49)/RelA(p65) or NFKB1(p49)/RelA(p65) heterodimers, both DNA-binding activities were strongly inhibited (Fig. 5, lanes 5 versus 6 and 9 versus 10), suggesting that neither NFKB1 nor NFKB2 affected the ability of MAD-3 to interact with its target sequence on RelA(p65). Addition of MAD-3 to p49/p65 or p50/p65 heterodimers, while inhibiting the binding of the heterodimeric complex, also stimulated the appearance of nucleoprotein complexes identical in mobility to the respective homodimer, again suggesting that MAD-3 can interact directly with NFKB2(p49) and NFKB1(p50) to enhance their DNA-binding properties. MAD-3 had no effect on the binding of recombinant Sp1 protein to its cognate site (Fig. 5, lanes 11 and 12), confirming that these inhibitory and stimulatory effects were specific.

Transcriptional activation of the HIV enhancer by NFKB2(p49)/RelA(p65) in transfected Jurkat cells is inhibited by MAD-3. To determine whether transcriptional activation by p49/p65 heterodimers could be inhibited by MAD-3 in a T cell, expression vectors encoding different NF-κB subunits and MAD-3 were cotransfected into Jurkat T-lymphoma cells together with reporter plasmids containing the HIV enhancer linked to the CAT gene. Cotransfection of equal amounts of p49 and p65 plasmids stimulated transcription mediated through the HIV LTR approximately 13-fold. Consistent with previous reports, this transactivation was more potent with NFKB2(p49)/RelA(p65) than with NFKB1(p50)/RelA(p65) expression vectors (38, 43). Co-

transfection of an expression plasmid encoding MAD-3 almost completely inhibited the stimulation observed with both p49/p65 and p50/p65 (Fig. 6A). This effect was also seen with a reporter plasmid bearing the heterologous simian virus 40 promoter containing multiple κB sites (data not shown). MAD-3 had no inhibitory effect on the κB-independent transcription stimulation of HIV by the cytomegalovirus immediate-early gene product and did not significantly inhibit CAT transcription mediated by the RSV LTR (Fig. 6B). In addition, this effect was observed in a dose-responsive manner (Fig. 6C). These data demonstrate that MAD-3 exerts its inhibitory effect specifically through NF-κB, most likely through its interaction with RelA(p65).

DISCUSSION

We have examined the biochemical basis for NFKB2 binding to κB and its ability to dimerize and interact functionally with p65 and MAD-3. We find that the affinities and specificities of DNA binding by NFKB2(p49) and NFKB1(p50) differ considerably. Notably, binding of NFKB2(p49)/RelA(p65) to κB shows more than fivefold-greater affinity than does binding of NFKB2(p49), and this heterodimer stimulates transcription of the HIV enhancer in vitro. The interaction of heterodimers of NF-κB, NFKB2(p49)/RelA(p65) and NFKB1(p50)/RelA(p65), was inhibited by the MAD-3 IκB, but neither NFKB2(p49) nor NFKB1(p50) binding was inhibited by MAD-3. The finding that NFKB2(p49)/RelA(p65) stimulates transcription of the HIV enhancer in vitro (Fig. 4) documents that NFKB2(p49) and RelA(p65) act in concert to stimulate HIV transcription. Previous studies had shown that overexpression of NFKB2(p49) and RelA(p65) stimulated κB-dependent reporter gene transcription in transfected cells, but the possibility remained that this effect was indirect, (e.g., due to the initiation of a cascade of stimulatory events or an association with endogenous cellular factors). The data reported here suggest that NFKB2(p49)/RelA(p65) exerts a direct stimulatory effect on transcriptional initiation. Recent studies have suggested that homodimeric NFKB1(p50) protein may stimulate κB-dependent transcription independently of RelA(p65) (11, 24); the in vitro transcription data presented here suggest that this is not the case for NFKB2(p49), since transcription of the HIV enhancer was not significantly stimulated by NFKB2(p49) alone (Fig. 4, lanes 1 and 2).

The weak affinity of homodimeric NFKB2(p49) for κB motifs (Fig. 2 and 3) relative to NFKB1(p50), as well as its site specificity, highlight significant differences between NFKB2(p49) and NFKB1(p50). The affinity of recombinant NFKB2(p49) for the HIV κB site (K_D = 69.1 pM) is 17.7 times lower than that of NFKB1(p50) (K_D = 3.9 pM) under the same conditions. The binding K_D of NFKB1(p50) for the HIV κB site compares very favorably with results of a previous report (11; 3.9 pM versus 6.7 pM) which used nearly identical experimental conditions. While the affinities of NFKB1(p50) for the H-2 κB and HIV κB sites are very similar (10a, 11), NFKB2(p49) has a four- to fivefold-higher affinity for the H-2 site (K_D = 15.7 pM) than for the HIV κB site (K_D = 69.1 pM). Heterodimeric NFKB2(p49)/RelA(p65) also binds to the HIV κB site with ~6-fold-higher affinity (K_D = 11.8 pM) than does NFKB2(p49) alone. This difference in affinity is not observed with NFKB1(p50), whose affinities for the HIV element as either a homodimer or a NFKB1(p50)/(RelA(p65) heterodimer (6.7 pM versus 5.7 pm [11]) are very similar (10a, 24, 38). The relatively lower
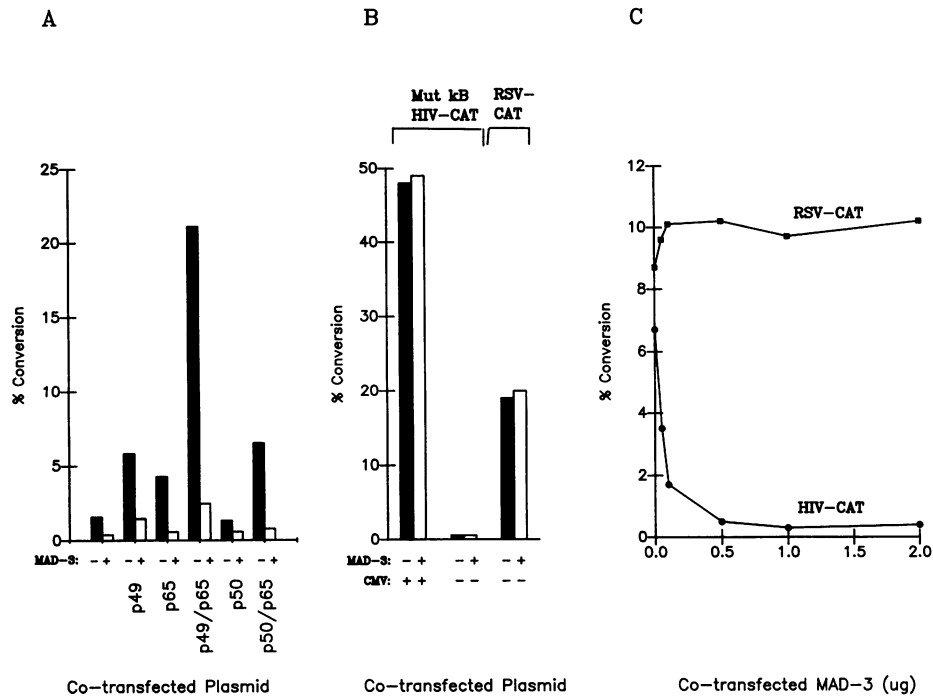
FIG. 6. Evidence that stimulation of κB-dependent CAT activity by NFKB2(p49) and RelA(p65) expression vectors is blocked by coexpression of MAD-3 in vivo. (A) Jurkat cells were cotransfected with 5 μg of HIV-CAT reporter plasmid, 0.2 μg of an expression vector encoding NFKB2(p49), NFKB1(p50), or RelA(p65) as indicated, 0.5 μg of RSV–MAD-3 expression vector as indicated (open bars), or 0.5 μg of an RSV–β-galactosidase control (filled bars). Transfections and CAT assays were performed as described in the text; CAT activity is shown as percent conversion. (B) Effect of MAD-3 on κB-independent CAT transcription. A 0.5-μg sample of MAD-3 or a promoter control was cotransfected with 2 μg of RSV-CAT (15) or 5 μg of a κB-mutated HIV-CAT reporter plasmid with a plasmid expressing the cytomegalovirus (CMV) immediate-early gene (12) as indicated. (C) Dose-response inhibition of p49/p65-mediated activation by MAD-3. NFKB2(p49)/RelA(p65) vectors (0.2 μg) with HIV-CAT (5 μg) or RSV-CAT (2 μg) were cotransfected with the indicated amounts of MAD-3 or RSV promoter control and assayed for CAT activity as described in the text. All data are representative of at least four independent transfections.

affinity of the NFKB2(p49)/RelA(p65) complex for κB may explain why NFKB2 was not identified initially by primary amino acid sequence from affinity-purified NF-κB.

Previous studies have shown that the RelA(p65) subunit can bind to the 3' (CTTTCC) half-site of the HIV κB sequence, although with low affinity (45). It is therefore likely that the binding of NFKB2(p49) is stabilized by the affinity of RelA(p65) for this half-site. Although the affinity of NFKB2(p49) for κB has been shown to be considerably weaker than that of NFKB1(p50), NFKB2(p49) nonetheless stimulates the κB-directed transcription of several genes more potently than does NFKB1(p50) when associated with RelA(p65) (Fig. 6A) (38). Taken together, these findings suggest that the affinity of binding to DNA is not the only determinant of the specificity of transcriptional activation for the HIV enhancer.

Despite their differences in DNA-binding affinity, the interactions of both NFKB2(p49) and NFKB1(p50) with RelA(p65) and MAD-3 were indistinguishable. In DNA-binding experiments, MAD-3 inhibited the binding of any complex which contained RelA(p65), regardless of its associated subunit (Fig. 5). Surprisingly, the binding of homodimeric NFKB2(p49) and NFKB1(p50) was stimulated by MAD-3. Although MAD-3 inhibited the binding of heterodimeric NFKB2(p49)/Rel(p65) and NFKB1(p50)/RelA(p65) to DNA, in the same reaction it was found to stimulate the appearance of homodimeric NFKB2(p49) and NFKB1(p50), respectively. While MAD-3 may conceivably

exert these stimulatory effects by disrupting the heterodimeric complex and sequestering RelA(p65), allowing newly formed homodimeric NFKB2(p49) or NFKB1(p50) to bind, we believe it more likely that MAD-3 can directly stimulate the binding of NFKB2(p49) and NFKB1(p50), since the same effect is observed with homodimeric NFKB2(p49) and NFKB1(p50). We postulate that our preparations of heterodimeric protein contain a small amount of homodimeric NFKB2(p49) or NFKB1(p50), whose DNA-binding properties are enhanced in the presence of MAD-3. MAD-3 had no effect on the binding of the unrelated factor, Sp1, for its cognate site, and MAD-3 itself possessed no detectable DNA-binding properties. This observation suggests that MAD-3 is capable of interacting specifically with Rel-related proteins without necessarily inhibiting their ability to bind DNA. This possibility is supported by two earlier reports. The first of these (25) demonstrated that the ankyrin repeat-containing protein, GABP-β, can enhance the DNA binding of the Ets-related protein, GABP-α, and the second (21) reported that antibodies raised against IκB could recognize nuclear NF-κB. However, since overexpression of MAD-3 inhibited κB-dependent transactivation in all cases (Fig. 6), the stimulatory effect of MAD-3 on NFKB2(p49) and NFKB1(p50) binding does not correlate with its functional properties, and so the biological significance of this unexpected effect is unclear. MAD-3 also prevented functional activation of the HIV enhancer by NF-κB in Jurkat cells. In the absence of MAD-3, overexpression of either

NFKB2(p49), NFKB1(p50), or RelA(p65) slightly stimulated HIV-CAT through the κB site, and we have previously postulated that this low-level stimulation may be due to association with endogenous NF-κB subunits (38, 43). Cotransfection of NFKB2(p49) or NFKB1(p50) with RelA (p65), however, provided greater stimulation, particularly with NFKB2(p49). Cotransfection of a MAD-3 expression vector inhibited NFKB2(p49)/RelA(p65) or NFKB1(p50)/ RelA(p65) stimulation by 80 to 90% in all cases (Fig. 6A).

In summary, these data suggest that the protein encoded by NFKB2 plays an important role in regulation of the HIV enhancer. The role of this NF-κB subunit in the control of other viral and cellular genes is not yet fully understood. The recent identification of NFKB2 in association with translocation in a B-cell lymphoma suggests that NFKB2 may also contribute to the regulation of genes involved in cell cycle regulation or oncogenesis. Despite the considerable structural similarity of NFKB1 and NFKB2, the data presented here show that these gene products have distinct biochemical properties. These biochemical differences provide a molecular basis to explain functional differences between NFKB1 and NFKB2. These results also demonstrate that despite their similarity, these DNA-binding subunits of NF-κB differ significantly in the ability to stimulate transcription. The interactions between alternative NF-κB complexes and other cellular transcription factors would thus provide a mechanism to selectively regulate transcriptional activation of the HIV enhancer, and they suggest that the basis for the difference between the ability of NFKB2(p49) and NFKB1(p50) to activate transcription is independent of DNA-binding affinity.

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