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Mutational Analysis of the p50 Subunit of NF-κB and Inhibition of NF-κB Activity by *trans*-Dominant p50 Mutants

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The NF-κB family of DNA-binding proteins regulates the expression of many cellular and viral genes. Each of these proteins has an N-terminal region that is homologous to the c-Rel proto-oncogene product, and this Rel homology region defines both DNA binding and protein dimerization properties of the individual proteins. Most of the NF-κB family members have been shown to associate with themselves or with each other to form homodimers or heterodimers, and previous studies have shown that dimerization of NF-κB factors is necessary to provide a functional DNA binding domain. We have used site-directed mutagenesis to identify regions in the Rel homology domain of the p50/NF-κB protein that are important for DNA binding and protein dimerization. Our studies have identified mutations of p50 that interfere with DNA binding only and those that interfere with protein dimerization. Mutations of p50 which disrupt only DNA binding were still able to associate with other members of the NF-κB protein family. We demonstrate that such heterodimeric complexes inhibit transcriptional activation mediated in *trans* through a *cis*-acting κB motif; therefore, we have identified *trans*-dominant negative mutants of p50.

Recent studies have shown that the NF-κB family of DNA-binding proteins regulates the inducible expression of several human and murine genes, including those for cytokines, cell surface receptors, acute-phase proteins, and viruses (including human immunodeficiency virus), by binding to the κB elements in the promoters or enhancers of these genes (reviewed in references 2, 11, and 17). The prototypic model of NF-κB is a complex consisting of a p50-p65 heterodimer that exists in the cytoplasm of unstimulated cells and is rendered inactive by its association with one (or perhaps more) of a group of inhibitors collectively referred to as IκB. Several proteins that can bind to the complex as inhibitors have been described previously (1, 8, 15, 32). Upon stimulation of cells, IκB dissociates from the complex (probably by phosphorylation of IκB) (9) and the active p50-p65 dimer is then free to translocate to the nucleus, bind to its specific recognition sequence, and enhance transcription. Thus, the activation of transcription is mediated by a posttranscriptional event that releases the preformed NF-κB dimer from its inhibitor.

Although NF-κB was initially characterized as a complex consisting of p50 and p65, the cloning of several other closely related factors led to the discovery that a number of homo- and heterodimeric complexes may form an active NF-κB complex. Members of the NF-κB family of proteins whose genes have been cloned include p50 (5, 10, 16, 19), p50B (also referred to as Lyt-10 and p49) (4, 20, 27), RelB (25), c-Rel (and the related oncogene product, v-Rel) (12, 31), and p65 (22, 23). The *Drosophila* protein dorsal, which is important in determining ventral-dorsal polarity, is also related to the NF-κB family of proteins (13, 29). The approximately 320-amino-acid-long N-terminal regions (Rel homology regions) of these factors are highly homologous to one another and confer DNA binding, protein dimerization, and nuclear translocation properties on the respective proteins. The carboxy termini of these factors differ but may be classified into two subgroups. p50 and p50B are derived from longer precursors that contain a glycine-rich region and several so-called ankyrin repeats that prevent binding of the precursor to DNA. The mechanism that produces the active factors p50 and p50B from their precursors is unknown, but they may be cleaved from their precursor proteins by proteolysis. No glycine-rich regions or ankyrin repeats are present in the other family members; however, unique regions of the C-terminal portion of p65, RelB, and c-Rel contain sequences that mediate *trans* activation (4, 7, 18, 24, 25, 28).

When the NF-κB family members associate to form homodimers or heterodimers, the efficiency of DNA binding and the ability to *trans* activate a target sequence are determined by the specific factors that make up the complex. Dimerization of two proteins is required to provide an effective DNA-binding domain. Homodimers of p50 bind to DNA strongly but are essentially unable to transactivate because p50 lacks an activation domain (4, 24, 28). Conversely, homodimers of p65 weakly bind to DNA and are capable of transactivation (22, 24, 25, 27, 28). In vitro, heterodimers of p50 or p50B with p65, c-Rel, or RelB have been described elsewhere (3, 4, 24, 25, 27, 28, 30). A previous study (18) has shown that a deletion of up to the first 200 amino acids of the active p50 protein could still dimerize and form a *trans*-dominant negative mutant. Another report identified a region of p65 (residues 222 to 231) that contributes to multimerization (24). In this study, we have used site-directed mutagenesis to determine more specifically which regions of the Rel-homologous part of p50 are important for DNA binding and protein dimerization.

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MATERIALS AND METHODS

Site-directed mutagenesis of p50. In vitro mutagenesis was performed with a MutaGene Phagemid kit (Bio-Rad Laboratories, Richmond, Calif.) with minor modifications. A Bluescript plasmid containing nucleotides 201 to 1406 of the p50 gene was transformed into CJ236 competent cells, and uracil-containing phagemids were grown essentially as described in the kit's protocol. Extraction of single-stranded DNA was performed, and the DNA was used as a template for synthesis of the mutagenic strand. Oligonucleotides containing the desired mutation (which created a novel restriction site) were synthesized and treated with polynucleotide kinase and ATP under standard conditions. The kinase-treated oligonucleotides were annealed to the single-stranded template, and a mutagenic strand was synthesized and ligated as described in the MutaGene kit protocol except that Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio) was used instead of T4 DNA polymerase. One microliter of the reaction mixture was transformed into Library Efficiency DH5-α competent cells (Life Technologies, Inc., Grand Island, N.Y.). To screen individual colonies, plasmid DNA was isolated and mutants were identified by the presence of the appropriate new restriction site. Alternatively, colonies were screened by colony hybridization (26) using a γ-^{32}P-end-labeled oligonucleotide that contained a sequence complementary to the mutation. All mutations were confirmed by sequencing with a Sequenase 2.0 kit (U.S. Biochemicals) as described in the kit's instructions.

Subcloning of mutants into PMT2T. Fragments of the p50 gene containing mutations were cloned into the expression vector PMT2T (14) in two steps. An *Asp* 718-*Xba*I fragment from a Bluescript plasmid containing nucleotides 201 to 1716 of p50 was blunt end ligated into the single *Eco*RI site of PMT2T (now designated PMT2T wild-type p50). Subsequently an *Xho*I-*Spe*I fragment of each mutant (in Bluescript) was inserted in place of the *Xho*I-*Spe*I region of PMT2T wild-type p50. Plasmids were prepared with a plasmid kit (Qiagen, Studio City, Calif.).

In vitro transcription and in vitro translation. In vitro transcription was performed on linearized plasmid constructs with an RNA transcription kit (Stratagene, La Jolla, Calif.). Four micrograms of plasmid DNA was transcribed in 50 μl of reaction mix, and the RNA pellet was resuspended in 25 μl of 1 mM Tris (pH 7.5)–0.1 mM EDTA. The amount of transcript was estimated by running an aliquot on a Tris-borate-EDTA-agarose gel and staining the gel with ethidium bromide. In vitro translation was carried out in wheat germ extract (Promega, Madison, Wis.) with 2 to 4 μl of in vitro-transcribed RNA in 50 μl of reaction mixture. For labeled transcriptions, 50 μCi of [^{35}S]methionine (translation grade, 1,000 Ci/mmol; New England Nuclear/Dupont, Wilmington, Del.) was added. The reaction products were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to ensure approximately equivalent translation for each RNA sample.

Mobility shift assay. Mobility shift assays were carried out as previously described (6). The oligonucleotide probe used was identical to the human immunodeficiency virus κB element with the sequence (5′ to 3′) GCTACAAGGGACTT TCCGCTGGGGACTTTCCAGG. The oligonucleotide was annealed to its complementary strand and end labeled with [γ-^{32}P]ATP. Two microliters of the in vitro translation reaction mixture was used for each assay.

Immunoprecipitation of p50-RelB complexes. Prior to immunoprecipitation, the individual RNA transcripts were titrated such that approximately equal amounts of each protein were translated in vitro. For this assay, translation was performed with nuclease-treated rabbit reticulocyte lysate (Promega). RelB and p50 RNAs were cotranslated (with [^{35}S]methionine labeling) and immunoprecipitated with an anti-RelB antibody as previously described (4, 25). The translation reaction products (before and after immunoprecipitation) were run in adjacent lanes on a 29:1 SDS–10% PAGE gel under denaturing conditions.

Transient transfections and CAT assay. NTERA-2 cells were maintained in Dulbecco modified Eagle medium with 4,500 mg of D-glucose (Life Technologies) per liter, 10% fetal calf serum, 22 μg of gentamicin (Life Technologies) per ml, and 1× Pen-Strep (Biofluids, Rockville, Md.). Transient transfections and chloramphenicol acetyltransferase (CAT) assays were performed as described elsewhere (4, 21). Plasmid J-16 (kindly provided by M. Lenardo), which was used as an indicator plasmid, contains the κB site from the immunoglobulin κ light chain enhancer, a minimal *fos* promoter, and a CAT indicator gene. PMT2T p65 is an expression plasmid for the NF-κB protein p65 and has been described previously (4). The amount of each plasmid used in the transfections was chosen to yield strong *trans* activation by p65 and included 10 μg of J-16, 1.2 μg of PMT2T p65, and 2 μg of PMT2T vector or wild-type or mutant p50 per ml of transfection solution. The transfection solution (0.5 ml) was added to 60-mm-diameter tissue culture plates containing NTERA-2 cells that were seeded the day prior to transfection at 7.5×10^5 cells per plate in a total volume of 5 ml of media. Cell extracts were harvested 2 days after transfection by washing the plates three times with phosphate-buffered saline (without calcium and magnesium) and by adding 1 ml of TEN buffer (40 mM Tris [pH 7.5], 1 mM EDTA, 150 mM NaCl). Cells were harvested by gentle scraping, then pelleted, resuspended in 110 μl of 100 mM Tris, pH 7.5, and freeze-thawed three times on dry ice and at 37°C. The cell debris was pelleted, and the protein concentration of the supernatant was measured with a protein assay kit (Bio-Rad). Each CAT assay was adjusted to contain an equal amount of protein from each individual transfection. CAT assays were performed as described previously (4, 21) in a total volume of about 150 μl and were generally performed in triplicate. Fold induction was calculated by dividing the counts per minute of the test samples by the baseline counts per minute of samples from cells transfected with the J-16 construct alone.

In vivo labeling and immunoprecipitation of p50-p65 complexes. Wild-type or mutant p50 DNA subcloned into the expression vector PMT2T was cotransfected with p65 into NTERA-2 cells (5 μg of DNA each) as described above. Cells were labeled 3 days after transfection by incubation for 2 h in growth medium lacking methionine and cysteine and containing [^{35}S]methionine plus [^{35}S]cysteine (NEN; 300 μCi each per ml). Cells were harvested as described above and extracted by freeze-thawing in 100 μl of buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 0.2 mM EDTA, 0.5 mM dithiothreitol, 1.5 mM $MgCl_2$, 0.4 M NaCl, 25% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged at 40,000 rpm at 4°C for 30 min, and aliquots were immunoprecipitated with a polyclonal rabbit anti-p65 antibody as previously described (4, 25). Immunoprecipitated proteins were run on an SDS–10% PAGE gel.

TABLE 1. Identification of p50 mutations[a]

Amino acid(s)	Nucleic acid sequence		Amino acid(s)		New restriction site
	Wild type	Mutant	Wild type	Mutant	
56-57	CGT TTC	AAA GCC	Arg-Phe	Lys-Ala	*Dra*I
59-60	TAT GTA	GCT AGC	Tyr-Val	Ala-Ser	*Nhe*I
111	CAC	GCT	His	Ala	*Nhe*I
114-115	GTG GGA	GCT AGC	Val-Gly	Ala-Ser	*Nhe*I
136-137	TTC GCA	GCT AGC	Phe-Ala	Ala-Ser	*Nhe*I
137-138	GCA AAC	GCT AGC	Ala-Asn	Ala-Ser	*Nhe*I
149-150	GTA TTT	CCG CGG	Val-Phe	Pro-Arg	*Sac*II
153-154	CTG GAA	CCC GGG	Leu-Glu	Pro-Gly	*Sma*I
193-194	AAA GAG	CCC GGG	Lys-Glu	Pro-Gly	*Sma*I
197-198	CGC CAA	CCC GGG	Arg-Gln	Pro-Gly	*Sma*I
274-275	AAA GTT	GCT AGC	Lys-Val	Ala-Ser	*Nhe*I
276-277	CAG AAA	GCG CGC	Gln-Lys	Ala-Arg	*Bss*HII
320	TTT TCC	GCT AGC	Phe	Ala	*Nhe*I
326-327	CAT AGA	GCT AGC	His-Arg	Ala-Ser	*Nhe*I

[a] Mutations are named by their positions in the amino acid sequence of p50.

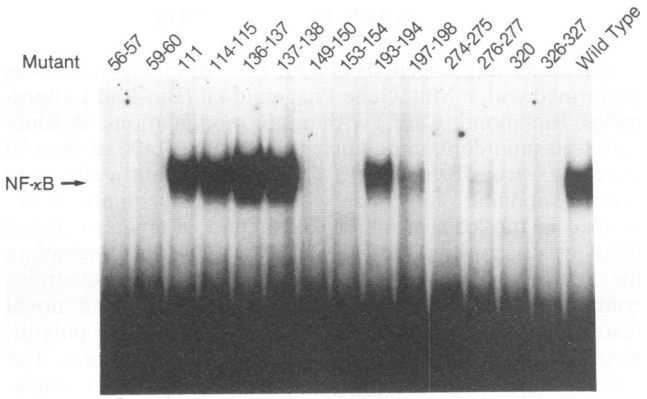

FIG. 2. Mobility shift assay of p50 mutations. In vitro translation reactions of each mutant were assayed for NF-κB binding by incubation with the NF-κB binding site from the human immunodeficiency virus enhancer. The mutant or wild-type translation mix that was used in each reaction is indicated above each lane.

RESULTS

Site-directed mutagenesis of the NF-κB p50 protein. To determine the regions of the NF-κB p50 protein important for DNA binding and protein dimerization, we used site-directed mutagenesis to make amino acid substitutions in regions that are highly conserved among the NF-κB family of proteins. Generally double amino acid changes were made throughout the sequence and were designed to introduce a new restriction enzyme site that would allow detection of the particular mutation. The locations, amino acid changes, and new restriction sites of the different mutations are shown in Table 1 and Fig. 1. Seven regions of the p50 protein were targeted with pairs of mutations very close to one another (Fig. 1). In some regions, fairly conservative changes were made; however, in a region which may be α helical (mutants 149-150, 153-154, 193-194, and 197-198) more drastic mutations were made with the introduction of helix-disrupting proline residues. In all cases the mutations were confirmed by sequencing.

Mutations in three different regions of p50 inhibit DNA binding. The mutants were first analyzed by their DNA binding properties in a mobility shift assay. RNA was transcribed in vitro from either wild-type plasmid or mutant plasmid and then translated individually in vitro.

The translation products were analyzed by mobility shift assay with the κB sequence of the human immunodeficiency virus long terminal repeat as a probe (Fig. 2). DNA binding was abrogated by mutants 56-57, 59-60, 149-150, 153-154, 274-275, 320, and 326-327. Very faint DNA binding was seen with mutant 276-277 (with longer exposures of the gel [data not shown]), and some decrease in binding was seen with mutant 197-198. Thus, DNA binding activity was affected by mutations in three different regions of the NF-κB protein. Since dimer formation of p50 is necessary to form an intact DNA binding domain (4, 10, 16), it may be anticipated that mutations which disrupt either the DNA binding domain itself or the protein dimerization region will inhibit binding to DNA.

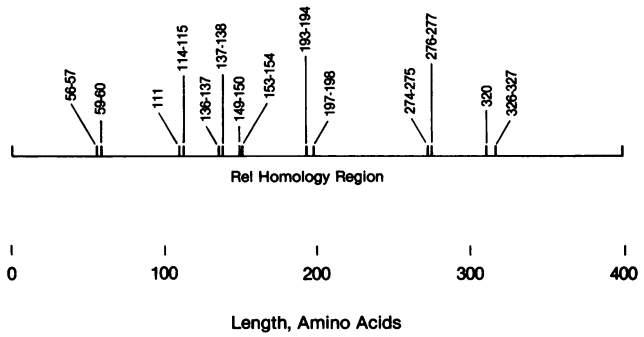

FIG. 1. Locations of mutations in the p50 protein. Amino acid mutations are named according to their position in p50. Numbers indicate the amino acid residues affected. Amino acid 1 is the initiator methionine (4, 16).

Identification of regions of p50 that are involved in protein dimerization. To determine whether certain mutations affected DNA binding only or protein dimerization, we tested the ability of each p50 mutant to coimmunoprecipitate with RelB, another member of the NF-κB family. RelB was chosen because it has been shown to heterodimerize efficiently with p50 (25) and its larger size allows it to be distinguished from p50 on SDS-PAGE gels. RelB and p50 mutant RNAs were cotranslated in a rabbit reticulocyte lysate mixture, and the labeled products were immunoprecipitated with RelB antibody and protein A-Sepharose. The immunoprecipitates were analyzed by SDS-PAGE (Fig. 3). In this figure, the labeled translation products before and after immunoprecipitation are shown for each reaction in adjacent lanes. RelB coimmunoprecipitated with wild-type p50 and all p50 mutants except for 274-275, 320, and 326-327. These results indicate that mutants 274-275, 320, and 326-327 interfered with p50/RelB dimerization. The loss of DNA binding activity on mobility shift assay by these mutations was probably the result of inhibition of p50 homodimerization. Because mutants 56-57, 59-60, 149-150, and 153-154 were able to dimerize efficiently in the immunoprecipitation assay but were unable to bind DNA on mobility shift assay, they most likely prevented DNA binding only. Our results are in agreement with those of a previous study (18) that showed a putative DNA-binding domain in the 5′ region of p50 and a protein dimerization region in the 3′ region of p50. In another recent report (24), amino acid residues 222 to 231 of p65 were found to be essential for dimerization of p65 with p50. These residues in p65 lie within the corresponding, homologous region of p50 spanned by residues 274 to 327

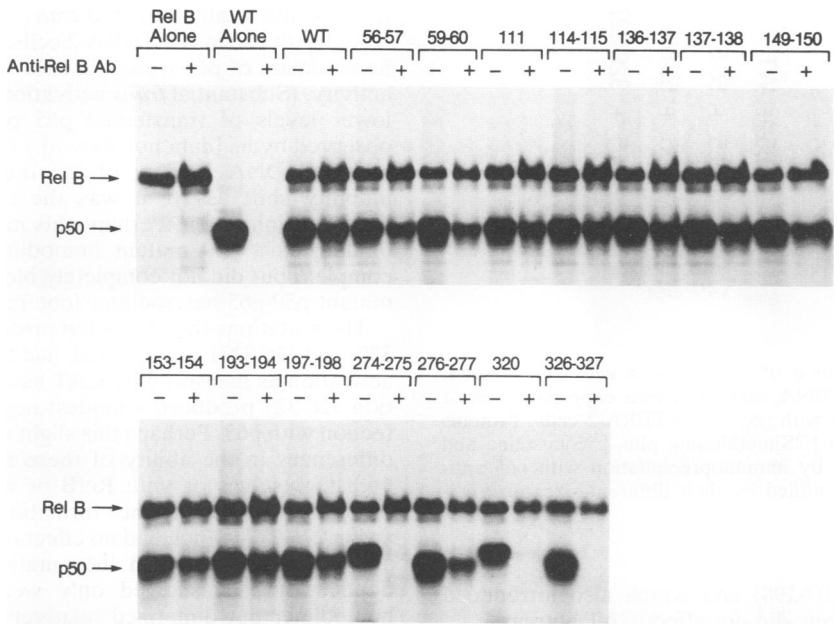

FIG. 3. Assay for heterodimerization of p50 mutants with another NF-κB protein, RelB. Mutant or wild-type p50 RNA was cotranslated with RelB RNA and analyzed for dimerization by immunoprecipitation of the complex with RelB antibody. RelB and p50 are identified by their different sizes on SDS-PAGE. The translations before (−) and after (+) immunoprecipitation are indicated. The first two lanes are RelB translations, and the next two lanes are wild-type p50 translations. These four lanes demonstrate the specificity of the RelB antibody.

(defined by our mutations). Thus, the same region controls dimerization of both p50 and p65.

Mutations that affect DNA binding but not protein dimerization can *trans*-dominantly inhibit NF-κB activity. In theory, mutations that interrupt the DNA binding domain but still permit protein dimerization could associate with wild type NF-κB protein. These complexes would be unable to bind to DNA (since they would lack a functional DNA binding domain), and they potentially could *trans*-dominantly inhibit NF-κB activity by complexing with other wild-type proteins of the NF-κB family.

The functional properties of our mutants were analyzed by transient transfections of NTERA-2 cells that express no detectable constitutive NF-κB activity (4). Wild-type and mutant p50 constructs were subcloned into an expression vector, PMT2T. Prior to subcloning, the entire sequence of the subcloned fragment was confirmed by sequencing for each of the constructs that inhibited DNA binding or protein dimerization. The effect of each mutation was analyzed by cotransfection into NTERA-2 cells with another member of the NF-κB family, p65, and an indicator CAT plasmid, J-16, that contains an NF-κB enhancer. This system was chosen for transfection since p50 lacks a *trans*-activation domain and transfection of p50 alone did not induce CAT expression. Furthermore, transfection of p65 alone yielded strong induction of CAT expression that could then be tested for *trans*-dominant inhibition by the p50 mutants. The extent of induction of CAT activity above that seen with the vector alone was determined for p65 cotransfected with wild-type p50 and each of the mutants (Fig. 4). The amount of each plasmid used in the transfections was adjusted so that the amount of p65 synthesized did not saturate induction of the indicator plasmid, J-16 (data not shown). Three of the mutations (56-57, 59-60, and 153-154) that affected DNA binding but not dimerization caused a highly significant decrease in CAT activity and hence were *trans*-dominant negative mutants; however, mutation 149-150 (that also affected the DNA binding domain) resulted in only a modest reduction of CAT expression. Mutations 149-150 and 153-154 were potentially more disruptive to the overall protein conformation, since they introduced proline residues into the sequence. Other mutations which did not affect DNA

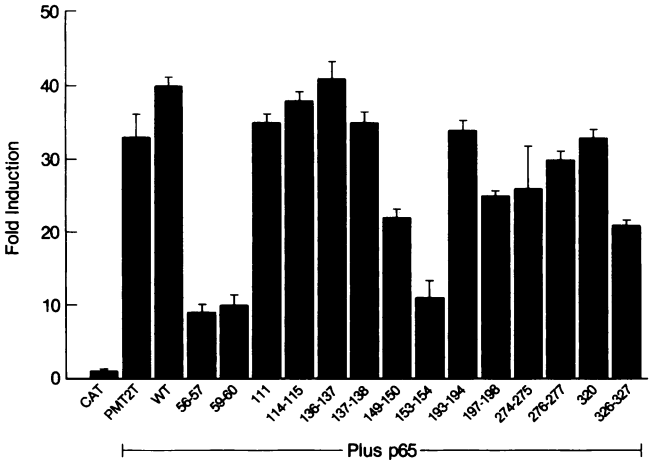

FIG. 4. Functional analysis of p50 mutants by CAT assay. Each mutant was subcloned into an expression vector and cotransfected with a p65 expression vector plus an NF-κB-CAT reporter plasmid into NTERA-2 cells. p65 is present in all transfections except the first sample (CAT). PMT2T denotes transfection of the parental plasmid construct with no insert. Induction of CAT activity was measured by dividing the counts per minute of each sample by the mean counts per minute of the reporter CAT construct alone (first bar). Each transfection was performed in triplicate, and the standard deviation is indicated on each bar. These results are representative of two independent experiments.

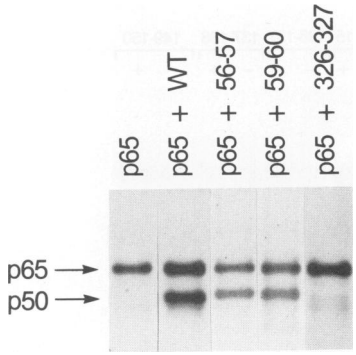

FIG. 5. Heterodimerization of p50 mutants with p65 in vivo. Mutant or wild-type p50 DNA subcloned into expression vector PMT2T was cotransfected with p65 into NTERA-2 cells. Proteins were labeled in vivo with [^{35}S]methionine plus [^{35}S]cysteine and analyzed for dimerization by immunoprecipitation with p65 antibody. p65 and p50 are identified by their different sizes on SDS-PAGE.

binding (193-194 and 197-198) and which also introduced proline residues, however, did not affect CAT activity.

To verify that the *trans*-dominant mutants (56-57, 59-60, 149-150, 153-154) dimerized with p65 in vivo, transfected cells were grown in media containing [^{35}S]methionine plus [^{35}S]cysteine, and then p65-mutant p50 ^{35}S-labeled complexes were immunoprecipitated with anti-p65 specific antibody. As seen in Fig. 5, this antibody coprecipitated mutants 56-57 and 59-60 but not mutant 326-327 (which interfered with protein dimerization). In this assay, mutants 149-150 and 153-154 also dimerized with p65 in vivo (data not shown). Thus, as was seen in vitro with RelB, the *trans*-dominant mutants dimerized efficiently with p65 in vivo.

With the possible exception of mutation 326-327, mutations that interfered with protein dimerization had little or no effect on CAT activity (274-275 and 320). These results indicate that mutants bearing several specific amino acid changes within the p50 protein which inhibit DNA binding (but not dimerization) acted as *trans*-dominant inhibitors of NF-κB activity of the p65 protein.

DISCUSSION

We have analyzed the effect of specific amino acid mutations of the Rel-homologous region of the NF-κB p50 protein. Mutations in two different areas of the 5′ Rel-homologous region of the gene resulted in a loss of DNA binding activity but did not affect protein dimerization; therefore, two separate regions of p50 may be involved in the formation of a DNA binding domain. From these studies it cannot be determined whether the mutations affected contact between the protein and its DNA recognition sequence or the tertiary structure of the protein. It is possible that the more conservative mutations in one region (56-57 and 59-60) interfered with the actual DNA binding domain whereas the more drastic mutations of the second region, which lies within a putative α-helical region (149-150 and 153-154), altered protein conformation and thus affected DNA binding indirectly. These four mutations inhibited DNA binding of p50 homodimers in mobility shift assays and exhibited *trans*-dominant inhibition of NF-κB p50/p65 in vivo, although to a variable degree. In the CAT assay, the effect of each mutant was evaluated by its ability to heterodimerize with p65 and inhibit *trans*-activation of a CAT reporter plasmid. None of the mutants totally inhibited *trans* activation when cotransfected with p65 into NTERA-2 cells, probably because some homodimers of p65 were still able to form and induce CAT activity. (Substantial *trans* activation by p65 alone at 40-fold-lower levels of transfected p65 plasmid DNA has been observed by us [data not shown].) Although mutant 149-150 inhibited DNA binding of the homodimeric complex on mobility shift assay, it was the least effective in *trans*-dominant inhibition. Perhaps this mutation prevented DNA binding as a p50 mutant homodimer (two mutations per complex) but did not completely block DNA binding by the mutant p50-p65 heterodimer (one mutation per complex).

The mutations that disrupted protein association (274-275, 320, and 326-327), as expected, had little or no effect on *trans* activation as measured by CAT assay. Of this group, mutation 326-327 produced a modest negative effect on cotransfection with p65. Perhaps this slight variability reflects subtle differences in the ability of these mutants to form dimers with themselves or with RelB or with p65. Similar subtle differences of heterodimer formation may explain why mutation 276-277, which had no effect on transactivation by p65 and which is located in the putative protein dimerization domain of p50, showed only weak DNA binding as a homodimer but dimerized relatively well with RelB in the immunoprecipitation assay. It is also conceivable that changes in the dimerization domain which have no apparent effect on dimerization may nonetheless have an indirect effect on DNA binding by altering the overall conformation of the heterodimeric complex.

Despite these considerations, our data clearly identified mutations in two separate areas of the Rel homology region of p50 that potently interrupted DNA binding and a third region in the carboxy terminus of the Rel homology region of p50 which appears essential for protein dimerization. The locations and effects specifically identified by our mutations are consistent with previous reports (18, 24) that suggested similar effects by mutations or by deletions of several amino acids. Our data further demonstrate that mutations which affect DNA binding by p50 but not dimerization can act as *trans*-dominant inhibitors of NF-κB activity, although the degree of inhibition varies. These mutations provide tools to analyze further the role of NF-κB-binding proteins in the activation of many genes that are regulated by these factors.

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REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1989. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242**:540–546.
2. **Baeuerle, P. A., and D. Baltimore.** 1991. The physiology of the NF-κB transcription factor, p. 423–446. *In* P. Cohen and J. G. Foulkes (ed.), The hormonal control regulation of gene transcription. Elsevier Science Publishers, Amsterdam.
3. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63**:803–814.
4. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R.-P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. Mol. Cell. Biol.

12:685–695.
5. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. Nature (London) **348:**76–80.
6. **Bressler, P., G. Pantaleo, A. DeMaria, and A. S. Fauci.** 1991. Anti-CD2 receptor antibodies activate the HIV long terminal repeat in T lymphocytes. J. Immunol. **147:**2290–2294.
7. **Bull, P., K. L. Morley, M. F. Hoekstra, T. Hunter, and I. M. Verma.** 1990. The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Mol. Cell. Biol. **10:**5473–5485.
8. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the Rel family of transcription factors. Science **253:**1268–1271.
9. **Ghosh, S., and D. Baltimore.** 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **334:** 678–682.
10. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62:**1019–1029.
11. **Gilmore, T. D.** 1990. NF-κB, KBF1, *dorsal*, and *rel*ated matters. Cell **62:**841–843.
12. **Grumont, R. J., and S. Gerondakis.** 1989. Structure of a mammalian c-*rel* protein deduced from the nucleotide sequence of murine cDNA clones. Oncogene Res. **4:**1–8.
13. **Ip, Y. T., R. Kraut, M. Levin, and C. A. Rushlow.** 1991. The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in Drosophila. Cell **64:**439–446.
14. **Israel, D. I., and R. J. Kaufman.** 1989. Highly inducible expression from vectors containing multiple GRE's in CHO cells overexpressing the glucocorticoid receptor. Nucleic Acids Res. **17:**4589–4604.
15. **Kerr, L. D., J.-I. Inoue, N. Davis, E. Link, P. A. Baeuerle, H. R. Bose, Jr., and I. M. Verma.** 1991. The rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev. **5:**1464–1476.
16. **Kieran, M., V. Blank, F. Logeat, J. Vanderkerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
17. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58:**227–229.
18. **Logeat, F., N. Israël, R. Ten, B. Blank, O. Le Bail, P. Kourilsky, and A. Israël.** 1991. Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant. EMBO J. **10:**1827–1832.
19. **Meyer, R., E. N. Hatada, H.-P. Hohmann, M. Haiker, C. Bartsch, U. Röthlisberger, H.-W. Lahm, E. J. Schlaeger, A. P. G. M. van Loon, and C. Scheidereit.** 1991. Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. Proc. Natl. Acad. Sci. USA **88:**966–970.
20. **Neri, A., C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. K. Chaganti, and R. Dalla-Favera.** 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene *lyt*-10, homologous to NF-κB p50. Cell **67:**1075–1087.
21. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–447.
22. **Nolan, G. P., S. Ghosh, H. C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell **64:**961–969.
23. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C.-H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251:**1490–1493.
24. **Ruben, S. M., R. Narayanan, J. F. Klement, C.-H. Chen, and C. A. Rosen.** 1992. Functional characterization of the NF-κB p65 transcriptional activator and an alternatively spliced derivative. Mol. Cell. Biol. **12:**444–454.
25. **Ryseck, R.-P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12:**674–684.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.
28. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
29. **Thisse, C., F. Perrin-Schmitt, C. Stoetzel, and B. Thisse.** 1991. Sequence-specific transactivation of the *Drosophila twist* gene by the *dorsal* gene product. Cell **65:**1191–1201.
30. **Urban, M. B., R. Schreck, and P. A. Baeuerle.** 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. **10:**1817–1825.
31. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. J. Virol. **52:**172–182.
32. **Zabel, U., and P. A. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61:**255–265.