The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression

DEAN W. BALLARD*,†, ERIC P. DIXON, NANCY J. PEFFER, HAL BOGERD, STEFAN DOERRE, BERND STEIN, and WARNER C. GREENE*

Howard Hughes Medical Institute, Department of Medicine and Department of Microbiology and Immunology, Duke University Medical Center, Durham, NC 27710

Communicated by Harold E. Varmus, November 6, 1991

ABSTRACT Molecular cloning of the polypeptide component of the Rel-related human p75 nucleoprotein complex has revealed its identity with the 65-kDa (p65) subunit of NF-κB. Functional analyses of chimeric proteins composed of NF-κB p65 C-terminal sequences linked to the DNA-binding domain of the yeast GAL4 polypeptide have indicated that the final 101 amino acids of NF-κB p65 comprise a potent transcriptional activation domain. Transient transfection of human T cells with an expression vector encoding NF-κB p65, but not NF-κB p50, produced marked transcriptional activation of a basal promoter containing duplicated κB enhancer motifs from the long terminal repeat of type 1 human immunodeficiency virus. These stimulatory effects of NF-κB p65 were synergistically enhanced by coexpression of NF-κB p50 but were completely inhibited by coexpression of the v-rel oncogene product. Together, these functional studies demonstrate that NF-κB p65 is a transactivating subunit of the heterodimeric NF-κB complex and serves as one cellular target for v-Rel-mediated transcriptional repression.

The NF-κB transcription factor complex plays a central role in the activation of type 1 human immunodeficiency virus (HIV-1) and many immunologically relevant cellular genes (1). Nuclear NF-κB DNA-binding activity is constitutively expressed in mature B cells (2) and induced in T cells by phorbol esters (3, 4), tumor necrosis factor α (5, 6), and the Tax protein of the type 1 human T-cell leukemia virus (HTLV-I) (7–9). Nuclear NF-κB expression is regulated in part at a posttranslational level (3) involving its dissociation from the cytoplasmic inhibitor IκB (10, 11). This dissociation reaction is apparently catalyzed by the phosphorylation of IκB, which in turn allows the rapid translocation of NF-κB to the nucleus (10–12).

Sequence analyses of human and murine cDNAs encoding the DNA-binding subunit (NF-κB p50) (13, 14) and the IκB-binding subunit (NF-κB p65) (15, 16) of NF-κB have revealed extensive homology between the N-terminal 300 amino acids of these two polypeptides, the v-Rel oncoprotein, and the dorsal gene product, a ventral morphogen in Drosophila (17). v-Rel specifically engages the κB enhancer and acts as a dominant negative repressor of NF-κB in mature T cells (18–20). However, the molecular basis for the transcriptional activation mediated by the NF-κB p50/p65 complex or transcriptional repression mediated by v-Rel has remained unknown.

Although gel retardation analyses with purified NF-κB originally suggested that only NF-κB p50 had intrinsic DNA-binding activity (21, 22), recent DNA/protein crosslinking studies with human T cell extracts have demonstrated four Rel-related κB-specific proteins that are expressed in a temporally biphasic manner (p55/p75, early; p50/p85, late) (7, 18, 23). The p55 and p50 adducts contain NF-κB p50 and a closely related protein, respectively (18), while the p85 adduct is composed of the c-rel protooncogene product (c-Rel) (18), which has been shown to contain a functional transactivation domain (20, 24, 25).

The ability of p75 to dimerize with NF-κB p50 suggested a potential relationship to NF-κB p65 (18); however, the lack of intrinsic DNA-binding activity reported for NF-κB p65 (21, 22) implied that these two proteins were distinct. In the present study, we have cloned p75 cDNAs based on peptide sequence information derived from the affinity-purified protein (18). We have demonstrated the complete identity of p75 and NF-κB p65 at the protein level. Transient transfection studies show that this enhancer-binding protein functions as a powerful transcriptional transactivator and a target for v-Rel-mediated repression.

MATERIALS AND METHODS

Isolation of p75 cDNA. Human p75 was partially purified from HeLa cytosolic extracts (18) and further fractionated by preparative SDS/PAGE (26) prior to digestion with cyanogen bromide (18 hr at 25°C) (27). Cleavage products were fractionated by reverse-phase HPLC and analyzed on an Applied Biosystems gas-phase sequenator. A degenerate antisense oligonucleotide probe (5′-GTCACTGTRTCGGIAGRTAC-TGGAAA-3′) was designed (28) based on peptide B (see text) and used to screen (29) a human HL-60 cDNA library. Nucleotide sequences of cross-hybridizing clones were determined by the dyeode chain-termination method (30).

DNA Binding and Peptide Mapping Studies. UV crosslinking and peptide mapping were performed (18, 31, 32) using a palindromic variant of the κB enhancer of the interleukin 2 receptor α-chain gene (5′-CAAGCCAGG-GAATTCCCTCTCCT-3′); this variant serves as a particularly potent binding site (18). Endoproteases used for peptide mapping (23) were added at an enzyme/protein ratio of 1:25.

Expression Vectors. To produce chimeric GAL4 expression vectors, C-terminal coding sequences of various Rel family members were ligated downstream of the first 147 codons of GAL4 in the pSG424 expression vector (33). The GAL4/VP16 expression vector has been described (33). The human c-rel cDNA clone (34) was generated by RNA amplification (18). All Rel expression vectors, including those encoding NF-κB p50 (amino acids 1–462; ref. 14), NF-κB p65 (16), and v-Rel (35), were constructed in pCMV4 (36). The chimeric gene encoding the NF-κB p50 Rel domain fused to the NF-κB p65 C terminus was constructed by amplification with over-

Abbreviations: CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus; LTR, long terminal repeat.
*Present address: Gladstone Institute of Virology and Immunology, P.O. Box 41900, San Francisco, CA 94141-9100.
†To whom reprint requests should be addressed at *.

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lapping primers (37). A blunt-ended Esp I-EcoRI fragment encoding the C-terminal 214 amino acids of human NF-κB p65 was fused to the HindII site in the v-Rel coding sequence to create the v-Rel/NF-κB p65 expression vector. An expression vector encoding the C-terminal 226 amino acids of NF-κB p65 alone was constructed by amplification of the corresponding 3′ cDNA sequences with oligonucleotide primers that introduced optimal translation initiation sequences (38).

Cell Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. COS cells or human Jurkat T cells were transfected with plasmid DNA by the DEAE-dextran method (39) and were assayed for CAT activity as described (40, 41). Reporter plasmids containing either the full-length HIV-1 long terminal repeat (LTR) (42) or five GAL4 binding sites (pG5BCAT; ref. 33) have been described. The pBLCAT-based (43) constructs contained either two HIV-1 κB enhancer repeats (~105 to ~79; κB-TATA-CAT) or five tandem phorbol ester-responsive elements (~72 to ~65; AP1-TATA-CAT) cloned immediately upstream of a basal albumin promoter (TATA-CAT) (44).

RESULTS

Molecular Cloning of p75 and Its Relationship to NF-κB p65.

To determine the primary structure of p75, microsequencing was performed on three peptides derived from the affinity-purified protein. Peptides A and B corresponded precisely to sequences present in human NF-κB p65 (16) (Fig. 1A). In contrast, peptide C was lacking in the original reported sequence of NF-κB p65. However, sequencing of an independently isolated p75 cDNA revealed the presence of three undetected nucleotides that alter the amino acid sequence between residues 372 and 380 to conform precisely with that of peptide C (Fig. 1B). This correction has been confirmed (C. Rosen, personal communication) and leads to 551 amino acids rather than 550 in the full-length NF-κB p65 polypeptide. These primary structural correlates suggested that p75 was either identical to NF-κB p65, a posttranslational variant, or the product of an alternatively spliced transcript.

To further explore the relationship between p75 and NF-κB p65, DNA/protein crosslinking studies were performed with partially purified p75 (18) and extracts from COS cells transfected with an NF-κB p65 cDNA expression vector. First, radiolabeled DNA/protein adducts were subjected to immunoprecipitation with antiserum specific for peptide A. Radiolabeled adducts containing NF-κB p65 (Fig. 2A, lane 6) and p75 (lane 7) were specifically immunoprecipitated with this antiserum, but not by preimmune serum (lanes 3 and 4). Notably, the immunoreactive adducts (70–75 kDa) formed with p75 and NF-κB p65 displayed indistinguishable electrophoretic mobilities in the presence of the bound DNA probe.

![Fig. 1. Comparison of the primary structures of p75 and NF-κB p65. (A): Sequence analysis was performed on three HPLC-purified p75 peptides (peptides A, B, and C). Relative positions of the resultant amino acid sequences in the predicted primary structure of human NF-κB p65 (16) are indicated. Numbers above the NF-κB p65 block diagram refer to amino acid position. (B) DNA sequence analysis of a partial p75 cDNA. Previously undetected nucleotides that alter the original published amino acid sequence for NF-κB p65 (underlined residues), but that are consistent with the amino acid sequences of peptide C, are shown in bold type.]

![Fig. 2. Human NF-κB p65 corresponds to a κB enhancer-binding protein identical to p75. (A) Partially purified HeLa p75 (lanes 4 and 7) or extracts from COS cells transfected with a pCMV4 expression vector lacking (lanes 2 and 5) or containing (lanes 3 and 6) an NF-κB p65 cDNA were mixed with a 32P-radioabeled κB enhancer probe and UV-irradiated. Radiolabeled crosslinked adducts were immunoprecipitated with either preimmune serum (lanes 2–4) or anti-peptide A antiserum (lanes 5–7) and analyzed by SDS/PAGE. Molecular weight standards are shown in lane 1 (Mr, × 10–3 at left). (B) Radiolabeled adducts corresponding to HeLa p75 (lanes 2, 4, 6, and 8) and COS NF-κB p65 (lanes 1, 3, 5, and 7) were subjected to partial proteolysis with the indicated endoproteases. Cleavage products retaining covalently bound 32P-labeled DNA were separated by SDS/PAGE and detected by autoradiography. Control samples run in lanes 1 and 2 correspond to undigested adducts.]

Based on renaturation studies, the factors giving rise to these two adducts were each capable of independently engaging the κB enhancer and comigrated during SDS/PAGE (ref. 18 and data not shown). Furthermore, the partial cleavage patterns for these two nucleoprotein adducts produced by digestion with Arg-C, Asp-N, or Lys-C endoproteinase proved identical (Fig. 2B). These findings indicate that NF-κB p65 mediates the formation of the p75 crosslinked adduct and, together with NF-κB p50, corresponds to the two κB enhancer-binding proteins that are rapidly expressed as a heterodimeric complex in the nucleus following human T-cell activation (18, 23).

**NF-κB p65 Contains a Potent Transcriptional Activation Domain.** To determine whether NF-κB p65 contains a func-
tional transactivation domain, chimeric proteins containing the DNA-binding domain (amino acids 1–147) (45) of the yeast transcription factor GAL4 were coexpressed in COS cells together with a CAT reporter construct containing five GAL4 binding sites. Fusion of the GAL4 DNA-binding domain with the acidic activation domain of the herpes simplex virus activator VP16 (46) produced a predictably strong chimeric activator (33) that stimulated transcription 40- to 50-fold above basal levels (Fig. 3A). However, replacement of the acidic activation domain of VP16 with the C terminus of NF-κB p65 (amino acids 313–551) yielded a chimeric protein that activated GAL4-directed transcription even more potently than GAL4/VP16. Consistent with previous findings (20, 24, 25), hybrid GAL4 proteins containing the C-terminal half of human c-Rel (amino acids 297–587) also activated transcription, albeit at 6- to 10-fold lower levels than that observed with GAL4/NF-κB p65. In contrast, GAL4 fusion constructs containing the C-terminal portion of v-Rel (amino acids 305–503) failed to significantly activate transcription from this promoter (20).

To localize the NF-κB p65 transactivation domain, we next constructed a series of GAL4/NF-κB p65 hybrids with deletions between amino acids 313 and 551 (Fig. 3B). Progressive deletion from amino acid 313 to 450 resulted in no loss of function. In contrast, further deletion of amino acids 451–501 markedly reduced (∼90%) the capacity of the resulting hybrid protein to activate GAL4-directed transcription. In reciprocal 3' deletion studies, removal of amino acids 502 to 551 from the C terminus of NF-κB p65 diminished the transactivation capacity conferred to GAL4 by ∼50%. Further truncation to amino acid 451 virtually abolished this residual activity. These studies suggest that the C-terminal 101 amino acids of NF-κB p65 are required for maximal activation. However, C-terminal deletion analysis of the full-length NF-κB p65 protein indicated that additional sequences between amino acids 414 and 450 also are important for the activation of κB-directed transcription (data not shown).

**Fig. 3.** Transcriptional activation by GAL4/NF-κB p65 chimeras. (A) COS cells were cotransfected with a CAT reporter plasmid containing five GAL4 binding sites (pG-B-CAT, 2 μg) and expression vectors (4 μg) encoding various chimeras (schematically represented on left) containing the DNA-binding domain of GAL4 (amino acids 1–147) fused to either the transactivation domain of VP16 or the C terminus of NF-κB p65, c-Rel, or v-Rel. Cells were assayed for CAT activity after 48 hr and the data are presented (histogram on right) as fold induction over basal levels obtained with the truncated GAL4 DNA-binding domain. Results were normalized for protein recovery and expressed as the mean fold increase in CAT activity ± SEM. (B) COS cells were cotransfected with pG-B-CAT (2 μg) and GAL4 expression vectors (4 μg) encoding truncated forms of the NF-κB p65 C terminus (amino acids 313–551) fused to the DNA-binding domain of GAL4 (amino acids 1–147). The N- and C-terminal endpoints of these deletion mutants are schematically depicted on the left. CAT activities are expressed as the mean fold increase in CAT activity ± SEM relative to basal levels measured with GAL4 alone. Comparable levels of protein expression were confirmed by immunoprecipitation with anti-GAL4 antibodies (data not shown).

**Fig. 4.** Functional analysis of chimeric and native Rel-related polypeptides. Jurkat T cells were cotransfected with CAT reporter plasmids (5 μg) containing either two tandem κB enhancer elements from the HIV-1 LTR (κB–TATA–CAT), five tandem AP1 binding sites (AP1–TATA–CAT), or the enhancer-deleted basal promoter (TATA–CAT) in the presence or absence of pCMV4 expression vectors encoding various Rel-related polypeptides. (A) Fusion and deletion endpoints of each chimeric or truncated protein, which were expressed at comparable levels when introduced into COS cells (data not shown). (B) Relative CAT activities measured after 48 hr of culture and expressed as the mean fold increase in CAT activity ± SEM.

Fusion with NF-κB p65 Converts v-Rel or NF-κB p50 into Strong κB-Specific Transactivators. To extend this functional analysis, expression vectors encoding various Rel-related polypeptides (Fig. 4A) were cotransfected in Jurkat human T cells with a CAT reporter plasmid containing two HIV-1 κB enhancers (κB–TATA–CAT). Cotransfection of expression vectors encoding full-length or truncated forms of the v-Rel oncoprotein failed to stimulate transcription from this promoter (Fig. 4B). However, replacement of v-Rel sequences downstream of the Rel homology domain with NF-κB p65 C-terminal sequences resulted in a chimeric protein that induced κB-specific promoter activity by >50-fold. While NF-κB p50 alone produced ≤5-fold stimulation, fusion of the C terminus of NF-κB p65 to the Rel homology domain of NF-κB p50 produced 55-fold stimulation. Neither of these chimeric Rel polypeptides activated CAT reporter constructs containing five AP1 binding sites (AP1–TATA–CAT) or the enhancer-deleted basal promoter (TATA–CAT), suggesting
that these transactivation events are contingent upon \( \kappa B \) enhancer binding by the N-terminal Rel homology domain. Consistent with this interpretation, transient expression of the NF-\( \kappa B \) p65 C terminus (residues 286–551), which lacks a DNA-binding domain, failed to stimulate \( \kappa B \)-directed transcrip-
tion. However, fusion of this disarmed C-terminal segment to the Rel homology domain of NF-\( \kappa B \) p65 reconsti-
tuted a potent native transactivator protein.

NF-\( \kappa B \) p50 Functionally Synergizes with NF-\( \kappa B \) p65, Whereas v-Rel Represses NF-\( \kappa B \) p65-Mediated Activation.

The predominant inducible nucleoprotein complex detected with crude nuclear extracts corresponds to an NF-\( \kappa B \) p50/ p65 heterodimer (18, 21, 23). These findings prompted us to examine whether coexpression of these two NF-\( \kappa B \) subunits might synergistically activate \( \kappa B \)-dependent transcription. To test this hypothesis, Jurkat T cells were transfected with either \( \kappa B \)-TATA-CAT or the HIV-1 LTR-CAT reporter plasmid and graded doses of an NF-\( \kappa B \) p50 expression vector in the presence or absence of a fixed suboptimal amount of the NF-\( \kappa B \) p65 expression vector (Fig. 5 A and B). In the absence of NF-\( \kappa B \) p50, the \( \kappa B \)-TATA-CAT and HIV-1

**FIG. 5.** Divergent functional effects of NF-\( \kappa B \) p50 and the v-Rel oncoprotein on NF-\( \kappa B \) p65-activated transcription. (A and B) Synergistic activation of \( \kappa B \)-dependent transcription by NF-\( \kappa B \) p50 and NF-\( \kappa B \) p65. Jurkat T cells were cotransfected with either the \( \kappa B \)-TATA-CAT or the HIV-1 LTR-CAT reporter plasmid and graded amounts of an NF-\( \kappa B \) p50 CDNA expression vector (pCMV4- p50) in the presence (\( a \)) or absence (\( c \)) of a suboptimal amount (0.5 \( \mu g \)) of the NF-\( \kappa B \) p65 CDNA expression vector. (C and D) Repression of NF-\( \kappa B \) p65-activated transcription by v-Rel. Jurkat T cells were cotransfected with either the \( \kappa B \)-TATA-CAT or the HIV-1 LTR-CAT reporter plasmid and graded amounts of a v-Rel CDNA expression vector (pCMV4-v-Rel) in the presence (\( a \)) or absence (\( c \)) of a fixed amount of the NF-\( \kappa B \) p65 CDNA expression vector (0.5–2 \( \mu g \)). The amount of DNA for all transfections was held constant by the addition of unmodified pCMV4 expression vector. Results from duplicate assays are expressed as the mean fold increase in CAT activity relative to that measured with CAT reporter constructs alone.

LTR–CAT transcription units were induced approximately 24- and 3-fold, respectively, by the limited amounts of NF-\( \kappa B \) p65. When transfected alone, NF-\( \kappa B \) p50 produced only weak activation of the \( \kappa B \)-TATA-CAT and essentially no stimulation of the HIV-1 LTR, compared with NF-\( \kappa B \) p65. However, combinations of NF-\( \kappa B \) p50 and NF-\( \kappa B \) p65 produced synergistic activation of both reporter units in a dose-dependent fashion (Fig. 5 A and B). At high concentra-
tions of p50, moderate inhibition of this response was ob-
tained, presumably due to the formation of transcriptionally inactive p50 homodimers.

v-Rel is a dominant negative repressor of NF-\( \kappa B \)-
dependent transcription induced by cellular activation with either phorbol ester or the Tax protein of HTLV-I (18, 19). We therefore assessed the ability of v-Rel to specifically repress NF-\( \kappa B \) p65-activated transcription in the absence of these cellular activation agents. In contrast to the costimulatory effects of NF-\( \kappa B \) p50 and NF-\( \kappa B \) p65, cotransfection of NF-\( \kappa B \) p65 with a v-rel expression vector encoding the full-length oncoprotein completely inhibited NF-\( \kappa B \) p65-
mediated activation of these same \( \kappa B \)-directed transcription units in a dose-dependent manner (Fig. 5 C and D). These observed antagonistic effects suggest that the 65-kDa subunit of NF-\( \kappa B \) represents one cellular target for v-Rel-mediated transcrip-
tional repression.

**DISCUSSION**

Human NF-\( \kappa B \) p65 is a Potent Transcriptional Activator. Transient CDNA expression studies have revealed that NF-\( \kappa B \) p65, but not NF-\( \kappa B \) p50, functions as a potent activator of \( \kappa B \) enhancer-directed transcription in human Jurkat T cells. The domain required for this transactivation function has been localized to the C-terminal 101 amino acids of NF-\( \kappa B \) p65. Consistent with the modular organization of eukaryotic transcription factors (47, 48), this transactivation function is fully preserved when the C-terminal half of NF-\( \kappa B \) p65 is fused to a heterologous DNA-binding domain (GAL4) or to the Rel homology domains of either v-Rel or NF-\( \kappa B \) p50. However, the Rel homology domain could indirectly modu-
late this transactivation function through its capacity to mediate dimerization with other Rel-related polypeptides (17). In this regard, we found that coexpression of NF-\( \kappa B \) p65 and NF-\( \kappa B \) p50 led to synergistic functional activation of \( \kappa B \)-dependent transcription units, relative to the expression of either protein alone. These synergistic functional effects most likely reflect the ability of NF-\( \kappa B \) p65 and NF-\( \kappa B \) p50 to form a heterodimeric complex that more avidly engages the \( \kappa B \) enhancer than does the NF-\( \kappa B \) p65 protein alone (18, 21).

Human NF-\( \kappa B \) p65 is a Cellular Target for v-Rel-Mediated Transcriptional Repression. We have previously shown that v-Rel, but not nontransforming v-Rel mutants, binds to the \( \kappa B \) enhancer and inhibits NF-\( \kappa B \)-activated transcription from the interleukin 2 receptor \( \alpha \)-chain promoter and the HIV-1 LTR in human T cells (18). These findings suggested a potential functional link between this NF-\( \kappa B \) inhibitory activity and cellular transcription mediated by v-Rel. We show here that fusion of the NF-\( \kappa B \) p65 transactivation domain to the N-terminal 331 amino acids of v-Rel converts this protein into a potent transactivator of \( \kappa B \)-directed transcrip-
tion. Furthermore, v-Rel completely inhibits NF-\( \kappa B \) p65-activated transcription when provided in trans, thus establish-
ing NF-\( \kappa B \) p65 as at least one cellular target for v-Rel-mediated transcriptional repression. Given their related DNA-binding activities (18), v-Rel may act as a com-
petitive inhibitor of NF-\( \kappa B \) p65 binding to the \( \kappa B \) enhancer. This potential mechanism for v-Rel-mediated repression is supported by the finding that mutations which disrupt its DNA-binding function but not heterodimerization with
NF-kB also abolishes its repressor activity (ref. 18 and unpublished data).

Potential Implications for the Regulation of NF-kB Function.

The data presented here and in prior reports (13–16, 18) establish that the predominant kB enhancer-binding proteins detected in DNA/protein crosslinking assays of human T-cell extracts correspond to at least three unique gene products, including NF-kB p50, NF-kB p65, and c-Rel. Our results demonstrate that NF-kB p65, but not NF-kB p50, functions as a potent transcriptional activator of kB enhancer-containing promoters. Of note, c-Rel also contains a transcription activation domain in its C terminus (20, 24, 25) but appears able to only weakly activate kB-specific transcription (19). Furthermore, in contrast to the rapid nuclear induction of NF-kB p65 occurring during T-cell activation, the nuclear expression of c-Rel is induced with delayed kinetics and, in part, is dependent upon de novo protein synthesis (23).

These findings suggest an attractive mechanism to regulate the functional activities of NF-kB, which involves the ability of NF-kB p50 to dimerize with either NF-kB p65 or c-Rel. Specifically, following T-cell stimulation, NF-kB p50/p65 heterodimers are rapidly mobilized to the nucleus to participate in transcriptional activation of early genes under NF-kB control (23). The extent of this induced expression, and perhaps the transcriptional activation of late genes under NF-kB control, may be temporally governed by the delayed nuclear import and action of distinct NF-kB p50/c-Rel heterodimers (23). Further studies on the biological relationship between these differentially induced heterodimers should provide important insights concerning their regulatory roles in the genetic program controlling T-cell activation and growth.

We thank J. Kadonaga, M. Hnatowich, and J. Ostrowski for critical discussions regarding this work; S. Ballard and J. Nevins for HeLa cytotoxicity extracts; M. Ptashne for GAL4 plasmids; S. Ruben and C. Rosen for the NF-kB p65 cDNA clone; R. Randall for amino acid sequence analysis; J. Didsbury for the HL-60 cDNA library; and B. Kissell for manuscript preparation.