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The c-*rel* protooncogene product represses NF-κB p65-mediated transcriptional activation of the long terminal repeat of type 1 human immunodeficiency virus

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ABSTRACT **The long terminal repeat (LTR) of the type 1 human immunodeficiency virus (HIV-1) and the 5′ regulatory region of the gene encoding the interleukin 2 receptor α subunit (IL-2Rα) share functional κB enhancer elements involved in the regulation of these inducible transcription units during T-cell activation. These κB enhancer elements are recognized by a structurally related family of interactive proteins that includes p50, p65, and the product of the c-*rel* protooncogene (c-Rel). Recent biochemical studies have shown that p65 and p50 form the prototypical NF-κB complex, which is rapidly translocated from the cytoplasm to the nucleus during T-cell activation. This intracellular signaling complex potently stimulates κB-directed transcription from either the HIV-1 LTR or the IL-2Rα promoter via the strong transactivation domain present in p65. We now demonstrate that nuclear expression of human c-Rel, which is induced by either phorbol ester or tumor necrosis factor α with delayed kinetics relative to p65, markedly represses p65-mediated activation of these transcription units. These inhibitory effects of c-Rel correlate with its DNA-binding activity but not with its ability to heterodimerize with p50, suggesting that c-Rel inhibition involves competition with p50/p65 for occupancy of the κB enhancer element. Together, these findings suggest that one function of c-Rel is as a physiologic repressor of the HIV-1 LTR and IL-2Rα promoters, serving to efficiently counter the strong transcriptional activating effects of p65.**

The NF-κB transcription factor (1–3) has been implicated in the transcriptional control of a variety of genes that are induced during T-cell activation, including those encoding the α subunit of the interleukin 2 receptor (IL-2Rα) (4) and the type 1 human immunodeficiency virus (HIV-1) (5). The expression of these transcription units can be triggered by multiple stimulants, including phorbol esters (2, 4), tumor necrosis factor α (6, 7), and the Tax protein of the type I human T-cell leukemia virus (HTLV-I) (8–10). These same activation agents also induce the nuclear expression of NF-κB, at least in part, through a posttranslational mechanism involving its dissociation from a cytoplasmic inhibitor(s) termed IκB (11–13).

The active nuclear form of NF-κB is a heterodimeric complex composed of two DNA-binding subunits, termed p50 and p65 (14). Both of these subunits share extensive (≈300 amino acids) N-terminal sequence homology with the c-*rel* protooncogene product (c-Rel) (refs. 15–19; reviewed in ref. 20). Like p65, c-Rel is present in nuclear and cytoplasmic NF-κB complexes (21, 22), is capable of binding directly to the κB enhancer (22), and can associate with p50 (23). However, while p65 is expressed in the nucleus within minutes following T-cell activation with phorbol esters, the induced expression of nuclear c-Rel is largely delayed for several hours (21). Recent functional studies with cloned cDNAs encoding p50 and p65 have demonstrated that NF-κB exerts strong activating effects on the HIV-1 long terminal repeat (LTR) and IL-2Rα promoter principally through a potent transactivation domain located in the C-terminal portion of p65 (24–26). In this regard, c-Rel also appears to contain a C-terminal transactivation domain (27–30); however, this domain lacks homology with the corresponding functional domain present in p65.

In this report, we provide evidence that p65 and c-Rel function as integral components of a counter-regulatory network of Rel-related transcription factors in human T lymphocytes. Specifically, these two sequentially induced factors display striking differences in their ability to activate κB enhancer-mediated transcription from the HIV-1 LTR. Further, when these Rel proteins are coexpressed, c-Rel markedly inhibits p65-activated transcription from either the HIV-1 LTR or the IL-2Rα promoter. Mutational analyses indicate that this dominant repressor activity of c-Rel involves its DNA-binding function rather than dimerization properties. Thus, in addition to its weak transcriptional activating effects, c-Rel also has the capacity to act as a specific inhibitor of nuclear p65 function. As such, competition between these two Rel proteins at the κB enhancer site may constitute a dynamic mechanism for positive and negative regulation of NF-κB-directed transcription.

MATERIALS AND METHODS

Expression Vectors. All *rel*-related cDNAs were inserted into pCMV4 (31) downstream of the human cytomegalovirus (CMV) immediate-early promoter. The wild-type human c-Rel (hc-Rel) expression vector contained a full-length cDNA generated by RNA amplification (19, 22). Nested C-terminal deletions of hc-Rel were produced by cloning truncated forms of this cDNA into a modified pCMV4 vector containing a universal translational terminator. Plasmids denoted c-Rel(1–298) and c-Rel(1–289) were created by using the polymerase chain reaction (PCR) to introduce a translational termination codon after codons 298 and 289, respectively. Plasmids expressing the N-terminal deletion series c-Rel(33–587), c-Rel(125–587), c-Rel(184–587), and c-Rel(291–587) were constructed by amplification with oligonucleotide primers that introduced a consensus translation initiation site (32). The human p50 expression plasmid was generated by inserting into

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Abbreviations: CAT, chloramphenicol acetyltransferase; hc-Rel, human c-Rel; HIV-1, type 1 human immunodeficiency virus; HTLV-I, type I human T-cell leukemia virus; IL-2Rα, interleukin 2 receptor α subunit; LTR, long terminal repeat.

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pCMV4 a *Rsa* 1-truncated form of the full-length KBF1 cDNA (16) which encodes an ≈43-kDa protein (aa 1–400). Expression vectors encoding human p65 (18, 26), the Tax protein of HTLV-1 (cTAX) (33), and the Tat protein of HIV-1 (cTAT) (33) were as previously described.

Transient Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. Human Jurkat T cells were transfected with effector and reporter plasmids by using DEAE-dextran and were assayed for CAT activity as described (26). Reporter plasmids contained the bacterial CAT gene positioned immediately downstream of either the full-length LTRs of HIV-1 or HTLV-I (34), the IL-2Rα promoter (nt −317 to +109) (35), or the HIV-1 κB enhancer (nt −105 to −79) inserted immediately upstream of a basal albumin promoter (κB-TATA-CAT) (36).

DNA–Protein Interactions. Expression vectors encoding wild-type and mutated Rel proteins were transfected into monkey COS-7 cells by a modified DEAE-dextran procedure (37). Recipient cells were metabolically radiolabeled with [^{35}S]cysteine 40–48 hr after transfection (37) and lysed in a low-stringency buffer containing 50 mM Hepes (pH 7.0), 250 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. DNA–protein crosslinking reactions (20 μl) were performed using a ^{32}P-labeled palindromic κB enhancer probe (κB-pd; 5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′) (22). Radiolabeled DNA–protein adducts were immunoprecipitated with hc-Rel-specific antiserum and analyzed by SDS/10% PAGE.

Protein–Protein Interactions. ^{35}S-labeled proteins were synthesized by *in vitro* transcription/translation using rabbit reticulocyte lysates (Promega). C-terminal deletions of hc-Rel were translated from run-off transcripts synthesized from linearized c-*rel* cDNA templates cloned into pGEM-3 (22). Similarly, pGEM-3 expression vectors encoding N-terminal c-Rel deletion mutants were generated by PCR as described above. Human p50 was expressed from a pBluescript (Stratagene) vector containing the full-length KBF1 cDNA (a gift from A. Israel; ref. 16) truncated at an internal *Rsa* I site. Protein–protein interactions were assessed by immunoprecipitation of *in vitro* translation mixtures with rabbit anti-peptide antibodies specific for either human c-Rel (aa 132–150 or 573–587), or p50 (aa 1–21). All immunoprecipitations were analyzed by SDS/10% PAGE.

Immunoblot Analyses. Nuclear extracts were prepared from Jurkat T cells as described (38). Protein samples (20 μg) were fractionated under reducing conditions by SDS/7.5% PAGE followed by electrotransfer onto nitrocellulose membranes. Rel polypeptides were detected with anti-peptide antisera raised against the C terminus of either c-Rel (aa 573–587) or p65 (aa 529–551), using enhanced chemiluminescence (Amersham).

RESULTS

Human p65 and c-Rel Exhibit Markedly Different κB-Specific Transcriptional Activating Properties. To directly compare the transactivation potentials of p65 and c-Rel in human T lymphocytes, various amounts of pCMV4 expression plasmids encoding each of these proteins were transiently expressed in Jurkat T cells together with CAT reporter plasmids containing either the full-length HIV-1 LTR (HIV-1 LTR-CAT; Fig. 1*A*) or the HIV-1 κB enhancer (κB-TATA-CAT; Fig. 1*B*). Consistent with prior reports (24–26), p65 markedly induced transcription from both promoters (10- to 100-fold), even at low doses (1–2 μg) of input effector plasmid. In contrast, cotransfection with even higher doses (5 μg) of the c-Rel expression vector failed to activate the HIV-1 LTR (Fig. 1*A*) and stimulated transcription from the more sensitive κB-TATA-CAT template at least an order of magnitude less than that observed with p65 (Fig. 1*B*). Of note, deletion of the C-terminal 72 aa from the wild-type c-Rel protein, a subregion which overlaps sequences previously implicated in c-Rel-mediated transactivation (27), virtually abolished these modest stimulatory effects on the HIV-1 κB enhancer (Fig. 1*C*).

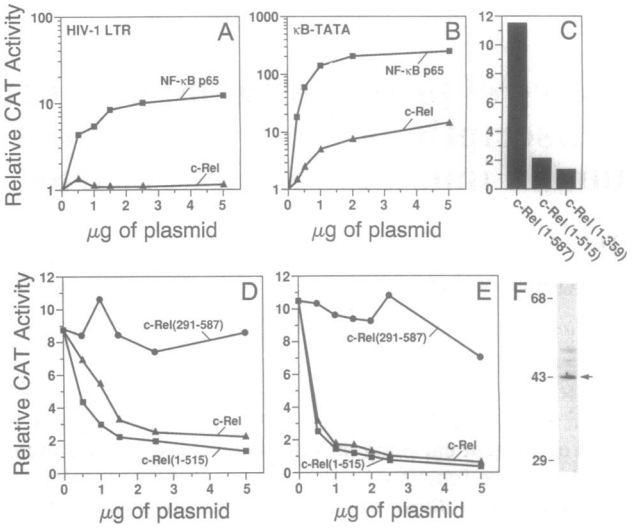

FIG. 1. Relative transcriptional activities of p65 and c-Rel. (*A* and *B*) Jurkat T cells (5 × 10^6) were transfected with CAT reporter plasmids (2.5 μg) containing either the full-length HIV-1 LTR (HIV-1 LTR-CAT) (*A*) or the HIV-1 enhancer (κB-TATA-CAT) (*B*) and various amounts of a c-Rel (▲) or p65 (■) cDNA expression vector. Input DNA concentration was held constant in all transfections by inclusion of the parental pCMV4 expression vector lacking a cDNA insert. CAT activities were determined after 40 to 48 hr and are presented as fold induction relative to the basal levels measured in the absence of either effector plasmid. Similar results were obtained in two independent experiments. (*C*) Expression plasmids (5 μg) encoding wild-type c-Rel (aa 1–587) or C-terminal deletion mutants truncated at aa 515 and 359 were cotransfected with the κB-TATA-CAT reporter plasmid into Jurkat cells. Relative CAT activities were determined as indicated above. (*D* and *E*) Jurkat T cells (5 × 10^6) were cotransfected with HIV-1 LTR-CAT (2.5 μg) and either a mixture of p50 (1 μg) and p65 (2.5 μg) expression vectors (*D*) or 2 μg of an HTLV-I Tax expression vector (*E*) along with various doses of plasmids encoding wild-type (▲) or truncated forms (aa 291–587, ●; aa 1–515, ■) of c-Rel. CAT activities were determined after 48 hr of culture and are expressed as fold induction relative to basal levels measured in the absence of added effector. (*F*) COS-7 cells were transfected with the c-Rel(291–587) expression plasmid and metabolically radiolabeled with [^{35}S]cysteine. Recipient cell lysates were subjected to immunoprecipitation with c-Rel-specific anti-peptide antiserum (aa 573–587) and analyzed by SDS/PAGE. Molecular size (kDa) markers are indicated.

Human c-Rel Represses p65-Mediated Transcription. The distinct transactivation potentials and the previously described delayed nuclear appearance of c-Rel versus p65 DNA-binding activity (21) prompted investigation of whether c-Rel might negatively regulate p65-mediated transcriptional activation. In this regard, immunoblotting studies of nuclear c-Rel and p65 expressed in Jurkat T cells stimulated with either phorbol 12-myristate 13-acetate (Fig. 2*A*) or tumor necrosis factor α (Fig. 2*B*) clearly showed distinct but overlapping induction kinetics for these two Rel proteins. To explore the potential functional consequences of the simultaneous expression of c-Rel and p65, Jurkat cells were cotransfected with the HIV-1 LTR-CAT reporter, a mixture of p50 and p65 expression vectors, and various c-Rel constructs (Fig. 1*D*). In the absence of c-Rel, the NF-κB p50/p65 complex activated transcription from the HIV-1 LTR by ≈9-fold over basal levels. However, coexpression of c-Rel in these cells markedly repressed these stimulatory effects in a

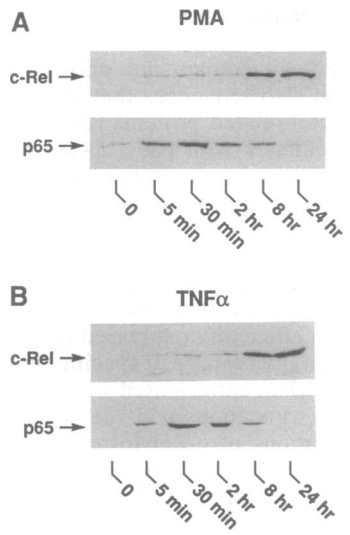

FIG. 2. Time course for induction of c-Rel and p65. Jurkat T cells were stimulated with either phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) (*A*) or tumor necrosis factor α (TNFα, 140 units/ml) (*B*) for the indicated times (0–24 hr). Nuclear extracts (20 μg) from these cells were fractionated by SDS/PAGE and subjected to immunoblot analysis with c-Rel and p65-specific anti-peptide antibodies as described (see *Materials and Methods*).

dose-dependent manner. Similar antagonistic effects were observed with a deletion mutant of c-Rel (aa 1–515) lacking the C-terminal subregion required for its weak transactivation activity (Fig. 1*D*). However, selective removal of the Rel homology domain from the wild-type c-Rel protein yielded an efficiently expressed N-terminal deletion mutant (aa 291–587; Fig. 1*F*) that failed to inhibit NF-κB-mediated activation of the HIV-1 LTR (Fig. 1*D*). These inhibitory effects of c-Rel on NF-κB-dependent transcription from the HIV-1 LTR were fully recapitulated in independent transfection experiments performed with an expression vector encoding the HTLV-I Tax protein (Fig. 1*E*), which potently activates the nuclear expression of endogeneous p50/p65 heterodimers (8–10).

To confirm the specificity of these biological effects, additional functional transfection studies were performed using CAT reporter constructs driven by well-characterized promoters that can be activated in either a κB-dependent or a κB-independent manner (Fig. 3). HTLV-I Tax-mediated activation of the IL-2Rα promoter, which requires NF-κB DNA binding events (8–10), was inhibited >80% by coexpression with either wild-type c-Rel or the c-Rel(1–515) deletion mutant lacking the C-terminal 72 aa (Fig. 3*A*). In contrast, Tax-mediated activation of the HTLV-I LTR, which proceeds by an NF-κB-independent mechanism (39–41), was not significantly inhibited by either c-Rel or c-Rel(1–515) (Fig. 3*B*). Similarly, κB-independent activation of the HIV-1 LTR by the HIV-1 Tat protein was not negatively affected by coexpression of either of these c-Rel proteins (Fig. 3*C*).

Mutational Analysis of c-Rel Repressor Function. To define more precisely which peptide sequences within c-Rel were required for its repressor activity, mutant c-*rel* expression vectors encoding a series of N- or C-terminally truncated forms of the protein were prepared. These mutants were first transiently expressed in Jurkat human T cells containing the HIV-1 LTR-CAT construct, with p65/p50 as the activator (Fig. 3 *D* and *E*). In the absence of c-Rel, the HIV-1 LTR was activated 4- to 5-fold by the cotransfected p50 and p65 expression vectors. These NF-κB-mediated stimulatory effects were completely inhibited by wild-type c-Rel (aa 1–587), as well as by a C-terminal deletion mutant of c-Rel containing aa 1–289 (Fig. 3*D*). However, removal of an additional 11 aa from this c-Rel mutant significantly impaired its ability to inhibit the NF-κB response, indicating that the C-terminal boundary of the involved functional domain(s) lies between aa 289 and 278. In reciprocal experiments with a series of N-terminal deletion mutants (Fig. 3*E*), removal of aa 1–32 from the full-length c-Rel protein proved sufficient to largely disrupt the wild-type inhibitory phenotype. Together, these functional studies localized the domain(s) involved in c-Rel-mediated transcriptional repression between aa 1 and 289, which corresponds with the Rel homology region.

The finding that the inhibitory effects exerted by c-Rel require the presence of sequences within its Rel homology domain suggested that the mechanism of repression might involve the DNA-binding properties of c-Rel. To localize the c-Rel DNA-binding domain, extracts from COS cells overexpressing these various deletion mutants were subjected to

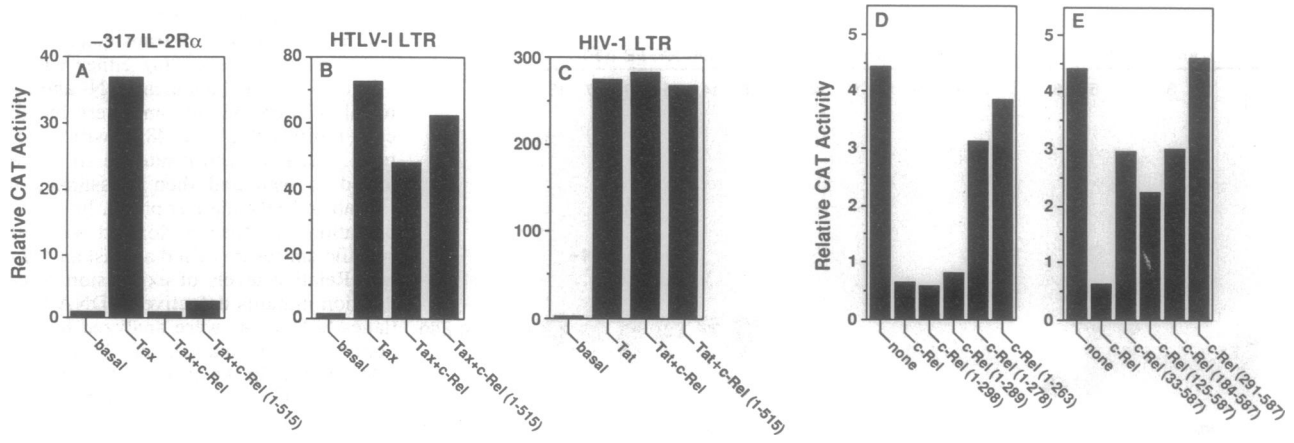

FIG. 3. (*A–C*) Specificity of c-Rel-mediated repression. Jurkat T cells (5×10^6) were transfected with CAT reporter plasmids containing either the IL-2Rα promoter (5 μg) (*A*), HTLV-I LTR (2.5 μg) (*B*), or HIV-1 LTR (2.5 μg) (*C*) in the presence or absence (basal) of cDNA expression vectors encoding the indicated proteins (Tax, 1 μg; Tat, 1 μg; c-Rel, 5 μg). CAT activities were determined after 40–48 hr of culture and are presented as fold induction relative to basal levels measured in the absence of the effector plasmids. Similar results were obtained in at least two independent transfection experiments. (*D* and *E*) Deletion mapping of c-Rel sequences required for repression. Jurkat cells were transiently transfected with HIV-1 LTR-CAT (2.5 μg) and a mixture of p50 (1 μg) and p65 (2.5 μg) expression vectors in the absence or presence of c-Rel expression vectors (5 μg) encoding the indicated C-terminal (*D*) or N-terminal (*E*) deletion mutants. CAT activities were determined after 40–48 hr of culture and represent mean values for duplicate experiments (<20% variation). Similar results were obtained in two to four independent transfection assays.

UV-crosslinking analysis with a ^{32}P-labeled κB enhancer probe (22). The resultant DNA–protein adducts were immunoprecipitated with c-Rel-specific antibodies and fractionated by SDS/PAGE. Extracts from vector-only-transfected cells had virtually no κB binding activity (Fig. 4*A*, lane 1). In contrast, extracts from recipient cells overexpressing wild-type c-Rel (lane 2) or C-terminal deletion mutants truncated at aa 515, 359, 307, 296, or 289 supported DNA binding, leading to the formation of smaller crosslinked adducts (lanes 3–7). However, C-terminal truncations extending to either aa 278 or aa 263 (lanes 8 and 9) completely disrupted this DNA binding function of c-Rel. In reciprocal studies, N-terminal deletion mutants truncated at aa 33 and 125 were examined, and both were found to lack binding to the κB enhancer probe (lanes 10 and 11). Control immunoprecipitation experiments performed with these two N-terminal deletion mutants, as well as the C-terminal mutants truncated at residues 278 or 263 lacking DNA-binding activity, confirmed that each of these proteins was efficiently expressed (Fig. 4*B*, lanes 1–4). Thus, the c-Rel subregion required for κB enhancer binding maps between aa 1 and 289 and appears coincident with those c-Rel sequences required for repression of p65-induced transcription (Fig. 3).

The Rel homology domain not only contains a DNA-binding motif but also sequences that mediate oligomerization with other Rel-related proteins (20), including the formation of heterodimers with p50 (15–18, 23). To examine whether c-Rel-mediated repression of p65 activation might involve competition for heterodimer formation with p50, a series of six c-Rel deletion mutants were translated *in vitro* by using rabbit reticulocyte lysate either alone or in combination with p50. Immunoprecipitation analyses performed with ^{35}S-labeled lysates confirmed that when translated alone, each c-Rel mutant was efficiently expressed (Fig. 4*C*, lanes 1–6) and lacked reactivity with p50-specific antiserum (lanes 7–12). However, when cotranslated with p50, the 1–359 and 1–278 C-terminal truncations and the 33–587 and 125–587 N-terminal truncations of c-Rel formed heteromeric complexes with p50, as evidenced by their coimmunoprecipitation with anti-p50 antiserum (lanes 13, 14, 16, and 17). In contrast, the 1–263 C-terminal deletion mutant and the 184–587 N-terminal deletion mutant failed to associate with p50, indicating that the extreme boundaries for the heterodimer-

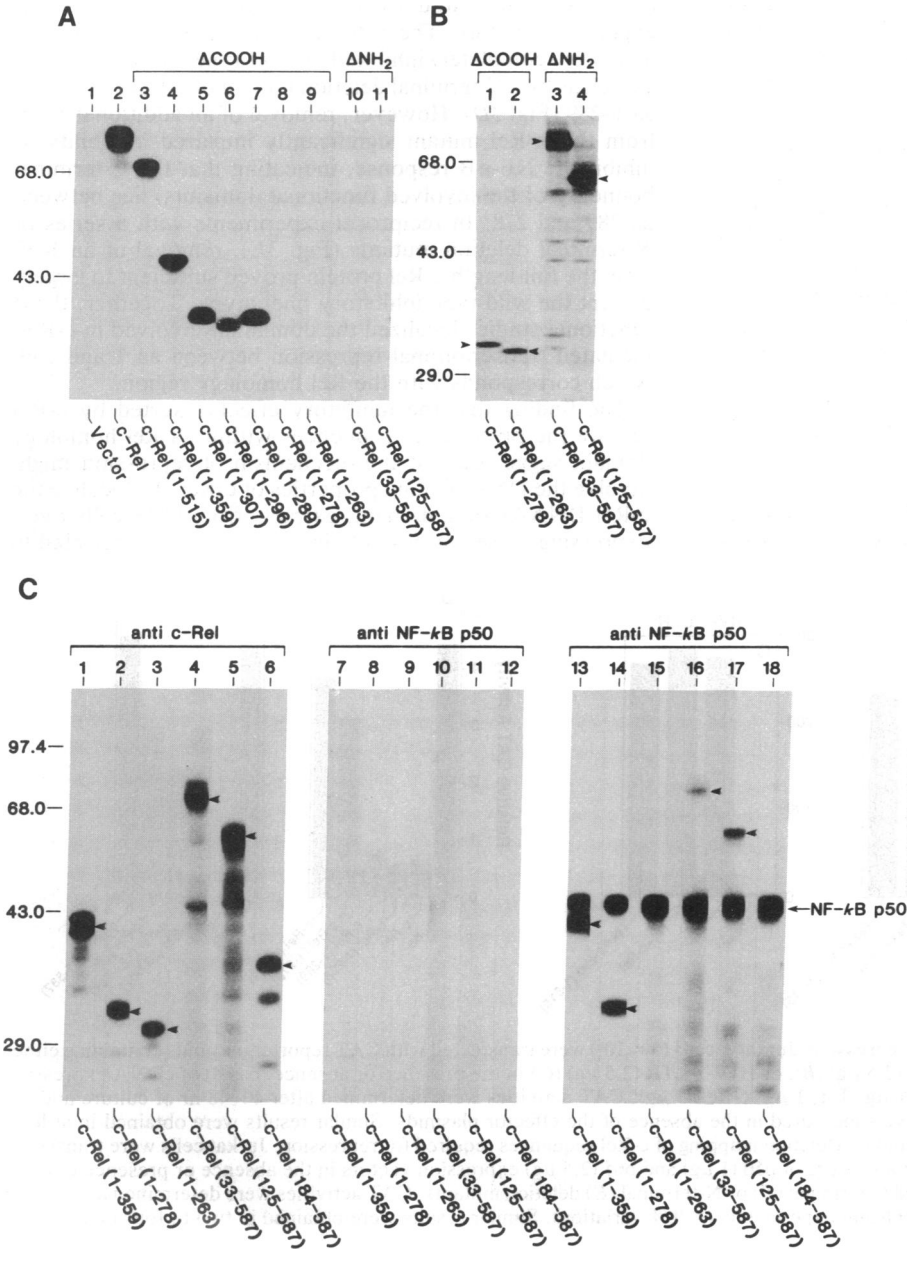

FIG. 4. Deletion mapping of the c-Rel DNA-binding and dimerization domains. (*A* and *B*) COS cells were transfected with expression vectors encoding either wild-type c-Rel (lane 1) or the indicated N- and C-terminal deletion mutants and were metabolically radiolabeled after 48 hr with [^{35}S]cysteine. Recipient cell lysates (1 μl) were allowed to bind and then crosslinked to a ^{32}P-labeled κB enhancer probe. Immunoprecipitation was then performed with c-Rel-specific antiserum raised against aa 132–150 (*A*). Relative levels of expression of c-Rel deletion mutants defective in DNA binding (lanes 8–11 of *A*) were analyzed by immunoprecipitation analysis of ^{35}S-labeled lysates (100 μl) in the absence of radiolabeled DNA (*B*, lanes 1–4). (*C*) Run-off transcripts encoding the indicated C- and N-terminal deletion mutants of c-Rel were translated *in vitro* in either the absence (lanes 1–12) or the presence (lanes 13–18) of p50 transcripts. Programmed lysates were subjected to immunoprecipitation analysis with either c-Rel-specific (lanes 1–6) or p50-specific (lanes 7–18) antiserum and analyzed by SDS/PAGE. The position of cotranslated p50 revealed in lanes 13–18 is indicated by an arrow.

ization domain of c-Rel are located between aa 125 and 278. As such, these experiments specifically identify a class of c-Rel mutants, including c-Rel(1–278) (lane 14) and c-Rel(33–587) (lane 16), which retain the ability to dimerize with p50 yet fail to repress NF-κB-dependent transcription (see Fig. 3). Thus, the c-Rel subregion involved in heterodimer formation with p50 appears to be distinct from the functional domains required for both transcriptional repression and κB-specific DNA binding (aa 1–289).

DISCUSSION

Prior DNA–protein crosslinking studies with phorbol ester-activated Jurkat cells have suggested that p65 (early) and c-Rel (late) are expressed with distinct but overlapping kinetics during the course of T-cell activation (21). Consistent with these prior studies, immunoblotting experiments revealed a similar delayed pattern of nuclear c-Rel expression relative to p65 following treatment of Jurkat T cells with tumor necrosis factor α. Based on these findings, *in vivo* transient transfection studies were performed to directly compare the transcriptional activities of these two Rel family members. Our results indicate that while p65 is a potent activator of both the HIV-1 LTR and a synthetic HIV-1 enhancer–CAT construct, c-Rel fails to activate the HIV-1 LTR and produces at least 10-fold weaker activating effects on the synthetic κB reporter plasmid. Further, when these transcription factors are coexpressed, c-Rel markedly represses the strong activating effects of p65 on the HIV-1 LTR. These inhibitory effects of c-Rel are not dependent on its unique C-terminal sequences (20) but instead are correlated with sequences in the Rel homology segment that mediate DNA binding. In contrast, analyses of N- and C-terminal deletion mutants of c-Rel clearly segregated repression from the overlapping but distinct dimerization domain within c-Rel. Together, these findings suggest that c-Rel represses NF-κB-dependent transcription through interference with p65 binding to the κB enhancer. These findings thus highlight the apparently opposing functions of two Rel family members which are differentially induced during T-cell activation.

In addition to its repressive properties, c-Rel may also positively regulate the expression of select cellular genes, perhaps including those whose protein products are required in the later phases of the T-cell activation process. In this regard, it has been shown that c-Rel contains a relatively weak transcriptional activation domain that is functional in several cell types (26–30), including human T lymphocytes (Fig. 1). The precise factors that determine whether a specific κB enhancer-containing transcription unit will be positively or negatively regulated by c-Rel remain unclear. It is possible that subtle sequence variations in the κB enhancer motif, or associated promoter elements, may dictate the functional consequences of c-Rel binding to a given target gene (42, 43). As well, the cellular context in which c-Rel acts could have important effects on its ultimate function. For example, v-Rel, the oncogenic counterpart of c-Rel, exerts strikingly different functional effects on κB-directed transcription, dependent upon the state of cellular differentiation (44).

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