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Chronic Human Immunodeficiency Virus Type 1 Infection Stimulates Distinct NF-κB/*rel* DNA Binding Activities in Myelomonoblastic Cells

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The relationship between human immunodeficiency virus type 1 (HIV-1) infection and the induction of NF-κB binding activity was examined in a myeloid cell model of HIV-1 infection derived from the PLB-985 cell line. Chronic infection of PLB-985 cells led to increased monocyte-specific surface marker expression, increased c-*fms* gene transcription, and morphological alterations consistent with differentiation along the monocytic pathway. PLB-IIIB cells displayed a constitutive NF-κB-like binding activity that was distinct from that induced by tumor necrosis factor alpha or phorbol 12-myristate 13-acetate treatment of the parental PLB-985 cell line. This unique DNA binding activity consisted of proteins of 70, 90, and 100 kDa with a high degree of binding specificity for the NF-κB site within the PRDII domain of beta interferon. In this report, we characterize the nature of these proteins and demonstrate that binding of these proteins is also induced following Sendai paramyxovirus infection. The 70-kDa protein corresponds to the NF-κB RelA (p65) subunit, which is activated in response to an acute paramyxovirus infection or a chronic HIV-1 infection. Virus infection does not appear to alter the amount of RelA (p65) or NFKB1 (p50) but rather affects the capacity of IκBα to sequester RelA (p65), therefore leading to constitutive levels of RelA DNA binding activity and to increased levels of NF-κB-dependent gene activity. The virally induced 90- to 100-kDa proteins have a distinct binding specificity for the PRDII domain and an AT-rich sequence but do not cross-react with NF-κB subunit-specific antisera directed against NFKB1 (p105 or p50), NFKB2 (p100 or p52), RelA (p65), or c-*rel*. DNA binding of the 90- to 100-kDa proteins was not inhibited by recombinant IκBα/MAD-3 and was resistant to tryptic digestion, suggesting that these proteins may not be NF-κB related. Transient cotransfection experiments demonstrated that RelA and NFKB1 expression maximally stimulated HIV-1 LTR- and NF-κB-dependent reporter genes; differences in NF-κB-like binding activity were also reflected in higher constitutive levels of NF-κB-regulated gene expression in HIV-1-infected myeloid cells.

$CD4^+$ cells of the monocyte-macrophage lineage, including myeloid progenitor cells in the bone marrow, can be infected with human immunodeficiency virus type 1 (HIV-1) (14, 53); these cells may serve as intracellular reservoirs for virus and contribute to the spread of HIV-1 to peripheral tissues, such as the skin, lungs, brain, and lymph nodes (39). One of the physiological consequences associated with HIV-1 infection of monocytic cells is altered regulation of inflammatory cytokine production (12, 13, 54); alterations in the expression of cytokines in HIV-1-infected myeloid cells may involve either increased or decreased cytokine levels, depending on a number of interrelated variables: the inducing agent, the cell types used in the study, and the state of differentiation of the cells. Conversely, cytokines exert differential effects on HIV-1 gene expression; interleukin-1 (IL-1), IL-3, IL-6, tumor necrosis factor alpha/beta (TNF-α/β), and granulocyte-monocyte colony-stimulating factor (GM-CSF) have been shown to stimulate HIV-1 replication, whereas alpha/beta interferon (IFN-α/β) decreases HIV-1 activity (49). Cytokines have also been implicated in the pathogenesis of AIDS-associated malignancies, such as Kaposi's sarcoma and B-cell lymphoma (20), and may contribute to fever, cachexia, and other clinical symptoms (7, 40).

One pathway by which cytokine expression and viral gene expression may be coordinately regulated is through the activation of the NF-κB/*rel* family of transcription factors (reviewed in references 4, 8, and 9). NF-κB/*rel* participates in the activation of many immunoregulatory cytokine genes and cell surface receptors. In addition, several viral enhancers, including those of cytomegalovirus, simian virus 40, and HIV-1, contain NF-κB recognition sites. The consensus recognition site is a decamer (5′-GGGANNYYCC-3′) with two pentameric half sites, each of which participates in the recognition and stabilization of the binding of the NF-κB dimer (70). While constitutive NF-κB binding activity has been detected in mature B cells and monocytes (23, 50), NF-κB expression is inducible in most cell types by viruses, double-stranded RNA, bacterial lipopolysaccharides, cytokines, phorbol esters, and oxygen radical intermediates (26, 37, 62, 63). NF-κB was initially described as two subunits, p50 (NFKB1) and p65 (RelA), that existed in the cytoplasm in a latent form complexed to an inhibitory subunit, IκB. Induction resulted in the release of IκB from p50-p65 and nuclear translocation of the DNA binding heterodimer (3, 18).

It is now apparent that NF-κB proteins constitute a family of transcription factors whose genes share homology in the N-terminal DNA binding domain with the c-*rel* proto-oncogene and with the *Drosophila* morphogen *dorsal* (4, 8, 9, 21).

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The family includes several members: NFKB1 (p50) is synthesized as a 105-kDa non-DNA binding precursor that is proteolytically cleaved to yield the mature p50 product (10, 19, 32, 41). The RelA (p65) protein contains in addition to the N-terminal *rel* homology domain a *trans*-activation domain in its C-terminal portion (21, 45, 56). Recently, an alternatively spliced form of RelA, termed ΔRelA (p65Δ), was identified; it inhibits NF-κB binding activity and has a distinct *trans*-dominant negative effect on transcription (43, 58). c-*rel* (p85) is the cellular homolog of v-*rel*, the transforming gene of the reticuloendotheliosis virus, an avian retrovirus that induces acute fatal lymphoma in young birds (9). The NFKB2 (p100, p52, and lyt-10) gene encodes a 100-kDa precursor (p100) and a 52-kDa product and corresponds to *lyt-10*, originally identified in a chromosomal translocation associated with B-cell lymphoma (44, 60). Recently, a 68-kDa protein referred to as relB (I-rel) was cloned; relB appears to form activating heterodimers with the p50 protein (57, 59).

Multiple ankyrin repeat-containing IκB proteins that regulate cytoplasmic anchoring and nuclear uptake of NF-κB proteins have also been defined (4, 8, 9): IκBα (MAD-3/pp40/RL/IF-1), cloned as an immediate-early gene in monocytes (25), interacts with high affinity with RelA and inhibits NFKB1 (p50)-RelA (p65) or NFKB2 (p52)-RelA heterodimers and RelA (p65) homodimers; IκBα is also capable of interacting with NFKB1 (p50) and c-*rel* with low affinity (6). The association of IκBα with NF-κB proteins occurs via the nuclear localization sequence, and in the uninduced state, IκB masks the nuclear localization sequence (NLS) to prevent nuclear translocation (6, 29, 31, 72). Bcl3 (and ΔBcl3) is the product of a gene associated with the t(14;19) chromosomal translocation in chronic lymphocytic leukemia. Bcl3 specifically inhibits the DNA binding activity of NFKB1 (p50) or NFKB2 (p52) homodimers (15, 30, 71). IκBγ is a murine 70-kDa protein derived from the carboxy-terminal 607 amino acids of p105 by alternative splicing. This protein inhibits the DNA binding of NFKB1 (p50)-RelA heterodimers, NFKB1 (p50) homodimers, and c-*rel* (27). Recently, the p105 precursor itself was shown to inhibit the nuclear translocation of c-*rel* and RelA; additionally, p105 generates nuclear RelA-p50 and p50–c-*rel* complexes through proteolytic processing (51).

Several studies have now examined the relationship among HIV-1 infection of myeloid cells, induction of NF-κB binding activity (2, 23, 47, 50, 64), cellular differentiation (1, 17, 46), and HIV-1 gene expression (38, 67). Previously, we established a population of HIV-1-infected myelomonoblastic cells (PLB-IIIB) derived from the PLB-985 cell line (55). Chronic infection of PLB-985 cells led to increased monocyte-specific surface marker expression, increased c-*fms* gene transcription, and morphological alterations consistent with differentiation along the monocytic pathway (55). PLB-IIIB cells displayed a constitutive NFκB-like binding activity consisting of proteins of 70, 90, and 100 kDa with a high degree of binding specificity for the NF-κB site within the PRDII domain of IFN-β (P2) (55).

In this report, we characterize the nature of these proteins and demonstrate that the binding of these proteins is also induced following Sendai paramyxovirus infection of PLB-985 cells. The 70-kDa protein corresponds to the NF-κB RelA (p65) subunit, which is specifically activated in response to a viral infection. The virally induced 90- to 100-kDa proteins have a distinct binding specificity for the PRDII domain and an AT-rich sequence but do not cross-react with NF-κB subunit-specific antisera directed against RelA, NFKB1 (p105 or p50), NFKB2 (p100 or p52), or c-*rel*. Transient transfection experiments demonstrated that differences in NF-κB-like binding activity were reflected in higher constitutive levels of NF-κB-regulated gene expression in HIV-1-infected myeloid cells.

MATERIALS AND METHODS

Cell culture and transfections. PLB-985 cells (66) were infected with HIV-1 strain IIIB as previously described by use of supernatants from HIV-1-infected U937 cells to establish the chronically infected PLB-IIIB cell line (55). PLB-985 and PLB-IIIB cells were maintained in RPMI 1640 (GIBCO, Life Technologies Inc., Grand Island, N.Y.) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah), 2 mM L-glutamine, and 20 μg of gentamicin (Schering Canada, Pointe Claire, Quebec, Canada) per ml. Exponentially growing cells were treated as follows, depending on the individual experimental protocol: addition of phorbol 12-myristate 13-acetate (PMA) at a final concentration of 25 ng/ml (Sigma Chemical Co., St. Louis, Mo.); addition of TNF-α at 100 U/ml (Genzyme Inc., Kneeland, Mass.); and priming with IFN-α2 (a gift from Schering Canada) for 4 h at 250 IU/ml and then Sendai virus infection at 1,000 hemagglutination units (HAU)/ml (Sendai virus was a kind gift from Kari Cantell). PLB-985 and PLB-IIIB cells were transfected by the DEAE-dextran procedure as previously described (24). Cells were induced 24 h later and harvested 36 h following transfection, protein was collected, and protein concentrations were determined by the Bio-Rad protein assay. The chloramphenicol acetyltransferase (CAT) reporter constructs [HIV-LTR, P2(2), 5'κB(3), and HIV mut] were described previously (26, 37). The NF-κB expression plasmids were produced by subcloning different NF-κB genes into the SVK3 vector: (i) for NFKB1 (p50), a 1,381-bp *Eco*RI-*Rsa*I fragment from KBF-1 (32) was subcloned into the *Eco*RI-*Sma*I site of SVK3; (ii) for ΔRelA (p65Δ), a 2,572-bp *Xba*I-*Xho*I fragment from plasmid BL-SK (58) was subcloned into the *Bam*HI-*Xho*I site of SVK3; (iii) for c-*rel*, a 2,340-bp *Eco*RI fragment of c-*rel* cDNA (11) was cloned into the SVK3 *Eco*RI site; and (iv) for IκBα, a 1,190-bp *Eco*RI fragment from pGEX-2T (see below) was subcloned into the *Eco*RI site of SVK3. The CMIN-p65 vector (58) was used to express the RelA (p65) subunit. CAT activity was measured with 80 μg of protein assayed for 2 h at 37°C as described by Gorman et al. (22). The percent acetylation was determined, and the relative inducibility was obtained by dividing the percent conversion of treated samples by that of untreated samples.

Expression and purification of recombinant proteins. The cDNA clone encoding p105 was obtained from A. Israel, and the region corresponding to p50 nucleotides 211 to 1687 (encoding amino acids 10 to 502 of p105) (32) was inserted into the pGEX-3X expression vector (Pharmacia). The cDNA encoding IκBα/MAD-3 was obtained by reverse transcriptase-polymerase chain reaction of total cellular RNA from Jurkat cells with specific primers corresponding to nucleotides 81 to 99 (5'-ACGTGAATTCAGCTGGTC CGCGCC-3') and 1151 to 1171 (5'-ATATAGGTGTGACGT GTGACCTTAAG-3'), which encompassed the entire coding region of MAD-3 (25). The 5' end of each oligonucleotide primer contained an *Eco*RI site used for ligation of the polymerase chain reaction-amplified cDNA into the pGEX-2T expression vector (Pharmacia). Both fusion proteins were expressed in *Escherichia coli* (DH5α; GIBCO/BRL) following 3 h of induction with 1 mM isopropyl-β-D-

thiogalactopyranoside (IPTG) (Pharmacia) at 30°C. Protein was isolated following bacterial lysis by six 30-s cycles of sonication in lysis buffer: 20 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol. After ammonium sulfate precipitation (30% saturation), proteins were resolubilized in lysis buffer containing 1% Triton X-100 and incubated with glutathione-agarose beads (Pharmacia) for 20 min. After a rinse with phosphate-buffered saline, fusion proteins were enzymatically cleaved as follows: p50, in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–1 mM $CaCl_2$–factor Xa (Boehringer GmbH, Mannheim, Germany); and IκBα, in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–2.5 mM $CaCl_2$–thrombin (Sigma) for 3 to 4 h at room temperature. The final cleaved protein products were eluted from the beads with 50 mM Tris-HCl (pH 7.5)–150 mM NaCl and stored with the addition of 1 mM dithiothreitol and protease inhibitors.

WCE preparations. Whole-cell extracts (WCE) were prepared from untreated PLB-985 and PLB-IIIB cells or from cells treated with PMA (25 ng/ml for 4 or 16 h) or TNF-α (100 U/ml for 4 or 16 h) or primed with IFN-α2 (250 IU/ml for 4 h) and infected with Sendai virus (1,000 HAU/ml for 6 h). Procedures for extract preparation were those described previously (35).

EMSA. Cell extracts (2.5 to 5.0 μg) were preincubated with the nonspecific DNA competitor poly(dI-dC) (5 μg; Pharmacia) for 10 min on ice in a total volume of 15 to 20 μl of WCE buffer containing 0.1% Nonidet P-40 (35). Binding activity was assessed with 0.2 ng of ^{32}P-end-labelled probes that corresponded to the PRDII domain of the IFN-β promoter (5′-GGGAAATTCCGGGAAATTCC-3′), the HIV-1 enhancer (5′-AGGGACTTTCCGCTGGGGACTTTCC-3′), and a nonspecific AT-rich oligonucleotide (5′-AAATTTAA ATTTAAATTTAAATTT-3′) and that were incubated with extracts for 30 min at room temperature. Protein-DNA complexes were then separated on a 5% native polyacrylamide gel (60:1 cross-link) with Tris-glycine (25 mM Tris, 195 mM glycine [pH 8.5]). In the competition analysis, a 125 M excess of unlabelled oligonucleotide was incubated with WCE for 10 min on ice prior to the addition of the probe. For examination of the individual proteins present in the complex, polyclonal subunit-specific antisera against p50 or p105 (antiserum 1141, N-terminus specific) (51), RelA (antiserum 1226, C-terminus specific) (51), c-*rel* (antiserum 265, C-terminus specific) (11), and p52 or p100 (antiserum 1267, N-terminus specific) were used in combination with the electrophoretic mobility shift assay (EMSA). Antisera (1:10) were incubated with WCE (2.5 μg) in the presence or absence of a specific competitor peptide (0.1 μg/μl) for 20 min at room temperature prior to the addition of poly(dI-dC) and radiolabelled probe as described for the EMSA.

Immunoprecipitation of RelA and IκBα. PLB-985 and PLB-IIIB cells were washed with phosphate-buffered saline, resuspended in methionine-free RPMI 1640 for 1 h, and labelled for 3 h with 0.5 mCi of Trans^{35}S label (ICN) per ml. Preparation of cell lysates, preclearing with normal rabbit serum, and immunoprecipitation with specific antibodies were carried out essentially as described previously (51).

UV cross-linking analysis. UV cross-linking analysis was performed in situ with a bromodeoxyuridine (BrdU)-substituted ^{32}P-labelled P2 probe in a standard mobility shift analysis, scaled up twofold and then continued as previously described (34).

Tryptic digestion of protein-DNA complexes. Tryptic digestion was performed by incubating recombinant p50 (50 ng) or WCE (5 μg) in 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Promega, Madison, Wis.) per ml for 1, 4, 15, and 30 min at room temperature as described by Baeuerle and Baltimore (3). Digestion was stopped with a 10-fold molar excess of soybean trypsin inhibitor (GIBCO), and a mobility shift analysis was performed with the digested extracts.

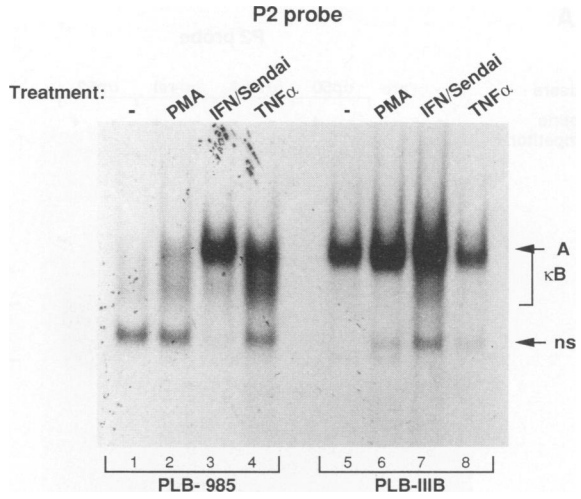
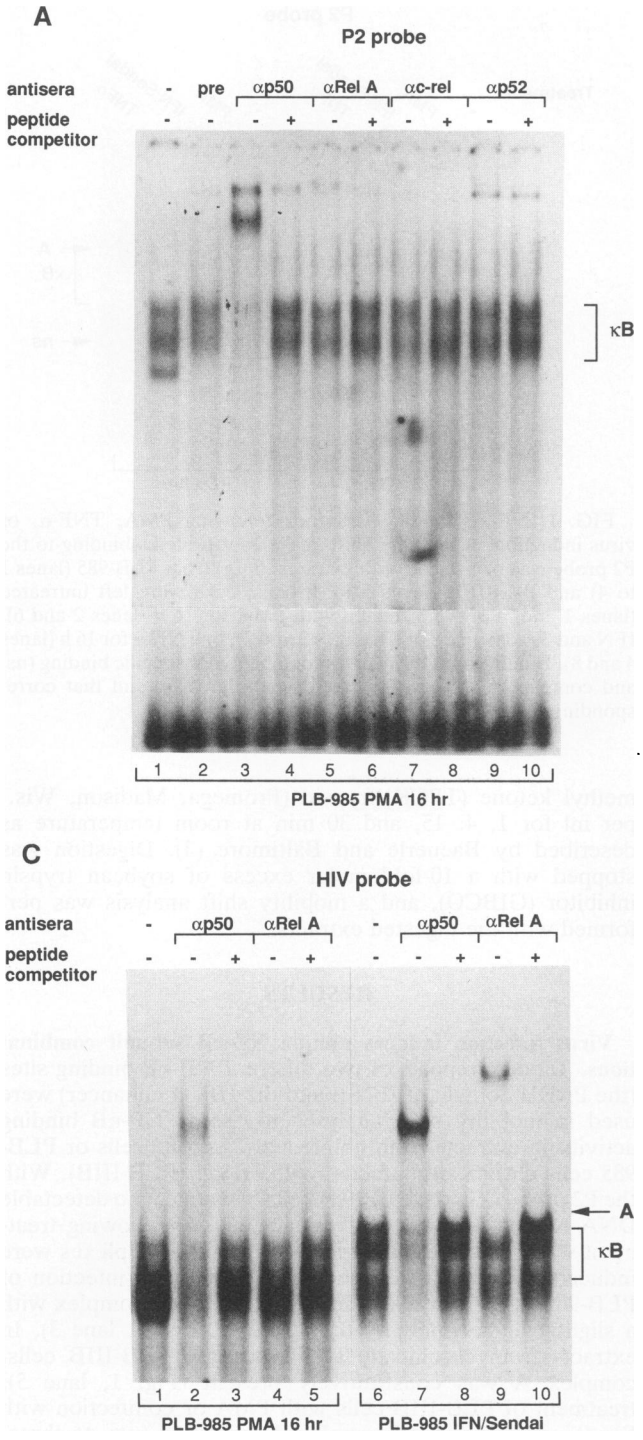


FIG. 1. NF-κB binding activities following PMA, TNF-α, or virus induction. A mobility shift analysis of proteins binding to the P2 probe was performed with extracts (5 μg) from PLB-985 (lanes 1 to 4) and PLB-IIIB (lanes 5 to 8) cells; cells were left untreated (lanes 1 and 5) or were treated with PMA for 16 h (lanes 2 and 6), IFN and Sendai virus for 6 h (lanes 3 and 7), or TNF-α for 16 h (lanes 4 and 8). Binding activities corresponding to nonspecific binding (ns) and complex A (A) are indicated by the arrows, and that corresponding to NF-κB (κB) is indicated by the bracket.

RESULTS

Virus infection induces unique NF-κB subunit combinations. Tandem repeats of two different NF-κB binding sites (the PRDII domain of IFN-β and the HIV-1 enhancer) were used in mobility shift analyses to assess NF-κB binding activity in extracts from uninfected PLB-985 cells or PLB-985 cells chronically infected with HIV-1 (PLB-IIIB). With the P2 probe (Fig. 1), PLB-985 cells had almost no detectable DNA binding activity (Fig. 1, lane 1), but following treatment with PMA or TNF-α for 4 h, NF-κB complexes were induced (Fig. 1, lanes 2 and 4); Sendai virus infection of PLB-985 cells for 6 h also induced an NF-κB complex with a slightly higher mobility (complex A) (Fig. 1, lane 3). In extracts from chronically HIV-1-infected PLB-IIIB cells, complex A was constitutively present (Fig. 1, lane 5); treatment of PLB-IIIB cells with PMA or coinfection with Sendai virus increased complex A formation two- to threefold (Fig. 1, lanes 6 and 7). The specificity of these complexes for the NF-κB site was demonstrated previously in competition and binding site mutagenesis studies (55). Complex A was also detected within 5 days of de novo HIV-1 infection in PLB-985 cells (data not shown). Similar NF-κB complexes were obtained when the HIV-1 enhancer was used as a probe (55; see also Fig. 2C, lanes 1 and 6). Thus, distinct NF-κB-related activities observed in HIV-1-infected cells were induced not only by chronic HIV-1 infection but also by acute Sendai virus infection.

RelA (p65) binding activity is induced in response to viral



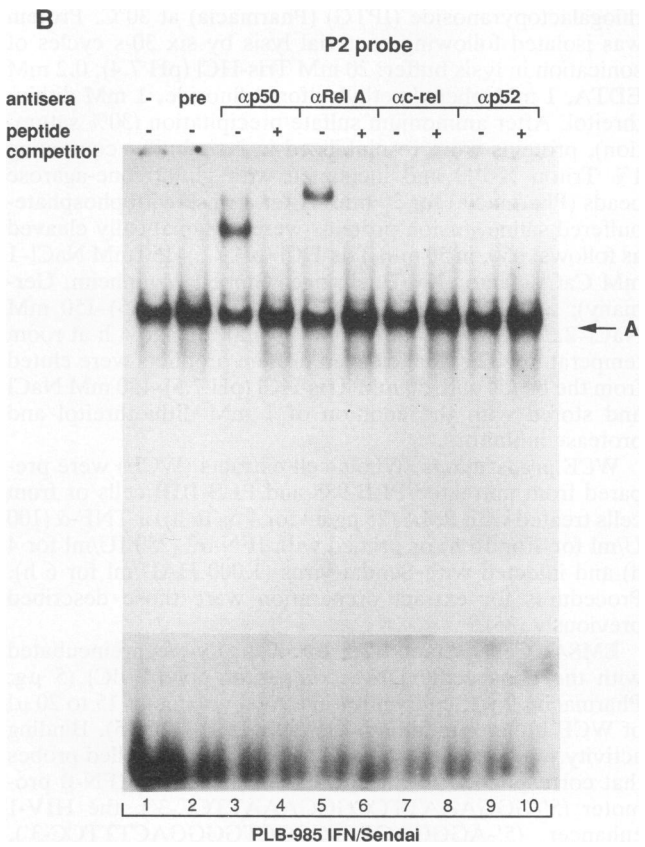


FIG. 2. Identification of NF-κB DNA binding subunits. WCE (2.5 μg) from PMA-treated PLB-985 cells (A) or Sendai virus-infected PLB-985 cells (B) were preincubated with or without NF-κB-specific antisera (indicated above the lanes) for 10 min and then allowed to interact with a ^{32}P-labelled P2 probe under standard EMSA conditions. Antisera specific for N-terminal NF-κB p50 or p105 (1141) (lanes 3 and 4), C-terminal RelA (1226) (lanes 5 and 6), C-terminal c-*rel* (265) (lanes 7 and 8), and N-terminal p52 or lyt-10 (1267) (lanes 9 and 10) were used (51). The specificity of the antibody-protein complex was confirmed by the addition of excess peptide as competitor (lanes 4, 6, 8, and 10). Similar analyses were performed with the HIV-1 probe (C). Extracts from PMA-treated PLB-985 cells (lanes 1 to 5) and IFN-treated and Sendai virus-infected cells (lanes 6 to 10) were preincubated with antisera to p50 (lanes 2, 3, 7, and 8) and RelA (lanes 4, 5, 9, and 10).

infection. For identification of the individual NF-κB proteins associated with the protein-DNA complexes, antisera specific for different NF-κB subunits, NFKB1 (p105 and p50), NFKB2 (p100 and p52), RelA (p65), and c-*rel*, were preincubated with PLB cellular extracts, and the products were analyzed by an EMSA (Fig. 2). In extracts from PMA-treated PLB-985 cells, the disappearance of the NF-κB-specific complex following preincubation with the N-terminus-reactive anti-p105 or anti-p50 antiserum (Fig. 2A, lane 3) was accompanied by the appearance of a shifted slowly migrating complex; the addition of excess peptide used for antibody production inhibited the formation of the shifted complex, thus confirming the specificity of the antibody (Fig. 2A, lane 4). No shifted complex was obtained with antisera to RelA or other subunits (Fig. 2A, lanes 5, 7, and 9); the same results were obtained with extracts from TNF-α-treated PLB-985 cells (data not shown). In most lanes, a weak nonspecific shifted band that was also seen with preimmune serum (Fig. 2A, lane 2) and that was not inhibited by the specific peptides used to prepare the antibodies (Fig. 2A, lanes 4, 6, 8, and 10) was observed.

In contrast, shifted complexes corresponding to both p50 and RelA were detected in extracts from Sendai virus-infected cells (Fig. 2B, lanes 3 and 5); the addition of excess p50 or RelA peptide to the reactions completely inhibited the formation of the shifted antibody-protein-DNA complex (Fig. 2B, lanes 4 and 6). No evidence for the involvement of

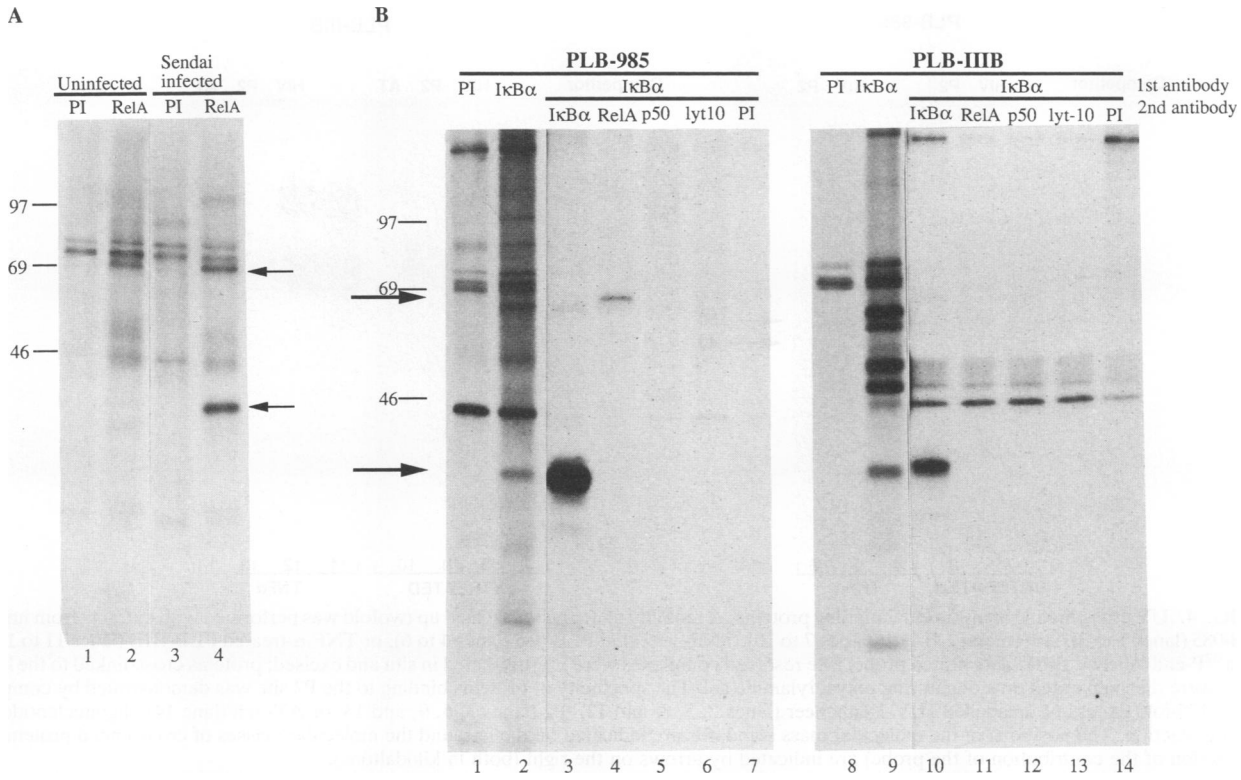


FIG. 3. Immunoprecipitation of RelA and IκBα. (A) Immunoprecipitation of a virus-inducible 40-kDa protein by anti-RelA antiserum. Cell lysates from PLB-985 cells (lanes 1 and 2) and Sendai virus-infected PLB-985 cells (lanes 3 and 4) were immunoprecipitated with preimmune serum (lanes 1 and 3) and with anti-RelA antiserum (lanes 2 and 4). PLB-985 cells (10^7) were infected with Sendai virus (100 HAU/ml) for 2 h. Cells were labelled for 3 h in methionine-free RPMI 1640 containing 0.5 mCi of Trans^{35}S label per ml. At the end of the labelling, PLB-985 cells were resuspended in RPMI 1640; after a 3-h chase, proteins were extracted and immunoprecipitated with preimmune (PI) serum and anti-RelA antiserum. (B) Coprecipitation of RelA with IκBα. Extracts from PLB-985 (lanes 1 and 2) and PLB-IIIB (lanes 8 and 9) cells were labelled for 3 h in methionine-free RPMI 1640; proteins were extracted and immunoprecipitated with preimmune serum (lanes 1 and 8) and with anti-IκBα antiserum (lanes 2 and 9). Extracts from PLB-985 (lanes 3 to 7) and PLB-IIIB (lanes 10 to 14) cells were also immunoprecipitated with anti-IκBα antiserum, and the precipitates were collected on protein A-Sepharose beads and extensively washed. Following boiling in 1% sodium dodecyl sulfate–0.5% β-mercaptoethanol, the initial precipitates were reprecipitated with antisera to IκBα (lanes 3 and 10), RelA (lanes 4 and 11), NFKB1 (p105 or p50) (lanes 5 and 12), and NFKB2 (lyt-10, p100, or p52) (lanes 6 and 13) and with preimmune (PI) serum (lanes 7 and 14). The positions of molecular mass markers are indicated (in kilodaltons); the arrows indicate RelA (p65) and IκBα.

c-*rel* or NFKB2 (p100 or p52) protein in complex formation was obtained (Fig. 2B, lanes 7 to 10). Similarly, shifted complexes corresponding to both p50 and RelA subunits were found in extracts from HIV-1-infected PLB-IIIB cells (data not shown). Thus, in myeloblastic PLB-985 cells, NF-κB RelA DNA binding is specifically induced as a consequence of either acute Sendai virus infection or chronic HIV-1 infection. The addition of anti-NFKB1 or anti-RelA antiserum did not result in the complete disappearance of the NF-κB complex, even with titration of the antiserum (data not shown), suggesting that other DNA binding proteins unrelated to NF-κB may be present in the complex.

When the HIV-1 enhancer probe was used, anti-p50 completely inhibited the NF-κB complexes in both PMA- and Sendai virus-induced cell extracts and resulted in the formation of a shifted protein-DNA complex (Fig. 2C, lanes 2 and 7). The addition of the p50 peptide used to produce the antiserum completely blocked the shift (Fig. 2C, lanes 3 and 8). Again, NF-κB RelA DNA binding was stimulated only in virus-infected cells (Fig. 2C, lane 9). Anti-RelA antiserum resulted in the specific disappearance of the uppermost protein-DNA complex; the addition of RelA peptide restored the uppermost NF-κB complex (Fig. 2C, lane 10). Since the upper complex also disappeared when anti-p50 antiserum was included in the reactions (Fig. 2C, lane 7), this complex represents a p50-RelA heterodimer. These data indicate that the proteins associated with the HIV-1 enhancer probe were mainly RelA and p50, whereas the P2 probe interacted with RelA and p50 as well as other proteins.

Immunoprecipitation of RelA and IκBα. The relative amounts of RelA and NFKB1 (p50) were not increased in virus-infected PLB-985 cells, as determined by Western immunoblot analysis (data not shown). To assess whether a change in the association between RelA and IκBα may be responsible for constitutive NF-κB binding activity, coimmunoprecipitation was performed with extracts from uninfected and virus-infected PLB-985 cells and with anti-RelA and anti-IκBα antisera (Fig. 3). PLB-985 cells were infected with Sendai virus and pulse labelled with [^{35}S]methionine for 3 h prior to preparation of cell lysates and immunoprecipitation with anti-RelA antiserum. From PLB-985 cells, a protein of 68 kDa was specifically immunoprecipitated by anti-RelA antiserum (Fig. 3A, compare lanes 1 and 2); from

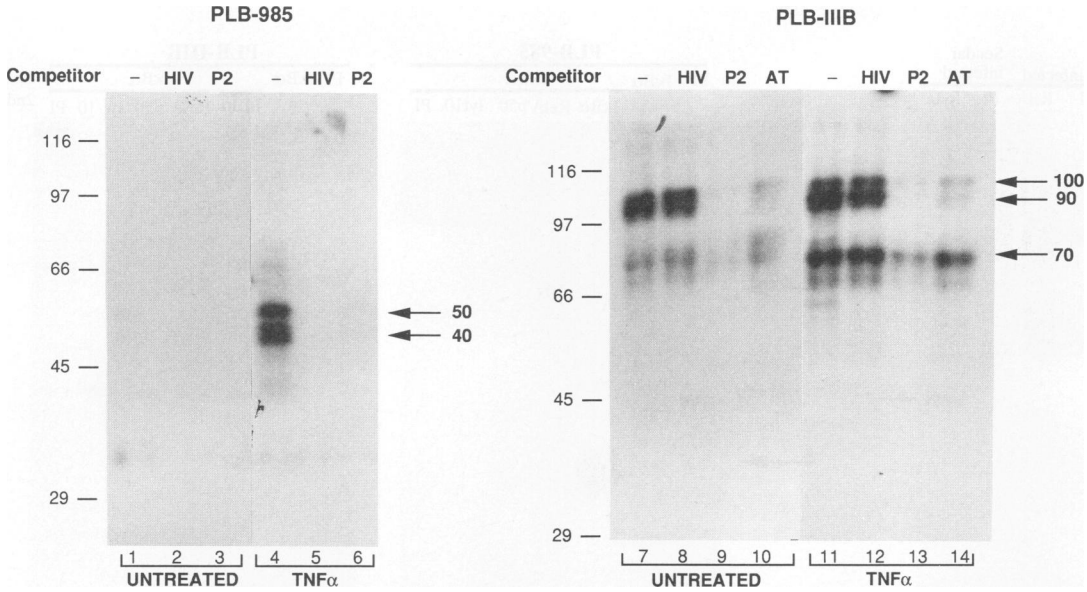


FIG. 4. UV cross-linking analysis of P2 binding proteins. A mobility shift analysis scaled up twofold was performed with extracts from untreated PLB-985 (lanes 1 to 3), untreated PLB-IIIB (lanes 7 to 10), TNF-α-treated PLB-985 (lanes 4 to 6), or TNF-α-treated PLB-IIIB (lanes 11 to 14) cells and a ^{32}P-end-labelled BrdU-substituted probe. The resolved complexes were UV irradiated in situ and excised; proteins cross-linked to the labelled probe were then separated on a denaturing polyacrylamide gel. The specificity of proteins binding to the P2 site was demonstrated by competition with a 125-fold excess of unlabelled HIV-1 enhancer (lanes 2, 5, 8, and 12), P2 (lanes 3, 6, 9, and 13) or AT-rich (lane 14) oligonucleotide in the binding reaction. The positions of the molecular mass standards are indicated on the left, and the molecular masses of cross-linked proteins (after subtraction of the contribution of the probe) are indicated by arrows on the right (both in kilodaltons).

Sendai virus-infected cells, the same amount of the 68-kDa protein was precipitated, but in addition, a 40-kDa protein was coprecipitated by anti-RelA antiserum (Fig. 3A, compare lanes 3 and 4). The 40-kDa protein was independently precipitated by anti-IκBα antiserum (Fig. 3B). Similarly, from PLB-IIIB cells, anti-RelA antiserum specifically coprecipitated a RelA-IκBα complex (data not shown). When anti-IκBα antiserum was used to immunoprecipitate complexes from PLB-985 or PLB-IIIB cells, several additional proteins, of 50 to 60 kDa, were precipitated (Fig. 3B, lanes 2 and 9). To identify these proteins, the immunoprecipitates were boiled and reprecipitated either with IκBα or different NF-κB antibodies. RelA was the only NF-κB-specific protein coprecipitated with anti-IκBα antiserum from PLB-985 cells (Fig. 3B, lanes 4 to 6). No detectable RelA was coprecipitated with anti-IκBα antiserum from PLB-IIIB cells (Fig. 3B, lanes 11 to 13). These results suggest that a portion of IκBα is not complexed with RelA in PLB-IIIB cells and that the rate of IκBα synthesis is higher in virus-infected than uninfected cells. Changes in the stability or phosphorylation of IκBα could result in the constitutive translocation of a fraction of RelA to the nucleus.

UV cross-linking analysis and sequence specificity of P2 binding proteins. UV cross-linking analysis with a BrdU-substituted DNA probe was used to identify the individual proteins interacting with P2. Unstimulated cells did not contain detectable levels of NF-κB binding proteins in this assay (Fig. 4, lanes 1 to 3); however, complexes induced in response to TNF-α (Fig. 4, lanes 4 to 6) or PMA (data not shown) treatment resolved into two major proteins, of 40 and 50 kDa, and a minor protein, of 70 kDa (Fig. 4, lane 4). These proteins could be inhibited through competition by an excess of either P2 or HIV-1 oligonucleotide (Fig. 4, lanes 5 and 6). In contrast, the DNA binding complex constitutively present in PLB-IIIB cells (55) and in Sendai virus-induced PLB-985 cells resolved into three proteins, of 70, 90, and 100 kDa (Fig. 4, lanes 7 to 10). These proteins exhibited a high degree of specificity for P2 and were inhibited through competition by an excess of unlabeled P2 probe (Fig. 4, lanes 9 and 13) but not the HIV-1 enhancer probe (Fig. 4, lanes 8 and 12). The 70-kDa protein was induced by TNF-α in HIV-1-infected cells (Fig. 4, compare lanes 7 and 11), and its binding was specifically inhibited by the addition of IκBα (data not shown). The 70-kDa protein therefore appears to be the NF-κB RelA (p65) subunit. Thus, distinct proteins with specificity for the P2 NF-κB site appear to be induced by either HIV-1 or Sendai virus infection of PLB-985 cells.

Specific binding to an AT-rich oligonucleotide. The P2 NF-κB site differs from the HIV-1 enhancer NF-κB site by only 2 bases, at positions 5 and 6, resulting in a 5-bp central AT-rich region. To determine whether virus-inducible complex A could bind to such an AT-rich site, cellular extracts from TNF-α-induced PLB-985 (Fig. 5, lanes 1 to 4) and PLB-IIIB (Fig. 5, lanes 5 to 8) cells were incubated with a radiolabelled AT-rich probe, either alone or with competitor DNA. Interestingly, binding to the AT-rich probe was detected only in virus-infected cells (Fig. 5, lanes 1 and 4); the AT binding activity was specifically inhibited by the addition of excess P2 (Fig. 5, lane 7) or AT-rich (Fig. 5, lane 8) oligonucleotide but not HIV-1 oligonucleotide (Fig. 5, lane 6). The same AT binding complex was also generated in extracts from Sendai virus-infected PLB-985 cells (data not shown). For determination of which proteins were involved in binding to this AT-rich region, competition analysis revealed that only the 90- to 100-kDa proteins could be specifically inhibited through competition by the addition of excess unlabelled AT-rich oligonucleotide (Fig. 4, lanes 10 and 14). This unique binding specificity of the 90- to 100-kDa

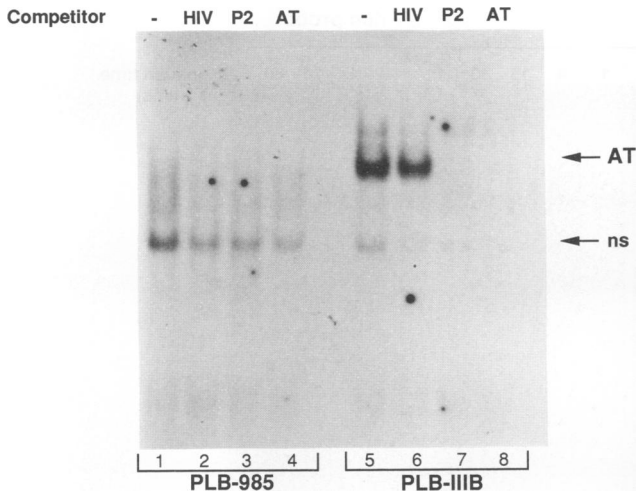


FIG. 5. Analysis of proteins binding to an AT-rich sequence. Extracts from PLB-985 (lanes 1 to 4) and PLB-IIIB (lanes 5 to 8) cells treated for 4 h with TNF-α were incubated with a ^{32}P-labelled AT-rich probe in the presence or absence of a 125-fold excess of unlabelled HIV-1 (lanes 2 and 6), P2 (lanes 3 and 7), or AT-rich (lanes 4 and 8) oligonucleotide as a competitor. Both the nonspecific (ns) band and the specific AT-rich complex are indicated with arrows.

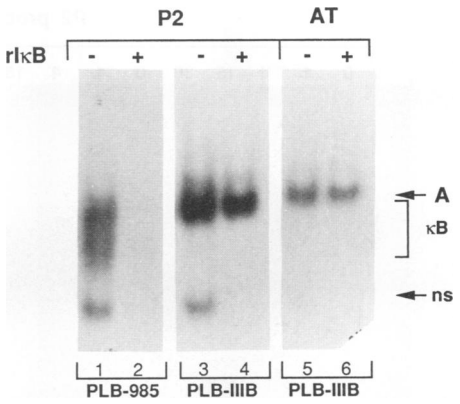


FIG. 6. Effect of rIκBα on inhibition of DNA binding activity. WCE (5 μg) from TNF-α treated PLB-985 (lanes 1 and 2) or PLB-IIIB (lanes 3, 4, 5, and 6) cells were incubated in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of rIκBα purified from *E. coli* for 10 min prior to the addition of a ^{32}P-end-labelled P2 (lanes 1 to 4) or AT-rich (lanes 5 and 6) probe and analyzed by mobility shift analysis. Nonspecific (ns) binding and complex A are indicated by arrows, while the NF-κB-specific complexes in lane 1 are indicated by the bracket.

species distinguishes them from the 70-kDa protein. Furthermore, the virus-induced complex binding to AT did not react with any NF-κB-specific antisera (p105 or p50, p100 or p52, RelA, or c-*rel*; data not shown).

Effect of rIκBα on DNA binding activity. To determine whether protein complexes induced by TNF-α or viral infection could be inhibited by IκBα, recombinant IκBα (rIκBα)/MAD-3 was preincubated with extracts from PLB-985 or PLB-IIIB cells before binding to the P2 probe (Fig. 6). rIκBα inhibited the TNF-α-induced NF-κB complexes in PLB-985 cells (Fig. 6, lane 2) but only partially decreased the level of complex A in PLB-IIIB cells (Fig. 6, lane 4). The protein-DNA complex formed with the AT-rich probe and extracts from PLB-IIIB cells (Fig. 6, lane 5) also was not inhibited by the addition of rIκBα (Fig. 6, lane 6). This result also indicates that virus-induced complex A was not regulated by IκBα and may not be an NF-κB-related complex.

Tryptic digestion analysis of proteins binding to P2. Previous studies demonstrated that NF-κB p50 had a trypsin-resistant core that maintained DNA binding specificity (3). To eliminate the possibility that the UV cross-linked 90- to 100-kDa adducts were p50 homodimers irreversibly bound to the probe and as an additional test of the NF-κB relatedness of the AT-rich binding 90- to 100-kDa proteins, tryptic digestion analysis of the different complexes was performed (Fig. 7). Recombinant p50 and extracts from TNF-α-treated PLB-985 and PLB-IIIB cells were digested with trypsin for 1 to 30 min and then analyzed for DNA binding activity to P2 (Fig. 7, lanes 1 to 15) and AT-rich (Fig. 7, lanes 16 to 20) probes. Purified recombinant p50 was reduced to a trypsin-resistant core in about 4 min (Fig. 7, lanes 1 to 5), whereas the complexes from TNF-α-treated PLB-985 cells (Fig. 7, lanes 6 to 10) were degraded more slowly but also generated the trypsin-resistant core. Interestingly, complexes from TNF-α-treated PLB-IIIB cells had trypsin-sensitive and trypsin-resistant components (Fig. 7, lanes 11 to 15), consistent with the fact that a portion of these complexes corresponds to NF-κB and a portion corresponds to the 90- to 100-kDa species. The protein complex that was from virus-infected cells and that specifically bound to AT-rich DNA was completely trypsin resistant (Fig. 7, lanes 16 to 20). As summarized in Table 1, the 90- to 100-kDa proteins observed in chronically HIV-1-infected PLB-IIIB cells or in Sendai virus-infected PLB-985 cells possessed a number of features that distinguished them from the NF-κB-specific complexes observed in TNF-α- or PMA-induced PLB-985 cells.

Expression of NF-κB-dependent plasmids in PLB cells. To

TABLE 1. Summary of NF-κB-like binding activities in PLB-985 cells

Assays used to characterize binding activity	Phorbol ester- or TNF-α-induced binding proteins using probe:			HIV- or Sendai virus-induced binding proteins using probe:		
	HIV enhancer	P2	AT	HIV enhancer	P2	AT
UV-cross-linked proteins (kDa)	40, 50[a]	40, 50	None	–[a]	70, 90, 100	90, 100
Cross-reactivity to NF-κB antisera[b]	p50	p50	None	p50, RelA	p50, RelA	None
rIκBα/MAD-3 inhibition[c]	++	++	–	++	+/–	–
Trypsin sensitivity[d]	ND	++	–	ND	+/–	–

[a] Refer to reference 55.
[b] Antisera against p50 and p105, p52 and p100, RelA, and c-*rel* were tested.
[c] ++, complete; ±, partial; – none.
[d] ++, complete; ± partial; – resistant; ND, not determined.

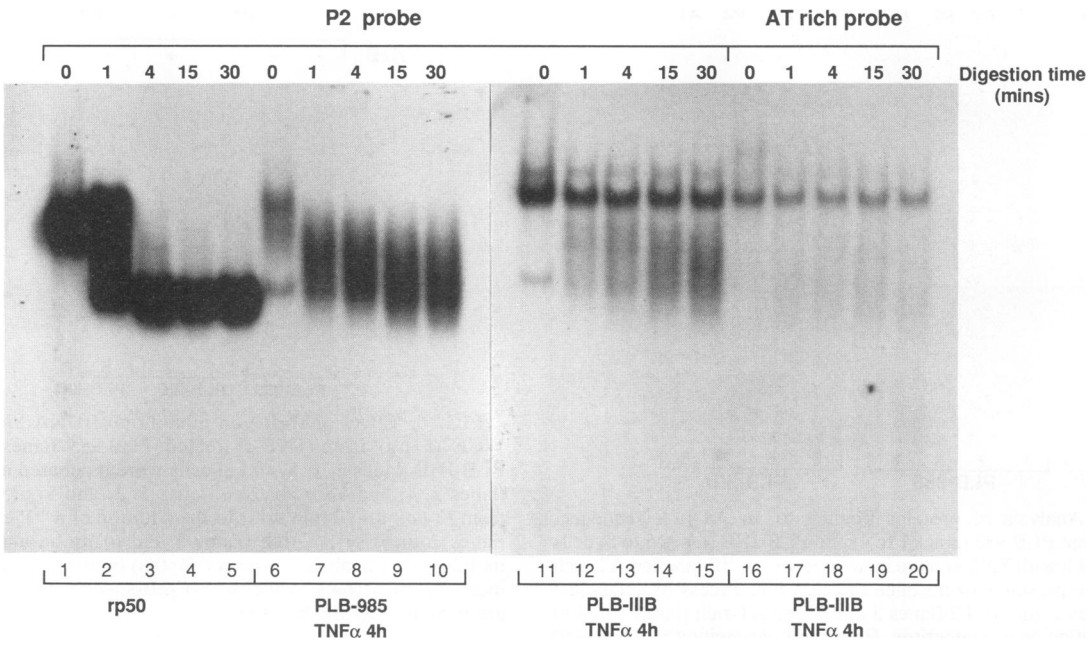


FIG. 7. Tryptic digestion of proteins binding to the P2 probe. Proteins binding to P2 (lanes 1 to 15) or to AT-rich (lanes 16 to 20) sequences were analyzed by an EMSA following tryptic digestion. Recombinant NF-κB p50 purified from *E. coli* (lanes 1 to 5) and WCE from TNF-α (4-h)-treated PLB-985 (lanes 6 to 10) or PLB-IIIB (lanes 11 to 20) cells were subjected to tryptic digestion analysis. Protein-DNA complexes were either untreated (lanes 1, 6, 11, and 16) or treated with TPCK-trypsin for 1 min (lanes 2, 7, 12, and 17), for 4 min (lanes 3, 8, 13, and 18), for 15 min (lanes 4, 9, 14, and 19), or 30 min (lanes 5, 10, 15, and 20), and then digestion was stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor.

demonstrate the functional consequences of these distinct binding species in vivo, transient transfection assays were performed in PLB-985 cells, using NF-κB-driven constructs expressing the CAT reporter gene (Fig. 8). Constructs consisted of the HIV-1 LTR linked to the CAT gene (HIV-LTR), four copies of the PRDII/NF-κB site linked to the minimal simian virus 40 promoter [P2(2)], three copies of the HIV-1 enhancer [5′κB(3)], and a mutant HIV-1 enhancer (HIV mut) (26, 37). Following DEAE-dextran-mediated transfection into PLB-985 cells, all constructs had a low level of activity; for all plasmids except the HIV mut plasmid, PMA treatment at 24 h after transfection resulted in a 4- to 12-fold inducibility of CAT activity. In contrast, when the same plasmids were introduced into PLB-IIIB cells, a high basal level of CAT activity was observed with the HIV-LTR, P2(2), and 5′κB(3) plasmids; this level of CAT activity was not increased significantly by PMA treatment of the cells. The high level of CAT activity was due to functional NF-κB binding sites, since the HIV mut plasmid had a low basal level of activity and no inducible activity in PLB-IIIB cells. In cotransfection experiments, the effects of overexpression of individual NF-κB subunits—NFKB1 (p50), RelA, c-*rel*, ΔRelA, or NFKB1 (p50) and RelA together—on HIV-LTR- or 5′κB(3)-dependent gene expression were measured in a Jurkat T-cell background (Table 2). The expression of either RelA alone or RelA in combination with NFKB1 (p50) stimulated the HIV-LTR, 5′κB(3), and P2(2) constructs but did not stimulate the HIV mut construct. Taken together, these results indicate that alterations in the NF-κB binding activities observed in HIV-1-infected cells may result in higher levels of NF-κB-regulated gene activity.

DISCUSSION

Two distinct types of virus infection—one an acute paramyxovirus infection and the other a chronic lentivirus infection—result in the appearance of multiple NF-κB-related protein-DNA complexes. Chronic HIV-1 infection sustains constitutive levels of NF-κB-like DNA binding activity in the myeloid model of HIV-1 infection. As summarized in Table 1, virus infection by either Sendai virus or HIV-1 induced DNA binding complexes consisting of 70-, 90-, and 100-kDa proteins; binding assays performed in the presence of subunit-specific antisera demonstrated that NFKB1 (p50) and RelA (p65) were present in the virus-induced complexes and bound to both HIV-1 enhancer and PRDII/NF-κB probes, although p50 was only weakly detected by cross-linking. On the basis of antibody cross-reactivity, molecular weight, TNF inducibility and inhibition by rIκBα, the 70-kDa protein corresponds to RelA (p65). Since the amounts of RelA and NFKB1 (p50) proteins were not increased in virus-infected cells, pulse-chase and coimmunoprecipitation experiments were undertaken to examine the interactions between RelA and IκBα. These experiments indicated that Sendai virus infection of PLB-985 cells induced de novo synthesis of IκBα, and the newly synthesized IκBα was chased into a complex that could be immunoprecipitated with either anti-RelA (p65) or anti-IκBα antiserum. In PLB-IIIB cells, anti-RelA (p65) antiserum immunoprecipitated a RelA-IκBα complex, whereas no detectable RelA was coprecipitated with anti-IκBα antiserum. These results suggest that in PLB-IIIB cells, two types of IκBα complexes may exist—one composed of RelA and IκBα and the other composed of IκBα without RelA (possibly complexed to other induced cellular proteins). As shown recently by Sun

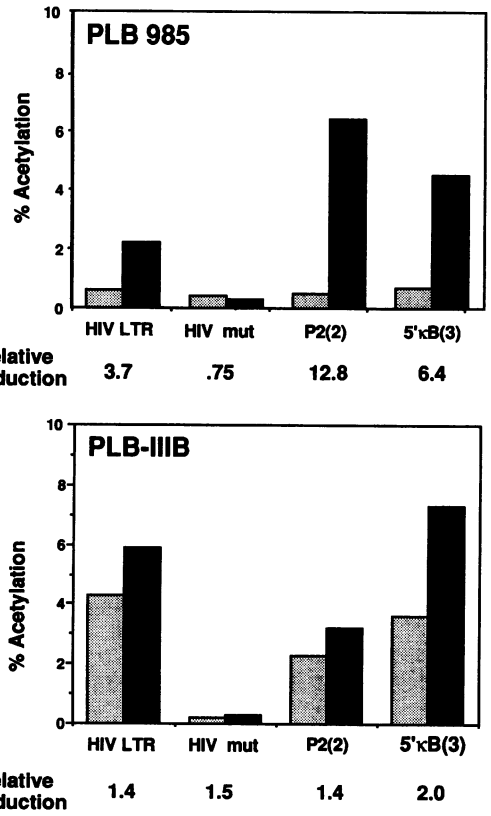


FIG. 8. Transcriptional activity of NF-κB-dependent reporter constructs in PLB-985 and PLB-IIIB cells. PLB-985 and PLB-IIIB cells were transfected by use of DEAE-dextran with 8 μg of a reporter plasmid containing the CAT gene under the control of the HIV-1-LTR, the HIV-1 enhancer [5'κB(3)], the P2 domain [P2(2)], or a mutant HIV-1 enhancer (HIV mut). Cells were treated with PMA (25 ng/ml) for 24 h following transfection and then harvested 12 h after induction. Chloramphenicol acetylation was assessed with 80 μg of total protein for 2 h; transcriptional activity is represented as percent acetylation. Symbols: ▨, uninduced cells; ■, PMA-induced cells.

et al. (63a), rapid degradation of a portion of the intracellular IκBα pool could result in constitutive translocation of a fraction of RelA to the nucleus. Nuclear RelA protein could in turn stimulate IκBα synthesis by an autoregulatory mechanism (63a).

TABLE 2. Transcriptional activity of individual NF-κB proteins on NF-κB-driven promoters

Inducer or NF-κB subunit	Activity[a] of CAT reporter construct:			
	HIV-LTR	5'κB(3)	HIV mut	P2(2)
None	3.3 (1.0)	3.5 (1.0)	0.3 (1.0)	1.4 (1.0)
PMA	30.1 (9.1)	52.5 (15)	0.5 (1.7)	20.1 (14.3)
NFKB1 (p50)	3.1 (0.9)	15.8 (4.5)	0.4 (1.3)	3.9 (2.8)
RelA (p65)	6.9 (2.1)	44.8 (12.8)	0.4 (1.3)	25.6 (18.2)
c-*rel*	2.0 (0.6)	6.4 (1.8)	0.3 (1.0)	4.6 (3.3)
NFKB1 + RelA	16.1 (4.9)	74.7 (21.3)	0.4 (1.3)	49.2 (35.1)
ΔRelA	1.8 (0.5)	4.3 (1.2)	ND	1.2 (0.9)
IκBα	0.8 (0.2)	1.7 (0.5)	ND	2.3 (1.6)

[a] Data are reported as percent acetylation (relative induction). ND, not determined.

Other studies have demonstrated the induction of NF-κB DNA binding activity in HIV-1-infected U937 cells (2), via a mechanism involving increased transcription and processing of the p105 precursor (47, 52). In THP-1 cells, HIV-1 infection resulted in two populations of chronically infected cells (50). Nuclear extracts from productively infected cells contained increased p50-RelA heterodimer binding activity, whereas extracts from cells expressing low levels of virus (restricted expression) displayed only p50 homodimer binding activity. Increased NF-κB p50-RelA binding activity was also observed in HIV-1-infected peripheral blood monocytes and macrophages, suggesting that a certain level of myeloid maturation was required prior to NF-κB induction (64).

The PRDII binding complex (A) identified in Sendai virus-infected cells and in HIV-1-infected PLB-IIIB cells was not completely shifted with the addition of p50 or RelA antibody or other NF-κB-specific antibodies, suggesting the presence of another, unrelated complex. This complex appears to be composed of the 90- to 100-kDa proteins, which possess a high affinity for binding to AT-rich sequences, including the IFN-β PRDII domain. Several experiments illustrate the lack of similarity between these proteins and previously characterized NF-κB subunits. First, the 90- to 100-kDa proteins associated with either the P2 or the AT-rich probe were not inhibited by the addition of excess rIκBα. Second, the formation of the 90- to 100-kDa protein complex with the P2 or the AT-rich probe was not blocked in the presence of several different NF-κB subunit-specific antisera. Third, the virus-induced 90- to 100-kDa protein complex was trypsin resistant, whereas recombinant p50 or p50-RelA from cell extracts was trypsin sensitive. The protein complex binding to the AT probe was also trypsin resistant. On the basis of the unique sequence specificity of the 90- to 100-kDa protein complex, the inability of IκBα to block DNA binding, and the trypsin resistance of the complex, these proteins do not appear to be NF-κB related.

One possibility is that the 90- to 100-kDa proteins are related to other transcription factors that bind AT-rich sequences or to the chromosome-associated HMG I/Y proteins, which have been shown to interact with the PRDII domain (65). HMG I/Y proteins of approximately 15 kDa bind to the minor groove of PRDII and interact with the central AAATT sequence on the coding strand. NF-κB binding to the major groove increased by 10- to 20-fold in the presence of HMG I/Y and resulted in a complex of retarded mobility that contributed to PRDII-dependent, virus-inducible expression of IFN-β (65). At present, we cannot rule out the possibility that the 90- to 100-kDa proteins represent a complex between HMG I/Y and the RelA subunit. The association of RelA with HMG I/Y may be highly stable and sufficient to alter the biochemical characteristics of the protein-DNA complex. However, mixing experiments with recombinant RelA and HMG I and Y (a kind gift from D. Thanos and T. Maniatis) failed to generate complex A-like binding activity (54a).

Recently, Nakayama et al. (42) characterized a high-molecular-weight NF-κB-like binding activity that was constitutively present in lymphoid cells and that interacted with the IL-6 κB motif, which also has a central 5-bp AT-rich region. Although the sequence specificity of IL-6 κB BFII was similar to that of the 90- to 100-kDa proteins described here, the BFII binding activity was partially inhibited by c-*rel* antisera (42). Similarly Lattion et al. (36) characterized a high-molecular-weight lymphoid cell-specific factor, NP-TCII, which bound to the simian virus 40 κB site but was distinct from NF-κB on the basis of several criteria: lack of

inhibition by IκBα, inability to be stimulated by deoxycholate treatment, distinct tryptic digestion products, and lack of reactivity to antisera against p50, v-*rel*, or c-*rel* (36). The level of expression of this factor was high in pre-B and immature T cells and was thought to be involved in lymphocytic development (36). The biochemical identity of these proteins, as well as a comparison with the 90- to 100-kDa proteins, will require further analysis.

Distinct combinations of NF-κB binding proteins contribute to differential gene activation (5, 28, 33, 35, 48, 68–70). In vitro transcription experiments demonstrated that NFKB1 (p50) homodimers stimulated transcription from a template controlled by the immunoglobulin κ element but not by the PRDII element, whereas RelA homodimers or NFKB1-RelA heterodimers stimulated in vitro transcription from both the immunoglobulin κ and the PRDII templates by 10-fold. Conformational differences may contribute to the degree of transcriptional activation by different subunit combinations (16). These results are consistent with those of cotransfection studies demonstrating that NF-κB heterodimer combinations are transcriptionally more potent than p50 homodimers (48, 61).

With regard to HIV-1 infection, an increase in transcriptionally active NF-κB DNA binding complexes in general and constitutive posttranslational induction of RelA in particular appears to provide an intranuclear environment that maintains a high level of HIV-1 LTR-mediated gene expression. Enhanced HIV-1 replication in myeloid cells may activate other NF-κB-regulated cytokine genes, including TNF, IL-1, IL-2, IL-6, and GM-CSF (49). In addition to an autostimulatory effect on HIV-1 replication and NF-κB induction, cytokine induction may produce a range of pleiotropic effects in the host, including inflammation, fever, and cachexia (54). The constitutive activation of specific members of the NF-κB/*rel* family of transcription proteins may thus contribute to the pathogenesis of HIV-1 infection in myeloid cells.

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