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Regulation of IκBα and p105 in Monocytes and Macrophages Persistently Infected with Human Immunodeficiency Virus

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The mechanisms regulating human immunodeficiency virus (HIV) persistence in human monocytes/macrophages are partially understood. Persistent HIV infection of U937 monocytic cells results in NF-κB activation. Whether virus-induced NF-κB activation is a mechanism that favors continuous viral replication in macrophages remains unknown. To further delineate the molecular mechanisms involved in the activation of NF-κB in HIV-infected monocytes and macrophages, we have focused on the regulation of the IκB molecules. First, we show that persistent HIV infection results in the activation of NF-κB not only in monocytic cells but also in macrophages. In HIV-infected cells, IκBα protein levels are decreased secondary to enhanced protein degradation. This parallels the increased IκBα synthesis secondary to increased IκBα gene transcription, i.e., increased RNA and transcriptional activity of its promoter-enhancer. Another protein with IκB function, p105, is also modified in HIV-infected cells: p105 and p50 steady-state protein levels are increased as a result of increased synthesis and proteolytic processing of p105. Transcriptional activity of p105 is also increased in infected cells and is also mediated by NF-κB through a specific κB motif. These results demonstrate the existence of a triple autoregulatory loop in monocytes and macrophages involving HIV, p105 and p50, and MAD3, with the end result of persistent NF-κB activation and viral persistence. Furthermore, persistent HIV infection of monocytes and macrophages provides a useful model with which to study concomitant modifications of different IκB molecules.

Human monocytes and macrophages are persistent reservoirs of human immunodeficiency virus (HIV) (20, 26, 27, 39, 47, 48, 54). While high numbers of productively infected macrophages have been found in the brain (39) and lymph nodes (54) of infected patients, little is known about the mechanisms regulating HIV persistence in these relevant host cells. Viral replication is in part controlled by specific viral proteins. Tat, a regulatory viral protein generated in the first cycles of viral transcription, increases and sustains viral replication by acting through the *tar* region in the HIV long terminal repeat (LTR) (16, 22, 35, 44). In some models, *tat* requires the interaction with *cis*-acting sequences present in the LTR and is known to bind cellular transcription factors such as NF-κB and Sp1 (8, 25, 36, 56, 68, 72). While both of these transcription factors play an essential role in the initial LTR-driven transcription of HIV, their potential function in regulating viral persistence in macrophages remains unknown.

NF-κB is an inducible transcription factor that regulates the expression of a large number of genes, including that of HIV (29, 51). The classic NF-κB complex is a heterodimer of p50 (NF-κB1) and p65 (RelA) (38, 52, 66). Each subunit belongs to a family of proteins with homology to the product of the c-*rel* proto-oncogene (67). Other members include p52 (NF-κB2) (10, 63), RelB (19, 61), and the *Drosophila* homolog, dorsal (33). Different homodimeric and heterodimeric combinations of these c-Rel-related molecules transactivate specific genes in response to different cellular activation signals (3). Much of this activation specificity is regulated by a variety of inhibitory molecules known as IκBs (42). Ankyrin-containing IκBs inhibit the transactivating potential of Rel-related molecules by their sequestration in the cytoplasm. IκB molecules with these features include IκBα (2, 4, 28, 30), IκBβ (74), pp40 (17), IκBγ (32), and the precursor proteins of p50 and p52, p105 (NF-κB1) and p100 (NF-κB2), respectively (43, 58, 62, 71). The cDNA of IκBα (MAD3) was isolated from a macrophage library, and the protein was shown to specifically inhibit the DNA binding of p50 and p65 (30). Such inhibition is mediated by physically masking the nuclear localization signal within p65, resulting in the cytoplasmic retention of the p50/p65 heterodimeric complex (7). NF-κB translocation to the nucleus requires at least its dissociation from IκBα (2, 4, 5). While the mechanisms leading to NF-κB/IκBα dissociation in the cytosol remain to be fully characterized, phosphorylation of IκBα (MAD3) occurring at the time of its dissociation from NF-κB has been documented in vitro (26) and in vivo (6, 15, 24, 69, 71) preceding its degradation by cellular proteases (6, 12, 15, 24, 31, 53, 70, 71). Whether phosphorylation is necessary for subsequent degradation remains to be clarified. p105 and p100 generate p50 and p52 upon proteolytic cleavage but also p65/p50 or p65/p52 complexes when p105 or p100, respectively, binds p65 (43, 58, 62, 71). Which stimuli and through which mechanisms the pool of p65 anchored by p105 and p100 can be translocated to the nucleus are unknown. Therefore, p65/p50 or p65/p52 complexes can be regulated in the cytosol by at least two independent mechanisms, one regulated by IκBα and the other regulated by p105 and p100.

Within the nucleus, NF-κB regulates the transcriptional activity of products of many viral and cellular genes (3, 41), including members of the *rel* family (p105, the precursor of p50 [62, 73]) and IκBα (12, 13, 40, 57, 65, 70). The promoter regions of the genes encoding p105 and IκBα contain functional NF-κB consensus binding sequences (40, 73), and p100 transcription is NF-κB dependent (71). Therefore, transloca-

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tion of NF-κB following cell activation increases IκBα, p105, and p100 transcription, replenishing the cytoplasmic store of such proteins. Newly assembled NF-κB will bind IκBα and/or p105, becoming anchored in the cytosol and restoring the cell to a prestimulatory (resting) state.

HIV infection of U937 monocytic cells results in increased nuclear translocation of NF-κB (1, 55, 60). The impact of this interaction in favoring viral persistence is unknown. To determine whether NF-κB activation by HIV plays a role in the regulation of HIV persistence in human monocytes and macrophages, we need to more fully understand the mechanisms by which HIV activates NF-κB, and in particular, if these mechanisms are similar to the ones previously described for transient stimuli (phorbol myristate acetate [PMA], tumor necrosis factor [TNF], etc.). Furthermore, it is important to investigate such mechanisms in more physiologically and relevant human host cells of HIV such as macrophages (29).

In this study, we have performed a systematic analysis of the different levels of NF-κB regulation in HIV-infected monocytes and, more importantly, in human macrophages by focusing in the transcriptional and posttranslational regulation of IκBα and p105. Here we show that HIV infection enhances NF-κB DNA binding not only in U937 monocytic cells but also in human macrophages. Analysis of p105 and p50 demonstrates increased steady-state protein levels secondary to increased protein synthesis of p105 and subsequent proteolysis to p50 in HIV-infected cells. This is secondary to increased transcription of the p105 promoter through a specific κB motif. On the contrary, steady-state protein levels of IκBα are decreased in HIV-infected monocytes and macrophages as a result of increased degradation. Increased IκBα synthesis regulated by NF-κB dependent transcriptional mechanisms compensates for the increased proteolysis. These results indicate that HIV infection in monocytes/macrophages results in a chronic and persistent activation of NF-κB by means of a triple positive autoregulatory loop composed of p105, IκBα, and HIV.

MATERIALS AND METHODS

Cells and HIV infection. U937 promonocytic cells (purchased from the American Type Culture Collection) were grown in RPMI with 5% heat-inactivated fetal bovine serum (Intergen), infected with the HIV LAV-Bru strain as previously described (1, 55), and studied from days 30 to 90 postinfection. During this period, infected cells are over 95% viable. Human macrophages were isolated by Ficoll-Hypaque gradient separation from buffy coats obtained from our blood bank. A total of 75×10^6 peripheral blood mononuclear cells were incubated in 10% human AB serum (Gibco) for 5 days in T75 flasks. Thereafter, nonadherent cells were removed, and the adherent population was infected with monocytotropic HIV strains (JR/FL and SF/162) obtained from the AIDS Reference and Reagent Repository. Over 95% of adherent cells are positive for nonspecific sterase and CD14 surface expression. Macrophages were harvested 3 to 4 weeks postinfection. HIV infection of U937 cells and human macrophages was monitored by reverse transcriptase activity and p24 assays (Cellular Products, Inc., Buffalo, N.Y.). The percentage of U937 cells and macrophages infected at the time of harvesting was determined by permeabilizing the cells and labeling them with fluorescein isothiocyanate-conjugated p24 (Virostat, Portland, Maine). Fluorescence-activated cell sorting analysis revealed a significant shift in the entire population (a single shifted peak), indicating that over 80% of cells were HIV infected. As previously described (1, 49, 50, 55), HIV infection of U937 cells and macrophages did not result in the production of cytokines known to activate NF-κB (TNF-α, interleukin-1, and interleukin-6).

Reagents. Lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 was obtained from Difco (Detroit, Mich.) and stored in water at −20°C. PMA was obtained from Sigma Chemical Co. (St. Louis, Mo.) and stored in dimethy sulfoxide at −20°C. TNF was obtained from Genzyme (Cambridge, Mass.) and stored in aliquots at −70°C.

Nuclear extraction and gel mobility shift assay. Nuclear protein was extracted by using a modification of the method of Dignam et al. (18). Briefly, 10^7 cells were washed with phosphate-buffered saline (PBS) and then buffer A (10 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES] 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT], 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml). Cells were then lysed with buffer A containing 0.1% Nonidet P-40 for 4 min at 4°C and washed twice with buffer A. The nuclear pellet was resuspended in 20 μl of buffer C (20 mM HEPES, 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, aprotinin, leupeptin, pepstatin) and incubated at 4°C for 30 min. Following centrifugation, the resultant supernatant was diluted with 50 μl of buffer D (20 mM HEPES, 20% [vol/vol] glycerol, 0.05 M KCl, 0.2 M EDTA, 0.5 mM PMSF, 0.5 mM DTT, aprotinin, leupeptin, pepstatin) and stored at −70°C. For mobility shift assays, 4 to 6 μg of protein extract was incubated with [γ^{32}P]ATP-labeled double-stranded oligodeoxynucleotide probe at room temperature for 15 min in 10 to 15 μl of binding buffer as previously described (1, 55). The binding reaction was analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel and visualized by autoradiography. DNA binding competition was assessed by preincubating the extract with a 40-fold excess of unlabeled oligonucleotide. The oligonucleotide used in the binding reaction corresponded to the NF-κB binding sequence present within the enhancer of the HIV LTR (5′-ACAAGGGACTTTCCGCT GGGGACTTTCCAGGGA-3′). The double-stranded probe was end labeled with [α-^{32}P]dCTP by using polynucleotide kinase.

Cytosolic extracts and Western blots (immunoblots). Cytosolic proteins were obtained from 0.5×10^7 to 1×10^7 cells. Cells were washed with cold PBS, resuspended in lysis buffer (10 mM HEPES, 50 mM NaCl, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 25% glycerol, 1 mM β-mercaptoethanol BME, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml) and incubated on ice for 4 min. The resultant supernatant contained cytosolic proteins (18). Amount of proteins was calculated as previously described (11), using the Bio-Rad protein assay. Equal amounts of protein were loaded in each sample. These proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes (Millipore) by standard procedures. Immunoblotting was done and visualized by using an ECL Western blotting detection kit (Amersham).

RNA extraction and Northern (RNA) blot analysis. Cells 10^7 were washed with 0.14 M NaCl and resuspended in 20 mM sodium acetate to 0.5% SDS. The same volume of water-saturated phenol was added and mixed by shaking in a 65°C water bath (3 min). The sample was then placed in an ethanol-dry ice bath until it started to freeze, warmed up to room temperature, and centrifuged. The phenol treatment was repeated on the upper phase, and the RNA was precipitated with sodium acetate and ethanol. It was then electrophoresed, transferred to a Hybond-N+ membrane (Amersham), and hybridized with ^{32}P-labeled probes. The IκBα (MAD3) probe consisted of a 1.3-kb *Eco*RI-*Xho*I fragment of the MAD3 cDNA (provided by Chiron Corporation), and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe consisted of a 1.3-kb *Pst*I fragment (23). Both probes were labeled with [α-^{32}P]dCTP by using a random primer DNA labeling kit (Boehringer Mannheim).

Plasmids. Plasmid 1.2 HN-luc (wild-type p105) consisted of the 1.2-kb *Hin*dIII-*Nco*I fragment of the p105 genomic clone inserted into the *Hin*dIII site of plasmid pc-luc upstream of the luciferase gene, as described before (73). ΔκB1-1.2 HN-luc, ΔκB2-1.2 HN-luc, and ΔHIP1 1.2 HN-luc contained the same insert as 1.2 HN-luc with deletions of κB1 (−72 to −61), κB2 (118 to 128), and κB3/HIP1 (213 to 227), respectively. κB3m-1.2 HN-luc and HIP1m-1.2 HN-luc contained the same insert as described above, with mutations 5′ of the κB3 (213 to 217) and 3′ of the HIP1 (223 to 227) sites, respectively. *Hin*dIII-*Xba*I-generated 1.2-kb fragments were subcloned into the *Hin*dIII site of plasmid pc-luc. Orientation of the inserts was verified by restriction mapping and confirmed by DNA sequencing as described above.

The κB1, κB2, and HIP1 deletions and the κB3 and HIP1 mutations were introduced into the 1.2-kb *Hin*dIII-*Nco*I fragment (cloned into Bluescript SK+ [Stratagene, La Jolla, Calif.]) by using the Mutagene kit (Bio-Rad, Richmond, Calif.) as instructed by the manufacturer. The oligonucleotides used as primers were synthesized in an Applied Biosystems model 394 DNA synthesizer and consisted of 23 to 39 nucleotides flanking the target sites for deletion or mutation: ΔκB1 [5′CAGGCGCTTCCT(GGGGGCTTCCC)TACCGGCTCCAG3′], ΔκB2 [5′CCCTAGAAGTGC(GGGCTTCCCC)CACCCCCGGC3′], ΔHIP1 [5′ACGTCAGTGGGA(ATTTCCAGCCA)GGAAGTGAGAGA3′], κB3m (5′CAGT***CTCC***ATTTCCAGCCAGGAA3′), and HIP1m (5′GTGGGAATTTC C***GCTAG***GGAAGTGAGA3′) (lowercase) letters and underlining indicate the native sequence to be deleted, and italicized boldface letters indicate mutations to be introduced in the native sequence). The mutated or deleted clones were verified by sequencing using Sequenase 2.0 (United States Biochemical, Cleveland, Ohio).

Plasmid κB-luc contains three tandem copies of the κB motif of the HIV LTR cloned upstream of plasmid cona-luc.

Plasmid MAD3-luc contains nucleotides −385 to +78 of the MAD3 cDNA cloned into the *Sac*I-*Kpn*I site of pGL2-Basic vector (Promega). Plasmid MAD3ΔκB is the same with a deletion of the κB motif (40). The thymidine kinase (TK)–β-galactosidase (β-Gal) plasmid is a mammalian reporter vector designed for expression of β-Gal in mammalian cells as transcribed by the herpes simplex virus TK minimal promoter (Clontech).

Antibodies. The antibodies used to identify the proteins present in the gel shift assay included polyclonal anti-p50 (28) and anti-c-Rel and anti-p65 (Santa Cruz Biotechnology Antibodies, Santa Cruz, Calif.) antibodies. Western blotting and immunoprecipitation were performed with polyclonal anti-p50, anti-p65, and anti-IκBα MAD3 (Santa Cruz Biotechnology Antibodies) sera. In some exper-

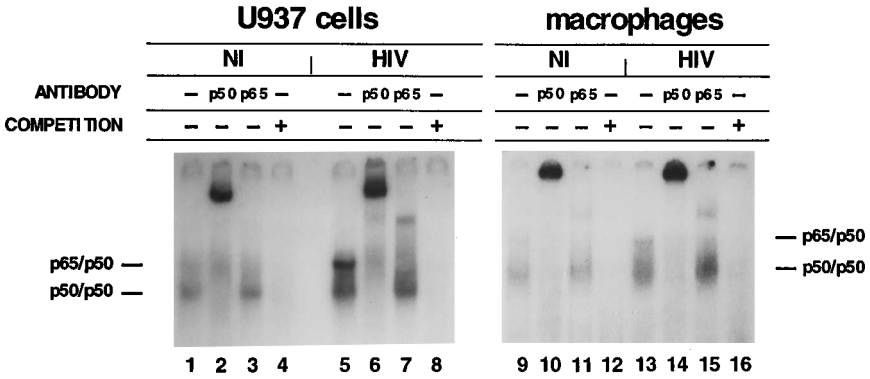

FIG. 1. Gel shift analysis of NF-κB DNA binding. A double-stranded oligonucleotide corresponding to the NF-κB-binding sequences of the enhancer region of the HIV LTR was used as a probe and incubated with nuclear proteins extracted from non infected (NI) and HIV-infected U937 cells (6 μg per sample) and with nuclear extracts from human macrophages (4 μg per sample). Lanes: 1, 6, 9, and 13, nuclear extract only; 2, 7, 10, and 14, nuclear extract preincubated with anti-p50; 3, 8, 11, and 15, nuclear extract preincubated with anti-p65; 4, 9, 12, and 16, nuclear extract preincubated with a 40-fold excess of unlabeled oligonucleotide. The composition of each bands is indicated at the left and right.

iments, a polyclonal anti-IκBα (MAD3) serum generated by using a glutathione *S*-transferase–MAD3 fusion protein or an anti-p105/p50 serum raised against a glutathione S-transferase–p105 fusion was also used.

Transfections. U937 cells (10^7 per point) were electroporated with 5 to 10 μg of luciferase reporter plasmid and 10 μg of control TK–β-Gal, using a Bethesda Research Laboratories Cell-Porator (800 μF and 300 V); 2×10^6 transfected cells were harvested 8 h later, using cell culture lysis reagent (Promega, Madison, Wis.), and β-Gal activity was detected by using the Tropix Galacto-Light reporter assay (34). The rest of the sample (8×10^6 cells) was harvested by the same procedure 16 to 20 h later, and luciferase levels were measured by using the Promega luciferase assay system. Both β-Gal and luciferase were measured in a Berthold Lumat. Total protein in an aliquot of each sample was measured by the Bradford technique (Bio-Rad) as described above. Results are presented as luciferase units/β-Gal units.

[^{35}S]methionine labeling and immunoprecipitation. U937 cells (10^7 per point) were incubated in methionine-free medium (ICN) for 30 min and then labeled with 1 mCi of [^{35}S]methionine-cysteine (32×10^6) cells for different time periods. For chase experiments, cells were centrifuged and resuspended in regular (methionine- and cysteine-containing) medium for different lengths of time. Cytosolic fractions were obtained by lysing cells in a lysis buffer (pH 7.5; 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml). Total cell extracts were obtained by using a radioimmunoprecipitation assay lysis buffer (0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris [pH 7.5], 1 mM PMSF, 1 mM sodium orthovanadate, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml). After centrifugation, precleared cell extracts were incubated overnight at 4°C with the specific antibody. Protein G-agarose beads (Gibco-BRL) were added to each sample, the samples were incubated for 2 h and washed, and the protein was eluted. These eluants were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore) as described above, and visualized by autoradiography and/or immunoblotting.

RESULTS

Increased NF-κB DNA-binding activity in HIV-Infected monocytes and macrophages. Increased NF-κB DNA binding has been described in monocytic cell lines infected with HIV (1, 55, 60). We first wanted to determine whether this phenomenon is present in a more relevant host cell such as macrophages. Human macrophages derived from peripheral blood monocytes were mock infected (supernatant of phytohemagglutinin–interleukin-2-treated peripheral blood lymphocytes in the absence of virus) or infected with the HIV strain SF/162. HIV-infected macrophages contained increased NF-κB DNA binding as shown by electrophoretic mobility shift assay (Fig. 1). Infection with other monocytotropic strains such as JR/FL, Ada, and BaL also resulted in NF-κB activation, while mock infection or infection with LAV-Bru did not result in HIV infection or NF-κB activation (not shown). The HIV-dependent NF-κB activation present in human macrophages is increased over the baseline NF-κB DNA-binding activity normally present in human macrophages (29). Preincubation of nuclear extracts with antibodies specific for the different NF-κB subunits as well as competition with an excess of unlabeled NF-κB DNA-binding oligonucleotide demonstrated that the NF-κB-like complex present in both HIV-infected macrophages and monocytic cell lines consists of a p50/p65 heterodimer. The anti-c-Rel antibody did not modify the nuclear DNA-binding complex, while it upshifted a DNA-binding complex present in the nuclei of the B-cell line BJAB (not shown).

IκBα undergoes posttranslational modifications in HIV-infected monocytes and macrophages. Having shown that HIV-induced NF-κB activation occurs not only in monocytic cell lines but also in a more relevant host cell target such as human macrophages, we next investigated whether IκB molecules are regulated by persistent HIV infection in these cells. IκBα is one key molecule in regulating NF-κB translocation from the cytosol to the nuclei, as shown by the fact that transient cell stimuli such as TNF, LPS, or PMA (12, 70) result in its phosphorylation and subsequent degradation (6, 12, 15, 24, 57, 70, 71). Immunoblotting experiments of cytosolic fractions from HIV-infected and uninfected U937 cells and human macrophages demonstrate that the steady-state levels of IκBα are persistently decreased in infected compared with uninfected cells (Fig. 2A, lanes 3 and 5). We next investigated whether decreased cytosolic IκBα in infected cells is secondary to decreased protein synthesis or, alternatively, results from increased protein degradation as would be expected following treatment with stimuli such as LPS (Fig. 2A, lane 2). Results of pulse-chase [^{35}S]methionine-cysteine labeling experiments followed by IκBα immunoprecipitation (Fig. 2B) indicate that both the synthesis rate (pulse time, 0 min) and IκBα degradation (60- and 180-min chases are increased in infected cells compared with uninfected cells. To specifically analyze whether the IκBα molecule susceptible to degradation was associated with NF-κB, additional ^{35}S pulse-chase experiments were performed. The entire NF-κB/IκBα complex was immunoprecipitated with the anti-p65 or anti-p105/p50 antibody. Increased IκBα synthesis (pulse time, 0 min) and degradation (180-mm chase) were present while the protein was associated with NF-κB, as determined by immunoprecipitating p65 (Fig. 2C) or p105/p50 (see Fig. 4B). The half-lives of IκBα calculated from Fig. 2B were 187 mm in uninfected cells and 103 min in infected cells. Similar results were obtained from Fig. 2C and 4B. Whether increased IκBα degradation in infected cells is secondary to its phosphorylation by a specific kinase(s)

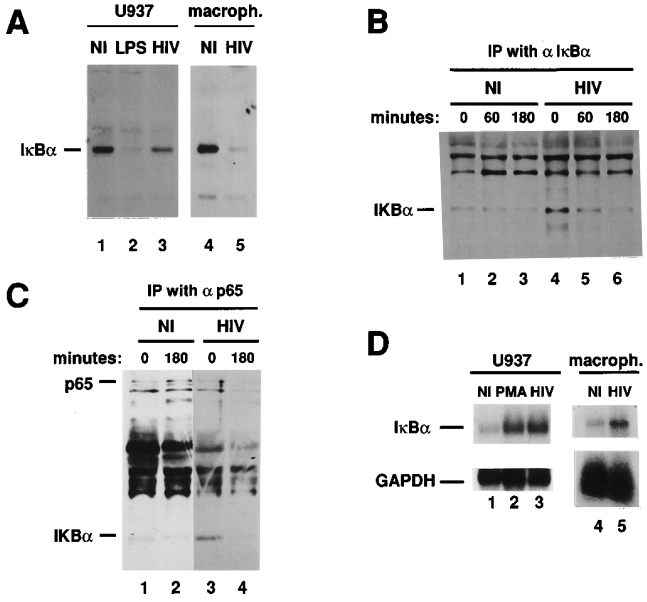

FIG. 2. Cellular sublocalization, synthesis and processing, and steady-state RNA levels of IκBα (MAD3). (A) Cytosolic proteins from U937 cells and human macrophages were extracted, subjected to SDS-PAGE, transferred, and immunoblotted with anti-IκBα (MAD3) antibodies. Equal amounts of protein were run in all lanes. LPS stimulation of U937 cells was done with a concentration of 1 μg/ml for 2 h. NI, noninfected. (B) U937 cells were labeled with [^{35}S]methionine for 2 h, chased with unlabeled methionine-containing medium for the indicated time, lysed, immunoprecipitated (IP) with anti-IκBα (MAD3), and analyzed by SDS-PAGE. The amounts of ^{35}S labeling of non-IκBα-related proteins were equal in all lanes. (C) U937 cells were labeled with [^{35}S]methionine for 20 min, chased with unlabeled methionine-containing medium for the indicated times, lysed, immunoprecipitated with anti-p65, and analyzed by SDS-PAGE. (D) RNA from U937 cells and human macrophages was extracted, electrophoresed, transferred, and hybridized with a ^{32}P-labeled MAD3 cDNA probe and subsequently with a ^{32}P-labeled GAPDH probe. PMA stimulation of U937 cells was done with a concentration of 20 ng/ml for 2 h.

or is directly caused by a cellular and/or viral protease independently from phosphorylation remains unknown.

Transcriptional regulation of IκBα in HIV-infected monocytes and macrophages. We next assessed whether increased IκBα synthesis in HIV-infected monocytic cells and macrophages is secondary to increased gene transcription. Northern blot analysis indicates that steady-state levels of IκBα RNA are also increased in HIV-infected monocytes and macrophages compared with uninfected cells (Fig. 2D). To further determine whether such increase is a result of enhanced gene transcription of the MAD3 enhancer-promoter, we performed transient transfections of the IκBα promoter cloned upstream of a luciferase reporter gene in uninfected and HIV-infected monocytic cells. A TK–β-Gal reporter gene was cotransfected to control for transfection efficiency. As shown in Fig. 3A, increased NF-κB activity is present in HIV-infected cells compared with uninfected cells as determined by increased luciferase production of an NF-κB concatemer-luciferase reporter gene which is also upregulated by LPS in uninfected cells (Fig. 3B). Similarly, the IκBα enhancer-promoter-luciferase construct is transcriptionally upregulated both in HIV-infected (compared with uninfected) cells (Fig. 3C) and in uninfected cells treated with LPS (Fig. 3D). To determine whether the IκBα upregulation by HIV is NF-κB dependent, a deleted IκBα luciferase construct was transfected into uninfected and HIV-infected cells. As shown in Fig. 3C, deletion of the κB site within the IκBα promoter significantly reduces the HIV-mediated upregulation of the wild-type IκBα promoter as well as that induced by LPS (Fig. 3D). Altogether, these results indicate that increased IκBα synthesis in HIV-infected cells is secondary to transcriptional upregulation of the promoter of the gene by NF-κB itself.

Regulation of p105 in HIV-infected cells. Other molecules aside from IκBα are involved in the regulation of NF-κB. p105 and p100 anchor p65 in the cytosol, resulting in an inactive pool of p65. Proteolytic cleavage of each precursor potentially leads to the formation of p65/p50 and p65/p52 complexes, respectively, that theoretically, are then free to undergo nuclear translocation. Even though our data point to IκBα as a key regulatory molecule targeted by persistent HIV infection to result in continuous NF-κB translocation, we cannot exclude that this chronic stimulus also regulates other functional IκB proteins such as p105. To further investigate this possibility, we first analyzed the steady-state protein level of p105 in uninfected and HIV-infected monocytic cells and human macrophages. Cytosolic fractions of infected monocytic cells and human macrophages contain increased steady-state levels of p105 and p50 (Fig. 4A). To investigate whether p105 synthesis and, possibly, the proteolytic cleavage of p105 into p50 are upregulated, we performed metabolic pulse-chase labeling experiments using [^{35}S]methionine-cysteine followed by p105/p50 immunoprecipitation. As shown in Fig. 4B, increased synthesis of p105 is already present in total cell extracts of HIV-infected cells after 20 min of pulse-labeling (time 0) compared with uninfected cells. Chase with unlabelled methionine and cysteine indicates that the amount of ^{35}S-labeled p105 is not modified in uninfected cells after 120 min, while a reduction of ^{35}S-labeled p105 is observed within the same period in HIV-infected cells. ^{35}S-p50, presumably resulting from p105 proteolytic processing, is already present in the 20-min pulse time in both uninfected and HIV-infected cells. However, a moderate increase in ^{35}S-p50 is observed in the chase point in HIV-infected cells compared with uninfected cells. To confirm this result, p105 and p50 ^{35}S-labeled bands were scanned by densitometry. The ratios of p105 to p50 in uninfected cells were 0.492 and 0.486 for the pulse and chase points, respectively; while in the HIV-infected cells, the ratios were 0.661 (pulse) and 0.501 (chase). These results suggest that p105 synthesis is increased in HIV-infected cells and that the processing of p105 into p50 is moderately enhanced in these cells compared with uninfected controls. Whether induced p105 proteolysis is mediated by a viral protease directly, as suggested previously in acute HIV infection in T lymphocytes (59), or by cellular protease(s) such as via activation of the ubiquitin-proteasome pathway (53) by HIV, remains unknown. Regardless of the mechanism, these results provide evidence that a persistent stimulus such as HIV can induce the modification of at least two inhibitory molecules to result in continuous NF-κB activation.

p105 transcriptional activity is upregulated in HIV-infected monocytes; role of the κB3 motif in the p105 enhancer-promoter. Increased synthesis of p105 is probably regulated by transcriptional mechanisms as shown to be the case of IκBα regulation in infected cells. Our group had previously shown that the native p105 enhancer-promoter is upregulated by NF-κB and that its transcriptional activity in HIV-infected monocytic cells is also increased (55, 73). Moreover, as shown in Fig. 4C, Northern blot analysis of RNA from uninfected and HIV-infected monocytic cells indicates increased steady-state RNA of p105 in HIV-infected cells. We next investigated through which region(s) of the promoter-enhancer of the p105 gene was mediating its upregulation via HIV infection. To do so, a detailed study of the different κB and other *cis*-acting motifs present in the p105 promoter was performed. Rese-

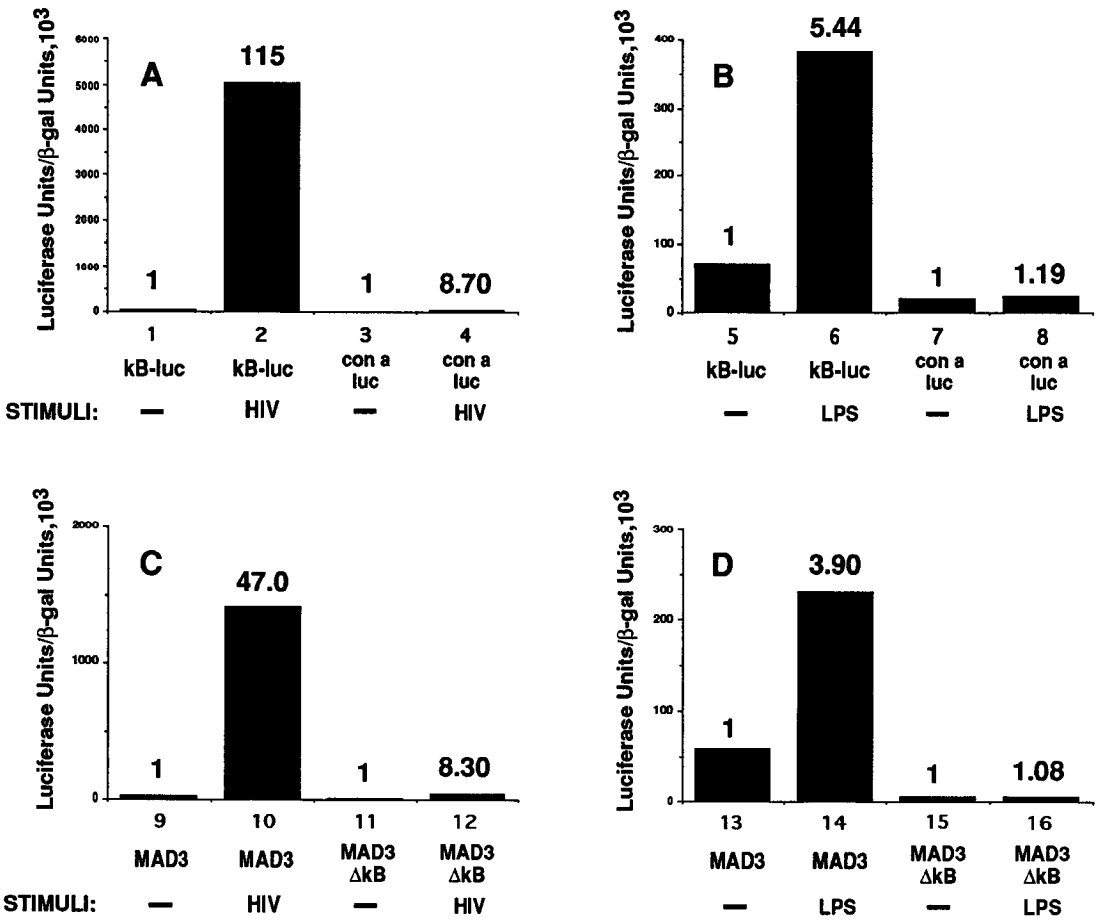

FIG. 3. Transcriptional activity of the IκBα (MAD3) enhancer-promoter. U937 cells were transfected as described in Materials and Methods. (A) Uninfected (−) and HIV-infected (HIV) cells were transfected with a κB-luc concatemer or the minimal cona-luc promoter. (B) Same plasmids transfected into uninfected cells stimulated or not with LPS (1 μg/ml). (C) The wild-type IκBα (MAD3) enhancer-promoter cloned upstream of a luciferase reporter gene (MAD3) or with a mutation of the κB motif (MAD3 ΔκB) was transfected into uninfected (−) or HIV-infected (HIV) cells. (D) The same MAD3 reporter genes were transfected into uninfected cells treated or not with LPS (1 μg/ml). In all experiments, luciferase activity was normalized to the β-Gal activity of a TK–β-Gal plasmid. The relative fold induction by HIV or LPS is indicated over each bar. This is a representative experiment of at least three additional experiments.

quencing of the promoter identified the two already described κB sequences (κB1 and κB2) and an additional one (κB3) which overlaps a putative HIP1/E2F motif (Fig. 5A). The DNA-binding activity of each of these four motifs (κB1, κB2, κB3, and HIP1/E2F) was analyzed in electrophoretic mobility shift assays using labeled oligonucleotides of each of these sequences. Results from these experiments indicate that the three κB motifs but not HIP1/E2F bind a nuclear protein(s) induced by LPS or HIV in U937 cells. This DNA-binding activity was identified as NF-κB, the κB3 motif being the one that conferred the highest DNA-binding activity of the three κB motifs (data not shown). Using transfection experiments, we next tested which of the four *cis*-acting motifs in the p105 promoter mediates its upregulation by stimuli that activate NF-κB in uninfected and in HIV-infected cells (see Fig. 5B for constructs). Deletions of the four motifs in the wild-type p105 promoter (see Materials and Methods) were introduced by site-directed mutagenesis and tested in transfection experiments. In uninfected cells, the wild-type p105 and the ΔκB2 and ΔHIP1 constructs were responsive to a cotransfected p65 expression plasmid, while the ΔκB1 motif and, to a higher degree, the ΔκB3 motif were less responsive, suggesting that κB3 (and to a lesser degree κB1) mediates p65 transactivation of the wild-type p105 (Fig. 6A). Similar

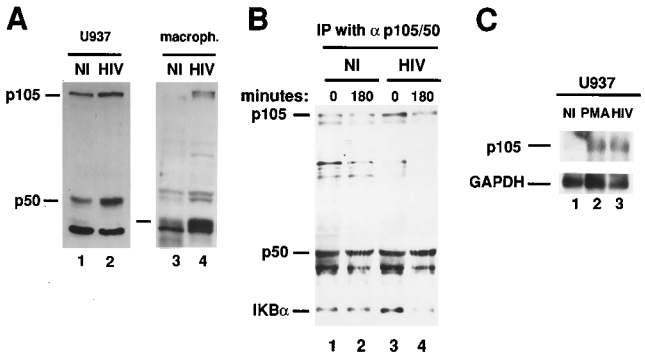

FIG. 4. Cellular sublocalization, synthesis and processing, and steady-state RNA levels of p105 and p50. (A) Cytosolic proteins from U937 cells and human macrophages were extracted, subjected to SDS-PAGE, transferred, and immunoblotted with anti-p105/p50 antibodies. Equal amounts of protein were run in all lanes. NI, noninfected. (B) U937 cells were labeled with [^{35}S]methionine for 20 min, chased with unlabeled methionine-containing medium for the indicated time, lysed, immunoprecipitated (IP) with anti-p105/p50, and analyzed by SDS-PAGE. The amounts of ^{35}S labeling on non-p105/p50-related proteins were equal in all lanes. (C) RNA from U937 cells was extracted, electrophoresed, transferred, and hybridized with a ^{32}P-labeled p105 cDNA probe and subsequently with a ^{32}P-labeled GAPDH probe. PMA stimulation of U937 cells was done with a concentration of 20 ng/ml for 2 h.

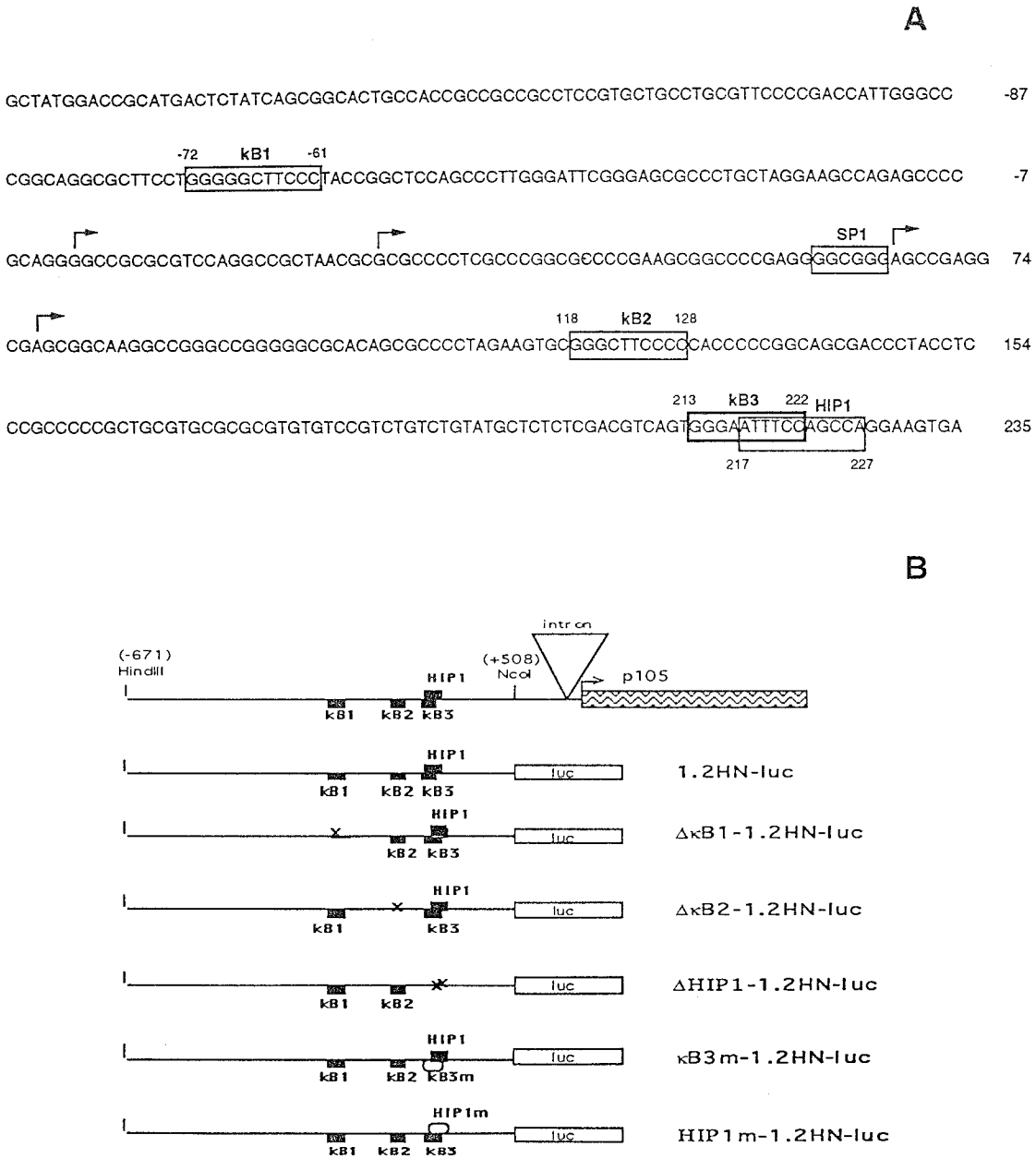

FIG. 5. p105 enhancer-promoter sequence and p105-luciferase constructs. (A) Sequence of the p105 enhancer-promoter (modified from reference 73). Arrows indicate transcription initiation sites. Boxes indicate specific DNA motifs. Numbering of nucleotides is from the major transcription initiation site. (B) p105-luciferase constructs. Deletions (X) or mutations (open ovals) were made by using oligonucleotides (see Materials and Methods) based on sequences in panel A.

results were observedin uninfected cells stimulated with LPS and PMA: κB3 but not the other motifs mediates NF-κB activation of a wild-type p105-luciferase construct (Fig. 6B). We next tested the basal activity of the wild-type and deleted p105-luciferase constructs in HIV-infected and uninfected cells. As shown in Fig. 6C, the κB3 motif but not the other DNA-binding motifs also mediates p105 transactivation by HIV.

DISCUSSION

Human monocytes and macrophages are a main reservoir of persistent HIV replication in infected individuals (20, 26, 27). The precise mechanisms regulating viral persistence in these host cells still remain elusive. NF-κB is an important participant in the transcriptional regulation of the HIV LTR (51). Whether it plays a role in sustaining viral replication through interactions with regulatory viral proteins such as Tat is not known. It has been previously shown that persistent HIV infection of monocytic cells results in NF-κB activation (1, 55, 60). We hypothesize that this unique virus-host cell transcription factor interaction is important for the regulation of viral persistence in these cells. In order to interrupt this interaction that could potentially lead to decreased viral replication, it is mandatory to understand (i) how NF-κB is regulated in human monocytic cells and (ii) how HIV infection influences NF-κB regulation. In this study, we show that NF-κB activation by

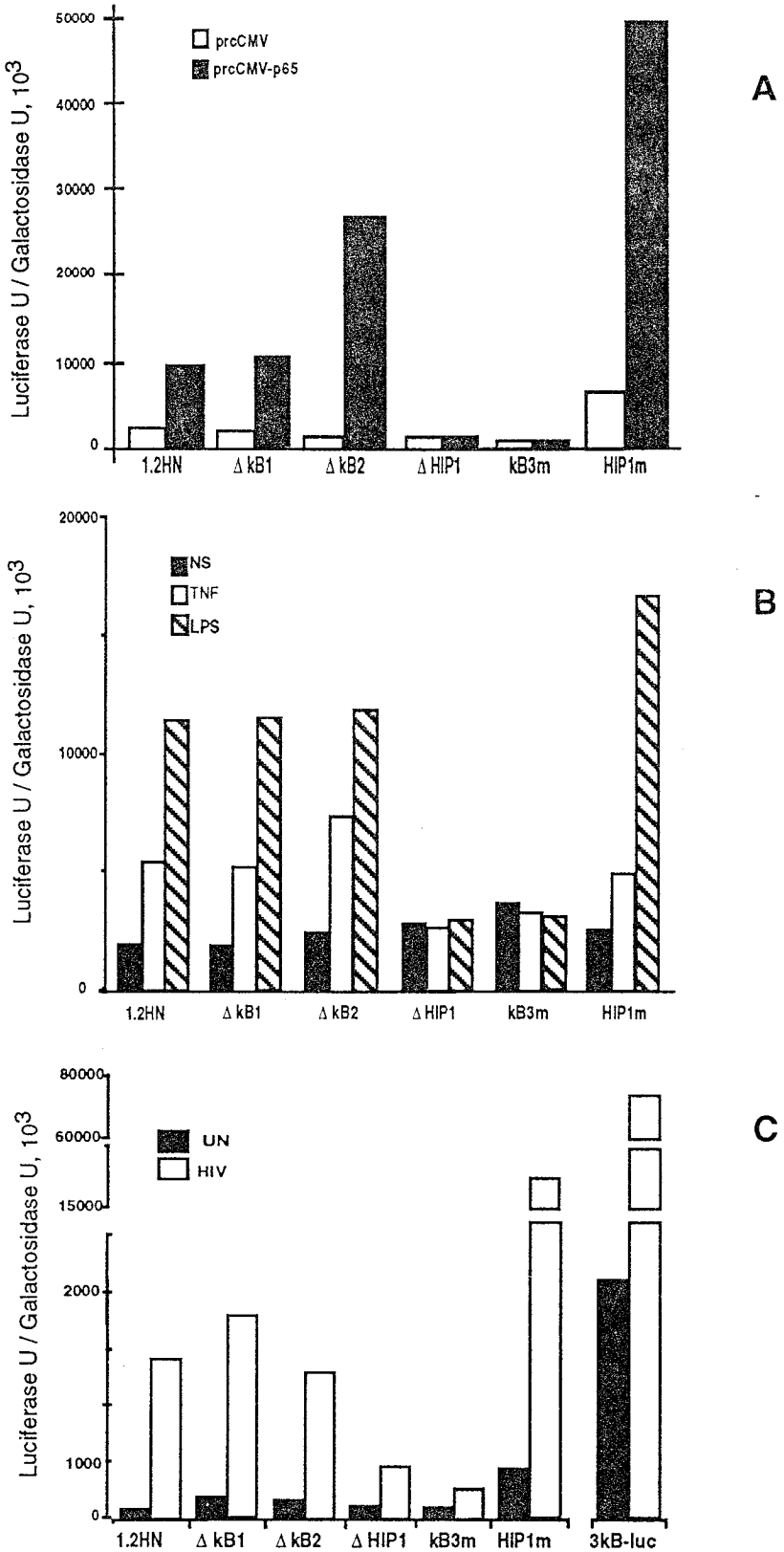

FIG. 6. Transfection of p105-luciferase constructs into U937 cells. (A) Uninfected U937 cells were transfected as described in Materials and Methods with p105-luciferase constructs and a control prCMV or prCMV p65 expression vector. (B) Uninfected U937 cells transfected with p105 constructs followed by no stimulation (NS), TNF (40 μg/ml), or LPS (1 μg/ml) for 18 h. (C) U937 uninfected (UN) and HIV-infected (HIV) cells were transfected with p105 constructs and a 3-kb cona-luc reporter gene. In all experiments, luciferase activity was normalized to the β-Gal activity of a TK–-Gal plasmid. Representative experiments of at least three experiments are shown.

HIV occurs not only in monocytic cells but more relevant, in human macrophages, and that two different IκB molecules, IκBα and p105, are selectively modified by persistent HIV infection. This would appear to result in continuous nuclear translocation of NF-κB and increased transcription of NF-κB-dependent promoters, such as p105, IκBα, and HIV promoters.

Our studies indicate that the NF-κB complex translocated into the nucleus of infected monocytes and macrophages is a bona fide p50/p65 complex in which c-Rel is absent. Because NF-κB and related family members are regulated by complex interactions with a variety of IκB molecules, we first investigated those IκBs which have been shown in vitro and in vivo to interact with p50 and p65. Candidate IκB molecules potentially implicated in regulating p65 and p50 in monocytic cells include IκBα and p105 or p100. Results from our studies indicate that persistent HIV infection is an example of a chronic stimulus which targets at least two different IκBs, IκBα and p105.

IκBα undergoes posttranslational modifications (degradation with or without phosphorylation) induced by signal transduction pathways triggered by a cellular stimulus such as TNF, LPS, or PMA. Although the relevance of IκBα phosphorylation as a requisite for subsequent protein degradation remains to be shown, all of the punctual stimuli mentioned above have been unequivocally shown to result in IκBα degradation concomitantly with NF-κB translocation (6, 12, 15, 24, 31, 53, 70, 71). In the model of persistent HIV infection in which continuous NF-κB translocation is present, our results indicate that IκBα levels are persistently decreased within infected monocytic cells and macrophages, suggesting that IκBα is a target of HIV-dependent pathways that lead to its posttranslational modification(s). At this point, it is unclear whether HIV infection results in IκBα phosphorylation rendering the molecule susceptible to subsequent degradation or whether proteolysis occurs in the absence of specific phosphorylation. Recently, persistent expression of human T-cell leukemia virus type I *tax* has been shown to increase IκBα degradation (37, 69) preceded by its phosphorylation (69). Our preliminary data derived from immunoprecipitation experiments of $^{32}P_i$ and [^{35}S]methionine-labeled cells indicate that IκBα is a phosphoprotein in the resting state and that an additional, more slowly migrating phosphorylated form of IκBα is present in infected cells. Confirming these results would imply that models of chronic NF-κB activation such as the one presented here may facilitate the study of the phosphorylation characteristics of IκBα and its regulation by different signal transduction pathways. If phosphorylation and/or other posttranslational modifications are not involved in predisposing IκBα to subsequent degradation, our data would imply that at least a proteolytic mechanism is activated in HIV-infected cells. Whether a cellular protease is upregulated by HIV infection such as via the ubiquitin-proteasome pathway or whether the HIV protease itself (53, 59) regulates IκBα degradation remains unknown. Alternatively, continuous IκBα phosphorylation by HIV-dependent signal transduction pathways would suffice to render the molecule susceptible to normally functional cell proteolytic pathways. The fact that newly synthesized IκBα undergoes some degree of proteolysis in resting nonactivated cells as shown in other studies (57) suggests that proteolytic pathways are functional. The notion that increased IκBα proteolysis in HIV-infected cells occurs in the face of a compensatory but apparently insufficient enhanced protein synthesis is novel and helps to explain that persistently decreased IκBα levels result in continuous NF-κB translocation.

The transcriptional regulation of IκBα by NF-κB has been clearly demonstrated and supports that concept of autoregulation between NF-κB and IκBα (12, 13, 40, 57, 65, 70, 73). Transient stimuli that result in NF-κB translocation by inducing degradation of IκBα also increase its transcription. This mechanism compensates the utilization (degradation) of IκBα and interrupts continuous NF-κB activation: newly synthesized IκBα will anchor NF-κB in the cytosol, avoiding its subsequent nuclear translocation unless new cell stimulation occurs. In our model, in which HIV behaves as a persistent stimulus, this autoregulatory shutoff mechanism between NF-κB and IκBα is only partially functional, as demonstrated by the continuous NF-κB translocation. Thus, it is not unexpected that increased levels of IκBα RNA and, more importantly, increased transcription of the IκBα enhancer-promoter via an NF-κB motif are present in HIV-infected cells in an attempt to compensate for the unbalanced IκBα degradation.

Other IκB molecules have been found to be functional in cells of monocytic origin. Because p50/p65 is the main complex translocated to the nucleus in cells persistently infected with HIV, IκB molecules such as p105 and p100 could also participate in the regulation of NF-κB by HIV in monocytic cells. In order for any of these molecules to generate p50- or p52-containing p65 heterodimers, proteolytical cleavage of the precursor needs to occur (9, 21, 38, 53). Although few data as to what physiological stimuli result in this cleavage are available, an underlying functional processing is continuously present in any resting cell to explain the continuous formation of p50 or p52. Although some reports indicate that processing of p105 or p100 can be increased by different stimuli (TNF and LPS) to result in p50 (46, 75), this has not been confirmed by others (71), leaving the issue of p105 and p100 regulation open to further studies. Our observation that a chronic stimulus such as HIV increases the processing of p105 implies that p50/p65 heterodimers aside from p50 homodimers can be generated by this mechanism independently targeting IκBα. Because in our model IκBα levels are decreased, it is unlikely that p50/p65 heterodimers generated from p105 processing would be anchored by IκBα, therefore freeing them for nuclear translocation in the infected cells. Preliminary data indicate that steady-state levels of p100 and p52 are also increased in HIV-infected compared with uninfected cells, although it is unknown if the processing of p100 as that described here for p105 is also increased. Importantly, if functional differences in IκB activity exist between p105 and p100, as already shown for differences in the transactivational activity of their proteolytic products p50 and p52, respectively (63), it will be important to perform detailed analysis of the regulation of both precursors by chronic stimuli such as HIV. As in the case of IκBα, it remains unknown by which mechanisms persistent HIV infection increases the normal ongoing processing of p105. Although the HIV protease has been shown to cleave p105 in a model of acute HIV infection in T-lymphoblastoid cells, it is also possible that activation of already functional proteolytic pathways is upregulated by HIV-dependent mechanisms.

Lastly, our studies provide additional information on the transcriptional regulation of the p105 enhancer-promoter by stimuli that activate NF-κB, especially HIV. Although our group has previously shown that NF-κB and HIV upregulate the transcription of the enhancer-promoter of this gene (55), we have been interested in further characterizing whether HIV requires DNA-binding motifs different from those binding NF-κB. To achieve this, we performed site-directed mutagenesis of the different NF-κB-binding motifs as well as an additional one, HIP1/E2F (45). By sequencing the mutated constructs, an extra NF-κB motif (κB3) overlapping the 5′ region of the HIP1/E2F motif was noted. A similar sequence was documented, although not mentioned or analyzed, in a second

report describing the enhancer-promoter of p105 (14). By performing electrophoretic mobility shift assays and transfection experiments addressing the role of each of the three κB motifs and the single HIP1 motif, we demonstrated that the κB3 motif and not the HIP1 motif is the main regulatory region of the enhancer-promoter of the p105 gene by NF-κB and HIV in monocytic cells, implying that its upregulation of HIV is mainly mediated by its activation of NF-κB and not other transcription factors. Of interest, the κB3 motif confers cell specificity. Preliminary studies in B-cell lines indicate that the Epstein-Barr virus LMP-1 protein and LPS, both of which activate NF-κB and p105 transcription in these cells, upregulate the p105 promoter independently from the κB3 motif (44a).

Viruses and specific viral proteins have been listed among the different stimuli known to activate NF-κB (3). At least in the case of HIV and to a certain degree with Epstein-Barr virus, our data indicate that the increased NF-κB activity triggered by persistent viral infection accompanies upregulation of cellular receptors and functions which may in part be NF-κB dependent, suggesting a functional role for the activation of this transcriptional factor. Furthermore, persistent HIV infection of macrophages provides an example of how a continuous stimulus such as HIV can modify and upregulate cellular pathways that lead to NF-κB activation, specifically by modulating at least two separate and independent IκB molecules such as IκBα and p105. Advancing in our understanding of NF-κB regulation by HIV will allow determination of the role of this crucial transcription factor in the regulation of HIV persistence in human macrophages.

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