IκB-α Represses the Transcriptional Activity of the HIV-1 Tat Transactivator by Promoting Its Nuclear Export

Antimina Puca‡, Giuseppe Fiume†, Camillo Palmieri‡, Francesca Trimboli‡, Francesco Olimpico‡, Giuseppe Scala§, and Ileana Quinto‡§

From the ‡Department of Biochemistry and Medical Biotechnology, University of Naples “Federico II”, 80131 Naples, Italy, and the §Department of Experimental and Clinical Medicine, University of Catanzaro “Magna Graecia,” 88100 Catanzaro, Italy

The long terminal repeat of human immunodeficiency virus, type 1 (HIV-1) contains an NF-κB enhancer and is potently inhibited by IκB-α532/36A, a proteolysis-resistant inhibitor of NF-κB transacting factors. The evidence that NF-κB is dispensable for HIV-1 expression raises the question of whether IκB-α represses the HIV-1 transcription by mechanisms distinct from NF-κB inhibition. Here, we report that IκB-α negatively regulates the HIV-1 expression and replication in an NF-κB-independent manner by directly binding to Tat, which results in the nuclear export and cytoplasmic sequestration of the viral transactivator. The sequence of IκB-α required for Tat inhibition spans from amino acids 72 to 287 and includes the nuclear localization signal, the carboxy-terminal nuclear export signal, and the binding site for the arginine-rich domain of Tat. This novel mechanism of cross-talk between Tat and IκB-α provides further insights into the mechanisms of HIV-1 regulation and could assist in the development of novel strategies for AIDS therapy.

The expression of HIV-13 is dependent on the RNA polymerase II complex and is regulated by cellular transacting factors that interact with the viral promoter (1). The HIV-1 LTR contains a basal promoter (nucleotides −78 to −1) with a TATAA box and three Sp1-binding sites, an enhancer (nucleotides −105 to −79) with two tandem κB sites, and a negative regulatory region (nucleotides −454 to −106) (2). The HIV-1 encoded Tat protein dramatically increases the viral gene expression by binding to the transactivation-responsive region at the 5′ leader HIV-1 RNA (nucleotides +1 to +59) (3) and promoting transcription initiation (4). In particular, Tat promotes the assembly of the preinitiation complex and nucleosomal remodeling through the interaction with several transcription factors and cofactors including TBP (5, 6), NF-κB (7), Sp1 (8), and the histone acetyltransferases p300/CBP and P/CAF (9–11). In addition, Tat recruits the cyclin T1/CDK9 complex, which hyperphosphorylates the carboxy-terminal domain of the large subunit of RNA polymerase II, resulting in processive transcription (12–15).

The NF-κB transacting factors enhance the HIV-1 transcription by binding to the κB sites of the HIV-1 LTR (16, 17). The NF-κB proteins, namely p105/p50, p100/52, p65/RelA, c-Rel, and RelB, share a Rel homology domain that is required for subunit dimerization, nuclear localization, and DNA binding (18, 19). The NF-κB transcriptional activity is negatively regulated through the association with the IκB proteins (20). IκB-α (21), the best characterized and ubiquitous member of the IκB family, contains six ankyrins, a nuclear localization signal (NLS) (22–24) and two nuclear export signals located at the amino terminus (N-NES) (25–28) and carboxyl terminus (C-NES) (29, 30). In unstimulated cells, IκB-α associates with the p50/p65 NF-κB complex and inhibits the DNA binding and transcriptional activity of NF-κB (18, 20). The IκB-α/NF-κB complex shuttles in and out of the nucleus and is prevalently retained in the cytoplasm (25–27, 31). Upon NF-κB-activating stimuli, IκB-α is phosphorylated at serines 32 and 36 by the IκB kinase complex, ubiquitinated at lysines 21 and 22, and proteolyzed by the 26 S proteasome (18, 20). Following the proteolysis of IκB-α, the free NF-κB complex translocates to the nucleus, where it binds to the κB sites and activates the transcription of the NF-κB-dependent genes, including the IκB-α gene (18, 20). The newly synthesized IκB-α migrates to the nucleus, where it displaces the NF-κB complex from DNA and promotes its nuclear export, thus terminating the NF-κB transcriptional activity (29, 31, 32).

IκB-α represses the Tat-mediated transactivation of the HIV-1 LTR upon cell transfection (33). Moreover, the mutant IκB-α532/36A, which is resistant to the signaling-induced proteolysis (34, 35), inhibits the expression of HIV-1 (36). Consistent with these findings, we showed that NL-IκB-M, a recombinant HIV-1 strain expressing the IκB-α532/36A gene integrated in the nef region, is strongly repressed at the transcriptional level and highly attenuated (37). In that study, the inhibition of HIV-1 by IκB-α532/36A was ascribed to the...
Physical and Functional Interaction of \( \kappa B-\alpha \) with HIV-1 Tat

repression of the NF-\( \kappa B \)-dependent expression of HIV-1. However, this mechanism accounts partially for the HIV-1 transcriptional inhibition because viral strains lacking the NF-\( \kappa B \) enhancer are competent for transcription and replication (38–40). The evidence that NF-\( \kappa B \) is dispensable for the transcriptional activation of HIV-1 raises the question of whether I\( \kappa B-\alpha \) represses other transcription factors, which differ from NF-\( \kappa B \) and are required for HIV-1 expression. To test this possibility, we have analyzed the functional and physical interactions of I\( \kappa B-\alpha \) with the HIV-1 Tat transactivator, which is dispensable for viral replication (41, 42). Here, we report that I\( \kappa B-\alpha \) binds to Tat and promotes its nuclear export to the cytoplasm. According to this novel evidence, I\( \kappa B-\alpha \) acts as a potent repressor of HIV-1 transcription by inhibiting both the NF-\( \kappa B \) and Tat transacting factors, which are major players in the transcriptional activation and elongation of HIV-1 transcripts.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pLTrLuc contains the U3 and R regions of the pNL4-3 molecular clone of HIV-1 upstream of the luciferase gene (37). pSV-\( \beta \)-gal was purchased from Promega. To generate p3XFLAG-CMV-Tat, p3XFLAG-CMV-Tat C(22,25,27)A, and p3XFLAG-CMV-Tat R(49–57)A, the sequence of Tat was amplified from the pGEX-2T-Tat expressing vectors (43) and p3XFLAG-CMV-Tat R(49–57)A, the sequence of Tat was amplified from the pGEX-2T-Tat expressing vectors (43) and p3XFLAG-CMV-Tat R(49–57)A. The mutated nucleotides are shown in bold type. The pGEX-2T-I\( L(272,274,277)A \). The mutated triplets indicated in bold type. The mutant I\( \kappa B-\alpha \) N/C NES was generated by site-directed mutagenesis of I\( \kappa B-\alpha \) at the level of the N-NES with the forward primer AAGGAGCTGCAG GAGCGC

**Physical and Functional Interaction of I\( \kappa B-\alpha \) with HIV-1 Tat**

Luciferase and \( \beta \)-gal assays, pSV-\( \beta \)-gal plasmid (0.2 \( \mu \)g) was co-transfected with the pTRLuc plasmids to monitor the transfection efficiency. Forty-eight hours post-transfection, the cells were lysed in lysis buffer of Dual Light Luciferase System (Tropix, Bedford, MA). The luciferase and \( \beta \)-galactosidase activities were evaluated by using the Dual Light luciferase system (Tropix, Bedford, MA) in a bioluminometer (Turner Biosystem, Sunnyvale, CA). The ratio of firefly luciferase activity to \( \beta \)-galactosidase activity was expressed as relative light units.

**Pseudotyped Virions and Single-round Infection**—293-T cells were transfected with wild type or NF-\( \kappa B \)-deleted pNL4-3.Luc.R\( \beta \)-Gal (600 nm) and pVSV.G (10 \( \mu \)g) expressing the G protein of the vesicular stomatitis virus. Forty-eight hours post-transfection, the cells supernatants were collected, and the virions were measured by p24 enzyme-linked immunosorbent assay. Jurkat cells (4 \times 10\(^6\)) were transfected by electroporation with pCMV4-\( \kappa B-\alpha \) or empty vector (30 \( \mu \)g) with I\( \kappa B-\alpha \) siRNA or control siRNA (500 pmol) (Dharmacon, Lafayette, CO) and 48 h later were infected with VSV-Luc virions (500 ng of p24) by spinoculation (45). The luciferase activity was measured in cell extracts 48 h post-infection.

**Viral Integration**—Genomic DNA was extracted from aliquots of infected cells (2 \times 10\(^6\)) using TRIzol (Invitrogen) and amplified with primers that annealed in the U5 region of the LTR (MH 531) and in the 5’ end of the gag gene (MH 532). The reaction mixture (25 \( \mu \)l) contained genomic DNA (200 ng), primers (600 nm), and 1 X iQ SYBR Green Supermix (Bio-Rad). Real time PCR was performed by using iCycler Apparatus (Bio-Rad). After an initial denaturation step (95 °C for 8 min), the cycling profile for total HIV-1 DNA was 50 cycles consisting of...
Physical and Functional Interaction of IκB-α with HIV-1 Tat

95°C for 10 s, 60°C for 10 s, and 72°C for 6 s. Viral DNA was normalized to cellular genomic glyceraldehyde-3-phosphate dehydrogenase. Primers were as follows: MH531, TGTTGC-CGGTCTTGTGTGT; MH532, GAGTCTCAGTGAGAG-AGC; glyceraldehyde-3-phosphate dehydrogenase forward, GAAGGTGAAGTCC; glycerinaldehyde-3-phosphate dehydrogenase reverse, GAAGGTGAATGGAGAT-TTC. The HIV-1 DNA copy number was measured as reported (46).

Viral Stocks and Cell Culture Infection—293-T cells were transfected with viral plasmids, and the viral production was measured by p24 enzyme-linked immunosorbent assay. Jurkat cells (5 × 10⁶ cells) were infected with p24 (0.3 ng) of viral stocks, and the cell supernatants were collected every 3 days for p24 assay; equal volumes of fresh medium were replaced into the cultures at the same time.

Statistical Analysis—The data were reported as the means ± S.E. and the statistical significance of differences between means was assessed by using the two-tail unpaired Student’s t test. The differences between the means were accepted as statistically significant at the 95% level (p = 0.05).

Cell Extracts and Western Blotting—Cells (5 × 10⁶) were harvested, washed in cold PBS, and lysed on ice in 500 μl of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 1% Triton X-100, 5 mM DTT, 1× protease inhibitor mixture EDTA-free (Roche Applied Science). After centrifugation at 15,000 × g for 15 min at 4°C, the supernatant was collected, and aliquots of proteins were resuspended in loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 1% bromphenol blue, 10% β-mercaptoethanol, 25% glycerol), resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and incubated with primary antibodies (1:1000) followed by incubation with horseradish-peroxidase-linked mouse or rabbit IgG (1:2000) (Amersham Biosciences) in PBS containing 5% nonfat dry milk (Bio-Rad). The proteins were detected by chemiluminescence using the Amersham Biosciences ECL system. The primary antibodies were as follows: anti-HA (F7), anti-GST (B-14), anti-IκB-α (C-15), and normal mouse serum from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG M2 and anti-γ-tubulin from Sigma-Aldrich; anti-caspase-3 and cleaved poly(ADP-ribose)-polymerase (Asp214) antibody from Cell Signaling Technology, Inc. (Danvers, MA).

GST Pulldown—GST fusion proteins were produced in Escherichia coli strain BL21 as previously described (43). Bacterial cultures (500 ml) were grown to exponential phase and induced with 0.25 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich) for 3 h to express GST fusion proteins. The bacteria were lysed by sonication in buffer A (1× PBS, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture EDTA-free), and the lysate was clarified by centrifugation at 27,000 × g for 30 min at 4°C. The supernatant was incubated with 1 ml of a 50% (v/v) slurry of glutathione-Sepharose beads (Amersham Biosciences) previously equilibrated in buffer A. After incubation on a rotating wheel at 4°C for 2 h, the beads were washed five times with buffer A and subjected to a high salt wash (0.8 M NaCl) to free the fusion proteins from contaminating bacterial nucleic acids (47). GST fusion proteins were eluted with 500 μl of 50 mM Tris-HCl containing 10 mM glutathione and 1 mM DTT. The eluted GST fusion proteins were dialyzed against dialysis buffer (1× PBS, 1 mM DTT, 10% glycerol), and aliquots (5–10 μg) were conjugated with glutathione-Sepharose (20 μl) in 500 μl of binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 3% Triton X-100, 5 mM DTT, 1× protease inhibitor mixture EDTA-free). The GST fusion proteins conjugated with glutathione-Sepharose were collected by centrifugation at 700 × g for 5 min at 4°C, and aliquots (5–10 μg) were incubated with cell extracts (200 μg) in 500 μl of binding buffer supplemented with 1 μg/μl of bovine serum albumin on a rocking platform for 3 h at 4°C. To remove nucleic acids, the cell
FIGURE 2. IκB-α inhibits the expression and replication of NF-κB-deleted viruses. A, p50−/− p65−/− MEFs (3 × 10^5) were transfected with the NF-κB-deleted pLTRluc (0.5 μg), with or without p3XFLAG-CMV-Tat (0.5 μg), pRC/CMV-hCycT1 (0.5 μg), and pCMV4-HA-IκB-α (0.5, 1, and 2 μg). The luciferase activity was measured 48 h post-transfection. Fold activation was calculated relative to transfection in the absence of Tat, hCycT1, and IκB-α expression plasmids. The mean values ± S.E. (n = 4) are shown. The asterisks indicate a statistically significant inhibition according to Student’s t test (without hCycT1: *, p = 0.008; **, p = 0.0009; ***, p = 0.0002; with hCycT1: *, p = 0.006; **, p = 0.001; ***, p = 0.0001). B, Jurkat cells (4 × 10^6) were electroporated with pCMV4-HA-IκB-α or empty vector (30 μg), IκB-α siRNA, or control siRNA (500 pmol) and infected with VSV-G-pseudotyped NL4-3.Luc.R E + virions that carry the wild type LTR (left panel) or the NF-κB-deleted LTR (right panel) (500 ng of p24); virus production was monitored by measuring the luciferase activity in cell extracts 48 h post-infection (top). The expression level of IκB-α was detected in cell extracts by Western blotting with anti-IκB-α C-15 (bottom). C, schematic representation of the viral genome of NL-IκB-M and NL-IκB-as carrying the wild type LTR or NL-IκB-M and NL-IκB-as carrying the NF-κB-deleted LTR. D, Jurkat cells (5 × 10^6) were infected with equal amounts (0.3 ng of p24) of the wild type LTR viruses, NL-IκB-M and NL-IκB-as (left panel), or NF-κB-deleted LTR viruses, NL-ΔκB-IκB-M and NL-ΔκB-IκB-as (right panel). The viral production was measured as p24 level in culture supernatants.
at room temperature. Following GST pulldown, the proteins were separated by 12% SDS-PAGE and analyzed by autoradiography and immunoblotting with antibodies.

**Co-immunoprecipitation**—Cell extracts were performed in PBS containing 1% Triton X-100 and 1× Protease Inhibitor Mixture EDTA-free. Antibodies (2.5 μg) were preincubated with protein G-Sepharose (Amersham Biosciences) (20 μl) in 50 μl of immunoprecipitation buffer (PBS containing 2% Triton X-100, 300 mM NaCl, 5 mM DTT, 1× Protease Inhibitor Mixture EDTA-free) overnight at 4 °C on a rocking platform. The protein G-Sepharose-coupled antibodies were incubated with cell extract (500 μg) in 500 μl of immunoprecipitation buffer overnight at 4 °C on a rocking platform. The immunocomplexes were collected by centrifugation at 700 × g for 5 min at 4 °C, washed in immunoprecipitation buffer, and resuspended in SDS gel loading buffer. The proteins were separated on 10% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and analyzed by immunoblotting with antibodies.

**Confocal Microscopy**—Confocal microscopy was performed as previously described (48). HeLa cells were seeded on poly-1-lysinetreated glass coverslips, fixed, and permeabilized using Cytofix/Cytoperm kit (BD Biosciences Pharmingen, San Diego, CA). To visualize FLAG-Tat and HA-IκB-α, the immunostaining was performed with anti-FLAG-M2-FITC mAb (F-4049; Sigma-Aldrich) and anti-HA rabbit antiserum (SC-805; Santa Cruz Biotechnologies) followed by goat anti-rabbit Alexa Fluor568 (A11011; Molecular Probes, Eugene, OR). The nuclei were stained with TO-PRO-3 iodide (T3605; Molecular Probes). The coverslips were mounted on glass slides by using ProLong Antifade Kit (P7481; Molecular Probes). The images were collected on a Leica TCS-SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63× APO PL A oil immersion objective (NA 1.4) and 60-μm aperture. Z stacks of images were collected using a step increment of 0.2 μm between planes. FLAG-Tat was visualized by excitation with an argon laser at 488 nm and photomultiplier tube voltage of 420 mV. HA-IκB-α was detected using a krypton laser at 568 nm and photomultiplier tube voltage of 650 mV. The nuclei were detected using a

extracts were treated with micrococcal nuclease (0.2 unit/μl) for 30 min at 28 °C. Protein complexes were collected by centrifugation at 700 × g for 5 min at 4 °C, washed in binding buffer, and resuspended in loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 1% bromphenol blue, 10% β-mercaptoethanol, 25% glycerol). The proteins were resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by immunoblotting with the indicated antibodies. The pcDNA3 plasmids expressing the IκB-α genes under the T7 promoter were used as templates to translate in vitro the [35S]methionine-labeled IκB-α proteins by using the TnT Quick Coupled transcription/translation systems (Promega). Aliquots (10 μl) of translation mixture were incubated with GST-Tat or GST proteins (10 μg) in 500 μl of binding buffer supplemented with 1 μg/ml of bovine serum albumin on a rocking platform for 3 h
RESULTS

IkB-α Represses the Tat-mediated Transactivation and Replication of HIV-1 Independently of NF-κB Activity—To determine the effect of IkB-α on the transcriptional activity of Tat, HeLa cells were transiently transfected with the luciferase gene under the control of the wild type or NF-κB- or Sp1-deleted HIV-1 LTR in the presence or absence of Tat and IkB-α. In agreement with previous observations (39), the deletion of the NF-κB or Sp1 sites significantly reduced the basal expression (Fig. 1A) and the Tat-mediated transactivation (Fig. 1, B–D) of the HIV-1 LTR. IkB-α inhibited the Tat transcriptional activity in a dose-dependent manner up to 80% for the wild type LTR (Fig. 1B) and 60% in the case of NF-κB-deleted LTR (Fig. 1C). The evidence that IkB-α inhibited the Tat-mediated transactivation of the LTR in the absence of the NF-κB enhancer underscored the existence of mechanisms of LTR inhibition distinct from NF-κB repression. IkB-α completely re-
Physical and Functional Interaction of IκB-α with HIV-1 Tat

pressed the Tat-mediated transactivation of the Sp1-deleted LTR (Fig. 1D); this strong inhibition was likely caused by repression of both NF-κB-dependent and independent transactivation of the LTR. The LTR inhibition was not a consequence of pro-apoptotic activity of IκB-α because the cleavage of caspase-3 and poly(ADP-ribose)polymerase was undetected in the wild type or NF-κB-deleted LTR. The virion production was significantly reduced by hyperexpression of IκB-α and increased by knocking down the endogenous IκB-α with IκB-α siRNA in both infections with the wild type (Fig. 2B, left panel) or the NF-κB-deleted virus (Fig. 2B, right panel). No difference in the number of integrated virus among the different samples was observed (supplemental Fig. S2). These results suggest that the levels of endogenous IκB-α inversely affected the expression of the integrated HIV-1 genome independently of the presence of the NF-κB-binding sites in the HIV-1 LTR.

To analyze the effect of IκB-α on the HIV-1 replication in the absence of the NF-κB-binding sites of the HIV-1 LTR, we generated the viral plasmids NL-ΔκB-IκB-M and pNL-ΔκB-IκB-as, which carry the IκB-αS32/36A-FLAG cDNA inserted into the nef region in sense or antisense orientation, respectively, and were deleted of the two tandem κB sites in the LTR (Fig. 2C). These recombinant HIV-1 plasmids were the NF-κB-deleted derivatives of pNL-IκB-M and pNL-IκB-as (37), which express or do not express, respectively, IκB-αS32/36A-FLAG. Jurkat cells were infected with the wild type LTR viruses (NL-IκB-M and NL-IκB-as) or the NF-κB-deleted LTR viruses (NL-ΔκB-IκB-M and NL-ΔκB-IκB-as), and the viral production was measured by p24 detection in culture supernatants. As previously reported (37), NL-IκB-M was potently attenuated as compared with the control NL-IκB-as because of the IκB-αS32/36A expression (Fig. 2D, left panel). In the case of NF-κB-deleted viruses, a significant attenuation of NL-ΔκB-IκB-M was also observed as compared with the control NL-ΔκB-IκB-as (Fig. 2D, right panel). These results indicate that IκB-α inhibited the HIV-1 replication independently of the NF-κB enhancer in the HIV-1 LTR and supported the evidence of additional mechanisms of HIV-1 inhibition by IκB-α other than NF-κB repression.

The Sequence of IκB-α from Amino Acids 72 to 287 Is Required for Tat Inhibition—The sequence of IκB-α encompassing amino acids 1–317 contains six ankyrins (amino acids 72–287), the NLS (amino acids 110–120), the N-NES (amino acids 265–277) (Fig. 3A). To map the IκB-α domains required for Tat inhibition independently of NF-κB repression, the activity of IκB-α mutants was analyzed in p50−/− p65−/− MEFs by transient expression of the NF-κB-deleted LTR and Tat. The IκB-α mutants that were progressively deleted of the carboxyl-terminal from
amino acids 317 to 280 significantly inhibited the Tat activity, whereas no inhibition was induced by IκB-α 1–269 deleted of the C-NES (Fig. 3B). Further, deletions of the carboxyl-terminal of IκB-α from amino acids 269 to 242 did not affect the Tat activity (Fig. 3B). IκB-α 72–317 lacking the amino-terminal sequence from amino acids 1 to 72 significantly inhibited Tat, whereas IκB-α 120–317, which was deleted of the NLS, lost the inhibitory activity (Fig. 3B). These results indicated that the sequences of IκB-α from amino acids 72 to 120 (overlapping the NLS) and from amino acids 269 to 280 (overlapping the C-NES) were both required for Tat inhibition. This was confirmed by experiments where the mutant IκB-α 72–287, which contains both the NLS and C-NES, inhibited Tat, whereas the mutants IκB-α 72–269 and IκB-α 72–287 L(272,274,277)A, which carry deletion or base pair substitutions of critical leucine residues of the C-NES sequence (29), respectively, failed to inhibit Tat (Fig. 3B). Lack of inhibition was confirmed at higher doses of IκB-α 120–317, IκB-α 72–269, and IκB-α 72–287 L(272,274,277)A (supplemental Fig. S3). The IκB-α mutants were all expressed in cell extracts, and no correlation was found between the level of expression and the inhibitory activity (Fig. 3C). These results demonstrated that the minimal sequence of IκB-α required for Tat inhibition spanned from amino acids 72 to 287; this region encompasses the six ankyrins of IκB-α including the NLS and C-NES.

IκB-α Binds to the Arginine-rich Domain of Tat—To test whether IκB-α physically interacts with Tat, the GST pulldown assay was performed with extracts from cells transfected with pCMV4-HA-IκB-α. GST-Tat retained IκB-α expressed in HeLa and MEFs (Fig. 4A, lanes 1 and 2). The binding of Tat with IκB-α was also observed in p50/p65 MEFs (Fig. 4A, lane 3), which ruled out that IκB-α and Tat were recruited in the same complex by associating with the p50 and p65 subunits of NF-κB. IκB-α was not retained by GST protein (Fig. 4A, lanes 4–6). The association of endogenous IκB-α with GST-Tat was also observed in HeLa extracts (Fig. 4B, lane 1). The treatment of the cellular extracts with micrococcal nuclease did not affect the binding of IκB-α with Tat (Fig. 4C, lane 2), thus ruling out the possibility that the association of the two proteins was bridged by nucleic acids.

To map the Tat domain that binds to IκB-α, GST-IκB-α was incubated with extracts from HeLa cells transfected with the wild type Tat or the mutants Tat C(22,25,27)A and Tat R(49–57)A fused to the FLAG epitope (Fig. 4D). In pulldown assay, GST-IκB-α retained the wild type Tat and Tat C(22,25,27)A (Fig. 4E, lanes 2 and 4), whereas it did not bind to Tat R(49–57)A (Fig. 4E, lane 3); Tat was not retained by GST alone (Fig. 4E, lanes 6–8). The association of IκB-α with Tat was further tested by in vivo immunoprecipitation with extracts from HeLa cells transfected with the plasmids expressing FLAG-Tat and HA-IκB-α. IκB-α immunoprecipitated with the wild type Tat and Tat C(22,25,27)A (Fig. 4F, lanes 2 and 3), whereas it did not associate with Tat R(49–57)A (Fig. 4F, lane 4). Altogether, these results indicate that the arginine-rich region of Tat encompassing amino acids 49–57 is required for the association with IκB-α.

Tat Binds to the Sixth Ankyrin of IκB-α—To determine the sequence of IκB-α binding to Tat, [35S]methionine-labeled IκB-α mutants (Fig. 5A) were incubated with GST-Tat or GST. Tat retained IκB-α 1–317 and IκB-α 1–269 (Fig. 5B, lanes 1 and 2), whereas it did not bind to IκB-α 1–263 (Fig. 5B, lane 3). The
mutants IκB-α 72–287, IκB-α 120–317, IκB-α 243–317, and IκB-α 72–287 L(272, 274, 277)A were efficient binders of Tat (Fig. 5B, lanes 4–7). As control, GST tested negative for the binding to labeled proteins (Fig. 5B, lanes 8–14). These results indicated that the IκB-α sequence from amino acids 263 to 269 within the sixth ankyrin of IκB-α was required for binding to Tat.

By CLUSTALW-based multiple sequence alignment (align. genome.jp), the amino acid sequence of the IκB-α sixth ankyrin is very divergent from the other five ankyrins of IκB-α and the ankyrins of the human IκB family (p100, p105, IκB-γ, IκB-ε, and Bcl-3) (supplemental Fig. S4, A and B). In particular, the sequence TRIQQQL (amino acids 263–269 of IκB-α), which is present in the sixth ankyrin and is required for the binding to Tat (Fig. 5B), is absent in ankyrins 1–5 of IκB-α as well as in the ankyrins of the IκB family members (supplemental Fig. S4, A and B). A more extended analysis by using FUZZPRO (bioweb.pasteur.fr/seqanal/interfaces/fuzzpro.html) failed to identify the TRIQQQL motif in the ankyrins of the human proteome except in the sixth ankyrin of IκB-α. Accordingly, p100 and p105, two members of the IκB family showing the highest identity with the sixth ankyrin of IκB-α, were unable to bind to Tat (supplemental Fig. S4C, lanes 1 and 4). These results suggest that the sixth ankyrin of IκB-α contains a unique diverged sequence as compared with other ankyrins, which might represent a privileged target site for Tat binding. Alternatively, this sequence might contribute to stabilize a peculiar structural domain required for the binding to Tat.

IκB-α Exports Tat from the Nucleus to the Cytoplasm—The cellular distribution of IκB-α and Tat was visualized by confocal fluorescence microscopy. HeLa cells were transfected with plasmids expressing FLAG-Tat and HA-IκB-α. When singularly transfected, Tat was nuclear, whereas IκB-α 1–317 was mostly cytoplasmic (Fig. 6A); this was confirmed by the fluorescence-based analysis of 30 cells for each transfection (Fig. 7). When co-transfected, Tat and IκB-α 1–317 co-localized within the cytoplasmic and perinuclear regions (Figs. 6A and 7). IκB-α 120–317, lacking both the N-NES and the NLS, and IκB-α 1–269, lacking the C-NES, were prevalently cytoplasmic and did not affect the nuclear location of Tat (Figs. 6A and 7). IκB-α 72–287, lacking the N-NES, was mostly cytoplasmic and promoted the translocation of Tat from the nucleus to the cytoplasm in 50% of the analyzed cells (Figs. 6A and 7). IκB-α 72–269 and IκB-α 72–287 L(272,274,277)A, which lacked the N-NES and C-NES, were distributed both in the nucleus and cytoplasm and did not affect the nuclear location of Tat (Figs. 6A and 7). No significant differences in the intracellular expression levels of the IκB-α mutants were observed in transfected cells (Fig. 6B). These results suggested that IκB-α promoted the displacement of Tat from nucleus to cytoplasm and that this activity required the integrity of the NLS and C-NES of IκB-α.

To analyze the role of the nuclear export activity of IκB-α in Tat inhibition, we generated the mutant IκB-α 1–317 N/C NES, which carries crucial base pair substitutions of both the N-NES (I52A,L54A) and C-NES (I272,274,277A), which inactivate the nuclear export activity (25, 29). IκB-α N/C NES was prevalently distributed in the nucleus and did not affect the nuclear location of Tat (Fig. 8, A and B). Moreover, IκB-α N/C NES did not repress the Tat-mediated transactivation of the NF-κB-deleted LTR (Fig. 8C), although it was able to bind to Tat in GST-pull
Physical and Functional Interaction of IκB-α with HIV-1 Tat

of Tat inhibition by the transfected IκB-α (supplemental Fig. S5, compare lanes 2 and 3 with lanes 5 and 6). These results indicate that the leptomycin B-mediated arrest of nuclear export released Tat from the IκB-α inhibition.

DISCUSSION

This study provides further insight into the mechanisms of HIV-1 inhibition by the IκB-α repressor. We have shown that IκB-α represses the Tat activity independently of the NFKB inhibitory activity by physical association and displacement of Tat from the nucleus to the cytoplasm. The association of IκB-α with the arginine-rich domain of Tat is not sufficient to interfere with the nuclear distribution and the transcriptional activity of Tat. In fact, the mutants IκB-α 120–317 and IκB-α 1–269 bind to Tat without affecting the nuclear location and transcriptional activity of the viral transactivator (Fig. 9A). Instead, the inhibition of Tat correlates with the nuclear export activity of IκB-α, which requires both the NLS (amino acids 110–120) and the C-NES (amino acids 265–277) together with the binding site for Tat (amino acids 263–269) (Fig. 9A). Consistent with this evidence, the mutant IκB-α N/C NES, which contains the full-length sequence of IκB-α but lacks the nuclear export signals, does not affect the nuclear location and the transcriptional activity of Tat (Fig. 9A). Altogether, these results suggest that IκB-α binds to Tat in the nucleus and exports the viral transactivator to the cytoplasm, where the complex IκB-α/Tat is mostly retained (Fig. 9B).

Relevance of IκB-α/Tat Interaction in the Viral Cycle—The evidence that IκB-α inhibits the transcriptional activity of Tat raises the question of why the endogenous IκB-α does not counteract the viral expression in HIV-1-infected cells. Indeed, IκB-α is subjected to persistent proteolysis in the course of HIV-1 infection (55–57). The HIV-1 entry through the gp120 envelope protein binding to CD4 receptor activates the IκB kinase complex, which promotes the proteolysis of IκB-α (58). This event leads to the transcriptional activation of NFKB-dependent genes,
including the HIV-1 genome and pro-inflammatory genes, which in turn sustain the proteolysis of IκB-α and the activation of NF-κB (59). In particular, Tat activates NF-κB by inducing the degradation of IκB-α (47), the up-regulation of NIK (60), and the transactivation of inflammatory cytokines (61–63).

The physical and functional interaction of IκB-α with Tat discloses a novel mechanism of HIV-1 transcriptional regulation. In fact, the ratio between IκB-α and Tat could determine the level of expression of the target genes, including HIV-1. In this scenario, whereas the endogenous IκB-α does not block the viral expression because it undergoes proteolysis in the course of HIV-1 infection (55–57), a proteolysis-resistant IκB-α mutant, such as IκB-αS32/36A, would subvert this equilibrium and repress HIV-1 by constitutive inhibition of both NF-κB and Tat transcriptional activities (36, 37). In this regard, the novel mechanism of Tat inhibition by IκB-α described here could assist in the development of a novel class of HIV-1 inhibitors. In particular, the inhibitory sequence of IκB-α (amino acids 72–287) represents a model structure to design peptide-based inhibitors acting at the transcriptional step of the HIV-1 life cycle.

The mechanism of Tat inhibition here described mimics the one of NF-κB inhibition, because in both cases IκB-α associates with these transcriptional factors in the nucleus and exports them to the cytoplasm. These events result in the down-regulation of the NF-κB and Tat transcriptional activities and explain the strong attenuation of HIV-1 by IκB-α. In this regard, compounds that increase the stability of endogenous IκB-α, such as inhibitors of IκB kinase and proteasomes, could be additional tools of conventional anti-retroviral therapies by inhibiting the NF-κB-Tat-dependent HIV-1 transcription.

Acknowledgments—We thank Dr. K.-T. Jeang for pRc/CMV-HA-CycT1 and pLTRLuc plasmids; Dr. W. C. Greene for pCMV-4-HA expressing IκB-α 1–317, elide 72–317, 1–287, and 1–263; Dr. M. Giaccia for the pGEX-2T-Tat C(22,25,27)A and pGEX-2T-Tat R(49–57)A; Dr. A. A. Beg for p50−/− p65−/− MEFs; Dr. N. Rice for anti-p105 antibody; and the NIH AIDS Research & Reference Program for the pGEX-2T-Tat (1–86) and pNL4-3.Luc.R’E’.

REFERENCES

1. Wu, Y. (2004) Retrovirology 1, 13
2. Pereira, L. A., Bentley, K., Peeters, A., Churchill, M. J., and Deacon, N. J. (2000) Nucleic Acids Res. 28, 663–668
3. Berkhourt, B., Silverman, R. H., and Jeang, K. T. (1989) Cell 59, 273–282
4. Brady, J., and Kashanchi, F. (2005) Retrovirology 2, 69
5. Kashanchi, F., Piras, G., Radonovich, M. F., Duvall, J. F., Fattaey, A., Chiang, C. M., Roeder, R. G., and Brady, J. N. (1994) Nature 367, 295–299
6. Raha, T., Cheng, S. W., and Green, M. R. (2000) PLoS Biol. 3, e44
7. Liu, J., Perkins, N. D., Schmid, R. M., and Nabel, G. J. (1992) J. Biol. Chem. 266, 3883–3897
8. Jeang, K. T., Chun, R., Lin, N. H., Gatignol, A., Glabe, C. G., and Fan, H. (1993) J. Biol. Chem. 67, 6224–6233
9. Benkiran, M., Chun, R. F., Xiao, H., Ogyrzko, V. V., Howard, B. H., Nakatanai, Y., and Jeang, K. T. (1998) J. Biol. Chem. 273, 24898–24905
10. Marzio, G., Tyagi, M., Gutierrez, M. I., and Giacca, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13519–13524
11. Hottinger, M. O., and Nabel, G. J. (1998) J. Virol. 72, 8252–8256
12. Hermann, C. H., and Rice, A. P. (1995) J. Biol. Chem. 69, 1612–1620
13. Zhu, Y., Pe’ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997) Genes Dev. 11, 2622–2632
14. Mancebo, H. S., Lee, G., Flygare, J., Tomassini, J., Luu, P., Zhu, Y., Peng, J., Blau, C., Hazuda, D., Price, D., and Flores, O. (1997) Genes Dev. 11, 2633–2644
15. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998) Cell 92, 451–462
16. Nabel, G., and Baltimore, D. (1987) Nature 326, 711–713
17. Mallard, M., Drangetti, E., Baldassarre, F., Ambrosino, C., Scala, G., and Quinto, I. (1996) J. Biol. Chem. 271, 20820–20827
18. Ghosh, S., and Karin, M. (2002) J. Biol. Chem. 109, (suppl.) S81–S96
19. Perkins, N. D., and Gilmore, T. D. (2006) Cell Death Differ. 13, 759–772
20. Hayden, M. S., and Ghosh, S. (2004) Genes Dev. 18, 2195–2224
21. Haskell, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. (1991) Cell 65, 1281–1289
22. Sachdev, S., Hoffmann, A., and Hannink, M. (1998) Mol. Cell. Biol. 18, 2524–2534
23. Turpin, P., Hay, R. T., and Dargemont, C. (1999) J. Biol. Chem. 274, 6804–6812
24. Sachdev, S., Bagchi, S., Zhang, D. D., Mings, A. C., and Hannink, M. (2000) Mol. Cell. Biol. 20, 1571–1582
25. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) EMBO J. 18, 6682–6693
26. Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000) Proc. Natl.
Physical and Functional Interaction of \( \kappa B-\alpha \) with HIV-1 Tat