

# Human immunodeficiency virus-1 Tat activates NF- $\kappa$ B via physical interaction with I $\kappa$ B- $\alpha$ and p65

Giuseppe Fiume<sup>1</sup>, Eleonora Vecchio<sup>1</sup>, Annamaria De Laurentiis<sup>1</sup>, Francesca Trimboli<sup>1</sup>, Camillo Palmieri<sup>1</sup>, Antonio Pisano<sup>1</sup>, Cristina Falcone<sup>1</sup>, Marilena Pontoriero<sup>1</sup>, Annalisa Rossi<sup>1</sup>, Annarita Scialdone<sup>1</sup>, Francesca Fasanella Masci<sup>1</sup>, Giuseppe Scala<sup>1,\*</sup> and Ileana Quinto<sup>1,2,\*</sup>

<sup>1</sup>Department of Experimental and Clinical Medicine, University of Catanzaro 'Magna Graecia', Viale Europa-Germaneto, 88100 Catanzaro and <sup>2</sup>Department of Biochemistry and Medical Biotechnology, University of Naples 'Federico II', Via Sergio Pansini 5, 80131 Naples, Italy

Received October 20, 2010; Revised November 19, 2011; Accepted November 22, 2011

## ABSTRACT

**Nuclear factor (NF)- $\kappa$ B is a master regulator of pro-inflammatory genes and is upregulated in human immunodeficiency virus 1 (HIV-1) infection. Mechanisms underlying the NF- $\kappa$ B deregulation by HIV-1 are relevant for immune dysfunction in AIDS. We report that in single round HIV-1 infection, or single-pulse PMA stimulation, the HIV-1 Tat transactivator activated NF- $\kappa$ B by hijacking the inhibitor I $\kappa$ B- $\alpha$  and by preventing the repressor binding to the NF- $\kappa$ B complex. Moreover, Tat associated with the p65 subunit of NF- $\kappa$ B and increased the p65 DNA-binding affinity and transcriptional activity. The arginine- and cysteine-rich domains of Tat were required for I $\kappa$ B- $\alpha$  and p65 association, respectively, and for sustaining the NF- $\kappa$ B activity. Among an array of NF- $\kappa$ B-responsive genes, Tat mostly activated the *MIP-1 $\alpha$*  expression in a p65-dependent manner, and bound to the *MIP-1 $\alpha$*  NF- $\kappa$ B enhancer thus promoting the recruitment of p65 with displacement of I $\kappa$ B- $\alpha$ ; similar findings were obtained for the NF- $\kappa$ B-responsive genes *CSF3*, *LTA*, *NFKBIA* and *TLR2*. Our results support a novel mechanism of NF- $\kappa$ B activation via physical interaction of Tat with I $\kappa$ B- $\alpha$  and p65, and may contribute to further insights into the deregulation of the inflammatory response by HIV-1.**

## INTRODUCTION

Nuclear factor (NF)- $\kappa$ B transcription factors regulate the transcription of genes that are involved in the immune and inflammatory response (1). The NF- $\kappa$ B family includes

RelA/p65, c-Rel, RelB, p50 and p52 that share a highly conserved 300-amino acid Rel homology domain (RHD) for homo- or hetero-dimerization and DNA-binding. The transcriptional activity of the NF- $\kappa$ B complex depends on dimer composition since C-terminal unrelated transcriptional activation domains are present exclusively in p65, RelB and c-Rel (2).

Inhibitors of NF- $\kappa$ B (I $\kappa$ B) associate with the NF- $\kappa$ B complex and interfere with its binding to DNA (3). In the canonical pathway of NF- $\kappa$ B activation, the activated I $\kappa$ B kinase (IKK) phosphorylates I $\kappa$ B at specific serine residues that target the protein to ubiquitination and proteasomal degradation, which releases the functional NF- $\kappa$ B complex in the nucleus. I $\kappa$ B- $\alpha$ , the most abundant inhibitor of NF- $\kappa$ B (4), is phosphorylated by IKK at Ser32 and Ser36 (5), and subsequently ubiquitylated at Lys21 and Lys22 to be degraded by the 26S proteasome (6). The NF- $\kappa$ B activity is enhanced by phosphorylation of p65 at Ser276 by PKA and MSK1 (7,8), Ser311 by PKC $\zeta$  (9) and Ser536 by IKK $\alpha$  (10,11). Acetylation of p65 at Lys218 and Lys221 increases the DNA binding and impairs the association with I $\kappa$ B- $\alpha$ , and acetylation at Lys310 enhances the p65 transcriptional activity (12,13). Post-activation turn off of NF- $\kappa$ B is regulated by negative feedback loop through inhibitors under the transcriptional control of NF- $\kappa$ B, such as I $\kappa$ B- $\alpha$  and ubiquitin-editing protein A20 (14–20). Deacetylation of p65 by histone deacetylase-3 or SIRT1, or acetylation of p65 at Lys122 and Lys123 down-regulate the NF- $\kappa$ B activity (12,21,22).

Persistent activation of NF- $\kappa$ B occurs in human immunodeficiency virus-1 (HIV-1)-infected monocytes, macrophages and microglia, and enhances the expression of NF- $\kappa$ B-responsive genes, including pro-inflammatory cytokines, cell adhesion molecules and chemokines (23–25). Chronic inflammation is a major cause of immune and neuron dysfunction in AIDS (26,27).

\*To whom correspondence should be addressed. Tel: +39 0961 3694058; Fax: +39 0961 3694090; Email: quinto@unicz.it  
Correspondence may also be addressed to Giuseppe Scala. Tel: +39 0961 3694059; Fax: +39 0961 3694090; Email: scala@unicz.it

Consistently, non-human primate hosts for simian immunodeficiency virus, such as African green monkeys and sooty mangabey, lack aberrant immune activation and do not develop AIDS despite high virus replication (28,29). Thus, understanding the mechanisms of NF- $\kappa$ B deregulation by HIV-1 may provide further insights into AIDS pathogenesis.

In HIV-1 entry, the binding of the gp120 viral envelope to CD4 induces the NF- $\kappa$ B activity by activation of IKK (30) and procaspase 8 (31). Following viral integration, the early encoded HIV-1 Tat protein interacts with the HIV-1 RNA and host cell factors to sustain the viral replication. Tat binds to RNA stem-loop structures generated by the 5' end of target transcripts, including the HIV-1 transactivation-responsive element (TAR) (32), tumor necrosis factor  $\beta$  (TNF $\beta$ ) (33) and interleukin-6 (IL-6) (34) to activate gene transcription. Indeed, Tat promotes the transcriptional initiation and elongation by interacting with transacting factors and cofactors, such as Sp1 (35), TFIID (36), E2F-4 (37), C/EBP $\beta$  (38), cyclin T1/CDK9 (39,40) and the histone acetyltransferases p300/CBP and P/CAF (41–43). When released from HIV-1-infected cells, Tat deregulates the cell signaling by binding to cell receptors, such as integrins (44), Flk1/KDR receptor (45) and chemokine receptors (46).

We first reported that NF- $\kappa$ B was constitutively active in Jurkat cells that stably expressed the Tat gene (47). Following gene transfection or protein transduction, Tat induced the IKK activity and proteasomal degradation of I $\kappa$ B- $\alpha$  (48), and increased the p65 transcriptional activity by inhibiting the SIRT-1-mediated deacetylation of p65 Lys310 (49). These findings suggested that Tat modulates crucial enzymes involved in NF- $\kappa$ B signaling; however, it was unclear how Tat could subvert the negative feedback of NF- $\kappa$ B, which is mainly dependent on *de novo* synthesis of I $\kappa$ B- $\alpha$  (15,17). We previously found that I $\kappa$ B- $\alpha$  binds to Tat and promotes the nuclear export of the viral transactivator (50,51). In this study, we report that Tat counteracts the post-activation turn off of NF- $\kappa$ B through direct interaction with I $\kappa$ B- $\alpha$  and p65, which enhances the DNA binding and transcriptional activity of the NF- $\kappa$ B complex. The new mechanism of NF- $\kappa$ B deregulation here described may provide further insights into the chronic immune activation of HIV-1 infection.

## MATERIALS AND METHODS

### Plasmids

The plasmids pcDNA-3xHA-I $\kappa$ B- $\alpha$ , p3xFLAG-CMV-Tat, p3xFLAG-CMV-Tat C(22,25,27)A, p3xFLAG-CMV-Tat R(49,52,53,55,56,57)A, pGEX-2T-Tat, pGEX-2T-Tat C(22,25,27)A and pGEX-2T-Tat R(49,52,53,55,56,57)A were previously described (50). The plasmids pNL4-3.Luc.R-E- and pHXB2-env were obtained from the AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH, USA; p $\kappa$ Bluc and pSV- $\beta$ -Gal were purchased from Promega (Madison, WI, USA). The plasmids pRc/CMV-3xHA-p65, pRc/CMV-3xHA-p65 $\Delta$ C(1–318), pRc/CMV-3xHA-p65 $\Delta$ N(122–551), p3xFLAG-CMV-Tat T,N(23,24)A,

p3xFLAG-CMV-Tat K(50,51)A, pGEX-2T-Tat T,N(23,24)A, pGEX-2T-Tat K(50,51)A and pNL4-3.FLAG-Tat.R-E- were generated as described in Supplementary Data.

### Cells, transfection, treatments and luciferase assay

HeLa, p50<sup>-/-</sup>p65<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) (52) and 293T cells were cultured in Dulbecco's modified Eagle's medium; Jurkat, U937 cells and human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640. PBMCs were isolated as previously described (53). Media were supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (Lonza Cologne AG, Germany). HeLa, p50<sup>-/-</sup>p65<sup>-/-</sup> MEFs and 293T were transfected with DNA by using FuGENE HD (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's protocol; total DNA amounts were equalized by transfection of pRc/CMV empty vector (Invitrogen, Carlsbad, CA, USA). For pulse-stimulation, HeLa cells were treated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) (20 ng/ml) for 5 min, or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; Sigma-Aldrich) (20 ng/ml) for 30 min, washed twice in complete culture medium and then returned to culture. For luciferase assays, pSV- $\beta$ -Gal was co-transfected with p $\kappa$ Bluc to monitor the transfection efficiency. Forty-eight-hour post-transfection, cells were lysed in lysis buffer of Dual Light Luciferase System (Tropix, Bedford, MA, USA) and the luciferase and  $\beta$ -galactosidase activities were evaluated by using Dual Light Luciferase System (Tropix) in a bioluminometer (Turner Biosystem, Sunnyvale, CA, USA). The ratio of firefly luciferase activity to  $\beta$ -galactosidase activity was expressed as relative light units.

### RNA interference

Jurkat or U937 cells were transfected by electroporation using a Bio-Rad apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, aliquots ( $5 \times 10^6$  cells) were suspended in 0.3 ml of RPMI 1640 supplemented with 20% fetal calf serum and subjected to a double electrical pulse (0.22 V, 960  $\mu$ F) in the presence of annealed siRNA (200 pmol); electroporated cells were washed and cultured in complete medium. RNA interference was performed with: siRNA Tat sense, CUGCUUGUACCAUUGCUAAU and siRNA Tat antisense, UAGCAAUUGGUACAAGCAGUU; siRNA control sense, CUGCUUGUACA AUUGCUAU and siRNA control antisense, UAGCAAUUGUACAAGCAGUU. RNA interference of p65 and I $\kappa$ B- $\alpha$  was performed with SMART pool siRNA p65 and I $\kappa$ B- $\alpha$  (Dharmacon, Chicago, IL, USA).

### Pseudotyped virions and single round infection

293T cells ( $1 \times 10^7$ ) were transfected with pNL4-3.Luc.R-E- or pNL4-3.FLAG-Tat.R-E- (10  $\mu$ g) together with pHXB2 Env (10  $\mu$ g), and 48-h post-transfection cell supernatant was collected. Enzyme-linked immunosorbent assay (ELISA) using anti-p24 antibody measured virion

concentration. PBMCs, Jurkat or U937 cells ( $5 \times 10^7$ ) were infected with HXB2 Env-pseudotyped virions (500 ng of p24) by spinoculation, as previously described (50).

#### Cell extracts, western blotting, IKK activity and NF- $\kappa$ B DNA binding

Total, nuclear and cytosolic extracts were performed as previously described (54); details are reported in Supplementary Data. Western blotting analysis was performed by resuspending protein aliquots in loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 1% bromophenol blue, 10%  $\beta$ -mercaptoethanol, 25% glycerol), resolved on 12% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and incubated with primary antibodies (1:1000) followed by incubation with horseradish-peroxidase-linked mouse or rabbit IgG (1:2000) (GE Healthcare Amersham, Little Chalfont, Buckinghamshire, UK) in PBS containing 5% non-fat dry milk (Bio-Rad Laboratories). Proteins were detected by chemiluminescence using the ECL System (GE Healthcare Amersham). Primary antibodies were purchased from: Santa Cruz Biotechnology, Santa Cruz, CA, USA (anti-HA F7, anti-I $\kappa$ B- $\alpha$  C15, anti-Histone H1, anti-Hexokinase-II); Sigma-Aldrich (anti-FLAG M2, anti- $\gamma$ -Tubulin); Upstate, Lake Placid, NY, USA (anti-p65). Densitometry of single bands was analysed by ImageJ software package (NIH, USA). IKK activity was evaluated in cytosolic extracts using the HTScan IKK kinase assay kit (Cell Signaling Technology, Danvers, MA, USA). Binding of p65, p50 and FLAG-Tat to the double-stranded NF- $\kappa$ B oligonucleotide was measured using NF- $\kappa$ B Combo Transcription Factor Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Electrophoretic Mobility Shift Assay (EMSA) was performed as previously described (55); details are described in Supplementary Data.

#### *In vitro* translation

HA-I $\kappa$ B- $\alpha$ , p65 and Tat were expressed under the T7 promoter and *in vitro* translated using the TnT quick coupled transcription/translation system (Promega), as previously reported (50). Details are described in Supplementary Data.

#### Immunoprecipitation assay and GST-pull down

Immunoprecipitation, GST-pull down, and production of GST proteins in *Escherichia coli* strain BL21 were performed as previously reported (50). Details are described in Supplementary Data.

#### Real-time PCR

Total RNA was extracted from cells by using the TRIzol reagent (Invitrogen); RNA aliquots (200 ng) were reverse transcribed using Random Examers (Roche) and Superscript III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. Real-time PCR was performed with the iQ Green Super mix (Bio-Rad Laboratories) and carried out with the iCycler iQ

Real-Time detection system (Bio-Rad Laboratories) under the following conditions: 95°C, 1 min; (94°C, 10 s; 60°C, 30 s)  $\times$  40. Primers for Tat and *MIP-1 $\alpha$*  are listed in Supplementary Data. Real-time PCR of *CSF3*, *LTA*, *NFKBIA*, *TLR2*, *GAPDH* and *ACTB* was performed using the RT<sup>2</sup> profiler PCR Array-Human NF- $\kappa$ B signaling pathway (QIAGEN Sciences, MD, USA). Reactions were carried out in triplicate, and gene expression levels were calculated relative to *GAPDH* mRNA levels as endogenous control. Relative expression was calculated as  $2^{-(Ct_{\text{gene under investigation}} - Ct_{\text{GAPDH}})}$ .

#### Chromatin immunoprecipitation assay

Cells were fixed by adding formaldehyde (Sigma-Aldrich) at the final concentration of 1%. After 10 min, ice-cold PBS plus 0.125 M glycine was added, and plates were transferred on ice, washed extensively with PBS, and scraped. After centrifugation, cells were 10 min lysed in lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40) supplemented with 1 $\times$  Complete Protease Inhibitor (Roche Diagnostic GmbH). Nuclei were pelleted (1000  $\times$  g, 5 min), and resuspended in sonication buffer (50 mM Tris-HCl pH 8.0, 1% SDS, 10 mM EDTA). Chromatin was sonicated using Bandelin Sonoplus GM70 (Bandelin Electronic, Berlin, Germany), centrifuged (14000  $\times$  g, 15 min), and supernatant was 10-fold diluted in dilution buffer (0.01% SDS, 16.7 mM Tris-HCl pH 8.0, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA). Samples were pre-cleared by 3-h incubation with 20  $\mu$ l of protein G agarose beads followed by incubation with antibodies against the analysed proteins. Primary antibodies were: anti-p65 (sc-372), anti-I $\kappa$ B $\alpha$  (sc-203) and rabbit IgG (sc-2027) from Santa Cruz Biotechnology; anti-FLAG M2 from Sigma-Aldrich. Immunoprecipitations were carried out at 4°C overnight and immune complexes were collected with protein G agarose beads, washed five times with low salt buffer (20 mM Tris-HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), four times with high salt buffer (20 mM Tris-HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), once with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and extracted in TE buffer containing 2% SDS. Protein-DNA cross-links were reverted by heating at 65°C overnight. DNA was further purified by QIAquick PCR purification kit (QIAGEN) and eluted in 50  $\mu$ l sterile distilled water. Specific enrichment in NF- $\kappa$ B enhancer sequences was measured by real-time PCR of chromatin immunoprecipitation (ChIP) eluates using SYBR GreenER Master Mix (Invitrogen). Reactions were carried out with the iCycler iQ Real-Time detection system (Bio-Rad Laboratories) using the following conditions: 95°C, 1 min; (94°C, 10 s; 60°C, 30 s)  $\times$  40. Primers used for *MIP-1 $\alpha$* , *GAPDH* and *ACTB* are listed in Supplementary Data. Real-time PCR of *CSF3*, *LTA*, *NFKBIA* and *TLR2* were performed using the Custom ChIP array (QIAGEN). For each sample, values were normalized to input DNA and reported as % of input over the rabbit IgG control.



## Statistical analysis

Statistical analysis was performed by two-tail unpaired Student's *t*-test. Data were reported as means  $\pm$  SE. Differences between the means were considered as statistically significant at the 95% level ( $P < 0.05$ ).

## RESULTS

### Tat enhances the NF- $\kappa$ B activity by hijacking I $\kappa$ B- $\alpha$ and inhibiting the post-activation turn off of NF- $\kappa$ B

We analysed the kinetic of NF- $\kappa$ B activation in single round HIV-1 infection by modulating the expression of Tat with RNA interference. Jurkat cells were transfected with siRNA Tat, siRNA control, or left untransfected, and 24 h later cells were infected with HXB2 Env-pseudotyped NL4-3.Luc.R<sup>-</sup>E<sup>-</sup> virions. By cytofluorimetric analysis, ~40% of cells was siRNA-transfected and HIV-1-infected at 12-h post-infection (Supplementary Figure S1). The expression of Tat was detected at 1-h post-infection and progressively increased up to 12 h in untransfected and siRNA control-transfected cells, while it was barely detected in siRNA Tat-transfected cells (Figure 1A). The LTR-dependent luciferase expression was also progressively induced at 1- to 12-h post-infection in untransfected and siRNA control-transfected cells, while it was barely observed in siRNA Tat-transfected cells, as expected for the Tat-dependent transactivation of the HIV-1 LTR (Figure 1A).

The progressive increase in NF- $\kappa$ B activity, as measured by p65-p50 binding to an NF- $\kappa$ B consensus oligonucleotide (Figure 1B) and nuclear p65 (Figure 1C, nucleus), was observed at 1- to 3-h post-infection in both Tat-positive (no siRNA or siRNA control) and Tat-negative (siRNA Tat) cells; however, at 12-h post-infection, the p65 DNA binding and nuclear p65 persisted elevated in presence of Tat (no siRNA and siRNA control), while they dropped in absence of Tat (siRNA Tat) (Figure 1B and C). I $\kappa$ B- $\alpha$  was degraded in the cytosol within 3-h post-infection and *de novo* synthesized at 6 h with full replenishment at 12-h post-infection in both Tat-positive and Tat-negative cells (Figure 1C, cytosol). Consistently, the IKK activity was induced at 1-h post-infection, and decreased at 3-h post-infection independently of the presence of Tat (Figure 1D). Similar kinetics of NF- $\kappa$ B activity, IKK activation and I $\kappa$ B- $\alpha$  degradation/re-synthesis were observed in single-round HIV-1 infection of PBMCs using a cocktail of Azidothymidine (AZT) and Lamivudine (3TC) as reverse transcriptase inhibitors (Supplementary Figure S2). These results indicated that in single-round HIV-1 infection the NF- $\kappa$ B activation initially correlated with the IKK activation and I $\kappa$ B- $\alpha$  degradation independently of the presence of Tat, and it was kept elevated in presence of Tat following the decay of the IKK activity and new synthesis of I $\kappa$ B- $\alpha$ .

Next, we tested the single action of Tat on the kinetic of NF- $\kappa$ B activity induced by PMA. To this end, HeLa cells were transfected with p3 $\times$ -FLAG-Tat, or empty vector, stimulated with PMA for 5 min, and extensively washed

to avoid the oscillatory kinetic of NF- $\kappa$ B activation (17,18). In un-stimulated cells, the p65 DNA binding (Figure 2A) and nuclear p65 (Figure 2B, nucleus) were slightly enhanced by Tat, while the I $\kappa$ B- $\alpha$  content was not significantly affected (Figure 2B, cytosol). Transient stimulation with PMA increased the NF- $\kappa$ B DNA binding activity and nuclear p65 at 5 min peaking at 60–120 min independently of the presence of Tat (Figure 2A and B); however, while the p65 DNA binding and nuclear p65 persisted elevated at 240-min post-treatment in Tat-positive cells, they dropped in Tat-negative cells (Figure 2A and B). In addition, Tat bound to the NF- $\kappa$ B oligonucleotide in un-stimulated cells and its binding increased following PMA stimulation (Figure 2A), suggesting that the viral protein was a component of the NF- $\kappa$ B complex bound to DNA. Degradation of I $\kappa$ B- $\alpha$  was progressively induced at 5–30 min, followed by *de novo* synthesis at 60 min with full replenishment at 240 min in both Tat-positive and negative cells (Figure 2B, cytosol). Consistently, the IKK activation started at 5 min, and turned off at 30 min independently of the presence of Tat (Figure 2C). These results indicated that Tat enhanced the NF- $\kappa$ B activity in un-stimulated and PMA-stimulated cells without affecting the IKK activity and I $\kappa$ B- $\alpha$  content.

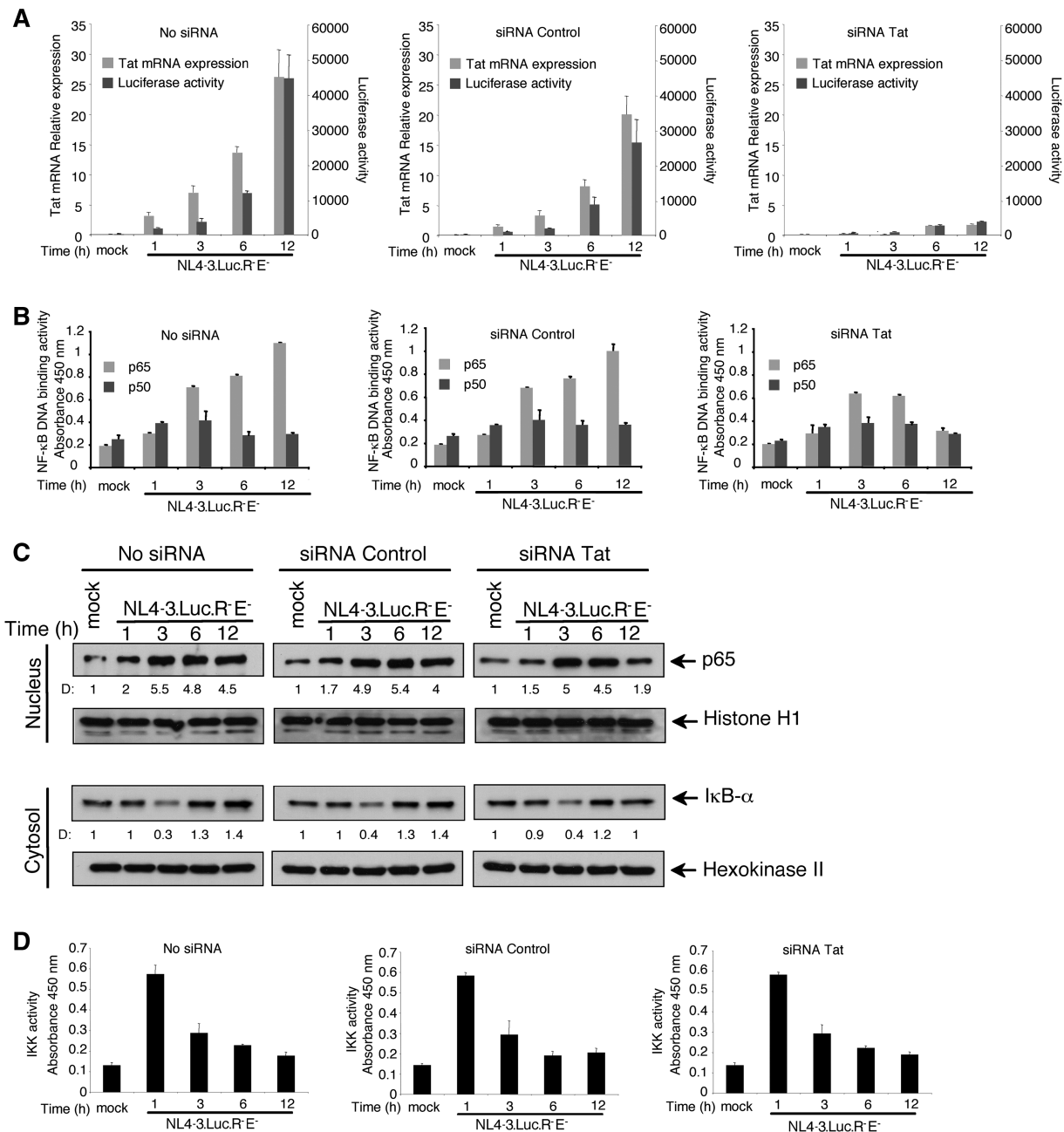
As we previously found that Tat binds to the sixth ankyrin of I $\kappa$ B- $\alpha$  (50,51), we tested whether Tat counteracted the generation of the I $\kappa$ B- $\alpha$ /NF- $\kappa$ B complex. To this end, the association of I $\kappa$ B- $\alpha$  with p65 was analysed *in vivo* at 0 and 240 min after short-pulse PMA, which corresponded to the time of un-stimulated condition and post-activation *de novo* synthesis of I $\kappa$ B- $\alpha$ , respectively. Coimmunoprecipitation of I $\kappa$ B- $\alpha$  with p65 was detected at 0- and 240-min post-treatment in Tat-negative cells (Figure 2D, lanes 1 and 3), and was halved in Tat-positive cells, where Tat coimmunoprecipitated with I $\kappa$ B- $\alpha$  (Figure 2D, lanes 2 and 4), indicating that Tat competed the I $\kappa$ B- $\alpha$  binding to p65.

### Tat counteracts the I $\kappa$ B- $\alpha$ inhibition of p65 by competing the repressor binding

We further investigated the physical interaction of Tat with I $\kappa$ B- $\alpha$  using *in vitro* translated proteins in coimmunoprecipitation assays. For mapping the interaction domains, we used the following Tat mutants: Tat T,N(23,24)A, Tat C(22,25,27)A, Tat K(50,51)A and Tat R(49–57)A (Figure 3A). I $\kappa$ B- $\alpha$  coimmunoprecipitated with all Tat proteins, except Tat R(49–57)A (Figure 3B). These results indicated that the arginine-rich domain of Tat was involved in the binding to I $\kappa$ B- $\alpha$ , which was consistent with previous observations (50,51). Moreover, Tat associating with I $\kappa$ B- $\alpha$  competed the binding of I $\kappa$ B- $\alpha$  to p65 in a dose-dependent manner (Figure 3C, lanes 3–5), while Tat R(49–57)A, which lacked the binding site for I $\kappa$ B- $\alpha$ , did not associate with I $\kappa$ B- $\alpha$  and did not compete the binding of I $\kappa$ B- $\alpha$  to p65 (Figure 3C, lanes 6–8).

Next, we evaluated whether Tat counteracted the I $\kappa$ B- $\alpha$ -mediated inhibition of p65 binding to DNA by incubating *in vitro* translated p65 and I $\kappa$ B- $\alpha$  proteins with the NF- $\kappa$ B probe in presence or absence of Tat followed by

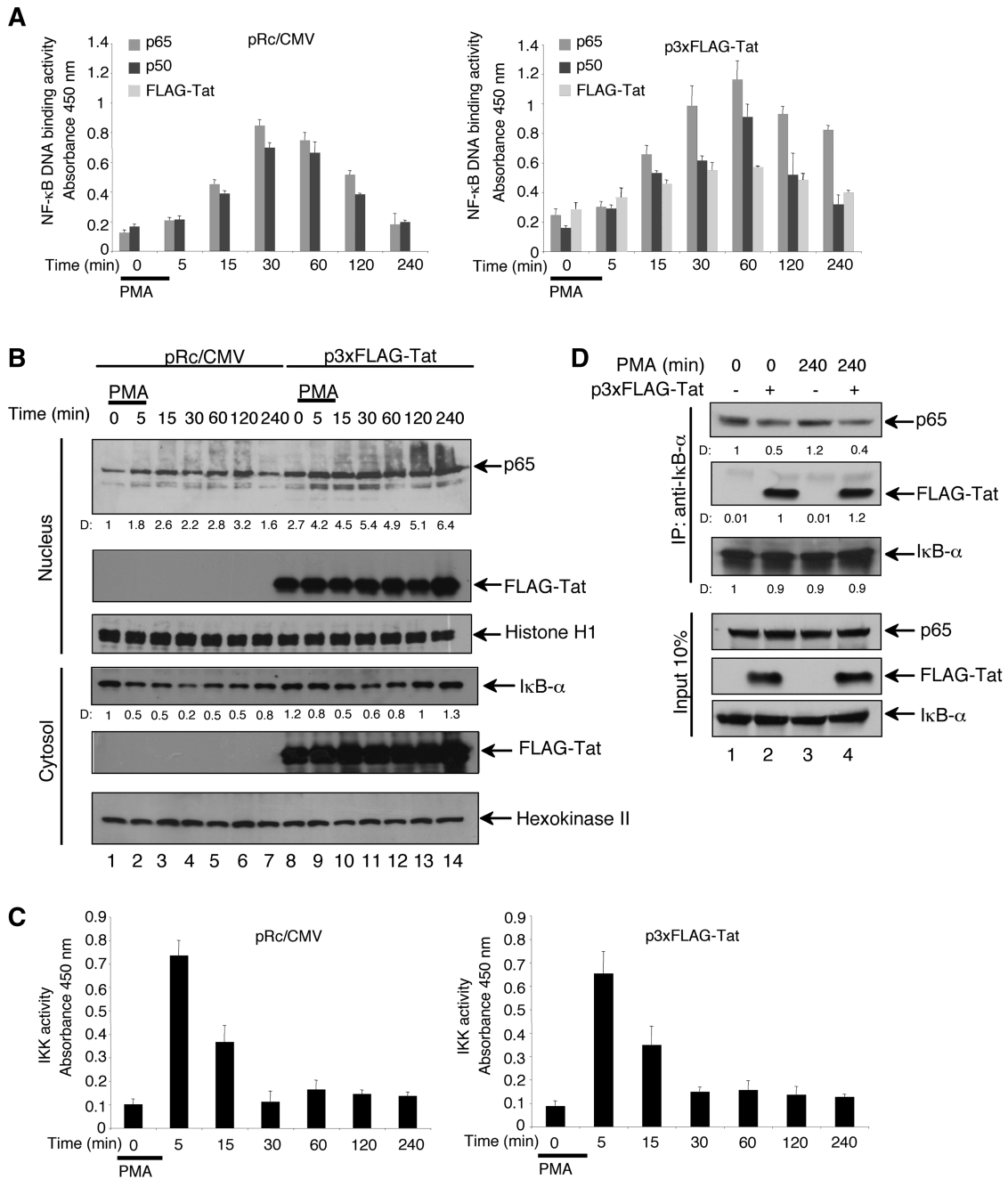




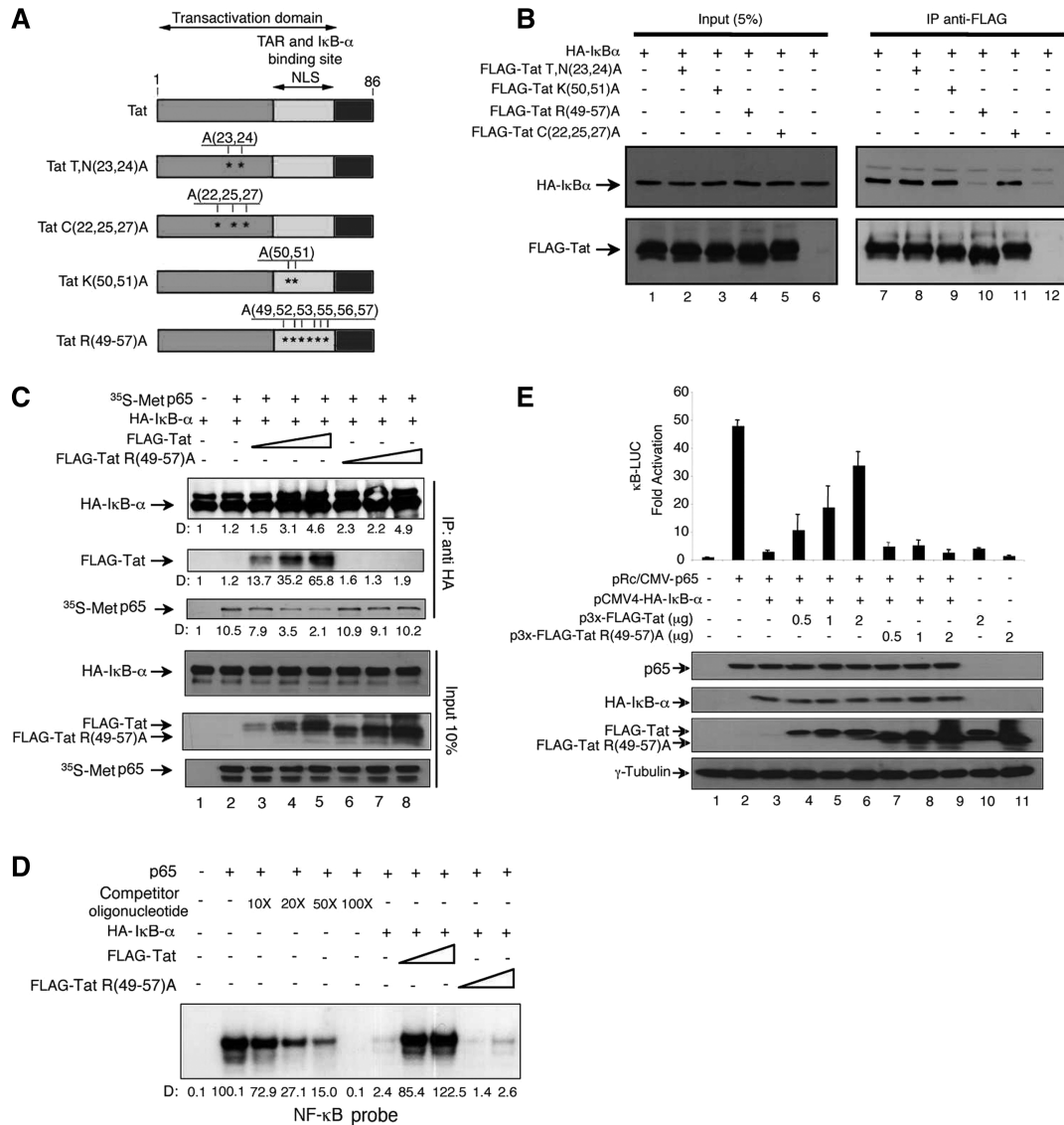
**Figure 1.** Tat counteracts the post-activation turn off of NF- $\kappa$ B in single round HIV-1-infection. Jurkat cells ( $5 \times 10^7$ ) were transfected with siRNA Tat or siRNA control (2 nmol), or left untransfected; 24 h later, cells were infected with HXB2-pseudotyped NL4-3.Luc.R<sup>-</sup>E<sup>-</sup> virions (500 ng of p24) and harvested at the indicated time. (A) Tat expression was measured in total RNA by real-time PCR, while the luciferase activity was measured in whole cell extracts. (B) Nuclear extracts (10  $\mu$ g) were analysed for the p65 and p50 binding to the NF- $\kappa$ B double-stranded oligonucleotide using the NF- $\kappa$ B Transcription Factor ELISA Assay kit (Cayman). (C) The expression of p65 and I $\kappa$ B- $\alpha$  was analysed by 12% SDS-PAGE and western blotting of nuclear or cytosolic extracts (20  $\mu$ g) using anti-p65 and anti-I $\kappa$ B- $\alpha$  antibodies. Histone H1 and Hexokinase II were detected with specific antibodies as markers of nuclear and cytosolic extracts, respectively. Densitometry values (D) of the bands were expressed as fold increase above the control (mock). (D) IKK activity was measured in cytosolic extracts (100  $\mu$ g) by using HTScan IKK Kinase Assay (Cell Signaling Technology). Values (mean  $\pm$  SE,  $n = 3$ ) are shown.

EMSA. The p65 DNA binding activity was inhibited by I $\kappa$ B- $\alpha$  and restored in a dose-dependent manner by wild-type Tat, and not by Tat R(49–57) (Figure 3D). Further, we analysed the Tat effect on the I $\kappa$ B- $\alpha$  repression of p65 transcriptional activity by transfecting p50<sup>-/-</sup>p65<sup>-/-</sup>MEFs with the NF- $\kappa$ B-Luc reporter together with expression vectors of p65 and I $\kappa$ B- $\alpha$ , in presence or absence of

Tat. I $\kappa$ B- $\alpha$  inhibited the p65-dependent expression of the *luciferase* gene, which was restored in a dose-dependent manner by wild-type Tat, and not by Tat R(49–57)A (Figure 3E). Altogether these results indicated that Tat counteracts the I $\kappa$ B- $\alpha$  repression of the p65 DNA-binding and transcriptional activity by associating with I $\kappa$ B- $\alpha$  and competing the repressor binding to p65.



**Figure 2.** Tat enhances the NF-κB activity following PMA stimulation by associating with IκB-α and counteracting the NF-κB repression. HeLa cells ( $5 \times 10^6$ ) were transfected with p3xFLAG-Tat or p3xFLAG empty vector (5 μg), and 48 h later were stimulated with PMA (5 min, 20 ng/ml) or left unstimulated, washed twice with DMEM and harvested at the indicated times. Nuclear and cytosolic extracts were prepared for further analysis. (A) Nuclear extracts were analysed for the p65, p50 and FLAG-Tat binding to the NF-κB double-stranded oligonucleotide using the NF-κB Transcription Factor ELISA assay kit (Cayman). (B) Nuclear and cytosolic extracts (20 μg) were separated by 12% SDS-PAGE and analysed by western blotting using the anti-p65, anti-FLAG, anti-Histone H1, anti-IκB-α and anti-Hexokinase-II antibodies. Densitometry values (D) of the bands were expressed as fold increase above the control (lane 1). (C) IKK activity was measured in cytosolic extracts (100 μg) by using HTScan IKK kinase assay (Cell Signaling Technology). (D) HeLa cells ( $5 \times 10^6$ ) were transfected with or without p3xFLAG-Tat (5 μg); 48 h later, cells were stimulated with PMA (5 min, 20 ng/ml) or left unstimulated, washed twice with DMEM, and harvested after 240 min. Whole cell extracts (1 mg) were immunoprecipitated with protein G-Sepharose-coupled anti-IκB-α antibody. Immunocomplexes were separated by 12% SDS-PAGE and analysed by western blotting with anti-p65, anti-FLAG and anti-IκB-α antibodies. Densitometry values (D) of the bands were expressed as fold increase above the control (lane 1). Values (mean ± SE,  $n = 3$ ) are shown.



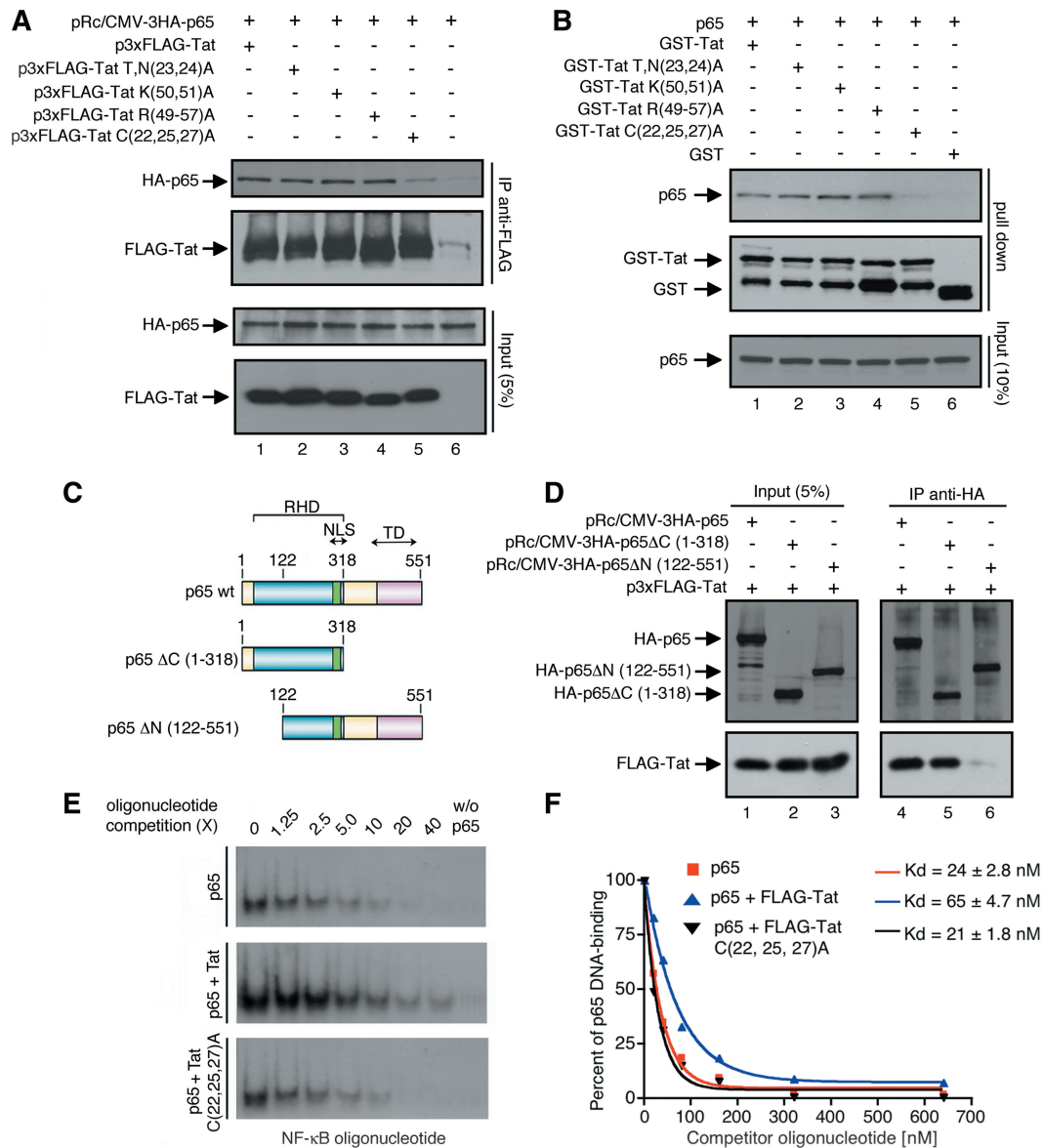
**Figure 3.** Tat relieves p65 from the IκB-α inhibition. **(A)** Schematic representation of wild-type and mutant Tat proteins. **(B)** HA-IκB-α (5 μl) was incubated in presence or absence of FLAG-Tat, FLAG-Tat T,N(23,24)A, FLAG-Tat K(50,51)A, FLAG-Tat R(49-57)A or FLAG-Tat C(22,25,27)A (10 μl) using *in vitro* translated proteins. Wild-type and mutant Tat proteins were immunoprecipitated with anti-FLAG antibody; immunocomplexes were separated by 12% SDS-PAGE and analysed by western blotting with anti-HA and anti-FLAG antibodies. **(C)** <sup>35</sup>S-methionine-labeled p65 (5 μl) was incubated with *in vitro* translated HA-IκB-α (5 μl) in presence or absence of FLAG-Tat or FLAG-Tat R(49-57)A (5, 10 or 20 μl). HA-IκB-α was immunoprecipitated with anti-HA antibody; immunocomplexes were separated by 12% SDS-PAGE and analysed by western blotting with anti-HA and anti-FLAG antibodies, or autoradiography (<sup>35</sup>S-Met-p65). Densitometry values (D) of the bands were expressed as fold increase above the control (lane 1). **(D)** The p65 DNA binding activity was analysed by EMSA using *in vitro* translated proteins. <sup>32</sup>P-labeled-NF-κB double-stranded oligonucleotide was incubated with p65 (0.5 μl) in presence or absence of HA-IκB-α (1 μl), FLAG-Tat or FLAG-Tat R(49-57)A (5 and 10 μl); competition of DNA binding was performed with 10- up to 100-fold molar excess of unlabeled NF-κB double-stranded oligonucleotide. DNA/protein complexes were run on 6% PAGE-TBE and analysed by autoradiography. **(E)** p50<sup>-/-</sup>p65<sup>-/-</sup>MEFs (3 × 10<sup>5</sup> cells) were transfected with pκBLuc (0.5 μg) and pSV-β-Gal (0.1 μg) with or without pRc/CMV-p65 (0.5 μg), pCMV4-HA-IκB-α (0.5 μg), p3xFLAG-Tat or p3xFLAG-Tat R(49-57)A (0.5, 1 and 2 μg). The luciferase activity was measured in cell extracts 48-h post-transfection and normalized to β-galactosidase activity. Fold activation was calculated relative to transfection of the pκBLuc plasmid alone. Values (mean ± SE, n = 3) are shown. As control of protein expression, aliquots of cell extracts (20 μg) were analysed by western blotting with anti-p65, anti-HA, anti-FLAG and anti-γ-tubulin antibodies.

### Tat increases the p65 affinity binding to DNA through association with p65

We tested whether Tat physically interacted with p65. HeLa cells were transfected with the expression vectors of HA-p65 and FLAG-Tat, and 24 h later Tat was immunoprecipitated from cell extracts. The p65 protein was coimmunoprecipitated with wild-type Tat or the

mutants Tat T,N(23,24)A, Tat K(50,51)A and Tat R(49-57)A at similar levels, while a significant decrease in coimmunoprecipitation was observed for Tat C(22,25,27)A (Figure 4A). Consistently, GST-pull down of *in vitro* translated proteins showed the direct binding of p65 to wild-type Tat, or the mutants Tat T,N(23,24)A, Tat K(50,51)A and Tat R(49-57)A, and lack of binding to





**Figure 4.** Tat associates with p65 increasing its DNA-affinity binding. (A) HeLa cells were transfected with pRc/CMV-3xHA-p65 (5 μg) in presence or absence of p3xFLAG-Tat, p3xFLAG-Tat T,N(23,24)A, p3xFLAG-Tat K(50,51)A, p3xFLAG-Tat R(49-57)A, or p3xFLAG-Tat C(22,25,27)A (5 μg). FLAG-Tat proteins were immunoprecipitated with anti-FLAG antibody; immunocomplexes were separated by 12% SDS-PAGE and analysed by western blotting with anti-FLAG or anti-HA antibodies. (B) *In vitro* translated p65 (5 μl) was incubated with GST-Tat, GST-Tat T,N(23,24)A, GST-Tat K(50,51)A, GST-Tat R(49-57)A, GST-Tat C(22,25,27)A, or GST (5 μg) conjugated with Glutathione-Sepharose. Protein complexes were recovered by GST-pull down, separated by 12% SDS-PAGE, and analysed by western blotting with anti-p65 or anti-GST antibodies. (C) Schematic representation of wild-type and mutant p65 proteins. (D) HeLa cells were transfected with p3xFLAG-Tat (5 μg) in the presence or absence of pRc/CMV-3xHA-p65, pRc/CMV-3xHA-p65ΔC (1-318), or pRc/CMV-3xHA-p65ΔN (122-551) (5 μg). Wild-type and mutant HA-p65 proteins were immunoprecipitated with anti-HA antibody; immunocomplexes were separated by 12% SDS-PAGE and analysed by western blotting with anti-FLAG or anti-HA antibodies. (E) Recombinant p65 protein (100 ng; Active Motif Carlsbad, CA, USA) was 20 min incubated with <sup>32</sup>P-labeled NF-κB double-stranded oligonucleotide in presence or absence of *in vitro* translated FLAG-Tat or FLAG-Tat C(22,25,27)A (5 μl); competitions were performed with 1.25- up to 40-fold molar excess of unlabeled oligonucleotide. DNA/protein complexes were separated by 6% PAGE in 0.5 × TBE buffer and analysed by autoradiography. (F) Densitometry of band-shifts shown in E.

Tat C(22,25,27)A (Figure 4B). These results indicated that Tat associated with p65 through the cysteine-rich domain and ruled-out the requirement of bridging proteins to mediate such interaction.

To identify the p65 domain required for binding to Tat, HeLa cells were transfected with FLAG-Tat

together with HA-p65 mutants, which were deleted of the transactivation domain [p65ΔC (1-318)], or the RHD [p65ΔN (122-551)] (Figure 4C). Tat coimmunoprecipitated with p65ΔC (1-318), and not with p65ΔN (122-551) (Figure 4D), indicating that the RHD of p65 was involved in the physical interaction with Tat.

To address the effect of Tat on the p65 DNA-binding affinity, purified recombinant p65 protein was incubated with <sup>32</sup>P-labeled-NF-κB double-stranded oligonucleotide in presence or absence of wild-type Tat or Tat C(22,25,27)A, and p65 DNA-binding was analysed by EMSA. Whereas wild-type Tat significantly increased the binding of p65 to DNA, the mutant Tat C(22,25,27)A, lacking the binding site for p65, was ineffective (Figure 4E). The p65 binding to DNA was evaluated by competition with increasing amounts of cold competitor NF-κB oligonucleotide, which showed that Tat significantly enhanced the p65 affinity binding to DNA (Figure 4E and F).

#### **Tat activates the p65-dependent expression of NF-κB-responsive genes, occupies the NF-κB enhancers and promotes the p65 recruitment with IκB-α displacement**

We analysed the action of Tat on the expression of NF-κB-responsive genes *in vivo*. To this end, HeLa cells were transfected with FLAG-Tat, FLAG-Tat C(22,25,27)A or FLAG-Tat R(49–57)A, and 48 h later the expression of a number of NF-κB-dependent genes was analysed by real-time PCR. Tat significantly increased the expression of *MIP-1α*, *CSF3*, *LTA*, *NFKB1A* and *TLR2*, while the mutants Tat C(22,25,27)A and Tat R(49–57)A were ineffective (Figure 5A–E). The Tat-dependent transactivation of the NF-κB-dependent genes was abolished when cells were transfected with siRNA p65, indicating that the Tat transcriptional activation was mediated by p65 (Figure 5A–E). Differently, the expression of the NF-κB-independent genes *GAPDH* and *ACTB* was unaffected by wild-type and Tat mutants (Figure 5F and G). These results were consistent with the requirement of both the cysteine-rich and arginine-rich domains of Tat to enhance the NF-κB activity, suggesting that the up-regulation of NF-κB-responsive genes by Tat might occur through physical interaction of the viral protein with p65 and IκB-α.

*MIP-1α*, which was the mostly activated gene by Tat, encodes for a chemokine that promotes the recruitment of pro-inflammatory cells (56). Consistently with our findings, *MIP-1α* expression was increased in glial cells and lymph nodes of AIDS patients (57,58); moreover, *MIP-1α* production was induced by HIV-1 infection (59,60) and Tat protein (57,61,62). The transcriptional regulation of *MIP-1α* has been poorly characterized. Jaspar-based analysis (<http://jaspar.genereg.net/>) predicted three putative NF-κB enhancers in the proximal promoter region of the *MIP-1α* gene: NF-κB1, –1023/–1014 nucleotides; NF-κB2, –661/–652 nucleotides; NF-κB3, +370/+379 nucleotides (Supplementary Figure S3A). To validate the predicted NF-κB binding sites of the *MIP-1α* promoter, we stimulated HeLa cells with TNF-α, a well-known NF-κB inducer, and measured the expression levels of *MIP-1α* by real-time PCR, and the p65 occupancy of the NF-κB sites by CHIP. TNF-α activated the expression of *MIP-1α* and induced the p65 recruitment to the NF-κB1 site of *MIP-1α* without affecting the occupancy of the putative NF-κB2 and NF-κB3 sites (Supplementary Figure S3B and S3C). Tat also

promoted the recruitment of p65 to the NF-κB1 site of *MIP-1α*, while it did not affect the putative NF-κB2 and NF-κB3 sites (Supplementary Figure S3D). Altogether these results indicated that the NF-κB1 site was the only effective NF-κB enhancer of the *MIP-1α* promoter in response to TNF-α and Tat.

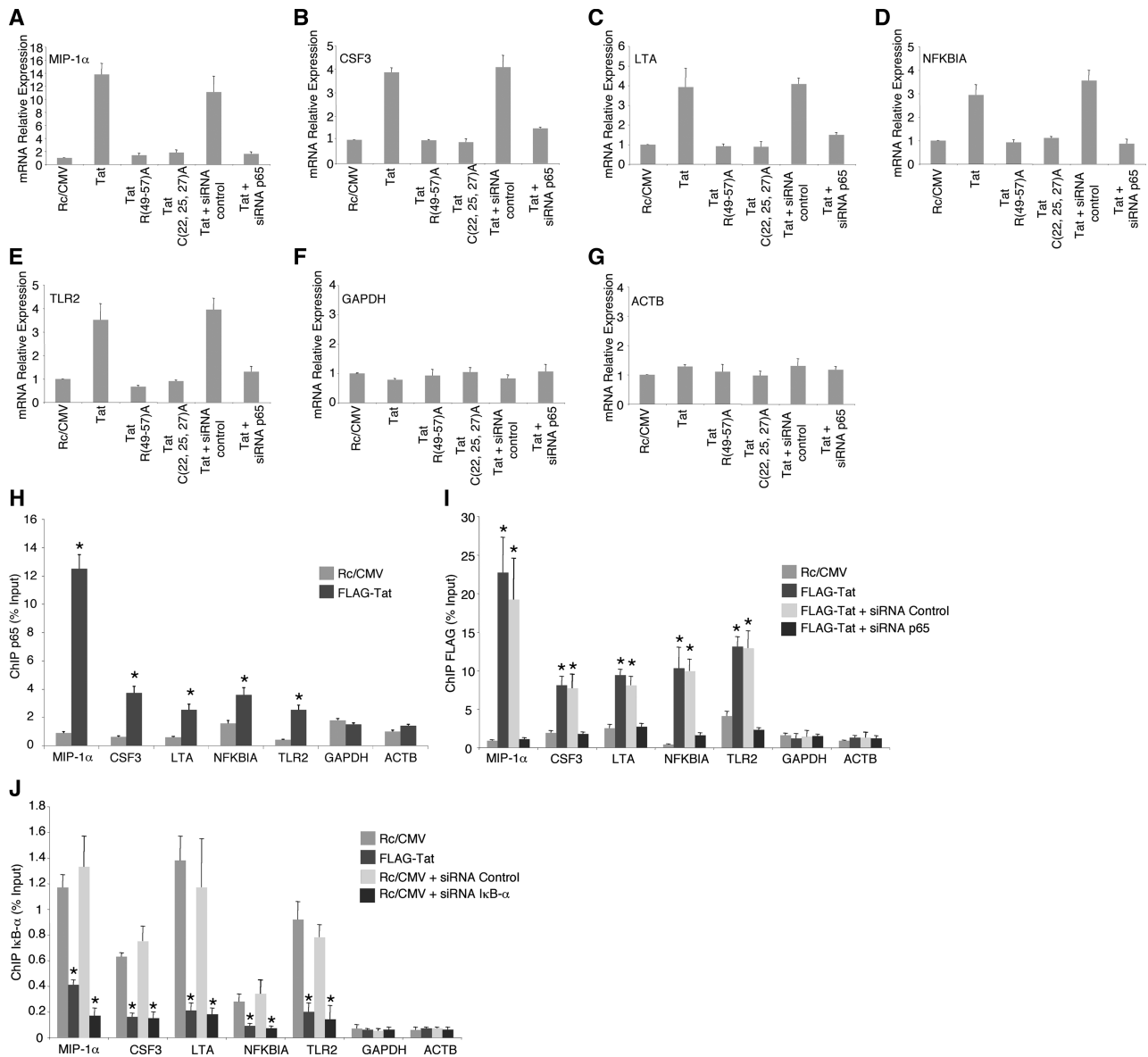
Similarly to *MIP-1α*, Tat increased the recruitment of p65 to the NF-κB enhancers of *CSF3*, *LTA*, *NFKB1A* and *TLR2*, while it was ineffective at the promoters of *GAPDH* and *ACTB* (Figure 5H). By CHIP, Tat bound to the NF-κB-responsive promoters, and not to the *GAPDH* and *ACTB* promoters, with loss of binding following p65 RNA interference (Figure 5I), suggesting that Tat occupancy occurred via p65 interaction. As additional findings, IκB-α was chromatin-immunoprecipitated at the NF-κB enhancers in absence of Tat, and it was significantly removed in presence of Tat, or following IκB-α RNA interference (Figure 5J), indicating that Tat displaced IκB-α from promoters.

#### **In HIV-1-infected monocytes Tat sustains the NF-κB activity and promotes the transcriptional activation of MIP-1α by interacting with IκB-α and p65**

We next analysed whether Tat affected the NF-κB activity and the expression of *MIP-1α* in the course of HIV-1 infection. To detect the Tat protein, we produced HXB2 Env-pseudotyped NL4-3.FLAG-Tat.R<sup>−</sup>E<sup>−</sup> virions, which were used in single-round infection of U937, a human monocytic cell line. Cells were transfected with siRNA Tat, siRNA control or left untransfected to modulate the Tat expression, and then analysed for the kinetic of NF-κB activation following viral infection. The expression of Tat and p24 was detected at 3- to 12-h post-infection, while it was barely detected in siRNA Tat-transfected cells (Figure 6A).

The NF-κB activity, as measured by p65 DNA binding, was induced at 3-h post-infection in both Tat-positive (no siRNA and siRNA control) and Tat-negative cells (siRNA Tat), and increased at 12h only in Tat-positive cells (Figure 6B). Degradation of IκB-α was observed at 3-h post-infection, and was followed by *de novo* synthesis of IκB-α at 12h independently of the Tat presence (Figure 6C). Consistently, the IKK activity was induced at 3-h post-infection and turned off at 12h independently of the Tat presence (Figure 6D). These results agreed with the kinetic of NF-κB activation in single-round HIV-1 infection of Jurkat cells (Figure 1), and further supported the requirement of viral expression to counteract the post-activation turn off of NF-κB.

In HIV-1-infected U937 cells, the endogenous Tat was coimmunoprecipitated with IκB-α and p65 (Figure 6E), and activated the *MIP-1α* expression in a p65-dependent manner, as shown by the lack of effect following RNA interference of Tat or p65 (Figure 6F). Moreover, Tat and p65 were both recruited to the NF-κB enhancer of *MIP-1α*, while the p65 occupancy was abolished by siRNA Tat (Figure 6G and H). Altogether these results indicated that in single round HIV-1 infection Tat associated with IκB-α and p65, and induced the



**Figure 5.** Tat activates the p65-dependent expression of NF-κB-responsive genes by occupying the NF-κB enhancers and promoting the p65 recruitment with IκB-α displacement. HeLa cells ( $5 \times 10^6$ ) were transfected with p3xFLAG-Tat, p3xFLAG-Tat C(22,25,27), p3xFLAG-Tat R(49-57) or empty vector (10 μg); for p65 RNA interference, cells ( $5 \times 10^6$ ) were transfected with p3xFLAG-Tat (10 μg) and siRNA p65 or siRNA control (200 pmol). Twenty-four-hour post-transfection, total RNA was extracted and analysed by real-time PCR to evaluate the expression of *MIP-1α* (A), *CSF3* (B), *LTA* (C), *NFKBIA* (D), *TLR2* (E), *GAPDH* (F), *ACTB* (G) genes. (H) HeLa cells ( $5 \times 10^6$ ) were transfected with p3xFLAG-Tat, or empty vector (10 μg) and 48 h later ChIP was performed with anti-p65 antibody. Real-time PCR was performed with primers specific for the indicated promoters. (I) HeLa cells ( $5 \times 10^6$ ) were transfected with empty vector, or p3xFLAG-Tat (10 μg) in presence or absence of siRNA p65, or siRNA control (200 pmol); 48 h later, ChIP was performed with anti-FLAG antibody. Real-time PCR was performed with primers specific for the indicated promoters. (J) HeLa cells ( $5 \times 10^6$ ) were transfected with empty vector, p3xFLAG-Tat (10 μg), or empty vector plus siRNA IκB-α or siRNA control (200 pmol); 48 h later, ChIP was performed with anti-IκB-α antibody. Real-time PCR was performed with primers specific for the indicated promoters. Values (mean ± SE,  $n = 3$ ) are shown. The asterisks indicate statistically significant differences compared to the control (empty vector) according to the Student's *t*-test ( $P < 0.05$ ).

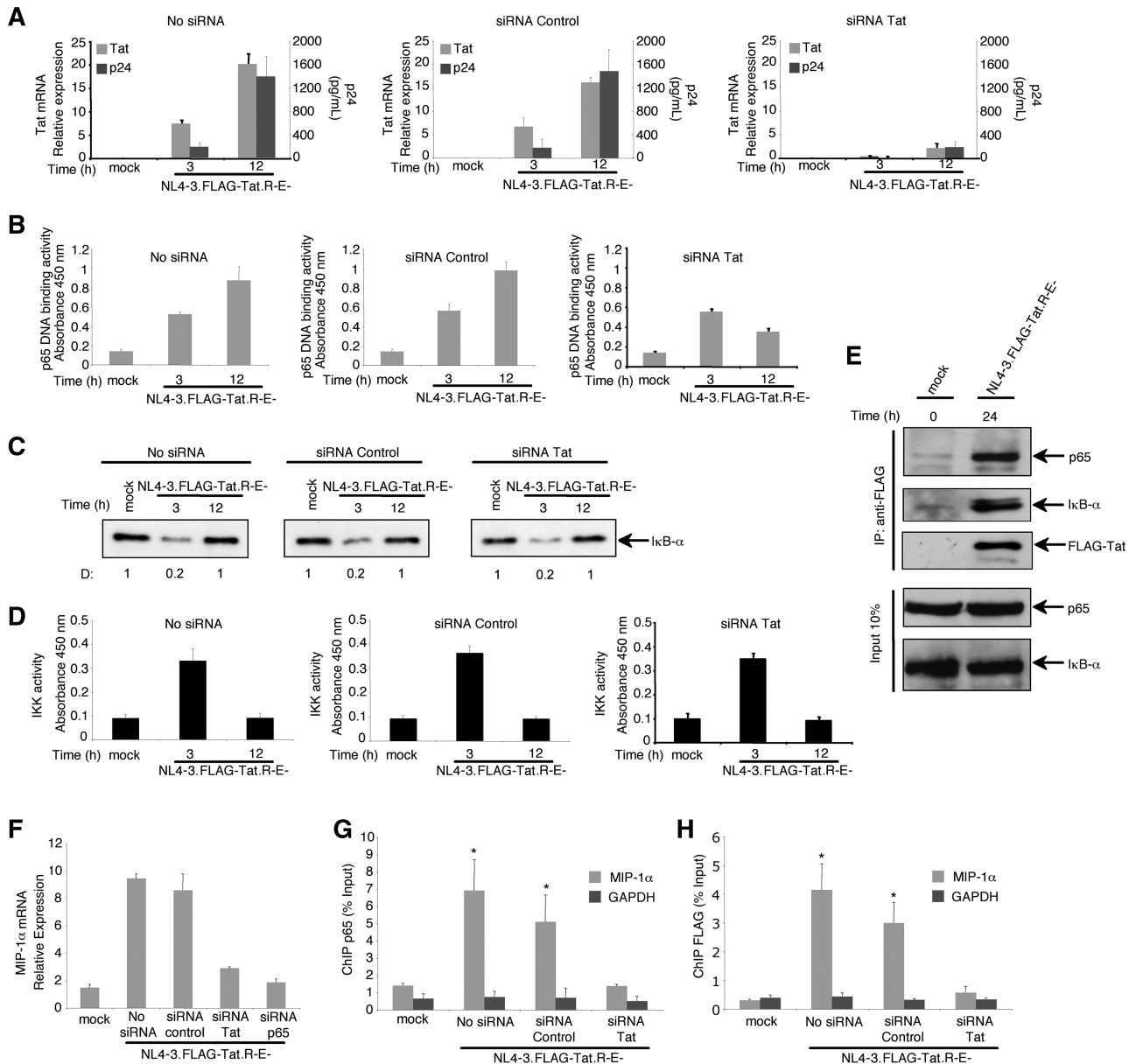
p65-dependent activation of *MIP-1α* expression through occupancy of the *MIP-1α* promoter and increased recruitment of p65.

## DISCUSSION

This study reports a novel mechanism of NF-κB activation by the HIV-1 Tat transactivator. Based on the

evidence that Tat enhanced the transcriptional activity of the p65 subunit of NF-κB (49,63,64), and physically interacted with the IκB-α repressor (50,51), we investigated the possibility that Tat could activate NF-κB via direct interaction with IκB-α and p65. To this end, the NF-κB activity was monitored in single round HIV-1 infection using RNA interference to silence the Tat expression. By this approach, we avoided the perpetuation of NF-κB activation signaling due to





**Figure 6.** In HIV-1 infection Tat sustains the NF- $\kappa$ B activity and enhances the *MIP-1 $\alpha$*  expression via interaction with I $\kappa$ B- $\alpha$  and p65. U937 cells ( $5 \times 10^7$ ) were transfected with siRNA Tat, siRNA control (2 nmol), or left untransfected; 24 h later cells were infected with HXB2-pseudotyped NL4-3.FLAG-Tat.R<sup>-</sup>E<sup>-</sup> virions (500 ng of p24) and harvested at the indicated time. (A) Real-time PCR of total RNA measured the Tat expression; ELISA measured the amount of p24 in whole cell extracts. (B) The binding of p65 to the NF- $\kappa$ B double-stranded oligonucleotide was measured in nuclear extracts (10  $\mu$ g) using the NF- $\kappa$ B Transcription Factor ELISA assay kit (Cayman). (C) The I $\kappa$ B- $\alpha$  content was analysed by 12% SDS-PAGE and western blotting of cytosolic extracts (20  $\mu$ g) using anti-I $\kappa$ B- $\alpha$  antibody. Densitometry values (D) of the bands were expressed as fold increase above the control (mock). (D) IKK activity was measured in cytosolic cell extracts (100  $\mu$ g) using HTScan IKK Kinase Assay (Cell Signaling Technology). (E) U937 cells ( $5 \times 10^7$ ) were infected with HXB2-pseudotyped NL4-3.FLAG-Tat.R<sup>-</sup>E<sup>-</sup> virions, or left uninfected. Twenty-four-hour post-infection, cell extracts (1 mg) were immunoprecipitated with protein G-Sepharose-coupled anti-FLAG antibody. Immunocomplexes were separated by 12% SDS-PAGE and analysed by western blotting with anti-p65, anti-FLAG and anti-I $\kappa$ B- $\alpha$  antibodies. (F) U937 cells ( $5 \times 10^7$ ) were transfected with siRNA Tat, siRNA p65, siRNA control (2 nmol), or left untransfected; 24 h later, cells were infected with HXB2-pseudotyped NL4-3.FLAG-Tat.R<sup>-</sup>E<sup>-</sup> virions, or left uninfected. Twenty-four-hour post-infection, total RNA was analysed for *MIP-1 $\alpha$*  expression by real-time PCR. (G and H) U937 cells ( $5 \times 10^7$ ) were transfected with siRNA Tat or siRNA control (2 nmol), or left untransfected; 24 h later, cells were infected with HXB2-pseudotyped NL4-3.FLAG-Tat.R<sup>-</sup>E<sup>-</sup> virions, or left uninfected. Twenty-four-hour post-infection, ChIP was performed with anti-p65 (G) or anti-FLAG (H). Real-time PCR was performed with primers specific for *MIP-1 $\alpha$*  and *GAPDH* promoters. Values (mean  $\pm$  SE,  $n = 3$ ) are shown. The asterisks indicate statistically significant differences compared to the control (mock), according to the Student's *t*-test ( $P < 0.05$ ).

subsequent rounds of viral entry in cell culture propagation (30,31). Upon HIV-1 infection, the early NF- $\kappa$ B activation occurred concomitantly with IKK activation and I $\kappa$ B- $\alpha$  degradation in the absence of Tat. Soon after the

shut off of IKK activity and new synthesis of I $\kappa$ B- $\alpha$ , the NF- $\kappa$ B activity was kept elevated in the presence of Tat, while it was down regulated upon silencing of the Tat gene. These findings indicate that in single round HIV-1

infection, Tat enhanced the NF- $\kappa$ B activity without affecting the IKK activity and the half-life of I $\kappa$ B- $\alpha$ . Similar kinetic of NF- $\kappa$ B activation occurred upon short-pulse of PMA in Tat-transfected HeLa cells, where Tat inhibited the post-activation turn off of NF- $\kappa$ B in presence of newly synthesized I $\kappa$ B- $\alpha$ .

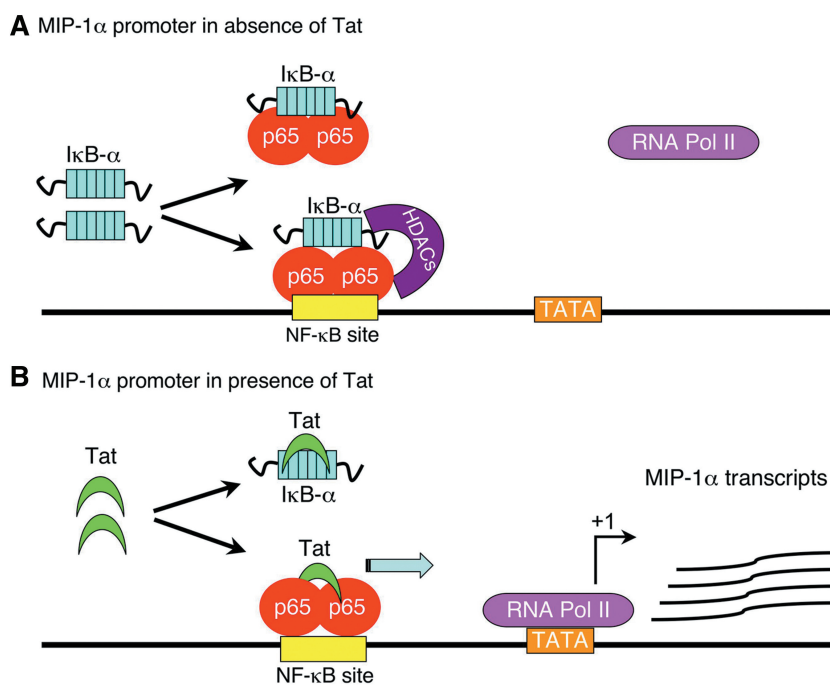
The Tat-dependent activation of NF- $\kappa$ B correlated with the association of the viral protein with I $\kappa$ B- $\alpha$ , which interfered with the generation of the I $\kappa$ B- $\alpha$ /p65 complex. This evidence demonstrated that Tat competed for the binding of I $\kappa$ B- $\alpha$  to p65 and prevented the I $\kappa$ B- $\alpha$  repression of p65. As additional mechanism of NF- $\kappa$ B activation, Tat associated with p65 and increased the p65 binding affinity to the NF- $\kappa$ B enhancer; this evidence was obtained with recombinant proteins indicating that the stronger binding of p65 to DNA was a consequence of direct association with Tat, which likely caused a conformational change of p65. We also demonstrated that the Tat cysteine-rich sequence (C22,25,27) was involved in the binding to the RHD of p65 and NF- $\kappa$ B activation. Differently, the Tat arginine-rich sequence (R49,52,53,55,56,57) was required for the binding to the sixth ankyrin of I $\kappa$ B- $\alpha$  (50,51), and for releasing p65 from the I $\kappa$ B- $\alpha$  inhibition. Altogether these results demonstrated that Tat abolished the negative feedback regulation of NF- $\kappa$ B by hijacking the I $\kappa$ B- $\alpha$  repressor, shielding p65 from the I $\kappa$ B- $\alpha$  embrace, and enforcing the p65 binding to DNA.

Further evidence of Tat activation of NF- $\kappa$ B showed that wild-type Tat, and not the Tat mutants lacking the arginine- or cysteine-rich domains, up-regulated *in vivo* the expression of a number of NF- $\kappa$ B-responsive genes, including *MIP-1 $\alpha$* , *CSF3*, *LTA*, *NFKB1A* and *TLR2*.

By ChIP analysis, we observed that Tat increased the recruitment of p65 to the NF- $\kappa$ B enhancers of the activated genes, and was recovered at the same sites. RNA interference of p65 abolished the Tat occupancy of the NF- $\kappa$ B enhancers indicating that the Tat binding to the NF- $\kappa$ B sites was mediated by p65. These results were consistent with the evidence that Tat displaced p65 from the binding to I $\kappa$ B- $\alpha$  (Figures 2D and 3C), and increased the p65 DNA binding affinity through association (Figure 4E and F). Thus, Tat likely increased the recruitment of p65 to the NF- $\kappa$ B-dependent promoters by associating with p65 and by interfering with the assembly of p65 with the repressor I $\kappa$ B- $\alpha$ .

Of interest, I $\kappa$ B- $\alpha$  was found associated to the NF- $\kappa$ B enhancers in absence of Tat, where it was displaced in presence of Tat. These results suggest a negative regulatory role of I $\kappa$ B- $\alpha$  in gene regulation through occupancy of specific promoters. Indeed, I $\kappa$ B- $\alpha$  was found associated with histone deacetylases at the *hes1* promoter, from where it was removed following TNF- $\alpha$  stimulation causing histone acetylation and *hes1* transcriptional activation (65). A similar mechanism of gene regulation could apply to the NF- $\kappa$ B-dependent genes analysed in this study, where Tat could activate the gene expression by removing I $\kappa$ B- $\alpha$  and promoting the p65 loading (Figure 7).

The physiological relevance of Tat cross talk with NF- $\kappa$ B was demonstrated in the HIV-1 infection of human monocytes, where HIV-1-encoded Tat protein counteracted post-activation turn off of NF- $\kappa$ B, as shown by: (i) sustained NF- $\kappa$ B activity by Tat in presence of newly synthesized I $\kappa$ B- $\alpha$ ; (ii) coimmunoprecipitation of Tat with I $\kappa$ B- $\alpha$  and p65; (iii) induction of



**Figure 7.** Model of *MIP-1 $\alpha$*  transcriptional activation by HIV-1 Tat. (A) In absence of Tat, I $\kappa$ B- $\alpha$  occupies the NF- $\kappa$ B enhancer of the *MIP-1 $\alpha$*  promoter and likely interacts with transcriptional repressors, such as HDACs (65), to inhibit gene transcription. (B) Tat activates the *MIP-1 $\alpha$*  expression by removing the I $\kappa$ B- $\alpha$  repressor from the NF- $\kappa$ B enhancer, and by increasing the binding of p65 NF- $\kappa$ B complex to the NF- $\kappa$ B enhancer.

*MIP-1 $\alpha$*  expression dependent on Tat and p65; (iv) Tat occupancy of the *MIP-1 $\alpha$*  NF- $\kappa$ B enhancer associated with increased recruitment of p65.

This study supports the pro-inflammatory action of Tat through physical interaction with p65 and I $\kappa$ B- $\alpha$ . Several inflammatory cytokines under the transcriptional control of NF- $\kappa$ B are hyper-expressed in HIV-1 infected individuals, including *IL-6* (66,67), *TNF- $\alpha$*  (68), and *MIP-1 $\alpha$*  (57–62,69–71). In the case of *TNF- $\alpha$*  and *IL-6*, Tat activated the cytokine expression by binding to the TAR-like stem loop of the 5' transcript, thus likely promoting the transcriptional elongation (33,37,47). Here, we have shown that the Tat-dependent transcriptional activation of a number of pro-inflammatory genes required the assembly of Tat with p65 at the NF- $\kappa$ B enhancer together with the displacement of I $\kappa$ B- $\alpha$  (Figure 5). Thus, in the set of distinct NF- $\kappa$ B-responsive genes, Tat acted as a component of the transcriptional initiation complex, which is consistent with previous reports on Tat promoting the transcription via DNA motifs (72,73).

Overproduction of pro-inflammatory cytokines is considered a major mechanism of immune deregulation (26) and neuron dysfunction in AIDS (74,75). In this scenario, inhibition of NF- $\kappa$ B activity could lead to a therapeutic strategy for counteracting the abnormal production of pro-inflammatory cytokines and chemokines in HIV-1 infection. NF- $\kappa$ B inhibitors, such as anti-oxidants, proteasome and IKK inhibitors, significantly reduced the HIV-1 replication and the associated inflammatory response (76,77); however, these NF- $\kappa$ B inhibitors acted indiscriminately in both HIV-1-infected and uninfected cells. The evidence that Tat activates NF- $\kappa$ B through direct interaction with I $\kappa$ B- $\alpha$  and p65 may lead to specific inhibitors to counteract the Tat pro-inflammatory action.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3 and Supplementary Materials and Methods.

## ACKNOWLEDGEMENTS

We thank the NIH AIDS Research & Reference Reagent Program for the plasmids pNL4-3.Luc.R- E- and pHXB2-env and the p24 antibody. We also thank Guido Poli for U937 cells.

## FUNDING

Istituto Superiore di Sanità-National Research Program on AIDS (Grant number 40C.77 to I.Q.); Ministero dell'Istruzione, dell'Università e della Ricerca (Grant number FIRB RBLA033WJX\_006 to I.Q.); EUROPRISE (Grant number LSHP-CT-2006-037611 to G.S.); FIRC fellowship (to A.R.). Funding for open access charge: EUROPRISE (grant number LSHP-CT-2006-037611).

*Conflict of interest statement.* None declared.

## REFERENCES

- Ghosh,S. and Hayden,M.S. (2008) New regulators of NF-kappaB in inflammation. *Nat. Rev. Immunol.*, **8**, 837–848.
- Vallabhapurapu,S. and Karin,M. (2009) Regulation and function of NF-kappaB transcription factors in the immune system. *Annu. Rev. Immunol.*, **27**, 693–733.
- Li,Q. and Verma,I.M. (2002) NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.*, **2**, 725–734.
- Haskill,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) Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell*, **65**, 1281–1289.
- Brown,K., Gerstberger,S., Carlson,L., Franzoso,G. and Siebenlist,U. (1995) Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science*, **267**, 1485–1488.
- Scherer,D.C., Brockman,J.A., Chen,Z., Maniatis,T. and Ballard,D.W. (1995) Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc. Natl Acad. Sci. USA*, **92**, 11259–11263.
- Zhong,H., Voll,R.E. and Ghosh,S. (1998) Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell.*, **1**, 661–671.
- Vermeulen,L., De Wilde,G., Van Damme,P., Vanden Berghe,W. and Haegeman,G. (2003) Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J.*, **22**, 1313–1324.
- Duran,A., Diaz-Meco,M.T. and Moscat,J. (2003) Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation. *EMBO J.*, **22**, 3910–3918.
- Sakurai,H., Chiba,H., Miyoshi,H., Sugita,T. and Toriumi,W. (1999) IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J. Biol. Chem.*, **274**, 30353–30356.
- O'Mahony,A.M., Montano,M., Van Beneden,K., Chen,L.F. and Greene,W.C. (2004) Human T-cell lymphotropic virus type 1 tax induction of biologically Active NF-kappaB requires IkappaB kinase-1-mediated phosphorylation of RelA/p65. *J. Biol. Chem.*, **279**, 18137–18145.
- Chen,L., Fischle,W., Verdin,E. and Greene,W.C. (2001) Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science*, **293**, 1653–1657.
- Chen,L.F., Mu,Y. and Greene,W.C. (2002) Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB. *EMBO J.*, **21**, 6539–6548.
- Krikos,A., Laherty,C.D. and Dixit,V.M. (1992) Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. *J. Biol. Chem.*, **267**, 2.
- Sun,S.C., Ganchi,P.A., Ballard,D.W. and Greene,W.C. (1993) NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science*, **259**, 1912–1915.
- Scott,M.L., Fujita,T., Liou,H.C., Nolan,G.P. and Baltimore,D. (1993) The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes Dev.*, **7**, 1266–1276.
- Hoffmann,A., Levchenko,A., Scott,M.L. and Baltimore,D. (2002) The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science*, **298**, 1241–1245.
- Nelson,D.E., Ihekwa,A.E., Elliott,M., Johnson,J.R., Gibney,C.A., Foreman,B.E., Nelson,G., See,V., Horton,C.A., Spiller,D.G. *et al.* (2004) Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science*, **306**, 704–708.
- Wertz,I.E., O'Rourke,K.M., Zhou,H., Eby,M., Aravind,L., Seshagiri,S., Wu,P., Wiesmann,C., Baker,R., Boone,D.L. *et al.* (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature*, **430**, 694–699.



20. Werner, S.L., Kearns, J.D., Zadorozhnaya, V., Lynch, C., O'Dea, E., Boldin, M.P., Ma, A., Baltimore, D. and Hoffmann, A. (2008) Encoding NF-kappaB temporal control in response to TNF: distinct roles for the negative regulators IkappaBalpha and A20. *Genes Dev.*, **22**, 2093–2101.
21. Kiernan, R., Bres, V., Ng, R.W., Coudart, M.P., El Messaoudi, S., Sardet, C., Jin, D.Y., Emiliani, S. and Benkirane, M. (2003) Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. *J. Biol. Chem.*, **278**, 2758–2766.
22. Yeung, F., Hoberg, J.E., Ramsey, C.S., Keller, M.D., Jones, D.R., Frye, R.A. and Mayo, M.W. (2004) Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.*, **23**, 2369–2380.
23. McElhinny, J.A., MacMorran, W.S., Bren, G.D., Ten, R.M., Israel, A. and Paya, C.V. (1995) Regulation of I kappa B alpha and p105 in monocytes and macrophages persistently infected with human immunodeficiency virus. *J. Virol.*, **69**, 1500–1509.
24. Hiscott, J., Kwon, H. and Genin, P. (2001) Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J. Clin. Invest.*, **107**, 143–151.
25. DeLuca, C., Roulston, A., Koromilas, A., Wainberg, M.A. and Hiscott, J. (1996) Chronic human immunodeficiency virus type 1 infection of myeloid cells disrupts the autoregulatory control of the NF-kappaB/Rel pathway via enhanced IkappaBalpha degradation. *J. Virol.*, **70**, 5183–5193.
26. Douek, D.C., Roederer, M. and Koup, R.A. (2009) Emerging concepts in the immunopathogenesis of AIDS. *Annu. Rev. Med.*, **60**, 471–484.
27. Kraft-Terry, S.D., Buch, S.J., Fox, H.S. and Gendelman, H.E. (2009) A coat of many colors: neuroimmune crosstalk in human immunodeficiency virus infection. *Neuron*, **64**, 133–145.
28. Broussard, S.R., Staprans, S.I., White, R., Whitehead, E.M., Feinberg, M.B. and Allan, J.S. (2001) Simian immunodeficiency virus replicates to high levels in naturally infected African green monkeys without inducing immunologic or neurologic disease. *J. Virol.*, **75**, 2262–2275.
29. Mandl, J.N., Barry, A.P., Vanderford, T.H., Kozyr, N., Chavan, R., Klucking, S., Barrat, F.J., Coffman, R.L., Staprans, S.I. and Feinberg, M.B. (2008) Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. *Nat. Med.*, **14**, 1077–1087.
30. Bossis, G., Salinas, S., Cartier, C., Devaux, C. and Briant, L. (2002) NF-kappaB activation upon interaction of HIV-1 envelope glycoproteins with cell surface CD4 involves IkappaB kinases. *FEBS Lett.*, **516**, 257–264.
31. Bren, G.D., Trushin, S.A., Whitman, J., Shepard, B. and Badley, A.D. (2009) HIV gp120 induces, NF-kappaB dependent, HIV replication that requires procaspase 8. *PLoS One*, **4**, e4875.
32. Berkhout, B., Silverman, R.H. and Jeang, K.T. (1989) Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell*, **59**, 273–282.
33. Buonaguro, L., Buonaguro, F.M., Giraldo, G. and Ensoli, B. (1994) The human immunodeficiency virus type 1 Tat protein transactivates tumor necrosis factor beta gene expression through a TAR-like structure. *J. Virol.*, **68**, 2677–2682.
34. Ambrosino, C., Ruocco, M.R., Chen, X., Mallardo, M., Baudi, F., Trematerra, S., Quinto, I., Venuta, S. and Scala, G. (1997) HIV-1 Tat induces the expression of the interleukin-6 (IL6) gene by binding to the IL6 leader RNA and by interacting with CAAT enhancer-binding protein beta (NF-IL6) transcription factors. *J. Biol. Chem.*, **272**, 14883–14892.
35. Jeang, K.T., Chun, R., Lin, N.H., Gagnon, A., Glabe, C.G. and Fan, H. (1993) In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor. *J. Virol.*, **67**, 6224–6233.
36. Kashanchi, F., Piras, G., Radonovich, M.F., Duvall, J.F., Fattaey, A., Chiang, C.M., Roeder, R.G. and Brady, J.N. (1994) Direct interaction of human TFIID with the HIV-1 transactivator tat. *Nature*, **367**, 295–299.
37. Ambrosino, C., Palmieri, C., Puca, A., Trimboli, F., Schiavone, M., Olimpico, F., Ruocco, M.R., di Leva, F., Toriello, M., Quinto, I. et al. (2002) Physical and functional interaction of HIV-1 Tat with E2F-4, a transcriptional regulator of mammalian cell cycle. *J. Biol. Chem.*, **277**, 31448–31458.
38. Abraham, S., Sweet, T., Sawaya, B.E., Rappaport, J., Khalili, K. and Amini, S. (2005) Cooperative interaction of C/EBP beta and Tat modulates MCP-1 gene transcription in astrocytes. *J. Neuroimmunol.*, **160**, 219–227.
39. Herrmann, C.H. and Rice, A.P. (1995) Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. *J. Virol.*, **69**, 1612–1620.
40. Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H. and Jones, K.A. (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell*, **92**, 451–462.
41. Marzio, G., Tyagi, M., Gutierrez, M.I. and Giacca, M. (1998) HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. *Proc. Natl Acad. Sci. USA*, **95**, 13519–13524.
42. Hottiger, M.O. and Nabel, G.J. (1998) Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein. *J. Virol.*, **72**, 8252–8256.
43. Benkirane, M., Chun, R.F., Xiao, H., Ogryzko, V.V., Howard, B.H., Nakatani, Y. and Jeang, K.T. (1998) Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. *J. Biol. Chem.*, **273**, 24898–24905.
44. Barillari, G., Sgadari, C., Fiorelli, V., Samanigo, F., Colombini, S., Manzari, V., Modesti, A., Nair, B.C., Cafaro, A., Sturzl, M. et al. (1999) The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the alpha5beta1 and alphavbeta3 integrins and by mobilizing sequestered basic fibroblast growth factor. *Blood*, **94**, 663–672.
45. Albin, A., Soldi, R., Giunciuglio, D., Giraudo, E., Benelli, R., Primo, L., Noonan, D., Salio, M., Camussi, G., Rockl, W. et al. (1996) The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nat. Med.*, **2**, 1371–1375.
46. Albin, A., Ferrini, S., Benelli, R., Sforzini, S., Giunciuglio, D., Aluigi, M.G., Proudfoot, A.E., Alouani, S., Wells, T.N., Mariani, G. et al. (1998) HIV-1 Tat protein mimicry of chemokines. *Proc. Natl Acad. Sci. USA*, **95**, 13153–13158.
47. Scala, G., Ruocco, M.R., Ambrosino, C., Mallardo, M., Giordano, V., Baldassarre, F., Dragonetti, E., Quinto, I. and Venuta, S. (1994) The expression of the interleukin 6 gene is induced by the human immunodeficiency virus 1 TAT protein. *J. Exp. Med.*, **179**, 961–971.
48. Demarchi, F., d'Adda di Fagagna, F., Falaschi, A. and Giacca, M. (1996) Activation of transcription factor NF-kappaB by the Tat protein of human immunodeficiency virus type 1. *J. Virol.*, **70**, 4427–4437.
49. Kwon, H.S., Brent, M.M., Getachew, R., Jayakumar, P., Chen, L.F., Schnolzer, M., McBurney, M.W., Marmorstein, R., Greene, W.C. and Ott, M. (2008) Human immunodeficiency virus type 1 Tat protein inhibits the SIRT1 deacetylase and induces T cell hyperactivation. *Cell Host Microbe*, **3**, 158–167.
50. Puca, A., Fiume, G., Palmieri, C., Trimboli, F., Olimpico, F., Scala, G. and Quinto, I. (2007) IkappaB-alpha represses the transcriptional activity of the HIV-1 Tat transactivator by promoting its nuclear export. *J. Biol. Chem.*, **282**, 37146–37157.
51. Vitagliano, L., Fiume, G., Scognamiglio, P.L., Doti, N., Cannavo, R., Puca, A., Pedone, C., Scala, G., Quinto, I. and Marasco, D. (2011) Structural and functional insights into IkappaB-alpha/HIV-1 Tat interaction. *Biochimie*, **93**, 1592–1600.
52. Hoffmann, A., Leung, T.H. and Baltimore, D. (2003) Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *EMBO J.*, **22**, 5530–5539.
53. Fiume, G., Rossi, A., Di Salle, E., Spatuzza, C., Mallardo, M., Scala, G. and Quinto, I. (2009) Computational analysis and in vivo validation of a microRNA encoded by the IBTK gene, a regulator of B-lymphocytes differentiation and survival. *Comput. Biol. Chem.*, **33**, 434–439.
54. Baldassarre, F., Mallardo, M., Mezza, E., Scala, G. and Quinto, I. (1995) Regulation of NF-kappa B through the nuclear processing of p105 (NF-kappa B1) in Epstein-Barr virus-immortalized B cell lines. *J. Biol. Chem.*, **270**, 31244–31248.

55. Palmieri, C., Trimboli, F., Puca, A., Fiume, G., Scala, G. and Quinto, I. (2004) Inhibition of HIV-1 replication in primary human monocytes by the IkappaB-alphaS32/36A repressor of NF-kappaB. *Retrovirology*, **1**, 45.
56. Maurer, M. and von Stebut, E. (2004) Macrophage inflammatory protein-1. *Int. J. Biochem. Cell. Biol.*, **36**, 1882–1886.
57. McManus, C.M., Weidenheim, K., Woodman, S.E., Nunez, J., Hesselgesser, J., Nath, A. and Berman, J.W. (2000) Chemokine and chemokine-receptor expression in human glial elements: induction by the HIV protein, Tat, and chemokine autoregulation. *Am. J. Pathol.*, **156**, 1441–1453.
58. Li, Q., Smith, A.J., Schacker, T.W., Carlis, J.V., Duan, L., Reilly, C.S. and Haase, A.T. (2009) Microarray analysis of lymphatic tissue reveals stage-specific, gene expression signatures in HIV-1 infection. *J. Immunol.*, **183**, 1975–1982.
59. Schmidt-mayerova, H., Nottet, H.S., Nuovo, G., Raabe, T., Flanagan, C.R., Dubrovsky, L., Gendelman, H.E., Cerami, A., Bukrinsky, M. and Sherry, B. (1996) Human immunodeficiency virus type 1 infection alters chemokine beta peptide expression in human monocytes: implications for recruitment of leukocytes into brain and lymph nodes. *Proc. Natl Acad. Sci. USA*, **93**, 700–704.
60. Choe, W., Volsky, D.J. and Potash, M.J. (2001) Induction of rapid and extensive beta-chemokine synthesis in macrophages by human immunodeficiency virus type 1 and gp120, independently of their coreceptor phenotype. *J. Virol.*, **75**, 10738–10745.
61. Sheng, W.S., Hu, S., Hegg, C.C., Thayer, S.A. and Peterson, P.K. (2000) Activation of human microglial cells by HIV-1 gp41 and Tat proteins. *Clin. Immunol.*, **96**, 243–251.
62. Hahn, Y.K., Vo, P., Fitting, S., Block, M.L., Hauser, K.F. and Knapp, P.E. (2010) beta-Chemokine production by neural and glial progenitor cells is enhanced by HIV-1 Tat: effects on microglial migration. *J. Neurochem.*, **114**, 97–109.
63. Liu, J., Perkins, N.D., Schmid, R.M. and Nabel, G.J. (1992) Specific NF-kappa B subunits act in concert with Tat to stimulate human immunodeficiency virus type 1 transcription. *J. Virol.*, **66**, 3883–3887.
64. Kelly, G.D., Morris, C.B. and Offermann, M.K. (1999) Lack of responsiveness of a nuclear factor-kappaB-regulated promoter to transactivation by human immunodeficiency virus 1 Tat in HeLa cells. *Virology*, **263**, 128–138.
65. Aguilera, C., Hoya-Arias, R., Haegeman, G., Espinosa, L. and Bigas, A. (2004) Recruitment of IkappaBalpha to the hes1 promoter is associated with transcriptional repression. *Proc. Natl Acad. Sci. USA*, **101**, 16537–16542.
66. Birx, D.L., Redfield, R.R., Tencer, K., Fowler, A., Burke, D.S. and Tosato, G. (1990) Induction of interleukin-6 during human immunodeficiency virus infection. *Blood*, **76**, 2303–2310.
67. Lafeuillade, A., Poizot-Martin, I., Quilichini, R., Gastaut, J.A., Kaplanski, S., Farnarier, C., Mege, J.L. and Bongrand, P. (1991) Increased interleukin-6 production is associated with disease progression in HIV infection. *Aids*, **5**, 1139–1140.
68. Emilie, D., Peuchmaur, M., Maillot, M.C., Crevon, M.C., Brousse, N., Delfraissy, J.F., Dormont, J. and Galanaud, P. (1990) Production of interleukins in human immunodeficiency virus-1-replicating lymph nodes. *J. Clin. Invest.*, **86**, 148–159.
69. Canque, B., Rosenzweig, M., Gey, A., Tartour, E., Fridman, W.H. and Gluckman, J.C. (1996) Macrophage inflammatory protein-1alpha is induced by human immunodeficiency virus infection of monocyte-derived macrophages. *Blood*, **87**, 2011–2019.
70. Tedla, N., Palladinetti, P., Kelly, M., Kumar, R.K., DiGirolamo, N., Chattopadhyay, U., Cooke, B., Truskett, P., Dwyer, J., Wakefield, D. et al. (1996) Chemokines and T lymphocyte recruitment to lymph nodes in HIV infection. *Am. J. Pathol.*, **148**, 1367–1373.
71. Cotter, R.L., Zheng, J., Che, M., Niemann, D., Liu, Y., He, J., Thomas, E. and Gendelman, H.E. (2001) Regulation of human immunodeficiency virus type 1 infection, beta-chemokine production, and CCR5 expression in CD40L-stimulated macrophages: immune control of viral entry. *J. Virol.*, **75**, 4308–4320.
72. Berkhout, B. and Jeang, K.T. (1992) Functional roles for the TATA promoter and enhancers in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. *J. Virol.*, **66**, 139–149.
73. Brady, J. and Kashanchi, F. (2005) Tat gets the “green” light on transcription initiation. *Retrovirology*, **2**, 69.
74. Bonwetsch, R., Croul, S., Richardson, M.W., Lorenzana, C., Del Valle, L., Sverstiuk, A.E., Amini, S., Morgello, S., Khalili, K. and Rappaport, J. (1999) Role of HIV-1 Tat and CC chemokine MIP-1alpha in the pathogenesis of HIV associated central nervous system disorders. *J. Neurovirol.*, **5**, 685–694.
75. King, J.E., Eugenin, E.A., Buckner, C.M. and Berman, J.W. (2006) HIV tat and neurotoxicity. *Microbes Infect.*, **8**, 1347–1357.
76. Mingyan, Y., Xinyong, L. and De Clercq, E. (2009) NF-kappaB: the inducible factors of HIV-1 transcription and their inhibitors. *Mini. Rev. Med. Chem.*, **9**, 60–69.
77. Yu, L., Mohanram, V., Simonson, O.E., Smith, C.I., Spetz, A.L. and Mohamed, A.J. (2009) Proteasome inhibitors block HIV-1 replication by affecting both cellular and viral targets. *Biochem. Biophys. Res. Commun.*, **385**, 100–105.