Tat Acetyl-acceptor Lysines Are Important for Human Immunodeficiency Virus Type-1 Replication*

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The human immunodeficiency virus type-1 trans-activator Tat is a transcription factor that activates the HIV-1 promoter through binding to the trans-activation-responsive region (TAR) localized at the 5'-end of all viral transcripts. We and others have recently shown that Tat is directly acetylated at lysine 28, within the activation domain, and lysine 50, in the TAR RNA binding domain, by Tat-associated histone acetyltransferases p300/CBP-associated factor, and hGCN5. Here, we show that mutation of acetyl-acceptor lysines to arginine or glutamine affects virus replication. Interestingly, mutation of lysine 28 and lysine 50 differentially affected Tat trans-activation of integrated versus nonintegrated long terminal repeat. Our results highlight the importance of lysine 28 and lysine 50 of Tat in virus replication and Tat-mediated trans-activation.

Human immunodeficiency virus type-1 (HIV-1)† Tat is a regulatory protein encoded by two exons localized on either side of the env gene. Tat is a multifunctional protein, absolutely required for virus replication and AIDS progression. Besides its primary function as the viral transcription factor, Tat has been proposed to be required for efficient reverse transcription (1). Tat is secreted from infected cells (2, 3), whereupon it binds to neighboring cells through electrostatic interactions, chemokine receptors (4), or cell surface integrins (5). Extracellular Tat is a cellular toxin that increases the efficiency of virus dissemination and reduces antiviral immunity to promote HIV-1 disease (6). Cells treated with Tat show increased expression of chemokine receptors (7, 8), decreased proliferation (9, 10), and apoptosis of bystander cells (11, 12). Furthermore, Tat has been shown to have chemokine-like properties that may serve to recruit chemokine receptor-expressing monocytes/macrophages toward HIV-producing cells and facilitate infection (3–5). Finally, extracellular HIV-1 Tat protein has been shown to selectively inhibit the entry and replication of T-cell tropic X4, but not macrophage-tropic R5, virus in peripheral blood mononuclear cells, which has been proposed as a mechanism to select against X4 viruses, thereby influencing the early course of HIV-1 disease (13).

The primary function attributed to Tat is its role in HIV-1 promoter activation. Tat is an atypical transcriptional activator that functions through binding, not to DNA, but to a short leader RNA, trans-activation responsive region (TAR) localized at the 5’ termini of all viral transcripts (14–16). Interaction between Tat and TAR is necessary for HIV-1 transcription both in vivo (17, 18) and in vitro (19, 20). Tat transcriptional activity on the HIV-1 promoter is tightly regulated by cellular factors (reviewed in Ref. 21): Tat-associated kinases (22–24) and Tat-associated histone acetyltransferases (25–28). Tat-associated kinase was identified as the kinase subunit of the positive transcription elongation factor b (29–33). Positive transcription elongation factor b is composed of a regulatory subunit, cyclin T1, and a catalytic subunit, CDK9, which phosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II (32, 34). Hyperphosphorylation of the RNA polymerase II carboxyl-terminal domain leads to productive elongation of transcription (24, 32, 33–37). Tat interacts with cyclin T1 to recruit positive transcription elongation factor b to the HIV-1 TAR element and to stimulate elongation of transcripts originating from the viral long terminal repeat (LTR) (32, 33). The other class of Tat co-activators, Tat-associated histone acetyltransferases, are composed of p300/CBP, p300/CBP-associating factor (PCAF) (25–27), and hGCN5 (28). Tat-associated histone acetyltransferases induce the activation of chromatinized HIV-1 LTRs (26, 27), presumably through acetylation of histones. Tat may also use the cellular acetylation pathway to control the expression of various cellular genes (38, 39). We and others have recently shown that Tat-associated histone acetyltransferases also directly acetylate the Tat protein in two different domains. Whereas p300 and hGCN5 acetylate lysine 50 within the RNA binding domain (28, 40–42), PCAF acetylates lysine 28 in the activation domain (40). Thus, this novel post-translational modification of Tat was found to govern two essential interactions necessary for HIV-1 transcription: binding of Tat to TAR and to positive transcription elongation factor b.

In the present work, we analyzed the role of Tat acetyl-acceptor lysines in virus replication. We show that mutation of lysine 28 and lysine 50 to either arginine or glutamine severely affected the replication of HIV-1 in a T-cell line. Additionally, the effect of Lys28 and Lys50 mutation on Tat trans-activation was dependent on the promoter context (integrated versus nonintegrated LTR). Our results highlight the importance of lysine 28 and lysine 50 of Tat in virus replication and the mechanism of Tat-mediated trans-activation.

EXPERIMENTAL PROCEDURES
Plasmid Constructs—pLTR-luc wild-type has been described (40). A FLAG sequence was introduced in the COOH terminus of pTat wild type, which was used as a template for mutagenesis. pTat-K28R, pTat-
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K28Q, pTat-K29R, pTat-K50R, and pTat-K50Q were constructed by the site-directed mutagenesis method using the QuikChange kit (Stratagene). Mutated clones were fully sequenced after identification. All proviral constructions were derived from the pNL4-3 infectious molecular clone (45). pNL4-3 Tat(-) was generated by introducing two consecutive stop codons at amino acid residues 28 and 50 into the plasmid pNL4-3. The plasmid designated pNLT(-) was inserted into the nef gene of pNLT(-) (44, 45). The resultant molecular genomes were designated pNLT, pNLT-K28Q, pNLT-K28R, pNLT-K29R, pNLT-K50Q, and pNLT-K50R.

**Transfection and Infection—**CEM cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and antibiotics. 293, HeLa, and HeLa P4 cells, that contain the lacZ gene under control of the integrated HIV-1 LTR (46), were propagated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected using calcium phosphate as described (40). Virus stocks were produced by transfection of 293 cells. Transfected cell supernatants were harvested at 48 h post-transfection and passed through 0.45-μm pore size filters. Viruses, normalized for reverse transcriptase (RT) activity, were used to infect CEM and HeLa P4 cells. Briefly, cells were incubated with virus for 2 h at 37°C, washed, and resuspended in medium. Virus production was monitored by RT assay of culture supernatants every 3 days.

**RT and Reporter Assays—**To measure RT activity, 10 μl of cell culture supernatants was mixed with 25 μl of RT buffer (60 mM Tris-HCl (pH 8), 75 mM KCl, 5 mM MgCl2, 0.1% IGEPAL CA-630, 1.04 mM EDTA, 5 μg/ml poly(A), 0.16 μg/ml oligo(dT), 40 mM dithiothreitol, and 10 μCi/ml [α-32P]dTTP (Amersham Biosciences)). The reactions were incubated for 2 h at 37°C, and 10 μl was spotted onto a DEAE filter, washed three times in 2× SSC, dried, and quantified using an Instant Imager (Packard). To assay luciferase activity, transfected HeLa cells were lysed and assayed for luciferase activity 48 h post-transfection, according to the manufacturer's protocol (Promega). β-galactosidase activity was measured in extracts of HeLa P4 cells 48 h post-transfection or 24 h postinfection, according to the manufacturer's protocol (Roche Molecular Biochemicals).

**In Vitro TAR/Tat Binding Assay—**Wild-type and mutant Tat proteins were translated in vitro in a coupled transcription-translation rabbit reticulocyte lysate system (Promega) in the presence of [35S]methionine according to the manufacturer's protocol. Synthetic biotinylated TAR RNA (2 μg) was immobilized on streptavidin-agarose beads and incubated with translated proteins for 2 h at 4°C. The complex was then washed, resolved by SDS-PAGE, and analyzed by autoradiography.

**Immunological Techniques and Western Blot Analysis—**HeLa cells were transfected with the indicated plasmids. At 24 h post-transfection, cells were washed twice in phosphate-buffered saline and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 8), 120 mM NaCl, 5 mM EDTA, 0.5% IGEPAL CA-630, 1 mM dithiothreitol, and protease inhibitor mixture). The cell lysates were clarified by centrifugation at 15,300 × g for 10 min at room temperature. The supernatants were subjected to immunoprecipitation with the indicated antibody following a preclarification step. Immunoprecipitates were then washed three times with lysis buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane using semidry blotting (Bio-Rad). Membranes were incubated with the primary antibody overnight at 4°C, washed, and incubated for 1 h with the appropriate secondary antibody (Amersham Pharmacia Biotech). Proteins were visualized by chemiluminescence (Amersham Biosciences) according to the manufacturer's protocol. For immunofluorescence, HeLa cells were transfected with plasmids expressing FLAG-tagged Tat wild type or mutants. Cells were fixed 24 h after transfection with 4% paraformaldehyde for 10 min at room temperature. The cells were then washed and permeabilized in 1× phosphate-buffered saline containing 5% fetal calf serum and 0.1% Triton X-100 for 10 min at room temperature. Cells were stained with anti-FLAG antibody followed by incubation with Texas Red-conjugated antibody.

**RESULTS**

**Mutation of Tat Acetyl-acceptor Lysines Affects HIV-1 Replication—**HIV-1 Tat is essential for virus replication and is a potent trans-activator of viral gene expression (20). We have recently shown that Tat lysines 28, within the activation domain, and lysine 50, within the RNA binding domain, are targeted for acetylation by PCAF and p300, respectively. Mutation of acetyl-acceptor residues Lys28 and Lys50 to alanine reduces Tat-mediated trans-activation of the HIV-1 promoter in transient transfection assays (40). Previous analysis has shown a discordance between residues that are important for virus replication and those important for trans-activation of a transiently transfected reporter gene under the control of the HIV-1 LTR (47). Thus, we investigated the role of Tat acetyl-acceptor lysines in HIV-1 replication. Lysine 28 and lysine 50 were mutated to either arginine or glutamine. As a control, lysine 29 that is not acetylated was also mutated to arginine. Using a previously described strategy (45), Tat wild-type or mutants were introduced in the nef frame of pNL4-3 Tat(-) in which the tat gene had been inactivated by engineering two consecutive stop codons at amino acids 11 and 12 (Fig. 1A). The different constructs were used to transf ect 293 cells, which express the adeno viral proteins E1A and E1B that strongly activate HIV-1 LTR and complement the defect in gene expression in viruses lacking Tat (45). Biochemical analysis of the resultant virions was performed. Fig. 1B shows that expression of viral proteins from recombinant genomes encoding wild-type or mutant Tat was identical. Furthermore, FLAG-tagged wild-type and mutant Tat proteins were readily detected in cells transfected with each of the respective viral genomes (Fig. 1C). The reverse transcriptase activity/β24 ratio was identical for all the molecular clones engineered (Fig. 1D). Thus, the engineered recombinant pNL4-3 molecular clones are competent for expression of the HIV-1 structural proteins with a normal RT/β24 ratio when transfected into 293 cells.

We then analyzed the replication of recombinant pNLT(-) encoding wild-type or mutant Tat in a T-cell line. Viruses, produced from 293 cells transfected with pNLT or pNLN mutants, were normalized for RT activity and used to infect CEM cells. Infected cells were monitored for virus replication by measuring supernatant RT activity every 2 or 3 days over a period of 21 days. Tat wild-type and Tat K29R viruses showed the same replication kinetics with an RT peak at day 13 postinfection (Fig. 1E). However, the mutations K28R, K28Q, and K50Q imposed significant replication defects. Tat K50R virus showed a 4-day delay to peak virus production when compared with the recombinant Tat wild-type virus. These results show that the acetyl-acceptor lysines within Tat play an important role in virus replication.

**Effect of Tat Acetyl-acceptor Lysines on Tat-mediated Activation of Integrated and Nonintegrated LTR—**To assess how mutation of Tat acetyl-acceptor lysines (Lys28 and Lys50) affects virus replication, we analyzed their effect on Tat-mediated trans-activation of an integrated and nonintegrated HIV-1 LTR. HeLa P4 cells that contain the lacZ gene under control of the integrated HIV-1 LTR (46) were infected with recombinant pNLT viruses encoding either Tat wild-type or mutants. β-Galactosidase activity was monitored 24 h after infection. Fig. 2A shows that transcriptional activity of Tat K50Q, Tat K28R, and Tat K28Q on the integrated LTR was severely reduced. Tat K50R transcriptional activity was slightly (1.6-fold) higher than the wild type. Similar results were obtained when the Tat-expressing constructs were transfected into HeLa P4 cells (data not shown). Taken together, these results show a correlation between the lack of transcriptional activity of Tat K28R, K28Q, and Tat K50Q on an integrated LTR and their inability to support virus replication. In contrast, the Tat K50R mutant, which showed delayed replication kinetics, was found to have slightly enhanced trans-activation.

We then analyzed the transcriptional activity of Tat wild-type and mutants in transient transfection assays. HeLa cells were transfected with an LTR luciferase reporter gene either alone or with Tat expression plasmids as indicated (Fig. 2B).
The transcriptional activity of Tat K50Q and Tat K28Q was reduced (3- and 6.5-fold, respectively), while that of Tat K50R and Tat K29R was comparable with Tat wild type. Interestingly, the transcriptional activity of Tat K28R, which was reduced by 5-fold on an integrated LTR, was comparable with that of wild-type in transient transfection assays (1.35-fold reduction). As previously shown, the transcriptional activity of the Tat K41A was significantly reduced (22, 23). Taken together, the experiments shown in Fig. 2 suggest that the effect of Tat acetyl-acceptor lysines on Tat-mediated transactivation is dependent on the promoter context. These experiments furthermore suggest that activation of an integrated versus nonintegrated LTR is not simply a function of the number of acceptor lysines in Tat. Although Tat K41A was significantly reduced in transfection assays, this result was not confirmed by experiments using integrated LTRs.

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integrated HIV-1 LTR by Tat may involve different mechanisms.

Tat trans-activation of the HIV-1 LTR minimally requires TAR, positive transcription elongation factor b, and Tat-associated histone acetyltransferases. Thus, we analyzed the effect of Tat acetyl-acceptor mutants on the interaction between Tat and TAR, cyclin T1, and PCAF. Fig. 3A shows that Tat wild type, Tat K28Q, Tat K28R, Tat K29R, and Tat K50R interact with TAR RNA. However, a weak interaction was observed between Tat K50Q and TAR RNA. This finding suggests that the reduced trans-activation function of Tat K50Q is probably due to a loss of its interaction with TAR RNA.

Because the positive transcription elongation factor b complex is required for Tat trans-activation, we investigated the role of lysine 28 and 50 in the interaction of Tat with cyclin T1. HeLa cells were transfected with FLAG-tagged Tat wild type or mutants. 24 h after transfection, cell extracts were prepared and subjected to immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were resolved on SDS-PAGE, and the presence of cyclin T1 was analyzed by immunoblotting using anti-cyclin T1 antibody. Fig. 3B shows that Tat wild-type and mutants were able to interact with cyclin T1. Tat K28R interaction with cyclin T1 was less efficient than Tat wild type (compare lanes 2 and 7). As previously reported, the Tat K41A mutant failed to interact with cyclin T1 (lane 3). We then analyzed the effect of human cyclin T1 on Tat-mediated trans-activation of the LTR in NIH 3T3 cells. As shown in Fig. 3C, human cyclin T1 enhanced Tat trans-activation by 3.4-fold. Although Tat K28Q and Tat K50Q were able to interact with cyclin T1, no synergistic activation of the LTR was observed. Human cyclin T1 enhanced Tat K28R and Tat K50R trans-activation by 2.3- and 1.9-fold, respectively. As expected, human cyclin T1 had no effect on Tat K41A transcriptional activity. These results suggest that binding of Tat to cyclin T1 is required but not sufficient for optimal trans-activation of the HIV-1 LTR.

Finally, because PCAF is known to assist Tat in activation of an integrated LTR (27), we analyzed the effect of Tat acetyl-acceptor lysines on the interaction between Tat and PCAF. Co-immunoprecipitation analysis showed that Tat wild type, Tat K50R, Tat K50Q, Tat K28Q, and Tat K29R were able to immunoprecipitate PCAF (Fig. 3D). However, Tat K41A and Tat K28R interacted weakly with PCAF. The expression level of Tat wild type and mutants is shown in the lower panel (Fig. 3D). This result may explain why Tat K28R is competent for activating the LTR in a transient transfection assay (Fig. 2B) but activates the integrated LTR poorly (Fig. 2A). Because Tat K28Q and Tat K50Q are transcriptionally incompetent yet bind to cyclin T1 and PCAF, we asked whether...
these mutants, by sequestering cyclin T1 and/or PCAF, are able to compete with Tat wild-type for LTR trans-activation. Thus, HeLa P4 cells were transfected with either Tat wild type alone or together with Tat mutants, and β-galactosidase activity was measured 24 h after transfection. As shown in Fig. 4, Tat K28Q, Tat K28R, and Tat K50Q reduced Tat wild-type transcriptional activity by 60, 40, and 70%, respectively. Tat K50R, Tat K29R, and Tat K41A showed no trans-dominant effect. Taken together, these experiments highlight the importance of cyclin T1 and PCAF in Tat-mediated trans-activation of the HIV-1 LTR.

**Mutation of Tat Acetyl-acceptor Lysines Affects Its Subcellular Localization**—Tat acetyl-acceptor lysines are localized to the activation domain (Lys28) that mediates the interaction between Tat and its co-activators and the RNA binding domain (Lys50) that also serves as a nuclear localization signal. Thus, we analyzed the effect of Lys50 and Lys28 mutations on the subcellular localization of Tat by immunofluorescence (Fig. 5). HeLa cells were transfected with either FLAG-tagged Tat wild type or mutants as indicated, and cells were stained with anti-FLAG antibody. Tat wild type showed a characteristic pattern consisting of diffuse nucleoplasmic fluorescence with intense nucleolar staining in 78% of transfected cells as observed previously (48, 49), whereas only 22% of transfected cells showed both nuclear and cytoplasmic staining. Tat K50Q was found to localize to both cytoplasm and nucleus in 90% of transfected cells. Tat K50R localized exclusively in the nucleus in 93% of transfected cells. Thus, the positive charge of Lys50 plays an important role in dictating Tat localization. Tat K29R showed a similar staining pattern to Tat wild-type. Mutation of Lys28 to glutamine increased the number of cells that showed both nuclear and cytoplasmic staining of Tat to 44%. Interestingly, Tat K28R, Tat K28Q, and Tat K41A showed a perinuclear, instead of diffuse nucleolar, localization as seen for the Tat wild type, Tat K50R, and Tat K29R. These results suggest that Lys41 and acetyl-acceptors Lys28 and Lys50 of Tat influence its subcellular localization.

**DISCUSSION**

The HIV-1 trans-activator Tat is absolutely required for virus replication and plays a critical role in AIDS pathogenesis. In this report, we have analyzed the role of acetyl-acceptors Lys28 and Lys50 in virus replication and Tat transcriptional activity. Thus, lysines 28 and 50 were mutated to either arginine (to conserve the positive charge) or glutamine (to neutralize the positive charge). By introducing these mutations in the pNL4-3 Tat minus background, using a previously described strategy (45, 47), we show that acetyl-acceptor Lys28 and Lys50...
play a critical role in virus replication. Mutation of Lys\textsuperscript{28} or Lys\textsuperscript{50} to glutamine results in replication-incompetent virus and transcriptionally inactive Tat on integrated and nonintegrated LTR. Interestingly, mutation of lysine 50 to arginine did not affect Tat-mediated trans-activation of either integrated or nonintegrated LTR but led to a 4-day delay in virus replication. Mutation of lysine 28 to arginine affected virus replication and activation of an integrated LTR without affecting LTR trans-activation in a transient transfection assay.

To investigate how mutation of Tat acetyl-acceptor lysines affects Tat transcriptional activity, we analyzed the effect of these mutations on Tat/TAR, Tat/cyclin T1, and Tat/PCAF interactions. Consistent with our previous report (40), mutation of lysine 50 to glutamine reduced the ability of Tat to bind TAR RNA without affecting its interaction with cyclin T1 or PCAF. Moreover, Tat K50Q has an altered cellular localization with nuclear and cytoplasmic staining in 90% of transfected cells. Thus, the lack of binding of Tat K50Q to TAR RNA and its abnormal cellular localization probably contribute to its inability to support virus replication and trans-activation of the LTR.

Tat K28R is able to interact with TAR and cyclin T1 but failed to interact with PCAF, one of the histone acetyltransferases that has been shown to assist Tat-mediated trans-activation of integrated LTR (27). Previously, it has been shown that the two-exon form of Tat activates an integrated LTR more efficiently than the Tat one-exon form (50). In the context of chromatin, Tat also has to overcome the chromatin repression exerted by the nucleosomal architecture of the integrated provirus (51, 52). In this respect, Tat has been shown to disrupt the repressive nucleosome 1 (nuc1) to activate the transcription from integrated LTR (27). Previously, it has been shown that mutation of Lys 28 or Lys 50 to arginine did not affect Tat-mediated transcription, whereas mutation of Lys 28 to glutamine affected virus replication and trans-activation of the LTR. Tat K28R activates both integrated and nonintegrated LTRs, while Tat K50Q showed an altered subcellular localization with 44% of transfected cells stained in both cytoplasm and nucleus. The inability of Tat K28R to activate the LTR and consequently to support virus replication may be explained, at least in part, by its altered subcellular localization. Additionally, K28Q mutation may affect the interaction between Tat and other cellular factors involved in trans-activation of the LTR and virus replication.

Mutation of Tat Lys\textsuperscript{28} to glutamine had no effect on Tat/TAR, Tat/cyclin T1, and Tat/PCAF interaction. Despite this, Tat K28Q was transcriptionally inactive on both integrated and nonintegrated LTR. Tat K28R showed an altered subcellular localization with 44% of transfected cells stained in both cytoplasm and nucleus. The inability of Tat K28R to activate the LTR and consequently to support virus replication may be explained, at least in part, by its altered subcellular localization. Additionally, K28Q mutation may affect the interaction between Tat and other cellular factors involved in trans-activation of the LTR and virus replication. Consistent with this hypothesis, only 7% of transfected cells showed both cytoplasmic and nuclear localization of Tat K50R compared with 22% of Tat wild type-transfected cells, suggesting a defect in its secretion. Taken together, these results suggest that the transcriptional activity of Tat is necessary but not sufficient to support virus replication.

Accumulating evidence suggests that Tat may also be an important virulence factor in vivo. Vaccination of nonhuman primates with Tat, either alone or in combination with other viral products, reduces virus replication, with lesions virus replication (6, 57–61). Whereas native Tat protein is cytotoxic, a modified Tat protein is considered an attractive target for HIV vaccine development. Thus, identification of Tat mutants able to compete with and inhibit wild-type Tat function will help to engineer the optimal Tat protein vaccine candidate.

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