Nuclear Targeting of Protein Phosphatase-1 by HIV-1 Tat Protein*

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Transcription of human immunodeficiency virus (HIV)-1 genes is activated by HIV-1 Tat protein, which induces phosphorylation of the C-terminal domain of RNA polymerase-II by CDK9/cyclin T1. We previously showed that Tat-induced HIV-1 transcription is regulated by protein phosphatase-1 (PP1). In the present study we demonstrate that Tat interacts with PP1 and that disruption of this interaction prevents induction of HIV-1 transcription. We show that PP1 interacts with Tat in part through the binding of Val\(^{36}\) and Phe\(^{38}\) of Tat to PP1 and that Tat is involved in the nuclear and subnuclear targeting of PP1. The PP1 binding mutant Tat-V36A/F38A displayed a decreased affinity for PP1 and was a poor activator of HIV-1 transcription. Surprisingly, Tat-Q35R mutant that had a higher affinity for PP1 was also a poor activator of HIV-1 transcription, because strong PP1 binding competed out binding of Tat to CDK9/cyclin T1. Our results suggest that Tat might function as a nuclear regulator of PP1 and that interaction of Tat with PP1 is critical for activation of HIV-1 transcription by Tat.

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that encodes transcriptional activator (Tat) protein. The activation domain of Tat (amino acids 1–48) interacts with host cell factors, whereas the positively charged RNA-binding domain (amino acids 49–57) interacts with HIV-1 transactivation response (TAR) RNA (1). In cell-free transcription assays Tat induces elongation of transcription (2, 3). In vivo, Tat also induces initiation of transcription from the integrated HIV-1 promoter (4–6). In a recent study Tat was shown to stimulate formation of a transcription complex containing TATA box-binding protein but not TATA box-binding protein-associated factors, thus indicating that Tat may enhance initiation of transcription (4). This finding apparently agrees with the early observation that Tat binds directly to the TATA box-binding protein-containing basal transcription factor TFIIID (7). Tat activates HIV-1 transcription by recruiting transcriptional co-activators that include positive transcription elongation factor b, containing CDK9/cyclin T1, an RNA polymerase II CTD kinase (3, 8, 9) and histone acetyltransferases (10–12). Whereas positive transcription elongation factor b induces HIV-1 transcription from non-integrated HIV-1 template (3, 8, 9), histone acetyltransferases allow induction of integrated HIV-1 provirus (10–12). In contrast to the well defined role of protein kinases, the role of protein phosphatases in Tat-mediated HIV-1 transcription is less well understood. FCP1, a CTD phosphatase that dephosphorylates Ser-2 during elongation of transcription (13), is inhibited by Tat and this inhibition may alleviate FCP1-mediated pausing of transcription (14, 15). In addition to FCP1, PP2A and PP1 were also shown to be involved in the regulation of HIV-1 transcription. Disregulation of cellular enzymatic activity of PP2A inhibited Tat-induced HIV-1 transcription (16). Expression of the catalytic subunit of PP2A enhanced activation of the HIV-1 promoter by phorbol myristate acetate, whereas inhibition of PP2A by okadaic acid and fostriecin prevented activation of the HIV-1 promoter (17). Thus, PP2A might positively regulate HIV-1 transcription. Our studies showed that PP1 is also a positive regulator of HIV-1 transcription in vitro (18, 19) and in vivo (20). Indeed, in a previous study we showed that inhibition of nuclear PP1 potently inhibited Tat-induced transcription (20). We concluded that the inhibition was because of sequestration of PP1 because the inhibition was alleviated by the co-expression of PP1 (20). We recently compared contributions of PP2A and PP1 to the dephosphorylation of CDK9 and to HIV-1 transcription in vitro and in vivo and found that although PP2A dephosphorylates CDK9 in vitro, in cultured cells PP1 is likely to dephosphorylate CDK9 and contribute to the regulation of HIV-1 transcription (21). PP1 belongs to the PPP family of serine-threonine protein phosphatases, which also includes PP2A, PP2B, and PP4–6 (22). PP1 is expressed in mammalian cells as three isoforms, α, β, δ, and γ1 (γ), and PP1γ shows a strong accumulation in the nucleolus, suggesting a role in the regulation of nucleolar processes including transcription (23). PP1 holoenzymes consist of a constant catalytic subunit and a variable regulatory subunit (R) that determines the localization, activity, and substrate specificity of the phosphatase (22, 24). Based on their effect on the activity of PP1, R subunits are divided into two major groups: (i) PP1 inhibitors, which block the activity of PP1 toward all substrates; and (ii) substrate specificities, which inhibit dephosphorylation of some substrates but enhance the dephosphorylation of other substrates (24). One of the nuclear R-subunits of PP1 is NIPP1 (nuclear inhibitor of PP1), which inhibits the dephosphorylation of a wide range of PP1 substrates including glycogen phosphorylase a (24). Tat-mediated HIV-1 transcription is blocked by inhibition of the nuclear pool of protein phosphatase-1 by HIV-1 Tat protein.
PP1, and the inhibition is reversed by co-expression of PP1 γ (20). In the present paper we show that Tat binds to PP1 in vitro and in vivo. We analyzed the interaction of Tat with PP1 using enzymatic assays. We identified the Val<sup>19</sup> and Phe<sup>38</sup> amino acids of Tat to mediate its binding to PP1. The V36A/F38A mutation of Tat prevented its binding to PP1 in vitro and in vivo. Moreover, Tat-V36A/F38A was inefficient in induction of HIV-1 transcription in cultured cells, and also did not translocate PP1 to the nucleus, as we observed for wild type Tat. Our results suggest that Tat might have a yet unrecognized function as a regulator of PP1, and that interaction of Tat with PP1 is critical for activation of HIV-1 transcription by Tat.

**EXPERIMENTAL PROCEDURES**

HEK293T and HeLa cells were purchased from ATCC (Manassas, VA). PP1 was purified from rabbit skeletal muscle as described in Ref. 25. Phosphorylase <i>b</i> was from Calzyme Laboratories (San Luis Obispo, CA). Peptides with Tat-derived sequences were chemically synthesized by Peptide Technologies (Gaithersburg, MD). Anti-Tat rabbit polyclonal antibodies and HIV-1 Clade B consensus Tat (15-mers) peptides, with 11-amino acid overlaps were received from the AIDS Research and Reference Reagents Program (National Institutes of Health). Anti-FLAG antibodies, trypsin (T-8642), and trypsin inhibitor (T-9003) were purchased from Sigma. HIV-1 Tat was expressed in Escherichia coli using the pGEM2 Tat bacterial expression vector and purified on an Aquapore RP-300 column (Applied Biosystems, Foster City, CA) by reversed-phase chromatography, as described (26). Protein G- and protein A-agarose were purchased form Upstate (Lake Placid, NY).

**Plasmas**—The reporter plasmid pK2 contained the HIV-1 LTR (−138 to +82), followed by nuclear localization signal and lacZ reporter gene (courtesy of Dr. Michael Emerman, Fred Hutchinson Cancer Institute, Seattle, WA). CMV-LacZ and β-actin-LacZ vectors were previously described (19). Tat expression plasmid was a gift of Dr. Ben Berkhourt (University of Amsterdam) (27). FLAG-Tat expression vector was a gift from Dr. Patricio Ray (Children’s National Research Institute, Washington, D.C.). The GST-Tat expression plasmids GST-Tat-(1–72), GST-Tat-(36–72), GST-Tat-(48), and also pGEM2Tat bacterial expression vector were obtained from the National Institutes of Health AIDS repository. GST-Tat fusion proteins were expressed in <i>E. coli</i> and purified on glutathione-agarose beads as described in Ref. 28. The V36A/F38A mutations and Q35R mutations of the 36QVC<sup>TM</sup> sequence of Tat were made according to the QuikChange site-directed mutagenesis protocol of Stratagene, using the appropriate primers and templates. The sequences of the DNA constructs were verified by sequencing using a commercial service from Macrogen (Korea). Expression vectors for PP1 γ-EGFP and PP1 γ-D64N-EGFP are described (29).

**Transient Transfections**—HEK293T cells were cultured in 96-well plates in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were transfected at 50% confluence with Lipofectamine Plus (Invitrogen), according to the manufacturer’s recommendations. Cells were cultured 48 h post-transfection and analyzed for β-galactosidase activity as described below. Transfections were normalized using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye conversion assay, which has a stable end point. The MTT reactions were analyzed at a wavelength of 620 nm (Lab Systems Multiscan MS).

**β-Galactosidase Assay**—Cells were washed with phosphate-buffered saline and lysed for 20 min at room temperature in 50 μl of lysis buffer containing 20 mM HEPES, pH 7.9, 0.1% Nonidet P-40, and 5 mM EDTA. Then, 100 μl of o-nitrophenyl-β-D-galactopyranoside solution (72 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.5, 1 mg/ml o-nitrophenyl-β-D-galactopyranoside, 12 mM MgCl<sub>2</sub>, 180 mM 2-mercaptoethanol) was added and incubated at room temperature until a yellow color developed. The reaction was stopped by addition of 100 μl of 1 M Na<sub>2</sub>CO₃. The 96-well plate was analyzed in a microplate reader at 414 nm (Lab Systems Multiscan MS).

**Phosphorylase a Dephosphorylation Assay**—The assay was carried out as described in Ref. 30. Protein phosphatase activities were measured with 4 nm catalytic subunit and 0.2 nmol of phosphorylase a as a substrate for PP1 or PP2A. The phosphorylase phosphatase assay (10 min at 30°C) was performed in a buffer containing 50 mM glycylglycine, pH 7.4, 0.5 mM dithiothreitol, and 5 mM β-mercaptoethanol. Where indicated, prior to the phosphorylase a assay, the samples were trypsinized (0.16 mg/ml for 10 min at 30°C) to liberate the fully active catalytic subunit of PP1. Trypsin was inhibited by the addition of 0.7 mg/ml trypsin inhibitor for 10 min at 30°C. Analysis of PP1 inhibition by Tat peptides was carried out with Prizm (GraphPad Software, San Diego, CA).

**GST Pull Down Assay**—PP1 (4 nM) was incubated with 10 μg of GST-Tat (0.5 μM) immobilized on glutathione-agarose beads for 1 h at 4°C in 20 μl of 50 mM glycylglycine, pH 7.4, 0.5 mM dithiothreitol, and 5 mM β-mercaptoethanol. The beads were washed with 1 ml of the glycylglycine buffer, subjected to trypsin treatment (0.16 mg/ml for 10 min at 30°C) followed by the addition of 0.7 mg/ml trypsin inhibitor for 10 min at 30°C. Liberated PP1 was measured by phosphorylase a phosphatase as described above.

**Co-immunoprecipitation and Western Blot**—HEK293T cells were transfected with the indicated expression vectors as described above. The whole cell extracts were prepared as described previously (30). About 100 μg of whole cell extract was supplemented with 5 μg of anti-FLAG antibodies. Then protein G-agarose beads preblocked with 5% bovine serum albumin and suspended in TNN buffer (50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl) were added, and the reaction was incubated in TNN buffer at 4°C for 2 h with rocking. The beads were precipitated and washed once with TNN buffer and once with the SDS-PAGE stacking kinase buffer (25 mM Tris-HCl, pH 6.8). The pellet was then resuspended in a 30 μl of 1× SDS loading buffer (25 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromphenol blue) and heated at 90°C for 3 min. The proteins were resolved on SDS-Tricine PAGE, 10%, to detect PP1 γ, or 12%, to detect Tat, and immuno blotted with corresponding antibodies. About 10 μg of total protein was used for the input.

**In Vitro Interaction of Biotinylated TAR RNA, Tat, and CDK9/Cyclin T1**—Biotin-TAR RNA (51 nucleotides) was purchased from Molecule Co. (molecule.com). To bind TAR RNA, streptavidin-agarose beads were washed with binding buffer (20 mM Tris-HCl, pH 7.5, 2.5 mM MgCl<sub>2</sub>, 100 mM NaCl) and incubated with TAR RNA (5 μg/reaction) or formamide-denatured TAR RNA for 30 min at 4°C. The beads were washed with the binding buffer and incubated with recombinant Tat protein (1.5 μg/reaction) for 30 min at 4°C. Beads were washed with binding buffer and then with TAK buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 10 mM ZnSO<sub>4</sub>, 1 mM dithiothreitol) containing 100 mM NaCl. Then 64 μg of yeast tRNA and 100 μg of bovine serum albumin were added per reaction to block the beads. Then recombinant CDK9/cyclin T1 (30 ng/reaction) was added in TAK buffer containing 100 mM NaCl. Protein phosphatase 1 was diluted in TAK buffer and added into the reaction where indicated at 0.1 unit of PP1 per reaction. Samples were incubated for 1 h on ice with occasional mixing. Then beads were washed 3 times with binding buffer, and bound proteins and RNA were eluted in 1× SDS-loading buffer. Proteins were separated on 12% SDS-PAGE, transferred to polyvinylidene difluoride membrane,
and immunoblotted with α-CDK9, α-Tat, or α-PP1y antibodies. Biotin TAR RNA was detected by Ponceau S staining.

**Immunofluorescent Microscopy**—HeLa cells were grown on slides to 50% confluence and transfected with Tat and PP1γ-EGFP or PP1α-EGFP expression vectors using Lipofectamine Plus (Invitrogen), according to the manufacturer’s recommendations. FLAG-Tat and FLAG-Tat-V36A/E38A were stained with anti-FLAG monoclonal antibodies (Sigma) and visualized by indirect immunostaining with TRITC-labeled goat anti-mouse antibodies. PP1-EGFP was visualized by green fluorescence. Slides were mounted in Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenylindole and photographed using a triple-band (red/green/blue) emission filter.

**RESULTS**

PP1 is a Critical Factor for HIV-1 Transcription—In accordance with previous data (20), showing that PP1γ is necessary for Tat-mediated HIV-1 transcription in cultured cells, we found that the transient expression in HEK293T cells of a catalytically dead mutant of PP1, i.e. PP1γ-D64N, inhibited the Tat-activated transcription of the HIV-1 LTR by 3-fold (Fig. 1A, lane 4). This inhibition was similar to the inhibition obtained by expression of the PP1 inhibitor NIPP1 (Fig. 1A, lane 5). In contrast, expression of wild-type PP1γ did not inhibit Tat-dependent transcription (Fig. 1A, lane 3). To determine whether the inhibition of PP1 affected promoters other than HIV-1, we analyzed whether transient expression of PP1γ-D64N has an effect on transcription from CMV or β-actin promoters. HEK293T cells were transfected with CMV-LacZ or β-actin-LacZ (19) expression vectors. Expression of wild-type PP1γ stimulated transcription from the CMV promoter but inhibited transcription from the β-actin promoter (Fig. 1B, lane 2). However, the expression of PP1γ-D64N (Fig. 1B, lane 3) or the PP1 inhibitor NIPP1 (Fig. 1B, lane 4) inhibited transcription. Thus, inhibition of PP1 had a specific effect on the activation of CMV promoter but not on the β-actin promoter. Analysis of the expression of EGFP-PP1γ WT and D64N showed that both proteins were expressed to a similar level (Fig. 1C, lanes 3 and 4). In contrast, Tat expression was decreased in the cells expressing NIPP1 or PP1γ-D64N (Fig. 1C, upper panel, lanes 4 and 5) in accord to the inhibitory effect of NIPP1 or PP1γ-D64N on the CMV promoter that drives the expression of Tat. Although, it is possible that the decreased level of Tat expression accounts for the inhibition of HIV-1 transcription, titration of the Tat-expressing plasmids showed either no effect on Tat transactivation in the case of untagged Tat or inverse dependence in the case of FLAG-Tat expression vector (data not shown). Thus, the amount of Tat needed for transactivation might be very small and the excess FLAG-Tat, which is more stable that untagged Tat, may be even inhibitory for HIV-1 transcription. Therefore we analyzed whether there is a direct interaction between Tat and PP1 disruption of which may be inhibitory for the HIV-1 transcription.

Tat Interacts with PP1—To assess whether Tat directly binds to PP1, immobilized GST-Tat (0.5 μM) was incubated with purified PP1 (4 nM) in glycyglycine, pH 7.4, 0.5 mM dithiothreitol, and 5 mM β-mercaptoethanol as described under “Experimental Procedures.” The binding of PP1 to Tat was detected by treatment of the Tat-associated PP1 with trypsin, which liberates the catalytically active part of PP1 (31). In this system, Tat-(1–72) bound about 60% of the input PP1 activity (Fig. 2A, lane 1), Tat-(1–48) bound about 35% of the input phosphatase activity, and Tat-(37–72) bound about 55% of the activity (Fig. 2A, lanes 2 and 3). In contrast, GST alone bound only 5% of the input phosphatase activity (Fig. 2A, lane 4). The inset (Fig. 2A) shows that equal levels of different Tat constructs were used. To investigate the interaction of Tat with PP1 in vivo, FLAG-tagged Tat was expressed in HEK293T cells and then immunoprecipitated from the 100 μg of whole cellular extract with anti-FLAG antibodies. Immunoblotting analysis showed that both CDK9, which served as a positive control, and PP1 co-precipitated with Tat from the cellular extracts (Fig. 2B, lane 3). EGFP-tagged PP1γ accumulates predominantly within the nucleus (23). Tat was also found to accumulate in the nucleus (32). To determine whether Tat and PP1 co-localize in the nucleus, FLAG-Tat was expressed along with PP1γ fused to EGFP in HeLa cells. Analysis by confocal microscopy showed co-localization of Tat and PP1 (Fig. 2C). Taken together, our results indicate that Tat might interact with PP1 in vitro and in vivo.

Two Separate Regions of Tat Are Involved in the Interaction with PP1—To determine whether Tat has an effect on the enzymatic activity of PP1 and PP2A we used glycogen phosphorylase α as a substrate (30). Tat inhibited the phosphorylase phosphatase activity of PP1 but not that of...
PP2A (Fig. 3A). HIV-1 Tat contains a positively charged RNA-binding region (amino acids 49–57, Fig. 3B) that could interact with a number of negatively charged proteins, including PP1. Therefore, we analyzed the regions of Tat involved in the inhibition of the phosphorylase phosphatase activity of PP1. Tat-(1–44) inhibited the phosphorylase phosphatase activity of PP1 with approximately half the efficiency of that of

![Graph](image-url)

**FIGURE 2.** Tat Interacts with PP1. A, Tat binds to PP1 in vitro. PP1 incubated as described under "Experimental Procedures" with GST-fused Tat-(1–72) (lane 1), Tat-(1–48) (lane 2), Tat-(37–72) (lane 3), and GST alone (lane 4). Beads were washed and treated with trypsin to liberate the catalytic subunit of PP1, and the amount of PP1 was quantified in phosphorylase phosphatase assay. Data are mean ± S.D. The inset shows purified GST-Tat-(1–72), GST-Tat-(1–48), GST-Tat-(36–72), or GST resolved by SDS 4–20%–PAGE and stained with Coomassie Blue. B, Tat binds to PP1 in cultured cells. FLAG-tagged Tat was expressed in HEK293T cells and precipitated with anti-FLAG antibodies. Lane 2, mock-transfected cells. Lane 3, cells transfected with FLAG-Tat. C, Tat and PP1 co-localize in the nucleus. Hela cells were grown 24 h on glass coverslips and then transfected with PP1-EGFP and FLAG-Tat. After 24 h cells were fixed and analyzed for the presence of EGFP by green fluorescence microscopy (left column), and the presence of FLAG by immunocytochemistry with anti-FLAG antibodies and TRITC-labeled secondary antibodies (middle column). The right column contains the overlay pictures.

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**FIGURE 3.** Two regions of Tat interact with PP1. A, Tat inhibits phosphorylase phosphatase activity of PP1 but not PP2A in vitro. Phosphorylase δ was used as a substrate for PP1 or PP2A in the presence of the indicated concentrations of purified Tat. Phosphorylase phosphatase activity presented as the percentage of control in the absence of Tat. B, schematic representation of HIV-1 Tat sequence. Two regions of Tat that interact with PP1 are shown in bold. C and D, inhibition of phosphorylase phosphatase activity of PP1 was determined in the presence of different concentrations of the indicated Tat peptides. IC50 were calculated using Prism. E and F, Tat-(29–43) is a non-competitive (E), whereas Tat-(45–59) is a competitive (F) inhibitor of PP1. Rate of PP1-mediated phosphorylase δ dephosphorylation (V0) was analyzed at different concentrations of phosphorylase δ ([S]) in the absence of Tat peptides, in the presence of 2 and 5 μM of corresponding Tat peptides. Plots are presented in reciprocal Lineweaver-Burk coordinates.
HIV-1 Tat Binds to PP1

FIGURE 4. The 35QVF38 sequence of Tat binds to PP1. A, Tat amino acids Val36 and Phe38 interact with the RVXF binding pocket of PP1. Inhibition of phosphorylase phosphatase activity of PP1 was determined in the presence of different concentrations of Tat-(29–52) WT and V36A/F38A mutant. Competition of PP1 inhibition by Tat-(29–52) was carried out in the presence of NIPP1-derived WT (KNSRVTFSED) or mutant (KNSRATASED) peptides. The results are the mean of three to four independent assays. B, Tat amino acids 42–70 inhibit PP1 but do not interact with the RVXF binding pocket of PP1. Inhibition of phosphorylase phosphatase activity of PP1 was determined in the presence of different concentrations of Tat-(42–70) without or with NIPP1-derived WT (KNSRVTFSED) or mutant (KNSRATASED) peptides. The results are the mean of three to four independent assays. C, the R200Q mutation of the 200RVTF203 sequence of the central domain of NIPP1 does not affect its inhibitory property. Inhibition of phosphorylase phosphatase activity of PP1 was determined in the presence of different concentrations of WT NIPP1-(143–224) (CD), mutant NIPP1-(143–224) R200Q (CD/R200Q), or mutant NIPP1-(143–224) V201A/F203A (CD/RATA). The results are the mean of four independent assays. D, conservancy of 35QVF38 sequence of Tat. Analysis of 370 sequences of Tat deposited to PubMed data base (www.ncbi.nlm.nih.gov/entrez). E, Tat amino acids Val36 and Phe38 mediate interaction of Tat with PP1 in vitro. PP1 was incubated with GST-fused WT Tat-(1–72) (lane 1), Tat-(1–72) V36A/F38A mutant (lane 2), or GST alone (lane 3). Beads were washed and treated with trypsin to liberate the catalytic subunit of PP1, and the amount of PP1 was quantified in phosphorylase phosphatase assay. Data are mean ± S.D. The inset shows purified GST Tat-(1–72), the V36A/F38A mutant of Tat-(1–72), or GST resolved by SDS 4–20%–PAGE and stained with Coomassie Blue. F, the V36A/F38A mutation of Tat does not affect its binding to TAR RNA and CDK9/cyclin T1. Biotinylated TAR RNA was preincubated with streptavidin-agarose beads, and then incubated with Tat, CDK9, and cyclin T1. The precipitated proteins and TAR RNA were eluted in SDS-loading buffer, resolved on 12% Tris-glycine SDS-PAGE, and stained with Coomassie Blue. GST resolved by SDS 4–20%–PAGE and stained with Ponceau-S. CDK9 resolved by SDS 4–20%–PAGE and stained with Ponceau-S. CDK9/cyclin T1. Biotinylated TAR RNA was preincubated with streptavidin-agarose beads, and then incubated with Tat, CDK9, and cyclin T1. The precipitated proteins and TAR RNA were eluted in SDS-loading buffer, resolved on 12% Tris-glycine SDS-PAGE, and immunoblotted with anti-CDK9 and anti-Tat antibodies. Biotinylated TAR RNA was stained with Ponceau-S. Lane 1, control without Tat. Lane 3, WT Tat. Lane 4, V36A/F38A mutant. The CDK9/cyclin T1 was added to all samples.

full-length Tat (Fig. 3C). Within this 44-amino acid N-terminal domain, Tat-(29–43) was an equally potent inhibitor as Tat-(1–44) (Fig. 3C), but Tat-(21–35) and Tat-(37–51) were much weaker inhibitors (Fig. 3C). Therefore the entire 29–43-amino acid sequence within the N-terminal activation domain of Tat appears to participate in the interaction with PP1. In addition to Tat-(1–44), Tat-(36–72) strongly inhibited the phosphorylase phosphatase activity of PP1 (Fig. 3D). This inhibition is likely to be mediated by residues 49–59, because Tat-(45–59) and Tat-(49–63) also strongly inhibited the phosphorylase phosphatase activity of PP1 (Fig. 3D). Tat-(41–55), Tat-(42–54), Tat-(52–60), and Tat-(53–67) were only about ½ to ½ as efficient as Tat-(45–59) in inhibiting the activity of PP1 (Fig. 3D). Therefore the entire Tat-(49–59) sequence appears to interact with PP1. Taken together, our results indicate that two sequences of Tat, 35KCCFHCVCF1KT1AL43 and 35KKRRQRRAH59, are likely to contain PP1-interacting amino acids (shown in Fig. 3B). This conclusion is in agreement with the results from the GST precipitation assays indicating that both N- and C-terminal domains of Tat-(1–72) bind PP1. To test whether both domains of Tat inhibit PP1 by the same mechanism, we analyzed the kinetics of the inhibition of PP1 by Tat-(29–43) and Tat-(45–59). Lineweaver–Burk plots of the inhibition of PP1 by Tat-(29–43) showed noncompetitive inhibition (Fig. 3E), whereas Tat-(45–59) clearly acted as a competitive inhibitor (Fig. 3F). Therefore, Tat-(29–43) probably does not bind at or near the catalytic site of PP1. In contrast, the peptide Tat-(45–59) might inhibit the phosphorylase phosphatase activity of PP1 by binding competitively at the active site of PP1. The polybasic sequence of Tat-(45–59) is similar to the NIPP1193KRKRKR198 sequence that is inhibitory to PP1 but that does not bind to the RVXF binding pocket of PP1 (33).

The Val36 and Phe38 Amino Acids of Tat Interact with the RVXF-binding Pocket of PP1—Nearly all regulatory subunits of PP1 contain at least one RVXF motif, which directly interacts with a hydrophobic surface on the catalytic site of PP1 that is remote from the catalytic site (34, 35). Tat-(29–43) contains the 35QVF38 sequence, which resembles the RVXF motif (Fig. 3B). To assess whether the 35QVF38 sequence is critical for the binding of Tat to PP1, Val36 and Phe38 were substituted to alanines within a Tat-(29–52) peptide. The substitution significantly reduced the potency of Tat-(29–52) to inhibit the phosphorylase phosphatase activity of PP1 (Fig. 4A). In contrast, the substitution of lysine at positions 50 and 51 to alanine in Tat-(29–52) had no significant effect on PP1 phosphorylase phosphatase activity (not shown). This observation suggests that amino acids Val36 and Phe38 are critical for the binding of Tat to PP1. To determine whether the 35QVF38 sequence of Tat
binds directly to the RVXF binding pocket of PP1, we did a competition assay with a peptide, KNSRVTFSED, corresponding to amino acids 197–206 of NIPP1 that contains the RVTF sequence and that is known to bind to the RVXF pocket of PP1 (34). Competition assays were performed with 4 nM PP1 in glycyglycine buffer as described under “Experimental Procedures.” Excess peptide completely blocked the inhibition of PP1 by Tat-(29–52) (Fig. 4A), unlike the mutant peptide KNSRATASED that did not have an effect on PP1 inhibition by Tat-(29–52) (Fig. 4A). In contrast, inhibition of PP1 by Tat-(42–70), although stronger than Tat-(29–52), was not affected by the RVXF-containing peptide (Fig. 4B).

The above results indicate that one of the mechanisms by which Tat interacts with PP1 involves binding of the \(35^\text{QVCF}^{38}\) sequence of Tat to the RVXF-binding pocket of PP1. The first residue of the \(35^\text{QVCF}^{38}\) sequence deviates from the consensus RVXF motif, which was defined as (RK)X_{0–2}(VI)P(FW) (36), where X is any residue and [P] any residue but proline. To determine whether a glutamine is tolerated at the first position of the RVXF motif, we made use of the previous finding that the inhibitory potency of NIPP1 variants is determined by the affinity of their RVXF motifs for PP1 (36). Interestingly, the central domain of NIPP1, NIPP1-(143–224) (Fig. 4C, CD), and the mutant central domain, NIPP1-(143–224)-R200Q (Fig. 4C, CD(R200Q)), were equally potent inhibitors of PP1 (Fig. 4C). In contrast, mutation of valine and phenylalanine at positions 2 and 4 of the RVXF motif of the central domain NIPP1-(143–224)-V201A/F203A (Fig. 4C, CD(RATA)), completely abolished the inhibitory potency. These data show that Gln is tolerated at position 1 of the PP1-binding RVXF motif.

To analyze the physiological importance of the \(35^\text{QVCF}^{38}\) sequence of Tat, we analyzed whether these residues are conserved among different HIV-1 isolates. We analyzed 370 sequences of Tat deposited to the PubMed data base (www.ncbi.nlm.nih.gov/entrez) and found Gln but not Arg or Lys at position 35, and also Phe but not Trp at position 38. To determine whether a glutamine is tolerated at the first position of the RVXF motif, we made use of the previous finding that the inhibitory potency of NIPP1 variants is determined by the affinity of their RVXF motifs for PP1 (36). Interestingly, the central domain of NIPP1, NIPP1-(143–224) (Fig. 4C, CD), and the mutant central domain, NIPP1-(143–224)-R200Q (Fig. 4C, CD(R200Q)), were equally potent inhibitors of PP1 (Fig. 4C). In contrast, mutation of valine and phenylalanine at positions 2 and 4 of the RVXF motif of the central domain NIPP1-(143–224)-V201A/F203A (Fig. 4C, CD(RATA)), completely abolished the inhibitory potency. These data show that Gln is tolerated at position 1 of the PP1-binding RVXF motif.

To further investigate the conservation of the \(35^\text{QVCF}^{38}\) sequence of Tat for the binding to PP1, immobilized GST-Tat, WT, and Tat-V36A/F38A mutant were incubated with purified PP1 and binding of PP1 to Tat was detected by protein phosphatase assays after the release of the Tat-associated PP1 by trypsin. The V36A/F38A mutation reduced binding of PP1 to Tat by 50% (Fig. 4E). This observation is consistent with the results in Fig. 3, C and D, that show an ~50% reduction in the inhibition of PP1 by Tat-(36–72) that lacks the N-terminal portion of Tat. To determine whether Tat-V36A/F38A was defective in promoting the binding of CDK9/cyclin T1 to TAR RNA, WT or mutant Tat were bound to biotinylated TAR RNA in the presence of recombinant CDK9/cyclin T1. Both WT Tat and Tat-V36A/F38A mutant equally well enhanced binding of CDK9/cyclin T1 to TAR RNA (Fig. 4F, lanes 3 and 4), a binding that did not take place in the absence of Tat (Fig. 4F, lane 2). Taken together, our results indicate that the \(35^\text{QVCF}^{38}\) sequence of Tat interacts with the RVXF binding pocket of PP1 and that Tat-V36A/F38A is defective in the binding to PP1 but not to TAR RNA and CDK9/cyclin T1 in vitro.

The \(35^\text{QVCF}^{38}\) Sequence of Tat Is Important for Activation of HIV-1 Transcription and Interaction with PP1 in Vivo—To determine whether the \(35^\text{QACA}^{38}\) mutant of Tat was functionally deficient in Tat-mediated HIV-1 transcription in cultured cells, we examined its effect on transcription of the HIV-1 LTR-lacZ reporter in HEK293T cells. In contrast to WT Tat, Tat \(35^\text{QACA}^{38}\) did not induce transactivation (Fig. 5A). Neither of the Tat constructs stimulates the expression of the reporter from a TAR-deleted mutant of HIV-1 LTR-lacZ (not shown). The inability of Tat \(35^\text{AACA}^{38}\) to induce transcription was not because of changes in its expression as both WT and mutant Tat were expressed equally well in HEK293T cells (Fig. 5B, lanes 3 and 4). Also, detection of wild-type and mutant Tat in HeLa cells transfected with the FLAG-Tat vector expressing reporter in HEK293T cells (Fig. 5B, lanes 3 and 4). This result shows that PP1 is bound to the \(35^\text{QVCF}^{38}\) sequence and also to the polybasic sequence of Tat. The Q35R Mutant of Tat Binds PP1 but Not CDK9 and Is Not Active in Inducing HIV-1 Transcription—Because binding of WT Tat to PP1 is relatively weak, we thought to create a mutant of Tat that would be more efficient in binding to PP1 and determine whether such a mutant is active as an activator of HIV-1 transcription. We mutated the Glu at position 35 of Tat into Arg to create a perfect “RVXF” sequence (\(35^\text{QVCF}^{38}\) sequence of Tat) to bind PP1. Although as we showed above the central domain of NIPP1 is an equally potent inhibitor without or with the R200Q mutation, both NIPP1 variants likely inhibit PP1 at stoichiometric concentrations and a “better” binding of the wild-type

Figure 5. The \(35^\text{QVCF}^{38}\) sequence of Tat is important for activation of HIV-1 transcription and interaction with PP1. A, the V36A/F38A mutation of Tat inhibits Tat-dependent HIV-1 transcription. HEK293T cells were transfected with HIV-1 LTR-lacZ expression vector, without or with the Tat (WT) or V36A/F38A mutant of Tat (QACA). For statistical evaluation, 4 samples were transfected independently in each experiment for each plasmid combination and each experiment was repeated 3 times. At 48 h posttransfection, the cells were lysed and analyzed for β-galactosidase activity with o-nitrophenyl-β-D-galactopyranoside. MTT assay was used to normalize the results. B, Tat-V36A/F38A mutant does not bind PP1 in vivo. HEK293T cells were transfected with FLAG-tagged WT or V36A/F38A mutant Tat (lanes 3 and 4). Tats were immunoprecipitated (IP) with anti-FLAG antibodies from HEK293T cells extracts in the absence (upper panel) or presence (medium panel) of TAR RNA and probed with antibodies against PP1 (ip) or Tat. Lane 1, untreated whole cell extract (10 μg). Lane 2, mock-transfected cells. C, WT Tat and Tat-V36A/F38A mutant have similar cellular localization. HeLa cells were grown 24 h on glass coverslips and then transfected with FLAG-Tat WT (left panel, FLAG-Tat) and V36A/F38A mutant (right panel, FLAG tat QACA). After 24 h cells were fixed and analyzed for the presence of FLAG-Tat by immunocytochemistry with anti-FLAG antibodies and TRITC-labeled secondary antibodies.
HIV-1 Tat Binds to PP1

FIGURE 6. Tat Q35R mutant is deficient in binding to CDK9/cyclin T1 and inactive in HIV-1 transcription. A, the Q35R mutation of Tat increases its binding to PP1 and decreases its binding to CDK9/cyclin T1. Recombinant Tat, WT, and the Q35R mutant, recombinant CDK9 and cyclin T1 and rabbit PP1 were purified as described under "Experimental Procedures." Biotinylated TAR RNA was preincubated with streptavidin-agarose beads, and then incubated with Tat, CDK9, cyclin T1, and PP1. The agarose beads were precipitated, and washed. The precipitated proteins and TAR RNA were eluted in SDS-loading buffer, resolved on 12% Tris-Tricine SDS-PAGE, and immunoblotted with anti-CDK9, anti-PP1, and anti-Tat antibodies. Biotinylated TAR RNA was stained with Porceau-S. Lane 1, control with denatured TAR RNA. Lane 2, control without Tat. Lane 3, WT Tat. Lane 4, Tat Q35R mutant. The CDK9/cyclin T1 and PP1 were added to all samples. B, Tat Q35R mutant does not bind CDK9/cyclin T1 in vivo. HEK293T cells were transfected with FLAG-tagged WT or Q35R-mutant Tat (lanes 3 and 4). Tats were immunoprecipitated (IP) with anti-FLAG antibodies from the cells extracts and probed with antibodies against CDK9, PP1, or Tat. Lane 1, untreated whole cell extract. Lane 2, mock-transfected cells. C, Tat Q35R mutant is inefficient in HIV-1 transcription. 293T cells were transfected with HIV-1 LTR-LacZ expression vector, without or with the Tat (wt) or Q35R-mutant of Tat (RVCF). For statistical evaluation, four samples were transfected independently in each experiment. At 48 h post-transfection, the cells lysed and analyzed for β-galactosidase activity with o-nitrophenyl-β-D-galactopyranoside. MTT assay was used to normalize the results.

cannot be detected by inhibition assays. As expected, Tat RVCF bound PP1 more efficiently than WT Tat (Fig. 6A, lanes 3 and 4). However, unexpectedly, in the presence of PP1, Tat RVCF did not bind CDK9/cyclin T1 (Fig. 6A, upper panel, lanes 3 and 4). To further investigate the effect of the Q35R mutation on the interaction of Tat with PP1, FLAG-tagged Tat was expressed in HEK293T cells and then immunoprecipitated from the cellular lysates with anti-FLAG antibodies. Again, there was a reduced amount of CDK9 co-precipitating with Tat RVCF (Fig. 6B, lanes 3 and 4). To determine whether the Tat RVCF mutant was functionally deficient in Tat-mediated HIV-1 transcription in cultured cells, we examined its effect on transcription of the HIV-1 LTR-LacZ reporter in HEK293T cells. In contrast to WT Tat, Tat RVCF induced transactivation to a lesser extent (Fig. 6C). This result shows that strong binding of PP1 may prevent interaction of Tat with CDK9/cyclin T1 and thus block Tat transactivation.

The 35QVCF<sup>α</sup> Sequence of Tat Is Important for Retargeting of PP1 in Vivo—To analyze whether Tat might re-target PP1 in cultured cells, we expressed wild-type or mutant Tat Q35QACA in HeLa cells by transfection and also co-transfected PP1α-EGFP. Unlike PP1α-EGFP, which is expressed in the nucleolus, PP1α-EGFP was located in both the cytoplasm and nucleus but was largely excluded from the nucleolus (Fig. 7A) as previously reported (23). To determine whether Tat, which is enriched in nucleoli (32), can re-target PP1α to nucleoli, we expressed FLAG-Tat, WT, or 35QACA<sup>α</sup> mutant, along with PP1α-EGFP. Co-expression of Tat with PP1α resulted in the migration of PP1α to the nucleoli (Fig. 7B). This was not seen with Tat-V36A/F38A (Fig. 7C), indicating that Tat binds to PP1 in vivo in the nucleolus and that the 35QVCF<sup>α</sup> motif is essential for this binding. In conclusion, our results indicate that residues Val<sup>36</sup> and Phe<sup>38</sup> of Tat are critical for the interaction of Tat with PP1 in vitro and in vivo and that mutation of these residues prevents Tat transactivation.

DISCUSSION

In agreement with our previous findings we show here that the over-expression of an inactive mutant of PP1 (PP1<sub>y</sub>-D64N) is inhibitory for Tat-induced transcription. This result confirms our previous finding that PP1 is involved in the regulation of HIV-1 transcription in cultured cells (20). We also found here that PP1<sub>y</sub>-D64N inhibits transcription from the CMV promoter and also β-actin promoter. We recently showed that PP1 dephosphorylates CDK9 in cultured cells (21) and thus the effect of PP1 on the CMV promoter can be explained by down-regulation of the activity of CDK9/cyclin T1, which is a critical factor for transcription from CMV promoter (37).

Analysis of the in vitro binding of Tat to PP1 showed that Tat directly associates with the catalytic subunit of PP1. In a previous study we found that GST-Tat binds a phosphatase from partially purified cellular extract and that this phosphatase was inhibited at a low concentration of okadaic acid, indicating that it was PP1 (18). In the present study we delineated two distinct regions of Tat that might interact with PP1 by using enzymatic kinetics and competition assays. The 35QVCF<sup>α</sup> sequence of Tat, which resembles the PP1-binding RVXF motif, interacted with the RVXF binding pocket of PP1. Previously, a herpes simplex virus protein γ<sup>34.5</sup> was shown to...
bind PP1 through an RVXF motif and to dephosphorylate eukaryotic initiation factor 2α to prevent PKR-mediated shut down of host protein synthesis (38, 39). Thus Tat may represent a viral regulatory protein that utilize host PP1 for the benefits of the viral replication.

Analysis of Tat interaction with PP1 in cultured cells showed (i) co-immunoprecipitation of Tat with endogenous PP1γ from cellular lysates; (ii) co-localization of Tat and PP1γ in nucleoli; (iii) nuclear targeting of PP1α in the presence of Tat. These results strongly support our major conclusion that Tat interacts with cellular PP1 and that the interaction is mediated by the 35QVC38 sequence of Tat because Tat-V36A/F38A mutations impaired these effects. Moreover, Tat-V36A/F38A mutant was incapable of activating HIV-1 transcription, which was not because of its altered expression. Phe38 was already shown to be crucial for Tat transactivation and viral replication (40). Moreover, the only compensatory mutation allowing virus replication was F38W substitution, which is allowed by the consensus RV motif (36). Our results show that the 35QVC38 sequence binds to PP1, thus explaining earlier findings of Verhoef and Berkhourt (40) that mutation of Phe38 dramatically reduced efficiency of HIV-1 replication. Our data also show that a Glu in position 1 is allowed. Lesser conservancy of the Glu35 and Val38 might indicate that additional determinants around the 35QVC38 sequence might also contribute to the interaction with PP1. It may also indicate that intrinsically weak interaction is required for Tat function. Indeed, mutation Q35R of Tat increased its binding to PP1, but also decreased its interaction with CDK9/cyclin T1 and reduced the transcriptional activity of the Tat Q35R mutant.

The catalytic subunit of PP1 lacks a nuclear localization signal and thus requires a regulatory subunit, such as NIPP1 and PNUTS, for targeting to the nucleus (41). We hypothesize that Tat functions as a nuclear targeting subunit of PP1 for delivery to specific nuclear substrate(s) important for the activation of HIV-1 transcription. Previously we have shown that PP1 dephosphorylates Ser2 residues of RNA polymerase II CTD. It is not clear at the moment whether dephosphorylation of RNAPII might benefit HIV-1 transcription, as Tat is generally believed to induce phosphorylation of RNAPII. In addition to the dephosphorylation of CTD, PP1 may be involved in dephosphorylation of other proteins, such as autophosphorylated CDK9 or a CDK9 regulatory subunit. Our recent study showed that PP1 dephosphorylates CDK9 in vivo, and that CDK9 dephosphorylation sites include Thr186 and also C-terminal autophosphorylation sites (21). Activation of CDK9 holoenzyme by PP1 in vitro was recently reported (42). Thus Tat may conceivably be involved in targeting of PP1 to CDK9/cyclin T1 or CDK9-regulatory proteins.

In conclusion, our results suggest that HIV-1 Tat may function as a targeting subunit of PP1. The Tat-PP1 interaction appears to be essential for HIV transactivation and thus represents a novel and attractive therapeutic target. The recent demonstration of the feasibility of selective pharmacological targeting of cellular dephosphorylation events (43) suggest that it might be possible to obtain synthetic compounds that disrupt the Tat-PP1 interaction.

Acknowledgments—The GST-Tat, pGEM2Tat bacterial expression vectors, and HIV-1 Clade B consensus Tat (15-mers) peptides were obtained through the National Institutes of Health AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, National Institutes of Health.

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Nuclear Targeting of Protein Phosphatase-1 by HIV-1 Tat Protein
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J. Biol. Chem. 2005, 280:36364-36371.
doi: 10.1074/jbc.M503673200 originally published online August 29, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503673200

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