Nuclear Protein Phosphatase-1 Regulates HIV-1 Transcription

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Summary

We recently reported that protein phosphatase 1 (PP1) dephosphorylates RNA polymerase II (RNAPII) C-terminal repeats and regulates HIV-1 transcription in vitro. Here we provide evidence that PP1 is also required for Tat-induced HIV-1 transcription and for viral replication in cultured cells. Inhibition of PP1 by overexpression of nuclear inhibitor of PP1 (NIPP1) inhibited Tat-induced HIV-1 transcription in transient transfection assays. A mutant of NIPP1 that was defective in binding to PP1 did not have this effect. Also, the co-expression of PP1γ reversed the inhibitory effect of NIPP1. Adeno-associated virus (AAV)-mediated delivery of NIPP1 significantly reduced HIV-1 transcription induced by Tat-expressing adenovirus in CD4+ HeLa cells that contained an integrated HIV-1 promoter (HeLa MAGI cells). In addition, infection of HeLa MAGI cells with AAV-NIPP1 prior to the infection with HIV-1 significantly reduced the level of HIV-1 replication. Our results indicate that PP1 might be a host cell factor that is required for HIV-1 viral transcription. Therefore, nuclear PP1 may represent a novel target for anti-HIV-1 therapeutics.
**Introduction**

The transcriptional activator (Tat) protein of the human immunodeficiency virus type 1 (HIV-1) induces elongation of transcription of HIV-1 viral genes (1). The activation domain of Tat (amino acids 1 to 48) interacts with the host Cdk9/cyclin T1, whereas the positively charged RNA-binding domain of Tat (amino acids 49-57) binds to the HIV-1 transactivation response (TAR) RNA (1). Cdk9/cyclin T1 is a protein kinase that enhances transcriptional elongation by phosphorylating the C-terminal domain (CTD) of the largest subunit of RNA Polymerase II (RNAPII) (2). The interaction of HIV-1 Tat with the bulge of TAR RNA and with cyclin T1 promotes binding of cyclin T1 to the loop of TAR RNA (3). As a result, Cdk9/cyclin T1 is recruited to the HIV-1 promoter (3, 4). Cdk9-mediated phosphorylation of RNAPII CTD directly stimulates transcription elongation (5). Cdk9 also stimulates elongation of transcription indirectly by relieving inhibition of transcription by several negative regulators, including the DRB sensitivity-inducing factor DSIF and the negative elongation factor NELF (6, 7).

We recently demonstrated that protein phosphatase-1 (PP1) serves as a CTD phosphatase (8). PP1 belongs to the PPP family of serine-threonine protein phosphatases, which also comprises PP2A (including PP4 and PP6), PP2B and PP5 (9). PP1 holoenzymes consist of a constant catalytic subunit and one or two variable regulatory subunits (R) that determine the localization, activity and substrate-specificity of the phosphatase (9). Nuclear inhibitor of PP1 (NIPP1) is an R-subunit of PP1 that inhibits the dephosphorylation of a variety of substrates including phosphorylase a (10). We recently showed that NIPP1 also inhibits the dephosphorylation of RNAPII CTD by PP1 (8). We also found that NIPP1 associates with a
CTD kinase complex (11). The previously identified CTD phosphatase, FCP1, inhibits HIV-1 transcription \textit{in vitro} (12, 13). In contrast, PP1 stimulates Tat-dependent transcription from HIV-1 LTR \textit{in vitro} (14). Therefore it was of interest to analyze whether PP1 is required for HIV-1 transcription and for viral replication \textit{in vivo}.

In the present study, we analyzed the effect of nuclear PP1 inhibition on Tat-induced HIV-1 transcription in cultured cells. PP1 was inhibited by overexpression of NIPP1 fused to the green fluorescent protein (EGFP). PP1 inhibition was shown to inhibit the Tat-dependent expression of the LacZ reporter under control of the HIV-1 LTR. NIPP1 also inhibited HIV-1 transcription induced by extracellularly added Tat. Expression of PP1\textgreek{g} rescued the NIPP1-mediated inhibition of Tat-dependent transcription. To further explore the effect of PP1 inhibition, we created recombinant adeno-associated viruses (AAV) which expressed either wild type or mutant NIPP1-EGFP fusion proteins. AAV-mediated delivery of NIPP1 but not the mutant NIPP1 into HeLa cells containing an integrated HIV-1 LTR-Lac Z reporter inhibited Adeno-Tat induced transcription from HIV-1 promoter. Moreover, AAV-mediated delivery of NIPP1 to CD4+ HeLa cells containing an integrated HIV-1 promoter reduced the replication of T-tropic HIV-1 virus. Taken together, our results indicate that PP1 is required for HIV-1 transcription.
Experimental Procedures

Materials - COS-7 cells, HEK 293, 84-31 and HeLa cells were purchased from ATCC (Manassas, VA). HeLa cells containing integrated HIV-1 LTR Lac Z and also expressing CD4 and T-tropic HIV-1 strain NL4-3 were obtained from AIDS Reagent Program (NIH). Anti-EGFP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of recombinant proteins - HIV-1 Tat was expressed in *Escherichia coli* and purified on an Aquapore RP-300 column (Applied Biosystems, Foster City, CA) by reversed-phase chromatography (15).

Plasmids - The reporter plasmid pJK2 contained HIV-1 LTR (-138 to +82) followed by a nuclear localization signal (NLS) and the LacZ reporter gene (courtesy of Dr. Michael Emmerman, Fred Hutchinson Cancer Institute, Seattle, WA). It expresses NLS-tagged β-galactosidase under the control of HIV-1 LTR (16). The reporter plasmid JK2ΔTAR contained a deletion of +19 to +87 nucleotides introduced by restriction digestion with BglII. The Tat expression plasmid was a gift from Dr. Ben Berkhout (University of Amsterdam) (17). The expression vectors for EGFP-fused NIPP1-(1-351) (WT NIPP1), NIPP1-(1-351) K193-197A/V201A/F203A/Y335D (PI mutant), NIPP1-(1-329) and NIPP1-(1-329) V201A/F203A (RATA mutant) were described (18). The expression vector for PP1γ-EGFP was prepared as described (19).

Transient transfections - HEK 293, 84-31, and COS-7 were cultured at 7x10^5 cells/well in DMEM containing 10% fetal bovine serum. Transfections were performed at 75% confluency using a Ca^{2+} – phosphate protocol and the indicated reporter plasmids. After transfection the cells were cultured for an additional 48 hours and then analyzed by FACS and by the assays of
β-galactosidase activity. Where indicated, Tat transactivation was induced 24 hours post transfection with JK2 by the addition of purified Tat (residues 1-72; 200 ng/ml) and 100 µM chloroquine, as previously described (20). HeLa cells were cultured in DMEM containing 10% fetal bovine serum. Cells were grown to 75% confluency and transfected with Lipofectamine Plus (Life Technologies, Rockville, MD) according to the recommendations of the manufacturer. Cells were lysed 48 hours post transfection and analyzed for β-galactosidase activity and by FACS.

Recombinant adeno-associated viruses – Recombinant AAV vectors containing either the EGFP-fused NIPP1 gene under the control of the CMV promoter (AAV.NIPP1) or mutant EGFP-fused NIPP1 K193-197A/V201A/F203A/Y335D (AAV.pA-RATA Y335D) were produced as described (21). Briefly, the cis plasmid (which contains the gene of interest with AAV ITRs), the trans plasmid (with the AAV rep and cap gene), and a helper plasmid (pFΔ6, which contains an essential region from the Ad genome) were co-transfected into HEP-293 cells at a ratio of 1:1:2 by calcium phosphate precipitation in an Ad-free system. The cells were harvested 96h later. The AAV was purified through two rounds of CsCl density gradient centrifugation, desalted by dialysis at 4°C against PBS, aliquoted, and kept at 4°C. The titer was determined by quantitative dot blot hybridization. The purity of the AAV vector preparation was addressed by colloidal brilliant blue G staining (Sigma, St. Louis, MO) and Western blotting of viral proteins separated by SDS-PAGE.

Preparation of Adeno-Tat - The E1-deleted recombinant Ad carrying Tat was generated as previously described (22). Briefly, a cDNA fragment encoding the full length HIV-1 Tat protein cloned into the plasmid pCXN was subcloned in the pAd.CMVlink plasmid. pAd.CMVlink
and Cla I digested adenoviral DNA were co-transfected into HEP-293 cells at a ratio of 3:1 by calcium phosphate precipitation to allow the recombination. The virus was purified through three rounds of plaque-purification. Viruses were replicated in HEK-293 cells and were purified from a cell lysate by two rounds of CsCl density gradient centrifugation. The purified virus was desalted on a Bio-Gel P-6 desalting column (Bio-Rad Laboratories, Hercules, CA) equilibrated with PBS. The titer of the virus preparation was determined both by absorbency at 260 nm and by plaque assay (23). The particle to plaque forming unit ratio was less than 100. Purified viruses were suspended in PBS at the desired concentrations.

β-galactosidase assays - Cells were washed with phosphate-buffered saline (PBS) and lysed for 20 min at room temperature in 50 µl of lysis buffer, containing 20 mM HEPES at pH 7.9, 0.1% NP-40 and 5 mM EDTA. Subsequently, 100 µl of o-nitrophenyl-β-D-galactopyranoside (ONPG) solution (72 mM Na₂ PO₄ at pH 7.5, 1 mg/ml ONPG, 12 mM MgCl₂, 180 mM 2-mercaptoethanol) was added and incubated at room temperature until a yellow color was developed. The reaction was stopped by addition of 100 µl of 1M Na₂CO₃. The 96-well plate was analyzed in a micro plate reader at 414 nm (Lab Systems Multiscan MS). For the X-gal assay cells were fixed in 0.5% glutaraldehyde for 10 min at room temperature, washed with PBS and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) solution (1 mg/ml X-gal, 4 mg/ml K₃Fe (CN)₆, 2.1 mg/ml K₄Fe (CN)₆ x 3H₂O and 1mM MgCl₂ dissolved in PBS) at 37°C for 30 min.

HIV-1 viral infections – HeLa cells containing LTR Lac Z and also expressing CD4 were subcultured 96-well plate at 10,000 cells per well. The cells were infected with AAV-NIPP1 or AAV-NIPP1 PI mutant viruses at 10⁶ Pfu per well while cells were still in suspension. After 24
hours of culturing the cells were infected with equal amounts of HIV-1<sub>NL4-3</sub> (MOI of 2.5) in 0.1 ml culture medium with 20 mg/ml polybrene. After 4 hours of incubation the medium was removed, cells were rinsed with phosphate buffered saline and incubated in complete DMEM with 10% fetal bovine serum. At 48 hours post infection, cells were fixed with 0.5% glutaraldehyde for 10 min at room temperature, washed with PBS and stained with X-gal at 37ºC for 30 min.
Results

Expression of NIPP1-EGFP blocks HIV-1 activated transcription in cultured cells

To analyze the contribution of PP1 to the control of HIV-1 transcription in intact cells, nuclear inhibitor of PP1 (NIPP1) was expressed as a fusion with the enhanced-green-fluorescent-protein (EGFP) in HEK 293, COS-7 or HeLa cells. It has been previously shown that the NIPP1-EGFP fusion protein was expressed to significantly higher levels than NIPP1 without this tag (18). As a control, we used a mutated version of NIPP1-EGFP NIPP1-(1-351) K193-197A/V201A/F203A/Y335D, PI-mutant) that no longer interacts with PP1 (24). In this mutant the PP1 binding sites in both the central and C-terminal domains of NIPP1 are mutated (24). To explore whether the effects of NIPP1 were mediated by its intrinsic endoribonuclease activity, we also used a C-terminally truncated version NIPP1-(1-329), which lacks the RNA-binding and hydrolyzing site (25). The EGFP tag allowed an analysis of the transfection efficiency by FACS or by fluorescence microscopy, which revealed that the WT and mutated NIPP1-EGFP were expressed in close to 100% of all cells (not shown). In accordance with previous observations (18), both the WT and mutated NIPP1-EGFP were exclusively nuclear, while EGFP was present in both the cytoplasm and the nucleus (not shown). The expression of the NIPP1-EGFP fusions was confirmed by immunoblotting of cell lysates with antibodies against EGFP (Fig. 1A). The expression of β-galactosidase from the HIV-1 LTR-LacZ vector (JK2) was analyzed using a qualitative X-gal-based assay, or a quantitative ONPG-based assay (Fig.1, panels B, C and D). In HEK 293 cells the co-transfection of the Tat-expressing vector and HIV-1 LTR-LacZ resulted in a 15-fold increase of β-galactosidase activity (Figs.1B and C, lane 2). The co-expression of NIPP1-EGFP blocked HIV-1 transcription nearly
completely (Figs. 1B and C, lane 3). In contrast, the co-expression of the PI-mutant of NIPP1-EGFP, which cannot bind PP1, did not have an inhibitory effect on the Tat-mediated transcription (Figs.1B and C, lane 4). The co-expression of NIPP1-(1-329) which lacks the endoribonuclease catalytic site, also inhibited Tat-induced HIV-1 transcription (Figs. 1B and C). In the absence of Tat, WT NIPP1 as well as NIPP1 PI and NIPP1-(1-329) moderately inhibited basal HIV-1 transcription (Fig.1D).

In COS-7 cells, Tat stimulated transcription from HIV-1 LTR even up to 40-fold (Fig.1E, lane 2). Again, the co-transfection of wild type NIPP1, but not the PI-mutant, inhibited Tat-activated transcription (Fig.1E, lanes 3 and 4). Transcription from a mutant HIV-1 LTR with a deletion of the fragment encoding TAR RNA (HIV-1 LTRΔTAR) was inhibited about 50% by both NIPPI and mutant NIPPI (Fig. 1E, lanes 7 and 8). In accordance with this result, in the absence of Tat, both NIPPI and the NIPPI PI-mutant inhibited the basal HIV-1 transcription by about 60% (Fig. 1E, lanes 9 to 11) indicating that this effect of NIPPI was not due to its ability to bind PP1.

In vitro, the central domain of NIPPI (residues 143-224) is an equally potent inhibitor of PP1 as full length NIPPI (24). We found that expression of EGFP fusion of NIPPI-(143-224) (Fig. 2A, lane 1) also inhibited Tat-induced HIV-transcription (Fig. 2B, lane 3). However, mutation of the PP1-binding RVXF motif, as in NIPPI-(143-224) V201AF203A (RATA mutant) (Fig.2A, lane 2) abolished the inhibition of Tat-induced HIV-1 transcription (Fig. 2B, lane 4). Collectively, our data indicate that the expression of NIPPI fairly specifically and potently blocks Tat-dependent transcription from HIV-1 LTR in cultured cells and that this inhibition depends on the binding of PP1.
The effects of NIPP1 on the Tat-dependent HIV-1 transcription are reversed by co-expression of PP1. To further analyze the role of PP1 in the NIPP1-mediated inhibition of induced HIV-1 transcription, we expressed a PP1 isoform, PP1γ-EGFP (Fig.2A, lanes 3). Expression of PP1 per se did not affect the Tat-dependent transcription (Fig. 2C, lane 3). But the NIPP1-EGFP mediated inhibition of Tat-dependent transcription (Fig. 2C, lane 4) was completely reversed by the expression of PP1 (Fig 2C, lane 5). Our results indicate that NIPP1 inhibits Tat-dependent HIV-transcription because it inhibits PP1, which appears to be necessary for transcription. Overexpression of PP1 titrates NIPP1, which explains why co-expression of NIPP1 and PP1 is not inhibitory.

The inhibition of Tat-dependent transcription by NIPP1 is not the result of a reduced expression of Tat – The inhibition of PP1 might deregulate the expression of Tat and in this way affect Tat-dependent transcription. To analyze whether Tat was a limiting factor in transfection experiments in this study, both the Tat-expressing vector as well as the HIV-1 LTR LacZ expression vector (JK2) were titrated. We found that even a 10-fold decrease in the amount of Tat-expressing plasmid did not reduce the level of transactivation in COS-7 cells or HEK 293 cells (data not shown, Supplemental Fig.1). In contrast, the Tat-activated HIV-1 LTR-LacZ expression was strictly dependent on the amount of HIV-1 LTR plasmid in COS-7 cells or HEK 293 cells (data not shown, Supplemental Fig. 1). These results suggest that the amount of HIV-1 LTR and not Tat was a limiting factor for LacZ expression in our transfection assays.
The expression of Tat was analyzed in COS-7 cells transfected with the Tat-expressing vector and immunostained with anti-Tat polyclonal antibodies (Fig. 3). Tat expression was visualized by indirect immunostaining with TRITC-labeled goat-anti-mouse antibodies (Fig. 3D). No staining was found in untransfected cells (Fig. 3E). We observed no significant difference in Tat expression in the cells transfected with Tat alone or co-transfected with Tat and either NIPP1-EGFP or mutant NIPP1-EGFP expression vectors (Figs. 3A and D). Expression of NIPP1, analyzed earlier in Western blot (Fig. 1A) was monitored here by fluorescence of EGFP (Fig. 3B).

To further investigate whether the inhibitory effect of NIPP1 on Tat-dependent transcription was dependent on the expression of Tat, HIV-1 transcription was activated by addition of purified Tat protein to the culture medium (20). The purified Tat increased HIV-1 transcription about 5-fold (Fig. 4, lanes 1 and 2). In contrast, HIV-1 LTRΔTAR, in which the TAR sequence was deleted, was only slightly (less than 2-fold) induced by Tat protein (Fig. 4, lanes 5 and 6). Again, the expression of NIPP1-EGFP but not the mutant NIPP1-EGFP blocked the Tat-induced transcription (Fig. 4, lanes 3 and 4). In contrast, neither NIPP1-EGFP nor mutant NIPP1-EGFP expression has an effect on transcription from the mutant HIV-1 LTRΔTAR (Fig. 4, lanes 5, 6 and 7). Taken together, our results indicate that the expression of NIPP1-EGFP blocked Tat-activated transcription and that this effect was not the result of a decreased expression of Tat.

Adeno-associated virus (AAV) – mediated delivery of NIPP1 inhibits induced HIV-1 transcription from an integrated HIV-1 promoter – In cells infected with HIV-1,
transcription from the integrated HIV-1 promoter is very inefficient in the absence of Tat as compared to the strong basal transcription from the naked DNA in vitro or transcription from the transiently transfected DNA containing HIV-1 promoter (1). Therefore we investigated the effect of NIPP1 expression on the induction of an integrated HIV-1 promoter using HeLa cells with an integrated HIV-1 LTR under control of the Lac Z reporter (16). To express NIPP1 and Tat, we used viral gene-mediated transfer. The WT or PI mutant of NIPP1 was expressed using AAV viral gene transfer, whereas Tat was expressed using Adeno Tat virus. Infection of the HeLa cells containing an integrated HIV-1 LTR Lac Z with adeno virus encoding Tat (Adeno-Tat) dramatically induced Tat-activated LacZ expression (Fig. 5A, lanes 1 and 2). Infection of the HeLa cells with AAV-NIPP1 but not AAV-NIPP1 PI mutant prior to the infection with Adeno-Tat virus reduced activated HIV-1 transcription by 50% (Fig. 5A, lanes 3 and 4). Because both NIPP1 and the PI-mutant of NIPP1 were expressed as fusions with EGFP, allowing equal infectivity to be ensured, their distinct effects cannot be ascribed to differences in gene delivery efficiency (Fig. 5B). These results indicate that PP1 is an essential factor also during HIV-1 transcription from the integrated HIV-1 promoter.

**AAV – mediated delivery of NIPP1 inhibits replication of HIV-1** - We further analyzed the effect of PP1 inhibition on HIV-1 transcription using a single HIV-1 replication assay. CD4+ HeLa cells containing an integrated HIV-1 LTR Lac Z (HeLa MAGI cells, (16) were infected with T-tropic HIV-1 strain NL4-3 virus. Following the infection by HIV-1, production of Tat leads to the expression of β-galactosidase detected by staining the cells with X-gal (Fig. 6A lane 1, and Fig.6B). AAV-mediated delivery of NIPP1 but not the PI-mutant of
NIPP1 decreased about 6-fold the number of X-gal positive cells (Fig. 6A, lanes 2 and 3, and Figs 6C and D). As a control to detect all cells containing HIV-1 LTR LacZ, HeLa MAGI cells were infected with Adeno-Tat virus (Fig.6A, lane 4, and Fig.6E). In non-infected cells, the expression of HIV-1 LTR was not detectable (Fig.6 F). These results indicate that inhibition of PP1 efficiently blocks HIV-1 viral replication.
Discussion

Recent studies firmly established that Tat-induced transcription from the HIV-1 promoter is regulated at the level of RNAPII CTD phosphorylation (1, 2). While the role of the Tat-associated CTD kinase Cdk9/cyclin T1 has been well established (2), the role of the protein phosphatases in the function of Tat is not very well understood. RNAP II CTD is dephosphorylated by a well-established CTD phosphatase, FCP1 (26). Tat inhibits FCP1, and this inhibition may alleviate FCP1-mediated pausing of transcription (12, 13). We have recently reported that PP1 also functions as a CTD phosphatase (8). We have shown that PP1 and FCP1 contribute equally to the dephosphorylation of RNAPII in vitro (8). In vivo a PPP-type phosphatase dephosphorylates approximately 40% of all RNAPII when cells are treated with high concentrations of flavopiridol (8). Interestingly, PP1 associates with the preinitiation complex and stimulates transcription from the HIV-1 promoter in vitro (14). Therefore we asked whether the inhibition of PP1 by overexpression of NIPP1, a nuclear inhibitor of PP1 (9), might have a regulatory effect in HIV-1 transcription. NIPP1 inhibits the dephosphorylation of a variety of substrates by PP1 including phosphorylase a (10). Reported NIPP1-mediated inhibition of splicing in vitro is independent of its ability to inhibit PP1 (27, 28). Recent studies showed that the expression of NIPP1 in Drosophila is lethal and that this phenotype is reversed by co-expression of PP1, indicating that the lethality stems from the inhibition of PP1 (29). It is unclear which nuclear process is affected and causes the embryonic lethality of the NIPP1 transgenic flies, but our studies ([14] and the present study) show that PP1 has an effect on transcription. Indeed, in the present paper we provide evidence that overexpression of NIPP1
inhibits HIV-1 transcription and that the inhibition correlates with the ability of NIPP1 to bind and inhibit PP1.

We have examined the role of NIPP1 in cultured cells by overexpression, using transient transfections and adeno-associated virus-mediated gene transfer. Overexpression of NIPP1 potently inhibited Tat-induced HIV-1 transcription. A NIPP1 mutant that does not bind PP1 was not inhibitory to transcription, whereas a mutant of NIPP1 that lacked the endoribonuclease active site was still inhibitory. Moreover, expression of the central domain of NIPP1 but not its RATA mutant, which is incapable of binding to PP1, also potently inhibited Tat-induced transcription. Therefore, the effect of NIPP1 on HIV-1 transcription is likely to be accounted for by inhibition of the nuclear pool of PP1. This conclusion is further supported by our observation that the effect of NIPP1 on HIV-1 transcription was reversed by the co-expression of PP1γ. This effect of co-expression of PP1γ is likely to be accounted for by titration of NIPP1, resulting in a lesser inhibition of the endogenous pools of PP1. Interestingly, the co-expression of PP1γ did not rescue the inhibition of basal transcription by NIPP1 (not shown). Therefore, it seems likely that PP1 is involved in the regulation of Tat-activated but not the basal HIV-1 transcription. It is possible that the mechanism of transcriptional stimulation by Tat includes a transient activation of PP1. Our preliminary results indicate that Cdk9/cyclin T1 phosphorylates NIPP1 and that this phosphorylation activates NIPP1-associated PP1 (unpublished data). Since Tat induces Cdk9/cyclin T1 to phosphorylate RNAP II CTD (19, 35), it will be of interest to investigate whether Tat induces phosphorylation of NIPP1 by Cdk9/cyclin T1.
It is not yet clear what are the substrates of PP1 during HIV-1 transcription. Because PP1 may function as a CTD phosphatase (8) and because CTD phosphorylation regulates transcriptional elongation (2), it is possible that PP1 catalyzes a transient dephosphorylation of the CTD during Tat-dependent transcription. Our preliminary studies using chromatin immunoprecipitation assays showed that Tat induces PP1 to dephosphorylate Ser-2 CTD of promoter proximal, but not promoter distal RNAPII (unpublished observations). In addition, PP1 may be involved in dephosphorylation of other proteins, such as autophosphorylated Cdk9. Cdk9 phosphorylation is important for the binding of Cdk9/cyclin T1:Tat complex to TAR RNA (30). Our preliminary experiments indicate that both PP2A and PP1 dephosphorylate autophosphorylated Cdk9 in vitro (unpublished observations), but it remains to be examined which of the two phosphatases dephosphorylates Cdk9 during transcription.

The finding that PP1, and perhaps also NIPP1, have a role in the regulation of HIV-1 transcription will open up a more general question of whether PP1 regulates the transcription of host cellular genes as well. It is possible that HIV-1 Tat, serving to recruit Cdk9, activates the NIPP1/PP1 holoenzyme in order to upregulate transcription of HIV-1 genes. Alternatively, PP1 may have an unrecognized role in the general regulation of the transcription of cellular genes. A more detailed study will address the questions of the regulation of different cellular promoters by PP1, the identification of the substrates for PP1 during HIV-1 and non-HIV-1 transcription, and the effects of Tat on dephosphorylation of these substrates by PP1. Uncovering a novel regulatory pathway of Tat-mediated transcription may provide additional valuable targets for HIV-1 therapeutics.
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References


LEGENDS OF THE FIGURES

Fig. 1. NIPPI Inhibits Tat-induced HIV-1 transcription. (A) Expression of EGFP-fused WT NIPPI-(1-351) (lane 1), C-terminally truncated NIPPI-(1-329) (lane 2) and NIPPI-(1-351) K193-197A/V201A/F203A/Y335D (NIPPI-(1-351) PI, lane 3) which is defective in PP1 binding. NIPPI and its mutants were expressed in COS-7 cells, proteins were resolved on 10% SDS-PAGE, transferred to a membrane and probed with antibodies against EGFP (B-C).

Expression of NIPPI inhibits Tat-dependent transcription in HEK 293 cells. Cells transfected with HIV-1 LTR-LacZ (JK2) expression vector (lane 1); JK2 and Tat expression vector (Tat) (lane 2); JK2, Tat and NIPPI-(1-351)-EGFP (NIPPI wt) (lane 3), JK2, Tat and NIPPI-(1-351) K193-197A/V201A/F203A/Y335D (NIPPI PI) (lane 4) or JK2, Tat and NIPPI-(1-329)-EGFP (NIPPI 1-329) (lane 5) expression vectors. At 48 hours posttransfection, the cells were either fixed and stained with X-gal (panel B), or lysed and analyzed for β-galactosidase activity with ONPG (panel C). (D) Expression of NIPPI wt, NIPPI PI and NIPPI-(1-329) mutants equally inhibits basal HIV-1 transcription in HEK 293 cells. Cells were transfected with JK2 (lane 1) and with the indicated amounts of expression vectors for EGFP-fused wild type NIPPI (lane 2), NIPPI-PI (lane 3) or NIPPI-(1-329) mutant (lane 4). (E) Expression of NIPPI inhibits Tat-dependent HIV-1 transcription in COS-7 cells. Cells were transfected with JK2 or with a TAR deleted construct (JK2ΔTAR), with or without the Tat expression vector and with the indicated amounts of expression vectors for EGFP-fused wild type NIPPI wt or NIPPI PI mutant. For statistical evaluation 4 to 5 samples were transfected independently in each experiment for each plasmid combination and each experiment was repeated 3-5 times.
Fig. 2. Inhibition of PP1 down regulates Tat-induced transcription. (A) Expression of EGFP-fused NIPP1 and PP1. The central domain of NIPP1 (NIPP1-(143-224), lane 1), NIPP1-(143-224) RATA (lane 2), and PP1γ (lane 3) were expressed in COS-7 cells, proteins were resolved on 10% SDS-PAGE, transferred to a membrane and probed with antibodies against EGFP. (B) Central domain of NIPP1 inhibits Tat-induced transcription. COS-7 cell were transfected with the HIV-1 LTR-LacZ (pJK2) expression vector alone (lane 1); JK2 and Tat expression vector (Tat) (lane 2); JK2, Tat and EGFP-NIPP1-(143-224) (wt) (lane 3); and JK2, Tat and EGFP-NIPP1-(143-224) V201A/F203A (RATA) (lane 4). (C) Expression of PP1γ rescues NIPP1 mediated inhibition of Tat-induced HIV-1 transcription. COS-7 cell were transfected with the HIV-1 LTR-LacZ (pJK2) expression vector alone (lane 1); JK2 and Tat expression vector (Tat) (lane 2); JK2, Tat and EGFP-PP1γ (lane 3); JK2, Tat and EGFP-NIPP1 (lane 4); and JK2, Tat, EGFP-PP1γ and EGFP-NIPPI (lane 5). At 48 hours posttransfection, the cells were lysed and analyzed for β-galactosidase activity with ONPG.

Fig. 3. Expression of NIPP1 does not inhibit expression of Tat. COS-7 cells grown on microscope slides were transfected with vectors expressing Tat and EGFP fused NIPP1-(1-351) (NIPP1-EGFP) or NIPP1-(1-351) K193-197A/V201A/F203A/Y335D-EGFP (NIPP1-PI-mutant-EGFP) (panels A to C). Panel D expression of Tat alone. Panel E, untransfected cells. Cells were fixed and immunostained with anti-Tat polyclonal antibodies.
Fig. 4. **NIPP1 inhibits Tat-transactivation induced by extracellular Tat.** COS-7 cell were transfected with the HIV-1 LTR-LacZ (pJK2) expression vector (lane 1 to 4) or with a TAR deleted construct (JK2ΔTAR) (lanes 5 to 8). In addition cells were cotransfected with expression vector for EGFP fused NIPP1-(1-351) (NIPP1 wt) (lanes 3 and 7) or NIPP1-(1-351) K193-197A/V201A/F203A/Y335D (NIPP1 PI) (lanes 4 and 8). Tat transactivation was induced by purified Tat protein added to the culture media of COS-7 cells (lanes 2, 3, 4 and 6). At 48 hours posttransfection, the cells were lysed and analyzed for β-galactosidase activity with ONPG.

Fig. 5. **Adeno-associated virus (AAV) –mediated delivery of NIPP1 inhibits Tat-induced HIV-1 transcription from integrated HIV-1 promoter.** (A), HeLa-MAGI cells containing stably integrated copy of HIV-1 LTR LacZ were infected with adeno-associated viruses expressing EGFP-fused NIPP1-(1-351) (AAV-NIPP1 wt) (lane 3) or NIPP1-(1-351) K193-197A/V201A/F203A/Y335D (AAV-NIPP1 PI) (lane 4). At 48 hours post infection Tat-transactivation was induced by infection with adenovirus encoding Tat (lanes 2 to 4). After 24 hours incubation cells were lysed and analyzed for β-galactosidase activity with ONPG.

(B) HeLa MAGI cells infected with AAV-NIPP1, AAV-NIPP1 PI or with adenovirus expressing EGFP (Adeno-EGFP). Cells were grown in 96-well plate and infected with the indicated recombinant adeno-associated virus. Photographs were taken 48 hours post infection using Olympus CKX41 microscope and Kodak DC120 digital camera.
Fig. 6. Adeno-associated virus (AAV)–mediated delivery of NIPPI inhibits HIV-1 viral replication. (A), HeLa-MAGI cells containing stably integrated copy of HIV-1 LTR LacZ and expressing CD4 receptor were infected with adeno-associated viruses expressing EGFP-fused NIPPI-(1-351) (AAV-NIPPI wt) (lane 2) or NIPPI-(1-351) K193-197A/V201A/F203A/Y335D (AAV-NIPPI PI) (lane 3). At 48 hours post infection, cells were infected with T-tropic HIV-1 (lanes 1 to 3) or with adenovirus encoding Tat (lane 4). After 48 hours incubation cells were fixed and stained with X-gal. Bars represent the number of blue cells counted in 3 to 4 non-overlapping fields. (B-F) Representative pictures of X-Gal stained cells from panel A. B. Shown are cells infected with HIV-1 alone (B); cells infected with AAV-NIPPI (C) or AAV-NIPPI PI (D) prior to infection with HIV-1; cells infected with Adeno-Tat (E) and control uninfected cells (F). Cells were fixed and stained with X-gal 48 hours after infection. Photographs were taken using Olympus CKX41 microscope and Kodak DC120 digital camera.

Supplement Fig.1 HIV-1 LTR and not Tat is a limiting factor in transcription. COS-7 (panels A and B) or HEK293 cells (panels C and D) grown in 96-well plate were transfected with a constant amount of JK2 (50 ng) and a variable amount of Tat expression vector (panels A and C); or with a constant amount of Tat expression vector (25 ng) and a variable amount of JK2 (panels B and C).
Ammosova et al., Fig. 3

A. Texas Red  B. EGFP-Fluorescence  C. TexasRed + EGFP-Fluorescence

Tat + NIPP1-EGFP

Tat + NIPP1 – PI-mutant -EGFP

Tat Alone

D.  E. Control
Ammosova et al., Fig. 4

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Transactivation, Fold

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7
A

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B

- AAV-NIPP1
- AAV-NIPP1 PI
- Adeno-EGFP

Transactivation, Fold

0 10 20 30 40 50 60 70 80

1 2 3 4
Ammosova et al., Fig. 6

A. Control
B. HIV-1
C. AAV-NIPP1 wt + HIV-1
D. AAV-NIPP1 PI + HIV-1
E. Adeno-Tat
F. Control

HIV-1  +  +  +  +  -
Adeno-Tat - - - +
AAV –NIPP1 - wt PI -

Blue cells, %
0  50  100
1  2  3  4

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Ammosova et al., Supplement Fig. 1

**A**

Titration of Tat

Transactivation, Fold

COS Cells

CMV-Tat, ng

0
20
40
60

0
20
40
60

**B**

Titration of JK2

Transactivation, Fold

0
20
40
80
120

0
40
100
200

**C**

Transactivation, Fold

293 Cells

CMV-Tat, ng

0
20
40
60

0
4
8
12

**D**

Transactivation, Fold

JK2, ng

0
40
80
120

0
4
8
12

Downloaded from http://www.jbc.org/ on January 28, 2018.
Nuclear protein phosphatase-1 regulates HIV-1 transcription
Tatyana Ammosova, Marina Jerebtsova, Monique Beullens, Yaroslav Voloshin, Patricio Ray, Ajit Kumar, Mathieu Bollen and Sergei Nekhai

J. Biol. Chem. published online June 4, 2003

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