Expression of a Protein Phosphatase 1 Inhibitor, cdNIPP1, Increases CDK9 Threonine 186 Phosphorylation and Inhibits HIV-1 Transcription*⒮

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CDK9/cyclin T1, a key enzyme in HIV-1 transcription, is negatively regulated by 7SK RNA and the HEXIM1 protein. Dephosphorylation of CDK9 on Thr[186] by protein phosphatase 1 (PP1) in stress-induced cells or by protein phosphatase M1A in normally growing cells activates CDK9. Our previous studies showed that HIV-1 Tat protein binds to PP1 through the Tat Q[35]VCF[38] sequence, which is similar to the PP1-binding RV_X_F motif and that this interaction facilitates HIV-1 transcription. In the present study, we analyzed the effect of expression of the central domain of nuclear inhibitor of PP1 (cdNIPP1) in an engineered cell line and also when cdNIPP1 was expressed as part of HIV-1 pNL4-3 in place of _nef_. Stable expression of cdNIPP1 increased CDK9 phosphorylation on Thr[186] and the association of CDK9 with 7SK RNA. The stable expression of cdNIPP1 disrupted the interaction of Tat and PP1 and inhibited HIV-1 transcription. Expression of cdNIPP1 as a part of the HIV-1 genome inhibited HIV-1 replication. Our study provides a proof-of-concept for the future development of PP1-targeting compounds as inhibitors of HIV-1 replication.

Cyclin-dependent kinase-9 (CDK9)[3]/cyclin T1 is a protein kinase that phosphorylates the C-terminal domain of the largest subunit of RNA polymerase II during transcription elongation (1). CDK9/cyclin T1 is part of positive transcription elongation complex (P-TEFb) and present in the cells within large and small complexes. The large complex contains 7SK RNA (2, 3) and the hexamethylene bisacetamide-induced protein (HEXIM1) (4, 5). The activity of CDK9 in the large complex is inhibited by the interaction with 7SK RNA and HEXIM1 (2–5). In stress-induced cells, CDK9/cyclin T1 dissociates from the 7SK RNA and HEXIM1 protein and then binds to the bromodomain protein 4 (Brd4) (6, 7), a member of bromodomain-containing extraterminal domain protein family that interact with acetylated histones. CDK9/cyclin T1 bound to Brd4 forms the transcriptionally active P-TEFb (7). Brd4 recruits P-TEFb to the promoters of cellular genes that are expressed at the end of mitosis (8). HIV-1 Tat protein can also recruit P-TEFb by binding directly to CDK9/cyclin T1 and targeting it to HIV-1 TAR RNA (7). Hence, Tat induces HIV-1 transcription by recruiting CDK9/cyclin T1 (9–11) and histone acetyltransferases to the HIV-1 promoter (7, 12–14). The HIV-1 Tat prevents the formation of the large complex and promotes the dissociation of CDK9/cyclin T1 from 7SK RNA/HEXIM1 complex (15). The mechanism of this Tat-mediated dissociation of CDK9/cyclin T1 and 7SK RNA/HEXIM1 involves the dephosphorylation of CDK9 on Thr[186] (16, 17). Protein phosphatase 1 (PP1) dephosphorylates Thr[186] of CDK9 *in vitro*, and the dephosphorylated CDK9/cyclin T1 does not associate with 7SK RNA (16). In stress-induced cells, PP1α dephosphorylates Thr[186] of CDK9 and in cooperation with protein phosphatase 2B (PP2B) disrupts the interaction between CDK9/cyclin T1 and 7SK RNA/HEXIM1 (18). In normally growing cells, protein phosphatase M1A (PPM1A) dephosphorylates CDK9 on Thr[186] (19).

Our previous studies showed that PP1 stimulates Tat-dependent HIV-1 transcription *in vitro* (20) and in cultured cells (21). PP1 holoenzymes consist of a constant catalytic subunit (PP1α, PP1β/δ, or PP1γ) and a variable regulatory subunit that determines the localization, activity, and substrate specificity of the phosphatase (22, 23). One of the major nuclear regulators of PP1 is NIPP1 (nuclear inhibitor of PP1), which inhibits the dephosphorylation of a wide range of PP1 substrates (22, 23). Tat-mediated HIV-1 transcription is blocked by the expression of NIPP1, and the inhibition is reversed by the co-expression of PP1γ (21). Tat contains a PP1-binding

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[3] The abbreviations used are: CDK9, cyclin-dependent kinase-9; Brd4, bromodomain protein 4; cdNIPP1, central domain NIPP1; EGFP, enhanced green fluorescent protein; HEXIM1, hexamethylene bisacetamide-induced protein; NIPP1, nuclear inhibitor of PP1; P-TEFb, positive transcription elongation complex; PP1, protein phosphatase 1; PP1C, catalytic subunit of PP1; PP2B, protein phosphatase 2B; PPM1A, protein phosphatase M1A; TAR RNA, transactivation response RNA.

motif and potentially functions as a PP1 regulatory subunit (24). Mutation of residues in the PP1-binding motif (V36A and F38A) prevents Tat from inducing HIV-1 transcription and the translocation of PP1 to the nucleus (24). We showed that in cultured cells, PP1, but not PP2A, likely dephosphorylates CDK9 and contributes to the regulation of activated HIV-1 transcription (25).

In the present paper, we analyzed the effect of the expression of cdNIPP1, an inhibitory 82-residue PP1-binding fragment of the central domain of NIPP (cdNIPP1), on the CDK9 phosphorylation and activity and HIV-1 transcription. We further analyzed the effect of cdNIPP1 expression on HIV-1 replication in a physiologically relevant manner by expressing cdNIPP1 as part of the HIV-1 genome in place of *nef*. Our results indicate that the expression of PP1 inhibitor suppresses HIV-1 replication.

EXPERIMENTAL PROCEDURES

Materials—293T cells were purchased from American Type Cultue Collection (Manassas, VA). The 84-31 line is a subclone of 293 cells that stably expresses E4 of Ad and that supports growth of adeno-associated virus (26). The catalytic subunit of PP1 (PP1C) was purified from rabbit skeletal muscle as previously described (27). WT Tat was expressed in *Escherichia coli* from pGEM2 Tat vector obtained from the NIH AIDS Research and Reference Reagent Program and purified on an Aquapore RP-300 column (Applied Biosystems) by reverse-phase chromatography as we described previously (28). Rabbit polyclonal antibodies to CDK9, rabbit polyclonal antibodies to cyclin T1, and rabbit polyclonal antibodies to enhanced green fluorescent protein (EGFP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tat rabbit polyclonal antibodies were received from AIDS Research and Reference Reagents Program (National Institutes of Health). Anti-α-tubulin and anti-FLAG monoclonal antibodies were purchased from Sigma. Rabbit anti-PP1γ antibodies were from Calbiochem. Anti-CDK9 phospho-Thr[186], anti-Brd4, and anti-HEXIM1 polyclonal antibodies were kind gifts from Dr. Qiang Zhou (University of California, Berkeley). Monoclonal antibodies specific to the unphosphorylated form of the C-terminal domain (8WG16) or C-terminal domain phosphorylated on serine 2 (H5) were purchased from BabCo (Richmond, CA). Rabbit polyclonal anti-NIPP1 antibodies were described in Ref. 29.

293T-cdNIPP1 Cells—293T cells stably expressing the central domain of NIPP1 (amino acids 143–224) were generated by transfection of NIPP1-(143–224)-EGFP and limiting dilution cloning. Genomic DNA was isolated from parental 293T cells and three different clones that were stably transformed with NIPP1-(143–224)-EGFP. About 500 ng of these genomic DNAs were used as a template in a PCR with the sense primer (5′-ATGGGTGGAGAGGATGATGAACTC-3′), corresponding to nucleotides 436–459 of bovine NIPP1 cDNA (accession number Z50748) and the antisense primer (5′-CGTCGCCGTCCAGCTCGACCAG-3′), corresponding to the inverse complement of nucleotides 724–745 of pEGFP-N1 vector (Clontech). The amplified PCR products were subcloned in

the pGEM-T Easy vector (Promega) and sequenced by T7 and SP6 primer.

Plasmids—The expression vectors for EGFP-fused central domain of NIPP1, NIPP1-(143–224), or NIPP1-(143–224) V201A/F203A (RATA mutant) were described previously (30). NIPP1 mutated within its RV*X*F motif has low binding affinity for PP1 (30). 7SK expression vector was a gift from Dr. Shona Murphy (Oxford University, UK). FLAG-CDK9 expression vectors were kind gifts from Dr. Qiang Zhou. Expression vectors for PP1γ-EGFP and for PP1γ-D64N-EGFP are described (31).

RT-PCR to Detect 7SK RNA—RNA was isolated from complexes that co-immunoprecipitated with anti-CDK9 antibodies using TRIzol reagent according the Invitrogen protocol. RNA was reverse-transcribed using a SuperScript II kit (Invitrogen) with the 7SK reverse primer. The following primer sequences were used: forward, 3′-GGATGTGAGGCGATCT-GGCTG-5′ and reverse, 3′-TAAAGAAAGGCAGACTGC-CAC-5′. These primers were used in a PCR that followed the RT reaction. In semiquantitative PCR, products were resolved on 2% agarose gel and photographed. In real time PCR, PCRs were run with Syber Green on a MioQ PCR machine and quantified using cdNIPP1 expression vector as control for the determination of copy number.

Fractionation of Cellular Lysates on Glycerol Gradient—293T and 293T-cdNIPP1 cells were grown in 100-mm plates and then lysed with 0.5 ml of whole cell lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1% Nonidet P-40, 0.1% SDS) supplemented with protease inhibitors mixture (Sigma). Cell lysates were clarified by centrifugation for 30 min at $10,000 \times g$ and loaded on top of a 10–30% glycerol (9-ml) gradient. Glycerol gradient buffer contained 20 mM HEPES-KOH, pH 7.9, 150 mM KCl, 200 μM EDTA. The gradient was spun in SW 41Ti rotor (Beckman) at 38,000 rpm for 18 h. Fractions (0.5 ml) were collected through a needle inserted into the bottom of the tube and analyzed by immunoblotting using the indicated antibodies. The x-ray films with the immunoblots for NIPP1 and CDK9 phosphorylated on Thr[186] were scanned and quantified using OptiQuant software from Packard Cyclone PhosphorImager (Perkin-Elmer). The pixels were adjusted to have the same total amount for the 293T and the cdNIPP1 extracts, and the normalized results were graphed.

Effect of cdNIPP1-derived Peptides on HIV-1 Transcription in CEM-GFP Cell Activated by Recombinant Tat—CEM cells containing integrated HIV-1 LTR *EGFP* (CEM-GFP obtained from the NIH AIDS Research and Reference Reagent Program) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% glutamine, 1% antibiotic solution (penicillin and streptomycin), and 500 μg/ml G418. CEM-GFP cells were treated in a 96-well plate with purified recombinant Tat added at 0.8 μg/300,000 cells per well in the presence of 100 μM chloroquine and, where indicated supplemented with 50 μM KNSRVTFSED or KNSRATASED peptides. After an 18-h incubation, the cells were precipitated by centrifugation and lysed for 20 min at room temperature in 50 μl of lysis buffer, containing 20 mM HEPES at pH 7.9, 0.1% Nonidet P-40, and 5 mM EDTA and transferred into 150 μl of PBS. The fluorescence was measured with 480-nm excitation

and 510-nm emission on a Luminescence Spectrometer LS50B (Perkin-Elmer Life Sciences) equipped with a robotic 96-well scanner.

Quantitative RT-PCR to Analyze NIPP1 Expression—RT was performed using the Superscript[TM] RT-PCR kit (Invitrogen). Random hexamers were used for RT-PCR. Primers for PCR were designed for β-actin (forward, GCGG-GAAATCGTGCGTGCGTGACATT; reverse, GATGGAGT-TGAAGGTAGTTTCGTG) and NIPP1 (forward, AGAAT-TCAACACTGCCACA; reverse, CACCCGCTTC TTCTTGACTG). Real-time PCR was performed with SYBR Green Super Mix (Bio-Rad) at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s for 45 cycles on a MyiQ real time PCR detection system (Bio-Rad). The obtained Ct numbers were converted to the cDNA copy numbers using as a calibration, amplification of NIPP1-expressing plasmid, and primers for NIPP1.

Cloning cdNIPP1 into pNL4-3—We used a 398-6 shuttle vector which was created by subcloning of BamHI-NcoI fragment from pNL4-3 containing *nef* sequence into a pUC vector and introducing CTCGAGTCTAGAGCGGCCGCTTCGA linker with XhoI, XbaI, and NotI sites into the XhoI site of the *nef* sequence. Then 398-6 shuttle vector was digested with BamHI and NcoI. The 2-kb fragment containing the linker was subcloned back into the Nco-BamH1 sites of pNL4-3 vector. The insert into the *nef* gene leads to the deletion of the first 36 amino acids of *nef*, which are critical for its function (32). The pNL4-3 398 vector was used as positive control in the infection experiment. To subclone cdNIPP1, pNL4-3 398 vector was digested with XhoI and NotI restriction enzymes, and the backbone was used to subclone the NIPP1-EGFP fragment that was obtained by digestion with XhoI and NotI from the WT and mutant NIPP1-(143–224)-EGFP expression vector. Correct cloning was verified by sequencing. Nuclear EGFP expression was verified by fluorescence in transfected 293 cells, and cdNIPP1 expression was verified by Western blotting using anti-NIPP1 antibodies.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay—LDH release, used as an indicator of cell membrane damage, was measured in the culture medium using an LDH assay kit (LDH Cytotoxicity Assay kit; BioVision, Mountain View, CA). Briefly, the medium was collected and mixed with 100 μl of reaction mixture provided by the manufacturer and incubated for 30 min at room temperature protected from light. High control was generated by treating cells with 1% Triton X-100 for 2 h prior to the beginning of the assay. The colorimetric change was measured with a microtiter reader (Labsystem Multiskan) at 450 nm.

Analysis of HIV-1 Replication—HIV-1 viruses were prepared by transfecting HeLa cells with genomic pNL4-3, pNL4-3 398, pNL4-3-cdNIPP1-EGFP, and pNL4-3-cdNIPP1mut-EGFP and collecting media at 72 h after transfection. Viral RT activity was determined and adjusted in the collected media. RT was measured in 10 μl incubated in a 96-well plate with RT reaction mixture containing $1\times$ RT buffer (50 mM Tris-HCl, 1 mM DTT, 5 mM MgCl$_2$, 20 mM KCl), 0.1% Triton X-100, 10^{-2} units poly(A), 10^{-2} units poly(dT), and [³H]TTP. The mixture was incubated overnight at 37 °C, and 5 μl of the reaction mix was spotted on a DEAE Filtermat paper, washed four times with 5% Na$_2$HPO$_4$ and three times with water, and then dried completely. RT activity was measured in a Betaplate counter (PerkinElmer Life Sciences). MT4 cells were grown to 70% confluence, inoculated with pNL4-3 viruses, and medium was collected at the indicated time points. The RT activity was determined as described above.

RESULTS

Expression of cdNIPP1 Redistributes Cellular Endogenous NIPP1 and Increases CDK9 Phosphorylation and Its Association with 7SK RNA—We analyzed the effect of the expression of a PP1 inhibitor in an engineered cell line that stably expressed the central, inhibitory domain of NIPP1 (residues 143–224) fused to EGFP (Fig. 1*A*, lane 2). By Western blotting analyses, the expression of CDK9 or PP1γ was not affected in these cells (Fig. 1*A*, lanes 1 and 2). Next, the distribution of NIPP1 and CDK9 phosphorylated on Thr[186] between small and large molecular weight complexes was analyzed in whole cell extracts prepared from 293T or 293T-cdNIPP1 cells after fractionation on glycerol gradients (Fig. 1*B*). In 293T-cdNIPP1 cells, cdNIPP1-EGFP expression was detected, and the WT NIPP1 was shifted toward the lower molecular weight complexes, compared with 293T cells (Fig. 1*B*, *NIPP1 panels*). Quantification of the band densities of NIPP1 immunoblots after adjusting the pixels to equalize the total amount of NIPP1 confirmed this observation (Fig. 1*B*). By comparison, there was no difference in the distribution of CDK9 (Fig. 1*B*, *CDK9 panel*, quantification not shown) and a slight difference in PP1 distribution (Fig. 1*B*, *PP1 panel*). The amount of CDK9 phosphorylated on Thr[186] was increased in the higher molecular weight fractions (fractions 7–10) of 293T-cdNIPP1 cells (Fig. 1*B*, *CDK9-T186P panel*). These results suggest that endogenous NIPP1 is redistributed in 293T-cdNIPP1 cells toward the smaller molecule fractions and that CDK9 phosphorylated on Thr[186] is present in higher molecular weight fractions. There were no changes in the distribution of HEXIM1, cyclin T1, or Brd4 in 293T-cdNIPP1 cells *versus* 293T cells (data not shown).

Because the phosphorylation of CDK9 on Thr[186] promotes the association of CDK9/cyclin T1 with 7SK RNA (16, 17), we analyzed whether the increased CDK9 phosphorylation affected the association of CDK9 with 7SK RNA. Analysis by quantitative real time PCR showed an increase in the amount of 7SK RNA that precipitated with CDK9 from 293T-cdNIPP1 cells compared with 293T cells (Fig. 1*C*). These results were replicated in a semiquantitative RT-PCR, which showed 7SK RNA co-precipitating with CDK9 from 293T-cdNIPP1 cell lysate (Fig. 1*D*, *lane 8*) in contrast to a little co-precipitation of 7SK RNA with CDK9 from 293T cells (Fig. 1*D*, *lane 4*). Pretreatment of the CDK9/7SK complex immunoprecipitated from 293T-cdNIPP1 cells with purified PP1c removed 7SK RNA (Fig. 1*D*, compare *lanes 8* and *9*), suggesting that association of 7SK RNA with CDK9 is phosphorylation-dependent and sensitive to dephosphorylation by PP1. Taken together, these results indicate that CDK9 is additionally phosphorylated on Thr[186] in 293T-cdNIPP1 cells and that

A

B

C

D
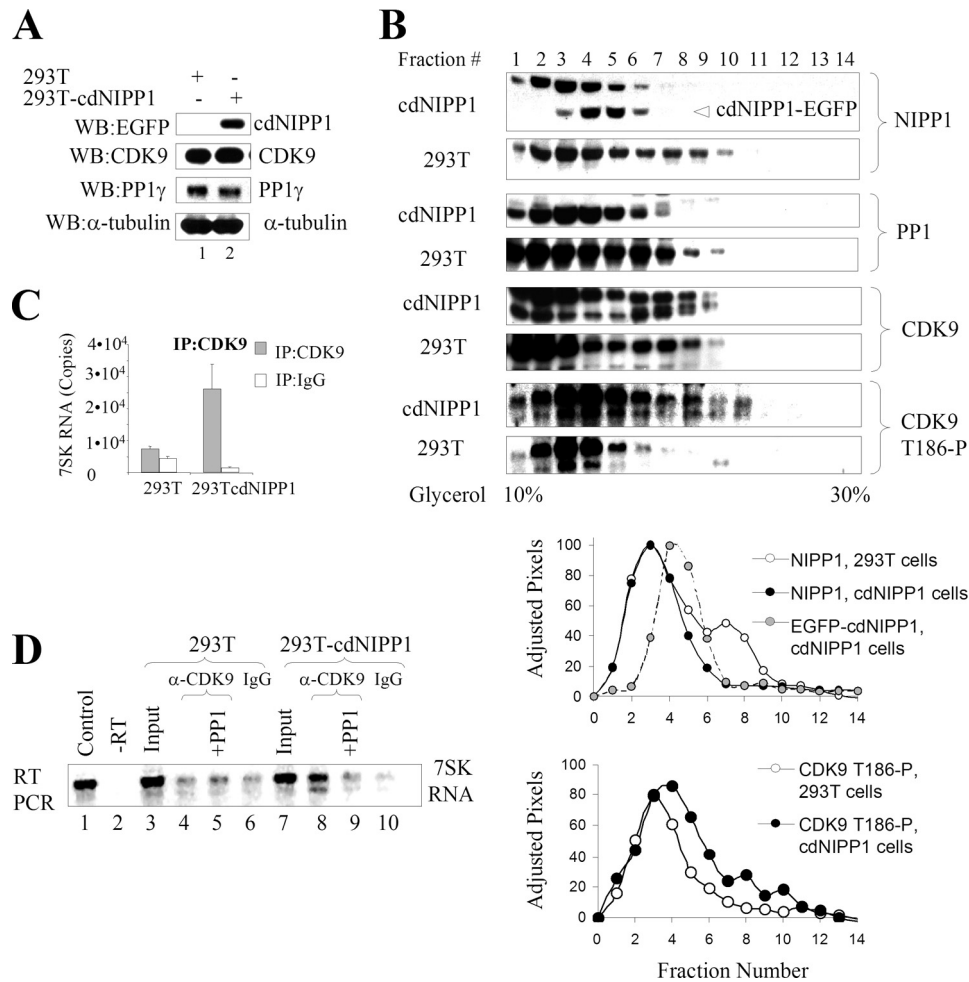
FIGURE 1. **Effect of cdNIPP1 expression on NIPP1 distribution, CDK9 phosphorylation, and association with 7SK RNA.** *A*, analysis of 293T-cdNIPP1 cells is shown. Western blot analysis of 293T and 293T-cdNIPP1 cells with antibodies for EGFP (to check for the expression of cdNIPP1-EGFP), CDK9, PP1γ, and α-tubulin is shown. *B*, expression of cdNIPP1 changes NIPP1 distribution and increases CDK9 phosphorylation on Thr[186]. Whole cell extracts were prepared from 293T and 293T-cdNIPP1 cells and separated on glycerol gradients by ultracentrifugation. The gradients were fractionated and analyzed by Western blotting for NIPP1, PP1, CDK9, and CDK9 phosphorylated on Thr[186]. The NIPP1 and CDK9 phosphorylated on Thr[186] immunoblots were scanned and quantified using PhosphorImager OptiQuant software. The pixels were adjusted to have the same total amount for the 293T and the cdNIPP1 extracts, and the normalized results are graphed (*lower panels*). *C*, expression of cdNIPP1 increases the association of CDK9 with 7SK RNA. RNA was isolated from material that co-precipitated with CDK9 from 239T or 293T-cdNIPP1 whole cell lysates. The RNA was reverse transcribed and analyzed by real time PCR. Results are presented as numbers of copies of 7SK RNA. *D*, PP1 removes CDK9-associated 7SK RNA. RNA was isolated from material that co-immunoprecipitated with CDK9 from 239T or 293T-cdNIPP1 cells, reverse-transcribed, and analyzed by semiquantitative PCR (26 cycles) using 7SK-specific primers. *Lane 1*, control using 7SK expression vector. *Lane 2*, control that was not reverse-transcribed. *Lanes 3* and *7* are inputs. *Lanes 4* and *8*, 7SK RNA associated with CDK9 in 293T or 293T-cdNIPP1 whole cell lysates. *Lanes 5* and *9*, immunoprecipitated CDK9 was treated with purified rabbit muscular PP1C prior to the RNA extraction. *Lanes 6* and *10*, controls in which the immunoprecipitations were carried out with nonspecific rabbit IgG.

phosphorylated CDK9 is associated with 7SK RNA in high molecular weight fractions.

Interaction of Tat with PP1 Is Altered in 293T-cdNIPP1 Cells—To investigate the interaction of Tat with PP1, FLAG-tagged Tat was expressed in 293T and 293T-cdNIPP1 cells and then immunoprecipitated from cellular extracts with anti-FLAG antibodies (Fig. 2*A*). The amount of FLAG-Tat expressed in 293T-cdNIPP1 cells was less than in 293T, and less Tat protein was immunoprecipitated from mutant cell line whole cell lysate (Fig. 2*A*, compare *lanes 3* and *4*). The amount of CDK9 co-precipitated with Tat was slightly reduced in 293T-cdNIPP1 cells (Fig. 2*A*, *lanes 3* and *4*). In contrast, the amount of PP1 co-precipitated with Tat was reduced substantially in 293T-cdNIP1 cells (Fig. 2*A*, *lanes 3* and *4*), even comparing with a reduced amount of Tat precipitated

from the mutated cell line. Thus, the expression of cdNIPP1 reduced the amount of PP1 available to bind Tat.

HIV-1 Transcription Is Inhibited in 293T-cdNIPP1 Cells—To investigate the effect of the stable expression of cdNIPP1 on HIV-1 transcription, 293T and 293T-cdNIPP1 cells were transiently transfected with HIV-1 LTR-Lac Z expression vector alone or in combination with Tat (Fig. 2*B*), and the β-galactosidase results were normalized to the amount of plasmid DNA in the cells, which was determined by real time PCR. Tat-induced HIV-1 transcription was reduced significantly in 293T-cdNIPP1 cells compared with parental 293T cells (Fig. 2*B*, *lane 2*). Basal HIV-1 transcription from a mutant HIV-1 LTR with a deletion of the TAR RNA sequence (HIV-1 LTRΔTAR) was also reduced in 293T-cdNIPP1 (Fig. 2*B*, *lane 3*). Although the CMV-*LacZ* transcription was also reduced,
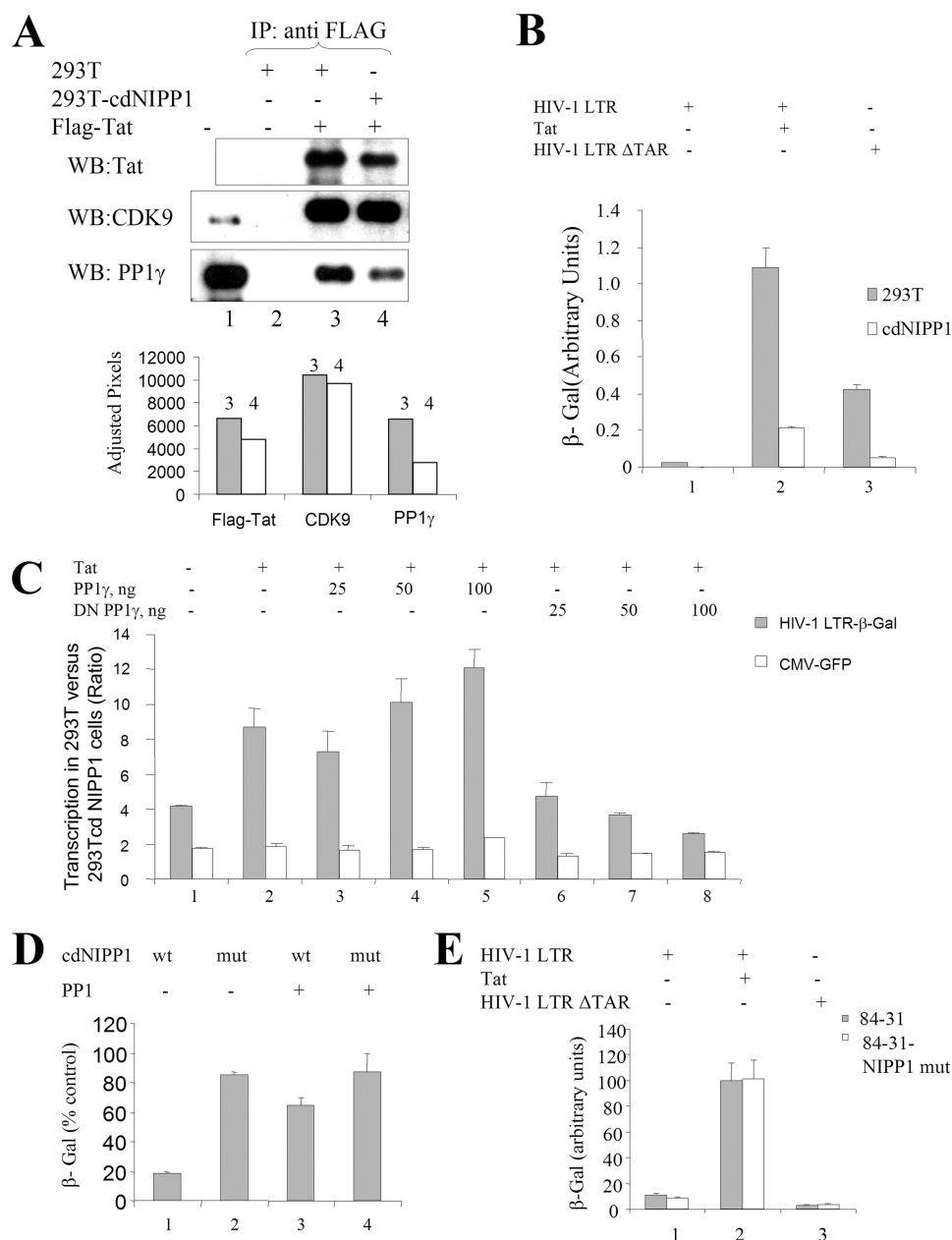
FIGURE 2. **Expression of cdNIPP1 inhibits HIV-1 transcription.** *A*, expression of cdNIPP1 disrupts the interaction of Tat and PP1. 293T and 293T-cdNIPP1 cells were transfected with FLAG-Tat expression vectors. Tat and associated proteins were immunoprecipitated with anti-FLAG antibodies, resolved on SDS-PAGE, and probed with antibodies against Tat, CDK9, and PP1γ. *Lane 1*, inputs. *Lane 2*, 293T cells without FLAG-Tat expressed. *Lanes 3* and *4*, 293T and 293T-cdNIPP cells, respectively, transfected with FLAG-Tat. *B*, expression of cdNIPP1 inhibits Tat-induced HIV-1 transcription. 293T and 293T-cdNIPP1 cells were transiently transfected with HIV-1 LTR-*LacZ* without (*lane 1*) or with co-transfection of Tat expressing plasmid (*lane 2*); or a TAR deleted construct of HIV-1 LTR-*LacZ* (HIV-1 LTRΔTAR) (*lane 3*). At 24 h after the transfection, the cells were lysed and analyzed for β-galactosidase activity. HIV-1 LTR-expression plasmid was analyzed by real time PCR and used for normalization. *C*, co-expression of cdNIPP1 and PP1γ or PP1γ-D64N-EGFP (DN PP1γ-EGFP) affects basal and Tat-induced HIV-1 transcription. 293T and 293T-cdNIPP1 cells were transfected with HIV-1 LTR-*LacZ* (*lane 1*) or in combination with Tat expression vector (*lanes 2–8*) and the indicated amounts of PP1γ (*lanes 3–5*) or DN PP1γ (*lanes 6–8*) expression vectors and co-transfected with CMV-EGFP expression vector. At 24 h the cells were lysed and analyzed for β-galactosidase and EGFP fluorescence. *D*, expression of PP1γ rescues cdNIPP1-mediated inhibition of Tat-induced HIV-1 transcription. 293T cells were transfected with HIV-1 LTR-*LacZ* and Tat expression vectors and with WT cdNIPP1 (*lanes 1* and *3*) or mutant cd-NIPP1 (*lanes 2* and *4*) expression vectors and with PP11γ expression vector (*lanes 3* and *4*). At 48 h after transfection, the cells were lysed and analyzed for β-galactosidase activity. *E*, expression of mutant NIPP1 does not affect HIV-1 transcription. 84-31 cells and 84-31 cells stably expressing mutant NIPP1 were transfected with HIV-1 LTR-*LacZ* without (*lane 1*) or with co-transfection of Tat-expressing plasmid (*lane 2*); or HIV-1 LTRΔTAR (*lane 3*). At 24 h the cells were lysed and analyzed for β-galactosidase activity.

the effect on HIV-1 promoter was much stronger (factor 4–10 compared with factor 2 for CMV promoter, Fig. 2*C*) and exceeded the effect on CMV promoter. Overexpression of PP1γ increased HIV-1 transcription in 293T cells over cdNIPP1 cells, and overexpression of PP1γ-D64N-EGFP (DN

PP1γ) decreased the transcription (Fig. 2*C*, *lanes 3–5* and *lanes 6–8*), suggesting the involvement of PP1. Co-expression of PP1γ reduced the inhibitory effect of the cdNIPP1 expression on Tat-induced HIV-1 transcription (Fig. 2*D*). Stable expression of mutant NIPP1 that does not bind PP1 in 84-31

cells did not have an effect on basal or Tat-induced HIV-1 transcription (Fig. 2*E*). To rule out the possibility that expression of cdNIPP1 causes general cellular toxicity, we analyzed the effect of cdNIPP1 expression on the release of LDH, which is an indicator of cell membrane damage. Measurement of LDH in the medium of 239T cells transfected with EGFP alone was similar to the LDH levels released from cells transfected with EGFP-fused WT or mutant NIPP1, cdNIPP1 or WT or DN PP1γ (supplemental Fig. S1*A*). The effect of chemically synthesized KNSRVTFSED peptide derived from the central domain of NIPP1 was analyzed in CEM-GFP T cells containing integrated HIV-1 LTR-EGFP reported which was activated by the addition of purified Tat protein to the culture medium (33). Addition of purified peptides KNSRVTFSED had small inhibitory and KNSRATASED, slightly stimulatory effect on LTR-driven EGFP expression (supplemental Fig. S1*B*), confirming the results obtained above with stable expression of cdNIPP1.

Analysis by real time PCR showed no difference in the expression of endogenous NIPP1 in the WT and mutant cdNIPP1-expressing cells (supplemental Fig. S1*C*), suggesting that the expression of NIPP1 was not altered. Taken together, these results indicate that the expression of cdNIPP1 inhibited HIV-1 transcription and that this inhibition was not due to generalized cellular toxicity of the PP1 inhibitor.

HIV-1 Replication Is Inhibited When cdNIPP1 Is Expressed as Part of the Viral Genome—To determine directly the effect of cdNIPP1 on HIV-1 viral replication in a more physiologically relevant system, we subcloned the cdNIPP1-EGFP sequence in place of *nef* into the pNL4-3 vector (Fig. 3*A*). The expression of cdNIPP1-expressing molecular clones was verified by the nuclear fluorescence of cdNIPP1-EGFP in 293T cells transfected with pNL4-3 cdNIPP1 WT and mutant and compared with the expression of EGPF-AD8 molecular clone (34) (Fig. 3*B*). The recombinant HIV-1 viruses were created by transfecting HeLa cells with pNL4-3 cdNIPP1 (WT or mutant) and collecting culture supernatants at 48 h after transfection. The viruses were equalized for RT activities and used to infect cultured MT4 cells. As positive controls, we used WT pNL4-3 virus and also pNL4-3 398 virus that contained inactivating insert in the *nef* gene that deletes 36 N-terminal amino acids of *nef* critical for the *nef* function (32) (see "Experimental Procedures"). The expression of NIPP1 in the infected MT4 cells was monitored by Western blotting (not shown). The RT activity was analyzed over the course of 13 days to determine the kinetics of viral replication (Fig. 3*C*). Compared with the WT pNL4-3, the viruses with the *nef* inserts showed delayed replication kinetics, likely due to the impairment of the *nef* function. Expression of cdNIPP1 in the context of an HIV-1 genome significantly inhibited viral replication compared with the expression of a control mutant cdNIPP1 in the same genome (Fig. 3*B*) or expression of WT pNL4-3 or pNL4-3 398 virus with the inactivating insert in the *nef* gene. These results verified that productive HIV-1 replication is inhibited when cdNIPP1 is expressed during the course of the viral infection in T cells.
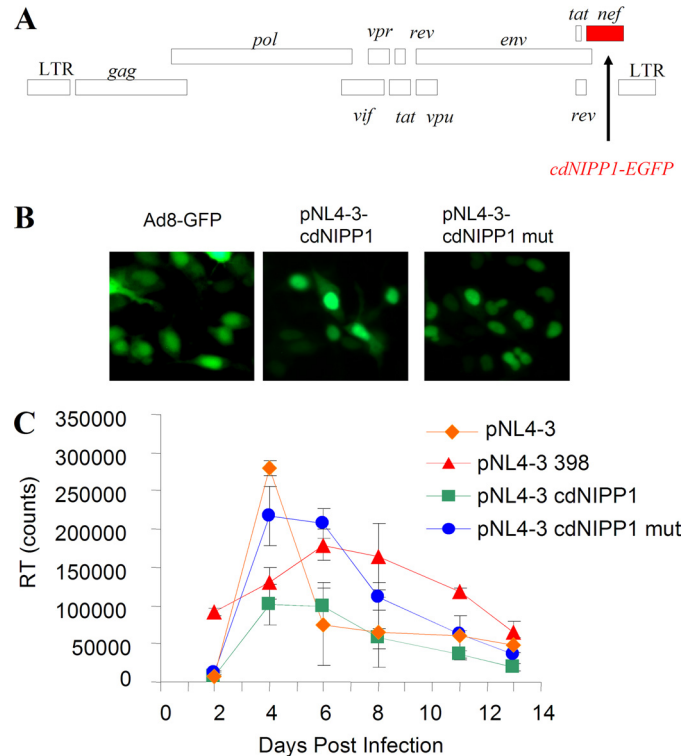
FIGURE 3. **Expression of cdNIPP1 inhibits HIV-1 replication.** *A*, cloning strategy. Diagram of the HIV-1 genome indicates the insertion of cdNIPP1-EGFP into *nef*. *B*, expression of cdNIPP1. EGFP-fused WT cdNIPP1 and mutant RATA cdNIPP1 were expressed in 293T cells transfected with recombinant pNL4-3 constructs or Ad8-GFP expression vector as control. *C*, replication of recombinant pNL4-3 cdNIPP1. Replication kinetics of WT pNL4-3 and control pNL4-3 398 and recombinant pNL4-3 expressing cdNIPP1 and mutant RATA cdNIPP1 in MT4 cells determined by RT measurement in cell media are shown. No new cells were added into the infected cultures.

DISCUSSION

Here, we show that expression of a relatively short, 82 residues PP1-binding peptide, derived from NIPP1 redistributed cellular PP1, modified CDK9 phosphorylation and association with 7SK RNA, and inhibited HIV-1 replication. Although the PP1 targets relevant to HIV-1 transcription are still not conclusively understood, one possibility is that PP1 dephosphorylates CDK9 (25). Thr^{186} of CDK9 can be dephosphorylated by PP1α *in vivo* (18). Our data are in agreement with this previous work because we documented an increased phosphorylation of CDK9 Thr^{186} and increased association of CDK9 with 7SK RNA when PP1 inhibitor was overexpressed. We previously hypothesized that during HIV-1 infection, the viral Tat protein serves as a PP1-targeting subunit. Here, we found that the expression of cdNIPP1 disrupts the association of Tat with PP1; this disruption could be a mechanism for inhibiting HIV-1 transcription, similar to the previously reported HIV-1 inhibitory effect of Tat mutations that prevented binding of Tat to PP1 (24). Accordingly, we observed the inhibition of HIV-1 transcription by a short peptide containing the RVTF sequence, although the effect was relatively small likely due to the problem with cell permeability and inefficient delivery.

Another possible biological target for PP1 dephosphorylation in HIV-1 transcription could be the transcription factor Sp1 because it is known to associate with PP1 at many cellular promoters (35). Sp1 is required by HIV-1 for basal transcrip-

tion (36) and can target cyclin T1 to the LTR in the absence of Tat or TAR RNA (37). An increase in Sp1 phosphorylation induced by Tat and DNA-PK can enhance HIV-1 transcription (38). RNA polymerase II could be a third PP1 substrate; we have previously shown that the C-terminal domain of RNA polymerase II is indeed dephosphorylated by PP1 (39). Our recent study showed that NIPP1 can serve as an RNA polymerase II-targeting subunit of PP1 (40), and thus altered RNA polymerase II dephosphorylation could also be considered as a potential inhibitory mechanism. Interestingly, the results presented here indicate that despite the expression of cdNIPP1, cells remained viable even though the association of CDK9 with 7SK RNA was increased. Also, a transient expression of cdNIPP1 was not toxic. Thus, our observations indicate a future possibility for the design of the HIV-1 inhibitory compounds that affect the interaction of Tat with PP1.

Here, we utilized an RVXF-binding peptide (*i.e.* cdNIPP1) to disrupt the interaction of PP1 with a subset of cellular regulatory subunits and inhibit HIV-1. Recently, a combined approach of *in silico* screening and a multistep biochemical validation procedure identified many novel PP1 interactors that contained novel PP1 binding motifs, "SILK" and "MyPhoNE" (41). Thus, small molecule compounds that disrupt either of these PP1-binding motifs or an RVXF motif might deregulate subsets of cellular PP1 holoenzymes and selectively target viral or cellular pathways. Taken together, previous studies and our findings here indicate that PP1 is involved in HIV-1 transcription. Efficient inhibition of HIV-1 transcription and replication through the redistribution of PP1 indicate that PP1 can be a future target for antiviral therapeutics.

REFERENCES

1. Nekhai, S., and Jeang, K. T. (2006) *Future Microbiol.* **1,** 417–426
2. Yang, Z., Zhu, Q., Luo, K., and Zhou, Q. (2001) *Nature* **414,** 317–322
3. Nguyen, V. T., Kiss, T., Michels, A. A., and Bensaude, O. (2001) *Nature* **414,** 322–325
4. Michels, A. A., Nguyen, V. T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L., and Bensaude, O. (2003) *Mol. Cell. Biol.* **23,** 4859–4869
5. Yik, J. H., Chen, R., Nishimura, R., Jennings, J. L., Link, A. J., and Zhou, Q. (2003) *Mol. Cell* **12,** 971–982
6. Jang, M. K., Mochizuki, K., Zhou, M., Jeong, H. S., Brady, J. N., and Ozato, K. (2005) *Mol. Cell* **19,** 523–534
7. Yang, Z., Yik, J. H., Chen, R., He, N., Jang, M. K., Ozato, K., and Zhou, Q. (2005) *Mol. Cell* **19,** 535–545
8. Dey, A., Nishiyama, A., Karpova, T., McNally, J., and Ozato, K. (2009) *Mol. Biol. Cell* **20,** 4899–4909
9. Garber, M. E., Wei, P., and Jones, K. A. (1998) *Cold Spring Harbor Symp. Quant. Biol.* **63,** 371–380
10. Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997) *Genes Dev.* **11,** 2622–2632
11. Yang, X., Gold, M. O., Tang, D. N., Lewis, D. E., Aguilar-Cordova, E., Rice, A. P., and Herrmann, C. H. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 12331–12336
12. Ott, M., Schnölzer, M., Garnica, J., Fischle, W., Emiliani, S., Rackwitz, H. R., and Verdin, E. (1999) *Curr. Biol.* **9,** 1489–1492
13. Kiernan, R. E., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calomme, C., Burny, A., Nakatani, Y., Jeang, K. T., Benkirane, M., and Van Lint, C. (1999) *EMBO J.* **18,** 6106–6118
14. Deng, L., de la Fuente, C., Fu, P., Wang, L., Donnelly, R., Wade, J. D., Lambert, P., Li, H., Lee, C. G., and Kashanchi, F. (2000) *Virology* **277,** 278–295
15. Barboric, M., Yik, J. H., Czudnochowski, N., Yang, Z., Chen, R., Contreras, X., Geyer, M., Matija Peterlin, B., and Zhou, Q. (2007) *Nucleic Acids Res.* **35,** 2003–2012
16. Chen, R., Yang, Z., and Zhou, Q. (2004) *J. Biol. Chem.* **279,** 4153–4160
17. Li, Q., Price, J. P., Byers, S. A., Cheng, D., Peng, J., and Price, D. H. (2005) *J. Biol. Chem.* **280,** 28819–28826
18. Chen, R., Liu, M., Li, H., Xue, Y., Ramney, W. N., He, N., Ai, N., Luo, H., Zhu, Y., Zhou, N., and Zhou, Q. (2008) *Genes Dev.* **22,** 1356–1368
19. Wang, Y., Dow, E. C., Liang, Y. Y., Ramakrishnan, R., Liu, H., Sung, T. L., Lin, X., and Rice, A. P. (2008) *J. Biol. Chem.* **283,** 33578–33584
20. Bharucha, D. C., Zhou, M., Nekhai, S., Brady, J. N., Shukla, R. R., and Kumar, A. (2002) *Virology* **296,** 6–16
21. Ammosova, T., Jerebtsova, M., Beullens, M., Voloshin, Y., Ray, P. E., Kumar, A., Bollen, M., and Nekhai, S. (2003) *J. Biol. Chem.* **278,** 32189–32194
22. Bollen, M., and Beullens, M. (2002) *Trends Cell Biol.* **12,** 138–145
23. Bollen, M. (2001) *Trends Biochem. Sci.* **26,** 426–431
24. Ammosova, T., Jerebtsova, M., Beullens, M., Lesage, B., Jackson, A., Kashanchi, F., Southerland, W., Gordeuk, V. R., Bollen, M., and Nekhai, S. (2005) *J. Biol. Chem.* **280,** 36364–36371
25. Ammosova, T., Washington, K., Debebe, Z., Brady, J., and Nekhai, S. (2005) *Retrovirology* **2,** 47
26. Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., and Wilson, J. (1999) *Gene Ther.* **6,** 1574–1583
27. DeGuzman, A., and Lee, E. Y. (1988) *Methods Enzymol.* **159,** 356–368
28. Deng, L., Ammosova, T., Pumfery, A., Kashanchi, F., and Nekhai, S. (2002) *J. Biol. Chem.* **277,** 33922–33929
29. Lesage, B., Beullens, M., Nuytten, M., Van Eynde, A., Keppens, S., Himpens, B., and Bollen, M. (2004) *J. Biol. Chem.* **279,** 55978–55984
30. Beullens, M., Vulsteke, V., Van Eynde, A., Jagiello, I., Stalmans, W., and Bollen, M. (2000) *Biochem. J.* **352,** 651–658
31. Ceulemans, H., Vulsteke, V., De Maeyer, M., Tatchell, K., Stalmans, W., and Bollen, M. (2002) *J. Biol. Chem.* **277,** 47331–47337
32. Ali, S. A., Huang, M. B., Campbell, P. E., Roth, W. W., Campbell, T., Khan, M., Newman, G., Villinger, F., Powell, M. D., and Bond, V. C. (2010) *AIDS Res. Hum. Retroviruses* **26,** 173–192
33. Nekhai, S., Bhat, U. G., Ammosova, T., Radhakrishnan, S. K., Jerebtsova, M., Niu, X., Foster, A., Layden, T. J., and Gartel, A. L. (2007) *Oncogene* **26,** 3899–3903
34. Vanham, G., Penne, L., Allemeersch, H., Kestens, L., Willems, B., van der Groen, G., Jeang, K. T., Toossi, Z., and Rich, E. (2000) *Aids* **14,** 2299–2311
35. Zhang, Y., Liao, M., and Dufau, M. L. (2008) *J. Biol. Chem.* **283,** 24039–24046
36. Jochmann, R., Thurau, M., Jung, S., Hofmann, C., Naschberger, E., Kremmer, E., Harrer, T., Miller, M., Schaft, N., and Stürzl, M. (2009) *J. Virol.* **83,** 3704–3718
37. Yedavalli, V. S., Benkirane, M., and Jeang, K. T. (2003) *J. Biol. Chem.* **278,** 6404–6410
38. Chun, R. F., Semmes, O. J., Neuveut, C., and Jeang, K. T. (1998) *J. Virol.* **72,** 2615–2629
39. Washington, K., Ammosova, T., Beullens, M., Jerebtsova, M., Kumar, A., Bollen, M., and Nekhai, S. (2002) *J. Biol. Chem.* **277,** 40442–40448
40. Jerebtsova, M., Klotchenko, S. A., Artamonova, T. O., Ammosova, T., Washington, K., Egorov, V. V., Shaldzhyan, A. A., Sergeeva, M. V., Zatulovskiy, E. A., Temkina, O. A., Petukhov, M. G., Vasin, A. V., Khodorkovskii, M. A., Orlov, Y. N., and Nekhai, S. (2010) *Mol. Cell Biochem.* **347,** 79–87
41. Hendrickx, A., Beullens, M., Ceulemans, H., Den Abt, T., Van Eynde, A., Nicolaescu, E., Lesage, B., and Bollen, M. (2009) *Chem. Biol.* **16,** 365–371