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Nicotinamide Phosphoribosyltransferase/Sirtuin 1 Pathway Is Involved in Human Immunodeficiency Virus Type 1 Tat-Mediated Long Terminal Repeat Transactivation

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ABSTRACT

Tat is a multifunctional transactivator encoded by human immunodeficiency virus type 1 (HIV-1). Tat transactivating activity is controlled by nicotinamide adenine nucleotide$^+$ (NAD^+)-dependent deacetylase sirtuin 1 (SIRT1). Nicotinamide phosphoribosyltransferase (Nampt) is a rate-limiting enzyme in the conversion of nicotinamide into NAD^+, which is crucial for SIRT1 activation. Thus, the effect of Nampt on Tat-regulated SIRT activity was studied in Hela-CD4-β-gal (MAGI) cells. We demonstrated that Tat caused NAD^+ depletion and inhibited Nampt mRNA and protein expression in MAGI cells. Resveratrol reversed Tat-induced NAD^+ depletion and inhibition of Nampt mRNA and protein expression. Further investigation revealed that Tat-induced inhibition of SIRT1 activity was potentiated in Nampt-knockdown by Nampt siRNA compared to treatment with Tat alone. Nampt siRNA potentiated Tat-induced HIV-1 transactivation in MAGI cells. Altogether, these results indicate that Nampt is critical in the regulation of Tat-induced inhibition of SIRT1 activity and long terminal repeat (LTR) transactivation. Nampt/SIRT1 pathway could be a novel therapeutic tool for the treatment of HIV-1 infection. J. Cell. Biochem. 110: 1464–1470, 2010.

KEY WORDS: TAT; SIRT1; NAMPT; LTR TRANSACTIVATION; HIV-1

Tat is a multifunctional transactivator encoded by human immunodeficiency virus type 1 (HIV-1). The full-length open reading frame of HIV-1 Tat is composed of the two exons of the viral tat gene and encodes a protein of approximately 101 amino acids [Karn, 1999]. Transcription of the HIV genome requires host cell transcription factors and the viral transactivator Tat [Harrich et al., 2006; Richter and Palu, 2006]. Tat potently activates HIV transcription and binds to an RNA stem-loop structure termed TAR that spontaneously forms at the 5′ extremities of all viral transcripts.

Tat undergoes multiple post-translational modifications including phosphorylation, methylation, acetylation, glycosylation, and ubiquitination that regulate the dynamics and complexity of interactions with RNA [Stevens et al., 2006]. Acetylation, an important posttranslational modification, is a reversible process mediated by enzymes that transfer acetyl groups from acetyl coenzyme A to the ε-amino group of lysines. In vitro, HIV-1 Tat is acetylated by p300, the human homologue of the yeast general control of amino acid synthesis 5 (GCN5) protein, p300/CBP-associated factor (PCAF), TAFII250 and Tat-interacting protein 60 [Ott et al., 2004; van Lint et al., 1996]. The acetylation site of p300 has been mapped to lysine 50, a highly conserved lysine within the arginine-rich motif of Tat. Deacetylases remove the acetyl moieties from lysines. Histone deacetylases (HDACs) are more sensitive to environmental or developmental cues than the histone acetylases (HATs) and can provide a regulatory role in transcription [Xu et al., 2007; Yang and Seto, 2007]. Tat binds deacetylases as well as acetyltransferases. Sirtuins, different from other type HDACs that inhibited by TSA, require nicotinamide adenine dinucleotide (NAD^+) as a cofactor [Pagans et al., 2005; Haigis and Guarente, 2006; Saunders and Verdin, 2007]. Lysine 50 in Tat is deacetylated by the class III deacetylase sirtuin 1 (SIRT1) [Pagans et al., 2005]. Tat transactivating activity is controlled by SIRT1 connects HIV transcription with the metabolic status of the cell. Changes in the cellular NAD^+/NADH ratio may, therefore, regulate Tat activity through deacetylation by SIRT1.

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Nicotinamide phosphoribosyltransferase (Nampt), also known as pre-B cell colony-enhancing factor (PBEF), was originally identified as a cytokine that facilitates the clonal expansion and differentiation of pre-B cells [Samal et al., 1994; Rongvaux et al., 2002]. Increased amounts of Nampt in mammalian cells cause upregulation of NAD^+. Nampt converts nicotinamide to nicotinamide mononucleotide, which is converted into NAD^+ by nicotinamide mononucleotide adenylyltransferase in the mammalian biosynthetic pathway. Nampt is a rate-limiting enzyme in the conversion of nicotinamide into NAD^+, which is crucial for SIRT1 activation and regulation of transcription. Nampt localizes and functions in both intra- and extracellular compartments [Revollo et al., 2007]. Intracellular Nampt generates NAD^+ and, consequently, has cell-protective benefits. NAD^+ shows functional activity in various signaling pathways. For instance, it regulates the transcription and function of NAD^+-dependent SIRT1, and increased expression of Nampt upregulates SIRT1 activity [Imai and Kiess, 2009; Zhang et al., 2009]. NAD^+-dependent SIRT1 regulates a variety of biological responses, such as stress response, metabolism, aging, and cell differentiation. In the early stage of HIV-1 infection, SIRT1 was enzymatically active on Tat and was released before the complex between P-TEFb and Tat could be recruited to TAR for its transcriptional effects [Pagans et al., 2005; Robinson, 2007]. In the late stage of HIV-1 infection, Tat directly interacts with the deacetylase domain of SIRT1 and blocks the ability of SIRT1 to deacetylate lysine 310 in the p65 subunit of NF-kB and inhibited LTR transactivation [Kwon et al., 2008].

In this study, Hela-CD4-β-gal cells, HeLa cells that expressed CD4 and were stably transfected with HIV-1 long terminal repeat (LTR)-β-galactosidase reporter DNA were selected. It was demonstrated that HIV-1 Tat depleted intracellular NAD^+ levels, and caused a decline in Nampt mRNA and protein expression. The results obtained here show that SIRT1 participated in the Tat-induced HIV-1 LTR transactivation and was dependent on the NAD^+/Nampt pathway. This indicates the Nampt/SIRT1 signaling pathway as a novel target of HIV Tat and provides evidence of a primary molecular response in a potential target cell of this important regulator of transcription activation.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin/EDTA were purchased from Hyclone (Logan, UT). Nicotinamide was purchased from Sigma (St. Louis, MO). Resveratrol was purchased form Calbiochem-Novabiochem Corporation (San Diego, CA). SIRT1-siRNA, Nampt-siRNA, control siRNA, antibodies for SIRT1, Nampt and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary Abs IR800 anti-rabbit and IR800 anti-mouse for visualization of immunoblots using the Odyssey infrared imager were obtained from LI-COR Biosciences (Lincoln, NE). Genejuice transfection and Genejuice siRNA reagents were obtained from Invitrogen (Carlsbad, CA). Tat plasmid [Sun et al., 2006] was kindly provided by Prof. Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing). All other chemicals were of the highest commercial grade available.

CELL CULTURE, TRANSFECTION, AND ASSESSMENT OF CELL VIABILITY

The HeLa-CD4-LTR-β-galactosidase indicator cell line is multinuclear activation of galactosidase indicator (MAGI) cells [Kimpton and Emerman, 1992]. MAGI cells were maintained in DMEM supplemented with 10% FCS, 200 μg/ml G418, 100 μg/ml hygromycin B, at 37°C in 5% CO_2 and 95% air in humidified atmosphere. For all transfections, MAGI cells were used at a confluency of 50–60%. Typically, cells were transfected with 100 nM of Tat plasmid per well in a 96-well dish by using Genejuice transfection reagent. Cell extracts were prepared 48 h after transfection. Cell viability was examined using MTT-based assays according to the manufacturer's instructions.

NAD^+ ASSAY

The NAD^+ levels were measured using a BioChain NAD^+ assay kit according to manufacturer's instructions (BioChain, Hayward, CA). NAD^+ levels were calculated according to the equivalent protein quantity (μg protein) per data point.

SIRT1 ACTIVITY ASSAY

SIRT1 was immunoprecipitated from whole cell extracts and SIRT1 activity was assayed using a deacetylase colorimetric activity assay kit (Biomol International, Plymouth Meeting, PA). Briefly, whole cell extracts were obtained from cells culture with the addition of 1 μM Trichostatin A (TSA). Endogenous SIRT1 from MAGI cells was obtained by immunoprecipitation and then incubated in deacetylase buffer with the substrate and 0.1 mM NAD^+ for 1 h at 37°C. As a negative control, 10 mM of NAM was also added to some extracts. The reactions were stopped by adding 50 μl of a developer solution supplemented with 1 μM TSA and 10 mM NAM. The plate was read after 10 min incubation at room temperature using a multi-well fluorometer (excitation 360 nm, emission 460 nm). A standard curve was generated using a deacetylated substrate Fluor-de-Lys (ranging from 1 to 40 μM). SIRT1 activity was determined as NAM-inhibitable, TSA-independent ability of cell extracts to deacetylate the specific fluorometric substrate. Calculation of net fluorescence included the subtraction of a blank consisting of buffer containing no NAD^+. The SIRT1-dependent activity was calculated after subtracting fluorescence values obtained in the absence of NAD^+. Experimental values were presented as pmol converted substrate/μg protein/min. The negative controls (10 mM NAM) were subtracted from each treatment to give the final values.

IMMUNOBLOT ANALYSIS

Cells were grown on 6-well plates. Following treatment of cells, the media was aspirated and the cells were washed twice in ice-cold PBS and lysed in 400 μl of lysis buffer. The samples were then briefly sonicated, heated for 5 min at 95°C, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8%) gels, and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature. The blots were incubated

overnight at room temperature with primary antibodies and then washed six times in TBS/0.1% Tween-20 prior to 1 h of incubation and visualized with secondary antibodies using Odyssey Infrared Imaging System (Li-Cor Biosciences). All immunoblots were visualized using the Odyssey infrared imaging system (LI-COR Biosciences) and secondary Abs according to the manufacturer's instructions. When using the infrared dye-labeled secondary antibody, the membranes were scanned directly after incubation and the band intensities were quantified using the Odyssey infrared imaging system (Li-Cor Biosciences) [Zhang and Wu, 2009].

SILENCING NAMPT OR SIRT1

MAGI cells were seeded in 6-well plates and allowed to grow to 50–60% confluence for 24 h. Transient transfections were performed with Genejuice siRNA transfection reagent according to the manufacturer's protocol. Likewise, a commercially available pool of three target-specific siRNAs were used to knock down Nampt gene or SIRT1 following the siRNA transfection protocol of the manufacturer. Untransfected cells and cells transfected with a negative control siRNA were included in all experiments. Five microliters siRNA and 1 μl transfection reagent were each diluted first with 94 μl reduced serum media (Opti-MEM) and then mixed. The mixtures were allowed to incubate for 30 min at room temperature and then were added by drop to each culture well containing 900 μl reduced serum media. Four hours after transfection, the medium was changed with completely fresh medium. The cells were cultivated for 24 h, were lysed, and the expression of Nampt or SIRT1 RNA or protein was assayed with RT-qPCR and Western blotting, respectively.

RT-QPCR

RNA from the MAGI cells was extracted using TRIzol Reagent. cDNA was synthesized using Thermoscript II cDNA Synthesis kit (Invitrogen). Two microliters cDNA were used for quantitative real time PCR. qPCR was performed using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA) with a SyberGreen PCR MasterMix (Applied Biosystems, Foster City, CA). All reactions were analyzed in triplicates. Data were normalized to the signal obtained using primers that amplified the GAPDH transcripts using the comparative CT method for relative quantification of gene expression.

MAGI CELL ASSAY

MAGI cells assays were performed according to the recommended protocol [Zhang et al., 2009]. Cells were transfected with Tat plasmid for 48 h. Two days after treatment, the cells were fixed for 5 min with 0.2% glutaraldehyde, 1% formaldehyde in phosphate-buffered saline. Cells were washed twice with phosphate-buffered saline and then staining solution (4 mM ferrocyanide, 4 mM ferricyanide, 2 mM $MgCl_2$, 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal)) was added and incubated at 37°C for 1 h. Blue cells were counted under microscopy.

STATISTICAL ANALYSIS

Each data bar represents the mean values ± standard deviations (SD) of at least three independent experiments in all cases. Results were analyzed using SPSS for Windows. Differences between groups were analyzed by one-way analysis of variance (ANOVA). If the *F*-value was significant, LSD post hoc test was used to compare multiple groups. A *P*-value of ≤ 0.05 was considered statistically significant in all cases.

RESULTS

TAT CAUSED DEPLETION OF NAD^+ LEVELS AND INHIBITED SIRT1 ACTIVITY, NAMPT MRNA, AND PROTEIN EXPRESSION IN MAGI CELLS

NAD^+ regulates the transcription and function of sirtuins (SIRTs), which are lysine deacetylases for histones and numerous transcription factors. Intracellular Nampt generates NAD^+ and, consequently, has cell-protective benefits. Nampt is a rate-limiting enzyme in the conversion of nicotinamide into NAD^+, which is crucial for SIRT1 activation and regulation of transcription. As shown in Figure 1, treatment with 100 nM Tat plasmid caused depletion of NAD^+ levels (42.1 ± 2.0 nmol/mg protein vs. 64.4 ± 3.7 nmol/mg protein, $n = 6$, $P < 0.01$) and inhibited SIRT1 activity (0.21 ± 0.02 pmol/μg protein vs. 0.32 ± 0.02 pmol/μg protein, $n = 6$, $P < 0.01$) in MAGI cells. Consistent with decline in NAD^+ levels, treatment with 100 nM Tat plasmid caused a decline in Nampt mRNA (~0.26 fold compared with control, $n = 3$, $P < 0.01$) and protein (Fig. 1D) expression.

TAT INHIBITED SIRT1 ACTIVITY VIA INHIBITION OF NAMPT EXPRESSION

To further address the role of Nampt in signaling SIRT1 activity, transfection of MAGI cells with 50 nM siRNA specific to Nampt inhibited >75% of Nampt expression compared to control whereas control siRNA had no effect (Fig. 2A,B). Tat-induced inhibition of SIRT1 activity was potentiated in Nampt-knockdown cells compared to treatment with Tat alone (0.14 ± 0.02 pmol/μg protein vs. 0.21 ± 0.02 pmol/μg protein, $n = 6$, $P < 0.01$). Control siRNA had no significant effect (0.34 ± 0.02 pmol/μg protein). Thus, Nampt was required for optimal in the regulation of Tat-induced inhibition of SIRT1 activity in MAGI cells.

RESVERATROL REVERSED TAT-INDUCED INHIBITION OF NAMPT AND NAD^+ IN MAGI CELLS

Nampt could co-regulate the sirtuins and other NAD^+-dependent processes. NAD^+ regulates the transcription and function of NAD^+-dependent SIRTs, and increased expression of Nampt upregulates SIRT1 activity. As shown in Figure 3, Treatment with 10, 20, 50 μM resveratrol significantly blunted Tat-induced inhibition of Nampt mRNA (~0.36 fold, ~0.54 fold, ~0.73 fold, vs. ~0.26 fold compared with control, $n = 3$, $P < 0.05$) and protein levels (Fig. 3B) in MAGI cells in a concentration manner. Treatment with 50 μM resveratrol alone had no effect on Nampt mRNA and protein levels in MAGI cells compared with control cells. Similar results were found with increase in NAD^+ levels after 10, 20, 50 μM resveratrol treatment (51.9 ± 1.7 nmol/mg protein, 56.1 ± 1.1 nmol/mg protein, 61.0 ± 1.5 nmol/mg protein vs. 43.1 ± 1.3 nmol/mg protein, $n = 6$, $P < 0.01$). Treatment with 50 μM resveratrol alone had no effect on NAD^+ levels in MAGI cells compared with control cells.

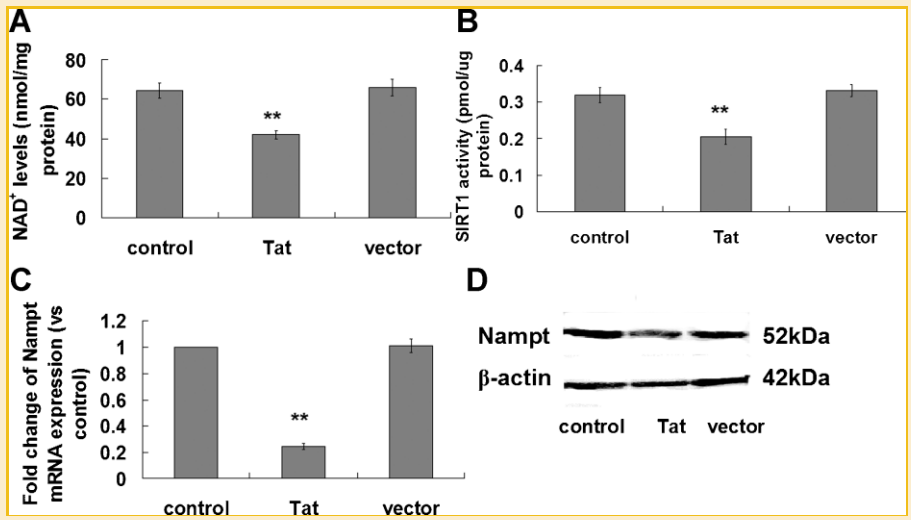

Fig. 1. Effects of Tat on NAD^+ levels and inhibited SIRT1 activity, Nampt mRNA and protein expression in MAGI cells. MAGI cells were transfected with 100 nM Tat plasmid for 48 h. A: Intracellular NAD^+ levels were assayed as described in the Materials and Methods Section. The empty expression vector was used in mock transfected controls. The data shown were the means of six independent experiments. $^{**}P < 0.01$ (compared to control). B: Cell extracts were tested for SIRT1 activity. The empty expression vector pCI was used in mock transfected controls. The data shown were the means of six independent experiments. $^{**}P < 0.01$ (compared to control). C: The cells were prepared as described in the Materials and Methods Section and the Nampt mRNA levels detected by reverse transcription and real time-PCR. Relative mRNA expression levels in various treatments were calculated after normalization with GAPDH mRNA levels as determined by real time-PCR. Fold changes were calculated from GAPDH-normalized Ct values; error bars represented SD of triplicate experiments. $^{**}P < 0.01$ (compared to control). D: The cells were prepared and the Nampt protein levels detected by western blotting analysis. β-Actin was used as a loading control. The blots shown were representative of three independent experiments with similar results.

NAMPT IS INVOLVED IN TAT-INDUCED LTR TRANSACTIVATION IN MAGI CELLS

HeLa cells, that express CD4 and are stably transfected with HIV-1 long terminal repeat-β-galactosidase reporter DNA, were transfected with Tat plasmid. The number of β-galactosidase-positive blue cells that are determined using light microscopy was scored to determine the affectivity of Tat-induced HIV-1 LTR transactivation.

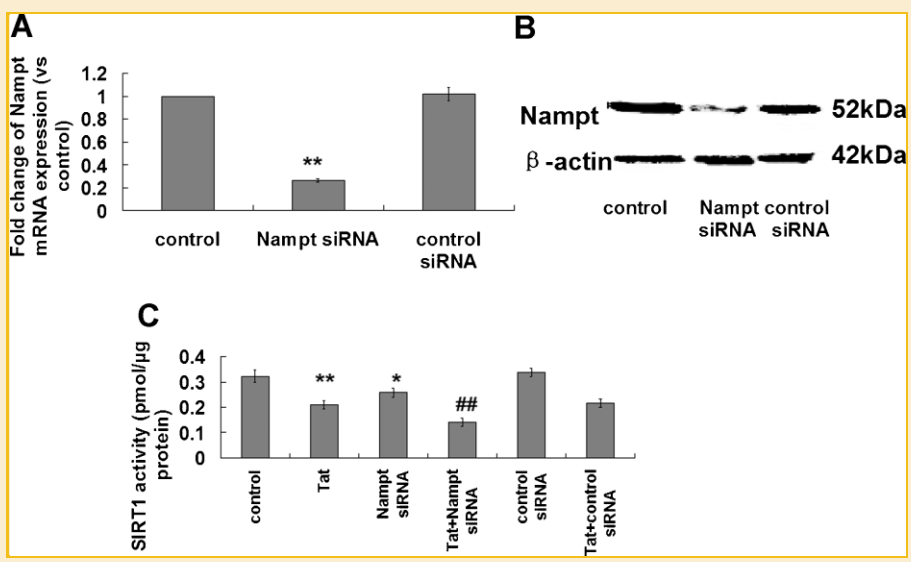

Fig. 2. Effects of Nampt siRNA on Tat-mediated inhibition of SIRT1 activity in MAGI cells. MAGI cells were transfected with 50 nM Nampt siRNA or control siRNA for 48 h. A: The cells were prepared as described in the Materials and Methods Section and the Nampt mRNA levels detected by reverse transcription and real time-PCR. Relative mRNA expression levels in various treatments were calculated after normalization with GAPDH mRNA levels as determined by real time-PCR. Fold changes were calculated from GAPDH-normalized Ct values; error bars represented SD of triplicate experiments. $^{**}P < 0.01$ (compared to control). B: The cells were prepared and the Nampt protein levels detected by western blotting analysis. β-Actin was used as loading controls. The blots shown are representative of three independent experiments with similar results. C: Cell extracts were tested for SIRT1 activity. The data shown were the means of six independent experiments. $^{*}P < 0.05$ (compared to control). $^{**}P < 0.01$ (compared to control). $^{\#\#}P < 0.01$ (compared to Tat).

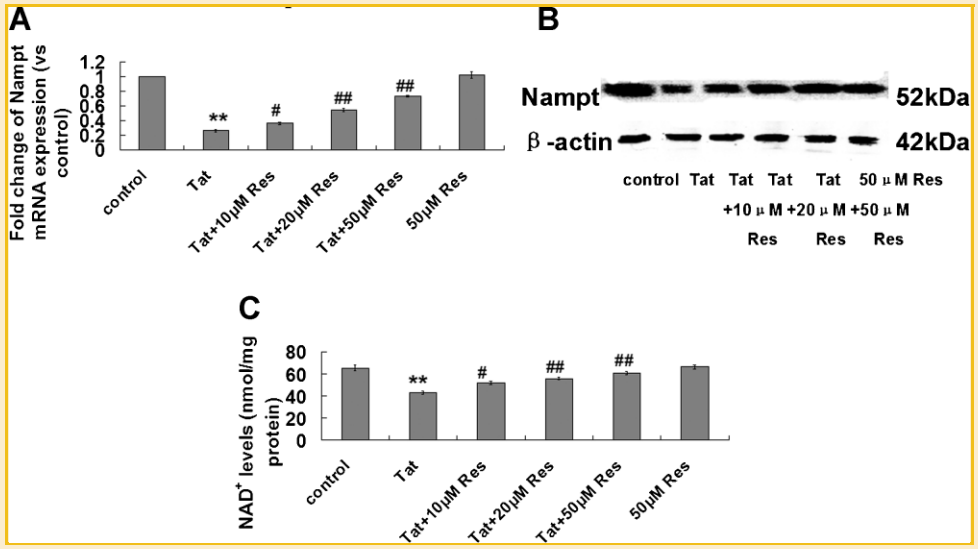

Fig. 3. Effects of Resveratrol on Tat-induced inhibition of Nampt and NAD^+ in MAGI cells. MAGI cells were pretreated with 10, 20, 50 μM Resveratrol (Res) for 1 h, then transfected with Tat plasmid for 48 h. A: The cells were prepared as described in the Materials and Methods Section and the Nampt mRNA levels detected by reverse transcription and real time-PCR. Relative mRNA expression levels in various treatments were calculated after normalization with GAPDH mRNA levels as determined by real time-PCR. Fold changes were calculated from GAPDH-normalized Ct values; error bars represented SD of triplicate experiments. $^{**}P < 0.01$ (compared to control). $^{\#}P < 0.05$ (compared to Tat), $^{\#\#}P < 0.01$ (compared to Tat). B: The cells were prepared and the Nampt protein levels detected by Western blotting analysis. β-Actin was used as a loading control. The blots shown were representative of three independent experiments with similar results. C: Intracellular NAD^+ levels were assayed as described in the Materials and Methods Section. The data shown were the means of six independent experiments. $^{**}P < 0.01$ (compared to control), $^{\#}P < 0.05$ (compared to Tat), $^{\#\#}P < 0.01$ (compared to Tat).

We explore whether SIRT1 was involved in Tat-induced LTR transactivation. Transfection of MAGI cells with siRNA specific to SIRT1 inhibited >75% of Nampt expression compared to control whereas control siRNA had no effect (Fig. 4A,B). As shown in Figure 4C, SIRT1 inhibitor nicotinamide (NAM) or splitomycin and SIRT1 siRNA, but not control siRNA, potentiated Tat-induced LTR transactivation (1.56 ± 0.03 fold, 1.66 ± 0.02 fold, 1.70 ± 0.03 fold compared with Tat treatment, $n = 6$, $P < 0.05$). Treatment with

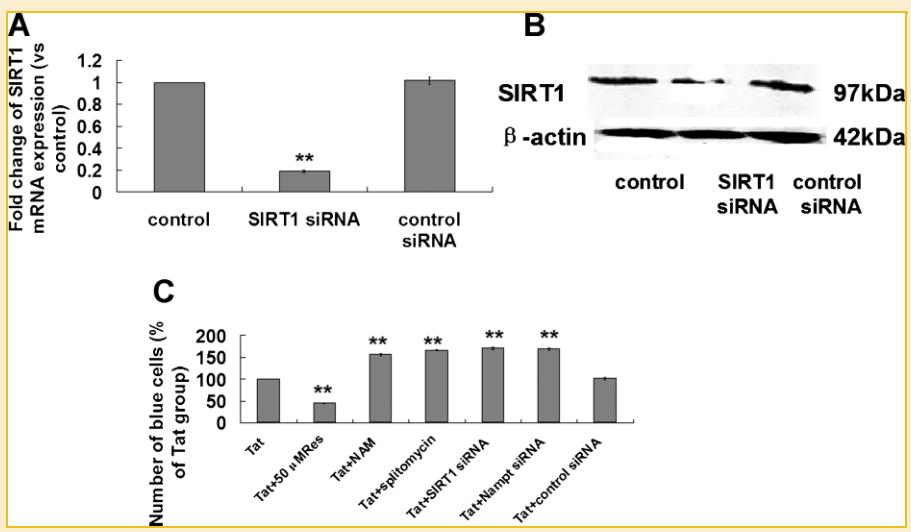

Fig. 4. Effect of Resveratrol, nicotinamide, splitomycin, SIRT1 siRNA or Nampt siRNA on Tat-induced LTR transactivation in MAGI cells. A: MAGI cells were transfected with 50 nM SIRT1 siRNA or control siRNA for 48 h. The cells were prepared as described in the Materials and Methods Section and the SIRT1 mRNA levels detected by reverse transcription and real time-PCR. Relative mRNA expression levels in various treatments were calculated after normalization with GAPDH mRNA levels as determined by real time-PCR. Fold changes were calculated from GAPDH-normalized Ct values; error bars represented SD of triplicate experiments. $^{**}P < 0.01$ (compared to control). B: MAGI cells were transfected with 50 nM SIRT1 siRNA or control siRNA for 48 h. The cells were prepared and the SIRT1 protein levels detected by western blotting analysis. β-Actin was used as loading controls. The blots shown were representative of three independent experiments with similar results. C: MAGI cells were pretreated with 50 μM Resveratrol (Res), 1 mM nicotinamide (NAM) or 10 μM splitomycin for 1 h, then transfected with the Tat plasmid or co-transfected with 50 nM SIRT1 siRNA or Nampt siRNA, control siRNA for 48 h. The cells were fixed and stained with X-gal. Photographs of blue cells were taken 48 h post-infection using Olympus microscope and Kodak digital camera. The data shown were means ± SD of six independent experiments. **Significantly different when compared with Tat group ($P < 0.01$).

nicotinamide or splitomycin or SIRT1 siRNA alone did not detect any blue cells in MAGI cells (data not shown).

To further address the role of Nampt in Tat-induced HIV-1 LTR transactivation, we used small interfering RNA (siRNA) to knockdown endogenous Nampt in MAGI cells. Tat-induced HIV-1 LTR transactivation was potentiated in Nampt-knockdown cells compared to control cells (1.69 ± 0.03 fold compared with Tat treatment, $n = 6$, $P < 0.05$). Treatment with siRNA Nampt or control siRNA alone did not detect any blue cells in MAGI cells.

DISCUSSION

NAD^+ is a classic coenzyme with a well-established role in cellular redox reactions, as well as a degradable substrate for several important enzymes including poly (ADP-ribose) polymerases (PARPs) and sirtuins [Imai, 2009a,b,c]. NAD^+ metabolism is considerably more complicated than previously thought. The mechanisms by which NAD^+ connects cellular metabolism with gene regulation remain to be established. NAD^+ shows functional activity in various signaling pathways. For instance, it regulates the transcription and function of NAD^+-dependent SIRTs, and increased expression of Nampt upregulates SIRT1 activity [Nakahata et al., 2009; Skokowa et al., 2009; van Gool et al., 2009]. In the present study, it was demonstrated that NAD^+/Nampt-dependent SIRT1 was also involved in HIV-1 Tat protein-mediated LTR transactivation.

Increased amounts of Nampt in mammalian cells cause upregulation of NAD^+, which supports the hypothesis that this enzyme is the rate-limiting determinant for NAD^+ production [Borradaile and Pickering, 2009; Garten et al., 2009; Imai, 2009c]. The redox reactions are not accompanied by any net consumption of the nucleotides. However, cells require ongoing NAD^+ synthesis because NAD^+ is consumed as a substrate by enzymes that break the glycosidic bond between the nicotinamide (NAM) moiety and ADP ribose moiety. A common feature of these reactions is that NAD^+ donates its ADP-ribose group, which breaks the glycosidic bond between nicotinamide and ribose, destroying the parent NAD^+ molecule. Nampt has drawn much attention in several different fields, including NAD^+ biology, metabolism, and immunomodulatory response [Nakahata et al., 2009; Skokowa et al., 2009; van Gool et al., 2009]. As a NAD^+ biosynthetic enzyme, Nampt regulates the activity of NAD^+-consuming enzymes such as sirtuins and influences a variety of metabolic and stress responses. Nampt is crucial for SIRT1 activation and regulation of transcription. In our study, it was demonstrated that Tat caused NAD^+ depletion and a decline in Nampt mRNA and protein expression. Moreover, knockdown of Nampt by siRNA potentiated Tat-induced LTR transactivation. This finding supports the hypothesis that Nampt is able to generate NAD^+ and that the biological effects of Nampt are indeed tightly linked to downstream NAD^+ synthesis and SIRT1 activation.

HIV-1 depends on the host transcription machinery to complete its life cycle. The level of transcription from HIV-1 genome is an important factor in determining the rate of viral replication [Baba, 2006; Stevens et al., 2006]. Binding of cellular transcription factors and host activators to the LTR results in the assembly of a transcriptional complex which, once modified by the viral factor Tat, can rapidly undergo efficient transcriptional elongation and reinitiation. The HIV Tat protein has been shown to be a powerful activator of integrated LTR expression. In addition, numerous cellular transcription factors were found to regulate transcription from the LTR, including Sp1, NF-kB, NFAT, HMG protein, C/EBP, AP-1, Ets-1, LEF-1, and YY1 [Stevens et al., 2006]. Acetylation is reversed by histone deacetylases (HDACs), a family of enzymes that removes acetyl groups from the tails of acetylated core histones. The HIV Tat protein has been shown to be a powerful activator of integrated LTR expression. It has been suggested that Tat can interact with histone acetyltransferases. A positioned nucleosome (nuc 1) spanning the region from 1 to 155 with respect to the transcription start site of HIV-1 LTR has been mapped in DNase I protection studies. Experiments examining the accessibility of integrated HIV-1 LTR to restriction endonucleases at this region suggest that disruption of this nucleosome accompanies transcriptional activation of integrated LTR by the viral factor Tat or the HDAC inhibitors trichostatin A (TSA) and trapoxin. While recruitment of selected host transcription activators and viral activator Tat to the LTR allows powerful activation of HIV transcription and viral replication, restriction of LTR expression by host repressors may allow activated, infected lymphocytes to return to the nonproductive resting state and establish viral quiescence. In our study, Tat-induced HIV-1 LTR transactivation was inhibited by NAD^+-dependent SIRT1, which has been confirmed by its activator, inhibitor and knockdown of SIRT1 by siRNA.

Modifications in NAD^+ and NAM levels are likely to be the most important regulators of sirtuins activity [Grubisha et al., 2005]. Despite a wealth of information on the molecules and mechanisms that mediate the effects of SIRT1 on several biological processes [Michan and Sinclair, 2007], the identification and mechanistic elucidation of the signals that activate the NAD^+ salvage pathway and, as a consequence, regulate the deacetylase activity of SIRT1 and of other sirtuins in response to nutrient availability and oxidative stress in mammalian cells remain to be fully understood. In the study of Pagans et al. [2005], SIRT1 deacetylates Tat and that this unmodified Tat is recycled to transactivate HIV transcription. However, in the same group, the new study [Kwon et al., 2008] showed that Tat proteins bound the SIRT1 deacetylase domain for substantially longer periods of time and blocked the access of SIRT1 to nuclear factor such as p65 and inhibited LTR transactivation. This paradox might be resolved by considering the differences in Tat levels in the early and late stages of viral infection [Blazek and Peterlin, 2008]. In our previous study, we have shown: (1), Tat decreased the intracellular glutathione (GSH) levels and increased ROS production [Zhang et al., 2009a]. (2), Inhibition of SIRT1 activity by Tat is considered a critical step of Tat transactivation [Zhang et al., 2009b]. (3), The NAD^+-SIRT1-AMPK signaling pathway may be activated by reduced nutrient availability to prevent Tat-induced HIV-1 LTR transactivation [Zhang and Wu, 2009]. In this study, we have shown that Tat activation is associated with SIRT1 activity. The interaction of SIRT1 and Tat was complex and need further elucidate. Further, we have identified an inverse

relationship between Nampt expression and NAD^+ levels on SIRT1 occupancy upon activation at LTR by Tat.

In conclusion, this correlation is also observed when knockdown of Nampt expression by siRNA potentiated Tat-induced LTR transactivation in MAGI cells. These observations not only confirm that the transcriptional activation is associated with inhibition by SIRT1 but suggest the possibility that Nampt-dependent up-regulation of NAD^+ levels regulate Tat-induced LTR transactivation. The results support pharmaceutical control of Nampt/SIRT1 pathway as a strategy for controlling HIV-1 infections.

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