Arginine Methylation of the Human Immunodeficiency Virus Type 1 Tat Protein by PRMT6 Negatively Affects Tat Interactions with both Cyclin T1 and the Tat Transactivation Region[∇]

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Received 30 August 2006/Accepted 24 January 2007

Arginine methylation has been shown to regulate signal transduction, protein subcellular localization, gene transcription, and protein-protein interactions that ultimately alter gene expression. Although the role of cellular protein arginine methyltransferases (PRMT) in viral gene expression is largely unknown, we recently showed that the Tat protein of human immunodeficiency virus type 1 (HIV-1) is a substrate for one such enzyme, termed PRMT6. However, the mechanism by which arginine methylation impairs the transactivation potential of Tat and the sites of arginine methylation within Tat remain obscure. We now show that Tat is a specific *in vitro* and *in vivo* substrate of PRMT6 which targets the Tat R52 and R53 residues for arginine methylation. Such Tat methylation led to decreased interaction with the Tat transactivation region (TAR) of viral RNA. Furthermore, arginine methylation of Tat negatively affected Tat-TAR-cyclin T1 ternary complex formation and diminished cyclin T1-dependent Tat transcriptional activation. Overexpression of wild-type PRMT6, but not a methylase-inactive PRMT6 mutant, reduced levels of Tat transactivation of HIV-1 long terminal repeat chloramphenicol acetyltransferase and luciferase reporter plasmids in a dose-dependent manner. In cell-based assays, knockdown of PRMT6 resulted in increased HIV-1 production and faster viral replication. Thus, PRMT6 can compromise Tat transcriptional activation and may represent a form of innate cellular immunity in regard to HIV-1 replication. Finding a way of inhibiting or stimulating PRMT6 activity might help to drive quiescently infected cells out of latency or combat HIV-1 replication, respectively.

The human immunodeficiency virus type 1 (HIV-1) Tat protein is a key player in HIV replication by virtue of its ability to dramatically increase gene transcription efficiency from the viral 5' long terminal repeat (LTR) (30). This is accomplished by binding of Tat to a 57-nucleotide stem-loop RNA structure located at the 5' terminus of the nascent HIV RNA transcript, an element referred to as the Tat transactivation response region (TAR). To stimulate the elongation efficiency of RNA polymerase II that initiates RNA synthesis from the LTR, Tat interacts with cyclin T1, which in turn recruits cyclin-dependent kinase 9 (CDK9) close to the C-terminal domain of RNA polymerase II. Subsequently, CDK9 enacts the hyperphosphorylation of RNA polymerase II and, as a result, dramatically accelerates RNA transcription (28).

Arginine is a positively charged amino acid known to mediate hydrogen bonding and amino-aromatic interactions. The nitrogen atoms of arginine within polypeptides can be post-translationally modified to contain methyl groups, a process termed arginine methylation (2, 12, 21). Protein arginine methylation results in the addition of one or two methyl groups to the guanidino nitrogen atoms of arginine. Three main forms of methylated arginine have been identified in eukaryotes: ω -N^G-

monomethylarginine, ω -N^G,N^G-asymmetric dimethylarginine, and ω -N^G,N^G-symmetric dimethylarginine. These modifications do not change the positive charge of arginine residues but can increase their hydrophobicity. The increased steric hindrance and decreased hydrogen bonding of methylated arginine residues may affect interaction of proteins with other cellular substrates.

Arginine methylation has been implicated in RNA processing, transcriptional regulation, signal transduction, DNA repair, and cellular localization, and it regulates many different protein-protein interactions (1–3, 8, 10, 11, 18, 19, 21). For example, arginine methylation of the transcriptional elongation factor Spt5 regulates its interaction with RNA polymerase II, thereby affecting transcription elongation (17). These findings are consistent with our previous work that demonstrated arginine methylation to have a negative impact on the transactivation activity of Tat and viral RNA export mediated by Rev in HIV-1 (5, 15).

The present study extends our work on Tat substantially from a mechanistic standpoint by showing that PRMT6 methylates the R52 and R53 residues of Tat and that methylated Tat affects Tat-TAR interactions by decreasing Tat-TAR binding affinity and lowers levels of the Tat-TAR-cyclinT1 complex. Furthermore, HIV-1-infected Jurkat cells that express small interfering RNA (siRNA) directed against PRMT6 showed increased viral production and faster viral replication kinetics than mock siRNA-expressing cells. Thus, compounds that activate PRMT6 might function as a brake on Tat function and help to impede HIV replication. In contrast, molecules that

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[∇] Published ahead of print on 31 January 2007.

inhibit PRMT6 might be able to stimulate HIV replication and perhaps serve as a means of driving latently infected cells to become overt producers of viral progeny.

MATERIALS AND METHODS

Reagents. Recombinant histidine-tagged (His)-Tat72, glutathione-S-transferase (GST)-Tat72, and various mutant GST-Tat72 constructs containing substitutions in the ARM were purified as described previously (29). Recombinant wild-type and mutated (VLD-KLA) GST-PRMT6, the myc epitope-tagged wild-type PRMT6 expression vector (pmyc-PRMT6), the myc epitope-tagged mutant PRMT6 expression vector coding for VLD-to-KLA substitutions (pmyc-PRMT6m), and the pHA-Tat expression vector that encodes hemagglutinin (HA) epitope-tagged Tat86 have been described previously (5). FLAG-tagged Tat101 (pcDNA3.1/FLAG-Tat101) was a kind gift from the laboratory of Melanie Ott, University of California, San Francisco (24). The myc epitope-tagged PRMT1 and PRMT5 (pmyc-PRMT1 and pmyc-PRMT5) plasmids and the functional enzymes PRMT1, PRMT3, PRMT4, PRMT5 and PRMT7, as well as specific antibodies against PRMT1 and PRMT5, were contributed by the laboratory of Stéphane Richard. Anti-PRMT6 antibodies were purchased from IMGENEX (San Diego, CA). Anti-myc and anti-HA antibodies were purchased from Invitrogen (Carlsbad, CA). Control and PRMT6 double-stranded RNA oligonucleotides (siRNA) were purchased from Dharmacon Research (Lafayette, CO). PRMT6 siRNA was derived from the PRMT6 sequence (accession no. AY043278) that includes nucleotides 996 to 1114 (5'-GCA AGA CAC GCA CGU UUC A-3'). The sequence of mock siRNA was 5'-AAU UGC CAC AAC AGG GUC GUG-3'. Constructs to create stable cell lines with down-regulated PRMT1 or PRMT5 were obtained from the laboratory of Sara Nakielnny, Lincoln's Inn Fields Laboratories, London, United Kingdom (27). The HIV-1 LTR luciferase reporter plasmid was a kind gift from the laboratory of Alan Frankel, University of California, San Francisco.

In vitro methylations. Purified His-Tat72 or GST-Tat72 and the mutant variant Tat (0.6 μ g) proteins were incubated with 1 μ g of wild-type or mutated GST-PRMT6 or PRMT1, -3, -4, -5, and -7 in the presence of 0.55 μ Ci of methyl-[³H]S-adenosyl-L-methionine (Amersham Biosciences, Piscataway, NJ) and TE buffer (1.67 mM Tris, 0.33 mM EDTA, pH 7.4) for 3 h at 37°C in a final volume of 10 μ l. Reactions were stopped by adding 10 μ l of 2 $\times$ Lämmli buffer, followed by boiling for 5 min. Samples were loaded on 12 or 15% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and a high level of N,N,N',N'-tetramethylethylenediamine and stained with Coomassie blue. The destained gels were visualized by fluorography with Amplify (Amersham Biosciences) according to the manufacturer's instructions.

In vivo methylations. Similar in vivo methylation experiments were previously described (5). Briefly, 293T cells were transfected with His-Tat72 and pmyc-PRMT1, -5, or -6. At 24 h posttransfection, the cells were pulse-labeled with L-methyl-[³H]methionine (Amersham Biosciences) for 3 h in the presence of cycloheximide (100 μ g/ml) and chloramphenicol (40 μ g/ml) (both from Sigma) in Dulbecco's modified Eagle's medium lacking the amino acids methionine, cysteine, and glutamine to prevent protein synthesis. The cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, pH 8) containing complete mini EDTA-free protease inhibitor (Roche) and processed as described for the in vitro methylations.

Coimmunoprecipitations. 293T cells were cotransfected with HA-Tat86 and pmyc-PRMT1 or -6. At 24 h posttransfection, cells were lysed and an aliquot was used for immediate immunodetection of PRMT1, PRMT6, and Tat86 with either anti-myc or anti-HA antibodies, respectively. Cell lysates were immunoprecipitated with anti-HA antibody. Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with anti-myc antibody.

CAT and luciferase reporter assays. Tat activation assays were performed with Tat fusion proteins and HIV-1 LTR chloramphenicol acetyltransferase (CAT) or HIV-1 LTR luciferase reporters containing HIV TAR sites (29). Where indicated, pSV2/Tat and variant Tat (100 ng) DNAs were transfected in the presence or absence of wild-type or mutated PRMT6, as well as the PRMT1 or PRMT5 (0.05 and 0.5 μ g) plasmids. For transfections, 100 ng of CAT reporter plasmid or 20 ng of luciferase reporter plasmid, 100 ng Tat-expressing plasmid, and any given pmyc-PRMT (50 to 500 ng) plasmid were cotransfected together with carrier plasmid (pBluescript DNA; total DNA was adjusted to 2 μ g) using 5 μ l Lipofectin (Life Technologies) in 3.8-cm² wells for 4 h. For NIH 3T3 cell transfections, 100 ng reporter plasmid, 100 ng Tat-expressing plasmid, and 300 ng pmyc-PRMT6 or pmyc-PRMT6m plasmids were cotransfected together with carrier plasmid (pBluescript DNA; total DNA was adjusted to 2 μ g) using 4 μ l

Lipofectamine 2000 (Life Technologies) for 5 h. For some experiments in 3T3 cells, 100 to 400 ng of a plasmid expressing human cyclin T1 (residues 1 to 272) (28) was also cotransfected. Cell extracts were assayed for CAT activity after 48 h as described previously (29). Activities were quantified using a Molecular Dynamics PhosphorImager, and activation relative to that for the reporter plasmid alone was calculated. For each experiment, CAT assays were performed in duplicate, and percentages of activation for the different reporters or protein mutants relative to those for the wild-type combination were calculated. Percentages of activation were then averaged over three or four separate transfection experiments. In the case of the luciferase reporter system, luciferase and *Renilla* activities were measured at 48 h posttransfection, using the dual-luciferase reporter assay system (Promega). Luciferase activity was then normalized to that of *Renilla*.

RNA-binding gel shift assays. Randomly labeled wild-type or mutant (UGGG in the loop changed to CAAA) TAR RNAs were transcribed by T7 RNA polymerase using synthetic oligonucleotide templates (22) and [³²P]CTP (3,000 Ci/mmol) and were purified on 15% polyacrylamide (8 M urea) gels and renatured as described elsewhere (26). To examine ternary complex formation with cyclin T1 and various GST-Tat proteins, GST-human cyclin T1 (1–272) was expressed and purified as previously described (28). The proteins (300 ng of GST-Tat or PRMT6-methylated GST-Tat together with 1.6 μ g of GST-cyclin T1) were incubated with various concentrations of RNA for 20 min at 30°C in 30 mM Tris-HCl, pH 8, 70 mM KCl, 0.01% NP-40, 5.5 mM MgCl₂, 1 mM dithiothreitol, and 12% glycerol (12 μ l final volume). RNA-protein complexes were resolved on 6% polyacrylamide Tris-glycine gels at 4°C, and bands were visualized by phosphorimaging.

Generation of cell lines stably expressing PRMT siRNAs. Oligonucleotides encoding siRNAs directed against PRMT mRNA have been described previously (5, 27) and were purchased from Invitrogen (California) for PRMT6. These oligonucleotides were annealed and ligated into pSUPER.retro (Oligo-engine) downstream of the H1 promoter, giving rise to the pSUPER.retro-PRMT6 retroviral vector. The latter was used to transfect Phoenix packaging cells with Lipofectamine 2000 (Invitrogen) to produce ecotropic retroviral supernatants which, at 48 h posttransfection, were filtered through a 0.45- μ m filter. This filtrate was then used to infect 293T, HeLa, and Jurkat cells, and infected cells were selected with puromycin (2 μ g/ml) for 2 weeks. Stable knockdown of the PRMT6 gene in the different cell lines was determined by Western blot analysis to ensure that knockdown, mediated by pSUPER.retro, was maintained over long periods.

HIV-1 virus production. 293T cells that stably express PRMT siRNAs, mock siRNA, or empty pSUPER vector were seeded into 100-mm plates and transfected with Lipofectamine 2000 (Invitrogen). Each plate received 10 μ g of BH10 proviral DNA. At 48 h posttransfection, the culture supernatants were collected and assayed for HIV-1 p24 (capsid) by an enzyme-linked immunosorption assay (Perkin-Elmer). Viral replication kinetics were monitored by reverse transcriptase activity assays using Jurkat cells stably expressing siRNA against PRMT6 or mock siRNA as a source of progeny HIV.

RESULTS

HIV-1 Tat is specifically arginine methylated by PRMT6. To prove the specificity of Tat arginine methylation by PRMT6, we carried out in vitro methylation assays with PRMTs 1, 3, 4, 5, 6, and 7 (Fig. 1A). Proteins were separated by SDS-PAGE, and equally loaded amounts of Tat were verified by Coomassie staining. On the fluorograph, only incubation with PRMT6 (lane 5) yielded an intense signal for Tat arginine methylation.

To further assess specificity, we established an in vivo methylation assay (Fig. 1B) involving PRMT1 and PRMT5, two other methyltransferases implicated in HIV-1 regulation (17), as well as PRMT6. HeLa cells were cotransfected with Tat and the different PRMTs and at 24 h posttransfection pulse-labeled with [³H]methionine, in the presence of translation inhibitors. Subsequent analysis of the cell lysates demonstrated that arginine methylation of Tat was only carried out by PRMT6 (lane 3).

Finally, we compared PRMT6 to PRMT1, the main PRMT in human cells (25), in coimmunoprecipitation experiments

FIG. 1. PRMT6 specifically methylates and interacts with HIV-1 Tat in vitro and in vivo. A. Recombinant histidine-tagged Tat72 was incubated with methyl- ^3H S-adenosyl-L-methionine in the presence of different PRMTs. Proteins were separated by SDS-PAGE and stained with Coomassie blue (upper panel), and tritium incorporation was monitored by fluorography (lower panel). The migratory positions are indicated by arrows on the left. B. 293T cells were transfected with histidine-tagged Tat72 (lanes 2 to 5) and myc epitope-tagged PRMTs (lanes 3 to 5). After 3 h pulse-labeling, cell lysates were separated by SDS-PAGE, Coomassie stained (upper panel), and fluorographed (lower panel). The migratory positions are indicated by arrows. C. 293T cells were transfected with HA epitope-tagged Tat86 (lanes 1 to 4) and myc epitope-tagged PRMT1 (lanes 2 and 4) or PRMT6 (lanes 1 and 3). A 5% portion of crude cell lysate was immunostained with either anti-myc (upper left panel) or anti-HA (lower left panel) antibodies. Coimmunoprecipitations were carried out with anti-HA antibody, and the eluates were immunostained with anti-myc antibody (right panel). All signals were detected with secondary antibodies coupled to horseradish peroxidase. IgG, immunoglobulin G. The migratory positions are indicated by arrows on the left.

(Fig. 1C). The controls showed the presence of Tat and either PRMT6 or PRMT1 in the crude cell lysates (lanes 1 and 2). However, only PRMT6 was detected after immunoprecipitation of Tat (lane 3), whereas PRMT1 did not apparently interact with Tat (lane 4).

The use of either Tat72 (Fig. 1A and B) or Tat86 (Fig. 1C) in these experiments did not reveal any significant differences and, hence, strengthened the notion that the arginine-rich motif of Tat is the only target site for PRMT6 within Tat. These results all demonstrate clearly the specificity of the Tat-PRMT6 interaction and arginine methylation of Tat by PRMT6.

PRMT6 overexpression quenches Tat transcriptional activation. CAT assays were used to monitor the transcriptional activation activity of methylated Tat. Accordingly, a pSV2/Tat72 expression vector was cotransfected into HeLa cells together with wild-type or mutated PRMT6 plasmids, the latter containing VLD-to-KLA substitutions in the active site that inactivate the enzyme. Transcriptional activation was monitored using an HIV LTR CAT reporter construct. The results

in Fig. 2A show that arginine methylation diminished the transactivation capacity of Tat. Notably, overexpression of wild-type PRMT6 inhibited HIV-1 LTR-directed expression of CAT in the presence of Tat (lanes 2 to 6), while overexpression of mutated PRMT6 minimally affected this function (lanes 8 to 12). These results confirm previous data by our group with an HIV-1 luciferase assay (5).

To demonstrate the specificity of this effect, we also tested PRMT1 and PRMT5 in an HIV-1 luciferase assay (Fig. 2B) and compared their impact on Tat transcriptional activation with wild-type and mutant PRMT6. As expected, the data revealed that a significant reduction in Tat transcriptional activation occurred only in the case of wild-type PRMT6 (lanes 9 to 12). Furthermore, these results also show that the site(s) of arginine methylation is located within Tat72, no matter which type of Tat (72, 86, or 101) is used. Therefore, all other experiments were performed using Tat72.

To rule out any effects of PRMT6 on other proteins, we repeated the HIV-1 luciferase assay in the absence of Tat and stimulated gene expression by adding 12-*O*-tetradecanoylphor-

FIG. 2. PRMT6 affects Tat transcriptional activation. A. HeLa cells were cotransfected with HIV LTR TAR CAT reporter plasmid (100 ng) and pSV2/Tat72 plasmid (100 ng) and pmc-PRMT6 or pmc-PRMT6m plasmids (50, 100, 200, and 500 ng) expressing wild-type or mutated PRMT6. CAT assays were performed 48 h after transfection. Tat activity was quantified using a Molecular Dynamics PhosphorImager. Transactivation levels were calculated as the amount of mono-acylated chloramphenicol by CAT in the presence of Tat divided by the amount of acylated chloramphenicol in the absence of Tat. B. HeLa cells were cotransfected with HIV LTR Luc reporter plasmid (20 ng), pFLAG-Tat101 (100 ng), and pmc-PRMTs (1, 5, 6, or 6m) plasmids (125, 250, and 500 ng) Luciferase activities were assayed at 48 h posttransfection and normalized to those of *Renilla*. C. NIH 3T3 cells were cotransfected with HIV LTR TAR CAT reporter plasmid (100 ng), pSV2/Tat72 plasmid (100 ng), pmc-PRMT6 or pmc-PRMT6m plasmids (300 ng), and human cyclin T1 (100, 200, 300, and 400 ng). CAT assays were performed as described for panel A. All assays were repeated at least two times.

bol-13-acetate (data not shown). The luciferase levels expressed were not affected by increasing amounts of PRMT1, PRMT5, or PRMT6, showing that the effects described above are specifically triggered through Tat arginine methylation by PRMT6.

The ability of PRMT6 to affect Tat transcriptional activation was cyclin T1 dependent (Fig. 2C). Toward this end, we co-transfected either wild-type or mutated PRMT6 together with pSV2/Tat72 and human cyclin T1 into murine NIH 3T3 cells, which require the addition of human cyclin T1 for proper Tat

transactivation. As expected, mutated PRMT6 did not affect cyclin T1-dependent Tat transcriptional activation (lanes 2 to 6). In contrast, wild-type PRMT6 down-regulated Tat transcriptional activation to basal levels, as if no human cyclin T1 had been added (lanes 8 to 12). Thus, these data demonstrate that the methyltransferase activity interferes with cyclin T1-dependent Tat transcriptional activation.

Arginine methylation of Tat diminishes Tat-TAR binding affinity and Tat-TAR-cyclin T1 complex formation. Prokaryotes do not contain any PRMTs (12). Therefore, to test the

FIG. 3. Tat methylated by PRMT6 affects Tat-TAR binding and formation of Tat-TAR-cyclin T1 ternary complexes. A. PRMT6 methylation of GST-Tat protein was carried out in vitro (see Material and Methods). Tat proteins (50, 100, 200, and 400 ng) previously subjected to wild-type, mutant, or no PRMT6 were incubated with radiolabeled HIV TAR RNA, and protein complexes were resolved by native gel electrophoresis. Control lanes with TAR alone (lane 1) and TAR with wild-type or mutated GST-PRMT6 (lanes 11 and 6, respectively) in which no complexes were observed are also shown. B. These experiments were carried out as described for panel A; however, human cyclin T1 (1.5 and 3 μ g) was additionally added. Control lanes with TAR only (lane 1) and TAR with cyclin T1, wild-type, or mutated PRMT6 (lanes 13, 9, and 5, respectively) are also shown. As a further control, a mutant TAR (UGGG in the loop mutated to CAAA) was used (lanes 14 to 20).

effect of arginine methylation on Tat-TAR RNA interactions, we purified GST-Tat from *Escherichia coli* and then compared in vitro-methylated Tat (wild-type PRMT6) to unmodified Tat (mutant or no PRMT6) in RNA-binding gel mobility shift experiments (Fig. 3A). Tat subjected to wild-type PRMT6 displayed a sharp loss in binding affinity for TAR RNA (lanes 14 and 15). In contrast, unmodified Tat treated with mutant PRMT6 (lanes 9 and 10) did not significantly differ from Tat that had been incubated under methylation conditions without any PRMT6 (lanes 4 and 5).

We next examined whether methylated Tat might affect binding to the cellular factor cyclin T1 (Fig. 3B). Tat methylated by wild-type PRMT6 was unable to form affinity Tat-TAR-cyclin T1 complexes (lanes 11 and 12). However, Tat treated with mutant PRMT6 (lanes 7 and 8) still formed complexes similar to those formed by Tat under conditions that did not include extraneous PRMT6 (lanes 3 and 4). Neither wild-type nor mutated PRMT6 was able to bind to TAR RNA directly (lanes 9 and 5, respectively). To prove specificity of

binding, we used a mutant TAR, in which the sequence UGGG of the TAR loop was replaced with CAAA. Such mutant TAR does not affect proper Tat-TAR binding but does prevent the association of cyclin T1 to the TAR loop and, hence, inhibits complex formation. As expected, Tat-TAR interactions were detected; however, neither methylated Tat (lanes 18 and 19) nor Tat treated with mutant PRMT6 (lanes 15 and 16) was able to form complexes involving cyclin T1. Taken together, these results demonstrate that methylated Tat can bind only weakly to TAR RNA or form Tat-TAR-cyclin T1 complexes, and this results in diminished transcriptional activation.

The Tat residues R52 and R53 are the targets of PRMT6. We next focused on the ability of the Tat ARM, more specifically the 49-RKKRR-53 stretch, to participate in high-affinity binding for HIV TAR RNA (Fig. 4A). CAT assay experiments demonstrated that both replacement of a wild-type Tat ARM with an RRRRRRRRR (9xR) sequence (lane 3) or introduction of an R49A substitution (lane 4) into wild-type Tat ARM resulted in strong Tat transcriptional activation similar to that of wild-type Tat (lane 2). Further mutational analysis with mutants containing single amino acid substitutions showed that the R53K mutant (lane 8) retained a transcriptional activation level similar to wild-type Tat, whereas transcriptional activity of the R52K mutant (lane 7) was reduced by fourfold. The double mutant R52K/R53K (lane 6) also revealed a severely impaired transcriptional activity. However, in the case of the double mutant R49A/R53A (lane 5) almost no transcriptional activity could be detected. This effect can be explained by the loss of positive charges in the ARM that are absolutely required for proper function in Tat transcriptional activation. These results demonstrate that R52 plays a key role in Tat transcriptional activation and confirm previous findings that a single arginine residue within the ARM of Tat is required for specific binding and transactivation (7).

In a different assay, we checked wild-type and mutant GST-Tat proteins purified from *E. coli* for in vitro methylation by PRMT6 (Fig. 4B). The results show that mutant Tat that was changed at either R52K or R53K resulted in clearly reduced methylation signals and that the R52K/R53K doubly mutated Tat was very poorly methylated by PRMT6. In the case of the R49A/R53A double mutant, we detected a weaker signal, but still stronger than that of the R53K mutant, which excludes R49 as a methyl-accepting residue. Taken together, these results demonstrate that both R52 and, to a lesser extent, R53 are targets for PRMT6.

Reduced expression of PRMT6 by siRNA stimulates Tat transactivation and HIV-1 viral production. In order to investigate whether PRMT6 might affect HIV-1 viral replication, we established PRMT6 knockdown 293T, HeLa, and Jurkat cell lines using the pSUPER.retro-PRMT6 retroviral vector. These cell lines stably express siRNA directed against PRMT6, leading to knockdown of PRMT6 gene expression (Fig. 5A), a state maintained by pSUPER.retro over long periods (data not shown). Furthermore, we also established PRMT1 and PRMT5 knockdown 293T cell lines for comparative purposes in Tat transactivation assays (Fig. 5B).

CAT reporter assays showed that PRMT6 knockdown HeLa cells displayed higher levels of Tat transcriptional activation than normal HeLa cells (Fig. 6A). In regard to Tat containing the R53K (Fig. 6B) and R52K (Fig. 6C) mutations, an increase

FIG. 4. Tat ARM residues affect transactivation, and PRMT6 methylates Tat at specific residues. **A**. The ARM basic region of Tat was replaced by nine arginine residues or point mutations in the mammalian Tat expression plasmid pSV2/Tat72. Each mutant plasmid (100 ng) was transfected into HeLa cells, and CAT activity was assayed and quantified as described for Fig. 2A. **B**. Recombinant variant GST-Tat proteins were purified from *E. coli* strain BL21; these proteins were incubated with methyl-[³H]S-adenosyl-L-methionine in the presence of GST-PRMT6 protein. For purposes of quantification, the methylation substrates were separated by SDS-PAGE and Coomassie stained (upper panel), and ³H incorporation was visualized by fluorography (lower panel). The migratory positions are indicated by arrows.

in transcriptional activity was detected in the case of PRMT6 knockdown HeLa cells. However, the difference between normal and knockdown cells was less than for wild-type Tat, which means that both the R52 and R53 residues are affected by PRMT6 methylation. In contrast, the R52K/R53K doubly mutated Tat (Fig. 6D) had only very weak transcriptional activity, and knockdown of PRMT6 did not increase its capacity to participate in transactivation. To demonstrate specificity of PRMT6 arginine methylation, we compared the transactivation capacities of wild-type Tat and mutant Tat R52K/R53K in HIV-1 luciferase assays in 293T cells that expressed siRNA against either PRMT1, PRMT5, or PRMT6 (Fig. 6E). As expected, the doubly mutated Tat showed significantly lower transcriptional activation levels than wild-type Tat; this was independent of any PRMT activity. In contrast, wild-type Tat was significantly increased in transactivation ability upon down-regulation of PRMT6, whereas knockdown of either PRMT1 or PRMT5 did not change the transactivation capacity

of Tat. All these results demonstrate that methylation of both R52 and R53 by PRMT6 reduces Tat transactivation.

As shown previously, HIV-1 p24 production was diminished concomitant with PRMT6 overexpression (5). Therefore, we transfected either PRMT1, PRMT5, or PRMT6 knockdown 293T cells with HIV-1 BH10 (Fig. 7A). Only down-regulation of PRMT6 resulted in higher production levels of HIV-1, whereas all other cell lines possessed similar p24 levels to those of ordinary 293T cells. We also studied virus replication kinetics in Jurkat cells (Fig. 7B). In the first experiment, we used HIV-1 derived from 293T cells expressing mock siRNA to in turn infect 10⁶ Jurkat cells either expressing siRNA against PRMT6 or a mock siRNA (Fig. 7B, left). The results showed that HIV-1 replication within the PRMT6 knockdown cells was detectable 2 to 3 days earlier than in mock siRNA Jurkat cells and that a threefold-higher production of HIV particles occurred at day 9 postinfection compared to mock siRNA Jurkat

FIG. 5. Knockdown of PRMTs by siRNA in different cell lines. **A**. PRMT6 knockdown 293T, HeLa, and Jurkat cell lines were established using the pSUPER.retro-PRMT6 retroviral vector. Cell lysates (40 μg) were separated on 10% SDS-PAGE. An immunoblot assay with PRMT6-specific antibody was performed; the bands corresponding to PRMT6 protein and the loading control β-actin are indicated. PRMT6 siRNA (6si), PRMT6 mock siRNA (6si m), and empty pSUPER.retro vector (pSUPER) cell lines are shown. **B**. PRMT1 and PRMT5 knockdown 293T cell lines (1si and 5si, respectively) were established as described for panel A. Immunoblot assays with PRMT-specific antibodies were performed.

FIG. 6. Reduced PRMT6 expression enhances Tat transcriptional activation. (A to D) HeLa cells and PRMT6 knockdown HeLa cells were transfected with HIV LTR CAT reporter plasmid (100 ng) as well as pSV2/Tat72 (wild type [wt]) (A), pSV2/Tat72 (R53K) (B), pSV2/Tat72 (R52K) (C), or pSV2/Tat72 (R52K/R53K) (D) plasmids (50, 100, 200, 300, and 500 ng). CAT activity was assayed and quantified as for Fig. 2A. (E) 293T cells in which the different PRMTs had been down-regulated (Fig. 5) were transfected with HIV LTR Luc reporter plasmid (20 ng) and pSV2/Tat72 (wt) or pSV2/Tat72 (R52K/R53K) plasmids. Luciferase was assayed and quantified as for Fig. 2B.

cells. When using HIV-1 collected from PRMT6 knockdown 293T cells for infection (Fig. 7B, right), viral growth studies showed even faster replication (3 to 4 days earlier) and four-fold-higher virus production in PRMT6 knockdown Jurkat cells compared to mock siRNA Jurkat cells. Thus, PRMT6 protein levels can regulate HIV-1 production.

DISCUSSION

Arginine methylation often takes place on proteins that contain a glycine- and arginine-rich motif (2, 12). Despite the fact that HIV-1 Tat does not harbor such a consensus sequence, our previous work has identified Tat as a substrate for PRMT6

FIG. 7. Knockdown of PRMT6 stimulates HIV-1 viral production. A. HIV-1 BH10 proviral cDNA (2 or 10 µg) was transfected into mock pSUPER 293T cells (pSUPER), PRMT1, -5, and -6 knockdown cells (1si, 5si, and 6si, respectively), and mock siRNA 293T cells (6si m). After 24 and 48 h, supernatant p24 levels were tested. B. HIV-1 virus growth kinetics in Jurkat cells. Viruses generated in mock siRNA (left) or PRMT6 knockdown (right) 293T cells were used to infect Jurkat cells either expressing mock siRNA (6si m) or siRNA against PRMT6 (6si). Numbers 1 and 2 in parentheses denote duplicates; 13 and 14 denote two different clones of PRMT6 knockdown Jurkat cells.

(5). The present study now identifies residues R52 and R53 within the ARM of Tat to be the targets for methylation by PRMT6 both in vitro and in vivo. This basic RNA-binding region of Tat, RKKRRQRRR (residues 49 to 57), is 9 amino acids long and contains a glutamine at position 54 that is not essential for Tat binding or activity (6).

Interaction of TAR with the ARM of Tat is mediated by a single arginine residue at position 52 (7). This residue forms a specific network of hydrogen bonds with the bulge region of TAR. Using a sensitive capillary electrophoresis mobility shift assay, others have shown that a synthetic ARM containing asymmetrically dimethylated arginine at position 52 is relatively unable to bind to TAR (23). Thus, methylation of R52 appears to change the pattern of hydrogen bonds created by its guanidinium group and modifies binding affinity to TAR. In the present study, we show that methylation of the arginine residues R52 and R53 within the ARM of Tat diminishes the affinity between Tat and TAR RNA. Presumably, distortion of the guanidinium group structure of R52 by methylation results in a modification of net hydrogen bonding and decreased Tat peptide affinity for TAR. We also confirmed that a high positive charge is important for RNA binding and that arginine residues are important for specific RNA recognition (29). Conceivably, methylation may also affect the localization of Tat.

Arginine methylation is generally thought to affect the function of methylated substrates by modulating their interactions with other proteins. Although methylation does not alter the overall charge on an arginine residue, the bulky methyl group

is likely to increase the steric hindrance on amino groups, which may lead to altered protein-protein interaction patterns. In the case of Tat, we asked whether methylation might affect its association with cellular factors, such as P-TEFb (cyclin T1 and CDK9). In the present study, our in vitro experiments clearly show that formation of Tat-TAR-cyclin T1 complexes was severely impaired when using methylated Tat. In vivo Tat transcriptional activation, which is associated with human cyclin T1 in murine 3T3 cells, was specifically diminished by overexpression of PRMT6 and, hence, Tat hypermethylation.

In experiments involving 293T and Jurkat cell lines, we showed that down-regulation of PRMT6 by siRNA significantly augmented Tat function. Furthermore, siRNA against PRMT6 significantly increased HIV-1 production and replication. This suggests that Tat activity might either need to be fine-tuned within cells in order to achieve optimal viral gene expression or that methylation represents a host antiviral defense mechanism against HIV-1.

Both PRMT1 and PRMT5 are implicated in HIV-1 regulation through action on cellular proteins (17). Comparing the effects of up- or down-regulation of these methyltransferases to that of PRMT6 demonstrates very clearly that only PRMT6 interacts specifically with Tat. Importantly, we did not find any significant change in virus production in our 293T cell assays (Fig. 7A) when using siRNA directed against PRMT1 or PRMT5. However, down-regulation of PRMT6 quickly led to increased production of virus. Our results all demonstrate that arginine methylation of Tat by PRMT6 is a very specific event

which has an impact on levels of virus production and replication.

Tat has previously been shown to be modified by histone acetyltransferase p300 at amino acid position K50 (16, 24). In the present study, we found that R52 and R53 are the two residues most likely to be methylated by PRMT6 in the context of the ARM. The proximity of the acetylation site at K50 to the methylated R52 and R53 residues raises the question as to whether these two distinct types of protein modification can occur on the same Tat molecule or whether one of these modifications on a single molecule excludes the other. Methylation of Tat may conceivably impede acetylation of K50 by p300, thus blocking Tat induction, an interplay that has already been described for histones (4, 18). We are currently studying this issue.

Overexpression of methyltransferases can inhibit transactivation, whereas methyltransferase inhibitors can increase transactivation (17). Indeed, both positive and negative small-molecule regulators of PRMT activity have recently been identified (9). Compounds that activate PRMT6, if identified, might function as a brake on Tat coactivator function and lead to diminished viral replication. In contrast, compounds that specifically block methylation by PRMT6 might be able to increase HIV replication and potentially drive latently infected cells to become efficient producers of progeny virus.

These and previous data (5, 15) suggest that PRMT6-mediated methylation of Tat may represent a form of innate cellular immunity that acts to restrict levels of HIV-1 replication. In this context, proteins of the APOBEC family are known to be potent inhibitors of HIV-1 replication as a result of deamination of cytosine residues in retroviral cDNA. However, HIV-1 Vif mediates APOBEC degradation and efficiently counteracts this cellular innate immunity (13, 14). It will be interesting to determine whether any HIV-1 proteins that are not PRMT6 substrates may be able to negatively impact on levels of PRMT6 methylation of Tat and Rev, as a means of offsetting the inhibitory effects of PRMT6 on viral replication.

ACKNOWLEDGMENTS

This research was supported by a grant from the Canadian Institutes of Health Research. C.F.I. was partially supported by a Swiss National Science Foundation fellowship award.

We thank Maureen Oliveira for technical assistance. We also thank Alan Frankel, Sara Nakielnny, and Melanie Ott for gifts of the various plasmid constructs described in Materials and Methods.

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