JOURNAL OF VIROLOGY, May 2009, p. 4749–4756 Vol. 83, No. 10
0022-538X/09/$08.00+0 doi:10.1128/JVI.02585-08

A Limited Group of Class I Histone Deacetylases Acts To Repress Human Immunodeficiency Virus Type 1 Expression▽

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Received 15 December 2008/Accepted 5 March 2009

Silencing of the integrated human immunodeficiency virus type 1 (HIV-1) genome in resting CD4$^+$ T cells is a significant contributor to the persistence of infection, allowing the virus to evade both immune detection and pharmaceutical attack. Nonselective histone deacetylase (HDAC) inhibitors are capable of inducing expression of quiescent HIV-1 in latently infected cells. However, potent global HDAC inhibition can induce host toxicity. To determine the specific HDACs that regulate HIV-1 transcription, we evaluated HDAC1 to HDAC11 RNA expression and protein expression and compartmentalization in the resting CD4$^+$ T cells of HIV-1-positive, aviremic patients. HDAC1, -3, and -7 had the highest mRNA expression levels in these cells. Although all HDACs were detected in resting CD4$^+$ T cells by Western blot analysis, HDAC5, -8, and -11 were primarily sequestered in the cytoplasm. Using chromatin immunoprecipitation assays, we detected HDAC1, -2, and -3 at the HIV-1 promoter in Jurkat J89GFP cells. Targeted inhibition of HDACs by small interfering RNA demonstrated that HDAC2 and HDAC3 contribute to repression of HIV-1 long terminal repeat expression in the HeLa P4/R5 cell line model of latency. Together, these results suggest that HDAC inhibitors specific for a limited number of class I HDACs may offer a targeted approach to the disruption of persistent HIV-1 infection.

Persistent proviral human immunodeficiency virus type 1 (HIV-1) infection, primarily within a small population of long-lived resting CD4$^+$ T cells, is a central obstacle to the clearance of established HIV-1 infection. This is due, in part, to the occasional silencing of proviral HIV-1 genomes in resting CD4$^+$ T cells, shielding this reservoir from immunological or pharmaceutical attack. To overcome proviral latency, potent and clinically tolerable agents capable of inducing expression of latent HIV-1 must be identified. Such efforts will be aided by an understanding of mechanisms that regulate transcription from the HIV-1 5′ long terminal repeat (LTR) promoter.

Once integrated, HIV-1 resides in the host chromatin environment, where its genome is packaged around histone octamers (25). The N-terminal tails of histones can be chemically modified by multiple enzymes including histone acetyltransferases and histone deacetylases (HDACs) (reviewed in reference 5). Acetylation of the lysine residues in histone tails by histone acetyltransferases allows transcriptional machinery access to the DNA template and serves as a signal for recruitment of transcription factors and complexes that upregulate gene expression. Conversely, HDACs are a family of lysine deacetylases that act on multiple targets, including histone tails. Deacetylation of histones creates a chromatin environment unfavorable to transcription, in part by creating a platform for the recruitment of histone methyltransferases and other factors that repress transcription. There are 18 known human HDACs, which are divided into four classes based on amino acid sequence and domain organization (reviewed in reference 5). Class I HDACs (HDAC1, -2, -3, and -8); class II HDACs (HDAC4, -5, -6, -7, -9, and -10); and the class IV HDAC, HDAC11, are all sensitive to global HDAC inhibitors such as trichostatin A (TSA). Class III HDACs, also known as the sirtuins, are structurally unrelated to the other classes and are not sensitive to traditional HDAC inhibitors.

Evidence suggests that mechanisms of acetylation and deacetylation regulate HIV-1 proviral expression (reviewed in reference 22). HDACs are recruited to the initiator and enhancer regions of the HIV-1 promoter by several transcription factors and corepressor complexes. The recruitment of HDAC1 to the latent HIV-1 LTR was first shown to be mediated by YY1 and LSF (4), and later studies demonstrated that NF-κB p50 homodimers (26), AP-4 (10), CTIP2 (20), Sp1 and c-Myc (12), and CBF-1 (23) could all participate in HDAC1 recruitment. CTIP2 was also found to recruit HDAC2 to the Sp1 binding site of the LTR in microglial cells (20). Furthermore, HDAC3 has been shown to associate with the HIV-1 LTR (18). HDAC3 occupancy at the LTR is further supported by evidence demonstrating recruitment of the HDAC3-containing corepressor complexes, N-CoR and SMRT, by unliganded thyroid hormone receptor in a *Xenopus laevis* oocyte model system for chromatin assembly (9).

The existence of multiple mechanisms that recruit HDACs to the proviral promoter may be of high therapeutic significance. Nonselective HDAC inhibition with potent agents such as TSA can induce HIV-1 expression in cell line models of latency (15, 16, 24). Furthermore, HDAC inhibitors induce viral expression in resting CD4$^+$ T cells obtained from aviremic, HIV-1-positive patients (1, 27). Inhibitors specific for the individual HDACs relevant to HIV-1 LTR regulation may

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▽ Published ahead of print on 11 March 2009.

provide a more selective targeting of the viral promoter and avoid toxicities that can accompany robust, global HDAC inhibition.

To define the specific HDACs that regulate HIV-1 transcription during latency, we characterized mRNA expression and protein expression and localization of HDAC1 to -11 in the resting $CD4^+$ T cells of HIV-1-positive patients. We evaluated HDAC occupancy at the HIV-1 LTR and the effect of targeted HDAC inhibition on HIV-1 transcriptional activation in cell line models of HIV-1 latency. We find that HDAC2 and HDAC3 play a prominent role in the regulation of HIV-1 expression. These enzymes may be important targets for selective antilatency therapies.

MATERIALS AND METHODS

Primary $CD4^+$ T cells and cell lines. Resting $CD4^+$ T cells were obtained from aviremic (<50 HIV-1 RNA copies/ml plasma), HIV-1-positive patients on stable antiretroviral therapy with $CD4^+$ T-cell counts of >300/μl via continuous-flow leukapheresis. Peripheral blood mononuclear cells were purified from leukapheresis products using a Ficoll gradient and subjected to a negative-selection purification using an antibody cocktail of anti-CD8, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-glycophorin A, anti-CD41, anti-CD25, and anti-HLA-DR as previously described (2) to obtain purified (>97%) resting $CD4^+$ T cells defined as a $CD4^+$ $CD45^+$ $CD3^+$ $CD69^-$ $CD25^-$ $CD8^-$ $CD14^-$ HLA-DR^- population by fluorescence-activated cell sorting analysis. J89GFP cells (16) were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). HeLa P4/R5 cells (14) were cultured in phenol red-free Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.5 mg/ml puromycin (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin.

RNA extraction and microarray analysis. RNA was extracted from purified resting $CD4^+$ T cells of aviremic, HIV-1-positive patients and hybridized to a microarray of 23,500 60-mer oligonucleotides from Agilent Technologies (Palo Alto, CA) as previously described (12). Intensities of HDAC mRNA expression were normalized to an Agilent internal standard and converted to log ratio values using the Rosetta Resolver system (Rosetta Biosoftware, Seattle, WA).

Western blot analysis of protein expression and localization. For fractionation of nuclear and cytoplasmic proteins, purified resting $CD4^+$ T or J89GFP cells were first lysed for 10 min on ice in a buffer containing 10 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1% NP-40, 10 mM NaF, and protease inhibitor cocktail (Sigma). Following centrifugation, cytoplasmic extracts were removed and nuclei were incubated in RIPA buffer with protease inhibitor cocktail (Sigma) and 10 mM NaF for 10 min on ice. Cellular debris was removed by centrifugation, and nuclear extracts were collected. Protein concentrations were determined using Bradford protein assays (Bio-Rad). To denature proteins, extracts were heated at 95°C for 5 min in NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Invitrogen). Protein extracts were separated on 4 to 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were probed with the following antibodies: anti-HDAC1 (sc-7872x), anti-HDAC2 (sc-7899x), anti-HDAC3 (sc-11417x), anti-HDAC6 (sc-11420), anti-HDAC7 (sc-11421x), anti-HDAC8 (sc-11405), and anti-HDAC9 (sc-28732) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HDAC4 (40969) and anti-HDAC5 (40970) (Active Motif, Carlsbad, CA); anti-HDAC10 (AP1110a) and anti-HDAC11 (AP1111b) (Abgent, San Diego, CA); anti-lamin B1 (ab16048) and anti-alpha-tubulin (ab7291) (Abcam, Cambridge, MA); and anti-glyceraldehyde-3-phosphate dehydrogenase (MAB374) (Millipore, Billerica, MA). Membranes were then washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies from ECL-Amersham (GE Healthcare, Piscataway, NJ). Proteins were visualized using ECL or ECL Plus detection reagent and developed on Hyperfilm ECL (GE Healthcare). Whole-cell protein extracts from resting $CD4^+$ T cells were collected by incubating cells in RIPA buffer as described above. Membranes were stripped by incubation in 62 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, and 2.7% β-mercaptoethanol for 25 min at 56°C and reprobed with antibodies as needed.

ChIP assays. J89GFP cells were fixed with 1% formaldehyde for 10 min at room temperature and subjected to the chromatin immunoprecipitation (ChIP) procedure using a ChIP assay kit (Millipore) following the manufacturer's protocol. Each immunoprecipitation was performed with approximately 1 million cells and 5 to 10 μg of the following ChIP-validated antibodies: anti-HDAC1 (sc-7872x), anti-HDAC2 (sc-7899x), and anti-HDAC3 (sc-11417x) from Santa Cruz; anti-HDAC4 (40969) or anti-HDAC6 (40971) from Active Motif; or anti-HDAC7 (ab1441) from Novus Biologicals (Littleton, CO). Rabbit immunoglobulin G (IgG) serum (5 to 24 μg; Sigma) was used to control for nonspecific immunoprecipitation of DNA. After formaldehyde de-cross-linking and proteinase K digestion (Roche, Nutley, NJ), DNA was purified using a QIAquick PCR purification kit (Qiagen, Germantown, MD) in accordance with the manufacturer's protocol.

Immunoprecipitated DNA was amplified using LTR −109F/LTR +82R (27) or LTR7F/LTR8R (12) primers to detect enrichment of the HIV-1 LTR and visualized by separating PCR products on an 8% acrylamide gel stained with ethidium bromide. Images were obtained using an InGenius L gel documentation system and GeneSnap software (Syngene, Frederick, MD). For quantitative PCR assays, ChIP DNA was amplified using LTRrt8 (5′-TAGCCAGAGAGCT CCCAGGCTCAGA-3′) and LTRrt9 (5′-AGCCCTCAGATGCTACATATAA GCA-3′) primers and Power SYBR green PCR Master Mix from Applied Biosystems (Foster City, CA). Values represent the enrichment over the IgG negative control using the threshold cycle ($2^{-\Delta\Delta CT}$) method.

siRNA transfection and β-galactosidase assays. Three distinct small interfering RNAs (siRNAs) were designed to each HDAC transcript based on predictors of on- and off-target activity (Sigma-Proligo). The sense sequences of siRNAs were as follows: HDAC1-1, CGCCAAGUGUGUGGAAUUU; HDAC1-2, CG AAUCCGCAUGACUCAUA; HDAC1-3, CUCAUAAUUUGCUGCUCAA; HDAC2-1, CAAAUACUAUGCUGUCAAU; HDAC2-2, CUCAUUAUCUG GUGAUAGA; HDAC2-3, CAGUGAUGAGUAUAUCAAA; HDAC3-1, CC AAGAGUCUUAAUGCCUU; HDAC3-2, GGCACCCAAUGAGUUCUAU; HDAC3-3, CAUUCAGGAUGGCAUACAA; HDAC8-1, CAUUCAGGAUG GCAUACAA; HDAC8-2, GACCGUGUCCCUGCACAAA; HDAC8-3, CAG UAUGGUGCAUUCUUUG. Individual siRNAs were reconstituted in nuclease-free water to achieve a 20 μM solution. The siRNAs targeting a single HDAC transcript were pooled at a 1:1:1 ratio prior to transfection. The siCONTROL Non-Silencing 1 (NS1) negative control was purchased from Dharmacon (Lafayette, CO) and reconstituted according to the manufacturer's protocol.

All siRNA transfection experiments were performed using HeLa P4/R5 cells. Briefly, cells were plated in 96-well, white tissue culture-treated plates (Corning, Corning, NY) in 80 μl of assay medium (phenol red-free Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 0.5 mg/ml puromycin [Sigma], 100 U/ml penicillin, and 100 μg/ml streptomycin [Invitrogen]) at 1×10^5 cells/ml and incubated overnight at 37°C and 5% CO_2. Prior to transfection, siRNA-Oligofectamine (Invitrogen) complexes were established according to the manufacturer's protocol and then added to each well to achieve a final concentration of 50 nM siRNA and 0.5% Oligofectamine. After incubation at 37°C and 5% CO_2 for 20 h, medium was removed from the cells and replaced with assay medium containing either 1 μM TSA or 1% dimethyl sulfoxide (DMSO). Following 20 h of incubation at 37°C and 5% CO_2, LTR-mediated β-galactosidase activity was measured using Gal-Screen (Applied Biosystems) according to the manufacturer's protocol.

Cell proliferation assays. Cell proliferation was evaluated following transfection with HDAC siRNA and incubation with TSA or DMSO using the alamarBlue assay (Trek Diagnostic Systems, Cleveland, OH) according to the manufacturer's protocol.

Statistical analysis. Normalized β-galactosidase activity and cell proliferation following siRNA-mediated HDAC knockdown were analyzed by the nonparametric Kruskal-Wallis test. To make individual comparisons against the mock control, the Mann-Whitney U test was applied post hoc with Bonferroni's correction. Analyses were performed using SPSS (version 16.0) software (Chicago, IL). A P value less than 0.05 was considered statistically significant.

RESULTS

HDAC1, -3, and -7 are highly expressed in resting $CD4^+$ T cells. To determine which HDACs are expressed in resting $CD4^+$ T cells, the primary reservoir for latent HIV-1 infection, we calculated the relative intensities of mRNA expression for HDAC1 to -11. Levels of HDAC mRNA expression were similar between patients. HDAC1, -3 and -7 had the highest mRNA expression levels (Fig. 1A). However, as these levels were derived from microarray data, they provide information

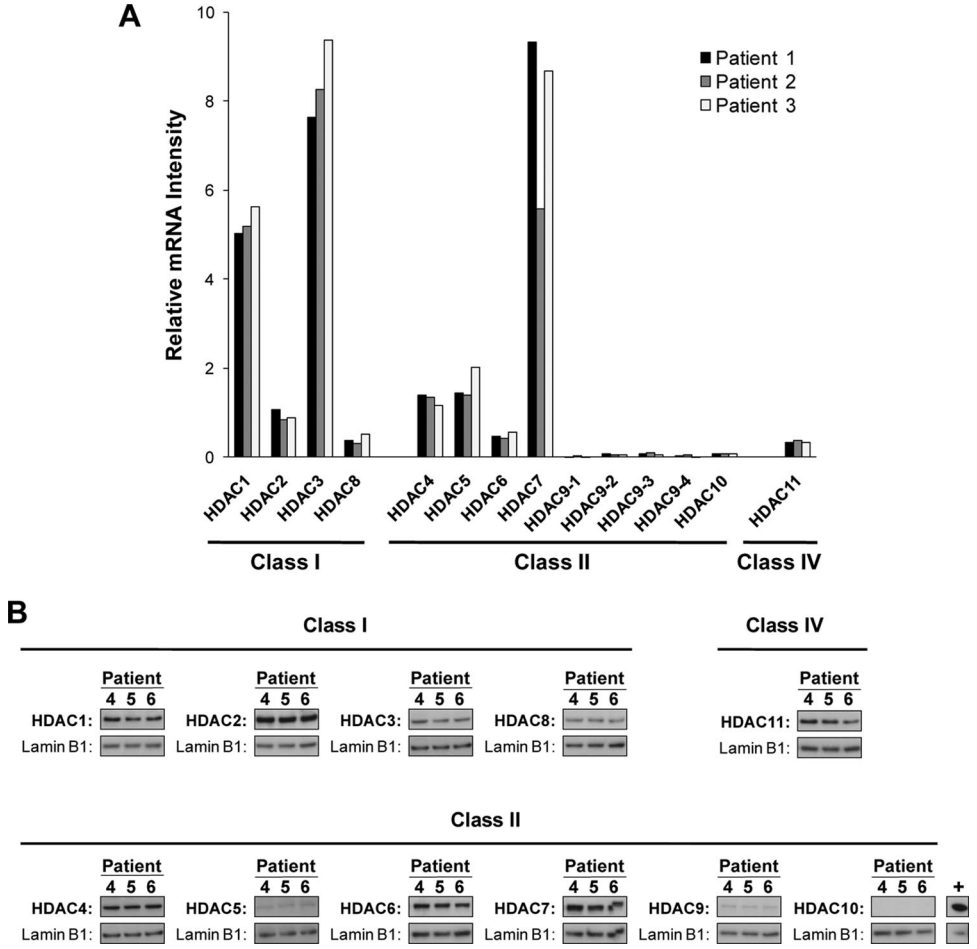

FIG. 1. HDAC mRNA expression in resting $CD4^+$ T cells from aviremic, HIV-1-positive patients. (A) HDAC1, -3, and -7 are the most highly expressed HDAC mRNAs in resting $CD4^+$ T cells. Microarray analysis of mRNA expression in the resting $CD4^+$ T cells from three HIV-1-positive patients provided relative intensities of HDAC mRNA expression. (B) HDAC1 to -11 are detectable in resting $CD4^+$ T cells. Whole-cell extracts were obtained from the resting $CD4^+$ T cells of HIV-1-positive patients, and 20 μg of protein was subjected to Western blot analysis with antibodies specific for HDAC1 to -11. An antibody against the nuclear envelope marker lamin B1 was used as a loading control. As a positive control (+) for the anti-HDAC10 antibody, 2 μg of whole-cell extracts from 293T cells transfected with an HDAC10 expression plasmid was subjected to Western blotting. Although HDAC10 was not detected in patient extracts in the experiment shown, it was detected when 40 μg of extracts was assayed (data not shown).

on only the relative intensities of HDAC expression in the resting $CD4^+$ T cells of the patients and not absolute expression levels.

To evaluate HDAC protein expression, we obtained whole-cell extracts from the resting $CD4^+$ T cells of three additional HIV-1-positive patients and performed Western blot analysis using antibodies specific for HDAC1 to -11. HDAC1 to -11 were detected in all three patients, and levels of HDAC protein expression were relatively constant between patients (Fig. 1B). As a positive control, we performed Western blotting on 2 μg of lysate from 293T cells transfected with an HDAC10 expression plasmid (lysate obtained by Abgent) to verify the ability of the HDAC10 antibody to function in Western blotting. Although HDAC10 was undetectable in 20 μg of whole-cell extracts as shown in Fig. 1B, HDAC10 was detected when Western blot analysis was performed on 40 μg of whole-cell extracts (data not shown). However, the low levels of HDAC10 expression in resting $CD4^+$ T cells suggest that it is unlikely to contribute to HIV-1 latency.

HDAC5, -8, and -11 are predominantly cytoplasmic in resting $CD4^+$ T cells. To determine the localization of HDACs within resting $CD4^+$ T cells, we separated cellular lysates into nuclear and cytoplasmic fractions and evaluated HDAC protein expression by Western blot analysis. Most HDACs were expressed in the nucleus, and many were detectable in both the nuclear and the cytoplasmic fractions (Fig. 2). However, HDAC5, -8, and -11 were primarily localized to the cytoplasm. These findings suggest that it is unlikely that HDAC5, -8, or -11 plays a direct role in regulating transcription driven by the LTR at the level of chromatin modification in the latent reservoir of resting $CD4^+$ T cells, although it is possible that some amounts of these enzymes reside in the nucleus below the level of detection of the antibodies used in this assay. Furthermore, it does not exclude a possible contribution to transcriptional

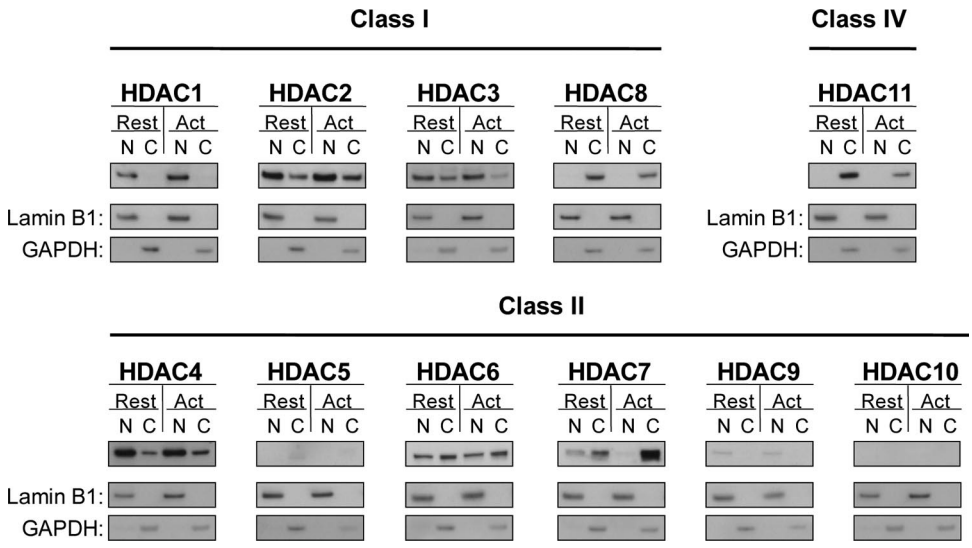

FIG. 2. HDAC5, -8, and -11 are excluded from the nuclei of resting $CD4^+$ T cells. Resting $CD4^+$ T cells from an aviremic, HIV-1-positive patient (patient 5 from Fig. 1A) were maintained in medium (Rest) or activated by incubation with 1 μg/ml of the mitogen PHA overnight (Act) before cellular lysates were harvested. Proteins were separated into nuclear (N) and cytoplasmic (C) fractions, and 15 μg of extracts was probed with antibodies targeting HDAC1 to -11 in Western blot analysis. Antibodies against lamin B1 and glyceraldehyde-3-phosphate dehydrogenase were used to assess loading of nuclear and cytoplasmic lysates. HDAC5, -8, and -11 were primarily localized to the cytoplasm in $CD4^+$ T cells. Following T-cell activation with PHA, HDAC7 expression increased and became sequestered in the cytoplasm, while HDAC11 expression decreased.

regulation by these HDACs in a pathway upstream of the LTR through deacetylation of cellular factors in the cytoplasm. HDAC10 expression levels were too low to permit appropriate analysis of localization in resting $CD4^+$ T cells.

Because latency is a phenomenon that occurs in resting $CD4^+$ T cells and not activated $CD4^+$ T cells, we examined whether there was a differential regulation of HDAC expression and/or localization in these two cell types. Most HDACs had similar expression levels and localization before and after mitogen stimulation with phytohemagglutinin (PHA) (Fig. 2). However, following T-cell activation, HDAC7 expression dramatically increased and became almost exclusively sequestered in the cytoplasm. Additionally, although HDAC11 remained cytoplasmic following T-cell activation, there was a noticeable decrease in HDAC11 protein expression. The differential regulation in the expression and localization of HDAC7 and HDAC11 in resting compared to activated $CD4^+$ T cells suggests that they may be serving different functions in the two cell populations. However, siRNA-mediated knockdown of HDAC7 or HDAC11 in the HeLa P4/R5 cell line model of HIV-1 latency did not induce LTR activation (data not shown). Thus, although HDAC7 and HDAC11 are differentially regulated in resting versus activated $CD4^+$ T cells, we found no evidence that they contribute to silencing of the proviral HIV-1 LTR.

HDAC1, -2, and -3 are resident at the HIV-1 LTR. Of HDAC1 to -11, there is evidence that HDAC1, -2, and -3 are recruited to the LTR in model cell lines of HIV-1 latency (4, 10, 12, 18, 20, 23, 26). Other HDACs could play an important role in HIV-1 regulation and could be critical for effective therapeutic targeting of latent infection. The frequency of latent HIV-1 in resting $CD4^+$ T cells has been estimated at less than 1 in 10^7 cells (3). Because proviral latency is an extremely rare event, it is impossible to perform ChIP assays on resting $CD4^+$ T cells obtained from patients, due to the small amount of target DNA. Thus, to address experimentally the question of which HDACs occupy the HIV-1 LTR, we utilized J89GFP cells. J89GFP cells are a Jurkat cell line that contains a stably integrated, full-length HIV-1 provirus (strain 89.6) with a green fluorescent protein (GFP) reporter incorporated into the viral genome (16). The viral genome in J89GFP cells is transcriptionally silent. However, upon appropriate stimulation, such as with the NF-κB inducer, tumor necrosis factor alpha, or the HDAC inhibitor TSA, viral transcription is activated and viral expression can be measured by GFP production (27). Critically, this cell line reproducibly returns to quiescence when stimuli are withdrawn.

First we determined the protein expression and cellular localization of HDAC1 to -11 in J89GFP cells by Western blot analysis of 30 μg of nuclear and cytoplasmic protein fractions. Due to the abundance of cell line extract, we were able to use twice as much protein in these Western blot assays as in those performed with scarce material extracted from available aviremic, HIV-infected patients' cells. We were able to detect all 11 HDACs in J89GFP cells (Fig. 3A). The nuclear and cytoplasmic distribution of HDACs in J89GFP cells mirrors that of resting $CD4^+$ T cells with the exception that HDAC1 and HDAC8 are detected in both the nucleus and the cytoplasm of J89GFP cells. Although these slight differences in HDAC expression between the J89GFP cells and resting $CD4^+$ T cells may be relevant to HIV-1 latency, importantly, all HDACs that are expressed in the nuclei of resting $CD4^+$ T cells are also present in the nuclei of J89GFP cells. Thus, J89GFP cells are a reasonable model cell line to evaluate HDAC recruitment to the integrated HIV-1 LTR by ChIP.

To assess the occupancy of the various HDACs at the HIV-1 LTR, we first performed ChIP assays using antibodies directed

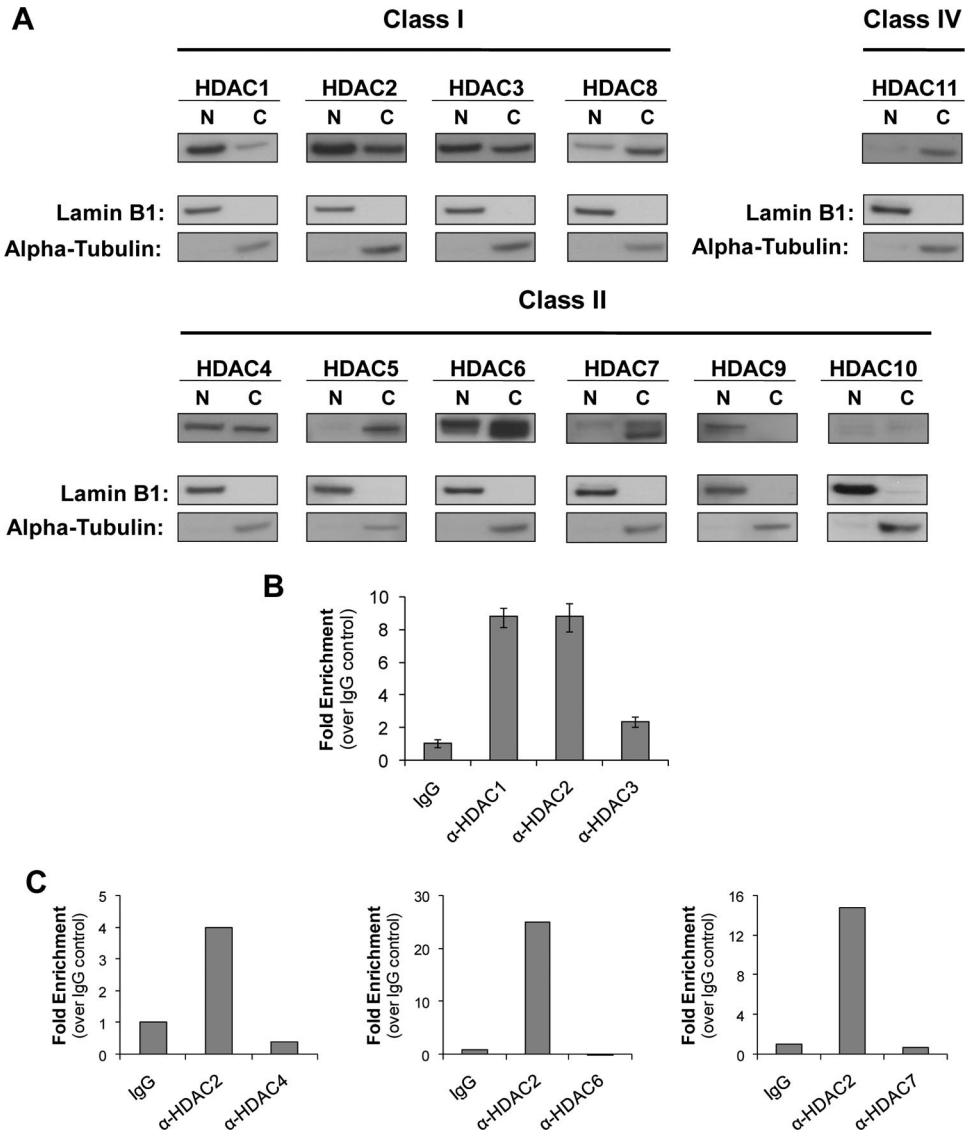

FIG. 3. HDAC1, -2, and -3 are recruited to the HIV-1 LTR in the J89GFP cell line model of latency. (A) HDAC localization in J89GFP cells is similar to that in resting $CD4^+$ T cells. Nuclear (N) and cytoplasmic (C) protein extracts (30 μg each) from J89GFP cells were probed with antibodies against HDAC1 to -11 in Western blot analysis. Antibodies targeting lamin B1 and alpha-tubulin were used as loading controls for nuclear and cytoplasmic extracts, respectively. (B) HDAC1, -2, and -3 associate with the HIV-1 LTR. Antibodies targeting the nuclear class I HDACs HDAC1, -2, and -3 were used in ChIP assays in J89GFP cells. Rabbit IgG serum was used to assay nonspecific immunoprecipitation of LTR DNA. (C) The nuclear class II HDACs HDAC4, -6, and -7 do not associate with the HIV-1 LTR. ChIP assays were performed in J89GFP cells. HDAC2 was used as a positive control, and rabbit IgG serum was used as a negative control. Values in panels B and C represent the enrichment of LTR DNA over the IgG negative control as determined by quantitative PCR. Experiments were performed on at least three occasions. Data are expressed as the means ± standard errors of the means.

against the class I HDACs that were detected in the nuclear protein extracts of resting $CD4^+$ T cells: HDAC1, -2, and -3. We observed HDAC1, -2, and -3 at the HIV-1 LTR (Fig. 3B) of J89GFP cells. These results agree with previous findings in diverse cell systems that HDAC1, -2, and -3 are recruited to the HIV-1 LTR (4, 10, 12, 18, 20, 23, 26). However, to our knowledge this is the first report of HDAC2 occupying the LTR in a T-lymphocytic cell line. Although the amount of HIV-1 LTR DNA immunoprecipitated with an HDAC3 antibody was smaller than the amount immunoprecipitated with HDAC1 or HDAC2 antibodies, it was a consistent finding observed over multiple experiments. Such results do not necessarily indicate that there are fewer HDAC3 molecules associated with the HIV-1 LTR, as they may simply reflect the ability of the particular antibody to perform in ChIP assays, the distance of the target enzyme from the DNA, or the availability of corresponding epitopes following formaldehyde fixation.

Next we examined the HIV-1 LTR occupancy levels of the predominant, nuclear class II HDACs in resting $CD4^+$ T cells, HDAC4, -6, and -7. We did not detect significant levels of HIV-1 LTR DNA enrichment over the IgG negative control following ChIP with ChIP-validated antibodies targeting

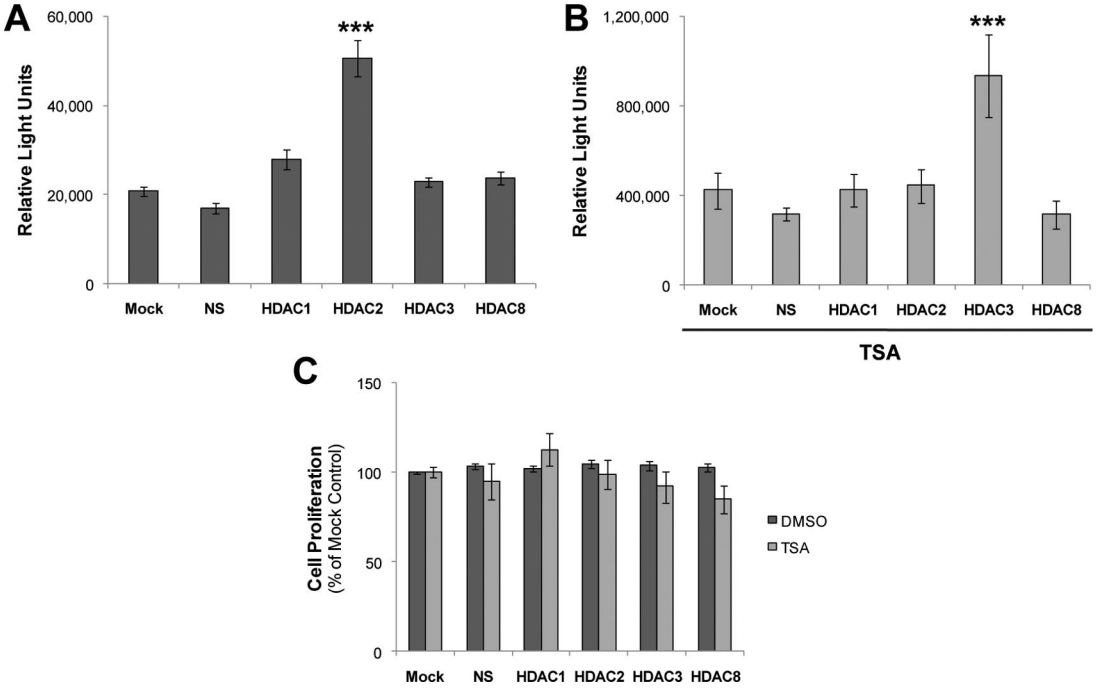

FIG. 4. HDAC2 and HDAC3 negatively regulate the HIV-1 LTR. (A) siRNA-mediated knockdown of HDAC2 induced a significant increase in LTR-driven *lacZ* expression compared to the mock control ($n = 64$; ***, $P < 0.001$). P4/R5 cells were transfected with siRNAs targeting the class I HDACs HDAC1, -2, -3, and -8. Twenty hours posttransfection, cells were incubated with 1% DMSO (vehicle control) for an additional 20 h. As a control, cells were transfected with nonspecific (NS) siRNA. Values are displayed in relative light units and were derived from Gal-Screen assays of cellular lysates. (B) siRNA knockdown of HDAC3 in conjunction with exposure to a submaximal concentration of the HDAC inhibitor TSA upregulated LTR-driven *lacZ* expression compared to cells that were mock transfected and exposed to TSA ($n = 32$; ***, $P < 0.001$). Cells were treated as for panel A except that 1 μM TSA was added in place of DMSO. (C) There were no differences in cell proliferation following HDAC knockdown compared to the mock controls ($n = 12$, $P = 0.522$ for DMSO experiment; $n = 12$, $P = 0.307$ for TSA experiment). alamarBlue assays were used to assess cell viability in panels A and B. Data in panels A to C are the combined results of at least three experiments and are expressed as the means ± standard errors of the means.

HDAC4, -6, or -7 (Fig. 3C). In these experiments, HDAC2 was used as a positive control to verify the success of the ChIP assay. However, as we are unaware of a positive-control region of DNA in J89GFP cells to verify the ability of these antibodies to work in ChIP assays in our hands, we cannot completely exclude the possibility that some levels of these enzymes may associate with the LTR. Taken together, these findings suggest that the class I HDACs HDAC1, -2, and -3 are the predominant HDACs to occupy the HIV-1 LTR in the J89GFP model of latency.

HDAC2 and HDAC3 regulate LTR-driven gene expression. To determine the impact of individual HDACs on the regulation of HIV-1 transcription, we treated the HIV-1 latency cell line model, HeLa P4/R5 cells (14), with siRNAs targeting the class I HDACs, HDAC1, -2, -3, and -8. HeLa P4/R5 cells are a variant of HeLa Magi cells that express both CXCR4 and CCR5. They contain an integrated *lacZ* gene under the control of the HIV-1 minimal LTR and were selected for low background β-galactosidase expression. As these cells contain only an integrated LTR promoter driving a LacZ reporter, they permit identification of factors that exert strong regulation over transcription from the LTR in the absence of Tat.

Silencing of HDAC2 mRNA led to a statistically significant increase in LacZ production as measured by β-galactosidase assays (Fig. 4A). Isolated knockdown of HDAC1, -3, and -8 did not result in a significant increase in LTR-driven LacZ expression. Using the alamarBlue assay, we evaluated cell proliferation following siRNA-mediated knockdown of HDAC1, -2, -3, and -8 and observed no differences in cell viability compared to the mock control (Fig. 4C). Thus, the lack of LTR induction seen with siRNA-mediated silencing of HDAC1, -3, and -8 is not due to a decrease in the viability or proliferation of cells following HDAC knockdown.

When proteins like HDAC3 are expressed at high levels in cells, it can be difficult to achieve a full knockdown of expression using siRNA alone. We observed substantial reductions (93%) of HDAC mRNA expression following siRNA knockdown as determined by reverse transcriptase PCR (data not shown). However, to overcome the effect of residual HDAC protein that may persist despite HDAC mRNA knockdown, we combined chemical HDAC inhibition with siRNA-mediated knockdown. Based on the 50% to 90% effective concentration for TSA induction of the LTR in HeLa P4/R5 cells, we treated P4/R5 cells with a submaximal concentration of TSA in conjunction with individual siRNA-mediated knockdowns of the class I HDACs. Combining TSA with individual siRNAs against HDAC1, -2, or -8 did not lead to a significant increase in LTR activation above that induced by TSA alone (Fig. 4B). Activation of the LTR by HDAC2 siRNA was not observed in this experiment because mock transfection combined with TSA treatment induced higher levels of β-galactosidase activity (~18-fold) than did isolated HDAC2 knockdown; thus, the

backgrounds of the two experiments are different. Exposure to global HDAC enzymatic inhibition along with a targeted knockdown of HDAC3, however, led to a significant increase in LTR activation over that in the mock-transfected cells that were treated with TSA alone (Fig. 4B). There were no differences in cell proliferation following TSA treatment with targeted HDAC knockdowns compared to the mock control (Fig. 4C). Taken together, these findings suggest that both HDAC2 and HDAC3 contribute to the restriction of HIV-1 LTR expression in HeLa P4/R5 cells.

DISCUSSION

In this study, we provide the first comprehensive assessment of the contribution of HDAC1 to -11 to HIV-1 latency in resting $CD4^+$ T cells. Our results suggest that the class I HDACs HDAC1, -2, and -3 are recruited to the HIV-1 LTR and that HDAC2 and HDAC3 regulate HIV-1 transcriptional repression in cell line models of HIV-1 latency. HDAC1, -2, and -3 are expressed in the nuclei of resting $CD4^+$ T cells (Fig. 1A and B and 2); thus, their contribution to HIV-1 transcriptional repression may extend to a clinically relevant viral reservoir.

Our data suggest that HDAC2 may be particularly important to the regulation of HIV-1 expression. Because ChIP is not feasible in resting $CD4^+$ T cells from HIV-1-positive patients due to the rarity of target DNA, we performed ChIP assays in the J89GFP cell line model of HIV-1 latency. We observed HDAC2 associating with HIV-1 LTR DNA. Although HDAC2 was previously reported to be recruited to the LTR in microglial cells (20), to our knowledge this is the first report of HDAC2 being detected at the HIV-1 LTR in a T-lymphocytic cell line (Fig. 3B). Isolated knockdown of HDAC2 by siRNA resulted in a significant increase in LTR-driven gene expression (Fig. 4A) in HeLa P4/R5 cells. These results are similar to those of Marban et al. (20), who detected a modest 1.5-fold increase in LTR-driven expression of a luciferase reporter when HDAC2 was targeted by short hairpin RNA.

As previously reported (18), we found that HDAC3 was recruited to the HIV-1 LTR (Fig. 3B). Additionally, in combination with global HDAC enzymatic inhibition by TSA, siRNA-mediated knockdown of HDAC3 resulted in a synergistic increase in HIV-1 expression (Fig. 4B). We achieved a substantial knockdown of HDAC3 mRNA (93%; data not shown), but LTR upregulation was seen only when the HDAC3 knockdown was combined with submaximal global HDAC inhibition. Persistent activity of a large cellular pool of HDAC protein, despite mRNA inhibition, could explain this finding. Alternatively, as has been reported with other HDACs, HDAC3 may function as a transcriptional repressor via a function that does not depend on its deacetylase activity (7, 17, 28). In the presence of global HDAC enzymatic inhibition, HDAC3 may continue to restrict LTR expression. This repression could be relieved by HDAC3 knockdown but would not be apparent without inhibition of other resident HDACs, e.g., HDAC1 and HDAC2.

It has been demonstrated by five independent research groups that HDAC1 is recruited to the LTR in cell line models of HIV-1 latency by an array of transcription factors (Fig. 3B) (4, 10, 12, 20, 23, 26). The existence of multiple mechanisms for the recruitment of this HDAC implies that it plays a role in maintaining LTR repression. Furthermore, in a chemical library screen of small-molecule HDAC inhibitors at Merck Laboratories, a decreasing HDAC1 50% inhibitory concentration correlated with an increase of LTR activation in HeLa P4/R5 cells (8). However, targeted HDAC1 inhibition by siRNA did not substantially increase LTR expression (Fig. 4A). Thus, while HDAC1 may contribute to the maintenance of deacetylated histones at the latent LTR, other HDACs appear to compensate for the loss of HDAC1 when its expression is dampened.

Future efforts to evaluate the impact of targeted HDAC knockdown on latent viral outgrowth from the resting $CD4^+$ T cells of HIV-1-positive patients are warranted to verify the importance of these findings for HIV-1 latency. Targeting a combination of selected HDACs (e.g., HDAC2 and HDAC3 or HDAC1, -2, and -3) may prove to be a more effective strategy for inducing HIV-1 expression in a broad population of cells in vivo, as HDAC expression is cell and tissue type specific (5). The discovery and development of new compounds with selective HDAC-inhibitory abilities are an area of intense research (reviewed in references 11 and 13). HDAC inhibitors are currently in use in numerous clinical trials for cancer treatment (6, 21), and the selective HDAC inhibitor vorinostat has been approved for the treatment of subcutaneous T-cell lymphoma (19). We have recently shown that vorinostat can induce expression of HIV-1 from the resting $CD4^+$ T cells of HIV-1-infected patients (1). Thus, selective HDAC inhibitors are viable candidates to explore as potential antilatency therapies.

Our results indicate that HDAC1, -2, and -3 are expressed in the nuclei of the latent reservoir of resting $CD4^+$ T cells in HIV-1-positive patients. HDAC1, -2, and -3 occupy the HIV-1 LTR in the J89GFP model of HIV-1 latency, and siRNA-mediated knockdown of HDAC2 and HDAC3 can reactivate the integrated, quiescent LTR of HeLa P4/R5 cells. These observations suggest that the use of a class I selective HDAC inhibitor, in particular one that acts on HDAC1, -2, and -3, may prove to be an attractive antilatency strategy with fewer of the toxicities that can accompany global HDAC inhibition.

ACKNOWLEDGMENTS

This work was supported by amfAR (107168-44-RGRL); R00046 to the UNC General Clinical Research Center; U54RR024383 to the UNC Clinical and Translational Science Award; AI50410 to the UNC Center for AIDS Research; and National Institutes of Health grants AI045297 to D.M.M., T32 AI 07001-32 to K.S.K., and T32 AI07151-30 to N.M.A.

We are grateful for the technical assistance of Daniel Parker, Nancy Cheng, Manzoor Cheema, and the staff of the UNC Blood Bank and to Linh Ngo for study coordination. We thank the patient volunteers for their generous contributions to research.

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