

Histone deacetylase 3 represses HTLV-1 tax transcription

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Abstract. We examined the epigenetic mechanisms involved in human T-cell lymphotropic virus type 1 (HTLV-1) Tax expression. Blockade of histone deacetylation with trichostatin A (TSA) resulted in Tax upregulation. Using a chromatin immunoprecipitation (ChIP) assay, we verified local histone hyperacetylation at the HTLV-1 LTR in response to TSA. In agreement, HDAC3 transfection led to reductions in both Tax expression and histone acetylation. HDAC3 mutations and deletions spanning the catalytic site had variable ability to repress Tax, but HDAC activity was not essential for repression. Immunoprecipitation studies revealed that Tax co-exists in a complex containing both histone deacetylase 1 (HDAC1) and 3 (HDAC3). Our results suggest that HDACs may actively participate in the repression of HTLV-1 Tax transcription.

Introduction

The HTLV-1 viral Tax protein has been proposed to play crucial roles in the pathogenesis of autoimmune disorders and cancer (1,2). Transformation of T-cells is characterized by IL-2-independent growth (3), a phenomenon in which Tax (4) and JAK/STAT pathway proteins (5,6) have been implicated. In addition, Tax may also play a role in the phenomenon of T-cell spontaneous proliferation, observed in

HTLV-1-infected individuals (7). It is believed that some of these effects depend on the ability of Tax to deregulate expression of host-cell genes including cytokine and proliferation-promoting genes (8).

The Tax protein itself directly stimulates expression of its own mRNA by binding DNA (9,10) at the Tax-response elements (TREs) on the U3 region of the viral LTR (11-13). Tax protein recruits DNA-binding cellular proteins including ATF/CREB, which associates with the CREB-binding protein (CBP)/p300 (14,15), in complexes that facilitate access to the TREs. Tax protein also directly binds the transcriptional coactivators, CBP/p300 (16-20) and P/CAF, (21), both proteins containing intrinsic histone acetyltransferase (HAT) activity (22). It has been shown that HAT enzymes lead to histone acetylation and promote transcription in part by relaxing specific nucleosomal-DNA interactions, facilitating the binding of transcription factors to their target gene promoters. In contrast, HDAC corepressors promote nucleosomal-DNA condensation, thus limiting the accessibility of transcription factors and downregulating gene expression (23,24). Blockade of HDAC enzymes by the inhibitor drugs butyrate or trichostatin A (TSA), leads to transcriptional activation of multiple genes by de-repression. In this study, we sought to examine whether histone deacetylase enzyme HDAC3 represses HTLV-1 Tax transcription.

Materials and methods

Cell growth conditions and treatments. HTLV-1-infected C81 cells were grown in complete medium (RPMI supplemented with 10% FCS, 10 mM Hepes, 2 mM L-glutamine, and 100 U/100 µg/ml penicillin/streptomycin). T-cell clones were maintained at a density of $<1 \times 10^6$ cells/ml and stimulated with the polyclonal activator, PHA (1 µg/ml), and irradiated feeders for continuous growth. All cells were plated on sterile petri dishes or 96-well plates, cultured in complete medium plus 10% IL-2 containing T-Stim (Collaborative Biomedical Products) and incubated at 37°C with 6% CO₂ and 96% humidity. HTLV-1-infected human Du43 T-cell clones (kindly provided by Per Höllsberg, Harvard University) were treated for 24 h with 100 nM TSA (Wako Pure Chemical Industries). HTLV-1-infected human Mu16 T-cell clones

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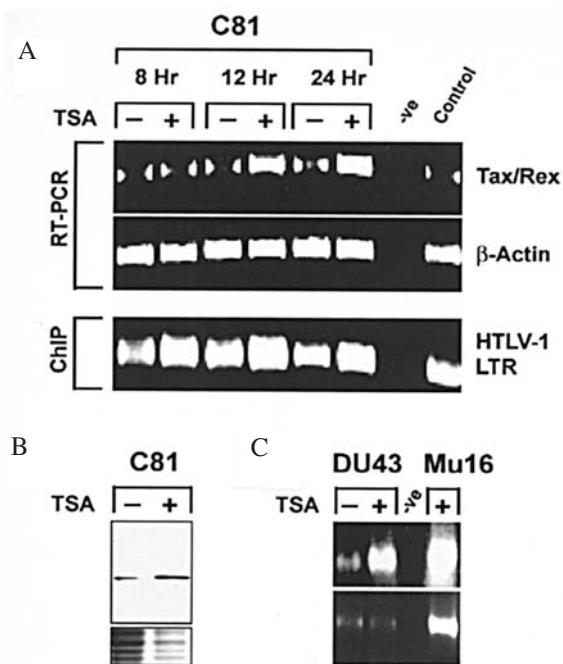


Figure 1. (A) Twelve hours after administration of 100 nM TSA, the mRNA for Tax/Rex is detected by RT-PCR in C81 cells. β -actin mRNA is shown as a control. Chromatin immunoprecipitation (ChIP) assay (lower panel) shows earlier (8 h) histone effects: higher PCR amplification of HTLV-1 LTR sequences immunoprecipitated with α -acH3 Abs in the TSA-stimulated C81 cells. The persistence of the enhanced Tax mRNA expression at 24 h correlates with the persistently enhanced genomic acetylation of the LTR region. HUT102 cells are used as control. (B) Treatment of C81 cells with TSA (10 ng/ml) for 24 h leads to Tax protein upregulation, as shown by Western blotting. (C) TSA (100 nM) for 24 h induces higher Tax/Rex mRNA in Du43, an HTLV-1-infected human CD4⁺ T-cell clone. RNA isolated from Mu16 T-cells, which chronically express higher levels, was used as a Tax-Rex control mRNA. Control cells received vehicle.

(provided by Per Höllsberg) and HUT102 cells, both known to express high levels of Tax, were used as controls.

RNA isolation and RT-PCR. RNA was isolated using the Trizol method (Gibco BRL). We performed RT-PCR with the Retroscript kit (Ambion, Austin, TX) using primers FWD 5'-TGGTCTTAATAGCCGCCAGTGGAA-3', and REV 5'-AGGTGATCTGATGCTCTGGACAGG-3', spanning the second splice junction site of Tax/Rex mRNA (25). We used the β -actin-specific primers FWD 5'-AACCCCAA GGCCAACCGCGAGAAGATGACC-3', and REV 5'-GGTGATGACCTGGCCGTCAGGCAGCTCGTA-3'.

PCR conditions included denaturing at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 2 min x 35 or 40 cycles, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed in 1% agarose (Gibco BRL) gels. Bands were detected by ethidium bromide staining.

ChIP assay. For the ChIP assay, cells were treated with 1% formaldehyde for 10 min at 37°C and collected in lysis buffer [Upstate Biotechnology, Inc (UBI)] containing PMSF, pepstatin A and aprotinin (Sigma). After sonication, lysates were centrifuged, diluted 10-fold in ChIP dilution buffer (UBI),

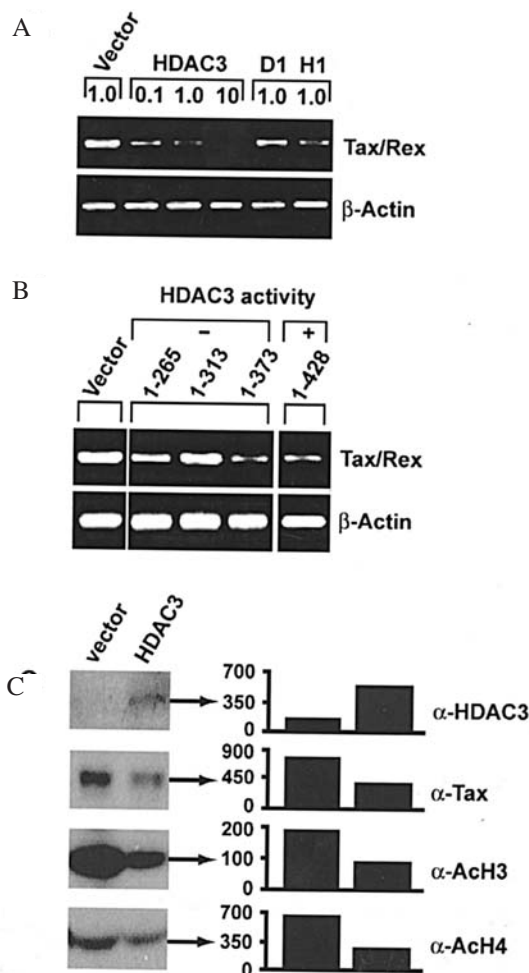


Figure 2. (A) HDAC3 transfection of C81 cells leads to a dose-dependent (0.1, 1.0 and 10 μ g of DNA) decrease in Tax mRNA expression, as shown by RT-PCR. HDAC3 constructs, D1 and H1, harboring mutations in the enzymatic site (tubular pocket) are less efficient at inducing repression. (B) Transfection of C81 cells with HDAC3 constructs that encode proteins devoid of enzymatic activity. Cells were collected at 48 h post-transfection for RNA isolation and RT-PCR. The experiment reveals that enzymatic activity is not essential for a repressor function on Tax mRNA expression. (C) Western blotting reveals WT HDAC3 (1-428) transfection for 24 h that correlates with decreased Tax and lower acetylation of histone proteins. Membranes were incubated with Abs against HDAC3, p40Tax, acH3 and acH4 histones. Densitometry on right panel shows relative units of intensity.

and precleared for 1 h at 4°C with agitation using salmon sperm DNA/Protein A agarose-50% slurry (UBI). Five μ g of α -acetylated histone H3 (UBI) Ab was added to the supernatant fraction and incubated overnight at 4°C with rotation. Following washes and elution, histone-DNA cross-linked complexes were reversed by heating at 65°C for 4 h. DNA was treated with EDTA, Tris HCL and proteinase K (UBI), extracted with phenol-chloroform, precipitated, and resuspended in 15 μ l of DEPC water. PCR detection of a 301-base HTLV-1 LTR DNA sequence was performed using the following primers: FWD 5'-CCGTCCTCAGGCGTTGA-3' and REV 5'-CCGGCTGAGTCTAGGTAGGCT-3'.

Western blotting. Following TSA treatment, C81 cells were harvested, washed, and lysed in extraction buffer (50 mM Tris, 10% glycerol, 150 mM NaCl, pH 8.0) by rocking at 4°C for 30 min. Following centrifugation the whole cell extracts were removed and stored at -80°C prior to use. Proteins were

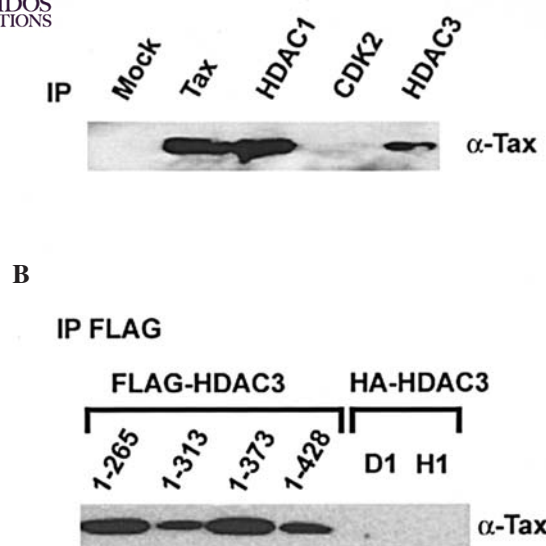


Figure 3. Immunoprecipitation of Tax and HDACs. (A) To examine whether endogenous HDACs and Tax physically associate in molecular complexes, we performed immunoprecipitation with Abs against Tax, HDACs 1 and 3 and CDK2, followed by Western blotting with anti-Tax antibodies. As shown, endogenous HDAC1 and HDAC3 but not CDK2 bind Tax in C81 cells. (B) C81 cell transfection with FLAG-HDAC3 constructs that encode proteins devoid of HDAC activity was performed for 48 h, prior to whole cell extract isolation. Control HA-tagged D1 and H1 point mutant constructs were also transfected. IPs with anti-FLAG Abs followed by Western blotting with anti-Tax were then performed. The experiment reveals that all FLAG-tagged deletion constructs used in the study have preserved ability to bind Tax, suggesting that the region important for binding is localized in the N-terminal half.

resolved by electrophoresis in 4-20% gradient Tris-Glycine gel (NOVEX, San Diego, CA), and transferred to a nitrocellulose membrane filter (Millipore, Bedford, MA). Coomassie staining was used to assess sample loading. The membranes were blocked with 5% nonfat milk and probed with Abs against p40Tax (AIDS Research and Reference Reagent Program, NIH) at a dilution of 1:1,000. HRP-conjugated goat α -rabbit secondary Ab (Affinity Bioreagents, Golden, CO) at a 1:10,000 dilution was used. The bands were visualized by the enhanced chemiluminescence system (NEN Life Science Products, Boston, MA) and exposure to X-ray film (Eastman Kodak, Rochester, NY). C81 cells were also transfected with WT HDAC3. From these cells, we isolated the acid-soluble fraction of whole proteins, using a histone isolation protocol recommended by the manufacturer, followed by Western blotting with α -acetylated histone 3 (ac-H3) and 4 (ac-H4) Abs (UBI).

Transfections. The HDAC3 enzymatic point mutant constructs, D1 and H1, used in transfections, were generated in our laboratory (details provided upon request) with the QuikChange site-directed Mutagenesis kit (Stratagene), following the manufacturer's instructions. The D1 aspartic acid for asparagine (D168N) and H1 histidine for phenylalanine (H135F) substitutions are both located deep in the tubular pocket that participates as the enzymatic site of HDAC3 and binding site for TSA (26). The D and H pocket residues are highly conserved throughout evolution, suggesting a critical

function. Cells were transfected by electroporation with a Biorad electroporator (300 V and 1,000 microFarads). FLAG-tagged HDAC3 deletion mutants (1-265, 1-313 and 1-373) known to encode proteins deficient in HDAC activity (27), were kindly provided by Dr Edward Seto (University of South Florida).

Immunoprecipitation (IP). IP was performed as previously described (28). Briefly, proteins were recovered in Laemmli buffer after high speed 20-min centrifugation. IP was performed with Abs against HDAC1 (Affinity Bio-reagents), HDAC3 (SantaCruz Biotechnology), CDK2 (Clontech) and a mock (anti-HA) Ab (SantaCruz Biotech-nology) as follows: protein extracts were incubated overnight at 4°C with the Ab, and immunoprecipitated proteins were recovered on protein A agarose (UBI) and washed for 5 min 3x at room temperature with 50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.1% NP-40. Gels were loaded with immunoprecipitates and run as described above and blots were probed with a primary Ab against Tax. In addition, IP with anti-FLAG Abs (Sigma) followed by Western blotting with anti-Tax Abs was performed in FLAG mutant-transfected C81 cells.

Results

We first treated C81 cells (1×10^6 per sample) for 8, 12 and 24 h (Fig. 1A). TSA led to increased expression of Tax/Rex mRNA at 12 and 24 h. To examine whether TSA-enhanced Tax mRNA expression was due to histone hyperacetylation at the HTLV-1 LTR and not due to nonspecific acetylation, we performed a parallel chromatin immunoprecipitation (ChIP) assay, as described in the Upstate Biotechnology protocol. As shown (Fig. 1A lower panel), TSA promotes local histone hyperacetylation at the HTLV-1 LTR as early as 8 h, a time that precedes the upregulation of Tax mRNA.

We then examined whether TSA treatment led to elevation of the Tax protein in C81 cells, using Western blotting. Analysis of C81 protein extracts with antibodies against Tax revealed upregulation of Tax protein by TSA (Fig. 1B). To examine if the ability of TSA to enhance Tax mRNA expression was not exclusive to C81 cells, we examined expression in HTLV-1-infected T-cell clones by RT-PCR. TSA treatment resulted in upregulation of Tax mRNA in HTLV-1-infected Du43 cells (Fig. 1C).

To examine if HDAC3 plays a role in repressing Tax, we transfected C81 cells with full-length, wild-type (WT) HDAC3, a 428 amino acid-encoding cDNA that we and others cloned and characterized (23,29,30), or the HDAC3 enzymatic site point mutants D1 and H1. Transfection with WT HDAC3 in a pCDNA3.1Zeo⁺ plasmid, as compared to vector alone, led to a dose-dependent decrease in Tax mRNA at 24 h (Fig. 2A). HDAC3 point mutants, D1 and H1, at a comparable dosage of 1 μ g DNA per transfection also repressed Tax but had a decreased ability to exert these effects when compared to WT HDAC3.

We then transfected cells with three FLAG-tagged HDAC3 deletion mutants (1-265, 1-313 and 1-373) known to encode proteins deficient in HDAC activity (27). Using these constructs, Yang *et al* have shown that the structural integrity

of the C-terminus of HDAC3 is required for enzymatic and repressor activity. As shown in Fig. 2B, several regions of HDAC3 maintain repressive activity on Tax mRNA expression that approximate that of the wild-type (428 amino acid-encoding) molecule, and the repression is more evident with the inclusion of the C-terminal domains of HDAC3. Interestingly, the 1-373 construct is devoid of HDAC activity but is shown to have preserved ability to repress Tax. The results indicate that the repressor function of HDAC3 on Tax mRNA can occur independently of its enzymatic activity. To verify if HDAC3 (1-428) transfection correlated with histone deacetylation, we then transfected C81 cells with WT HDAC3. From these cells, we isolated the acid-soluble fraction of whole proteins, using a histone isolation protocol recommended by the manufacturer of α -acetylated histone 3 (ac-H3) and 4 (ac-H4) Abs (UBI). Western blotting showed that, as compared to vector only-transfected cells, HDAC3 transfection led to decreased Tax protein expression that correlated with increased HDAC3 and decreased histone H3 and H4 acetylation (Fig. 2C).

To assess if Tax protein physically associates with HDAC proteins, we performed IPs. These experiments revealed that Tax is found complexed with HDAC1 or HDAC3 (Fig. 3A). To verify that the deletion mutants used in this study encoded proteins that retained the ability to bind Tax, we performed IPs on extracts from FLAG mutant-transfected C81 cells. IP with anti-FLAG Abs (Sigma) followed by Western blotting with anti-Tax Abs revealed that all the deletion constructs were capable of binding Tax (Fig. 3B). HA-tagged point mutant D1 and H1 constructs were used as negative IP controls for the anti-FLAG Abs.

Discussion

Lin *et al* (31) identified butyrate, a non-specific HDAC inhibitor, as a strong inducer of HTLV-1 RNA and Tax protein expression. HDAC inhibitors can upregulate expression of the feline foamy virus and other viruses *in vitro* (32,33). In this study, we show that the specific HDAC inhibitor TSA led to upregulation of Tax mRNA in cells known to chronically harbor the HTLV-1 virus. Our demonstration of increased histone hyperacetylation at the HTLV-1 LTR site following TSA administration supports a direct effect of this drug on the HTLV-1 proviral genome.

Recent studies have shown that repressor complexes containing HDACs are associated with the 5' or 3' promoter HTLV-1 LTR *in vivo* (34). Such recruitment of HDACs to a retroviral promoter site has also been shown for HIV (35). HDAC1 binds the HTLV-1 Tax protein (36) and HDAC1 overexpression downregulates HTLV-1 Tax transcription (34,37). Thus, Tax and HDAC1 may reciprocally inhibit each other's ability to interact with the HTLV-1 LTR (34,36). Our study demonstrates that transfection with HDAC3 also downregulates HTLV-1 Tax expression, highlighting another layer of complexity for this repressor mechanism.

Further, we demonstrate a physical association between HDAC3 and Tax proteins. Our use of point mutants indicated that the ability of HDAC3 to repress Tax may depend in part on the integrity of a region spanning the catalytic domain. This domain is composed of residues that contribute to the formation

of the enzymatic pocket. The HDAC3 residues mutated in this study (amino acid residues 135 and 168) occupy deep sites of the tubular pocket of this enzyme (26,38). However, the requirement for the structural integrity of the enzymatic region is not absolute, since enzymatically inactive HDAC3 fragments (27) remained capable of Tax repression. The region spanning amino acid residues 314-373 and 402-428 of HDAC3 had the strongest effects on Tax repression. The results suggest that other protein-protein interaction sites influence Tax repression independently of HDAC3's enzymatic function. These interactions may include recruitment of Tax to other sites of the HDAC3 protein, to enhance repression by physical proximity, or binding of other repressor molecules on the C-terminal half of the molecule. Of note, the Tax-binding domain of a closely related deacetylase, HDAC1, localizes between amino acid residues 29-97 of the N-terminus (37). All flagged HDAC3 constructs used for transfections in this study contained this N-terminal region and their encoded proteins bound Tax protein, as assessed via immunoprecipitation studies.

Promoter DNA methylation is another silencing mechanism proposed to play a modulatory role in retroviral latency (39,40). In fact, the HTLV-1 LTR has been shown to be highly methylated in Tax-nonexpressing cells derived from adult T-cell leukemia patients (40). In addition, HDACs bind DNA methyltransferases (DNMTs) (41), the chief enzymes responsible for DNA methylation. We have found that the DNA methylation inhibitor drug, 5-aza-2'-deoxycytidine (AZA), also upregulated Tax expression (results not shown). Thus, the ability of TSA to induce Tax expression suggests that pharmacologic HDAC inhibition may simultaneously affect multiple, possibly convergent, gene silencing mechanisms. Our results support a role for HDAC3 in molecular complexes that mediate Tax function, and provide a basis for further exploration of retroviral latency mechanisms.

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