

Tight Interplay among SAMHD1 Protein Level, Cellular dNTP Levels, and HIV-1 Proviral DNA Synthesis Kinetics in Human Primary Monocyte-derived Macrophages^{*[5]}

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Background: SAMHD1 is a host antiviral component that regulates cellular dNTP levels.

Results: We report a tight kinetic interplay between SAMHD1 level and the ability of HIV-1 to replicate in MDMs.

Conclusion: Very fast kinetics of SAMHD1 degradation by Vpx is mechanistically tied with HIV-1 DNA synthesis in MDMs.

Significance: The observed temporal relationship is central to better understanding HIV-1 infection in MDMs.

Recently, SAMHD1 has come under intense focus as a host anti-HIV factor. SAMHD1 is a dNTP triphosphohydrolase, which leads to the regulation of DNA metabolism in host cells. HIV-2/SIV (simian immunodeficiency virus) viral protein x (Vpx) has been shown to promote the degradation of SAMHD1. In this study, we examine the kinetics of SAMHD1 degradation, the increase in the dNTP pool level, and the efficiency of proviral DNA synthesis in Vpx+ virus-like particle (VLP)-treated monocyte-derived macrophages (MDMs). Our results indicate a very close temporal link with a reduction in SAMHD1 detected within the first few hours of Vpx+ VLP treatment. This loss of SAMHD1 is followed by a significant increase in cellular dNTP levels by 8 h after Vpx+ VLP addition, ultimately leading to the enhancement of the HIV proviral DNA synthesis rate and HIV infection in MDMs. Finally, the pretreatment of MDMs with the Vpx+ VLPs, which is a widely used protocol, displayed identical proviral DNA synthesis as compared with MDMs co-treated with Vpx+ VLP and HIV vector. These findings further indicate that Vpx degradation of SAMHD1 is sufficiently rapid to enable appropriate progression of reverse transcription in MDMs, even

when present at the time of infection. Overall, this study demonstrates a tight interplay between SAMHD1 level, dNTP levels, and HIV proviral DNA synthesis kinetics in MDMs.

Sterile α motif domain and HD domain-containing protein 1 (SAMHD1)³ has been linked to Aicardi-Goutières syndrome (1), which is a hereditary neurodegenerative disorder with similarities to systemic lupus erythematosus. The SAM domain has been reported to contribute to developmental regulation (2), whereas the HD domain is widely found in many proteins (3). SAMHD1, along with TREX1, has been suggested to regulate host cellular DNA metabolism and could also be an innate antiviral response (1). Two recent studies by Goldstone *et al.* (4) and Powell *et al.* (5) have demonstrated that SAMHD1 is a dNTP triphosphohydrolase. Several recent studies by Hrecka *et al.* (6) and Laguette *et al.* (7), as well as another study by Berger *et al.* (8), have revealed that the HIV-2/SIV accessory protein, Vpx, counteracts SAMHD1, a newly identified antiviral HIV restriction factor, specifically in macrophages and dendritic cells. It was hypothesized that SAMHD1 may delay the HIV/SIV reverse transcription step by lowering cellular dNTP pools.

In 2004, for the first time, we reported that human primary monocyte-derived macrophages (MDMs) harbor ~100 times lower cellular dNTP pools (20–40 nM) than activated CD4⁺ T cells (1–5 μ M) (9). These dNTP pool levels were further validated recently using LC-MS/MS analysis (10). Collectively, these data suggest that the reduced dNTP pool in MDMs is a biochemical host factor that restricts HIV-1 reverse transcription. Indeed, the series of recent studies indicated above, including our recent study (11), confirmed that SAMHD1 is responsible for the low dNTP concentrations found in MDMs. However, after our study, we immediately investigated how tightly regulated the three key reported molecular events are: 1) degradation of SAMHD1, 2) increase in dNTP levels, and 3) HIV-1 proviral DNA synthesis kinetics influenced by Vpx treatment in MDMs. These events essentially encompass the complete mechanistic parameters of the dNTP-HIV replication landscape in nondividing cells. Our laboratory has the capability to measure dNTP pools in nondividing cells such as MDMs using the HIV-1 RT-based dNTP assay (9), which is sensitive enough to detect the low dNTP levels and not ribonucleoside triphosphates in MDMs. Therefore, we simultaneously monitored these three molecular events after Vpx treatment in MDMs.

EXPERIMENTAL PROCEDURES

Ethics Statement—These experiments used primary human primary monocytes obtained from human buffy coats (New York Blood Services, Long Island, NY). These are preexisting

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³ The abbreviations used are: SAMHD1, sterile α motif domain and HD domain-containing protein 1; Vpx, viral protein x; Vpr, viral protein r; VLP, virus-like particle; MDM, monocyte-derived macrophage; SIV, simian immunodeficiency virus; qPCR, quantitative real-time PCR; TBST, Tris-buffered saline with Tween; RNR, ribonucleotide reductase.

materials that are publicly available, and there is no subject-identifying information associated with the cells. As such, the use of these samples does not represent human subjects research because: 1) materials were not collected specifically for this study, and 2) we are not able to identify the subjects.

Cells—Primary human monocytes were isolated from the peripheral blood buffy coats by positive selection using MACS® CD14+ beads as described previously (12). Monocytes were matured into MDMs in the presence of 5 ng/ml human GM-CSF (Miltenyi Biotec) treated at days 0 and 2 of maturation. MDMs were used at day 7 of maturation for experiments. RPMI medium (10% FBS with antibiotics) was replaced before transducing with virus-like particles (VLPs) and D3-HIV GFP vectors. For quantitative real-time PCR (qPCR) 2LTR circle copy number analysis, 1 million cells/well (6-well plates) were treated with VLPs (145 ng of p27/well), and total DNA was harvested using a Promega genomic purification kit. Two different VLP conditions were used: 2 h of pretreatment and cotreatment when treating with D3-HIV GFP vector (2.2×10^5 ng of p24/well, roughly a multiplicity of infection 0.1). For Western blot analysis and dNTP analysis, 2 million cells (3-cm dishes) were treated with 290 ng of p27/dish.

VLP Generation—Due to the requirement for large amounts of VLPs to concomitantly treat samples from the same donor for all the different assays, we subsequently modified the protocol from Berger *et al.* (8). Six T225 flasks containing 293T cells were transfected with 40 μ g of pVpx− VLP or pVpx+ VLP (kindly provided by Drs. Florence Margottin-Goguet and Nathaniel Landau) and 20 μ g of pVSVg at a ratio of 1 μ g of DNA to 5 μ l of polyethylenimine (1 mg/ml; PEI). The following day, medium were discarded and replaced with fresh DMEM medium (5% FBS and antibiotics). On days 2–4 after transfection, the medium was collected and replaced with fresh medium. On the day of collection, medium was centrifuged at 1200 rpm for 5 min to remove cells. Supernatant was subsequently filtered through a 0.45- μ m membrane (Corning Inc.). Thirty milliliters of supernatant were overlaid on top of 5 ml of a 25% sucrose cushion (25% (w/v) sucrose, 10 mM Tris-HCl, pH 7.5), 0.1 M NaCl and 1 mM EDTA). VLPs were concentrated at 28,000 rpm for 90 min by ultracentrifugation. Supernatant was aspirated, and pellets were suspended in 700 μ l of serum-free DMEM. Aliquots (50 μ l) were stored at -80°C . Each harvest was processed that day. The p27 antigen level was determined using an ELISA kit (Advanced BioScience Laboratories, Inc., Rockville MD).

D3-HIV GFP Vector Generation—D3-HIV GFP plasmid encodes the HIV-1 NL4-3 genome with the enhanced GFP gene in place of the HIV-1 *nef* gene and has a deleted envelope (9). To generate virus, 293T cells in T225 flasks were transfected with 40 μ g of pD3-HIV and 20 μ g of pVSV-g plasmids using PEI in 37 ml of DMEM medium/flask. At day 1 of HIV-1 production, media were discarded and replaced with fresh DMEM medium. At day 2, medium was harvested and replaced with fresh DMEM medium. The medium was centrifuged at 2500 rpm for 7 min to remove cellular debris and then stored at 4°C in a T75 flask. Day 3 medium was harvested and processed as described for day 2. D3-HIV GFP virus was concentrated using ultracentrifugation (22,000 rpm for 2 h in a SW28 rotor). Viral pellets

were DNase I-digested for 1 h at 37°C . Afterward, debris was removed by centrifugation (14,000 rpm for 5 min). Sample aliquots were frozen at -80°C until used. The level of p24 antigen was determined using an ELISA kit (Advanced BioScience Laboratories Inc.). Samples were analyzed for GFP expression at 24 or 48 h after transduction using an Accuri C6 flow cytometer (BD Biosciences). Data files were analyzed using the FlowJo software (TreeStar).

HIV-1 RT-based dNTP Assay—The nucleotide incorporation assay employs a 19-mer DNA template (3'-CAGGGAGA-AGCCCGCGGTN-5'). The template is annealed to a 5' end ^{32}P -labeled 18-mer DNA primer (5'-GTCCCTGTTCCGGCC-GCCA-3') for the HIV-1 RT-based assay used for this reaction, and the standard curves made with known amounts of dNTPs were used to convert the percentage of primer extension into the dNTP quantity used in each reaction as described previously (13). MDMs were collected and lysed with 60% cold methanol. Samples were incubated at 95°C for 3 min. Cellular debris was cleared by centrifugation at 14,000 rpm. Supernatant was dried in a SpeedVac. Pellets were resuspended in 20 μ l of reaction buffer (50 mM Tris-HCl, pH 8.0, and 10 mM MgCl_2). Two microliters of the sample extract were used in the primer extension assay. Reactions were resolved on 14% urea-PAGE. Data were captured using a PMI instrument (Bio-Rad) and then analyzed using the Quantity One software (Bio-Rad). The amounts of dNTPs per cell and the macrophage cell volume were used to calculate cellular dNTP concentration as described.

Western Blot Analysis—Samples were processed in radioimmune precipitation buffer containing 1 μM DTT, 10 μM PMSF, 10 $\mu\text{l/ml}$ phosphatase inhibitor (Sigma), and 10 $\mu\text{l/ml}$ protease inhibitor (Sigma). The cells were sonicated with three 5-s pulses to ensure complete lysis. Cellular debris was removed by 15,000 rpm centrifugation for 10 min. Supernatants were stored at -80°C before use. Cell lysates (20 μg) were resolved on 8% SDS-PAGE. Proteins were transferred to nitrocellulose membrane. The membrane was blocked with 2% nonfat milk in TBST for 1 h followed by the addition of primary SAMHD1 antibody (Abcam) and incubation overnight at 4°C . The next day, the membrane was washed (3 \times , 20 min with TBST) and stained with donkey anti-rabbit HRP for 1 h at room temperature. Membrane was washed (3 \times with TBST) and developed using the SuperSignal West Femto Kit (Thermo Scientific). The membrane was stripped and reprobed for actin. Images were captured using the Bio-Rad ChemiDoc Imager. Data were normalized to actin as a loading control.

RESULTS

Primary human MDMs from three donors were treated with VLPs with and without Vpx. MDMs were harvested for analysis at the indicated time points: 0, 2, 4, 8, 12, 18, 24, 36, and 48 h. We chose this intense sampling scheme to investigate the interplay among three key reported molecular events: 1) degradation of SAMHD1, 2) increase in dNTP levels, and 3) HIV-1 proviral DNA synthesis kinetics for each donor tested. A representative Western blot for SAMHD1 and actin is displayed (Fig. 1A). As can clearly be observed, Vpx− VLPs did not promote SAMHD1 degradation. However, by 4 h after Vpx+ VLP treatment, a noticeable decrease in SAMHD1 protein level was detected.

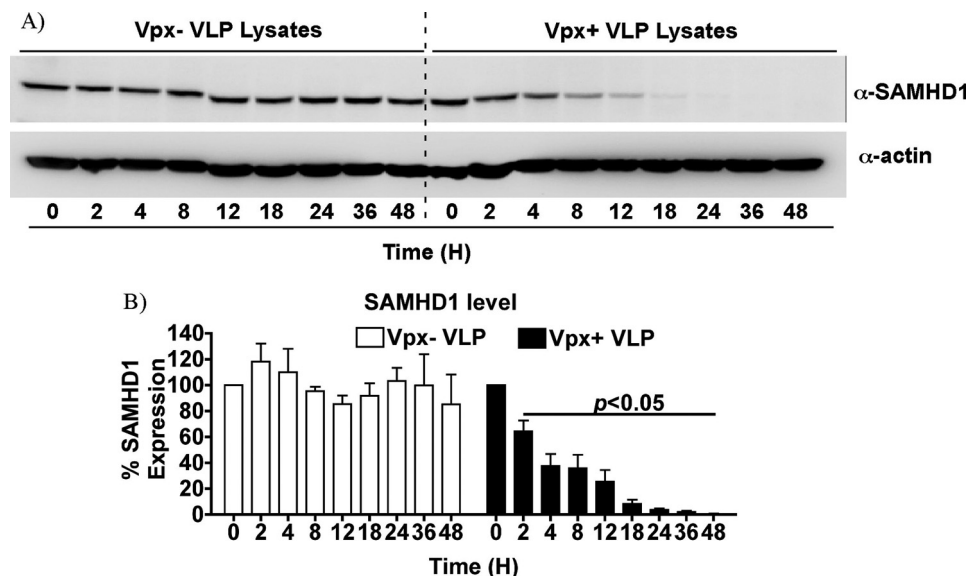


FIGURE 1. **Western blot analysis of MDM lysates.** *A*, a representative Western blot is shown for SAMHD1 and actin expression levels. Two million MDMs were treated with VLPs with and without Vpx (145 ng p27/million cells), and then harvested at the indicated time points. *B*, data are graphed as the mean and S.E. for three independent donors. Data were normalized to actin, used as a loading control. Student's *t* test analysis was performed to determine statistical significance ($p < 0.05$) for the different time points.

Data compilations for the three independent donors are shown in Fig. 1*B* and show significant reduction in SAMHD1 level at 2 h. Moreover, the SAMHD1 level continued to decrease to undetectable levels by 48 h after Vpx+ VLP addition.

Next, we examined changes in cellular dNTP levels for MDMs using the highly sensitive HIV RT-based assay (9). A representative gel for the HIV RT-based primer extension assay is shown in Fig. 2*A*. The *top panel* shows the controls: no dNTPs and a sample with exogenous dNTPs added to have no extension (*Primer*) and extension (*Primer +1*), respectively. MDM extracts from Vpx- VLPs treatment (Fig. 2*A, top*) show only moderate amounts of extended products over the time course. Importantly, this is in contrast to MDM extracts from Vpx+ VLP treatment (Fig. 2*A, bottom*), which show much more extended products at 4 h after VLP treatment. The data were quantitated and displayed in Fig. 2*B* as a graph for the three independent donors. We detected a significant difference ($p < 0.05$) with the dNTP levels starting 8 h after Vpx+ VLPs treatment. Importantly, the dNTP levels rapidly increased 8–18 h after treatment before reaching a plateau. Moreover, dNTP levels increased concomitantly with SAMHD1 degradation. This supports previous studies indicating that SAMHD1 degradation by Vpx directly results in a subsequent increase in cellular dNTP levels in MDMs (6, 7, 11, 14).

Next, because the dNTP abundance is directly related with DNA polymerization kinetics, we examined the time-dependent proviral DNA synthesis kinetics by measuring 2LTR circle copy numbers using the qPCR technique. 2LTR circles are defective proviral DNA products that have failed to integrate into the host genome. Moreover, they are commonly used as a surrogate to monitor late proviral synthesis steps in the HIV life cycle (15). Previous studies have used pretreatment of target cell populations, macrophages, and dendritic cells before transducing them with HIV vectors (6–8, 11, 16, 17). Indeed, as shown in Fig. 3*A*, the pretreatment of MDMs with the Vpx+

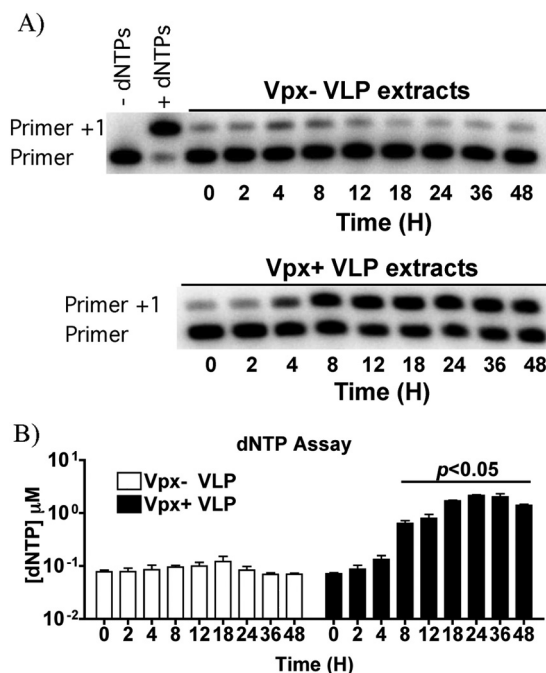


FIGURE 2. **HIV RT-based primer extension assay.** *A*, a representative HIV-1 RT-based primer extension gel is shown. Extension products are indicated by *Primer +1* lane. Extracts from Vpx- VLPs are shown at the top, whereas Vpx+ VLPs extracts are below. *B*, data collected are graphed as mean and S.E. for three independent donors. Student's *t* test analysis was performed to determine statistical significance between the different time points.

VLPs accelerated proviral DNA synthesis tracking with the increase in dNTP levels.

The 2-h pretreatment with the VLPs is a commonly used protocol to investigate the impact of Vpx on lentiviral vector transduction. Next, we compared the proviral DNA synthesis kinetics in MDMs with either the co-treatment with both VLPs and HIV vector or the 2-h pretreatment. As depicted in Fig. 3, *A* and *B*, both the pretreatment and the co-treatment with Vpx+

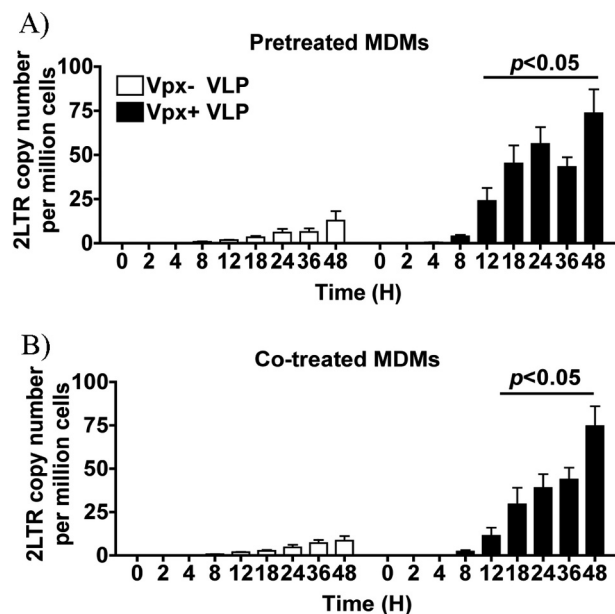


FIGURE 3. qPCR analysis for 2LTR circle copy numbers. A, MDMs were pretreated for 2 h with VLPs before the addition of D3-HIV GFP virus. At the indicated time points, cells were collected and processed for total DNA. qPCR for 2LTR circle copy numbers were performed in duplicate for three independent donors. B, MDMs were co-treated with VLPs and D3-HIV GFP virus. Data are plotted as mean and S.E., and paired Student's *t* test analysis was performed to determine statistical significance.

VLPs led to enhanced 2LTR circle copy numbers as compared with the MDMs treated with Vpx– VLPs. Both Vpx+ treatment groups were significantly higher than Vpx– treatment groups at 12 h after D3-HIV GFP addition and continuously increased even up to 48 h. Both Vpx+ VLPs treatment groups reached similar 2LTR copy numbers by 48 h. We also examined GFP expression by FACS and found comparable frequency of GFP+ cells in both the pretreated and the co-treated Vpx+ VLP groups (supplemental Fig. 1). Collectively, our data presented in this study strongly support that the accelerated reverse transcription kinetics is induced by the tight interplay between SAMHD1 degradation and dNTP pool increase, which greatly improves the overall success rate of proviral DNA synthesis completion in nondividing cells.

DISCUSSION

Vpx is believed to have arisen from gene duplication from Vpr (18). Although sequence similarities exist between Vpr and Vpx, they have different cellular functions (19–21). Vpx promotes the DCAF1 E3 ubiquitin ligase-mediated degradation of SAMHD1 (6, 7). Our recent study revealed that the Vpx-mediated degradation of SAMHD1 causes an increase in the cellular dNTP pool size (11), which, in turn, allows HIV-1 reverse transcriptase (RT) to efficiently carry out reverse transcription of the viral RNA genome into double-stranded proviral DNA. Moreover, a series of our extensive studies revealed that cellular dNTP concentration is critical for efficient reverse transcription by HIV-1 RT (9, 10, 22–26), especially in nondividing HIV target cell types.

Recently, Sunseri *et al.* (27) have shown that a modified Vpx can be incorporated into HIV-1 via p6. Although in this system, Vpx competes with Vpr for packaging into the virion, the

authors found enhancement of transduction efficiency. Moreover, Vpx proteins from different lentiviruses, SIV *versus* HIV-2, have different activities in promoting transduction of MDM and dendritic cell populations, with SIV being more effective at enhancing transduction. Alternatively, the amount of Vpx being packaged within the virion could also be the reason for this observation because packaging of HIV-2 Vpx is reduced as compared with SIV of macaque Vpx (27). This may be of importance when trying to directly compare SIV *versus* HIV-2 or HIV-1 infection in animal models and humans. Moreover, Vpr is known to be released by infected cells and may negatively influence bystander cells (28). Vpx may also be released from infected cells and influence bystander cells. However, a putative model would be that Vpx, unlike Vpr, would promote infection of macrophage and dendritic cells, both nondividing cells, by down-regulating SAMHD1 expression at the site of infection. In addition to SAMHD1 degradation by Vpx, we believe that intracellular dNTP biosynthesis is required. Briefly, dNTP biosynthesis occurs through ribonucleotide reductase (RNR), the *de novo* synthesis pathway, and the salvage pathway. In eukaryotes, RNR is a heterodimeric enzyme composed of R1 and either R2 or p53R2 subunits (29). This heterodimeric complex catalyzes the conversion of ribose nucleoside diphosphates into deoxynucleoside diphosphate (30) and is highly regulated within the cell by the expression of the R2 subunits, cellular localization of subunits, and substrate feedback control mechanisms (31–34). Deoxynucleoside diphosphates require an additional phosphorylation step to become dNTPs, which then serve as a substrate for DNA polymerases and HIV-1 RT. R2 is mainly expressed during the S phase of the cell cycle (35), whereas p53R2 is induced upon DNA damage (36). We have not detected an increase in either R2 or p53R2 levels within the Vpx+ VLP-treated MDMs (data not shown). However, the functional activity of this complex still remains to be measured at this time. We speculate that Vpx may be influencing the RNR and kinase activities concomitantly with promoting SAMHD1 degradation to rapidly increase cellular dNTP levels in nondividing cells.

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