Efficient Pre-catalytic Conformational Change of Reverse Transcriptases from SAMHD1 Non-counteracting Primate Lentiviruses During dNTP Incorporation

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Abstract

Unlike HIV-1, HIV-2 and some SIV strains replicate at high dNTP concentrations even in macrophages due to their accessory proteins, Vpx or Vpr, that target SAMHD1 dNTPase for proteasomal degradation. We previously reported that HIV-1 reverse transcriptase (RT) efficiently synthesizes DNA even at low dNTP concentrations because HIV-1 RT displays faster pre-steady state $k_{pol}$ values than SAMHD1 counteracting lentiviral RTs. Here, since the $k_{pol}$ step consists of two sequential sub-steps post dNTP binding, conformational change and chemistry, we investigated which of the two sub-steps RTs from SAMHD1 non-counteracting viruses accelerate in order to complete reverse transcription in the limited dNTP pools found in macrophages. Our study demonstrates that RTs of SAMHD1 non-counteracting lentiviruses have a faster conformational change rate during dNTP incorporation, supporting that these lentiviruses may have evolved to harbor RTs that can efficiently execute the conformational change step in order to circumvent SAMHD1 restriction and dNTP depletion in macrophages.

Keywords

reverse transcriptase; lentiviruses; SAMHD1; dNTPs; macrophages; conformational change
INTRODUCTION

During the course of its pathogenesis, HIV-1 infects both activated/dividing CD4+ T cells and terminally differentiated/nondividing myeloid cells such as macrophages and microglia (1–4). While activated CD4+ T cells support robust HIV-1 replication kinetics and undergo rapid cell death upon infection, HIV-1 replication kinetics in macrophages is greatly suppressed. HIV-1 infected myeloid cells display long cell survival, leading to the persistent production of low levels of HIV-1, particularly in the brain (5,6). A series of recent studies revealed that the observed suppressed HIV-1 replication kinetics in myeloid cells is due to host SAM domain and HD domain containing protein 1 (SAMHD1) which is a dNTP triphosphohydrolase (dNTPaSe) that depletes the dNTP substrates of reverse transcriptases (RT) in macrophages (7,8). However, some SIV strains replicate rapidly even in macrophages. The fast replication capability of these SIV strains is due to their accessory protein, viral protein X (Vpx) (9,10), which is a gene duplication product of another viral accessory protein, viral protein R (Vpr) (11,12). Lentiviral Vpx directly binds to host SAMHD1 protein and induces the E3-ligase mediated proteasomal degradation of SAMHD1 (9,13,14). Less abundant SAMHD1 leads to the elevation of cellular dNTP concentrations in macrophages and the acceleration of reverse transcription during the viral replication cycle (15). Several studies demonstrated that this SAMHD1 degradation capability already existed among SIV strains that encode Vpr, but not Vpx, such as the SIVagm strains (16). These SIV strains use their Vpr proteins to counteract their host SAMHD1 via the same proteasomal degradation pathway hijacked by Vpx (17,18). Importantly, while SAMHD1 sequence variations are observed among the many primate host species, Vpr/Vpx species specificity also recognizes host-specific SAMHD1 sequences for the proteasomal degradation (19,20).

Due to the presence of host SAMHD1, SAMHD1 non-counteracting lentiviruses such as HIV-1 replicate in limited dNTP pools during the infection of macrophages; conversely, SAMHD1 counteracting lentiviruses such as SIVmac239 replicate under abundant dNTP conditions even in macrophages. We previously observed that SAMHD1 non-counteracting lentiviral RTs can efficiently synthesize DNA even at the low dNTP concentrations found in macrophages. This suggests that the efficient DNA synthesis capability of SAMHD1 non-counteracting lentiviral RTs enables these lentiviruses to overcome SAMHD1-mediated viral restriction (21). The slower replication kinetics of HIV-1 compared to those of SAMHD1 counteracting strains such as HIV-2 and some SIVs, reveal that the potential RT-mediated mechanism to overcome low dNTP concentrations in macrophages is much less effective than Vpx/Vpr-mediated SAMHD1 degradation. However, this RT-mediated mechanism may enable HIV-1 to complete its reverse transcription step even in macrophages with limited dNTP pools. Pre-steady state kinetic analysis using a rapid quench instrument (22,23) can simultaneously measure $K_d$ (dNTP binding affinity) and $k_{pol}$ ($K_{conf} + K_{chem}$), lending insight into the molecular activities and mechanisms of various enzymes. Employing the use of pre-steady state kinetic analyses, we previously reported that while SAMHD1 non-counteracting lentiviral RTs display faster rates of incorporation ($k_{pol}$) when compared to SAMHD1 counteracting lentiviral RTs, these polymerases share similar $K_d$ values (23). This suggests that the faster $k_{pol}$ values of the SAMHD1 non-counteracting lentiviral RTs allow these...
viruses to complete proviral DNA synthesis even at the low dNTP concentrations found in macrophages.

In this study, we investigated the pre-steady state kinetics and elemental effect of RTs from various SAMHD1 counteracting and non-counteracting primate lentivirus strains in order to understand the differential relationship between RT kinetics and host SAMHD1 proteins among these lentiviruses. It was demonstrated with many DNA polymerases (24–27), including HIV-1 RT (28), that (i) the $K_{\text{conf}}$ step is the slowest/rate-limiting step during the pre-steady state dNTP incorporation reaction and (ii) the $K_{\text{chem}}$ step is very rapid, indicating that the $k_{\text{pol}}$ value is predominantly represented by the $K_{\text{conf}}$ step ($k_{\text{pol}} \approx K_{\text{conf}}$). This conclusion was experimentally made by the absence of the phosphorothioate elemental effect when using dNTP$\alpha_S$ substrates which contain sulfur instead of oxygen at the $\alpha$-phosphate position of dNTP. During the incorporation of dNTP$\alpha_S$, the sulfur atom present on the alpha-phosphate of the substrate slows only the chemistry step ($K_{\text{chem}}$), a phenomenon defined as an elemental effect.

In this study, we determined the elemental effect of RTs from SAMHD1 non-counteracting SIV strains, SIVcpz and SIVgor, and RTs from SAMHD1 counteracting SIV strains, SIVagm 9063–2 and SIVmne CL8. Overall, our kinetic analysis explains how the RTs of the SAMHD1 non-counteracting lentiviruses mechanistically gained faster $k_{\text{pol}}$ rates and how these lentiviruses became capable of circumventing SAMHD1-mediated restriction in order to complete proviral DNA synthesis even at the extremely low cellular dNTP concentrations found in nondividing macrophages.

**EXPERIMENTAL PROCEDURES**

**Cells, plasmids and chemicals**

The following full-length clones of various HIV-1 and SIV strains were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, National Institutes of Health: HIV-1 94UG114.1 Non-infectious Molecular Clone from Drs. Beatrice Hahn and Feng Gao, and the UNAIDS Network for HIV Isolation and Characterization (cat# 4001)(29); HIV-1 94CY017.41 Non-infectious Molecular Clone from Drs. Stanley A. Trask, Feng Gao, Beatrice H. Hahn, and the Aaron Diamond AIDS Research Center (cat# 6175)(30); pSIVgorCP2139 from Drs. Jun Takehisa, Matthias H. Kraus, and Beatrice H. Hahn (cat#11722)(31); SIV$\text{CPZ}\text{TAN2.69}$ from Drs. Jun Takehisa, Matthias H. Kraus and Beatrice H. Hahn (Cat #11497)(32). A full-length molecular clone of SIVmne CL8 was previously constructed (33), while a full-length molecular clone of SIVagm 9063–2 was kindly provided by V. Hirsh (34) (National Institutes of Health, Bethesda, MD). The aforementioned molecular clones were used to clone the flag tagged Vpr genes of HIV-1 Ug and HIV-1 Cy into pCDNA3.1/hygro (+) ($\text{HindIII}$ and $\text{Xhol}$, ThermoFisher) while the flag tagged Vpr genes of SIVgor, SIVcpz, and SIVagm 9063–2 were synthesized into pcDNA3.1/ Hygro (+) by GenScript (Piscataway, NJ). Full length molecular clones were also used to clone the RT genes of HIV-1 Cy (21), HIV-1 Ug (21), SIVagm 9063–2 (35), SIVgor, SIVcpz 2.69 into pET28a (NdeI and Xhol sites, Novagen) and SIVmneCL8 RT into pHis (NdeI and EcoRI). The following SAMHD1 proteins were synthesized into pLVX-IRES- mCherry with an N-terminal HA tag from NCBI Reference sequences NM_001280510.1 (chimpanzee) and

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NM_001279619.1 (gorilla). The hSAMHD1 gene encoded from the plasmid provided by Dr. Felipe Diaz-Griffero (36) was cloned into pLVX-IREs-mCherry with an N-terminal HA tag. African Green Monkey SAMHD1 haplotype IV in pLPCX was gifted from Dr. Michael Emerman (37) (Fred Hutchinson Cancer Research Center, Seattle, WA). Pigtail macaque SAMHD1 gene was amplified from pigtail macaque mRNAs and cloned into pcDNA3.1. Rhesus macaque SAMHD1 in pLenti was generously obtained from Dr. Nathaniel Landau (New York University, New York, NY). Also obtained from Dr. Nathaniel Landau were a plasmid expressing SIVmac251 proteins except Env (pSIV3 +Vpx) and pSIV3 with Vpx deletion (pSIV −Vpx) (38,39).

**SAMHD1 degradation assay**

The SAMHD1 degradation assay was conducted as previously reported (19,37,40). Briefly, using polyethylenimine, 293T cells (2 × 10^6 cells) were co-transfected with a plasmid expressing host specific HA-tagged SAMHD1 proteins (0.1 μg) and a plasmid expressing either flag-tagged (SIVgam 9063–2) or HA-tagged (HIV-1 Cy, HIV-1 Ug, SIVgor, and SIVcpz) viral accessory proteins Vpx/Vpr or the entire proviral genome (SIVmne CL8) (2 μg). The cell lysates were prepared by sonication from the transfected cells at 48 h post transfection and western blots were performed to visualize not only HA-tagged primate SAMHD1 proteins using an anti-HA antibody, but also hSAMHD1 using anti-hSAMHD1 antibody. GAPDH was used for as a loading control. Vpr and Vpx proteins were visualized using anti-flag tag and anti-HA tag antibodies. The mean relative SAMHD1 levels were calculated by densitometry analysis and normalized to the GAPDH loading control. The ratios of the normalized SAMHD1 levels with and without viral protein expression were calculated for determining the SAMHD1 degradation efficiency.

**RT protein expression and purification**

All six N-terminal His-tagged RTs were expressed in *E. coli* BL21 Rosetta2 DE3 (Millipore) and their p66/p66 homodimers were purified as described previously (41) with the following changes. For HIV-1 Cy and SIVgam 9063–2 RTs, clear lysate obtained through sonication was applied to HisBind resin (Millipore) equilibrated with a binding buffer containing 0.5M NaCl, 20mM Tris-HCl pH 7.9, and 5mM imidazole. The column was washed with 15 column volumes binding buffer prior to being eluted in 1mL fractions by a solution containing 20mM Tris-HCl pH 7.9, 0.5M NaCl, and 1M imidazole. Fractions containing the His tagged-p66/p66 were pooled and dialyzed for 16 hours in a buffer containing 50mM Tris-HCl pH 7.5, 200mM NaCl, 1mM EDTA, and 10% glycerol. The RTs then underwent an additional 3 hour dialysis in a solution containing 50mM Tris-HCl pH 7.5, 200mM NaCl, 1mM EDTA, 10% glycerol, and 1M DTT. Purification of HIV-1 Ug, SIVmne CL8, SIVgor, and SIVcpz 2.69 RTs required different binding, elution, and dialysis/storage buffers. The clear lysate of these RTs was loaded onto a His-resin bed equilibrated with binding buffer containing 40mM Tris-HCl pH 7.5, 250mM KCl, 5 mM MgCl₂, 5mM betamercaptoethanol, 20mM imidazole, and 10% glycerol. The proteins were eluted from the column using a solution containing 40mM Tris-HCl pH 7.5, 250mM KCl, 5mM MgCl₂, 240mM imidazole, and 10% glycerol before being dialyzed for 16 hours in a buffer containing 50mM Tris-HCl pH 7.5, 150mM KCl, 0.25mM EDTA, 1mM betamercaptoethanol, and 20% glycerol. To examine the purity of the proteins, the dialyzed RTs
were run on a 4–15% SDS-PAGE gel (BioRad)(Supplemental Figure 1). All RTs were determined to have at least 95% purity and were flash frozen in liquid nitrogen prior to being stored at −80°C for future use.

**Pre-steady state kinetic analysis**

To determine the active site concentration of the six RT proteins, we first performed pre-steady-state burst experiments using an RFQ-3 rapid quench-flow apparatus (KinTek Corporation). A 32P-labelled template:primer (T/P) was prepared by annealing a 5’-32P-labeled 17mer primer (5’-CGCGCCGAA TTCCCGCT-3’, Integrated DNA Technologies) to a 3-fold excess of 40mer RNA template (5’-AAGCUUGGCUGCAGAAUAUUUGCUAGCGGGAAUUCGGCGCG-3’, Integrated DNA Technologies). In burst experiments, 100nM RT pre-bound to 300nM T/P through a 10 min incubation at 37°C was rapidly mixed with a solution containing 300uM dATP, 10mM MgCl₂, 50mM Tris-HCl, pH 7.8 and 50mM NaCl at 37°C. The reactions were quenched at the following time points with EDTA: 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, and 3 seconds. Reaction products were separated on 14% polyacrylamide/8M urea gel, visualized using a PharosFX (Bio-Rad), and quantified with Image Lab Software (Bio-Rad). To determine the active site concentration of each purified RT, product formation was fit to the burst equation (Eq.1).

\[
[Product] = A\left[1 - \exp(-k_{obs} \times t)\right] + (k_{ss} \times t) \\
\text{Eq. 1}
\]

In this equation, A is the amplitude of the burst and reflects the concentration of enzyme that is in an active form, \(k_{obs}\) is the observed first-order burst rate for dNTP incorporation, and \(k_{ss}\) is the linear steady state rate constant (23,42,43). Active site titrations were performed in triplicate for each lentiviral RT.

Finally, to determine the pre-steady state kinetic activity of the six RTs, we employed single turnover experiments. For these experiments, a 32P-labelled template:primer (T/P) was prepared by annealing a 5’-32P-labeled 22mer primer (5’-CGCGCCGAATTCCCGCTAGCAA-3’, Integrated DNA Technologies) to a 3-fold excess of 40mer RNA template (5’-AAGCUUGGCUGCAGAAUAUUUGCUAGCGGGAAUUCGGCGCG-3’, Integrated DNA Technologies). In single turnover experiments, 250nM active RT enzyme pre-bound to 50nM T/P was rapidly mixed with a solution containing 10mM MgCl₂ and varying concentrations (1.6, 6.25, 12.5, 25, 50, and 100μM) of either dTTP or dNTPαS substrate in the presence of 50mM Tris-HCl, pH 7.8, and 50mM NaCl. The reactions were quenched at various time points (0, 0.01, 0.03, 0.05, 0.1, 0.5, and 2 seconds) with 3mM EDTA and visualized using the same methods as above. The amounts of product were quantified using Image Lab software and plotted as a function of time. The data were then fit to a single exponential equation (Eq. 2).

\[
[Product] = A\left[1 - e^{-k_{obs}t}\right] \\
\text{Eq. 2}
\]
In which \( A \) is the amplitude of product formation, \( k_{\text{obs}} \) is the observed pre-steady state rate for dNTP incorporation, and \( t \) is time. Next, \( k_{\text{obs}} \) was plotted as a function of substrate concentration and fit to a non-linear regression curve equation (Eq. 3).

\[
k_{\text{obs}} = \frac{k_{\text{pol}}[d\text{NTP}]}{K_d + [d\text{NTP}]}
\]

In which \( k_{\text{pol}} \) is the maximum rate of dNTP incorporation and \( K_d \) is the equilibrium dissociation constant for the dNTP substrate (44). Single turnover experiments were conducted in triplicate for both dTTP and dNTPaS substrates.

**Elemental effect analysis**

The elemental effect of each RT was determined using the following equation (45):

\[
\text{Elemental Effect} = \frac{k_{\text{pol}}(dTTP)}{k_{\text{pol}}(dTTPaS)}
\]

Eq. 4

If this value exceeds 4, it can be concluded that there is elemental effect. If this value is ≤4, then there is no elemental effect present (46,47).

**RESULTS and DISCUSSION**

**Test for SAMHD1 degradation capability of SIV strains**

While SAMHD1 non-counteracting HIV-1 replicates in the SAMHD1-mediated limited dNTP pools found in macrophages, SAMHD1 counteracting lentiviruses such as HIV-2 and some SIV strains replicate under abundant dNTP conditions even in macrophages (48,49). We previously reported that unlike RTs from SAMHD1 counteracting lentiviruses, HIV-1 RTs efficiently synthesize DNA even in the low dNTP concentrations found in macrophages (21). This led us to hypothesize that the efficient DNA synthesis capability of the SAMHD1 non-counteracting HIV-1 RTs enables these viruses to complete proviral DNA synthesis even in the SAMHD1 mediated limited dNTP concentrations found in infected macrophages. Our follow-up pre-steady state kinetic analysis reported that the efficient DNA synthesis kinetics of HIV-1 RTs is due to their performing a faster \( k_{\text{pol}} \) step than SAMHD1 counteracting lentiviral RTs (23) - a kinetic event which occurs after the binding of dNTP substrate \( (K_d \text{ step}) \) to the active site (50).

In this study, since the \( k_{\text{pol}} \) step consists of two sequential sub-steps (50): 1) a conformational step followed by a 2) chemistry step (28,42), we investigated which of these two post dNTP binding sub-steps the RTs of the SAMHD1 non-counteracting primate lentiviruses accelerate in order to execute the faster \( k_{\text{pol}} \) step than the RTs of the SAMHD1 counteracting lentiviruses. This study aimed at mechanistically elucidating how these two groups of lentiviruses evolutionarily adapted to the largely different cellular dNTP concentrations found in their nondividing myeloid target cells. For this investigation, we employed RTs of four different SIV strains: SIVgor, SIVcpz, SIVagm 9063–2 and SIVmne CL8.
Many SIV strains from various primate species counteract the anti-viral activity of SAMHD1 by targeting the host protein for E3-ligase dependent proteasomal degradation (9,13,16,17,51). The mechanism of counteracting SAMHD1 via proteasomal degradation was initially found in SIV strains encoding Vpx [i.e. SIVsm (10)], a protein which directly binds to SAMHD1 and recruits the host DDB1-CUL4-DCAF E3 ligase complex to induce the proteasomal degradation of SAMHD1 (9,10,14). Later works demonstrated that this anti-SAMHD1 mechanism already existed even in some of SIV strains that do not encode Vpx. However, another accessory protein of these SIV strains, Vpr, is capable of inducing SAMHD1 degradation in these primate lentiviruses. This suggests that there was a splitting of anti-SAMHD1 function during the gene duplication of Vpr that appears to have created Vpx and two populations of Vpr: one that is able to counteract SAMHD1 and one that is not (12,19). Therefore, we began by testing the SAMHD1 degradation activity of the four SIV strains (SIVgor, SIVcpz, SIVagm 9063–2, and SIVmne CL8) that we here investigated for their RT enzyme kinetics.

SIVcpz and SIVgor are considered to be the origin of HIV-1 (52,53), and like HIV-1, these two SIV strains encode Vpr, but not Vpx. Since it was previously reported that as observed with HIV-1 strains, SIVcpz Vpr does not proteasomal degrade chimpanzee SAMHD1 (16), we first verified these results and tested whether SIVgor Vpr can induce the proteasomal degradation of gorilla SAMHD1. Additionally, we tested the capability of SIVmne CL8 and SIVagm 9063–2 to degrade their host SAMHD1 proteins. Due to the host SAMHD1-lentivirus specificity (19) including SAMHD1 sequence variations among the host species, we expressed SAMHD1 proteins from the specific host species related to each of primate lentiviruses used in this study: gorilla, chimpanzee, pig-tailed macaque, and African green monkey (haplotype IV) (37) for the SAMHD1 degradation assay (19,37,40). The Vpr genes of SIVgor, SIVcpz and SIVagm 9063–2 were expressed in order to observe their ability to mediate the degradation of their host SAMHD1. As a control, Vpx of SIVmac251 (pSIV3) was tested. In the SAMHD1 degradation assay (19,37,40), 293T cells were co-transfected with a lentiviral plasmid expressing the host SAMHD1 protein and a mammalian plasmid expressing the corresponding Vpr or Vpx protein. SAMHD1 protein levels were monitored by western blots. Vpr, Vpx, and SAMHD1 proteins expressed in this assay were tagged with either HA- or Flag-tag at their N-terminal ends. The ratios of the SAMHD1 protein levels in each of the triplicated SAMHD1 degradation assay were calculated for comparison using densitometry analysis. Since SIVmne CL8 has never been assessed for its ability to degrade SAMHD1, SIVmne CL8 Vpr or Vpx could possess or lack the ability to Counteract SAMHD1. For this reason, we utilized a full-length molecular clone of SIVmne CL8, rather than a plasmid containing its Vpr or Vpx protein, to assess the SAMHD1 degradation capabilities of this virus.

First, as shown in Figure 1A, the level of the rhesus macaque SAMHD1 protein in the transfected 293T cells was markedly reduced when the cells were co-transfected with a SIVmac251 plasmid (pSIVmac +Vpx) that expresses all viral proteins except Env. However, this reduction was not observed when SAMHD1 was co-transfected with the same SIVmac251 plasmid containing a Vpx deletion (pSIVmac -Vpx). However, Vpr proteins of two HIV-1 strains, HIV-1 Cy (Figure 1B) and HIV-1 Ug (Figure 1C), could not degrade their host SAMHD1 proteins. In contrast, Vpr of SIVagm 9063–2 (Figure 1F) degraded African
green monkey haplotype IV SAMHD1 protein in this assay. It was previously reported that SIVagm strains from different subspecies of African green monkeys use their Vpr proteins to degrade their host haploid type specific SAMHD1 proteins (37). Also, the transfection of the molecular clone of Vpx-encoding SIVmne CL8 (Figure 1G) also degraded pig-tail macaque SAMHD1 protein. Importantly, Vpr proteins of both SIVcpz (Figure 1D) and SIVgor (Figure 1E) could not degrade their host SAMHD1 proteins. Collectively, the results in Figure 1 demonstrate that, as is the case for HIV-1, SIVgor and SIVcpz do not degrade their host SAMHD1 protein, whereas SIVagm 9063–2 and SIVmne CL8 proteasomally degrade their host SAMHD1 proteins. Therefore, this data suggests that while SIVgor and SIVcpz should replicate under SAMHD1-mediated limited dNTP pools in macrophages, both SIVagm 9063–2 and SIVmne CL8 should replicate under abundant dNTP conditions even in macrophages.

**Pre-steady state kinetic analysis of SIVgor and SIVcpz RTs**

The enzymatic dNTP incorporation by DNA polymerases including RTs follows a series of sequential mechanistic steps that can be separately measured for their kinetic rates (28,42,45,50). Typically, as illustrated in Figure 2A, first, RT binds to template:primer (T/P, $K_D$), forming a binary complex (RT:T/P), and this binary complex binds to a dNTP substrate ($K_D$, dNTP binding affinity), forming ternary complex (RT:T/P:dNTP). Next, the ternary complex will undergo the $k_{pol}$ step, which consists of two sequential sub-steps: 1) a conformational change of the complex (RT*:T/P:dNTP, $K_{conf}$) followed by 2) a chemistry step ($K_{chem}$) to complete the phosphodiester bond formation between 3' OH of the primer and α-phosphate of the dNTP substrate. Substrate incorporation is finally followed by the slow release of PPi product. Single round pre-steady steady state kinetic analysis has been extensively employed to determine the kinetic rate of these individual steps involved dNTP incorporation by DNA polymerases (23,28,42,45,50,54,55).

Previous studies have shown that RTs originating from SAMHD1 non-counteracting HIV-1 strains are characterized by higher $k_{pol}$ than RTs from various SAMHD1-counteracting SIV strains, which suggested that HIV-1 RTs might have evolved to have faster $k_{pol}$ step in order to complete proviral DNA synthesis even in macrophages harboring SAMHD1-mediated low dNTP pools (23). SIVcpz and SIVgor are the closest relatives of HIV-1 and, particularly, SIVcpz is considered as the origin of HIV-1. In addition, unlike the SAMHD1-counteracting SIV strains that we previously characterized, SIVcpz and SIVgor not only lack Vpx, but their Vpr proteins do not proteasomally degrade their host SAMHD1 proteins (Figure 1) (16). Therefore, we tested whether SIVcpz and SIVgor RTs also have higher dNTP incorporation efficiency ($k_{pol}/K_D$) and faster $k_{pol}$ rates, compared to the RTs of SAMHD1-counteracting SIVs (SIVagm 9063–2 and SIVmne CL8). We also employed RTs of two HIV-1 strains (HIV-1 Ug and HIV-1 Cy) as comparison controls.

In order to measure the pre-steady state kinetic values of the RT proteins, which requires a single round of incorporation, we first measured the active site concentration of each purified RT enzyme using pre-steady state burst experiments. Pre-steady state burst experiments, which are performed using an excess T/P to RT enzyme, provide a burst amplitude that defines the concentration of active RT:T/P complexes capable of dNTP
incorporation (Equation 1, Experimental Procedures) and is followed by a steady-state turnover rate as the product complex is released from the enzyme in the rate limiting step of the dNTP incorporation pathway (28). We observed typical burst kinetics for all six RTs and found they all possessed 20–75% active protein (Figure 2B–2G and Supplemental Figure 2).

Next, using all six RTs normalized for their active site concentrations, we employed pre-steady state single turnover experiments to determine the kinetic parameters, $K_d$ and $k_{pol}$, involved in single nucleotide incorporation by these RTs. In single turnover experiments, prebound RT:T/P binary complexes, created by pre-incubating fivefold excess active RT with radiolabeled T/P, are rapidly mixed with MgCl$_2$ and various concentrations of dTTP substrate ranging from 1–100μM for reactions ranging from 0–2 seconds. Every reaction was quenched at its designated time using EDTA. We first determined the rate of single nucleotide incorporation at each substrate concentration (Equation 2, Experimental Procedures). These rates were then plotted against substrate concentration to determine the dNTP binding affinity ($K_d$), maximum rate of dNTP incorporation ($k_{pol}$), and the dNTP incorporation efficiency ($k_{pol}/K_d$) for each enzyme (Equation 3, Experimental Procedures; Supplemental Figure 3). As summarized in Table 1 and Figure 3A, both HIV-1 Ug and HIV-1 Cy RTs displayed relatively fast $k_{pol}$ rates of incorporation (HIV-1 Ug: 594.7 s$^{-1}$, HIV-1 Cy: 139.8 s$^{-1}$) in comparison to their SIV SAMHD1-counteracting counterparts (SIVagm 9063–2: 42.92 s$^{-1}$, SIVmneCL8: 68.17 s$^{-1}$). The relative difference in $k_{pol}$ values between HIV-1 Ug and HIV-1 Cy was interesting to observe. This roughly four-fold difference between the two lentiviral RT $k_{pol}$ values was retained when conducting pre-steady state kinetic analysis of a C incorporation event at a different location along the same primer-template (data not shown). This suggests that the observed difference is not an effect of primer-template sequence, rather a product of RT activity. Amino acid sequence comparisons of HIV-1 Cy and HIV-1 Ug RTs did not reveal any striking differences outside of a number of proline residue variations that might affect overall protein structure. However, of the many residue differences HIV-1 Cy and HIV-1 Ug RTs possess, any of them could play a role in overall protein dynamics during polymerization. Importantly, RTs from SIVgor and SIVcpz also displayed fast $k_{pol}$ values similar to the HIV-1 RTs at 193.10 s$^{-1}$ and 355.70 s$^{-1}$ respectively. Consistent with previous studies, all six RTs displayed similar $K_d$ values during the incorporation of the dTTP substrate. This indicates that all RTs bind the dNTP substrate with similar affinity, whereas the RTs from the SAMHD1 non-counteracting lentiviruses displayed faster $k_{pol}$ values than the RTs of the SAMHD1 counteracting lentiviral origins (Figure 3A). We were surprised to find the $K_d$ of HIV-1 Ug RT to be relatively high in comparison to that HIV-1 Cy RT. When observing the aforementioned pre-steady state kinetics of a C incorporation event, HIV-1 Ug displayed $K_d$ values similar to that of HIV-1 Cy, suggesting that the elevated $K_d$ value reported here could possibly be an effect of the primer-template sequence (data not shown). In addition, the overall dNTP incorporation efficiency ($k_{pol}/K_d$; Figure 3B and Table 1) is 4–6 times higher in the RTs from the SAMHD1 non-counteracting lentiviruses compared to the RTs from the SAMHD1 counteracting lentiviruses. This data supports the idea that the RTs of the SAMHD1 non-counteracting lentiviruses (HIV-1, SIVgor, and SIVcpz) enable these lentiviruses to circumvent the SAMHD1 restriction and to complete the proviral DNA synthesis even in the limited dNTP pools found in nondividing myeloid cells.
Phosphorothioate elemental effect of RTs from SAMHD1 non-counteracting and counteracting primate lentiviruses

The $k_{pol}$ step consists of two sequential substeps, 1) conformational change ($K_{conf}$) and 2) chemistry ($K_{chem}$). Therefore, we next tested which of these two sub-steps RTs of SAMHD1 non-counteracting lentiviruses evolutionarily honed over time in order to gain a faster $k_{pol}$ step. The phosphorothioate elemental effect has been used to determine whether the chemical step of a polymerization reaction is rate-limiting and is evaluated by comparing the rates of incorporation of the natural dNTP substrate versus a dNTPαS substrate (Equation 4, Experimental Procedures) (46,50). The sulfur on the α-phosphate in dNTPαS significantly slows down the $K_{chem}$ rate, while not affecting $K_{conf}$. Therefore, if the $K_{chem}$ step is rate limiting, then $k_{pol}$ during the incorporation of dNTPαS also becomes slower than that of the natural dNTP substrate ($k_{pol}$ for dNTPαS is 4–11 times smaller than that for natural dNTPs: elemental effect = 4–11) (45–47). In contrast, if $K_{conf}$ is the rate limiting and $K_{chem}$ is fast (much smaller than $K_{conf}$ or close to 0), the delayed chemistry step by dNTPαS does not significantly affect the overall $k_{pol}$ step ($k_{pol}$ values for both dNTPs and dNTPαS are similar: elemental effect is less than 4), implying that the $k_{pol}$ rate predominantly represents its pre-catalytic conformational change rate, $K_{conf}$ ($k_{pol}$ ≈ $K_{conf}$) (24,46,50,56,57).

To determine whether an elemental effect was present among the six RTs studied here, single turnover experiments were conducted as described above using a dNTPαS substrate and pre-steady state parameters were calculated and compared to those determined with natural dTTP (Table 2; Supplemental Figure 4) for each RT. As shown in Figure 4 and Table 2, phosphorothioate elemental effects ($k_{pol}^{dTTP}/k_{pol}^{dNTPαS}$) of all six RT proteins examined in this study were less than 4. The absence of phosphorothioate elemental effect indicates that the conformational change step of these RTs, not their chemistry step, is rate limiting during their overall $k_{pol}$ step, and their $k_{pol}$ values are predominately represented by their conformational change rates. These findings suggest that the faster $k_{pol}$ rate observed with RTs of SAMHD1 non-counteracting lentiviruses (SIVcpz, SIV gor, and HIV-1 strains) is due to their faster rates of the conformational change that occurs post dNTP binding. As illustrated in our model (Figure 5), while SAMHD1 counteracting lentiviruses (SIVmac239, SIVagm 9063–2, and SIVmne CL8) utilize Vpr/Vpx to counteract SAMHD1 and overcome the kinetic barrier presented in macrophages, our study suggests that SAMHD1 non-counteracting lentiviruses may have evolved over time to harbor RTs that execute a faster conformational change step during nucleotide incorporation, thus enabling the viruses to complete reverse transcription even in the SAMHD1 mediated low dNTP pools of nondividing myeloid cells. In conclusion, while the mechanism employed by SAMHD1 non-counteracting lentiviruses of executing a faster RT conformational change is less effective in overcoming SAMHD1 restriction than the Vpx/Vpr mechanism employed by SAMHD1 counteracting lentiviruses, this RT-based mechanism is sufficient to complete reverse transcription, albeit at slower rates, in the SAMHD1 mediated low dNTP concentrations found in macrophages.

FRET-based measurements have been reported to monitor the post-dNTP binding finger-closing conformational change rate of several DNA polymerases such as DNA polymerase β (25) and Klenow fragment of E. coli DNA polymerase I (58). However, this type of the
measurement has not been fully established for any RT protein. It is possible that this FRET-based assay can be applied for directly comparing the pre-catalytic fingers-closing rates between SAMHD1 counteracting lentiviral RTs and SAMHD non-counteracting lentiviral RTs. There are two potential mechanistic pathways to explain the adaption of the more efficient post dNTP binding conformational change displayed by SAMHD1 non-counteracting lentiviral RTs. First, it is possible that the finger-closing conformational change rates of the SAMHD1 non-counteracting lentiviral RTs are simply faster than those of the SAMHD1 counteracting lentiviral RTs. This possibility can be tested by using the FRET-based assay as described for other DNA polymerases. Second, it is possible that the distance of the movement during the finger-closing conformational change is shorter for the RT proteins of the SAMHD1 non-counteracting lentiviruses, compared to the RT proteins of the SAMHD1 counteracting lentiviruses. This possibility can be investigated through the structural comparison of the ternary complexes of the RT proteins from the SAMHD1 non-counteracting lentiviruses and SAMHD1 counteracting lentiviruses.

Overall, these kinetic studies support the idea that SAMHD1 non-counteracting primate lentiviruses such as HIV-1, SIVgor and SIVcpz might have evolved over time to possess RTs that can more efficiently execute the conformational change step, which enables these lentiviruses to circumvent SAMHD1-mediated dNTP depletion and complete proviral DNA synthesis in nondividing myeloid target cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

| Abbreviation | Definition |
|--------------|------------|
| RT           | reverse transcriptase |
| HIV          | human immunodeficiency virus |
| SIV          | simian immunodeficiency virus |
| SAMHD1       | SAM domain and HD domain containing protein 1 |
| Vpx          | viral protein X |
| Vpr          | viral protein R |
| dNTP         | deoxynucleoside triphosphate |
| PP<sub>i</sub> | inorganic pyrophosphate |
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Figure 1: SAMHD1 degradation capability of primate lentiviruses.
SAMHD1 degradation capability by lentiviral proteins was determined using the SAMHD1 degradation assay (19,37,40). In this assay, 293T cells were co-transfected with a plasmid expressing host specific HA-tagged SAMHD1 proteins (0.1 μg) and a plasmid expressing either flag-tagged or HA-tagged viral accessory proteins (Vpx or Vpr), the entire (SIVmne CL8), or partial viral proteins (SIVmac251) (2μg). The levels of SAMHD1 were determined by western blots with anti-HA antibody (A, D-G) or anti-hSAMHD1 antibody (B and C), and the expression of the viral accessory protein (Vpx or Vpr) were determined by anti-flag
tag (SIVagm 9063–2) or anti-HA tag (HIV-1 Cy, HIV-1 Ug, SIVgor, and SIVcpz) antibody. GAPDH was used as a loading control. (A) Test for Rhesus macaque (Rh Mac) SAMHD1 degradation by SIVmac251 (−) and (+) Vpx. (B) Test for human SAMHD1 degradation by HIV-1 Cy Vpr protein. (C) Test for human SAMHD1 degradation by HIV-1 Ug Vpr. (D) Test for chimpanzee SAMHD1 degradation by SIVcpz Vpr. (E) Test for gorilla SAMHD1 degradation by SIVgor Vpr. (F) Test for African green monkey haploid type IV SAMHD1 (Agm SAMHD1 IV) degradation by SIVagm 9063–2 Vpr. (G) Test for pig-tail macaque (Pt Mac) SAMHD1 degradation by SIVmne CL8 full length molecular clone. pCDNA3.1-hygro, a plasmid that does not express viral proteins, was used as a negative (−) control in B-G. The molecular weight of each protein presented is marked. The data presented in this figure are representative data from two independent transfections. The mean relative SAMHD1 levels shown were calculated by densitometry analysis and normalized to the GAPDH loading control. The calculated mean±SD (standard deviation) values corresponding to the normalized SAMHD1 levels following challenge with Vpr/Vpx are (A) 0.024±0.025 (B) 1.042±0.485 (C) 0.766±0.101 and (D) 0.181±0.061 (E) 0.397±0.093 (F) 2.121±0.233 (G) 0.959±0.548.
Figure 2: Determination of active site concentrations of SIVgor and SIVcpz reverse transcriptase proteins by burst kinetic analysis.

(A) Scheme for dNTP incorporation by reverse transcriptase (RT). Free RT molecules initially bind to template:primer (T/P, $K_D$), forming the RT:T/P binary complex. Next, dNTP substrate binds to the binary complex, forming RT:T/P:dNTP ternary complex ($K_d$, dNTP binding affinity). The ternary complex then undergoes the $K_{pol}$ step which consists of two sequential sub-steps, 1) pre-catalytic conformational change ($K_{conf}$) forming RT*:T/P:dNTP and 2) chemistry ($K_{chem}$) extending T/P to T/P$_{n+1}$, followed by the PP$_i$ product release.

Burst kinetic analysis of (B) HIV-1 Ug, (C) HIV-1 Cy, (D) SIVagm 9063–2, (E)
SIVmneCL8, (F) SIVgor, and (G) SIVcpz RT proteins determined the active site concentration of these proteins. Active site titrations were performed in excess T/P conditions to ensure all RT active sites were occupied by T/P. Substrate (dATP) was added to the reaction to allow single nucleotide incorporation events to occur for 0–3 seconds. Reaction product was quantified and fit to a burst equation to determine the active site concentration of each RT (Equation 1, Experimental Procedures). Secondary burst kinetic curves for all RTs are shown in Supplemental Figure 2 to display the range of active site activity for each purified enzyme. Active site concentrations were determined from triplicate experiments for all six RT proteins and the calculated activities were found to range from 20–75%.
The pre-steady state kinetic $K_{pol}$ and $K_d$ values of HIV-1 Ug, HIV-1 Cy, SIVagm 9063–2, SIVmne CL8, SIVgor, and SIVcpz RT proteins were determined with dTTP (Table 1) as described in Experimental Procedures. Their $K_{pol}$ (A) and $K_{pol}/K_d$ (B) values were compared. The assays were conducted in triplicate. Statistical significance was determined by first grouping the lentiviral RTs into three groups for comparison: HIV-1 (HIV-1 Cy and HIV-1 Ug), SAMHD1-counteracting SIVs (SIVagm 9063–2 and SIVmne CL8), and SAMHD1 non-counteracting SIVs (SIVgor and SIVcpz). Statistical significance from unpaired two-tailed student’s t-tests is indicated as: *, p<.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Figure 3: Comparison of pre-steady state kinetic values among six lentiviral RT proteins.

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The $K_d$ and $K_{\text{pol}}$ values of the six lentiviral RT proteins with dTTP (Table 1) and dNTPαS (Table 2) were determined as described in Experimental procedures, and the phosphorothioate elemental effect of these proteins were calculated as the ratios between mean $K_{\text{pol}}$ values with natural dTTP and dNTPαS ($K_{\text{pol}}^{\text{dTTP}} / K_{\text{pol}}^{\text{dNTPαS}}$) (Equation 4, Experimental Procedures). An elemental effect value of less than 4 (dotted line) indicates that the conformational change sub-step of the $K_{\text{pol}}$ step is rate-limiting (46,47) and that the pre-catalytic conformational change rate predominantly represents the $K_{\text{pol}}$ step in these six RT proteins.

**Figure 4: Phosphorothioate elemental effect of six lentiviral RT proteins.**
Figure 5. Model for anti-SAMHD1 strategies employed by SAMHD1 non-counteracting and counteracting lentiviruses in non-dividing myeloid cells.

In nondividing myeloid cells, while SAMHD1 counteracting viruses utilize Vpx (HIV-2, SIVsm, SIVmac, and SIVmne, green) or Vpr (SIVagm, SIVmus, and SIVdeb, purple) to elevate intercellular dNTP pools and rapidly complete reverse transcription (green and purple lines respectively), SAMHD1 non-counteracting lentiviruses (HIV-1, SIVcpz, and SIVgor) have been evolutionarily honed to perform a faster conformational change step of their RT protein during dNTP incorporation (red dotted line) in order to complete reverse transcription even in the SAMHD1-mediated low dNTP pools. While the mechanism of executing a faster RT conformational change to overcome SAMHD1 restriction is not as effective as the Vpx/Vpr mechanism in overcoming SAMHD1 restriction, it is still sufficient to circumvent (red dotted line) SAMHD1 restriction and complete reverse transcription even at the SAMHD1 mediated low dNTP concentrations found in macrophages.

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Table 1: 
Pre-steady state kinetic values of six primate lentiviral RT proteins with dTTP.

Single turnover pre-steady state kinetic analysis was performed using a dTTP substrate. Experiments were conducted in triplicate. Representative plots from which this data derived can be found in Supplemental Figure 3.

| RT strains     | $k_{pol}$ (s$^{-1}$) | $K_a$ (μM) | $k_{pol}/K_a$(s$^{-1}$ * μM$^{-1}$) |
|----------------|----------------------|------------|-----------------------------------|
| HIV-1 Ug       | 594.70±144.00        | 167.90±69.47 | 3.71                              |
| HIV-1 Cy       | 139.80±52.40         | 41.54±21.96  | 3.58                              |
| SIVagm 9063–2 | 42.92±15.27          | 40.82±20.63  | 1.11                              |
| SIVmne CL8     | 68.17±4.46           | 90.37±37.14  | 0.83                              |
| SIVgor         | 193.10±77.67         | 45.21±22.62  | 5.18                              |
| SIVcpz         | 355.70±83.50         | 104.50±21.45 | 3.39                              |
Table 2:
Pres-steady state kinetic values of six primate lentiviral RT proteins with dNTPαS.

Single turnover pre-steady state kinetic analysis was performed using a dNTPαS substrate. Experiments were conducted in triplicate. Representative plots from which this data derived can be found in Supplemental Figure 4.

| RT strains   | $k_{pol}$ (s$^{-1}$) | $K_d$ (μM) | $k_{pol}/K_d$ (s$^{-1}$ * μM$^{-1}$) |
|--------------|----------------------|------------|--------------------------------------|
| HIV-1 Ug     | 182.2±12.22          | 105.6±16.43| 1.74                                 |
| HIV-1 Cy     | 140.3±36.36          | 102.5±66.65| 1.69                                 |
| SIVagm 9063–2 | 51.1±9.563           | 114.1±33.35| 0.46                                 |
| SIVmne CL8   | 50.7±24.43           | 119.5±41.73| 0.41                                 |
| SIVgor       | 265.3±92.09          | 141.8±57.33| 1.91                                 |
| SIVcpz       | 244.2±163.5          | 190.5±149.3| 1.36                                 |

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