2013

**Slower Uncoating Is Associated with Impaired Replicative Capability of Simian-Tropic HIV-1**

Kenn Kono
Eric Takeda
Hiromi Tsutsui
Ayumu Kuroishi
Amy E. Hulme

*See next page for additional authors*

Follow this and additional works at: [https://bearworks.missouristate.edu/articles-chhs](https://bearworks.missouristate.edu/articles-chhs)

**Recommended Citation**
Kono, Ken, Eri Takeda, Hiromi Tsutsui, Ayumu Kuroishi, Amy E. Hulme, Thomas J. Hope, Emi E. Nakayama, and Tatsuo Shioda. "Slower uncoating is associated with impaired replicative capability of simian-tropic HIV-1." PloS one 8, no. 8 (2013).

This article or document was made available through BearWorks, the institutional repository of Missouri State University. The work contained in it may be protected by copyright and require permission of the copyright holder for reuse or redistribution. For more information, please contact BearWorks@library.missouristate.edu.
Authors
Kenn Kono, Eric Takeda, Hiromi Tsutsui, Ayumu Kuroishi, Amy E. Hulme, Thomas J. Hope, Emi E. Nakayama, and Tatsuo Shioda

This article is available at BearWorks: https://bearworks.missouristate.edu/articles-chhs/184
Slower Uncoating Is Associated with Impaired Replicative Capability of Simian-Tropic HIV-1

Ken Kono†, Eri Takeda†, Hiromi Tsutsui1, Ayumu Kuroishi1, Amy E. Hulme2, Thomas J. Hope2, Emi E. Nakayama1, Tatsuo Shioda1*

1 Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University,Suita, Osaka, Japan, 2 Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America

Abstract

Human immunodeficiency virus type 1 (HIV-1) productively infects only humans and chimpanzees, but not Old World monkeys, such as rhesus and cynomolgus (CM) monkeys. To establish a monkey model of HIV-1/AIDS, several HIV-1 derivatives have been constructed. We previously generated a simian-tropic HIV-1 that replicates efficiently in CM cells. This virus encodes a capsid protein (CA) with SIVmac239-derived loops between α-helices 4 and 5 (L4/5) and between α-helices 6 and 7 (L6/7), along with the entire vif from SIVmac239 (NL-4/5S6/7vifS). These SIVmac239-derived sequences were expected to protect the virus from HIV-1 restriction factors in monkey cells. However, the replicative capability of NL-4/5S6/7vifS in human cells was severely impaired. By long-term cultivation of human CEM-SS cells infected with NL-4/5S6/7vifS, we succeeded in partially rescuing the impaired replicative capability of the virus in human cells. This adapted virus encoded a G-to-E substitution at the 116th position of the CA (NL-4/5SG116E6/7vifS). In the work described here, we explored the mechanism by which the replicative capability of NL-4/5S6/7vifS was impaired in human cells. Quantitative analysis (by real-time PCR) of viral DNA synthesis from infected cells revealed that NL-4/5S6/7vifS had a major defect in nuclear entry. Mutations in CA are known to affect viral core stability and result in deleterious effects in HIV-1 infection; therefore, we measured the kinetics of uncoating of these viruses. The uncoating of NL-4/5S6/7vifS was significantly slower than that of wild type HIV-1 (WT), whereas the uncoating of NL-4/5SG116E6/7vifS was similar to that of WT. Our results suggested that the lower replicative capability of NL-4/5S6/7vifS in human cells was, at least in part, due to the slower uncoating of this virus.

Introduction

HIV-1 infection begins with the interaction and fusion of viral and cellular membranes. After fusion, a conical core, consisting of the two viral genomic RNAs and several viral proteins, is released into the cytoplasm of the target cell. The major component of the core is the viral capsid protein (CA). In the cytoplasm, CA eventually dissociates from the viral complex in a process termed uncoating. During this time, reverse transcription (RT) of the viral genomes occurs. The resultant double-stranded DNA associates with viral and cellular proteins, constituting the pre-integration complex (PIC). The PIC migrates into the nucleus, where the viral DNA integrates into the chromosomal DNA of the target cell.

HIV-1 uncoating was thought to occur immediately following viral fusion, as CA was undetectable in RT complexes isolated from infected cells [1–3]. Thus, CA was thought to have only a minor role in HIV-1 infection. However, subsequent reports indicated that mutations in CA decreased HIV-1 infectivity. Most of these CA mutant viruses displayed decreased levels of RT products [4–10]. On the other hand, the mutant virus Q63/67A, which encodes two Gln-to-Ala substitutions in CA, exhibited a defect in nuclear entry [4,11,12]. Changes in core stability caused by some of these CA mutations seem to affect uncoating kinetics, which may result in impaired RT or nuclear
entry. Thus, timely uncoating was thought to be important for efficient HIV-1 infection. In agreement with this idea, anti-HIV factors TRIM5α and TRIMCyp were shown to bind viral core and accelerate uncoating, thus abrogating productive RT [13–17]. This observation suggests that the core persists as a defined structure for a certain period of time after fusion. Intriguingly, Yamashita et al. showed that CA is important for HIV-1 infection of non-dividing cells [11,18]. In addition, the transportin-SR2 (or TPQN3) -dependence of HIV-1 nuclear entry has been mapped to the HIV-1 CA [19,20]. These results also suggest a functional link between the HIV-1 CA and nuclear entry.

We previously generated simian-tropic HIV-1 that replicates efficiently in cynomolgus monkey (CM) cells [21]. This virus encodes a CA with SIVmac239-derived loops between α-helices 4 and 5 (L4/5) and between α-helices 6 and 7 (L6/7), along with the entire SIVmac239 vif. These SIVmac239-derived sequences allow HIV-1 to escape from restriction factors in monkey cells, including cyclophilin A (CypA), TRIM5α, and ApoB mRNA editing catalytic subunit (APOBEC) 3G. However, the replicative capability of this virus (NL-4/SS6/7SvifS) in human cells was severely impaired. By long-term cultivation of human CEM-SS cells infected with NL-4/SS6/7SvifS, we succeeded in partially rescuing the replicative capability of this virus in human cells [22]. This adapted virus encoded a G-to-E substitution at the 116th position of the CA (NL-4/SS5G116E6/7SvifS). Interestingly, this G116E mutation also occurred after adaptation in rhesus monkey cells [23].

In the work presented here, we examined the mechanism by which the replicative capability of NL-4/SS6/7SvifS was severely impaired in human cells.

Materials and Methods

Cells

The human kidney adherent 293T cells and the human cervical cancer HeLa cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells of the human T cell line CEM-SS were maintained in RPMI 1640 medium supplemented with 10% FBS.

Virus propagation

Virus stocks were prepared by transfection of 293T cells with HIV-1 derivatives described previously [21,22,24] using polyethylenimine (PEI) (molecular weight, 25,000; Polysciences). As shown in Figure 1A, NL-vifS possesses the entire vif of SIVmac239 in the background of HIV-1 NL4-3 (NL-SVR in reference [24]). NL-4/SS6/7SvifS encodes CA with the SIVmac239-derived L4/5 and L6/7 in the background of NL-vifS [21]. NL-4/SS5G116E6/7SvifS encodes CA with an additional G-to-E substitution at the 116th position, in the background of NL-4/SS6/7SvifS [22]. NL-Nhe is a mutant of the NL4-3 proviral clone in which an NheI restriction enzyme cleavage site was blunted and re-ligated, introducing frame-shift mutations in the env gene [25]. For NL-Nhe, GFP-expressing NL4-3-derived HIV-1 proviral clone MSMnG [25], and luciferase-expressing NL4-3-Luc-R-E- (NIH AIDS Research and Reference Reagent Program), the BssHII to Apal fragment (corresponding to the majority of the gag gene) was replaced with the corresponding fragment of NL-4/SS6/7SvifS or NL-4/SS5G116E6/7SvifS. Viral titers were measured with the RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Viral infections

CEM-SS cells (2 x 10^6 per reaction) were infected with HIV-1 derivatives at titers equivalent to 20 ng of p24 per reaction. Culture supernatants were collected periodically, and p24 levels were measured using an ELISA kit.

Real-time PCR analysis

CEM-SS cells (1 x 10^6 per reaction) were infected with DNase I-pretreated HIV-1 derivatives at titers equivalent to 80 ng of p24 per reaction. DNase I pretreatment consisted of incubation with DNase I (20 units/ml in 10 mM MgCl2) for 30 min at room temperature. After 2 hr on ice, infected cells were washed with PBS, resuspended in medium, and returned to 37°C until harvesting at the indicated time point post-infection. Genomic DNA was extracted by using the QiAamp DNA Blood Mini kit (Qiagen). After digestion with 1 unit/μl DpnI for 4 hr at 37°C, 30 ng of DNA was analyzed for US/gag, 2-LTR, and Alu-HIV by real-time PCR using published primers and TaqMan probes [26,27] in an Applied Biosystems 7500 Real-Time PCR System.

In situ uncoating assay

The in situ uncoating assay was conducted as previously described [11,28]. Briefly, the labeled virus was generated by cotransfecting 9 μg NL-Nh CA mutant proviral plasmid, 4 μg S15-dTomato-expressing plasmid, 4 μg vesicular stomatitis virus G protein (VSV-G)-expressing plasmid, and 1 μg GFP-Vpr-expressing plasmid into 10-cm plates of 293T cells using PEI. HeLa cells were spinoculated with the labeled virus for 2 hr at 16°C in the presence or absence of bafilomycin A (BafA) (Sigma). Virus-containing supernatant then was removed and replaced with 37°C medium in the presence or absence of BafA, shifted to 37°C, and fixed with 3.7% formaldehyde (Polysciences) in 0.1M PIPES buffer (pH 6.8) at the indicated time point post-infection. The fixed HeLa cells were permeabilized with blocking solution (0.1 M PIPES [pH 6.8], 10% normal donkey serum [Jackson ImmunoResearch Laboratories], 0.01% Triton X-100, 0.01% NaN3) for 5 min at room temperature, stained with anti-p24 mAb AG3.0 (NIH AIDS Research and Reference Reagent Program) in blocking solution without Triton X-100 for 1 hr at room temperature for primary staining, and secondarily stained with labeled Cy5 donkey anti-mouse antibodies (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. Images were collected and deconvoluted with a Deltavision microscope and software (Applied Precision). Following deconvolution, images were blinded for identity to remove bias during counting. The number of GFP-positive virions was assessed at each time point, and each virion was individually inspected for punctate dTomato fluorescent signal and p24 Cy-5 signal.
Figure 1. Structure of the simian-tropic HIV-1 clones and the replication properties in human cells. (A) White bars denote HIV-1 (NL4-3) and gray bars SIVmac239 sequences. (B) Equal amounts of NL-vifS (black diamonds), NL-4/5SviF (white circles), NL-4/5S6/7SviF (white squares), and NL-4/5SG116E6/7SviF (gray triangles) were inoculated into human CEM-SS cells, and culture supernatants were collected periodically. p24 antigen levels were measured by ELISA. Error bars reflect actual fluctuations of duplicate infections.

doi: 10.1371/journal.pone.0072531.g001
Statistical analysis

Differences in luciferase activities, amounts of late RT products, and uncoating kinetics were evaluated with unpaired t tests.

Results

The replicative capability of NL-4/5S6/7SvifS was impaired in human cells, while that of NL-4/5SG116E6/7SvifS was partially rescued by a single amino acid mutation in CA

Several HIV-1 derivatives have been constructed to establish a monkey model of HIV-1/AIDS (Figure 1A). NL-4/5SvifS could replicate in CM cells [24]. Introduction into NL-4/5SvifS of SIVmac239 L6/7, which is a determinant of HIV type 2 (HIV-2) CM TRIM5α sensitivity [29], improved viral growth in CM cells [21]. However, the replicative capability of the resultant virus (NL-4/5S6/7SvifS) in human cells was greatly attenuated. After long-term cultivation of human CEM-SS cells infected with NL-4/5S6/7SvifS, we succeeded in partially rescuing the impaired replicative capability of the virus [22]. This adapted virus (NL-4/5SG116E6/7SvifS) encoded a G-to-E substitution at the 116th position of NL4-3 CA sequence. Figure 1B shows the replication of NL-vifS that possesses the entire vif of SIVmac in the background of HIV-1, NL-4/5SvifS, NL-4/5S6/7SvifS, and NL-4/5SG116E6/7SvifS in human CEM-SS cells. Consistent with our previous report [22], the replicative capability of NL-4/5S6/7SvifS was severely impaired in human cells (Figure 1B). On the other hand, the replicative capability of NL-4/5SG116E6/7SvifS was improved compared with that of NL-4/5S6/7SvifS, and slightly better than that of NL-4/5SvifS, even though replication did not reach the levels seen with NL-vifS.

We then inoculated CEM-SS cells with VSV-G-pseudotyped luciferase-expressing HIV-1 vector encoding wild type (WT), 4/5S6/7S, or 4/5SG116E6/7S CA. As shown in Figure 2A, infectivity was significantly reduced by the 4/5S6/7S mutation (p<0.0001), and infectivity was restored by addition of the G116E mutation to 4/5S6/7S (p=0.0004). Similar results were obtained when we used a VSV-G-pseudotyped GFP-expressing version of the HIV-1 vector (Figure 2B). These results clearly indicated that the different replicative capability of the viruses was due mainly to effects at the early stage of viral replication.

Levels of NL-4/5S6/7SvifS RT products were decreased at 12 hours after infection

To determine which step of NL-4/5S6/7SvifS early infection stage was impaired, we first measured RT products of replication-competent viruses NL-vifS, NL-4/5S6/7SvifS, and NL-4/5SG116E6/7SvifS in CEM-SS cells. At 12 hr after infection, the amounts of U5/gag (late RT products) and 2-LTR circles (a surrogate for nuclear entry) of NL-4/5S6/7SvifS were 69.4% (p=0.0270) and 38.6% (p=0.0003) of those of NL-vifS, respectively (Figure 3A). These results suggested that NL-4/5S6/7SvifS has defects in both RT and nuclear entry. On the other hand, the amount of Alu-HIV (integrated viral DNA) of NL-4/5S6/7SvifS was 38.2% (p=0.0029) that of NL-vifS, being slightly higher than that of NL-4/5SvifS.
The levels of late RT product of NL-4/5S6/7SvifS were increased at the earlier time points of infection

To determine the mechanisms of the decreased RT production of NL-4/5S6/7SvifS and NL-4/5SG116E6/7SvifS, we analyzed RT at earlier time points after infection. Contrary to our expectation, the amount of late RT products of NL-4/5S6/7SvifS exceeded that of NL-vifS at 4 and 8 hr after infection (Figure 4A). This result indicated that the kinetics of RT product generation was faster for NL-4/5S6/7SvifS than for NL-vifS, despite the fact that the 12-hr levels of late RT products were lower with NL-4/5S6/7SvifS than with NL-vifS (Figures 3A and 4A). The late RT production of NL-4/5S6/7SvifS peaked at 8 hr after infection before decreasing at 12 hr after infection. In contrast, late RT products of NL-vifS gradually increased until 12 hr after infection. The peak amount of late RT products with NL-4/5S6/7SvifS was comparable to that with NL-vifS. Thus we conclude that NL-4/5S6/7SvifS had a defect not in RT but in nuclear entry, and that the synthesized viral cDNA that failed to enter the nucleus was degraded.

In a sharp contrast to NL-4/5S6/7SvifS, NL-4/5SG116E6/7SvifS yielded reduced amounts of late RT products compared to NL-vifS at the respective time points. Similar to NL-vifS, however, the late RT products of NL-4/5S6/7SvifS gradually increased until 12 hr after infection. These findings also were unexpected and indicated that the single G-to-E substitution (which at least partially rescued the impaired replicative capability of NL-4/5S6/7SvifS in human cells) also attenuated late RT at the earlier time points. Therefore, the mechanism underlying decreased late RT is expected to overcome the degradation of unproductive RT products of the initial infection. The difference in the level of late RT of NL-vifS at 24 hr after infection was almost the same as that at 12 hr after infection. This persistence is likely due to the degradation of unproductive RT products from the first round of infection and newly generated RT products from the second-round infection by the progeny viruses, since this experiment used replication-competent viruses. In the cases of NL-4/5S6/7SvifS and NL-4/5SG116E6/7SvifS, the RT products from the second-round infection also would be impaired. Thus, these viruses were not expected to overcome the degradation of unproductive RT products of the initial infection. The difference of late RT, 2-LTR, and Alu-HIV between NL-4/5S6/7SvifS and NL-4/5SG116E6/7SvifS gradually expanded at 48 and 72 hr after infection, presumably due to the effects of multiple rounds of infection. This result was in good agreement with that of the p24 production shown in Figure 1B.

The uncoating kinetics of NL-4/5S6/7S were slower than that of the virus with the NL4-3 CA

Several studies have reported that mutations in CA affected viral core stability and resulted in deleterious effects on RT [4] or nuclear entry [12]. To determine whether the CA mutations in NL-4/5S6/7SvifS or NL-4/5SG116E6/7SvifS affect the core stability, we performed an in situ uncoating assay according to the method described previously [11,28]. For our experiment, a replication-incompetent virus (NL-Nh), which carries a frameshift mutation in the env gene, was used as the wild type virus. The CA of NL-Nh was replaced with that of NL-4/5S6/7SvifS or NL-4/5SG116E6/7SvifS, since the virus had to be pseudotyped with VSV-G. NL-Nh, NL-Nh mutant encoding 4/5S6/7S CA, and NL-Nh mutant encoding 4/5SG116E6/7S CA were labeled with GFP-Vpr, while the viral membrane was labeled with S15-dTomato; the membrane label was expected to disappear after productive fusion of the virion into the cytoplasm. To provide a negative control reaction, bafilomycin A (BafA) was included to block fusion of the virus and cellular membranes. This control was used to confirm that unfused viral particles fail to undergo uncoating. Infection was synchronized and at various times after infection the cells were fixed and stained with an antibody to p24 CA. The total number of complexes that entered the cytoplasm (green spots that lost S15-dTomato) was counted.
Figure 3. Measurement of the reverse transcribed products of simian-tropic HIV-1 in human cells. (A) CEM-SS cells were infected with NL-vifS, NL-4/5S6/7SvifS, or NL-4/5SG116E6/7SvifS, and DNA was extracted at 12 hr after infection and subjected to real-time PCR assays using U5/gag primers for late reverse transcription (RT), 2-LTR primers for nuclear transported viral DNA, and Alu-HIV primers for integrated DNA. Mean relative amounts of U5/gag, 2-LTR, and Alu-HIV products obtained from three independent experiments (the amount in the NL-vifS sample at 12 hr after infection is set at 1) are indicated. Mean numbers of U5/gag, 2-LTR, and Alu-HIV copies per 30 ng of total DNA of NL-vifS-infected cells were 39695, 187, and 2.17, respectively. Error bars reflect the SD of the three independent experiments. (B) CEM-SS cells were infected with NL-vifS, NL-4/5S6/7SvifS, or NL-4/5SG116E6/7SvifS, and DNA was extracted at 12, 24, 48, and 72 hr after infection and subjected to real-time PCR assays as described above. The number of viral DNA (U5/gag and 2-LTR) copies per 30 ng of total DNA and relative amount of Alu-HIV products (the amount in the NL-vifS sample at 12 hr after infection is set at 1) is indicated. Error bars reflect the SD of triplicate values of real-time PCR. Presented data are representative of three independent experiments.

doi: 10.1371/journal.pone.0072531.g003
and the number of complexes that contained CA (coated) was compared to the number of complexes that lost CA staining (uncoated). The data was graphed at each time point as the % of fused CA-positive (coated) cytoplasmic particles (Figure 5). Actual numbers of counted dots are shown in Table S1. At 1 and 2 hr after infection, virus encoding 4/5S6/7S CA had a higher percentage of CA-positive particles than did the virus encoding NL4-3 CA; the difference was significant (p=0.018 and p=0.018 for 1 and 2 hr, respectively) at each time point. In comparison, virus encoding 4/5SG116E6/7S CA had amounts of CA-positive particles that were not significantly different from those seen with the virus encoding NL4-3 CA (p=0.18 and p=0.08 for 1 and 2 hr, respectively). The differences between 4/5S6/7S and 4/5SG116E6/7S viruses at 1 and 2 hr after infection were small but statistically significant (p=0.021 and p=0.037 respectively). These results suggested that the uncoating kinetics of NL-4/5S6/7SvifS was slower than that of NL-vifS, while the uncoating kinetics of NL-4/5SG116E6/7SvifS was similar to that of NL-vifS.

Discussion

We previously constructed a simian-tropic HIV-1 NL-4/5S6/7SvifS that can replicate well in CM cells [21,22].
However, the replicative capability of this virus in human cells was severely impaired. NL-4/5SS6/7SvifS showed nearly normal levels of Gag processing and human TRIM5α sensitivity similar to that of NL4-3 [22]. In the present study, we showed that the amount of RT products of NL-4/5SS6/7SvifS was reduced compared to those of NL-vifS at 12 hr after infection. Surprisingly, however, the amount of the RT products of NL-4/5SS6/7SvifS at 4 and 8 hr after infection was elevated compared to that of WT. Analysis of 2-LTR and integrated HIV DNA suggested that NL-4/5SS6/7SvifS had a defect in nuclear entry but not in integration. By contrast, NL-4/5SSG116E6/7SvifS, which encodes a single G116E substitution in CA, showed partial restoration of replicative capability, even though the amount of the RT products was apparently reduced. These results indicated that the G-to-E substitution at the 116th position of CA impaired RT production but restored the defect of NL-4/5SS6/7SvifS in the subsequent step.

Mutations in CA have been reported to affect viral core stability, resulting in deleterious effects on RT [4,10] or nuclear entry [12]. In the work described here, VSV-G-pseudotyped virus with NL-4/5SS6/7S CA showed slower uncoating kinetics. Thus, it is possible that the hyper-stable core of NL-4/5SS6/7SvifS affects nuclear entry, resulting in lower replicative capability in human cells. It remains unclear why the hyper-stable core would be deleterious for nuclear entry. One possible explanation is that the hyper-stable core masks viral nuclear localization signals of matrix, integrase, or Vpr [30–32], or masks a viral DNA structure, the central DNA flap, which is known to be important for nuclear targeting [33–36]. Another possibility is that host factors that are required for HIV-1 to enter the nucleus, such as importin α/importin β heterodimer [37–39], importin 7 [37,40,41], NUP153 [42], and TNPO3 [20], are unable to access the viral particles at the proper time or place. Although TNPO3 has been shown to bind HIV-1 integrase, Krishnan et al. recently showed that CA is the viral factor that dictates TNPO3 dependency [43]. Thus, it is also possible that mutations in CA of NL-4/5SS6/7SvifS affected the interaction between CA and TNPO3.

It is possible that the core of each HIV-1 CA mutant has its own optimal uncoating kinetics for RT production. For example, a virus with Q63/67A mutations in CA previously has been shown to uncoat more slowly than WT, but could synthesize cDNA at a level comparable to that of WT during single-round infection [4,11,12,44]. In the case of NL-4/5SS6/7SvifS, the slow uncoating may be optimal for its RT, since RT production by this virus was faster than that by WT, even though the slower uncoating might be deleterious for nuclear entry. If so, it is reasonable to assume that the G-to-E substitution at the 116th position of CA that reduced the core stability of NL-4/5SS6/7SvifS resulted in impaired RT. Further studies, including evaluation of physical core stability and more precise analysis of RT products, are necessary to substantiate this hypothesis.

It is known that drug-resistant HIV-1 often acquires mutations that have a negative effect on viral replicative capability [45–50]. In addition, some of the resistant viruses acquire secondary mutations that do not compensate directly for the negative effects caused by the primary mutations, but instead improve another step, resulting in better replicative capability [51,52]. Similarly, the G-to-E substitution at the 116th position of CA may impair RT production but compensate for a defect of NL-4/5SS6/7SvifS in a subsequent step. In the present study, however, we failed to resolve the step at which the G116E substitution of CA compensates for a defect of NL-4/5SS6/7SvifS, since no significant improvement was observed in the levels of 2-LTR circles (nuclear entry) nor HIV-Alu (integration) of NL-4/5SSG116E6/7S at 12 hr after infection (Figure 3). The addition of the G116E mutation to NL-4/5SS6/7SvifS may change the affinity of viral core for certain host factors and subsequently allow viral cDNA to be integrated at chromosome positions that are preferable for subsequent transcription. Alternatively, we might have failed (in Figure 3) to detect very small recoveries of 2-LTR circles and/or HIV-Alu levels, although these recoveries were sufficient to be detected after amplification by viral transcription (in Figure 2). Further studies would be required to elucidate the precise mechanisms by which the G116E mutation at least partially restored the impaired infectivity of NL-4/5SS6/7SvifS.

We note that the uncoating process was completed within 4 hr after infection (as shown in Figure 5), while the levels of the late-RT products continued to increase through 8–12 hr (as shown in Figures 3 and 4). Similar delay in accumulation of late-RT product compared with uncoating kinetics was reported previously [44]. Since a fluorescence-labeled antibody was used to detect assembled CAs of the pre-uncoating cores in the uncoating assay, it is likely that some cores undergoing uncoating became undetectable in this assay but still continued RT production. At present, the precise role of CA in nuclear entry and integration of HIV-1 remains to be elucidated. Further studies would be needed to determine the number of CA molecules required for efficient nuclear entry and integration of HIV-1 pre-integration complex.

It should be noted here that the amounts of p24 from culture supernatants of 293T cells transfected with NL-4/5SS6/7SvifS and NL-4/5SSG116E6/7SvifS plasmid constructs were approximately 75% of those of NL-vifS (data not shown). These results suggested that the viral assembly step also is impaired in NL-4/5SS6/7SvifS, and that the G-to-E substitution in NL-4/5SSG116E6/7SvifS fails to compensate for the mild defect in assembly of NL-4/5SS6/7SvifS. Therefore, defects in both early and late viral replication steps may contribute to the impaired replicative capabilities of NL-4/5SS6/7SvifS and NL-4/5SSG116E6/7SvifS in human cells. It is also possible that NL-4/5SS6/7SvifS has defects in steps other than those assessed in the present study.

In the study presented here, we showed that a simian-tropic HIV-1, NL-4/5SS6/7SvifS, exhibited both slower uncoating and a defect in nuclear entry. On the other hand, the adapted virus NL-4/5SSG116E6/7SvifS showed recovered uncoating kinetics. In addition to the Q63/67A mutant, 4/5SS6/7S is the second example showing the association of slower uncoating with a disadvantage in nuclear entry. However, it is too early to generalize from this conclusion, and further studies on various other CA mutants would be required to elucidate the precise role of uncoating kinetics in HIV-1 replication.
Conclusions

Our results suggest that the lower replicative capability of NL-4/55677S/WSF in human cells is due to the slower uncoating of this virus.

Supporting Information

Table S1. Actual numbers of dots in uncoating assay.

Acknowledgements

We are grateful to Cindy Danielson, Doug Dylla, and Z Kelley for their support with the in situ uncoating assay. We thank Tadashi Miyamoto for helping with experiments, and Setsuko Bandou and Noriko Teramoto for their assistance. pNL4-3-Luc-R-E- plasmid and AG3.0 antibody to p24CA were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. NL-Nh and MSmG plasmid were kind gifts from Dr. Jun-ichi Sakuragi.

Author Contributions

Conceived and designed the experiments: KK EEN TS. Performed the experiments: KK ET HT AK EEN. Analyzed the data: KK ET AEH TJH EEN TS. Contributed reagents/materials/analysis tools: AEH TJH. Wrote the manuscript: KK EEN TS.

References

1. Bukrinsky MI, Sharova N, McDonald TL, Pushkarskaya T, Tarpley WG et al. (1993) Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc Natl Acad Sci U S A 90: 6125-129. doi:10.1073/pnas.90.15.6125. PubMed: 7687060.

2. Fassati A, Goff SP (2001) Characterization of intracellular reverse transcriptase activity following propagation of HIV-1 in T cells. J Virol 75: 3626-3635. doi:10.1128/JVI.75.8.3626-3635.2001. PubMed: 11524352.

3. Miller MD, Farett CM, Bushman FD (1997) Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J Virol 71: 5382-5390. PubMed: 9188609.

4. Forshey BM, von Schwedler U, Sundquist WI, Aiken C (2002) Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. J Virol 76: 5667-5677. doi:10.1128/JVI.76.11.5667-5677.2002. PubMed: 11991995.

5. Fitzon T, Leschonsky B, Bieler K, Paulus C, Schröder J et al. (2000) Proline residues in the HIV-1 N-terminal capsid domain: structure determinants for proper core assembly and subsequent steps of early replication. Virology 268: 294-307. doi: 10.1006/viro.2000.1786.

6. Leschonsky B, Ludwig C, Bieler K, Wagner R (2007) Capsid stability and replication of human immunodeficiency virus type 1 are influenced critically by charge and size of Gag residue 183. J Gen Virol 88: 207-216. doi:10.1099/vir.0.81894-0. PubMed: 17170453.

7. Scholz I, Arvidson B, Husey D, Barklis E (2005) Virus particle core defects caused by mutations in the human immunodeficiency virus capsid N-terminal domain. J Virol 79: 1470-1479. doi: 10.1128/JVI.79.3.1470-1479.2005. PubMed: 15650173.

8. Tang S, Murakami T, Agresta BE, Campbell S, Freed EO et al. (2001) Human immunodeficiency virus type 1 N-terminal capsid mutants that exhibit aberrant core morphology and are blocked in initiation of reverse transcription in infected cells. J Virol 75: 9357-9366. doi: 10.1128/JVI.75.19.9357-9366.2001. PubMed: 11533199.

9. Tang S, Murakami T, Cheng N, Steven AC, Freed EO et al. (2003) Human immunodeficiency virus type 1 N-terminal capsid mutants containing cores with abnormally high levels of capsid protein and virtually no reverse transcriptase. J Virol 77: 12592-12602. doi:10.1128/JVI.77.23.12592-12602.2003. PubMed: 14610162.

10. Wacharapolpin P, Lauerhart D, Auewarakul P (2007) The effect of capsid mutations on HIV-1 uncoating. Virology 358: 48-54. doi:10.1016/j.virol.2006.08.031. PubMed: 16996553.

11. Yamashita M, Enomer M (2004) Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. J Virol 78: 5670-5678. doi: 10.1128/JVI.78.11.5670-5678.2004. PubMed: 15140094.

12. Brass AL, Dykhoom DM, Benita Y, Yan N, Engelman A et al. (2008) Identification of host proteins required for HIV infection through a functional genomic screen. Science 319: 921-926. doi:10.1126/science.1152725. PubMed: 18189820.

13. Christ F, Thys W, De Rijk J, Gijbers R, Albaneese A et al. (2008) Transportin-SR2 imports HIV into the nucleus. Curr Biol 18: 1192-1202. doi:10.1016/cub.2008.07.079. PubMed: 18722123.

14. Sayah DM, Sokolksia E, Berthoux L, Luban J (2004) Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature 430: 569-573. doi:10.1038/nature02777. PubMed: 15243620.

15. Sebastian S, Luban J (2005) TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. Retrovirology 2: 40. doi: 10.1186/1742-4690-2-40. PubMed: 15987037.

16. Forshey BM, Shi J, Aiken C (2005) Structural requirements for recognition of the human immunodeficiency virus type 1 core during host restriction in owl monkey cells. J Virol 79: 869-875. doi:10.1128/JVI.79.2.869-875.2005. PubMed: 15613315.

17. Shi J, Aiken C (2006) Saturation of TRIM5 alpha-mediated restriction of HIV-1 infection depends on the stability of the incoming viral capsid. Virology 350: 493-500. doi:10.1016/j.virol.2006.03.013. PubMed: 16624834.

18. Yamashita M, Enomer M (2004) Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. J Virol 78: 5670-5678. doi: 10.1128/JVI.78.11.5670-5678.2004. PubMed: 15140094.

19. Kuroishi A, Bozek K, Shiada T, Nakayama EE (2010) A single amino acid substitution in the HIV-1 capsid protein affects viral sensitivity to TRIM5 alpha. Retrovirology 7: 58. doi:10.1186/1742-4690-7-58. PubMed: 20609213.

20. Nomaguchi M, Yokoyama M, Kondo K, Nakayama EE, Shioda T et al. (2013) Gag-CAP Q110D mutation elicits TRIM5-independent enhancement of HIV-1 replication in macaque cells. Microbes Infect 15: 56-65. doi:10.1016/j.micinf.2012.10.013. PubMed: 23125344.

21. Kamada K, Igarashi T, Martin MA, Kamrski B, Hatcho K et al. (2006) Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. Proc Natl Acad Sci U S A 103: 16959-16964. doi:10.1073/pnas.0608289103. PubMed: 17065315.

22. Ohishi M, Shioda T, Sakuragi J (2007) Retro-transduction by virus pseudotyped with glycoprotein of vesicular stomatitis virus. Virology 362: 131-138. doi:10.1016/j.virol.2006.12.030. PubMed: 17258261.

23. Julias JG, Ferris AL, Boyer PL, Hughes SH (2001) Replication of HIV-1 in a human immunodeficiency virus type 1 (HIV-1) derivative that has similar simian immunodeficiency virus (SIVmac239) vif and CA-alpha-helices 4 and 5 loop improves replication in cynomolgus monkey cells. Retrovirology 6: 70. doi:10.1186/1742-4690-6-S2-P70. PubMed: 19650891.

24. Kamada K, Kojima A, Sato H, Shioda T, Nomaguchi M et al. (2009) Evidence for a functional link between uncoating of the human immunodeficiency virus type 1 core and nuclear import of the viral preintegration complex. J Virol 83: 3712-3720. doi:10.1128/JVI.80.7.3712-3720.2006. PubMed: 16571788.

25. Stremmel M, Perron M, Lee M, Li Y, Song B et al. (2006) Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. Proc Natl Acad Sci U S A 103: 5514-5519. doi:10.1073/pnas.0509996103. PubMed: 16540544.
28. Campbell EM, Perez O, Melar M, Hope TJ (2007) Labeling HIV-1 virions with two fluorescent proteins allows identification of virions that have productively entered the target cell. Virology 360: 286-293. doi: 10.1016/j.virol.2006.10.025. PubMed: 17123568.

29. Song H, Nakayama EE, Yokoyama M, Sato H, Levy JA et al. (2007) A single amino acid of the human immunodeficiency virus type 2 capsid affects its replication in the presence of cynomolgus monkey and human TRIM5α/β proteins. J Virol 81: 7280-7285. doi: 10.1128/JVI.00406-07. PubMed: 17475650.

30. Bovéjac-Bertoia M, Dvorin JD, Fouquier RA, Jenkins Y, Meyer BE et al. (2001) HIV-1 infection requires a functional integrase NLS. Mol Cell 7: 1025-1035. doi: 10.1016/S1097-2765(01)00240-4. PubMed: 11389849.

31. Bukrinsky MI, Haggerty S, Dempsey MP, Sharova N, Adzhubel A et al. (1993) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature 365: 666-669. doi: 10.1038/365666a0. PubMed: 8105392.

32. Popov S, Rexach M, Zybarth G, Reiling N, Lee MA et al. (1998) Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO J 17: 909-919. PubMed: 9463369.

33. Ao Z, Yao X, Cohen EA (2004) Assessment of the role of the central DNA flap in human immunodeficiency virus type 1 replication by using a single-cycle replication system. J Virol 78: 3170-3177. doi: 10.1128/JVI.78.6.3170-3177.2004. PubMed: 14990738.

34. Dvorin JD, Bell P, Maul GG, Yamashita M, Emerman M et al. (2002) Reassessment of the roles of integrase and the central DNA flap in human immunodeficiency virus type 1 nuclear import. J Virol 76: 12087-12096. doi: 10.1128/JVI.76.23.12087-12096.2002. PubMed: 12414950.

35. Limón A, Nakajima N, Lu R, Ghory HZ, Engelman A (2002) Wild-type levels of nuclear localization and human immunodeficiency virus type 1 replication in the absence of viral DNA flap. J Virol 76: 12078-12086. doi: 10.1128/JVI.76.23.12078-12086.2002. PubMed: 12414949.

36. Zennou V, Petit C, Guetard D, Nerblass U, Montagnier L et al. (2003) Human immunodeficiency virus type 1 genomic DNA can be imported by a central DNA flap. Cell 110: 173-185. doi: 10.1016/S0092-8674(00)08028-4. PubMed: 10786833.

37. Fassati A, Görlich D, Harrison I, Zaytseva L, Mingot JM (2003) Nuclear import of HIV-1 intracellular reverse transcription complexes is mediated by importin-7. EMBO J 22: 3675-3685. doi: 10.1093/emboj/cdg357. PubMed: 12853482.

38. Gallay P, Hope T, Chin D, Trono D (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc Natl Acad Sci U S A 94: 9825-9830. doi: 10.1073/pnas.94.18.9825. PubMed: 9275210.

39. Heaps AC, Jans DA (2006) HIV-1 integrase is capable of targeting DNA to the nucleus via an importin alpha/beta-dependent mechanism. Biochem J 398: 475-484. doi: 10.1042/BJ20060466. PubMed: 16716146.

40. Ao Z, Huang G, Yao H, Xu Z, Labine M et al. (2007) Interaction of human immunodeficiency virus type 1 integrase with cellular nuclear import receptor importin 7 and its impact on viral replication. J Biol Chem 282: 13456-13467. doi: 10.1074/jbc.M610546200. PubMed: 17360709.

41. Zaitseva L, Cherepanov P, Leyens L, Wilson SJ, Rasaiyaah J et al. (2009) HIV-1 exploits importin 7 to maximize nuclear import of its DNA genome. Retrovirology 6: 11. doi: 10.1186/1742-4690-6-11. PubMed: 19392229.

42. Woodward CL, Prakobwanakit S, Mossessian C, Chow SA (2009) Integrase interacts with nucleoporin NUP153 to mediate the nuclear import of human immunodeficiency virus type 1. J Virol 83: 6522-6533. doi: 10.1128/JVI.02061-08. PubMed: 19369352.

43. Krishnan L, Matreyek KA, Oztop I, Lee K, Tipper CH et al. (2010) The requirement for cellular transportin 3 (TNPO3 or TRN-SR2) during infection maps to human immunodeficiency virus type 1 capsid and not integrase. J Virol 84: 397-406. doi: 10.1128/JVI.01899-09. PubMed: 19846519.

44. Hulme AE, Perez O, Hope TJ (2011) Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. Proc Natl Acad Sci U S A 108: 9975-9980. doi: 10.1073/pnas.1014522108. PubMed: 21628558.

45. Condra JH, Schlief WA, Blahy OM, Gabryelksi LJ, Graham DJ et al. (1995) In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature 374: 569-571. doi: 10.1038/374569a0. PubMed: 7700387.

46. Condra JH, Holder DJ, Schlief WA, Blahy OM, Danovich RM et al. (1996) Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. J Virol 70: 8270-8276. PubMed: 8970546.

47. Croteau G, Doyon L, Thibeault D, McKercher G, Pilote L et al. (1997) Impaired fitness of human immunodeficiency virus type 1 variants with high-level resistance to protease inhibitors. J Virol 71: 1089-1096. PubMed: 8673921.

48. Martinez-Picado J, Savara AV, Sutton L, D’Aquila RT (1999) Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. J Virol 73: 3744-3752. PubMed: 9927435.

49. Molla A, Korneyeva M, Gao Q, Vasavanonda S, Schipper PJ et al. (1999) Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat Med 2: 760-766. doi: 10.1038/nm0796-760. PubMed: 8673921.

50. Zennou V, Mammano F, Paulous S, Mathez D, Clavel F (1998) Loss of viral fitness associated with multiple Gag and Gag-Pol processing defects in human immunodeficiency virus type 1 variants selected for resistance to protease inhibitors in vivo. J Virol 72: 3300-3306. PubMed: 9526567.

51. Myint L, Matsuda M, Matsuda Z, Yokomaku Y, Chiba T et al. (2004) Gag non-cleavage site mutations contribute to full recovery of viral fitness in protease inhibitor-resistant human immunodeficiency virus type 1. Antimicrob Agents Chemother 48: 444-452. doi: 10.1128/AAC.48.2.444-452.2004. PubMed: 14742193.

52. Nijhuis M, Schuurman R, de Jong D, Erickson J, Guschina I et al. (1999) Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. AIDS 13: 2349-2359. doi: 10.1097/00002030-199912030-00006. PubMed: 10597776.