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HIV-1 is more dependent on the K182 capsid residue than HIV-2 for interactions with CPSF6

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ABSTRACT

The HIV-1 capsid (CA) utilizes CPSF6 for nuclear entry and integration site targeting. Previous studies demonstrated that the HIV-1 CA C-terminal domain (CTD) contains a highly conserved K182 residue involved in interaction with CPSF6. In contrast, certain HIV-2 strains possess a substitution at this residue (K182R). To assess whether CA-CPSF6 interaction via the CA CTD is conserved among primate lentiviruses, we examined resistance of several HIV-1- and HIV-2-lineage viruses to a truncated form of CPSF6, CPSF6-358. The results demonstrated that viruses belonging to the HIV-2-lineage maintain interaction with CPSF6 regardless of the presence of the K182R substitution, in contrast to the case with HIV-1-lineage viruses. Our structure-guided mutagenesis indicated that the differential requirement for CA-CPSF6 interaction is regulated in part by residues near the 182nd amino acid of CA. These results demonstrate a previously unrecognized distinction between HIV-1 and HIV-2, which may reflect differences in their evolutionary histories.

1. Introduction

It is estimated that approximately one million people worldwide are living with HIV-2 infections. HIV-2 infection is found predominantly in West African nations, such as Guinea-Bissau, Gambia, Senegal, Cape Verde, Cote d'Ivoire, Mali, Sierra Leone, and Nigeria. While HIV-1 arose as a consequence of cross-species transmission of simian immunodeficiency virus (SIV) from chimpanzee (SIVcpz) (Gao et al., 1999), HIV-2 was derived from transmission of SIV from sooty mangabey (SIVsmm) (Chen et al., 1996; Hirsch et al., 1989; Santiago et al., 2005). In general, HIV-2 infection induces lower pathogenicity in infected individuals than does HIV-1 (MacNeil et al., 2007; Marlink et al., 1994; Popper et al., 1999).

HIV-2 is genetically classified into 8 groups, A to H (Gao et al., 1992, 1994). Furthermore, two recombinant forms of genetically divergent HIV-2 strains, 7312A and 510-03, have been identified in West Africa (Robertson et al., 1995; Sharp et al., 1995). In the late 2000s, Ibe et al. identified 3 infection cases of recombinant HIV-2 in Nagoya, Aichi, Japan, which was the same type of the AB-recombinant virus 7312A. These isolates were verified as the first circulating recombinant form of HIV-2 groups A and B (CRF01_AB) (Ibe et al., 2010). Importantly, all three patients suffered from AIDS-related symptoms with low $CD4^+$ cell counts and high viral loads. These findings suggested that the HIV-2 strains in these patients have the potential to induce rapid disease progression in infected individuals. In addition, our phylogenetic analysis has demonstrated that the HIV-2 CRF01_AB capsid (CA) proteins were genetically distant from the CA proteins of HIV-2 group A, HIV-2 group B, and SIV (Miyamoto et al., 2012). Furthermore, we have shown that the CRF01_AB viruses show higher levels of resistance to human TRIM5α than does an HIV-2 group-A virus (GH123 strain) (Miyamoto et al., 2012). However, it has been unclear yet whether any other differential interplay between the characteristic CA sequence of HIV-2 CRF01_AB and other host factors that might be associated with their different pathogenicity.

Cleavage and polyadenylation specificity factor subunit 6 (CPSF6) is a host factor associated with HIV-1 replication. Several lines of evidence have revealed that HIV-1 CA utilizes CPSF6 for nuclear entry and integration site targeting by the pre-integration complex (Lee et al., 2010; Saito et al., 2016b; Sowd et al., 2016; Zhou et al., 2015). It is well established that intact CPSF6 binding is conserved among primate lentiviruses (Price et al., 2012). Furthermore, a recent study has shown that intact CPSF6 binding is dispensable for *in vitro* viral replication, but is selected *in vivo* for HIV-1 replication (Saito et al., 2016b). These findings suggested that primate lentiviruses have selected for intact

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CPSF6 binding during viral evolution. Previous structural and genetic analyses have demonstrated that CPSF6 binds to CA via two distinct interfaces corresponding to the N-terminal domain (NTD) and the C-terminal domain (CTD) of CA. Substitutions in the CA NTD (e.g., N57A and N74D) abolish CPSF6 binding by the CAs of a wide range of primate lentiviruses, suggesting that the NTD contains one or more CPSF6 binding interface(s) (Lee et al., 2010; Price et al., 2012). In the case of the CA CTD, a lysine (K) at the 182nd amino acid residue is highly conserved; substitution of the lysine with arginine (K182R) decreased CPSF6 binding, highlighting the importance of this CTD residue for optimal CPSF6 binding by the CAs of HIV-1-lineage viruses (Bhattacharya et al., 2014; Price et al., 2014; Saito et al., 2016a). However, it is unknown whether CA-CPSF6 interaction via the CA CTD is conserved among HIV-2 viruses and other primate lentiviruses, as seen with the NTD.

To our surprise, we noticed that CRF01_AB HIV-2 strains (described above) uniquely carry a K182R substitution (HXB2 numbering) in CA (the NMC842 in Fig. 1A). The characteristic presence of K182R in CRF01_AB viruses prompted us to examine how this substitution affects CPSF6 binding. Here, we show the impact of the K182R substitution on CA-CPSF6 interaction by the CAs of HIV-2-lineage viruses by testing the resistance of CA mutants to the antiviral effect of a truncated form of CPSF6, CPSF6-358. Our analysis reveals that the NMC842 strain exhibits CPSF6-358 resistance similar to those seen with HIV-1, regardless

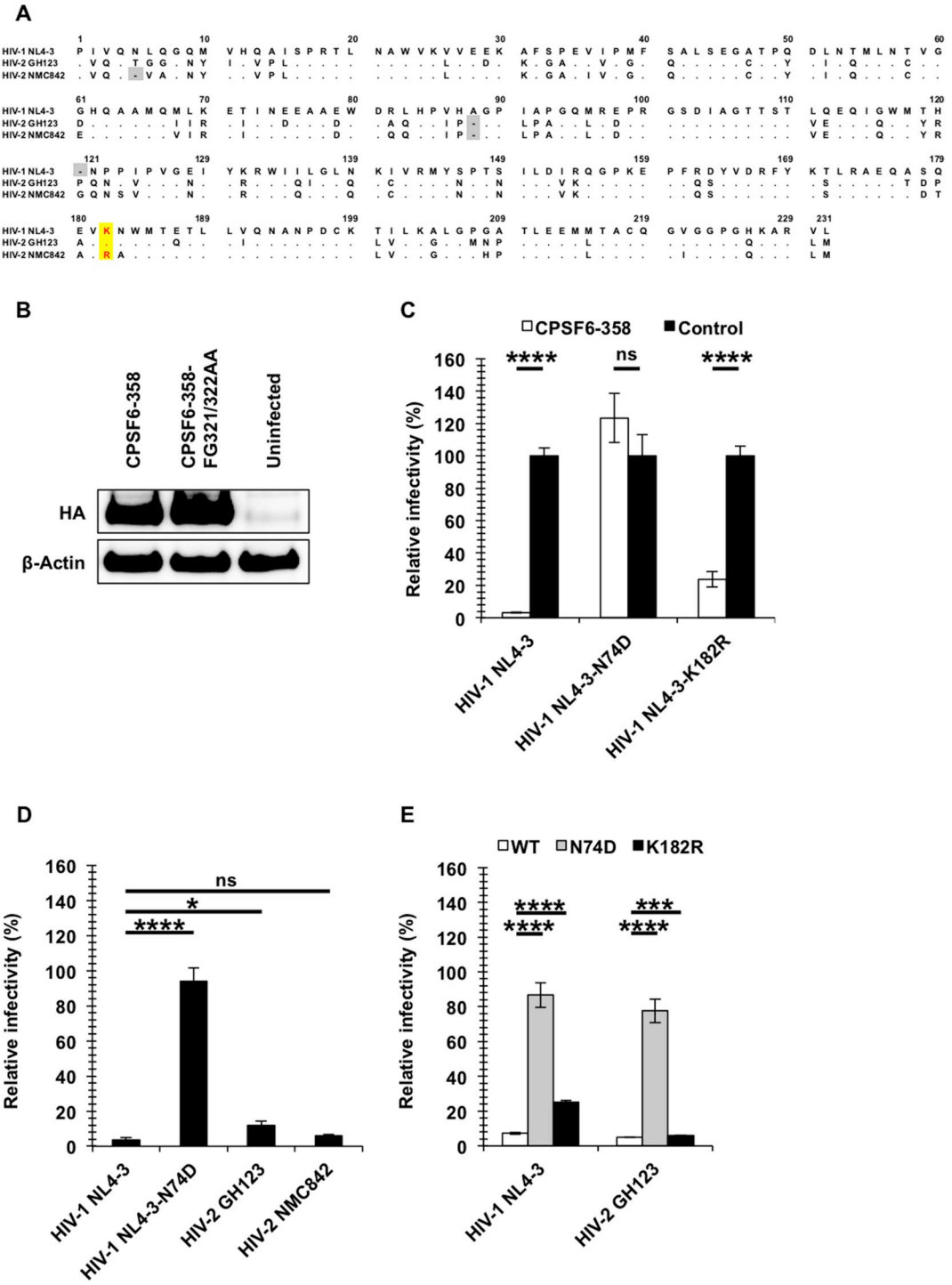

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Fig. 1. Identification of the naturally occurring K182R substitution in an HIV-2 isolate and its impact on resistance to CPSF6-358.
(A) Amino acid alignment of the HIV-1 NL4-3 CA, HIV-2 GH123 CA, and HIV-2 NMC842 CA. The 182nd position of the NL4-3 CA and the corresponding positions of the HIV-2 CAs are highlighted with red letters and yellow background. At the top of the alignment, the residue numbers are shown according to the HIV-1 HXB2 numbering scheme.
(B) Expression level of HA-tagged CPSF6-358 or CPSF6-358-FG321/322AA in recombinant Sendai virus-infected MT4 cells was evaluated using the rat anti-HA monoclonal antibody.
(C) MT4 cells expressing CPSF6-358 or CPSF6-358-FG321/322AA (Control) were superinfected with VSV-G-pseudotyped HIV-1 viruses harboring luciferase-encoding reporter genes. The degree of CPSF6-358 resistance was calculated by dividing the relative luciferase units (RLUs) of each virus in the presence of CPSF6-358 by that in the presence of CPSF6-358-FG321/322AA (Control). The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of at least three independent experiments. Differences were examined by a two-tailed, unpaired Student *t*-test. $^{****}P < 0.0001$, ns (not significant).
(D) MT4 cells expressing CPSF6-358 or CPSF6-358-FG321/322AA (Control) were superinfected with VSV-G-pseudotyped HIV-1 or HIV-2 viruses harboring GFP-encoding reporter genes. The degree of CPSF6-358 resistance was calculated by dividing the GFP positivity of each virus in the presence of CPSF6-358 by that in the presence of CPSF6-358-FG321/322AA (Control). The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of at least three independent experiments. Differences were examined by a two-tailed, unpaired Student *t*-test. $^{****}P < 0.0001$, $^{*}P < 0.05$, ns (not significant).
(E) MT4 cells expressing CPSF6-358 or CPSF6-358-FG321/322AA (Control) were superinfected with VSV-G-pseudotyped HIV-1 or HIV-2 harboring luciferase-encoding reporter genes. The degree of CPSF6-358 resistance was calculated by dividing the RLU of each virus in the presence of CPSF6-358 by that in the presence of CPSF6-358-FG321/322AA (Control). The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of three independent experiments. Differences were examined by a two-tailed, unpaired Student *t*-test. $^{****}P < 0.0001$, $^{***}P < 0.001$.

of the presence of the K182R substitution. Introduction of the K182R substitution within the CAs of viruses belonging to HIV-2-lineage, including HIV-2 GH123 strain and SIVmac239, had only a minimal effect on CPSF6-358 resistance, while the introduction of K182R within the CAs of HIV-1-lineage viruses greatly increased the resistance. The differential sequence requirement of HIV-1 and HIV-2 CA for interaction with CPSF6 is previously unrecognized evidence, which might reflect differences of these two viruses in their evolutionary histories.

2. Materials and methods

2.1. Plasmid DNAs

env-deleted molecular clones encoding the CA of the NL4-3 strain of HIV-1, and carrying a GFP-encoding reporter gene (pMSMnG (Ohishi et al., 2007)) or a luciferase-encoding reporter gene (pBru3oriΔEnv-luc2 (Yamashita and Emerman, 2004, 2005)) in place of the *nef* gene, were used in the present study. The CA-encoding sequences of six transmitted founder (TF) viruses (TF2474 (pWITO.c/2474), TF2625 (pCH040.c/2625), TF2960 (pCH058.c/2960), TF2627 (pCH077.t/2627), TF2633 (pCH106.c/2633), and TF2821 (pSUMA.c/2821)) (Keele et al., 2008; Salazar-Gonzalez et al., 2009), the SIVcpzMT145 strain (GenBank Accession no. DQ373066) (Keele et al., 2006), and the SIVgorCP2139 strain (GenBank Accession no. FJ424865) (Takehisa et al., 2009) were transferred to the pBru3oriΔEnv-luc2 plasmid by using the In-Fusion HD Cloning Kit (TaKaRa). Equivalent molecular clones based on the HIV-2 GH123 strain and carrying a GFP-encoding reporter gene (pROD-env(–)-GFP-GH123 (Miyamoto et al., 2012)) or a luciferase-encoding reporter gene (pGL-ANΔEnv-Luc (Ueno et al., 2003)) also were used. The pROD-env(–)-GFP-NMC842 plasmid encoding the CA sequence of the HIV-2 NMC842 strain (GenBank Accession no. AB499688) has been described previously (Miyamoto et al., 2012). The pV1-derived plasmid harboring the full-length SIVmac239 provirus (Zhang et al., 2009) was used as a template to generate the pV1-siv-ΔEnv-Luc plasmid carrying a luciferase-encoding reporter gene. Various CA mutations were introduced into these clones using standard cloning procedures. A plasmid DNA encoding the vesicular stomatitis virus G (VSV-G) glycoprotein (pMD2G) has been described previously (Dull et al., 1998). For preparation of recombinant Sendai virus (SeV) expressing CPSF6-358 (Lee et al., 2010), the cDNA of CPSF6-358 was PCR amplified from the pMRX-mCPSF6-358 plasmid (kind gift by Dr. Takeuchi) (Hori et al., 2013). Further, the CA binding-deficient mutant of CPSF6-358, CPSF6-358-FG321/322AA (Fricke et al., 2013; Lee et al., 2012), was constructed using standard mutagenesis procedures. The HA tag sequence was introduced into C-terminus of both molecules with PCR. The entire coding sequences of these CPSF6-358-HA molecules were transferred to the pSeV18 + b (+) plasmid (Nakayama et al., 2005).

2.2. Cell culture

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) and 1x penicillin-streptomycin (P/S) (Nacalai Tesque). MT4 cells were cultured in RPMI (Nacalai Tesque) supplemented with 10% FBS and 1x P/S.

2.3. Viruses

All viruses were generated by transfecting HEK293T cells using polyethylenimine (PEI, PolySciences). SeVs expressing CPSF6-358 or CPSF6-358-FG321/322AA were recovered using a previously described method (Nakayama et al., 2005). SeVs passaged a second time in embryonated chicken eggs were used as stocks for all experiments.

2.4. Infection

MT4 cells were infected (at a multiplicity of infection of 10) with SeVs expressing CPSF6-358 or CPSF6-358-FG321/322AA. After 6-h incubation, MT4 cells (at 5×10^5 cells per mL) were challenged with VSV-G-pseudotyped HIV-1, HIV-2 or SIV viruses encoding a luciferase reporter protein. The relative luciferase units (RLUs) were determined at 2 days after infection using the Bright-Glo Luciferase Assay reagent (Promega) on a luminometer. The degree of resistance to CPSF6-358 was calculated by dividing the RLU of each virus in the presence of CPSF6-358 by that in the presence of CPSF6-358-FG321/322AA.

2.5. Comparison of relative infectivity of CA mutants

To compare the relative infectivity of CA mutants, MT4 cells were infected with HIV-1 or HIV-2 viruses encoding a luciferase reporter protein. Virus inputs were normalized with reverse transcriptase (RT) activity of each virus stock by a SYBR Green PCR-enhanced RT assay (SG-PERT) previously described (Vermeire et al., 2012). Cells were lysed at 2 days after infection and the RLUs were measured. Relative infectivity (% compared with the wild-type (WT) virus) of the CA mutants was calculated by dividing the RLU of CA mutants by that of the respective WT virus.

2.6. Western blot

Pelleted cells were resuspended in 1x NuPAGE LDS Sample Buffer (Thermo) containing 2% β-mercaptoethanol. Expression of hemagglutinin (HA)-tagged host factors in SeV-infected MT4 cells was confirmed by western blotting using a rat anti-HA monoclonal antibody (Roche Diagnostics, clone 3F10, cat# 11867423001) followed by horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody (American Qualex, cat# A103PT). Chemiluminescence was detected using the Chemi-Lumi One Ultra reagent (Nacalai Tesque) according to the manufacturer's instructions.

2.7. Structure modeling of CA mutants

Hexameric CA structural models of HIV-1 and HIV-2 CA variants bound to partial CPSF6 protein ($CPSF6_{313–325}$ with sequence PVLFPGQPFGQPP) were constructed using the Modeller program (Eswar et al., 2006) based on a crystal structure of hexameric HIV-1 CA with a bound CPSF6 peptide (PDB 4U0B) (Price et al., 2014), since HIV-2 CA structure with a bound CPSF6 peptide has never been determined yet with X-ray crystallography and NMR. The figures of the model structures were generated with the PyMOL program (https://pymol.org/). The constructed model structures can be provided upon request.

2.8. Statistical analysis

Differences in infectivity between different conditions (e.g. between cells expressing host factors and cells expressing control molecule, between WT and CA mutants) were examined by a two-tailed, unpaired Student *t*-test. *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Identification of the K182R substitution in a clinical HIV-2 isolate and its impact on resistance to CPSF6-358

Recent advances in the understanding of the CPSF6 binding interface allowed us to re-evaluate the sequences of the CA proteins of primate lentiviruses to identify substitutions that might affect CPSF6 binding. Importantly, we identified the naturally-occurring K182R substitution in the CA (HXB2 numbering) of a HIV-2 CRF01_AB virus (strain NMC842; Fig. 1A). Of note, the CRF01_AB viruses were isolated from patients in the advanced stages of HIV disease (Ibe et al., 2010). Concerning the impact of the K182R alteration, previous reports have indicated that this substitution decreases the CPSF6 binding of HIV-1 (Price et al., 2014; Saito et al., 2016a). Based on these findings, we considered three possibilities. First, the CRF01_AB viruses may be able to grow well despite the decreased CPSF6 binding resulting from the K182R substitution. Second, the CRF01_AB viruses carrying the K182R substitution may still bind well to CPSF6, due to compensation by some second-site changes unique in the CRF01_AB viruses. Third, the K182R substitution might not affect CPSF6 binding by HIV-2 viruses. To test the first possibility, we examined the sensitivity or resistance of a CRF01_AB virus (the NMC842 isolate) to viral suppression by a truncated form of CPSF6, CPSF6-358 (Lee et al., 2010; Ning et al., 2018). Previous studies have shown that the sensitivity of CA mutants to CPSF6-358 generally correlates with *in vitro* protein binding between CA and the CPSF6 peptide, as assessed by isothermal titration calorimetry (ITC) analysis (Bhattacharya et al., 2014; Ning et al., 2018; Price et al., 2012, 2014; Saito et al., 2016b). We used Sendai virus (SeV) vectors to express CPSF6-358 or a control consisting of a CA-binding-deficient mutant (CPSF6-358-FG321/322AA) that harbors substitutions (F321A and G322A) in the region critical for interaction with CA (Fig. 1B). We used combination of SeV and MT4 cells in this study to achieve an efficient and reproducible expression of CPSF6 variants without apparent cytopathic effect. Consistent with previous reports (Ambrose et al., 2012; De Iaco et al., 2013; Fricke et al., 2013; Henning et al., 2014; Hori et al., 2013; Lee et al., 2010, 2012; Price et al., 2012; Saito et al., 2016a, 2016b; Shi et al., 2015), infection by the HIV-1 NL4-3 (WT) virus was efficiently blocked in CPSF6-358-expressing cells (Fig. 1C). In contrast, the N74D substitution conferred complete CPSF6-358 resistance to the HIV-1 NL4-3 (WT) virus, validating the specificity of this assay. Notably, we observed that the NMC842 virus possessed CPSF6-358 resistance comparable to that of the HIV-1 NL4-3 (WT) virus, despite the presence of the K182R substitution (Fig. 1D). The HIV-2 GH123 (WT) virus also showed CPSF6-358 resistance similar to that of the HIV-1 NL4-3 (WT) virus. These results suggested that the NMC842 virus grows well while retaining intact CA-CPSF6 interaction, indicating that the first possibility is not valid. We next tested the second and third possibilities. To this end, we generated an HIV-2 GH123-K182R mutant. We predicted that if the second possibility was true, the HIV-2 GH123-K182R mutant should exhibit increased resistance to CPSF6-358. In contrast, if the third possibility was true, comparable CPSF6-358 resistances should be observed in HIV-2 GH123 (WT) virus and its K182R mutant. Consistent with previous reports (Price et al., 2014; Saito et al., 2016a), the HIV-1 NL4-3-K182R virus showed increased resistance to CPSF6-358 compared to the HIV-1 NL4-3 (WT) virus (7.3% vs. 25.1% respectively) (Fig. 1E). Notably, we found that the HIV-2 GH123-K182R mutant showed CPSF6-358 resistance comparable to that of the HIV-2 GH123 (WT) virus (5.9% vs. 5.0% respectively), suggesting that the K182R substitution had only a limited effect on the HIV-2 GH123 virus. Therefore, the third possibility mentioned above seemed to be most plausible.

3.2. Limited impact of the K182R substitution on CPSF6-358 resistance of viruses belonging to the HIV-2-lineage

While the HIV-1 NL4-3-K182R mutant showed increased resistance to CPSF6-358 compared to the HIV-1 NL4-3 (WT) virus, the HIV-2 GH123-K182R mutant showed CPSF6-358 resistance comparable to that of the HIV-2 GH123 (WT) virus (Fig. 1E). To confirm the third possibility and to test if this difference is strain-specific or lineage-specific, we generated expanded panels of both HIV-1- and HIV-2-lineage viruses (Fig. S1). We examined the impact of the K182R substitution on CPSF6-358 resistance of the HIV-1-lineage viruses, including six transmitted founder (T/F) HIV-1 viruses, as well as the SIVcpzMT145 and SIVgorCP2139 viruses. Along with the HIV-2-lineage viruses, we included SIVmac239 virus in the analysis. Interestingly, we noticed that WT viruses of the HIV-1 lineage showed variable resistances to CPSF6-358 (Fig. 2A). Specifically, HIV-1 TF2960 and HIV-1 TF2627 exhibited higher infectivity in CPSF6-358-expressing cells, suggesting higher resistance of these viruses to CPSF6-358. The N74D substitution conferred complete CPSF6-358 resistance to all viruses (Fig. 2A and B), suggesting that the CA NTD plays a critical role in the CA-CPSF6 interaction of viruses belonging to both HIV-1- and HIV-2-lineages. Strikingly, we observed that the K182R substitution (HXB2 numbering) increased CPSF6-358 resistance of all the HIV-1-lineage viruses (Fig. 2A). In contrast, the K182R (HXB2 numbering) substitution did not change the CPSF6-358 resistance of SIVmac239 virus (3.3% vs. 3.6% for the K182R and WT viruses, respectively) (Fig. 2B). Although more HIV-2-lineage viruses should be tested in the future study, these results suggested that the distinct impact of the K182R substitution on CPSF6-358 resistance seemed lineage-specific rather than strain-specific.

Bhattacharya et al. (2014) showed that the K182A substitution also increased CPSF6-358 resistance of the HIV-1 NL4-3 virus. Therefore, we examined the influence of a K182A mutation on the CPSF6-358 resistance of the HIV-2 GH123 and SIVmac239 viruses. The results showed that the K182A substitution increased the CPSF6-358 resistance of viruses belonging to both the HIV-1- and the HIV-2-lineages (Fig. 2C). Thus, it appeared that the CPSF6-358 resistance of the HIV-1-

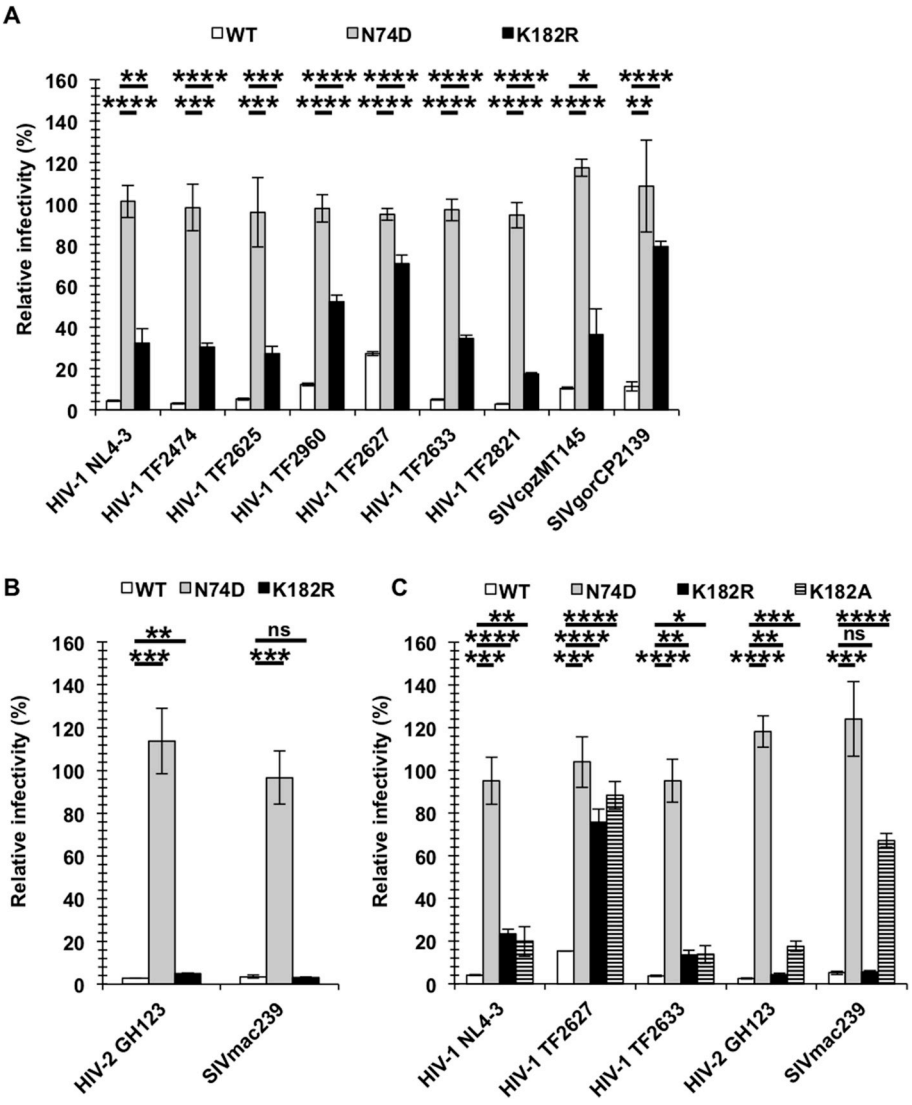

Fig. 2. Limited impact of the K182R substitution on CPSF6-358 resistance of viruses belonging to the HIV-2-lineage.
(A, B and C) MT4 cells expressing CPSF6-358 or CPSF6-358-FG321/322AA (Control) were super-infected with VSV-G-pseudotyped lentiviruses harboring luciferase-encoding reporter genes. The degree of CPSF6-358 resistance was calculated by dividing the RLU of each virus in the presence of CPSF6-358 by that in the presence of CPSF6-358-FG321/322AA (Control). The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of at least three independent experiments. Differences were examined by a two-tailed, unpaired Student *t*-test. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ns (not significant).

lineage viruses is increased by both K182A and K182R substitutions, whereas those of the viruses belonging to the HIV-2-lineage were greatly enhanced by the K182A substitution but only minimally affected by the K182R substitution. We infer that although the side chain of the 182nd amino acid residue in CA contributes to CA-CPSF6 interaction, amino acids permitting intact interaction differ between viruses belonging to HIV-1- and HIV-2-lineages.

Apart from its effects on CA-CPSF6 interaction, one might imagine that the K182R substitution specifically decreases infectivity of HIV-1-lineage viruses; this observation would explain why the lysine residue at the 182nd amino acid is conserved in HIV-1-lineage viruses. To test whether the K182R substitution differentially affects the infectivity of viruses belonging to HIV-1- or HIV-2-lineages, we tested the relative infectivity of WT, N74D, K182R, and K182A mutants of HIV-1 NL4-3 and HIV-2 GH123 viruses in MT4 cells. The relative infectivities of the K182R mutants of HIV-1 NL4-3 and HIV-2 GH123 viruses were comparable (approximately 20% of the WT virus) (Fig. 3). This result suggested that the characteristic presence of the K182R substitution in the HIV-2 strains is unlikely to be due to a differential effect of the K182R substitution on viral infectivity. Notably, the K182A mutation was detrimental for infectivity of both viruses, given that the K182A mutants had low relative infectivity (0.5–1.1% of the WT virus).

Taken together, these results demonstrated that the K182R substitution differentially affects the CPSF6-358 resistance of viruses belonging to HIV-1- or HIV-2-lineages.

3.3. Identification of possible domains determining differential effect of the K182R substitution

As shown above, the K182R substitution differentially affects the CPSF6-358 resistance of viruses belonging to HIV-1- or HIV-2-lineage viruses. However, it remains unclear how the CA of the HIV-2 interacts with CPSF6, given the lack of structural information regarding the complex formed by HIV-2 CA and the CPSF6 peptide. To address this issue, we performed molecular modeling of a complex between HIV-2 CA and the CPSF6 peptide, based on the crystal structure of the HIV-1 CA hexamer bound to the CPSF6 peptide. As reported previously (Price et al., 2014), this crystal structure shows that K182 in the HIV-1 CA has a direct interaction with a backbone atom in the CPSF6 peptide (Fig. 4A, **left panel**). Hence, in the case of the HIV-1 CA, the observed decrease in CPSF6 binding due to introduction of the K182R substitution might result from weakening of the interaction. A previous crystal structure study indicates that HIV-1 CA can also form an alternative conformation with extended L8-9 linker yielding a change in the configuration around amino acid residue 182 at the peptide-free state (Price et al., 2014). Therefore, HIV-1 CA K182R mutant might favor the alternative conformation at the CPSF6 peptide-binding state, resulting in loss of the direct interaction with backbone atom in the CPSF6

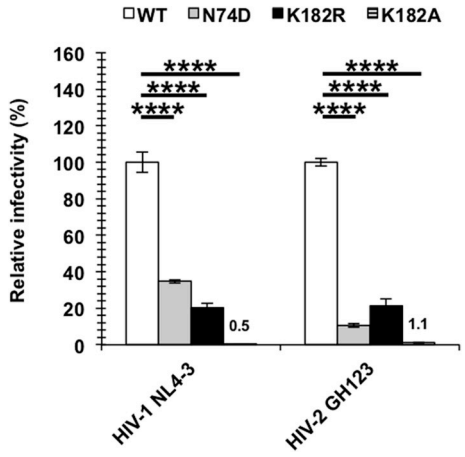

Fig. 3. Relative infectivity of CA mutants in MT4 cells.
MT4 cells were infected with HIV-1 or HIV-2 viruses harboring luciferase-encoding reporter genes. Virus inputs were normalized with reverse transcriptase (RT) activity of each virus stock by a SYBR Green PCR-enhanced RT assay (SG-PERT). The RLU was determined 2 days after infection. The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of three independent experiments. The relative values of the K182A mutants were shown in the graph. Differences were examined by a two-tailed, unpaired Student *t*-test. ****$P < 0.0001$.

peptide (Fig. 4A, right panel). In contrast, a structural model of the HIV-2 CA hexamer bound to CPSF6 indicated that K182 as well as R182 in HIV-2 CA might still be able to interact directly with CPSF6 (Fig. 4B). When we compared the CA sequences from HIV-1 and HIV-2 (Fig. 1A and Fig. S2), we noticed that the sequence identity of the HIV-1 and HIV-2 CAs is relatively low (~65%), with multiple different amino acid residues present in the region of the L8-9 linker. Hence, we hypothesized that residues in the area of the loop may regulate the distinct impact of the K182R substitution on CPSF6-358 resistance. To confirm this hypothesis, we examined the CPSF6-358 resistance of several HIV-1 NL4-3 mutants carrying substitutions at the L8-9 linker and/or residues that underpin the conformation of the linker (L8/9, L7/8+α8, and the combination thereof) (Fig. 4C and D**, orange and magenta**). We also examined the CPSF6-358 resistance of HIV-1 NL4-3 mutants carrying the other substitutions around CPSF6-binding sites (α4, α8+α11, L10/11, and the combination thereof) (Fig. 4C and D**, yellow, green, and cyan**).

Prior to testing CPSF6-358 resistance, we compared the relative infectivity of these mutants since modification of CA may greatly affect viral infectivity. In fact, the mutant virus harboring α4 of the HIV-2 GH123 strain was found to be non-infectious (Fig. S3). We observed that other CA mutants had variable infectivity as compared with HIV-1 NL4-3 (WT) virus (Fig. 4E). Interestingly, the K182R substitution differentially affected viral infectivity based on the backbone virus. Specifically, while the K182R substitution significantly decreased infectivity of WT, L7/8+α8 and L10/11 viruses, it did not affect or even enhanced the infectivity of L8/9, α8+α11 and L8/9 + L7/8+α8 viruses.

Importantly, the variation in baseline infectivity appears not to affect CPSF6-358 resistance. When we examined CPSF6-358 resistance of serially diluted HIV-1 NL4-3 (WT) and N74D viruses (Fig. S4A), the result showed that baseline infectivity did not affect CPSF6-358 resistance of these viruses (Fig. S4B). We therefore decided to test CPSF6-358 resistance of aforementioned CA mutants.

It should be noted that substitutions in the loops themselves (L8/9, L7/8+α8) increased resistance to CPSF6-358 (Fig. 4F). Nonetheless, the results showed that the introduction of the K182R substitution had only marginal effects on the CPSF6-358 resistance of these mutants. In contrast, other mutants carrying substitutions around the CPSF6 binding cavity (α8+α11, and L10/11) (Fig. 4C and D, **green and cyan, respectively)** exhibited increased resistance to CPSF6-358 upon introduction of the K182R substitution, a pattern similar to that seen for the HIV-1 NL4-3 (WT) virus (Fig. 4G). Taken together, these results suggested that residues in the L8-9 linker are, at least in part, involved in the distinct impact of the K182R substitution on CA-CPSF6 interaction when comparing between HIV-1 and HIV-2 CAs.

4. Discussion

In the present study, we demonstrated that the K182R substitution differentially affects the CPSF6-358 resistance of viruses belonging to HIV-1- or HIV-2-lineages. Structural and genetic analyses suggested that residues in the L8-9 linker of HIV-2 CAs likely are responsible for maintaining the interaction between the 182nd residue and CPSF6.

We first demonstrated that the HIV-2 NMC842 virus shows normal CPSF6-358 resistance despite the presence of a K182R substitution in the CA. We next showed that the K182R substitution differentially affects the CPSF6-358 resistance of viruses belonging to the HIV-1- or HIV-2-lineages. Notably, although more HIV-2-lineage viruses need to be examined in the future study, this difference seems conserved in each lineage, implying that this characteristic difference arose just after the divergence of these viruses from their common ancestor virus. From this point of view, it would be interesting to test the impact of the K182R substitution on the CPSF6-358 resistance of genetically distant primate lentiviruses such as SIVmon or SIVsyk, which infect mona monkeys or Sykes' monkeys, respectively.

It is interesting that the K182R substitution has distinct impacts on the CPSF6-358 resistance of viruses belonging to HIV-1- or HIV-2-lineages. Lysine (K) and arginine (R) are chemically similar, given that both are positively charged basic amino acids. However, these residues differ in their flexibility and in the size of their side chains; substitution from K to R (or vice versa) occasionally results in phenotypic differences, including effects on the function and stability of proteins (Sokalingam et al., 2012). For example, distinct impacts have been reported for K-to-R substitutions in the reverse transcriptase (RT) proteins of HIV-1 and HIV-2. Although the K65R mutation in both HIV-1 and HIV-2 RTs is associated with resistance to nucleoside RT inhibitors (NRTIs) such as tenofovir (Ntemgwa et al., 2009; Peterson et al., 2011; Wensing et al., 2017), this mutation increases the fidelity of HIV-1 RT but not that of HIV-2 RT (Alvarez et al., 2017). Similarly, Goujon et al. revealed that the full antiviral activity of Mx2 was retained with the presence of arginines at the triple-arginine motif (positions 11 to 13), but not when these residues were replaced with lysines (Goujon et al., 2015). Therefore, a similar K-to-R substitution may occasionally affect the functional phenotype in a different manner depending on the protein backbone. In the case of the primate lentiviral CA, the K182R substitution enhances the CPSF6-358 resistance of HIV-1-lineage viruses, likely by extension of the L8-9 linker region (Fig. 4A**, right panel**), while this substitution should have a decreased impact in viruses belonging to the HIV-2-lineage (Fig. 4B). Notably, given that the K182A substitution in HIV-2 resulted in increased resistance to CPSF6-358, HIV-2 requires positively charged residues at this position for interaction with CPSF6 (Fig. 2C). Previous reports have shown that CA-CA interactions are biochemically distinct between HIV-1 and HIV-2 (Miyazaki et al., 2017), despite the similar shapes of their matured cores (Briggs et al., 2003; Chrystie and Almeida, 1988; Fukui et al., 1993). Further analyses will be required to clarify the detailed mechanisms of the distinct phenotypic effect of the K182R substitution in the CAs of HIV-1 and HIV-2.

The HIV-2 NMC842 virus also is of interest because of its characteristic CA sequence. We previously reported that the HIV-2 NMC842 virus and its relatives have higher levels of resistance to human TRIM5α than do HIV-2 group-A viruses (notably, the GH123 strain) (Miyamoto et al., 2012). Separately, Mamede et al. identified an HIV-2 strain harboring the K182R substitution (Mamede et al., 2017). This virus,

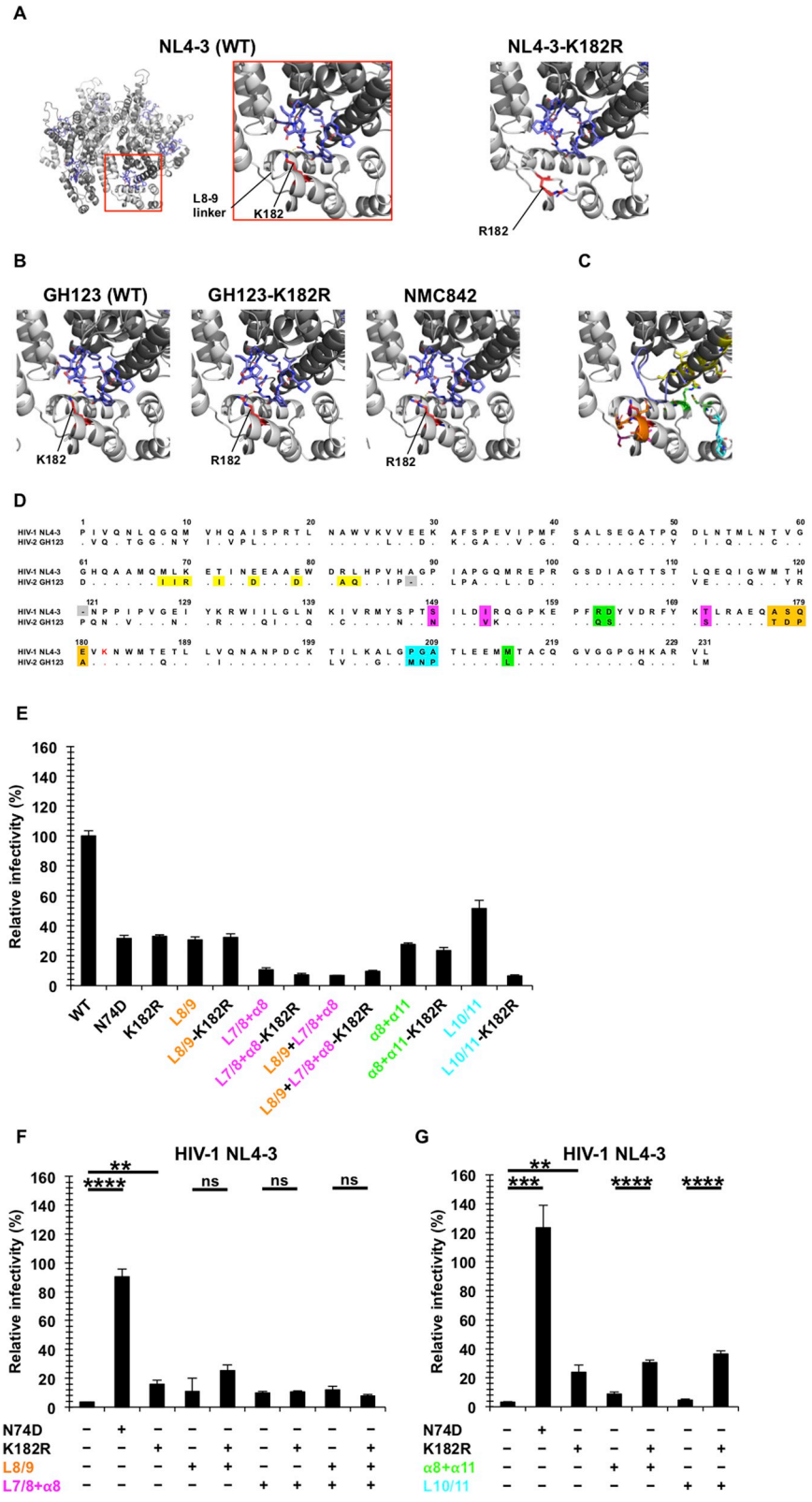

Fig. 4. Possible domains determining different effect of the K182R substitution.

(A) Interaction models of HIV-1 CA and a CPSF6 peptide. (left) Interaction between HIV-1 (NL4-3) CA (light or dark gray ribbons) and a CPSF6 peptide (navy sticks) in the crystal structure (PDB 4U0B). The K182 residue is highlighted with red sticks. (right) Putative interaction between HIV-1 (NL4-3) CA K182R mutant (light or dark gray ribbons) and a CPSF6 peptide (navy sticks). The K182R residue is highlighted with red sticks. The decreased CA-CPSF6 interaction by the K182R substitution might result from a different preference of conformation around the CA L8-9 linker. The K182R mutant might favor an alternative conformation with extended L8-9 linker, which disrupts direct interaction with the CPSF6 peptide.

(B) Putative interactions of CA in HIV-2 (GH123 or NMC842) (light or dark gray ribbons) with a CPSF6 peptide (navy sticks). The K182/R182 residues are shown with red sticks. The constructed model structures can be provided upon request.

(C) Positions of amino acid residues that differ between HIV-1 (NL4-3) and HIV-2 (GH123) CAs. The residues near the CPSF6 peptide (navy ribbon) are highlighted with colored sticks. We introduced mutations in these residues of HIV-1 (NL4-3): L8/9 (orange); A177T/S178D/Q179P/E180A, α4 (yellow); M68I/L69I/K70R/T72I/E75D/R82A/L83Q, α8 + α11 (green); R162Q/D163S/M215L, L10/L11 (cyan); P207M/G208N/A209P, L7/8 + α8 (magenta); S149N/I153V/T171S.

(D) Amino acid sequence comparison of the HIV-1 (NL4-3) and HIV-2 (GH123) CAs. The positions where mutations were introduced are highlighted with background colors similar to those used in Fig. 3C: L8/9 (orange), α4 (yellow), α8 + α11 (green), L10/L11 (cyan), L7/8 + α8 (magenta). At the top of the alignment, the residue numbers are shown according to the HXB2 numbering scheme.

(E) Relative infectivity of HIV-1 (NL4-3) variants. MT4 cells were infected with HIV-1 viruses harboring luciferase-encoding reporter genes. Virus inputs were normalized with RT activity of each virus stock. The RLU was determined 2 days after infection. The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of three independent experiments.

(F and G) MT4 cells expressing CPSF6-358 or CPSF6-358-FG321/322AA (Control) were superinfected with VSV-G-pseudotyped HIV-1 viruses harboring luciferase-encoding reporter genes. The degree of CPSF6-358 resistance was calculated by dividing the RLU of each virus in the presence of CPSF6-358 by that in the presence of CPSF6-358-FG321/322AA (Control). The results shown are the mean and standard deviation (SD) of triplicate measurements from one assay and were representative of three independent experiments. Differences were examined by a two-tailed, unpaired Student *t*-test. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, ns (not significant).

#13, has a sequence similar to that of the CRF01_AB viruses (Fig. S5). Notably, all of these viruses harbor the P120G substitution in CA (Fig. 1A and Fig. S5B), which confers elevated resistance to human TRIM5α (Miyamoto et al., 2012). Additionally, we note that these viruses contain unique amino acids, such as E187 and H208 (Fig. S5A). These results suggest that the unique substitutions in the CRF01_AB viruses and their relatives may help the viruses tolerate the K182R substitution. Our finding that the CRF01_AB viruses maintain CA-CPSF6 interaction despite the presence of the K182R substitution suggests that viruses belonging to both the HIV-1- and HIV-2-lineages have been under evolutionary pressure to maintain CA-CPSF6 interaction by any means, emphasizing the importance of CPSF6 binding for optimal viral replication.

From this point of view, it is of interest that WT viruses of the HIV-1-

lineage showed variable CPSF6-358 resistance (Fig. 2A). Specifically, HIV-1 TF2960 and HIV-1 TF2627 viruses showed higher resistance to CPSF6-358 than did other strains, suggesting impaired interaction with CPSF6. Although the reason for this phenomenon remains unclear, Henning et al. demonstrated that CA variants that emerged in hosts carrying the HLA-B27 allele were suppressed by endogenous CPSF6 (Henning et al., 2014). Therefore, HIV-1 TF2960 and HIV-1 TF2627 viruses might have been selected for "minimum" binding to CPSF6 during their evolution. Nevertheless, the K182R substitution further increased the CPSF6-358 resistance of these viruses (Fig. 2A), supporting our conclusion that the K182R substitution decreases CA-CPSF6 interaction by HIV-1-lineage viruses.

Although we do not have solid evidence regarding the reason for appearance of the K182R alteration in HIV-2 strains, we hypothesize that the K182R substitution in HIV-2 strains arose as a cytotoxic T lymphocyte (CTL) escape mutant. In fact, two groups (Jennes et al., 2008; Leligdowicz et al., 2007) have identified, in HIV-2-infected populations, CTL epitopes that include the 182nd amino acid residue. It should also be noted that we were able to find similar CTL epitopes in HIV-1-infected populations (Brodie et al., 1999; Kaul et al., 2001; Kunwar et al., 2013). Therefore, it is intriguing to speculate why the HIV-1-lineage viruses have avoided mutating the 182nd residue, in contrast to certain HIV-2 strains. We tested whether the K182R substitution affected infectivity of HIV-1 NL4-3 and HIV-2 GH123 viruses. The results showed that the K182R substitution yielded comparable decreases in the infectivity of both HIV-1 and HIV-2 (Fig. 3). Therefore, we conclude that the reason why HIV-1 did not select the K182R substitution is not due to different effects of the K182R substitution on viral infectivity. It is possible that the K182R substitution might be beneficial only in the CRF01_AB viruses. It should be noted that the NMC842-R182K virus was found to be non-infectious (Fig. S6), supporting this hypothesis. Therefore, it is tempting to speculate that there might be some link between the loss of flexibility at CA residue 182 and the potential for inducing rapid disease progression (like that seen with HIV-1) with HIV-2 CRF01_AB viruses. It is possible that the loss of flexibility at CA residue 182 in HIV-1 and HIV-2 CRF01_AB viruses might be due to the accumulation of CA mutations caused by more frequent transmission among individuals with different *HLA* alleles than that of viruses in other HIV-2 groups. Notably, ordinary HIV-2 infection is characterized by slower disease progression with lower viral load than that seen with HIV-1, and lower viral load would lead to lower frequency of virus transmission. However, further studies will be necessary to verify these speculations.

In conclusion, we presented evidence that the K182R substitution differentially affects CA-CPSF6 interaction in viruses of the HIV-1- and HIV-2-lineages. Furthermore, our results suggested that residues in the L8-9 linker are, at least in part, involved in the distinct impact of the K182R substitution on CA-CPSF6 interaction between HIV-1 and HIV-2 CAs. Future studies will be necessary to clarify the nature of CPSF6 binding by CA proteins of primate lentiviruses.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Conceived and designed the experiments: AS HOde. Performed the experiments: AS HOde KN HOhmori. Analyzed the data: AS HOde EEN YI TS. Contributed reagents/materials/analysis tools: EEN. Wrote the paper: AS HOde YI TS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.04.012.

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