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Original article

Distinct combinations of amino acid substitutions in *N*-terminal domain of Gag-capsid afford HIV-1 resistance to rhesus TRIM5α

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Abstract

TRIM5α is a potent anti-retroviral factor that interacts with viral capsid (CA) in a species-specific manner. Recently, we and others reported generation of two distinct HIV-1 CAs that effectively overcome rhesus TRIM5α-imposed species barrier. In this study, to directly compare the effect of different mutations in the two HIV-1 CAs on evasion from macaque TRIM5-restriction, we newly generated macaque-tropic HIV-1 (HIV-1mt) proviral clones carrying the distinct CAs in the same genomic backbone, and examined their replication abilities in macaque TRIM5-overexpressing human cells and in rhesus cells. Comparative analysis of amino acid sequences and homology modeling-based structures revealed that, while both CAs gained some mutated amino acids with similar physicochemical properties, their overall appearances of N-terminal domains were different. Experimentally, the two CAs exhibited incomplete TRIM5α-resistance relative to SIVmac239 CA and different degrees of susceptibility to various TRIM5 proteins. Finally, two HIV-1mt clones carrying a different combination of the CA mutations were found to grow to a comparable extent in established and primary rhesus cells. Our data show that there could be some distinct CA patterns to confer significant TRIM5-resistance on HIV-1.

Keywords: HIV-1; HIV-1mt; Capsid; Gag-CA; Rhesus macaque; TRIM5α

1. Introduction

TRIM5α interacts with retroviral Gag-capsid (CA) and inhibits viral replication in a species-specific manner [1–6]. TRIM5α acts as a pattern-recognition molecule via its C-terminal B30.2/SPRY domain on diverse retroviral CAs [7–12]. It is proposed that retroviruses overcome TRIM5α-restriction either by mutating CA to abolish recognition by TRIM5α B30.2/SPRY domain, or by altering a surface pattern of CA lattice [9]. Macaque TRIM5α is one of the major species-barriers for HIV-1. Evasion from macaque TRIM5α-restriction would facilitate establishing HIV-1/macaque models useful for basic and clinical AIDS studies [13,14]. Recently, we successfully generated rhesus macaque (RhM) TRIM5α-resistant HIV-1 CA, designated LSDQ (Fig. 1A), through comparative sequence/structure analyses of HIV and SIVmac239 CAs [15]. Soll et al. also constructed RhM TRIM5α-resistant HIV-1 CA, designated LNEIE (Fig. 1A), by "assisted evolution" method [16]. Interestingly, LSDQ and LNEIE CAs have different amino acid substitutions that contribute to TRIM5α-resistance. Furthermore, a virus carrying LSDQ CA or LNEIE CA grew best in RhM peripheral blood

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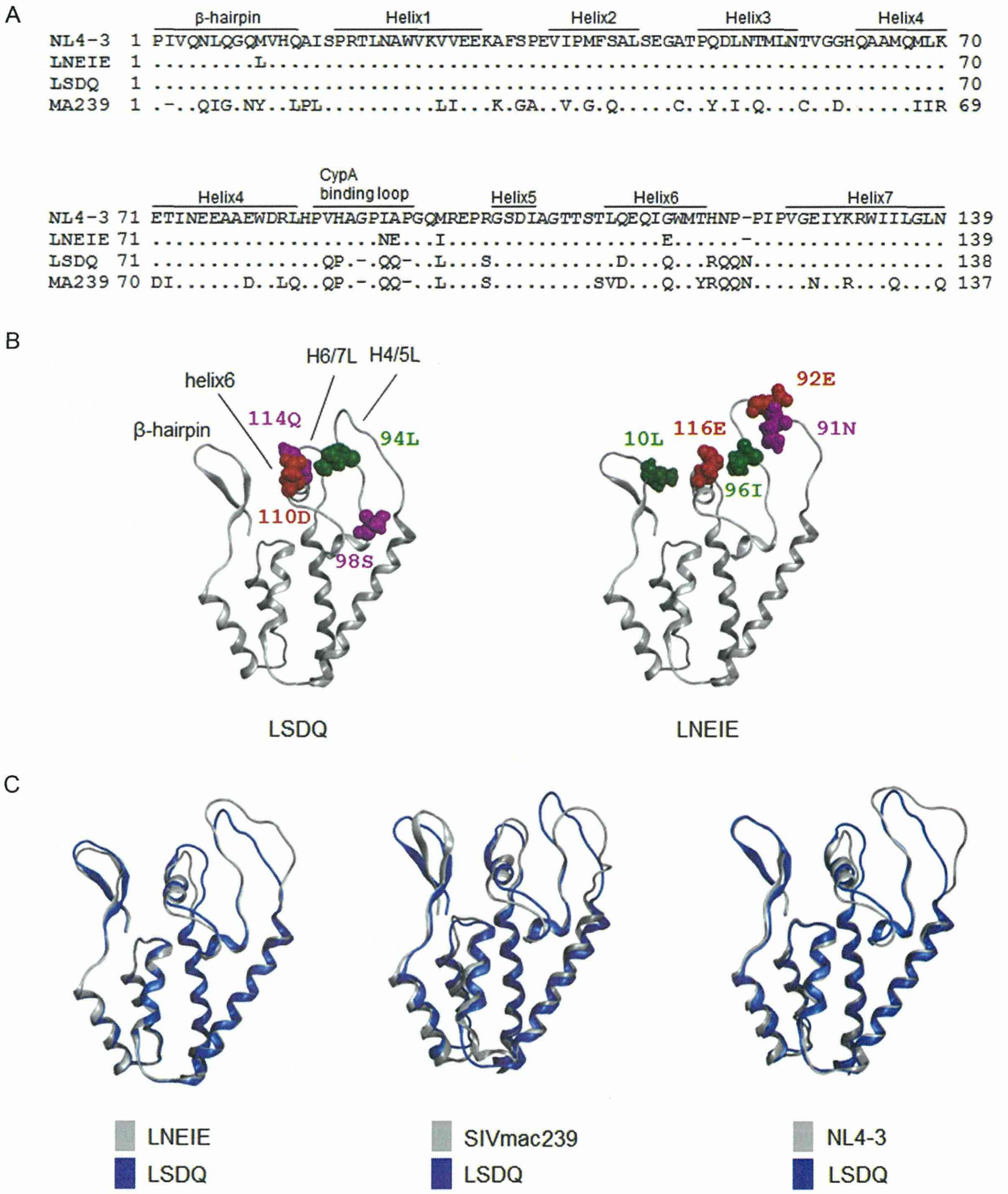

Fig. 1. Structure of CA NTD from two different HIV-1mt clones. (A) Alignment of CA sequences. Amino acid sequences in CA (amino acid residues 1 to 137/138/139) of HIV-1_{NL4-3} (GenBank: AF324493), LNEIE [16], LSDQ [15], and SIVmac239 (GenBank: M33262) were aligned by Genetix ver. 11. Dots show the same amino acid residues with those of HIV-1_{NL4-3}. Hyphens indicate the gap. The domains of β-hairpin and helices 1 to 7 are indicated based on the previous publication [37]. (B) Structural models for CA NTD from two distinct HIV-1mt clones LSDQ and LNEIE. Molecular models were constructed by homology modeling and were refined as previously described [15]. HIV-1 CA NTD at a resolution of 1.95Å (PDB code: 4LQW) [20] was used as modeling template. (C) Superimposition of the CA structures. Superposed structures of LNEIE/LSDQ CAs (left), SIVmac239 (modified structure of PDB code 4HTW)/LSDQ CAs (middle), and NL4-3 (PDB code 3GV2)/LSDQ CAs (right) are shown using two different colors indicated.

mononuclear cells (PBMCs) among the macaque-tropic HIV-1 (HIV-1mt) clones examined in each study [15,16]. In this work, we aimed to gain virological and structural insights into evasion from TRIM5α-restriction using the two distinct HIV-1 CAs.

2. Materials and methods

2.1. Plasmid DNA

An HIV-1mt clone designated MN4/LSDQgtu and a standard SIVmac clone designated SIVmac239 used in this study were described previously [15]. Clone pLNEIE was constructed by introduction of the five mutations [16] into the CA-coding region of a sub-genomic clone derived from pNL4-3 by QuickChange Site-Directed Mutagenesis kit (Agilent Technologies Inc., Santa Clara, CA). Clone pSCA was constructed from the above sub-genomic clone by overlapping PCR and QuickChange Site-Directed Mutagenesis kit to have Gag sequences as described for stHIV-1_{SCA} [16,17]. Proviral clones designated LSDQ+4gtu, LNEIE+4gtu, and SCA+4gtu were generated by replacement of the *Bss*HII-*Sbf*I DNA fragment of MN4/LSDQgtu with the corresponding fragments of "MN4/LSDQgtu", pLNEIE, and pSCA clones, respectively.

2.2. Cell culture, virus preparation, and reverse transcriptase (RT) assays

A human kidney cell line 293T, a RhM lymphocytic cell line M1.3S and RhM PBMCs were cultured as described previously [15]. The *TRIM5* genotypes of PBMCs, prepared from RhM individuals and used for infection experiments, were determined as described previously [15]. Virus stocks were prepared from 293T cells transfected with proviral clones on day 2 post-transfection. Virus stocks were assayed for RT activities, and used for infection experiments as previously described [15].

2.3. TRIM5 susceptibility assays

TRIM5 susceptibility assays in human MT4 cells were done by the recombinant Sendai virus (SeV)-TRIM5 expression system as described previously [15,18].

2.4. Multi-cycle virus replication assays

Infection of M1.3S cells was ordinarily performed as described previously [15]. For infection of RhM PBMCs, the spinoculation method [19] was used. Virus replication was monitored by RT activity released into the culture supernatants.

2.5. Structural analysis

Molecular models for HIV-1mt CA N-terminal domain (NTD) were constructed by homology modeling and were refined as described previously [15]. HIV-1 CA NTD at a resolution of 1.95Å (PDB code: 4LQW) [20] was used as modeling template. Superimpositions of the structures were done using the Protein Superpose module in MOE (Chemical Computing Group Inc., Quebec, Canada).

3. Results

3.1. Sequence and structure comparison of LSDQ and LNEIE CAs

Determinants in retroviral CA to modulate TRIM5α-susceptibility have been mapped to CA surface domains including β-hairpin, a loop between helices 4 and 5 (H4/5L), helix6, and H6/7L (Fig. 1A) [15,18,21–29]. LSDQ and LNEIE, the two RhM TRIM5α-resistant HIV-1 CAs, have different amino acid sequences, convergently in a cyclophillin A (CypA) binding loop within H4/5L and in H6/7L. The loop regions in LSDQ CA have been replaced with those in SIVmac239 CA (Fig. 1A). As indicated in Fig. 1B, LSDQ and LNEIE CAs commonly gained a negatively charged amino acid residue in helix6 (110D for LSDQ and 116E for LNEIE) and paired substitutions in helix6 and H4/5L (114Q/94L for LSDQ and 116E/96I for LNEIE). However, the overall appearance of CA NTD was different between the two clones mainly due to difference in H4/5L- and H6/7L-length, which could affect a surface pattern of viral core (Fig. 1C, left). In addition, the structure of LSDQ CA was different from those of its parental CAs, i.e., SIVmac239 and NL4-3 CAs, especially in the H4/5L region (Fig. 1C, middle and right). Moreover, the β-hairpin domain of SIVmac239 CA was structurally distinct from those of LSDQ, LNEIE, and NL4-3 CAs (Fig. 1C). Conclusively, LSDQ and LNEIE CAs are structurally unique to each other (Fig. 1), but both contribute to the TRIM5α-resistance [15,16].

3.2. LSDQ and LNEIE CAs exhibit different susceptibilities to the restriction mediated by various macaque TRIM5 proteins

To examine potentials of the two distinct CAs for evading TRIM5α-restriction and for viral replication, we constructed new proviral clones in the backbone of our best HIV-1mt designated MN4/LSDQgtu (Fig. 2A) [15]. The *Bss*HII-*Sbf*I DNA fragment of MN4/LSDQgtu was replaced with the corresponding fragments of LNEIE [16] and LSDQ [15] to generate LNEIE+4gtu and LSDQ+4gtu, respectively. The sequence differences between the two clones reside only in the CA NTD (Fig. 1A).

First, we determined susceptibility of LSDQ+4gtu and LNEIE+4gtu to various TRIM5 proteins expressed by SeV vectors. Ability of viral clones to evade TRIM5-restriction, in comparison with that of SIVmac239, can be readily determined by this recombinant SeV-TRIM5 overexpression system [15,18]. Macaque TRIM5 alleles are divided into three functionally different groups: $TRIM5\alpha^{TFP}$, $TRIM5\alpha^{Q}$, and $TRIM5^{CypA}$ [30–32]. TRIM5α proteins of both RhM and cynomolgus macaque (CyM), and CyM TRIM5CypA inhibit HIV-1 replication, but not RhM TRIM5CypA [33,34]. Thus,

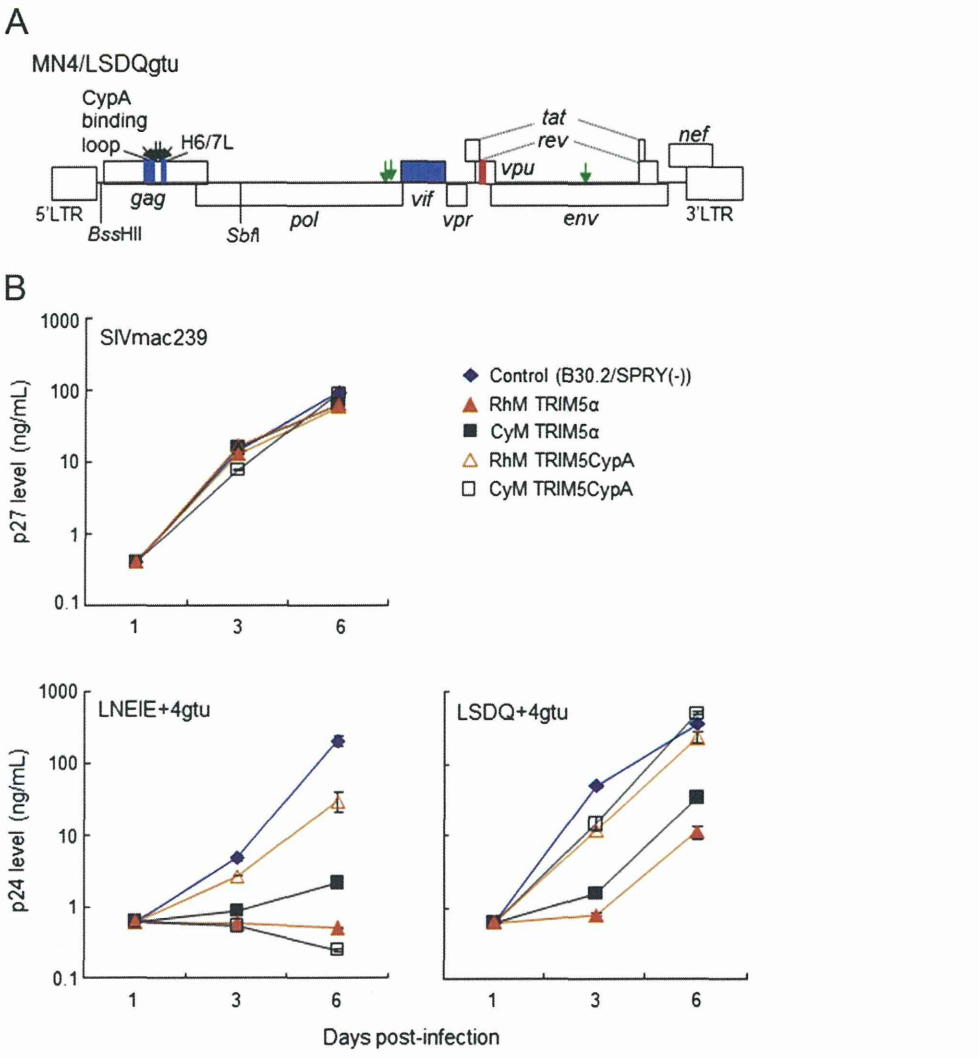

Fig. 2. Susceptibility of viral clones to various macaque TRIM5 proteins. (A) Proviral genome structure of an HIV-1mt clone MN4/LSDQgtu [15]. Blue and red areas show sequences from SIVmac239 and SIVgsn166 (SIV isolated from the greater spot-nosed monkey) (GenBank: AF468659), respectively. Green arrows show the adaptive mutations that enhance the viral growth potential [38]. Four amino acid substitutions (M94L/R98S/Q110D/G114Q) in CA that increase RhM TRIM5α-resistance are indicated by black arrows [15]. The *Bss*HII and *Sbf*I sites used for construction of MN4/LSDQgtu-based viral clones carrying distinct CAs are indicated. (B) TRIM5 susceptibility assays. Human MT4 cells (1.0×10^5) were infected with recombinant SeV expressing B30.2/SPRY (−) TRIM5, CyM TRIM5α (*TRIM5*α^{Q}), RhM TRIM5α (*TRIM5*α^{TFP}), CyM TRIM5CypA (*TRIM5*^{CypA}), or RhM TRIM5CypA (*TRIM5*^{CypA}). B30.2/SPRY (−) TRIM5 without the ability to restrict viral replication served as a control. Nine hours after infection with recombinant SeVs, cells were super-infected with 20 ng (Gag-p24) of HIV-1mt clones or 20 ng (Gag-p27) of SIVmac239. Virus replication was monitored by the amount of Gag-p24 (HIV-1mt clones) or Gag-p27 (SIVmac239) in the culture supernatants. Error bars show fluctuations between duplicate samples. Representative data from two independent experiments are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

we tested here four different TRIM5 alleles, i.e., RhM TRIM5α (*TRIM5*α^{TFP}), CyM TRIM5α (*TRIM5*α^{Q}), RhM TRIM5CypA (*TRIM5*^{CypA}), and CyM TRIM5CypA (*TRIM5*^{CypA}), using B30.2/SPRY(−) TRIM5 as a control. As shown in Fig. 2B, SIVmac239 replicated similarly well in the presence of RhM TRIM5α, CyM TRIM5α, RhM TRIM5-CypA, or CyM TRIM5CypA as in control cells expressing B30.2/SPRY(−) TRIM5. While not complete as compared with the case of SIVmac239 [15], LSDQ+4gtu showed more resistance to various RhM/CyM TRIM5 proteins than LNEIE+4gtu. In particular, consistent with previous observations, LSDQ+4gtu replicated well in the presence of CyM TRIMCypA, but not at all LNEIE+4gtu [15,16]. Furthermore, in the presence CyM/RhM TRIM5α, LNEIE+4gtu appeared to replicate (note the data in the presence of CyM TRIM5-CypA in Fig. 2B) but clearly more poorly than LSDQ+4gtu.

Table 1
Lethal mutations in CA of MN4/LSDQgtu.[a]

Mutants	CA mutations relative to LSDQ	CA domains	References
P37S-LSDQ	P38S	Helix2	[35]
LSVDQ	L109V	Helix6	[25]
LSDQY	T117Y	Helix6	
LSVDQY	L109V/T117Y	Helix6	
Mutants of β-hairpin domain	Amino acid sequences in β-hairpin[b]		
LSDQ (parental clone)	PIVQNLQGQMVHQAI		[15]
Wild-type SIVmac239	PVQQIGGNYVHLPL		
M10L-LSDQ	PIVQNLQGQ**L**VHQAI		[16]
Q13L-LSDQ	PIVQNLQGQMVH**L**AI		
IGGN-LSDQ	PIVQ**IGGN**MVHQAI		
Beta-1	P**VQ**Q**IGGN**MVHQAI		
Beta-2	PIVQ**IGGNY**VH**L**AI		
Beta-3	PIVQNLQGQMVH**LPL**		
Beta-4	P**VQ**QNLQGQMVHQAI		
Beta-5	PIVQ**IGGNY**VHQAI		
Beta-6	P**VQ**Q**IGGNY**VH**L**AI		
Beta-7	**PVQQIGGNYVHLPL**		
Beta-8	PIVQ**IGGNY**VH**LPL**		

[a] Lethal mutations as judged by viral replication in M1.3S cells during the observation period (15 days).
[b] Bold letters show the mutations introduced into LSDQ CA. For alignment of four CA NTD sequences, see Fig. 1A.

These results show that LSDQ and LNEIE have intrinsically different abilities to negotiate anti-viral effects of various macaque TRIM5 proteins.

Amino acid substitutions in CA contributing to escape from RhM TRIM5α-restriction have been identified by *in vivo* adaptation of SIVsm (SIV from the sooty mangabey) in RhM (P37S and R97S in SIVsm CA) [30,35], and by "gain-of-sensitivity assays" using SIVmac239 CA (L93M, S97R, V108L, D109Q, and Q113G) [25]. TRIM5-resistant LSDQ CA already has M94L, R98S, Q110D, and G114Q mutations corresponding to L93, S97, D109, and Q113 residues in SIVmac239 CA [15]. Therefore, it was possible that amino acid substitutions such as P38S (corresponding to P37S in SIVsm and SIVmac239 CAs) and L109V (corresponding to V108 in SIVmac239 CA) in LSDQ CA might enhance its TRIM5-resistance. The β-hairpin domain in retroviral CAs is also an important determinant for evasion from TRIM5α-restriction [18,25,27] (Fig. 1C). Based on these considerations, we introduced various amino acid substitutions into the MN4/LSDQgtu CA (Table 1) to increase TRIM5α-resistance, hopefully up to the SIVmac239 CA level. Resultant proviral clones were tested for their growth abilities in a RhM cell line M1.3S. However, our extensive attempts to obtain biologically active CAs, potentially more resistant to macaque TRIM5 proteins than MN4/LSDQgtu CA, were unsuccessful so far (Table 1). Thus, some mutation(s) and/or combination(s) of mutations in CA other than those in Table 1 may be necessary to confer full resistance to TRIM5α on the HIV-1mt.

3.3. HIV-1mt clones carrying LSDQ/LNEIE CA replicate well in RhM PBMCs

To compare the effects of a different spectrum of mutations in CAs on viral growth potential, we examined LSDQ+4gtu and LNEIE+4gtu for their replication in RhM cells. In M1.3S cells (*TRIM5α*$^{TFP/TFP}$) [36], LSDQ+4gtu replicated slightly better than LNEIE+4gtu (Fig. 3A). In PBMCs prepared from four RhM individuals (*TRIM5α*$^{TFP/Q}$), LSDQ+4gtu grew better (Fig. 3B, upper panel) than or similarly to LNEIE+4gtu (Fig. 3B, lower panel). Next, to compare the competence of the CAs to that of SIVmac239 CA in terms of multi-cycle virus replication in RhM PBMCs, we newly constructed a proviral HIV-1mt clone carrying SIVmac239 CA. Because insertion of the entire CA-coding sequence of SIVmac into the corresponding region of HIV-1 genome was lethal, we generated a new Gag clone (SCA) exactly as previously reported for stHIV-1$_{SCA}$ [16,17] (Fig. 4A), and then made a proviral clone designated SCA+4gtu as described to construct LSDQ+4gtu and LNEIE+4gtu (Fig. 2A) for infection experiments. Proviral clone SCA was more replication-competent than LSDQ [15] (~3-fold) as determined in feline CRFK cells stably expressing RhM-TRIM5α (*TRIM5α*$^{TFP/TFP}$), but showed a lower titer (~2-fold–4-fold) in CRFK-naïve cells and TZM-bl indicator cells relative to LSDQ (our unpublished results). As shown in Fig. 4B, while LSDQ+4gtu grew better than SCA+4gtu in all four PBMC preparations tested (*TRIM5α*$^{TFP/Q}$), LNEIE+4gtu did so in two preparations (PBMCs from RhMs 610 and 611). In these two PBMC preparations, LSDQ+4gtu and LNEIE+4gtu grew similarly well. In the other two preparations, of note, LSDQ+4gtu grew better than LNEIE+4gtu (PBMCs from RhMs 599 and 609 in Fig. 4B). It remains to be elusive whether the observed difference in growth potentials in some PBMC preparations of the two clones are attributable to TRIM5α-restriction, viral fitness (infectivity of LNEIE determined in TZM-bl indicator cells relative to that of LSDQ was 0.72 on average), unknown cellular factor(s), and/or cellular physiological state/environments.

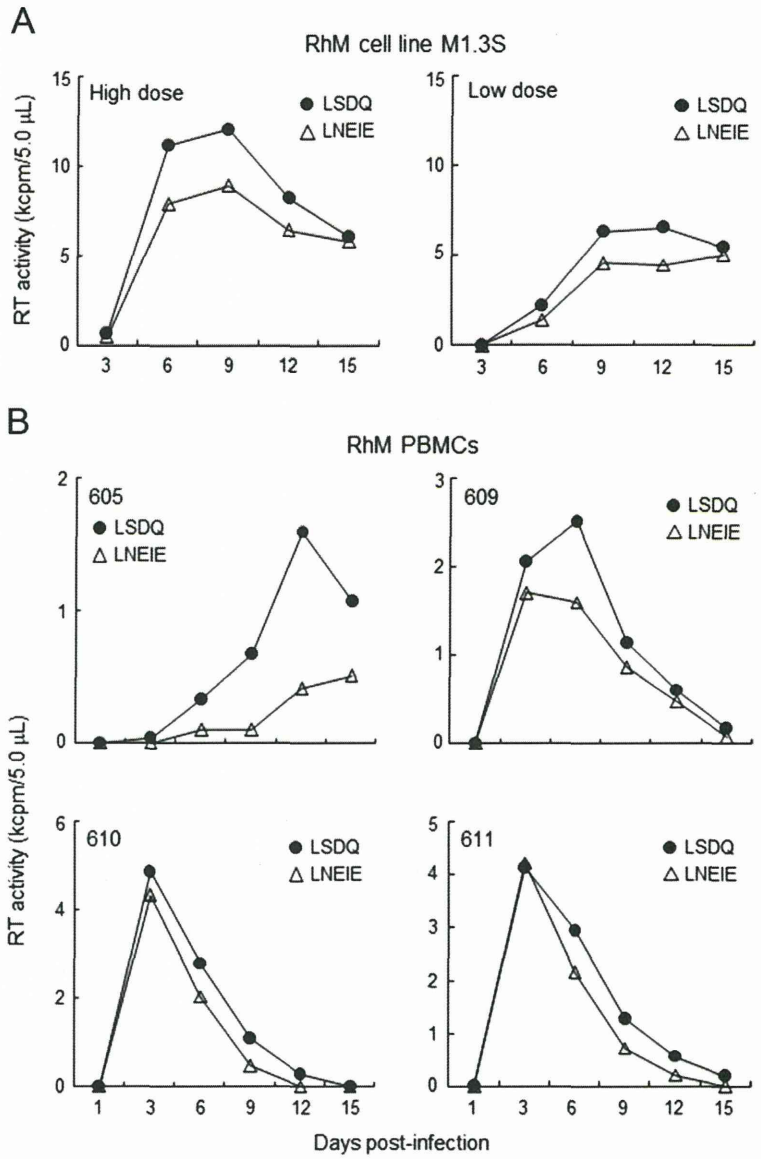

Fig. 3. Growth kinetics of two HIV-1mt clones with a distinct CA in RhM cells. Input viruses were prepared from 293T cells transfected with the indicated clones, and viral replication was monitored by RT activity released into the culture supernatants. LSDQ, LSDQ+4gtu; LNEIE, LNEIE+4gtu. (A) Infection of M1.3S cells (*TRIM5α*$^{TFP/TFP}$). Cells (2.0×10^5) were infected with equal virus amounts (High dose, 5.0×10^5 RT units; Low dose, 5.0×10^4 RT units). (B) Infection of PBMCs from four RhM individuals (*TRIM5α*$^{TFP/Q}$). Equal amounts of viruses were spin-infected into the PBMC preparations. Infection conditions were as follows: 2.4×10^6 RT units/1.0×10^6 cells for monkey 605; 4.0×10^6 RT units/2.0×10^6 cells for monkeys 609, 610, and 611.

4. Discussion

In this study, we performed side by side comparative analyses of the TRIM5-resistance/growth ability in RhM cells of HIV-1mt viruses carrying distinct CAs (LSDQ and LNEIE in Fig. 1) that are resistant to RhM TRIM5α [15,16]. LSDQ and LNEIE CAs exhibited various degrees of susceptibility to macaque TRIM5 proteins, and the former was generally more resistant to TRIM5-restriction than the latter in our TRIM5-overexpression system (Fig. 2). However, growth potentials of HIV-1mt viruses carrying LSDQ or LNEIE CA were similar in some preparations of RhM PBMCs, and varied among PBMCs from RhM individuals with *TRIM5*$^{TFP/Q}$ (Figs. 3 and 4). These results may only reflect a low endogenous expression level of TRIM5 proteins in PBMCs relative to that in cells infected with recombinant SeVs. The expression levels of TRIM5 proteins in various cells, however, can not be measured as yet due to the lack of appropriate anti-macaque

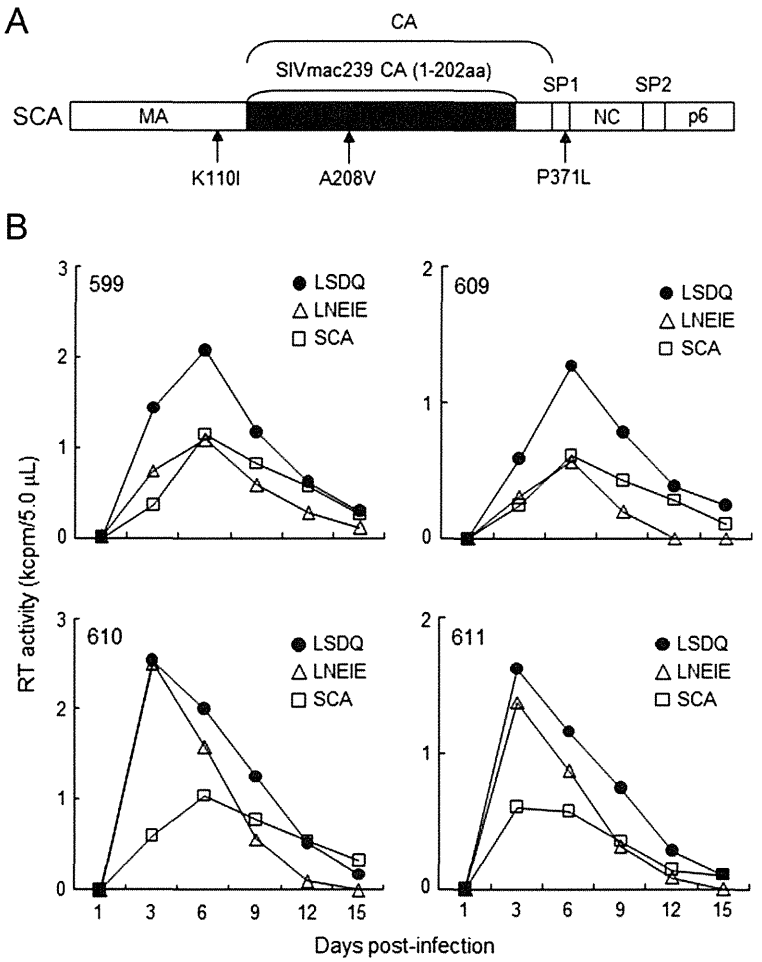

Fig. 4. Growth kinetics of various HIV-1mt clones with a distinct CA in RhM PBMCs. (A) Gag-coding region of pSCA. White and black areas show sequences from HIV-1_{NL4-3} and SIVmac239, respectively. Mutations introduced are indicated. MA, matrix; SP1, spacer peptide 1; NC, nucleocapsid; SP2, spacer peptide 2. (B) Infection of PBMCs from four RhM individuals (*TRIM5α*$^{TFP/Q}$). Input viruses were prepared from 293T cells transfected with the indicated clones, and equal amounts of viruses were spin-infected into the PBMCs. Infection conditions were as follows: 2.4 × 10^6 RT units/2.0 × 10^6 cells for monkey 599; 1.2 × 10^6 RT units/1.0 × 10^6 cells for monkeys 609, 610, and 611. Viral replication was monitored by RT activity released into the culture supernatants. LSDQ, LSDQ+4gtu; LNEIE, LNEIE+4gtu; SCA, SCA+4gtu.

TRIM5 antibodies. Alternatively, the above results suggest that overcoming TRIM5-restriction may not be enough for maximal virus growth of the HIV-1mt clones in RhM cells. Thus, a new generation of HIV-1mt clones that replicate constantly well in PBMCs from any RhM individuals like SIVmac239 would be necessary to establish the HIV-1-infected RhM model system. Of similar importance, detailed biological and structural analyses of the interaction between LSDQ/LNEIE CA and macaque TRIM5 proteins would contribute to better understand the underlying molecular mechanism for HIV-1 restriction by the proteins.

We previously suggested that R98S in HIV-1mt CA may be a key residue to circumvent macaque TRIM5α-restriction [15], since the corresponding residues in SIVsm and SIVmac239 CAs have been shown to contribute to the alteration of TRIM5α-susceptibility [25,30,35]. The coincidence of four amino acid residues important for evasion of RhM TRIM5-restriction in two independent studies on HIV-1 [15] and SIV [25] (L93, S97, D109, Q113 for SIVmac239 CA and L94, S98, D110, Q114 for HIV-1mt CA as described above) has raised a possible involvement of some specific amino acids in the TRIM5-regulation. However, comparative analysis of LSDQ and LNEIE clones here suggests that combinations of mutations in an appropriate context in CA rather than individual residues are critical for efficient escape from TRIM5α-restriction. As TRIM5α has evolved to target diverse retroviral CAs by flexibility of its B30.2/SPRY domain [7-9,12], HIV-1 can, in turn, gain RhM TRIM5α-resistance

through several distinct CAs with different amino acid sequences and/or CA surface patterns.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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