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Impact of a single amino acid in the variable region 2 of the Old World monkey TRIM5α SPRY (B30.2) domain on anti-human immunodeficiency virus type 2 activity

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ABSTRACT

Variable region 1 (V1) of the SPRY domain of TRIM5α is a major determinant for species-specific virus restriction in primates. We previously reported that a chimeric TRIM5α containing baboon V1 in the background of cynomolgus monkey TRIM5α showed potent anti-human immunodeficiency virus type 2 (HIV-2) activity. Since baboons are reportedly sensitive to HIV-2 infection, there was a discrepancy between the ability of baboon TRIM5α V1 to restrict HIV-2 and baboon sensitivity to HIV-2. In the study presented here, we examined the roles of V2 and V3 of the baboon TRIM5α SPRY domain in its anti-HIV-2 activity. A chimeric TRIM5α containing the entire baboon SPRY domain showed weak anti-HIV-2 activity. This attenuation of activity was caused by a single serine-to-proline substitution in baboon TRIM5α V2. These findings indicate that the combination of V1 with other variable regions of SPRY is important in anti-HIV-2 activity of primate TRIM5α.

Introduction

Human immunodeficiency virus type 1 (HIV-1) has a very narrow host range limited to humans, chimpanzees, gibbons, and pig-tailed monkeys *in vivo* (Lusso et al., 1988; Arthur et al., 1989; Agy et al., 1992), and gorillas *in vitro* (Locher et al., 1996). Previous experiments have demonstrated that Old World monkeys (OWM) such as rhesus and cynomolgus monkeys are not sensitive to HIV-1 infection. This block is partly explained by the presence of tripartite motif 5α (TRIM5α) (Stremlau et al., 2004) in cells of those monkeys. Rhesus and cynomolgus monkey TRIM5α restricts HIV-1 infection but not simian immunodeficiency virus isolated from macaque (SIVmac) (Stremlau et al., 2004; Nakayama et al., 2005). In contrast, human TRIM5α fails to restrict those viruses, but potently restricts N-tropic murine leukemia viruses (N-MLV) (Hatziioannou et al., 2004; Kecksova et al., 2004; Yap et al., 2004). Unlike human and other OWM, pig-tailed monkeys lack expression of TRIM5α, whereas express TRIM5θ and TRIM5η lacking anti-HIV-1 activity (Brennan et al., 2007). TRIM5α shares with other splicing variants a common amino-terminal TRIM motif, comprising RING, B-box and coiled-coil domains (which is called RBCC domain), and encodes a unique SPRY (B30.2) domain (Reymond et al., 2001). Studies on recombinant TRIM5αs between human and rhesus monkey have shown that the determinant of the species specificity resides in the SPRY domain (Perez-Caballero et al., 2005; Sawyer et al., 2005). Studies on recombinant TRIM5αs between African green monkey (AGM) and cynomolgus monkey demonstrated that 17-amino acid residues and adjacent AGM-specific 20-amino acid duplication in the SPRY domain determined species-specific restriction of SIVmac (Nakayama et al., 2005). Similarly, a study comparing orangutan and gorilla TRIM5α also showed that the amino acid residues at the 385th and 389th positions in the SPRY domain of orangutan TRIM5α are important for inhibiting HIV-1 and SIVmac (Ohkura et al., 2006). Furthermore, by comparing human and rhesus monkey TRIM5α restriction of N-MLV, the amino acid residues of human TRIM5α at the 409th and 410th positions in the SPRY domain are found to be important for inhibiting N-MLV (Peron et al., 2006). Interestingly, a study comparing human and rhesus monkey TRIM5α showed that a single arginine to proline (P) change at the 332nd position in the SPRY domain of human TRIM5α conferred potent restriction of not only HIV-1 but also SIVmac239 (Stremlau et al., 2005; Yap et al., 2005).

Human immunodeficiency virus type 2 (HIV-2) has a genome similar to that of SIVmac (Hahn et al., 2000), which is not restricted by rhesus monkey and cynomolgus monkey TRIM5α. We previously evaluated the ability of cynomolgus monkey TRIM5α to restrict eight different HIV-2 isolates and found that it could restrict viruses carrying P at the 119th or 120th position of the capsid protein (CA), whereas it failed to restrict those with either alanine or glutamine (Song et al., 2007). HIV-2 GH123 strain has P at the 120th position of CA and was restricted by cynomolgus monkey TRIM5α while its mutant HIV-2 GH123/Q carrying glutamine was not restricted. Subsequently, we found that rhesus monkey TRIM5α showed broad spectrum of HIV-2 restriction and could restrict HIV-2 strains that were not restricted by cynomolgus monkey TRIM5α (Kono et al., 2008). The variable region 1

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(V1) of the SPRY domain of rhesus monkey TRIM5α appeared to be a determinant for this restriction, since a chimeric TRIM5α containing cynomolgus monkey V1 in the background of rhesus monkey TRIM5α could not restrict HIV-2 GH123/Q, while a chimeric TRIM5α containing rhesus monkey V1 in the background of cynomolgus monkey could (Kono et al., 2008). On the other hand, we found that a chimeric TRIM5α containing baboon V1 in the background of cynomolgus monkey TRIM5α could restrict both HIV-2 GH123 and HIV-2 GH123/Q, despite the fact that baboons are sensitive to HIV-2 infection (Barnett et al., 1994; Locher et al., 1998, 2001). One possible explanation for this discrepancy is that variable region 2 or 3 (V2 or V3) of SPRY domain also contributes to anti-HIV-2 activity. In the study presented here, we examined the contribution of V2 and V3 of baboon TRIM5α SPRY domain to anti-HIV-2 activity and found that a single amino acid in V2 affects its restriction activity against HIV-2.

Results

Variable region 2 (V2) of baboon TRIM5α SPRY (B30.2) domain weakens anti-HIV-2 activity

In our previous study, we constructed a recombinant Sendai virus (SeV) expressing chimeric TRIM5α between cynomolgus monkey TRIM5α and baboon TRIM5α by using Sph I and BamH I restriction enzyme digestion. As can be seen in Fig. 1, the N-terminal fragment contains RING, B-box2, and coiled-coil domains, the central fragment contains V1 of the SPRY domain, and the C-terminal fragment contains V2 and V3 of the SPRY domains. In that study, we reported a chimeric TRIM5α containing baboon V1 in the background of cynomolgus monkey (2B2, but renamed CBC in this study) (Fig. 2A) could restrict HIV-2 GH123/Q, which was not restricted by cynomolgus monkey TRIM5α. To examine anti-HIV-2 activity of TRIM5α containing the entire baboon SPRY domain, we constructed a recombinant SeV expressing a chimeric TRIM5α containing V1, V2, and V3 of the SPRY domain of baboon TRIM5α (CBB) (Fig. 2A). Western blot analysis using an antibody against hemagglutinin (HA) tag showed that CBB chimeric TRIM5α was expressed in recombinant SeV infected human T-cell line MT4 cells at levels similar to those of rhesus and cynomolgus monkey TRIM5αs and CBC chimeric TRIM5α (Fig. 2B). Those TRIM5αs were tested for their ability to restrict X4-tropic HIV-1 strain NL43 and HIV-2 strains GH123 and GH123/Q. MT4 cells infected with recombinant SeV expressing each of the TRIM5αs were then superinfected with HIV-1 NL43, HIV-2 GH123, or HIV-2 GH123/Q. We used SeV expressing cynomolgus monkey TRIM5α lacking the SPRY domain, CM SPRY(−) TRIM5α, as a negative control for functional TRIM5α. Both CBC and CBB chimeric TRIM5αs as well as rhesus

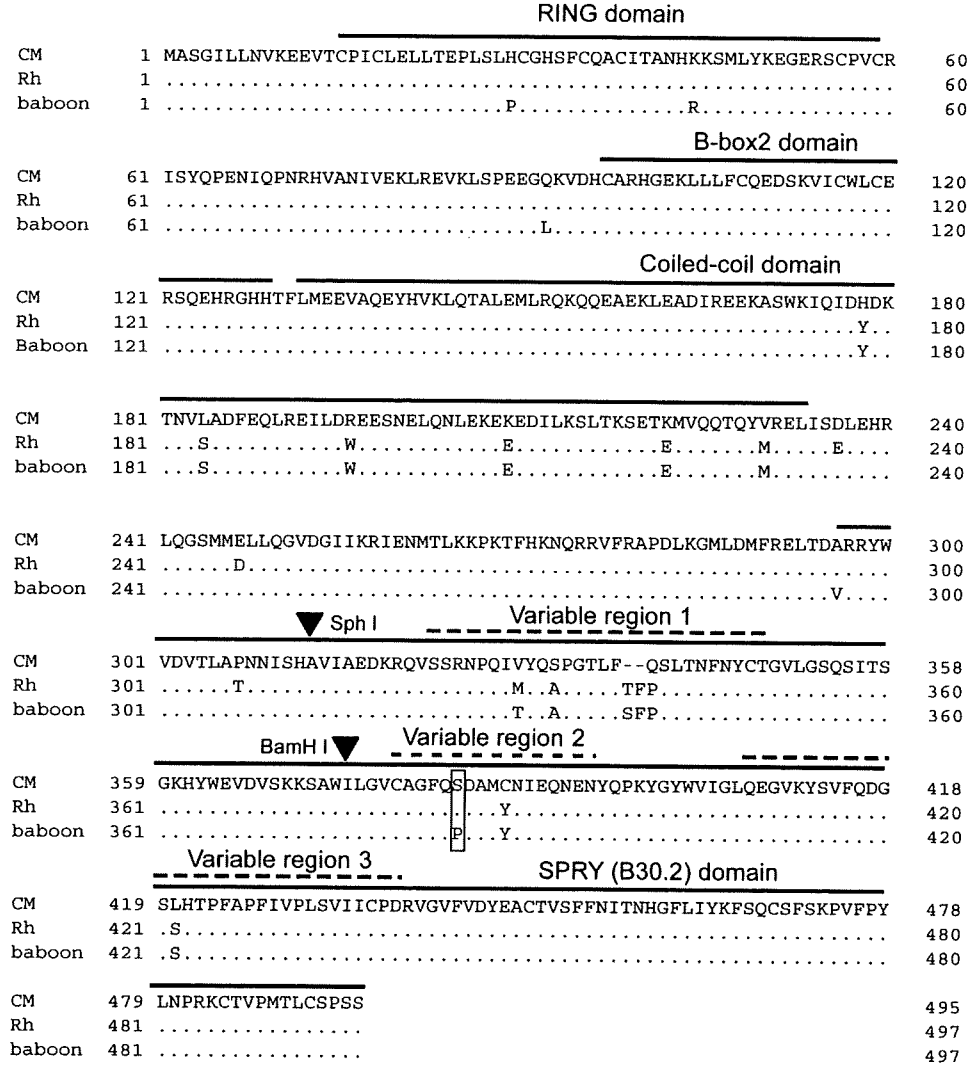


Fig. 1. Alignments of amino acid sequence of cynomolgus monkey (CM), rhesus monkey (Rh), and baboon TRIM5αs. The RING, B-box2, coiled-coil and SPRY (B30.2) domains are indicated by labeled bars over the sequences. Variable regions 1, 2, and 3 are indicated by a broken bar over the sequence. Inverted triangles denote Sph I and BamH I restriction enzyme site, respectively. Dots denote the amino acid residues identical to one of the cynomolgus monkey TRIM5αs and dashes denote a lack of amino acid residue that is present in rhesus monkey and baboon TRIM5αs. The box marks the amino acid residue that affects anti-HIV-2 activity of TRIM5α (see Results).

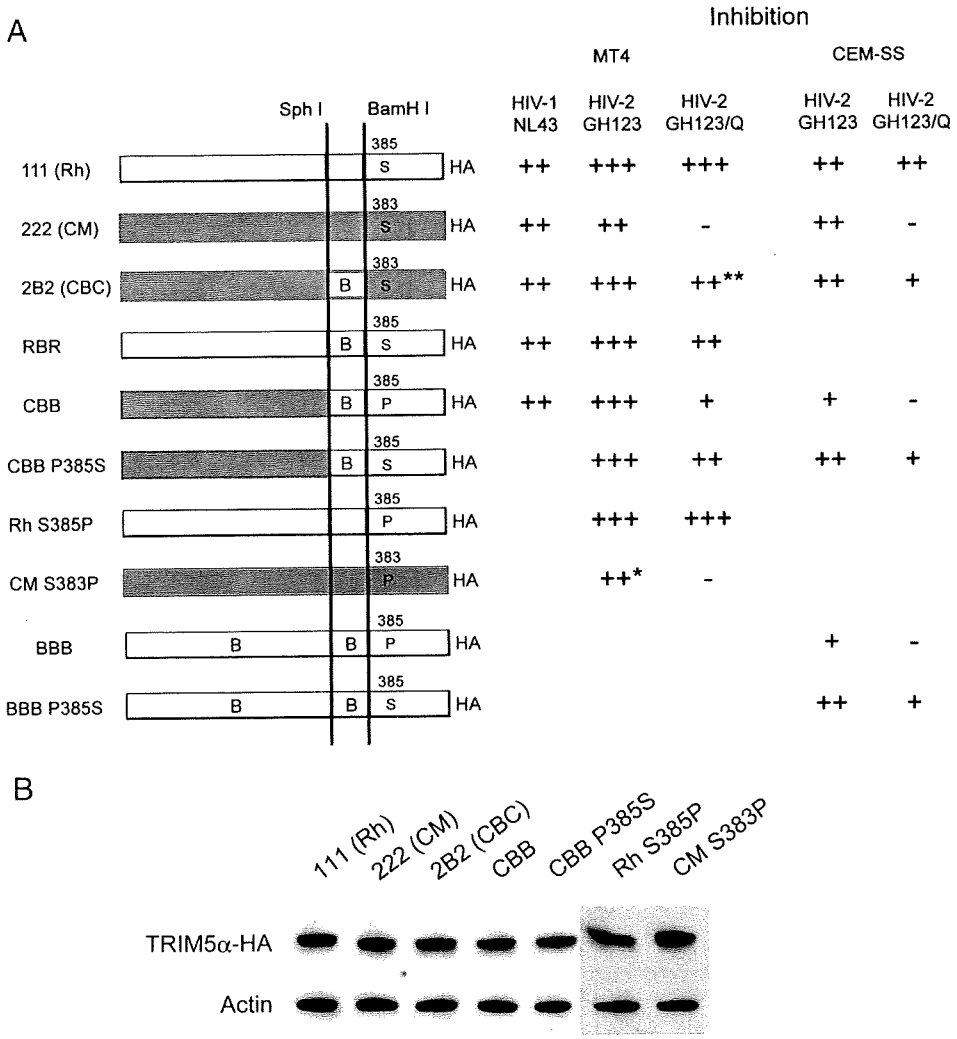
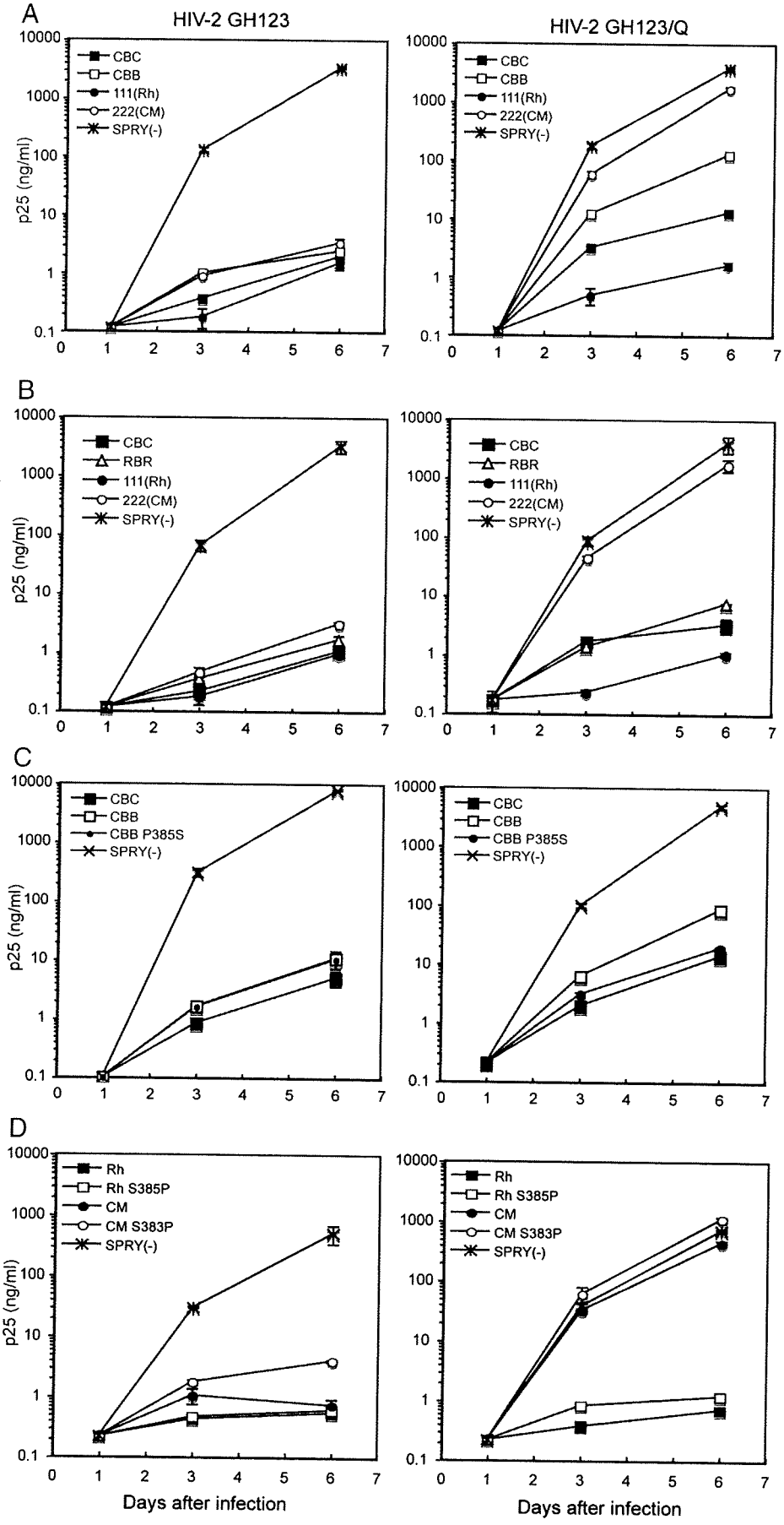


Fig. 2. (A) Schematic representation of chimeric and mutant TRIM5αs, and summary of the results. White and grey bars denote rhesus monkey (Rh) and cynomolgus monkey (CM) sequences, respectively. B denotes a baboon sequence. The amino acid at the 385th or 383rd position in V2 of the SPRY domain is indicated in the construct. +++, ++, +, and − denote more than 1000-fold, 100- to 1000-fold, 8- to 100-fold, and less than 8-fold suppression of virus growth, respectively, compared with the negative control on day 6. * denotes that anti-HIV-2 activity is slightly weaker than that of wild type CM TRIM5α. ** denotes that anti-HIV-2 GH123/Q activity of 2B2 (CBC) TRIM5α was assigned as +++ in our previous report (Kono et al., 2008), but it was assigned as ++ in the present study. Because the CBC TRIM5α suppressed HIV-2 GH123/Q approximately 1000-fold, a slight difference among experiments caused fluctuation of assignment. However, the order of anti-HIV-2 GH123/Q activity is fairly constant among different experiments (rhesus monkey TRIM5α is the most potent and CBC is the next). (B) Twenty-four hours after Sendai virus (SeV) infection, TRIM5α protein in lysates of MT4 cells infected with recombinant SeV expressing 111 (Rh), 222 (CM), 2B2 (CBC), CBB, CBB P385S, Rh S385P, or CM S383P TRIM5α were visualized by Western blotting with an antibody against HA tag.

monkey and cynomolgus monkey TRIM5αs almost completely restricted HIV-1 NL43 (data not shown) and HIV-2 GH123 (Fig. 3A left). In the case of HIV-2 GH123/Q (Fig. 3A right), rhesus monkey TRIM5α but not cynomolgus monkey TRIM5α restricted the virus growth as previously described (Kono et al., 2008). HIV-2 GH123/Q was restricted potently by CBC chimeric TRIM5α, although the virus grew at higher titer than in cells expressing rhesus monkey TRIM5α ($p<0.0005$, t-test, $n=8$), confirming the importance of V1 sequence of rhesus monkey TRIM5α to restrict HIV-2 GH123/Q (Kono et al., 2008). On the other hand, this virus was only moderately restricted by CBB chimeric TRIM5α, since the virus attained clearly higher titers in cells expressing CBB chimeric TRIM5α than in those expressing CBC chimeric TRIM5α ($p<0.0005$, t-test, $n=6$). This indicates that the anti-HIV-2 GH123/Q activity of CBB chimeric TRIM5α was weaker than that of CBC chimeric TRIM5α.

We further observed that rhesus monkey chimeric TRIM5α containing baboon V1 (RBR) restricted HIV-2 GH123/Q replication to the same extent as CBC chimeric TRIM5α did (Fig. 3B right). The difference between CBB and RBR chimeric TRIM5αs in the SPRY domain was detected only at the 385th amino acid residue in V2, where baboon TRIM5α carries P and rhesus monkey TRIM5α carries serine (S) (Fig. 1 box). CBC chimeric TRIM5α also carries S at the 385th position. These results thus strongly suggest that the amino acid residue at the 385th position of TRIM5α affects its restriction activity against HIV-2 GH123/Q infection. To confirm this hypothesis, we also constructed an SeV expressing mutant CBB TRIM5α in which amino

Fig. 3. (A) MT4 cells were infected with recombinant SeV expressing CBC (■), CBB (□), 111 (Rh) (●), 222 (CM) (○), or CM SPRY(−) (*) TRIM5α. (B) MT4 cells were infected with recombinant SeV expressing CBC (■), RBR (△), 111 (Rh) (●), 222 (CM) (○), or CM SPRY(−) (*) TRIM5α. (C) MT4 cells were infected with recombinant SeV expressing CBC (■), CBB (□), CBB P385S (●), or CM SPRY(−) (*) TRIM5α. (D) MT4 cells were infected with recombinant SeV expressing Rh (■), Rh S385P (□), CM (●), CM S383P (○), or CM SPRY(−) (*) TRIM5α. Nine hours after infection, cells were superinfected with HIV-2 GH123 (A–D, left) or HIV-2 GH123/Q viruses (A–D, right). Culture supernatants were separately assayed for levels of p25 from HIV-2. Error bars show actual fluctuations between levels of p25 in duplicate samples. A representative of two or three independent experiments is shown.


A
HIV-2 GH123
HIV-2 GH123/Q
CBC
CBB
111(Rh)
222(CM)
SPRY(-)
B
RBR
C
CBB P385S
D
Rh
Rh S385P
CM
CM S383P
p25 (ng/ml)
Days after infection

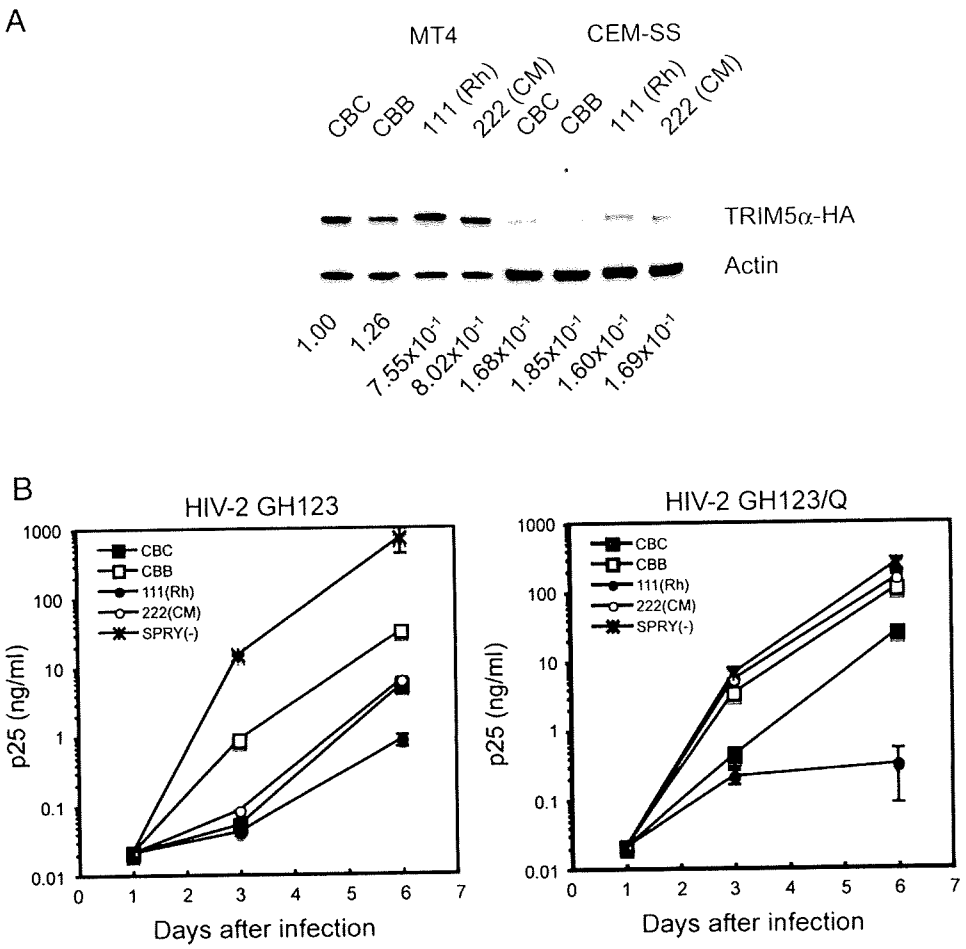


Fig. 4. (A) TRIM5α protein lysates of MT4 and CEM-SS cells infected with recombinant SeV expressing CBC, CBB, 111 (Rh), or 222 (CM) were visualized by Western blotting with antibodies against HA tag and actin. The relative TRIM5α expression obtained from the band intensity of TRIM5α divided by that of actin is shown. (B) CEM-SS cells were infected with recombinant SeV expressing CBC (■), CBB (□), 111 (Rh) (●), 222 (CM) (○), or CM SPRY(−) (*) TRIM5α. Nine hours after infection, cells were superinfected with HIV-2 GH123, or HIV-2 GH123/Q viruses. Culture supernatants were separately assayed for levels of p25. Error bars show actual fluctuations between levels of p25 in duplicate samples. A representative of three independent experiments is shown.

acid residue P at the 385th position was replaced with S (CBB P385S TRIM5α; Fig. 2A) and compared its ability to restrict HIV-2 GH123/Q with that of CBB TRIM5α. As expected, CBB P385S TRIM5α could inhibit HIV-2 GH123/Q replication to a larger extent than CBB chimeric TRIM5α did (Fig. 3C right) ($p<0.00005$, t-test, $n=4$). This finding shows that a single amino acid substitution from P to S at the 385th position of baboon TRIM5α strengthens its anti-HIV-2 activity.

A. single amino acid in V2 of the SPRY domain of TRIM5α affects its restriction activity against HIV-2

Cynomolgus monkey TRIM5α has S at the 383rd amino acid position, which corresponds to the 385th amino acid of baboon and rhesus monkey TRIM5αs (Fig. 1 box). To know whether an S-to-P amino acid substitution at the 385th or 383rd position also affects anti-HIV-2 activity of rhesus or cynomolgus monkey TRIM5α, we constructed SeVs expressing mutant rhesus monkey TRIM5α with amino acid residue S at the 385th position replaced with P (Rh S385P TRIM5α) and mutant cynomolgus monkey TRIM5α with S at the 383rd position replaced with P (CM S383P TRIM5α) (Fig. 2A). These mutant TRIM5αs were expressed at levels similar to those of the wild type TRIM5α (Fig. 2B). CM S383P TRIM5α and the wild type cynomolgus monkey TRIM5α restricted HIV-2 GH123 replication (Fig. 3D left), whereas they failed to restrict HIV-2 GH123/Q replication (Fig. 3D right). However, it should be noted that HIV-2 GH123 attained slightly higher titers on day 6 in cells expressing CM S383P TRIM5α than in those expressing the wild type cynomolgus monkey TRIM5α (Fig. 3D left). The difference was very small, but statistically significant in six independent samples ($p<0.0001$, t-test). In contrast, we could not detect any statistically significant differences in anti-HIV-2 activity between the wild type and mutant rhesus monkey TRIM5αs in six independent samples (Fig. 3D). It is possible that the effects of small amino acid changes in V2 on anti-viral activity vary depending on differences in V1.

Chimeric TRIM5α containing the baboon SPRY domain failed to restrict HIV-2 GH123/Q replication in human T cell line CEM-SS

After we obtained data presented in Fig. 3, we realized that the expression levels of TRIM5α protein by SeVs are much lower in another T cell line CEM-SS than those in MT4 cells. To examine the restriction ability of chimeric TRIM5αs with more physiological levels of expression, we repeated the experiment of Fig. 3A in CEM-SS cells. The expression levels of TRIM5α compared to those of actin protein in CEM-SS cells were approximately one-fifth of those in MT4 cells (Fig. 4A). As shown in Fig. 4B, all TRIM5αs inhibited HIV-2 GH123 replication, although the extent of inhibition varied among the TRIM5αs. CBB chimeric TRIM5α partially inhibited HIV-2 GH123 (Fig. 4B left) but failed to inhibit HIV-2 GH123/Q (Fig. 4 B right) in CEM-SS cells. These findings demonstrate that chimeric TRIM5α containing the baboon SPRY domain fails to restrict HIV-2 GH123/Q replication.

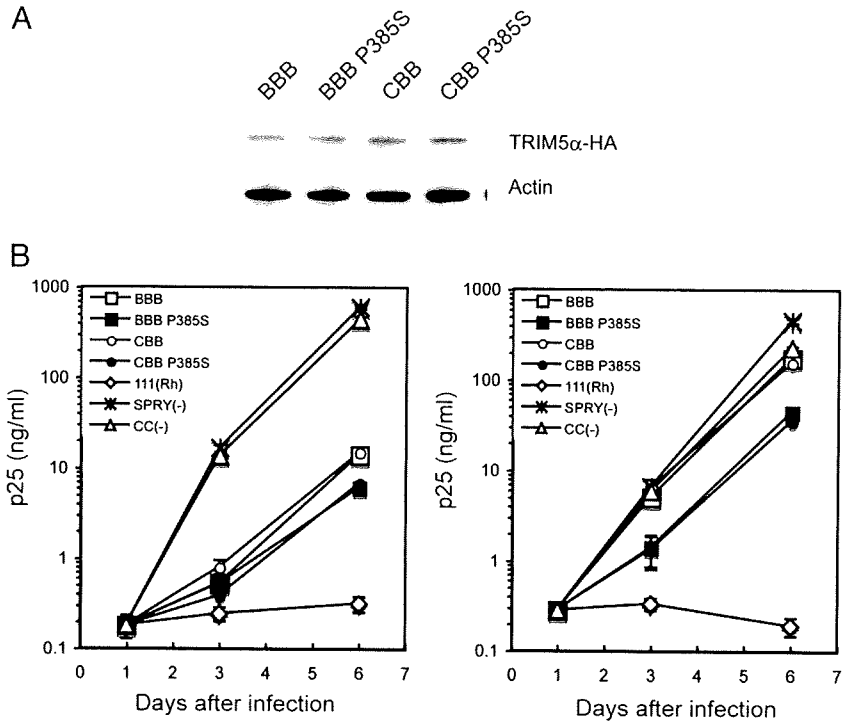


Fig. 5. (A) TRIM5α protein lysates of CEM-SS cells infected with recombinant SeV expressing BBB, BBB P385S, CBB, or CBB P385S TRIM5α were visualized by Western blotting with antibodies against HA tag and actin. (B) CEM-SS cells were infected with recombinant SeV expressing BBB (□), BBB P385S (■), CBB (○), CBB P385S (●), 111 (Rh) (◇), CM SPRY(−) (*), or AGM TRIM5α lacking coiled-coil domain (CC(−) TRIM5α) (Δ). Nine hours after infection, cells were superinfected with HIV-2 GH123, or HIV-2 GH123/Q viruses. Culture supernatants were separately assayed for levels of p25. Error bars show actual fluctuations between levels of p25 in duplicate samples. A representative of two independent experiments is shown.

The full-length baboon TRIM5α also failed to restrict HIV-2 GH123/Q replication in CEM-SS cells

Finally, we constructed SeV expressing the full-length baboon TRIM5α (BBB TRIM5α), and the mutant baboon TRIM5α in which amino acid residue P at the 385th position was replaced with S (BBB P385S TRIM5α) to confirm the effect of the 385th amino acid in the full-length baboon TRIM5α. Expression levels of BBB and BBB P385S TRIM5αs were comparable to those of CBB and CBB P385S TRIM5αs (Fig. 5A). Consistent with results presented in Fig. 4B, BBB TRIM5α restricted HIV-2 GH123 (Fig. 5B left), but failed to restrict HIV-2 GH123/Q (Fig. 5B right) in CEM-SS cells. Anti-HIV-2 GH123 and GH123/Q activities of BBB and BBB P385S TRIM5αs were comparable to those of CBB and CBB P385S TRIM5αs, respectively (Fig. 5B). These results excluded the possibility that the RBCC domain of baboon TRIM5α contains potency determinants for restriction of HIV-2. Furthermore, the difference in anti-HIV-2 GH123 and GH123/Q activity between BBB and BBB P385S TRIM5α was also observed in CEM-SS cells (Fig. 5B right) ($p<0.01$, $n=4$, t-test) and MT4 cells (data not shown) confirming that a single amino acid at the 385th position of the baboon TRIM5α affects its restriction activity against HIV-2. In Fig. 5B, there was no difference in the effects of HIV-2 GH123 and GH123/Q replication between CM SPRY(−) TRIM5α and AGM TRIM5α lacking coiled-coil domain (CC(−)TRIM5α). CM SPRY(−) TRIM5α suppressed activity of endogenous TRIM5α, while CC(−) TRIM5α was a non-interfering mutant (Nakayama et al., 2006; Maegawa et al., 2008). Therefore, these results indicated that effects of endogenous TRIM5α in CEM-SS cells could be neglected in our assay.

Structural model of the SPRY domain of baboon TRIM5α

To gain further insight into the structural effects of single amino acid substitution at the 385th position of the baboon SPRY domain, a three-dimensional (3-D) model of the baboon SPRY domain was constructed by a homology-modeling based on the recently published crystal structure of the PRYSPRY domain of mouse TRIM21 (protein data bank (PDB) ID: 2VOK and 2VOL) (Keeble et al., 2008) and the human PRYSPRY-19q13.4.1 domain (PDB ID: 2FBE) (Gruetter et al., 2006). A previous study described the 3-D model of the SPRY domain of TRIM5α based on the structure of the GUSTAVUS SPRY domain

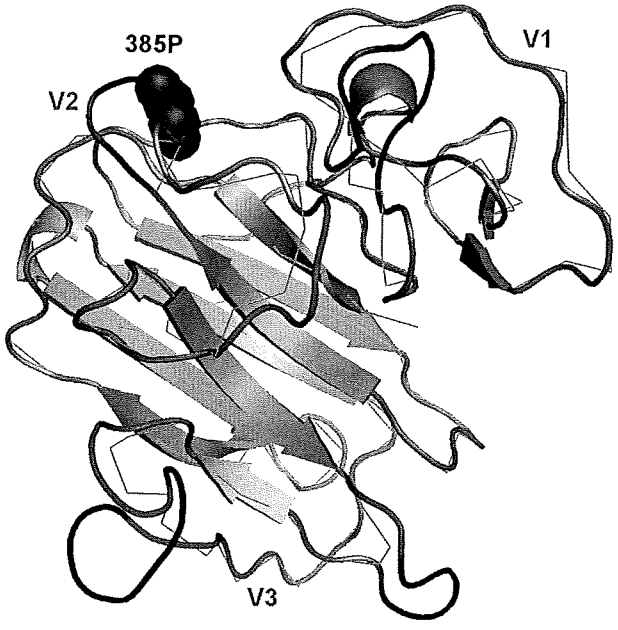


Fig. 6. Structural model of the SPRY domain of baboon TRIM5α. The 3-D model of the baboon SPRY domain was constructed with a homology-modeling technique using Modeller 9v4, and visualized with PyMol v1.0r2. The baboon SPRY model (green cartoon) is superimposed on the structure of the SPRY domain of mouse TRIM21 (orange backbone trace). Insertions in the baboon SPRY domain relative to the templates are shown in red, and V1, V2 and V3 in blue. The spheres indicate P at the 385th position in V2.

(PDB ID: 2FNJ) and 2FBE (Ohkura et al., 2006). The amino acid identity between the two more recently published SPRY domain structures (2VOK and 2VOL) and the SPRY domain of baboon TRIM5α is 35% and is over 10% larger than the domain identity with the templates used in the previous studies. Thus, owing to the use of more similar templates, we expect our model to be more reliable. In our model (Fig. 6), V1 and V2 are located in the loops at the surface of the SPRY domain structure. Unfortunately, P residue at the 385th position in V2 was one of the four amino acid insertions of the baboon SPRY domain sequence as compared to the templates. However, as the insertion is short, we do not expect major errors in this region of the model, and we can assume that the insertion is located at the protein surface. In the model, V2 is located near V1, and P residue at the 385th position of baboon TRIM5α faces toward V1. It was previously speculated that V1 and V2 are included in variable loops which form a canonical binding interface of TRIM and other protein families with the SPRY domain (James et al., 2007). A substitution involving P is expected to affect local backbone conformation, and a P-to-S substitution in particular results in an additional hydrogen bond donor–acceptor. Overall these findings indicate that position 385 is located at a putative binding site, where a P-to-S substitution is expected to affect the local structure and surface chemistry. This is consistent with the observed impact of these substitutions on TRIM5α anti-HIV-2 activity. Nevertheless, it is not yet clear how these substitutions affect TRIM5α restriction activity and binding affinity in particular, which calls for further investigation of the protein interactions involving TRIM5α.

Discussion

We previously reported that chimeric TRIM5α containing baboon V1 could restrict HIV-2 GH123/Q that was not restricted by cynomolgus monkey TRIM5α (Kono et al., 2008). Since baboons are reportedly sensitive to HIV-2 infection, there was a discrepancy between the ability of baboon TRIM5α V1 to restrict HIV-2 and the susceptibility of host to virus. In the study presented here, we showed that the anti-HIV-2 activities of full-length baboon TRIM5α and chimeric TRIM5α containing the entire baboon SPRY domain were weaker than that of chimeric TRIM5α containing baboon V1 alone, and that the full-length baboon TRIM5α and chimeric TRIM5α containing the entire baboon SPRY domain failed to restrict HIV-2 GH123/Q in CEM-SS cells. These results were consistent with those of the previous *in vivo* studies (Barnett et al., 1994; Locher et al., 1998, 2001). We also found that a single amino acid in V2 of the SPRY domain of TRIM5α affected its anti-HIV-2 activity.

There are several precedents that a single amino acid change in V1 and V3 of the SPRY domain of TRIM5α affects its restriction activity against retroviruses (Stremlau et al., 2005; Yap et al., 2005; Peron et al., 2006). A previous study comparing orangutan and gorilla TRIM5αs showed that the amino acid residues at the 385th and 389th positions (corresponding to the 389th and 393rd positions of baboon TRIM5α, the second and third boxes, respectively, indicated by white arrows in Fig. 7) in V2 of the SPRY domain of orangutan TRIM5α are important

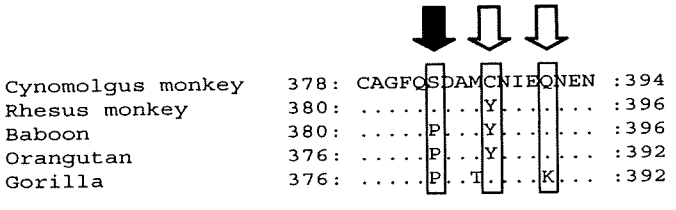


Fig. 7. Alignment of amino acid sequences of V2 within the SPRY domain of cynomolgus monkey, rhesus monkey, baboon, orangutan, and gorilla TRIM5αs. The first box indicated by the black arrow denotes the amino acid residue that affects anti-HIV-2 activity. The second and third boxes indicated by white arrows denote the amino acid residues that are important for anti-HIV-1 and anti-SIVmac activity of orangutan TRIM5α.

for inhibiting HIV-1 and SIVmac (Ohkura et al., 2006). Ours is the first report to demonstrate the importance of another amino acid residue at the 385th (baboon) or 383rd (cynomolgus monkey) position in V2 of the SPRY domain of TRIM5α (the first box indicated by a black arrow in Fig. 7) for restricting HIV-2. A structural model of the baboon TRIM5α SPRY domain revealed that this 385th amino acid residue located near V1, the major determinant of species-specific viral restriction of TRIM5α. In contrast to baboon and cynomolgus monkey TRIM5αs, there was no evidence of an obvious contribution by the 385th amino residue for anti-HIV-2 activity in the case of rhesus monkey TRIM5α background. It was reported that the specific combination of amino acids in V1 and V2 plays an important role in determining specificity of restriction (Ohkura et al., 2006). Therefore, it is possible that the combination of amino acids in V1 and V2 of the SPRY domain of baboon as well as cynomolgus monkey TRIM5α is more stringent in its anti-HIV-2 activity than that of rhesus monkey TRIM5α.

We showed that anti-HIV-2 activity of CBC chimeric TRIM5α was almost the same as that of CBB P385S TRIM5α. The amino acid differences between CBC chimeric TRIM5α and CBB P385S TRIM5α in the SPRY domain were located at two amino acids positions at the 389th in V2 (the second box in Fig. 7) and at the 422nd in V3 (Fig. 1). This indicates that these amino acid substitutions did not affect the restriction activity of TRIM5α against HIV-2, though the amino acid substitution at the former position of gorilla TRIM5α was reported to affect its anti-SIVmac activity (Ohkura et al., 2006). This is another example that the effect of amino acid substitution at the specific position in V2 could vary among different species of TRIM5α.

We observed that the CBB chimeric TRIM5α only partially inhibited HIV-2 GH123 when we used CEM-SS cells, while it almost completely did so when we used MT4 cells. We also found that the CBB chimeric TRIM5α failed to inhibit HIV-2 GH123/Q when we used CEM-SS cells, while it at least partially limited replication of HIV-2 GH123/Q when we used MT4 cells. The anti-viral effect of TRIM5α was apparently weaker in CEM-SS cells than in MT4 cells, which was at least in part due to lower levels of TRIM5α expression by SeV in CEM-SS cells than in MT4 cells. The reason for these lower expression levels is not clear, however. Since the levels of other proteins such as green fluorescence protein, TRIM22, and CCR5 expressed in CEM-SS cells by SeV were also low (data not shown), this is not specific to TRIM5α. We confirmed that the SeV could infect MT4 and CEM-SS cells with the same efficiency (data not shown). The low levels of TRIM5α expression in CEM-SS cells are therefore attributed to low efficiency in transcription or translation of the proteins expressed by SeV. When we used CEM-SS cells, the anti-HIV-2 GH123 activity of CBB TRIM5α was weaker than that of cynomolgus monkey TRIM5α. It is possible that baboon is sensitive to broader strains of HIV-2 than cynomolgus monkey.

Although we have clearly demonstrated that the full-length baboon TRIM5α cannot inhibit HIV-2 GH123/Q in CEM-SS cells, it is still possible that genomic polymorphisms in baboon TRIM5α could affect the sensitivity of baboons to HIV-2. In rhesus monkey TRIM5α V1, there is a 339th–TFP–341st to Q polymorphism, which reduces the strength of anti-HIV-2 activity (Newman et al., 2006; Kono et al., 2008). It would be interesting to investigate whether baboons TRIM5α have genomic polymorphism(s), which could alter the anti-viral activity.

Materials and methods

Cloning and expression of TRIM5α

Construction of cynomolgus monkey TRIM5α carrying an HA tag at the C-terminus (CM-TRIM5α-HA), rhesus monkey TRIM5α carrying an HA tag at the C-terminus (Rh-TRIM5α-HA), CBC chimeric TRIM5α carrying an HA tag at the C-terminus (CBC chimeric TRIM5α-HA), RBR chimeric TRIM5α carrying an HA tag at the C-terminus (RBR chimeric TRIM5α-HA), CM SPRY(−) TRIM5α, and CC(−) TRIM5α were described previously (Nakayama et al., 2005; Song et al., 2007; Kono

et al., 2008; Maegawa et al., 2008). To obtain the C-terminal portion of baboon TRIM5α fused with an HA tag (BamH I to Not I), we performed site-directed mutagenesis by using the PCR-mediated overlap primer extension method (Ho et al., 1989). Briefly, two DNA fragments with overlapping ends were generated by using the outer primers and the complementary primers with overlapping complementary nucleotides containing the desired mutations. The resultant two fragments were then combined in the subsequent fusion reaction to anneal the overlapping ends. This allows the 3′ overlap of each strand to serve as a primer for a 3′ extension of the complementary strand. Cloned RBR chimeric TRIM5α-HA was used as a template for PCR-amplification with outer primers (5′-GCGGCCGCTACTATGGCTTCTGC-3′ and 5′-GAATTCTCAAGAGCTTGGTGA-3′) and complementary primers (5′ GGCTTCCAACCTGATGCAATG-3′ and 5′-CATTGCATCAGGTTGGAAGCC-3′) containing nucleotides specific for baboon TRIM5α (underlined). To generate CBB chimeric TRIM5α, the previously generated C-terminal portion of baboon TRIM5α fused with HA tag (BamH I to Not I) and the N-terminal portion of CBC chimeric TRIM5α (Not I to BamH I) were assembled on the pcDNA3.1 (−) vector (Invitrogen, Carlsbad, CA). To generate CBB P385S TRIM5α, the N-terminal portion of CBC chimeric TRIM5α (Not I to BamH I) and the C-terminal portion of Rh-TRIM5α-HA (BamH I to Not I) were assembled on the pcDNA3.1 (−) vector.

To generate mutant rhesus monkey TRIM5α, in which an amino acid residue S at the 385th position was replaced with an amino acid P found in baboon TRIM5α (Rh S385P TRIM5α), the N-terminal portion of rhesus monkey TRIM5α (Not I to BamH I) and the C-terminal portion of CBB chimeric TRIM5α-HA (BamH I to Not I) were assembled on the pcDNA3.1 (−) vector. Mutant cynomolgus monkey TRIM5α, in which an amino acid residue S at the 383rd position was replaced with an amino acid P (CM S383P TRIM5α), was generated by site-directed mutagenesis with the PCR-mediated overlap primer extension method as described above. To obtain the BamH I–Not I fragment of CM S385P TRIM5α, cloned cynomolgus monkey TRIM5α was used as a template for PCR-amplification with the outer and complementary primers specified above. The resultant fragment and the N-terminal of cynomolgus monkey TRIM5α (Not I and Bam HI) were assembled on the pcDNA3.1 (−) vector.

Full-length baboon TRIM5α (BBB TRIM5α) was generated by the site-directed mutagenesis. To obtain the N-terminal portion, we performed seven successive PCR reactions by using cloned rhesus monkey and cynomolgus monkey TRIM5αs as templates. The primers used in these reactions are Not-TRIM primer (5′-GCGGCCGCTACTATGGCTTCTGG-3′), B1For primer (5′-CCTGAGTCTGCCCTGCGGCCACA-GCTTCTGCCAAGCGTGCATCACTGCGAACCACAGGAAGTCCATGC-3′), B1Rev primer (5′-GCATGGACTTCCTGTGGTTCGCAGTGATGCACGCTTGG-CAGAAGCTGTGGCCGCAGGGCAGACTCAGG-3′), B2For primer (5′-CCAGAAGAGGGACTGAAGGTTGATCACTGTGC-3′), B2Rev primer (5′-GCACAGTGATCAACCTTCAGTCCCTCTTCTGG-3′), B3For primer (5′-GAGAGAGCTCATCTCAGATCTGGAGC-3′), B3Rev primer (5′-GCTCCA-GATCTGAGATGAGCTCTCTC-3′), and B4Rev primer (5′-GCATGC-GAAATGTTGTTTGGAGCCAGTGTCACATCAACCCAGTAGCGTCGGA-CATCTGTTAGC-3′) containing nucleotides specific for baboon TRIM5α (underlined). The first PCR reaction was performed by using rhesus monkey TRIM5α as a template and Not-TRIM and B1Rev as primers. The second reaction was performed by using rhesus monkey TRIM5α as a template and B1For and B2Rev as primers. The resultant fragments were used as templates in the third PCR reaction with Not-TRIM and B2Rev as primers. The forth reaction was performed by using rhesus monkey TRIM5α as a template and B2For and B3Rev as primers. The resultant fragments were used as templates in the fifth reaction with Not-TRIM and B3Rev as primers. The sixth reaction was performed by using cynomolgus monkey TRIM5α as a template and B3For and B4Rev as primers. The fifth and sixth fragments were used as templates in the seventh reaction with Not-TRIM and B4 Rev as primers. The resultant fragment and the C-terminal of CBB TRIM5α (Sph I and Not I) were assembled on the pcDNA3.1 (−) vector. To generate mutant baboon TRIM5α (BBB P385S TRIM5α), the N-terminal portion of BBB TRIM5α (Not I and BamH I) and the C-terminal portion of rhesus monkey TRIM5α (BamH I and Not I) were assembled on the pcDNA3.1 (−) vector. The entire coding sequences of those TRIM5αs were verified and then transferred to the Not I site of pSeV18b+. Recombinant SeVs carrying various TRIM5αs were recovered with a previously described method (Nakayama et al., 2005). The viruses were passaged twice in embryonated chicken eggs and used as stock for all experiments.

Viral infection

2×10^5 MT4 or CEM-SS cells were infected with SeV expressing each of the TRIM5αs at a multiplicity of infection of 10 plaque-forming units per cell and incubated at 37 °C for 9 h. Cells were then superinfected with 20 ng of p25 from HIV-2 GH123, or GH123/Q strains. The culture supernatants were collected periodically, and the level of p25 was measured with a RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Statistical analysis

Differences in relative p25 levels to control CM SPRY(−) TRIM5α among chimeric TRIM5αs were evaluated by *t*-test.

Western blot analysis

MT4 or CEM-SS cells infected with recombinant SeVs expressing HA tagged TRIM5α proteins were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonident P40, 0.5% sodium deoxycholate). TRIM5α proteins in the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the gel were then electronically transferred to a membrane (Immobilion; Millipore, Billerica, MA). Blots were blocked and probed with anti-HA high affinity rat monoclonal antibody (Roche, Indianapolis, IN) and anti-actin affinity rabbit polyclonal antibody (Sigma, St. Louis, MO) overnight at 4 °C. Blots were then incubated with peroxidase-conjugated anti-rat IgG (American Qualex, San Clemente, CA) and peroxidase-linked protein A (Amersham, Piscataway, NJ), and bound antibodies were visualized with a Chemilumi-One chemiluminescent kit (Nacalai Tesque, Kyoto, Japan).

Modeling

Three protein domain structures were used as the templates for building the model: SPRY domains 2VOK and 2VOL of mouse TRIM21 (Keeble et al., 2008) and the human PRYSPRY domain 2FBE (Gruetter et al., 2006). To adjust the alignment for the variable positions of the TRIM5α SPRY domain in primates, five other primate TRIM5α SPRY domain sequences were used: human (NCBI accession number AK027593), african green monkey (AB210050), cynomolgus monkey (AB210052), rhesus monkey (AY523632) and sooty mangabey (AY710303, EF551344). Alignment of the baboon and other primate SPRY domain sequences to the three templates was obtained with the T-Coffee method (Notredame et al., 2000). Two additional methods (Edgar, 2004, Thompson et al., 1994) have also been used to generate multiple sequence alignments. Comparison of the alignments generated by the three methods revealed only minor differences. In particular, the three alignments differ by a shift of two to three positions in the placement of four insertions in the primate SPRY domain relative to the template sequences. This indicates that the alignment is reliable except in the regions of these four insertions since other parts of the sequences are aligned in the same manner independently of the method used. The model was built using Modeller 9v4 (Eswar et al., 2006; http://salilab.org/modeler) and visualized with PyMol v1.0r2 (DeLano WL, http://www.pymol.org).

TRIM5α cDNA sequences

TRIM5α cDNA sequences for baboon (AY843505), orangutan (DQ437601), and gorilla (DQ4307600) were obtained from the GenBank database.

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Modification of a loop sequence between α-helices 6 and 7 of virus capsid (CA) protein in a human immunodeficiency virus type 1 (HIV-1) derivative that has simian immunodeficiency virus (SIVmac239) *vif* and CA α-helices 4 and 5 loop improves replication in cynomolgus monkey cells

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Abstract

Background: Human immunodeficiency virus type 1 (HIV-1) productively infects only humans and chimpanzees but not cynomolgus or rhesus monkeys while simian immunodeficiency virus isolated from macaque (SIVmac) readily establishes infection in those monkeys. Several HIV-1 and SIVmac chimeric viruses have been constructed in order to develop an animal model for HIV-1 infection. Construction of an HIV-1 derivative which contains sequences of a SIVmac239 loop between α-helices 4 and 5 (L4/5) of capsid protein (CA) and the entire SIVmac239 *vif* gene was previously reported. Although this chimeric virus could grow in cynomolgus monkey cells, it did so much more slowly than did SIVmac. It was also reported that intrinsic TRIM5α restricts the post-entry step of HIV-1 replication in rhesus and cynomolgus monkey cells, and we previously demonstrated that a single amino acid in a loop between α-helices 6 and 7 (L6/7) of HIV type 2 (HIV-2) CA determines the susceptibility of HIV-2 to cynomolgus monkey TRIM5α.

Results: In the study presented here, we replaced L6/7 of HIV-1 CA in addition to L4/5 and *vif* with the corresponding segments of SIVmac. The resultant HIV-1 derivatives showed enhanced replication capability in established T cell lines as well as in CD8+ cell-depleted primary peripheral blood mononuclear cells from cynomolgus monkey. Compared with the wild type HIV-1 particles, the viral particles produced from a chimeric HIV-1 genome with those two SIVmac loops were less able to saturate the intrinsic restriction in rhesus monkey cells.

Conclusion: We have succeeded in making the replication of simian-tropic HIV-1 in cynomolgus monkey cells more efficient by introducing into HIV-1 the L6/7 CA loop from SIVmac. It would be of interest to determine whether HIV-1 derivatives with SIVmac CA L4/5 and L6/7 can establish infection of cynomolgus monkeys *in vivo*.

Background

Human immunodeficiency virus type 1 (HIV-1) productively infects only humans and chimpanzees but not Old World monkeys (OWM) such as cynomolgus (CM) and rhesus (Rh) monkeys [1]. Unlike the simian immunodeficiency virus isolated from macaques (SIVmac), HIV-1 replication is blocked early after viral entry, before the establishment of a provirus in OWM cells [1-3]. This restricted host range of HIV-1 has greatly hampered its use in animal experiments and has caused difficulties for developing prophylactic vaccines and understanding HIV-1 pathogenesis. In order to establish a monkey model of HIV-1/AIDS, various chimeric viral genomes between SIVmac and HIV-1 (SHIV) have been constructed and tested for their replicative capabilities in simian cells. The first SHIV was generated on a genetic background of SIVmac with HIV-1 *tat*, *rev*, *vpu*, and *env* genes [4]. Although such a SHIV is useful for the analysis of humoral immune responses against the Env protein [5-7], SHIVs containing other HIV-1 structural proteins, especially the Gag-Pol protein, have become highly desirable, since cellular immune response against Gag is generally believed to be important for disease control [8-10].

In recent years, several host factors involved in HIV-1 restriction in OWM cells have been identified. ApoB mRNA editing catalytic subunit (APOBEC) 3 G modifies the minus strand viral DNA during reverse transcription, resulting in an impairment of viral replication [11-13]. This activity could be counteracted with the viral protein Vif [14-17]. Although HIV-1 Vif can potently suppress human APOBEC3G, it is not effective against Rh APOBEC3G, which explains at least partly why HIV-1 replication is restricted in monkey cells. It is well known that Cyclophilin A (CypA) binds directly to the exposed loop between α-helices 4 and 5 (L4/5) of HIV-1 capsid protein (CA), but not to the SIVmac CA. Several studies have found that CypA augments HIV-1 infection in human cells but inhibits its replication in OWM cells [18-20]. A construction of a SHIV with a minimal segment of SIVmac was reported recently by Kamada et al. [21]. This SHIV was designed to evade the restrictions mediated by APOBEC3G and CypA in OWM cells and contains the 7-aa segment corresponding to the L4/5 of CA and the entire *vif* of SIVmac. The SHIV was found to be able to replicate in primary CD4+ T cells from pig-tailed monkey as well as in the CM HSC-F T cell line. Both in HSC-F and in primary CD4+ T cells, this chimeric virus grew to lower titers than did SIVmac [21]; and when inoculated into pig-tailed monkeys, this SHIV did not cause CD4+ T cell depletion or any clinical symptoms in the inoculated animals [22]. Another SHIV, stHIV-1 (a virus carrying 202 amino acid residues of SIVmac CA and *vif* generated by Hatziioannou et al.) could replicate efficiently in Rh cells [23]. However, long-term passaging in Rh cells was necessary to generate an efficiently replicating stHIV-1, and this adapted virus has not yet been fully characterized; so it may be that further modifications of the viral genome are necessary for optimal replication of HIV-1 genomes in OWM cells.

TRIM5α, a member of the tripartite motif (TRIM) family proteins, was identified in 2004 as another intrinsic restriction factor of HIV-1 in OWM cells [24]. Rh and CM TRIM5α were found to restrict HIV-1 but not SIVmac [25,26]. TRIM5α recognizes the multimerized CA of an incoming virus by its α-isoform specific SPRY domain [27-29] and is believed to be involved in innate immunity to control retroviral infection [30]. Previously, Ylinen et al. mapped one of the determinants of TRIM5α sensitivity in L4/5 of HIV type 2 (HIV-2) CA [31]. In addition, we identified a single amino acid of the surface-exposed loop between α-helices 6 and 7 (L6/7) of HIV-2 CA as a determinant of the susceptibility of HIV-2 to CM TRIM5α [32]. We hypothesized that the L6/7 of HIV-1 CA also determines susceptibility to CM TRIM5α. Here, we investigated whether an additional replacement of L6/7 of HIV-1 CA with that of SIVmac would enhance the replication capability of a SHIV genome in established T cell line HSC-F and in CD8+ cell depleted peripheral blood mononuclear cells (PBMCs) from CMs.

Materials and methods

DNA constructions

The HIV-1 derivatives were constructed on a background of infectious molecular clone NL4-3 [33]. NL-ScaVR, a virus containing SIVmac239 L4/5 and the entire vif gene, was constructed according to the procedure described by Kamada et al. [21]. A single amino acid His (H) at the 120th position of NL-ScaVR CA was replaced with Gln (Q) by means of site-directed mutagenesis with the PCR-mediated overlap primer extension method [34], and the resultant construct was designated NL-ScaVRA1. The L6/7 of CA (HNPPIP) of NL-SVR, NL-ScaVR, or NL-DT5R was also replaced with the corresponding segments of SIVmac239 CA (RQQNPIP) by means of site-directed mutagenesis, and the resultant constructs were designated NL-SVR6/7S, NL-ScaVR6/7S, or NL-DT5R6/7S, respectively. The BssHII-ApaI fragment of NL-ScaVR, NL-SVR6/7S, or NL-ScaVR6/7S, which corresponds to matrix (MA) and CA, was transferred to env deleted NL4-3 (NL-Nhe) to generate the env (-) version of each of the constructs.

Cells and Virus propagation

The 293 T (human kidney), LLC-MK2 (Rh kidney), and TK-ts13 (hamster kidney) adherent cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS. The CD4+ CXCR4+ CM T cell line HSC-F [35] was maintained in RPMI 1640 medium containing 10% FBS. Virus stocks were prepared by transfection of 293 T cells with HIV-1

NL4-3 derivatives using the calcium phosphate co-precipitation method. Viral titers were measured with the p24 or p27 RetroTek antigen ELISA kit (ZeptoMetrix, Buffalo, NY), and viral reverse transcriptase (RT) was quantified with the Reverse Transcriptase Assay kit (Roche Applied Science, Mannheim Germany).

Green fluorescence protein (GFP) vector

The HIV-1 vector expressing GFP was prepared as described previously [36,37]. To construct the HIV-1-WT-GFP and HIV-1-L4/5S-GFP vector, we replaced the Eco RI-Apa I fragment corresponding to MA and CA of the pMDLg/p.RRE packaging vector with those fragments from NL4-3 and NL-ScaVR, respectively. The GFP viruses were prepared from 293 T cells in a 15-cm dish by co-transfection with a combination of 24 μg of pMDLg/p.RRE derivatives, 36 μg of CS-CDF-CG-PRE (GFP encoding viral genomic plasmid), 10 μg of pMD.G (vesicular stomatitis virus glycoprotein (VSV-G) expressing plasmid), and 10 μg of pRSV-Rev (Rev expressing plasmid). Forty-eight hours after transfection, the culture supernatants were collected and used for infection.

Viral infections

3×10^5 MT4 or HSC-F cells were infected with 20 ng of p24 of NL4-3, NL-ScaV, NL-ScaVR, NL-ScaVR6/7S, NL-DT5R, or NL-DT5R6/7S. The culture supernatants were collected periodically, and p24 levels were measured with an ELISA kit.

Particle purification and Western blotting

The culture supernatant of 293 T cells transfected with plasmids encoding HIV-1 NL4-3 derivatives was clarified by means of low speed centrifugation. Nine ml of the resultant supernatants were layered onto a 2 ml cushion of 20% sucrose (made in PBS) and centrifuged at 35,000 rpm for 2 h in a Beckman SW41 rotor. After centrifugation, the virion pellets were resuspended in PBS, and p24 antigen concentrations were measured with ELISA. SDS-polyacrylamide gel electrophoresis was applied to 120 ng of p24 of HIV-1 derivatives, and virion-associated proteins were transferred to a PVDF membrane. CA and CypA proteins were visualized with the anti-p24 antibody (Biodesign International, Saco, ME) and the anti-CypA antibody (Affinity BioReagents, Golden, CO), respectively.

Saturation assay

HIV-1 derivatives or SIVmac particles were prepared by transfecting each of the env-deleted HIV-1 NL4-3 derivatives or SIVmac plasmids with a plasmid encoding VSV-G into 293 T cells, and culture supernatants were collected two days after transfection. One day before infection, Rh LLC-MK2 and hamster TK-ts13 were plated at a density of 5×10^4 cells per well in a 24-well plate. Prior to GFP virus infection, the cells were pretreated for 2 hours with 200 ng of p24 of each of the HIV-1 or SIVmac particles pseudotyped with VSV-G. Immediately after the pre-treatment, the cells were washed and infected with the HIV-1-WT-GFP or HIV-1-L4/5S-GFP virus. Two hours after infection, the inoculated GFP viruses were washed, and the cells were cultivated in fresh media. Two days after infection, the cells were fixed by formaldehyde, and GFP expressing cells were counted with a flowcytometer. To suppress endogenous TRIM5α activity, the cells were first infected with Sendai (SeV) expressing TRIM5 lacking the SPRY domain at a multiplicity of infection of 10 plaque forming units per cell. Sixteen hours after SeV infection, the cells were treated with 200 ng of p24 of the particles and then infected with the HIV-1-L4/5S-GFP vector as described above.

Preparation of CD8-depleted CM PBMCs and viral infection

CM PBMCs were suspended in RPMI medium 1640 supplemented with 10% (vol/vol) FBS, and the CD8+ cells were removed with a magnetic bead system (Miltenyi Biotec, Auburn, CA) and stimulated for 1 day with 1 μg/ml of PHA-L (Sigma, St. Louis. MO). For prolonged stimulation, CD8-depleted CM PBMCs were first stimulated with 1 μg/ml of PHA-L for 2 days and then with human IL2 100 U/ml for 2 more days. 3×10^5 cells were then inoculated with 200 ng of p24 of NL-DT5R, NL-DT5R6/7S or with 200 ng of p27 of SIVmac239 and incubated at 37°C in a medium containing 100 U/ml of human IL2. The culture supernatants were collected periodically, and the levels of p24 or p27 were measured with an antigen capture assay (Advanced BioScience Laboratories, Kensington, MD)

Results

Construction and characterization of HIV-1 molecular clones containing CA and Vif sequences from SIVmac239

Several proviral DNA constructs have been generated to counteract the restriction of HIV-1 replication in CM T cell line HSC-F [38] (Fig. 1). We first generated NL-SVR and NL-ScaVR according to the procedure described by Kamada et al. [21]. NL-ScaVR, a virus with SIVmac239 L4/5 CA and *vif*, could replicate slowly in HSC-F and replicated well in MT4 as previously reported (Fig. 2A). We recently discovered that the 120th amino acid of CA affected the sensitivity of HIV-2 to CM TRIM5α [32]. We, therefore, introduced an additional amino acid substitution, His to Gln, at this position in NL-ScaVR. The resultant virus was designated NL-ScaVRA1; but this virus unexpectedly showed less efficient replication than did the parental NL-ScaVR in both MT4 and HSC-F cells (Fig. 2A), probably due to a reduced viral fitness created by this mutation. We, therefore, replaced the entire L6/7 CA of NL-ScaVR (HNPPIP) with the corresponding loop from SIVmac239 (RQQNPIP), and the resultant virus was des-

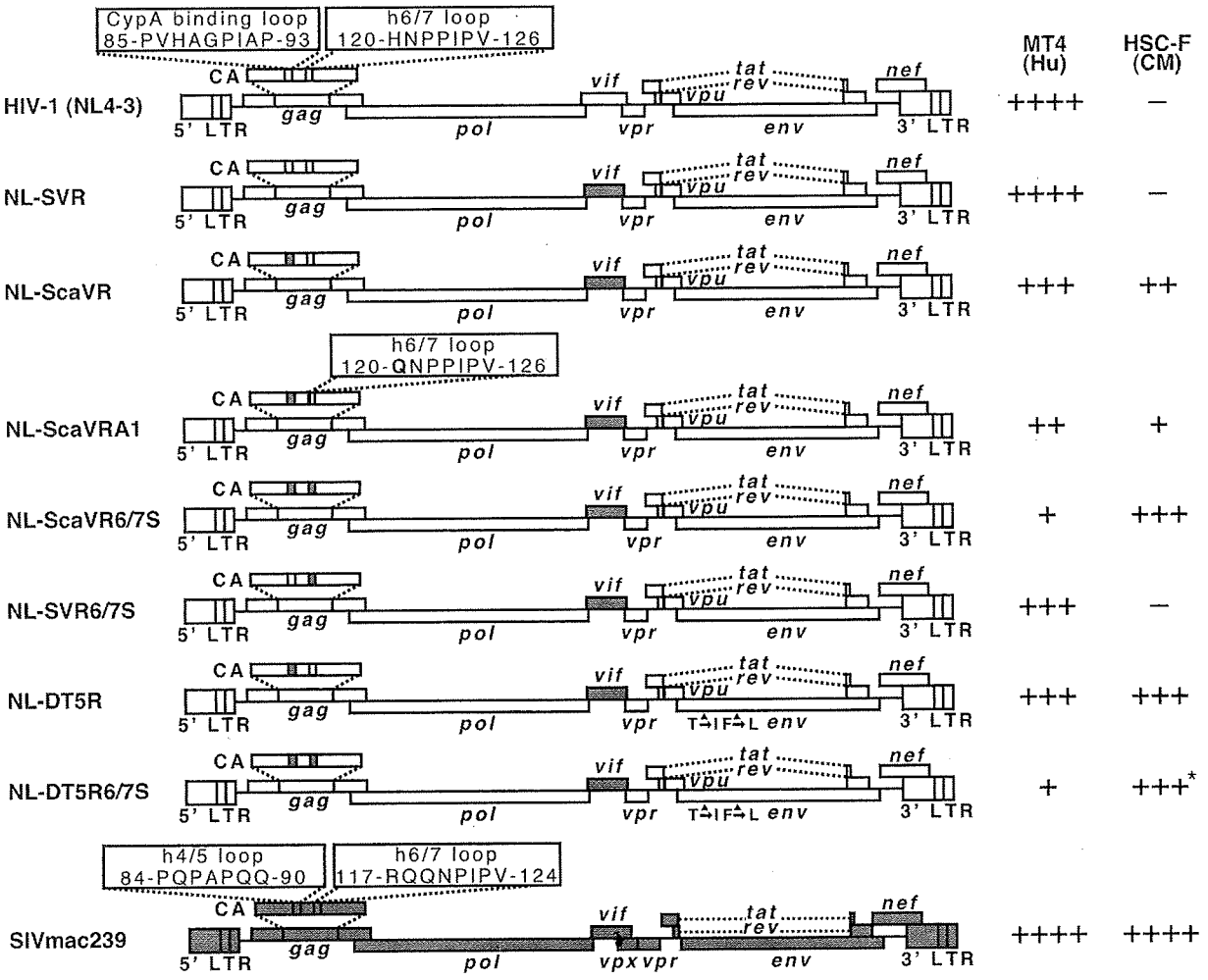

Figure 1

Structure of the chimeric HIV-1/SIVmac clones and a summary of their replication capabilities. White bars denote HIV-1 (NL4-3) and gray bars SIVmac239 sequences. ++++, +++, ++, +, and -denote the peak titer of virus growth in human (Hu) and cynomolgus monkey (CM) cells, respectively, to more than 1000 ng/ml, 100–1000 ng/ml, 10–100 ng/ml, 1–10 ng/ml, and less than 1 ng/ml concentration of capsid (CA) protein in the culture supernatants. * denotes that NL-DT5R6/7S replicated faster in HSC-F than did the parental NL-DT5R (see Fig. 2C).

ignated NL-ScaVR6/7S. The amount of RT per 1 ng of CA of NL-ScaVR (0.083 ng) was comparable to that of NL-ScaVR6/7S (0.081 ng), indicating that the replacement of L6/7 in HIV-1 with the corresponding loop of SIVmac did not affect the reactivity of CA antigen. Although NL-ScaVR6/7S grew slightly slower in MT4 cells, it could replicate more efficiently in HSC-F cells than the parental NL-ScaVR could (Fig. 2A). Similar results were obtained when we inoculated 20 ng of RT equivalent of NL-ScaVR or NL-ScaVR6/7S into HSC-F cells and measured the periodic RT production in culture supernatants (data not shown). These findings demonstrated that L6/7 CA of SIVmac improved the replication in CM cells of an HIV-1 derivative that already contained a SIVmac L4/5 and *vif*. We then generated NL-SVR6/7S, in which the L4/5 sequence was from HIV-1, but the L6/7 and *vif* came from SIVmac. NL-SVR6/7S showed better replication than NL-ScaVR6/7S in MT4 cells, but lost its replicative capability in HSC-F cells (Fig. 2B). NL-SVR, a virus with SIVmac *vif*, could replicate in MT4, but failed to do so in HSC-F (Fig. 2B). These results indicated that both L4/5 and L6/7 of SIVmac are required for efficient replication in HSC-F.

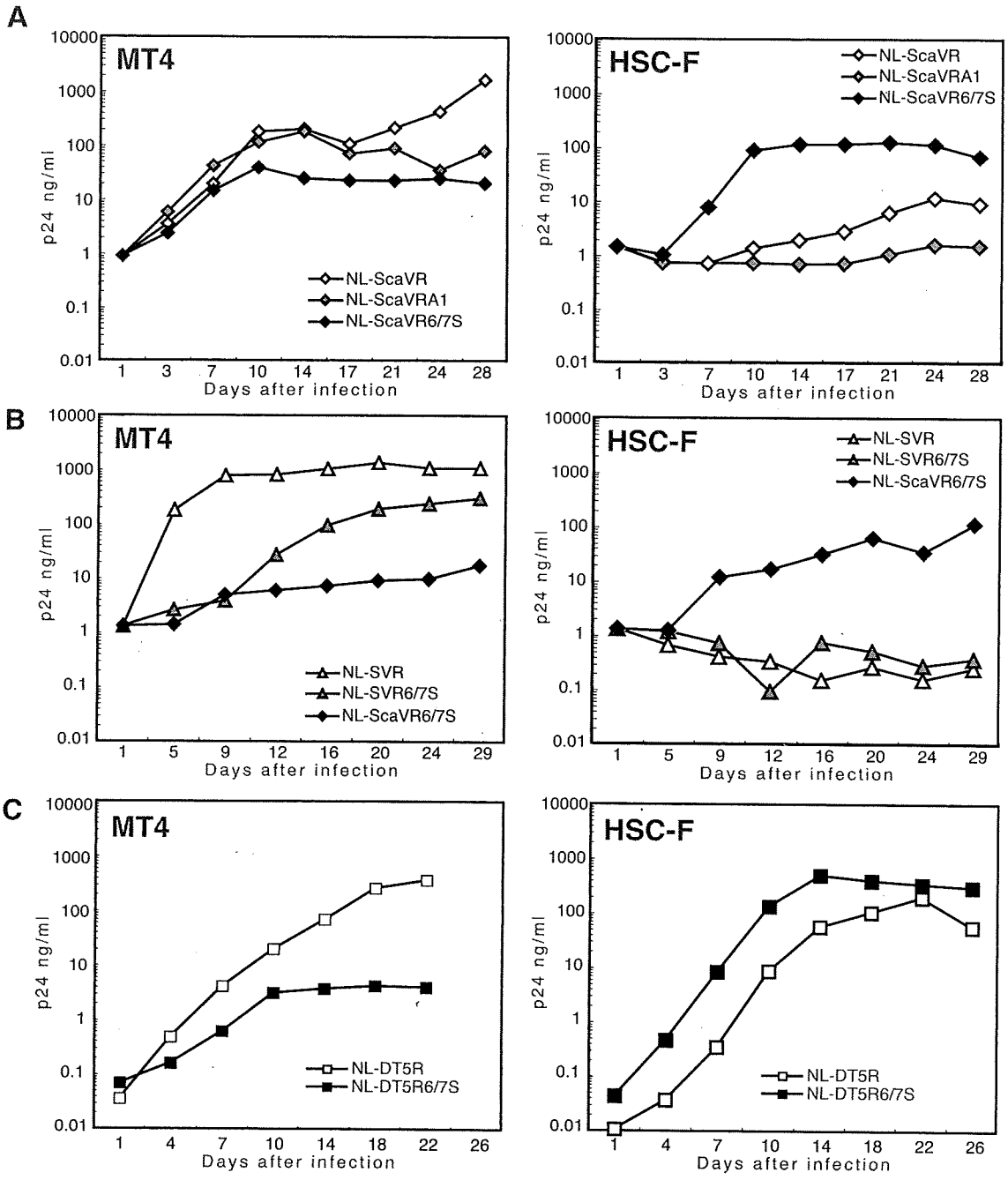

Figure 2

Replication properties of HIV-1 derivatives. Equal amounts of (A) NL-ScaVR (white diamonds: virus with SIVmac L4/5 and *vif*), and NL-ScaVRA1 (gray diamonds: virus with additional replacement of the 120th amino acid His with Gln in NL-ScaVR), and NL-ScaVR6/7S (black diamonds: virus with SIVmac L4/5, L6/7, and *vif*) (B) NL-SVR, NL-ScaVR6/7S, and NL-SVRS6/7S (gray diamonds: virus with SIVmac L6/7 and *vif*), and (C) NL-DT5R (white squares) and NL-DT5R6/7S (black squares), were inoculated into human MT4 or CM HSC-F cells, and culture supernatants were collected periodically. p24 antigen levels were measured by ELISA.

We then introduced SIVmac L6/7 into NL-DT5R, a molecularly cloned virus with two nonsynonymous changes in the *env* gene gained during long-term passages of NL-ScaVR in HSC-F cells [21]. The resultant virus was designated NL-DT5R6/7S. Although the peak titer of NL-DT5R6/7S was almost the same as that of NL-DT5R, NL-DT5R6/7S could replicate faster in HSC-F than the parental NL-DT5R (Fig. 2C). This finding confirmed that SIVmac L6/7 CA sequence improved the replication in CM cells of HIV-1 derivatives that contained SIVmac L4/5 and *vif*. The finding suggested that HIV-1 L6/7 and L4/5 CA sequences are important for intrinsic restriction in CM cells.

CypA incorporation into virus particles was not affected by replacement of HIV-1 L6/7 with that of SIVmac

Several studies have demonstrated that CypA augments HIV-1 infection in human cells [39], but inhibits its replication in OWM cells [18-20]. CypA was packaged in HIV-1 but not in SIVmac virus particles. To determine whether the replacement of HIV-1 L6/7 with that of SIVmac affects CypA binding of HIV-1 CA, we performed Western blot analysis of viral particles from HIV-1 derivatives. As shown in Fig. 3 (upper panel), CypA proteins were clearly detected in the NL-SVR particles (lane 1) but not in those of NL-ScaVR (lane 3), thus confirming that the L4/5 sequence of HIV-1 but not of SIVmac is required for CypA incorporation into viral particles. CypA proteins were detected in NL-SVR6/7S (lane 2) but not in NL-ScaVR6/7S (lane 4), indicating that the additional replacement of HIV-1 L6/7 with that of SIVmac had little effect on CypA incorporation. This finding suggests that the effect of L6/7 replacement on viral growth was independent from CypA binding of HIV-1 CA. When we used anti-p24 antibody (Fig. 3, lower panel), p55 Gag precursors and p24 proteins were clearly detected. There were no differences in the amount of p24 or the ratio of p24 to p55 among the four HIV-1 derivatives, indicating that the HIV-1 Gag precursor proteins with SIVmac L4/5 and L6/7 were processed normally by the viral protease.

Replacement of both L4/5 and L6/7 of HIV-1 CA with the corresponding loops from SIVmac impaired the CA binding activity of TRIM5 in Rh cells

It is known that the intrinsic restriction factors working against HIV-1 in CM and Rh cells can be saturated by inoculation of a high dose of HIV-1 particles [19,40-42]. To determine whether alteration in the CA of HIV-1 would affect its ability to saturate restriction factors, Rh LLC-MK2 cells were pre-treated with equal amounts of VSV-G pseudotyped HIV-1 particles that were with or without SIVmac L4/5 and/or L6/7 CA to saturate intrinsic restriction factor(s). The pre-treated cells were then infected with GFP-expressing HIV-1 carrying SIVmac L4/5 CA (HIV-1-L4/5S-GFP), since we wanted to exclude any effects of CypA on

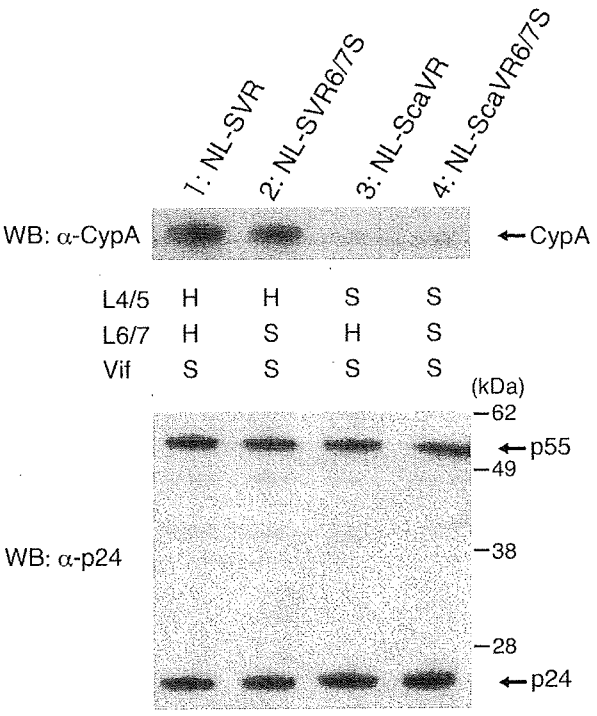

Figure 3
Western blot analysis of CA and CypA in particles of HIV-1 derivatives. The viral particles of NL-SVR (lane 1), NL-SVR6/7S (lane 2), NL-ScaVR (lane 3) and NL-ScaVR6/7S (lane 4) were purified by ultracentrifugation through a 20% sucrose cushion. CypA (upper panel) and p24 and p55 proteins (lower panel) were visualized by Western blotting (WB) using anti-CypA and anti-p24 antibody, respectively. "H" and "S" denote the amino acid sequences derived from HIV-1 and SIVmac, respectively.

the GFP expressing virus in LLC-MK2 cells. The susceptibility of particle-treated cells to virus infection was determined by the percentage of GFP-positive cells. The cells treated with the wild type (WT) particles showed greatly enhanced susceptibility to HIV-1 infection compared with non-treated cells (Fig. 4A, left), demonstrating that the intrinsic restriction factor(s) in LLC-MK2 cells were saturated by a high dose of particles. The cells treated with the particles carrying SIVmac L4/5 and those treated with particles carrying SIVmac L6/7 also showed enhanced susceptibility to HIV-1 infection (Fig. 4A, left). The cells treated with particles carrying both SIVmac L4/5 and L6/7 showed only slight enhancement of HIV-1 susceptibility (Fig. 4A, left; $p = 0.007$ compared by means of paired t test using all data points with the WT particle treated cells). Similarly, the cells treated with SIVmac particles showed only minor enhancement in HIV-1 susceptibility (Fig. 4A, left). Hamster TK-ts13 cells which lack TRIM5α expres-

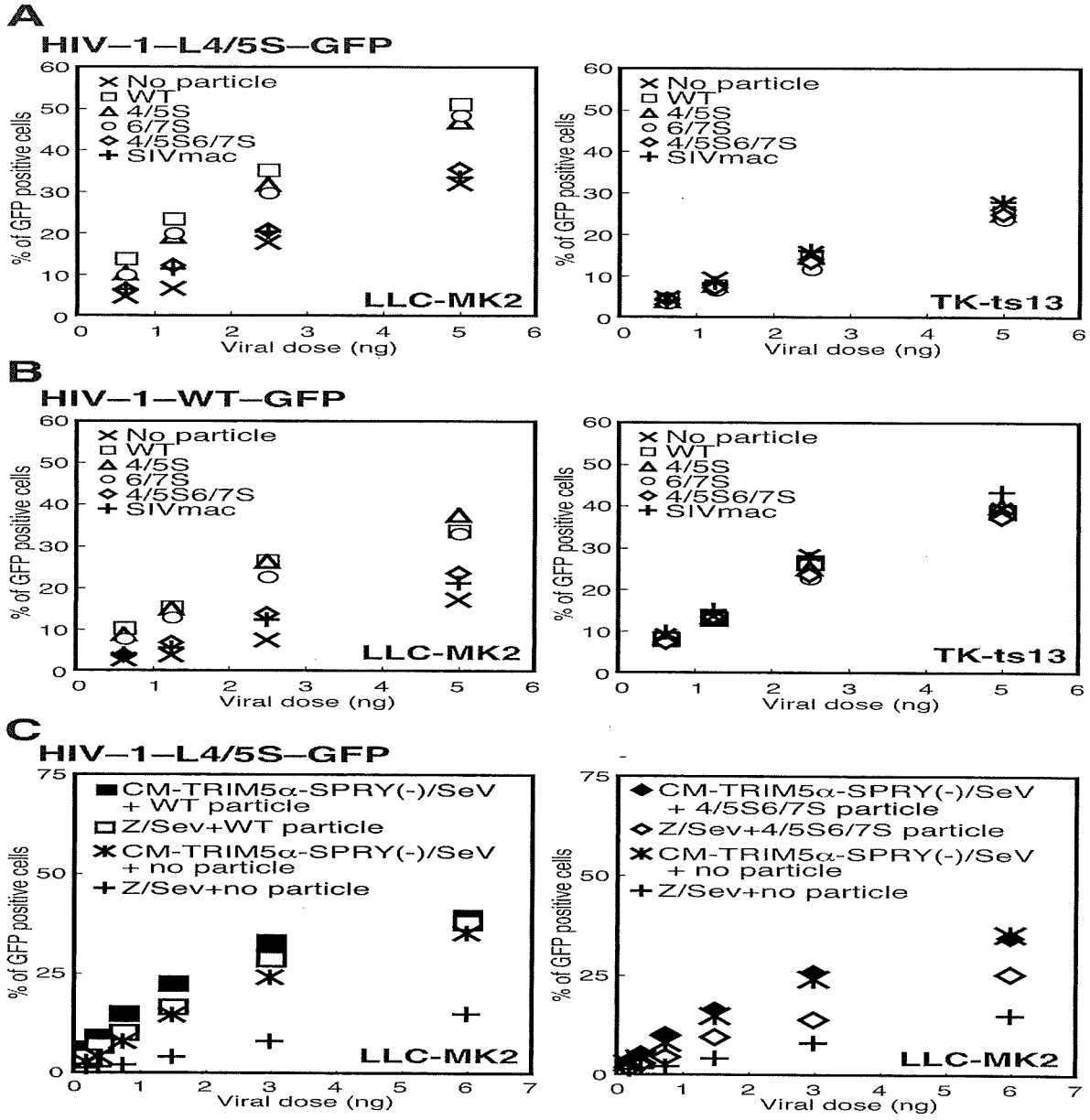

Figure 4

Saturation of intrinsic antiviral factors resulting from inoculation of high dose of virus particles. (A) Rhesus LLC-MK2 cells or hamster TK-ts13 cells were pre-treated with equal amounts of VSV-G pseudotyped particles with WT HIV-1 (white squares: Wt), with SIVmac L4/5 (white triangles: 4/5S), with SIVmac L6/7 (white circles: 6/7S), with SIVmac L4/5 and L6/7 (white diamonds: 4/5S6/7S), with SIVmac239 (pluses: SIVmac) or none (crosses) for 2 hours. The cells were then infected with the GFP expressing HIV-1 vector carrying SIVmac L4/5 (A: HIV-1-L4/5S-GFP) or GFP expressing HIV-1 vector with WT capsid (B: HIV-1-WT-GFP). Representative data of four independent experiments are shown. (C) Saturation activities were assessed in the presence or absence of functional TRIM5α. Before particle treatment, cells were infected with Sendai virus (SeV) expressing TRIM5 without the SPRY domain (black symbols), or an empty vector, parental Z strain of SeV (white symbols). Sixteen hours after SeV infection, cells were treated with particles for 2 hours and then infected with HIV-1-L4/5S-GFP. Representative data from six independent experiments are shown.

sion, on the other hand, showed no difference in HIV-1 susceptibility among cells treated with various HIV-1 derivatives or SIVmac particles (Fig. 4A, right). As shown in Fig. 4B, similar results were obtained when we used a GFP-expressing virus with WT HIV-1 capsid (HIV-1-WT-GFP). These results indicate that both HIV-1 L4/5 and L6/7 are important for CA binding to antiviral factor(s) in Rh cells. As described previously [20], HIV-1-WT-GFP could induce infection in only small numbers of LLC-MK2 cells. In contrast, more TK-ts13 cells were infected with HIV-1-WT-GFP than with HIV-1-L4/5-GFP. It is thus possible that CypA is a supporting factor for HIV-1 replication in hamster cells as well as in human cells.

Endogenous TRIM5α seems to be a likely candidate for the antiviral factor saturated by a high dose of HIV-1 particles (Fig. 4A and 4B). To confirm this, we assessed the ability of WT and mutant HIV-1 particles to saturate the intrinsic restriction factor in the presence or absence of functional TRIM5α. The dominant negative effect of an over-expressed TRIM5 mutant lacking SPRY domain [43] was used to suppress the function of cell endogenous TRIM5α. As shown in Fig. 4C, the infection of a recombinant SeV expressing TRIM5 without the SPRY domain caused marked enhancement of HIV-1-L4/5S-GFP virus infection without prior particle treatment (crosses vs. asterisks). This indicates that this dominant negative

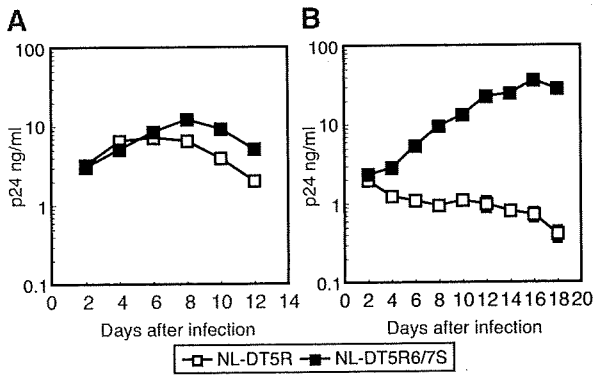

Figure 5
Replication capabilities of HIV-1 derivatives in peripheral blood mononuclear cells (PBMC) from CM. (A) PBMCs were obtained from CM, after which the CD8+ cells were removed, and the cells were stimulated with PHA-L for 1 day. (B) CD8-depleted CM PBMC were first stimulated with 1 μg/ml of PHA-L for 2 days and then with human IL2 100 U/ml for 2 more days. Equal amounts of p24 of NL-DT5R (white squares) or NL-DT5R6/7S (black squares) were inoculated, and the culture supernatants were collected periodically. p24 antigen levels were measured by ELISA. Values represent means with actual fluctuations of duplicate samples added. The values for mock infected cell culture supernatants were zero in the ELISA assay.

TRIM5 mutant successfully suppressed the restriction activity of endogenous TRIM5α. Treatment with the WT HIV-1 particles also saturated the restriction factors in the cells infected with the empty vector virus (parental Z strain of SeV), while the additional effect of the dominant negative mutant TRIM5α remained unclear (Fig. 4C left, white vs. black squares). These results suggest that the intrinsic factors saturated by the WT particles were mainly endogenous TRIM5α. In contrast to the effect of the WT particle treatment, the effect of the dominant negative TRIM5 mutant on HIV-1 infection was evident when we used particles with SIVmac L4/5 and L6/7 (Fig. 4C, right, white vs. black diamonds, $p = 0.007$, paired t test). These findings suggest that the diminished capability of particles with SIVmac L4/5 and L6/7 to saturate restriction factors was mainly due to their loss of interaction with TRIM5α. We, therefore, concluded that the ability of HIV-1 with SIVmac L4/5 and L6/7 to bind to TRIM5α is diminished in LLC-MK2 cells.

HIV-1 derivative with SIVmac L4/5, L6/7, and vif sequences can replicate efficiently in monkey primary cells

To verify the effect of additional replacement of HIV-1 L6/7 with that of SIVmac in primary CM cells, we prepared PBMCs from CM and removed CD8+ cells by means of magnetic beads. The cells were then stimulated for 1 day with 1 μg/ml of PHA-L. NL-DT5R6/7S showed more efficient replication than did the parental NL-DT5R in these cells and reached its peak titer 8 days after infection (Fig. 5A). For prolonged stimulation, CD8-depleted CM PBMCs were first stimulated with 1 μg/ml of PHA-L for 2 days and then with human IL2 100 U/ml for 2 more days. In these cells, NL-DT5R with HIV-1 L6/7 did not grow at all. On the other hand, NL-DT5R with SIVmac L6/7 (NL-DT5R6/7S) grew in CM primary cells in response to prolonged stimulation by PHA and IL-2 to reach titers, similar to those attained in cells with short stimulation, up to 8 days after infection (Fig. 5A and 5B). Furthermore, NL-DT5R6/7S continued to grow to much higher titers and reached its peak titer 16 days after infection; this higher peak may be due to better proliferation of these cells than those cells receiving short term stimulation (Fig. 5B). These results confirmed that the replicative capability of HIV-1 in CM cells was augmented by the additional replacement of L6/7 of CA with the corresponding sequence from SIVmac.

Discussion

We created simian-tropic HIV-1 with more efficient replication capability in CM cells using the knowledge obtained from our previous study of TRIM5α and HIV-2 capsid sequence variations [32] Introduction of the entire SIVmac L6/7 CA into the previously constructed version of HIV-1 derivatives containing SIVmac L4/5 CA and *vif* [21] caused only a four amino acid change in CA but

showed improved replication capability of HIV-1 in the CM cell line HSC-F. Introduction of the entire SIVmac L6/7 CA into NL-DT5R, which has two additional amino acid mutations in the *env* gene, enhanced replication in CD8+ cells-depleted CM PBMCs. After prolonged stimulation of CM PBMCs, replication of the original version of NL-DT5R was suppressed while that of NL-DT5R with SIVmac L6/7 was not. It would thus be of interest to test whether those HIV-1 derivatives with both L4/5 andL6/7 from SIVmac can induce infection of CM *in vivo*.

While the high-dose inoculation of WT HIV-1 particles into Rh cells saturated endogenous TRIM5α and enhanced subsequent infection with HIV-1, the introduction of HIV-1 particles that contained both L4/5 and L6/7 from SIVmac greatly impaired the ability of the particles to saturate TRIM5α. When we replaced either HIV-1 L4/5 or L6/7 with the corresponding sequence from SIVmac, these particles still saturated TRIM5α. These findings suggest that TRIM5α recognized the overall structure composed of both L4/5 and L6/7 of HIV-1 CA. Our previous results from computational 3D-structure modeling analysis of HIV-2 CA support this hypothesis [32]. The 120th amino acid of HIV-2 CA, which affects viral susceptibility to TRIM5α restriction, was located in L6/7. It is especially worth noting that the amino acid substitution at the 120th position was previously predicted to induce marked changes in the configuration of L6/7 and the L6/7 with the CM TRIM5α-sensitive Pro positioned most closely to L4/5 of HIV-2 [32]. It would, therefore, be interesting to investigate whether monkey TRIM5α proteins recognize CypA bound-L4/5 of HIV-1 CA.

During the preparation of our manuscript, Lin and Emerman reported that SIVagmTAN with both HIV-1 L4/5 and L6/7 was susceptible to Rh-TRIM5α restriction [44]. Our result is consistent with their finding, since the HIV-1 particles with both SIVmac L6/7 and SIVmac L4/5 showed reduced saturation activity for TRIM5α in Rh cells compared with HIV-1 particles with SIVmac L4/5 alone. Hatziioannou et al. very recently reported that stHIV-1 strains, which differ from HIV-1 only in the *vif* gene, could efficiently replicate in pig-tailed monkey and proposed a pig-tail monkey model of HIV-1 infection [45]. This is not surprising, since pig-tailed monkeys lack a TRIM5α protein, and the dominant form of TRIM5 expressed in this monkey species is a TRIMCyp fusion protein lacking anti-HIV-1 activity [46-48].

When we subjected CD8-depleted CM PBMC to prolonged stimulation, NL-DT5R6/7S grew efficiently but NL-DT5R did not. Since the expression levels of TRIM5α mRNA in human PBMC increased after stimulation with PHA and IL2 for 3 days (data not shown), we speculated that the higher expression levels of CM-TRIM5α in fully stimulated CM cells resulted in efficient restriction of NL-DT5R. However, no clear enhancement of CM TRIM5α mRNA expression could be detected in the CM cells subjected to prolonged stimulation (data not shown). The reason why NL-DT5R failed to grow in CM cells with prolonged stimulation is not yet clear, but it is possible that fully stimulated CM cells exerted stronger intrinsic inhibitory activity against HIV-1 infection than those with short-term stimulation.

NL-DT5R6/7S and NL-ScaVR6/7S replicated less efficiently in human MT4 cells than did the parental NL-DT5R and NL-ScaVR. One possible explanation is that the virus with SIVmac L6/7 became resistant to CM TRIM5α but became more sensitive to human TRIM5α, since the latter can restrict SIVmac more efficiently than HIV-1. Another possibility is that replacement of CA allowed the virus to evade the intrinsic inhibitory factors in CM cells but impaired viral replication *per se*.

We used the CM T cell line HSC-F and CD8+ cell-depleted PBMC from CM but not from Rh for our replication experiments. Although we observed an improvement of viral replication in CM cells, we cannot assume that the replacement of L4/5 and L6/7 is enough for HIV-1 to replicate to high titers in Rh cells since the CM TRIM5α resistant HIV-2 mutant virus GH123 (Q) was found to be restricted by Rh TRIM5α [34]. NL-DT5R6/7S and NL-ScaVR6/7S also showed less efficient replication capability than did SIVmac (Fig. 1). We are currently trying to adapt these viruses to CM and Rh cells by means of long-term passaging in the hope of introducing compensating mutations that can overcome these disadvantages and further augment their replicative capabilities in human and simian cells to reach a similar level as seen with SIVmac.

Conclusion

We have succeeded in improving simian-tropic HIV-1 for more efficient replication in CM cells by introduction of the SIVmac L6/7 CA sequence. It will be of interest to determine whether the HIV-1 derivatives with SIVmac L4/5 and L6/7 can induce infection in cynomolgus monkeys *in vivo*. Even if they fail to do so, further modification and/or adaptation of the current version of simian-tropic HIV-1 in monkey cells might be expected to lead to the development of an HIV-1 infection model in OWMs. This model has been long-awaited as a tool for vaccine development and as a model for better understanding of AIDS pathogenesis.

Abbreviations

OWM: old world monkey; CM: cynomolgus monkey; Rh: rhesus monkey; SHIV: HIV-1/SIV chimeric virus; CypA: cyclophilin A; TRIM: tripartite motif; CA: capsid; PBMC: peripheral blood mononuclear cell; GFP: green fluores-

cence protein; VSV-G: vesicular stomatitis virus glycoprotein; SeV: Sendai virus; L4/5: a loop between α-helices 4 and 5; L6/7: a loop between α-helices 6 and 7.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TS and EEN designed the research, AK, AS, YS, and EEN performed the research, TS, MN, AA, and EEN analyzed the data, and AA, HA, TS, and EEN wrote the paper.

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