shown). We used SeV expressing cynomolgus monkey TRIM5α lacking the SPRY domain, CM SPRY(−) TRIM5α, as a negative control. MT4 cells infected with recombinant SeV expressing rhesus monkey TRIM5α, cynomolgus monkey TRIM5α, or CM SPRY(−) TRIM5α were then superinfected with an X4-tropic HIV-1 strain NL43, SIVmac239, HIV-2 strain GH123, or GH123/Q which is a mutant HIV-2 carrying glutamine (Q) at the 120th position of CA. In agreement with the results of previous studies, rhesus and cynomolgus monkey TRIM5α could restrict HIV-1 NL43, but failed to restrict SIVmac239 (Fig. 2, upper panels). HIV-2 GH123, cynomolgus monkey TRIM5α-sensitive strain, was restricted by both rhesus and cynomolgus monkey TRIM5αs (Fig. 2, lower left panel), which is also consistent with the findings of previous studies (Song et al., 2007). Despite a high degree of sequence similarity between rhesus and cynomolgus monkey TRIM5αs, rhesus monkey TRIM5α could inhibit replication of HIV-2 GH123/Q, the strain resistant to cynomolgus monkey TRIM5α (Fig. 2, lower right panel).

Variable region 1 (V1) of SPRY (B30.2) domain of rhesus monkey TRIM5α is a determinant for restriction of HIV-2 GH123/Q infection

It is known that the variable region 1 (V1) (Song et al., 2005a,b) of the rhesus monkey TRIM5α SPRY domain is the major determinant of anti-HIV-1 potency (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). To determine the precise region of TRIM5α responsible for the anti HIV-2 GH123/Q activity of rhesus monkey TRIM5α, we constructed a recombinant SeV expressing chimeric TRIM5αs between rhesus and cynomolgus monkey TRIM5α by using *Sph*I and *Bam*HI restriction enzyme digestion (Fig. 3A). The central fragment comes from *Sph*I and *Bam*HI digestion contains the V1 of SPRY domain (Fig. 1).

As expected, all forms of chimeric TRIM5α inhibited HIV-1 NL43 and HIV-2 GH123 replication, although the extent of inhibition of HIV-2 GH123 varied among chimeric TRIM5αs (Fig. 3B, left and center panel). On the other hand, 212, 112, and 211 chimeric TRIM5α restricted HIV-2 GH123/Q infection, whereas 121, 221, and 122 chimeric TRIM5α did not (Fig. 3B, right panel), although the expression levels of the latter 1 day after SeV infection were not lower than those of parental and other chimeric TRIM5αs (Fig. 3C). Furthermore, these chimeric TRIM5α expression levels at day 6 after SeV infection were almost equivalent to those at day 1 after SeV infection (data not shown). These results confirmed that stability and kinetics of expression were similar among these chimeric TRIM5αs. Since 212,112, and 211 chimeric TRIM5α possess the rhesus monkey TRIM5α V1, those results indicated that the rhesus monkey TRIM5α V1 is a determinant of anti HIV-2 GH123/Q activity. It is noteworthy that cynomolgus monkey TRIM5α-sensitive HIV-2 GH123 grew to slightly higher titers in the cells

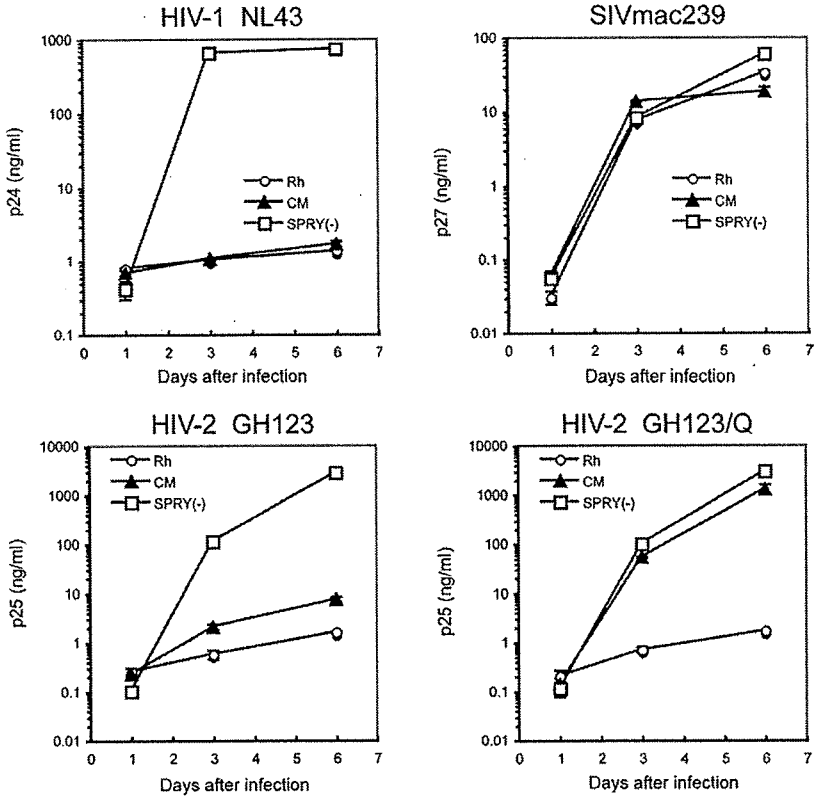

Fig. 2. MT4 cells infected with recombinant SeV expressing rhesus monkey (Rh; ○), cynomolgus monkey (CM; ▲), or CM SPRY(−) (□) TRIM5α were inoculated with HIV-1 NL43, SIVmac239, HIV-2 GH123, or HIV-2 GH123/Q. Culture supernatants were respectively assayed for levels of p24, p27, or p25. HIV-2 GH123/Q is a mutant virus carrying glutamine (Q) at the 120th position of HIV-1 GH123 capsid. CM SPRY(−) TRIM5α served as negative control.

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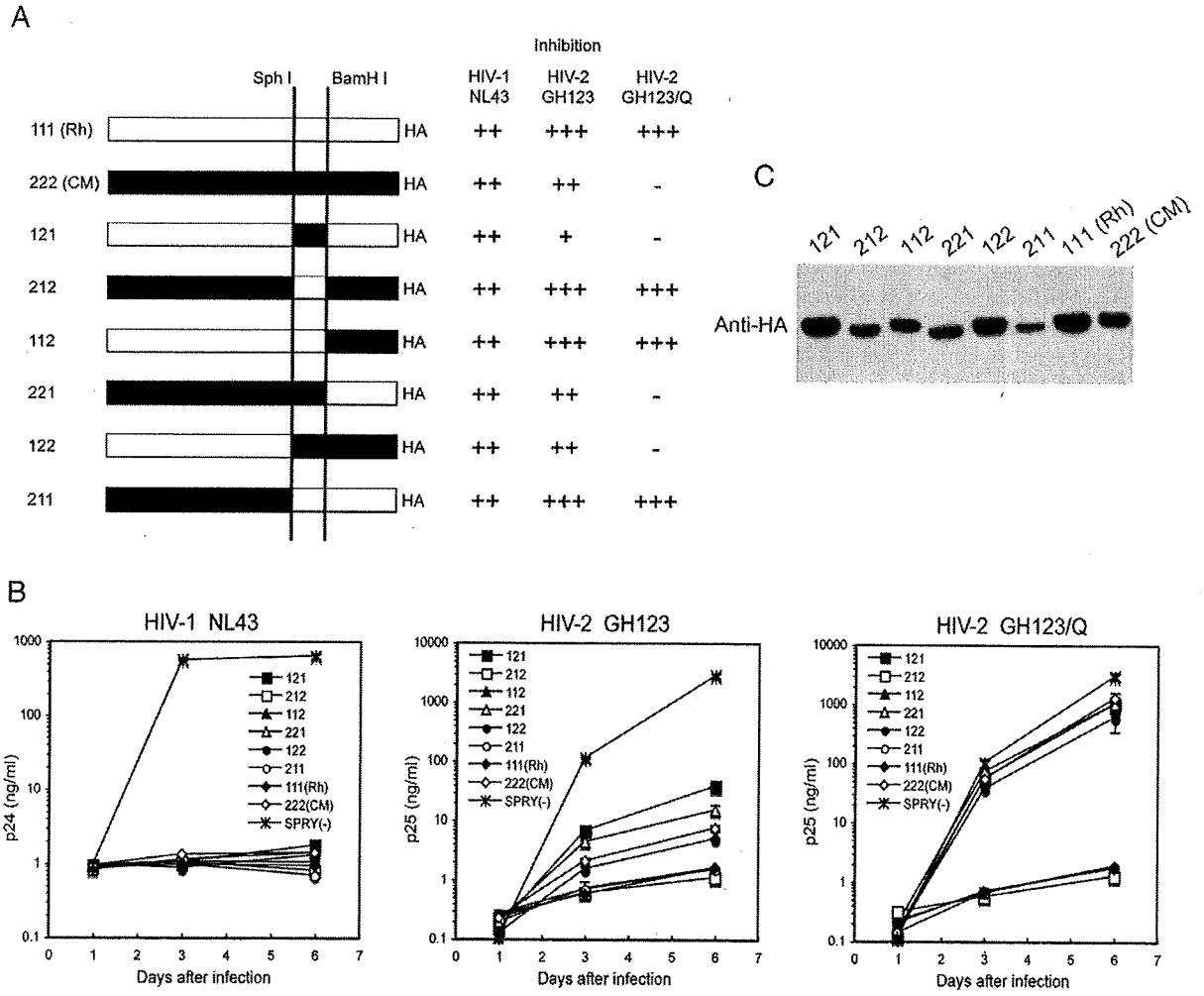

Fig. 3. (A) Schematic representation of chimeric TRIM5αs and summary of the results. White and black bars denote rhesus monkey (Rh) and cynomolgus monkey (CM) sequences, respectively. +++, ++, +, and − denote more than 1000-fold, 100- to 1000-fold, 8- to 100-fold, and less than 8-fold suppression of virus growth, respectively, compared with the negative control on day 6. (B) MT4 cells were infected with recombinant SeV expressing 121 (■), 212 (□), 112 (▲), 221 (△), 122 (●), 211 (○), 111 (Rh) (♦), 222 (CM) (◇), or CM SPRY(−) (*) TRIM5α. Nine hours after infection, cells were inoculated with HIV-1 NL43, HIV-2 GH123, or HIV-2 GH123/Q viruses. Culture supernatants were respectively assayed for levels of p24 or p25. (C) Twenty-four hours after SeV infection, TRIM5α proteins in lysates of MT4 cells infected with recombinant SeV expressing 121, 212, 112, 221, 122, 211, 111 (Rh), or 222 (CM) TRIM5α were visualized by Western blotting with an antibody against HA-tag.

expressing chimeric 121 or 221 TRIM5α than in those expressing parental cynomolgus monkey or 122 chimeric TRIM5α (Fig. 3B, center panel). These results indicate that the extent of inhibition of HIV-2 GH123 by chimeric 121 and 221 TRIM5αs is slightly less than that by parental cynomolgus monkey and 122 chimeric TRIM5α. It has been reported that all three variable regions (V1–V3) could contribute to the anti-viral activity of TRIM5α (Ohkura et al., 2006). It is possible that the combination of the cynomolgus monkey TRIM5α V1 and the rhesus monkey TRIM5α C-terminal portion of the SPRY domain slightly impairs the anti-HIV-2 function of TRIM5α.

V1 of SPRY domain of rhesus monkey TRIM5α is a determinant for broader and more potent anti-HIV-2 activity

To examine the restriction activities of rhesus monkey TRIM5α against other HIV-2 strains, we tested HIV-2 UC2, HIV-2 UC12, and HIV-2 UC14 for their growth potential in human T-cell line Hut78 infected with SeV expressing 121, 212, 111 (rhesus monkey; Rh), 222 (cynomolgus monkey; CM), or SPRY(−) TRIM5α. As shown in Fig. 4, rhesus monkey TRIM5α and cynomolgus monkey chimeric TRIM5α containing the V1 of rhesus monkey TRIM5α (212) completely restricted HIV-2 UC2 and HIV-2 UC14, whereas cynomolgus monkey TRIM5α and rhesus monkey chimeric TRIM5α containing the V1 of cynomolgus monkey TRIM5α (121) failed to do so. Rhesus monkey TRIM5α and 212 chimeric TRIM5α also completely restricted HIV-2 UC12, while cynomolgus monkey TRIM5α did so partially, which is consistent with our previous findings (Song et al., 2007). 121 chimeric TRIM5α showed very weak restriction activity on replication of HIV-2 UC12. Putting these findings together leads us to conclude that the V1 is a determinant for broader and more potent anti-HIV-2 activity of rhesus monkey TRIM5α.

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Amino acid residues TFP at the 339th to 341st positions of rhesus monkey TRIM5α are important for inhibiting HIV-2 GH123/Q replication

As shown in Fig. 1, the V1 of the SPRY domain of rhesus monkey TRIM5α contains two amino acid residues TF that are not present in cynomolgus monkey TRIM5α. To examine whether these two additional amino acid residues are responsible for the broad anti-HIV-2 activity of rhesus monkey TRIM5α, we constructed a recombinant SeV expressing rhesus monkey TRIM5α lacking these two amino acid residues (Rh deltaTF TRIM5α). We also constructed a SeV expressing mutant rhesus monkey TRIM5α in which amino acid residues TFP at the 339th to 341st positions were replaced with a single amino acid Q found in cynomolgus monkey TRIM5α (Rh TFP-Q TRIM5α) (Fig. 5A). Expression levels of mutant TRIM5αs in MT4 cells infected with these SeVs were comparable to those of parental TRIM5αs (Fig. 5B).

As shown in Fig. 5C, both of the mutant rhesus monkey TRIM5αs restricted HIV-1 infection. In the case of HIV-2, Rh deltaTF TRIM5α inhibited both HIV-2 GH123 and HIV-2 GH123/Q replications, although anti-HIV-2 activity was slightly weaker than that of parental rhesus monkey TRIM5α. On the other hand, Rh TFP-Q TRIM5α lost its inhibitory activity against HIV-2 GH123/Q, while it could suppress replication of HIV-2 GH123 to the same extent as it was suppressed by 121 chimeric TRIM5α. Conversely, cynomolgus monkey TRIM5α possessing TFP instead of Q at the 339th position (CM Q-TFP TRIM5α) completely restricted HIV-2 GH123/Q replication (Fig. 5D). These results indicated that these three amino acids are important for restricting HIV-2 GH123/Q by rhesus monkey TRIM5α.

Baboon TRIM5α V1 confers anti-viral activity against HIV-2 GH123/Q to cynomolgus monkey TRIM5α, whereas sooty mangabey and pig-tailed monkey TRIM5α V1 fail to do so

HIV-2 is thought to originate from the simian immunodeficiency virus of sooty mangabeys (Hahn et al., 2000). Several HIV-2 isolates could grow in baboon and pig-tailed monkey, and some of them were reported to cause an AIDS-like disease in these animals (Barnett et al., 1994; Locher et al., 1998; Locher et al., 2001; McClure et al., 2000). To assess anti-HIV-2 activity of these OWMs TRIM5αs, we constructed a recombinant SeV expressing cynomolgus monkey chimeric TRIM5α containing the V1 of baboon (2B2), sooty mangabey (2S2), or pig-tailed monkey (2P2) TRIM5α (Fig. 6A). Expression levels of these chimeric TRIM5αs in MT4 cells infected with the SeVs were comparable to those of parental cynomolgus monkey TRIM5α (Fig. 6C). The amino acid sequences of the V1 of baboon, sooty mangabey, and pig-tailed monkey TRIM5α are shown in Fig. 6B. Baboon and sooty mangabey TRIM5α show the SFP sequence at the 339th to 341st positions, whereas pig-tailed monkey TRIM5α has a single Q as cynomolgus monkey TRIM5α. As shown in Fig. 6D, all the chimeric TRIM5αs containing the V1 of baboon, sooty mangabey, and pig-tailed monkey TRIM5α restricted HIV-1 infection. These results are consistent with those of previous studies (Kaiser et al., 2007; Newman et al., 2006; Ohkura et al., 2006). In the case of HIV-2, there were wide variations in anti-HIV-2 activity by the chimeric TRIM5αs. Chimeric TRIM5α containing baboon V1 inhibited both HIV-2 GH123 and HIV-2 GH123/Q replications, while chimeric TRIM5α containing sooty mangabey V1 partially inhibited HIV-2 GH123 and only slightly inhibited HIV-2 GH123/Q replication. Finally, chimeric TRIM5α containing pig-tailed monkey V1 only slightly inhibited both HIV-2 GH123 and HIV-2 GH123/Q replication. This shows that there is a lack of correlation between the effects of various TRIM5αs on HIV-2

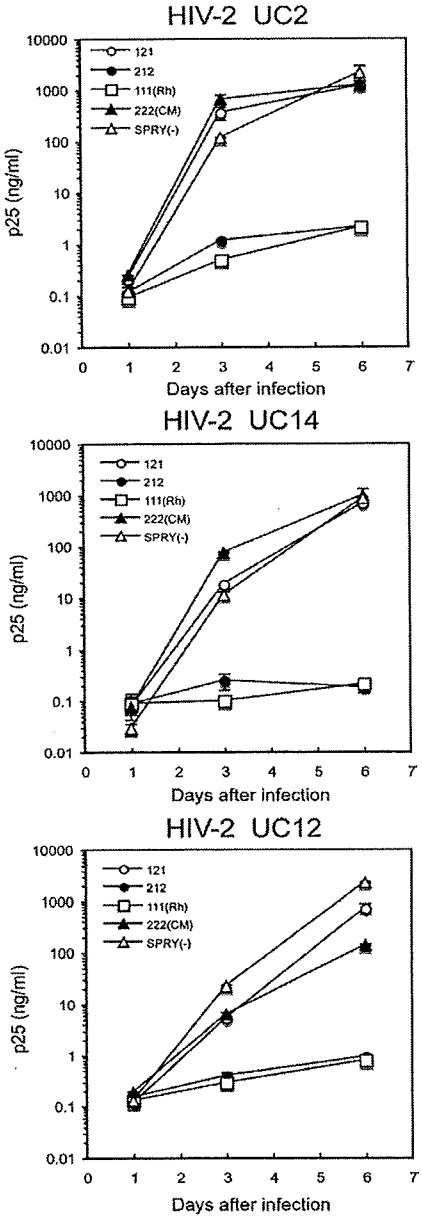

Fig. 4. Hut78 cells infected with recombinant SeV expressing 121 (○), 212 (●), 111 (Rh) (□), 222 (CM) (▲) or CM SPRY(−) (△) TRIM5α. Cells were superinfected with HIV-2 isolates, UC2, UC14, or UC12. Culture supernatants were assayed for levels of p25.

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A

	Amino acid sequence	Inhibition HIV-1 NL43	HIV-2 GH123	HIV-2 GH123/Q
Rh	QIMTQAPGTLFTFPSLN	++	+++	+++
CM	..V..S.....--Q...	++	++	-
Rh deltaTF	..........--....	++	++	++
Rh TFP-Q	..........--Q...	++	++	-
CM Q-TFP	..V..S..........	++	+++	+++

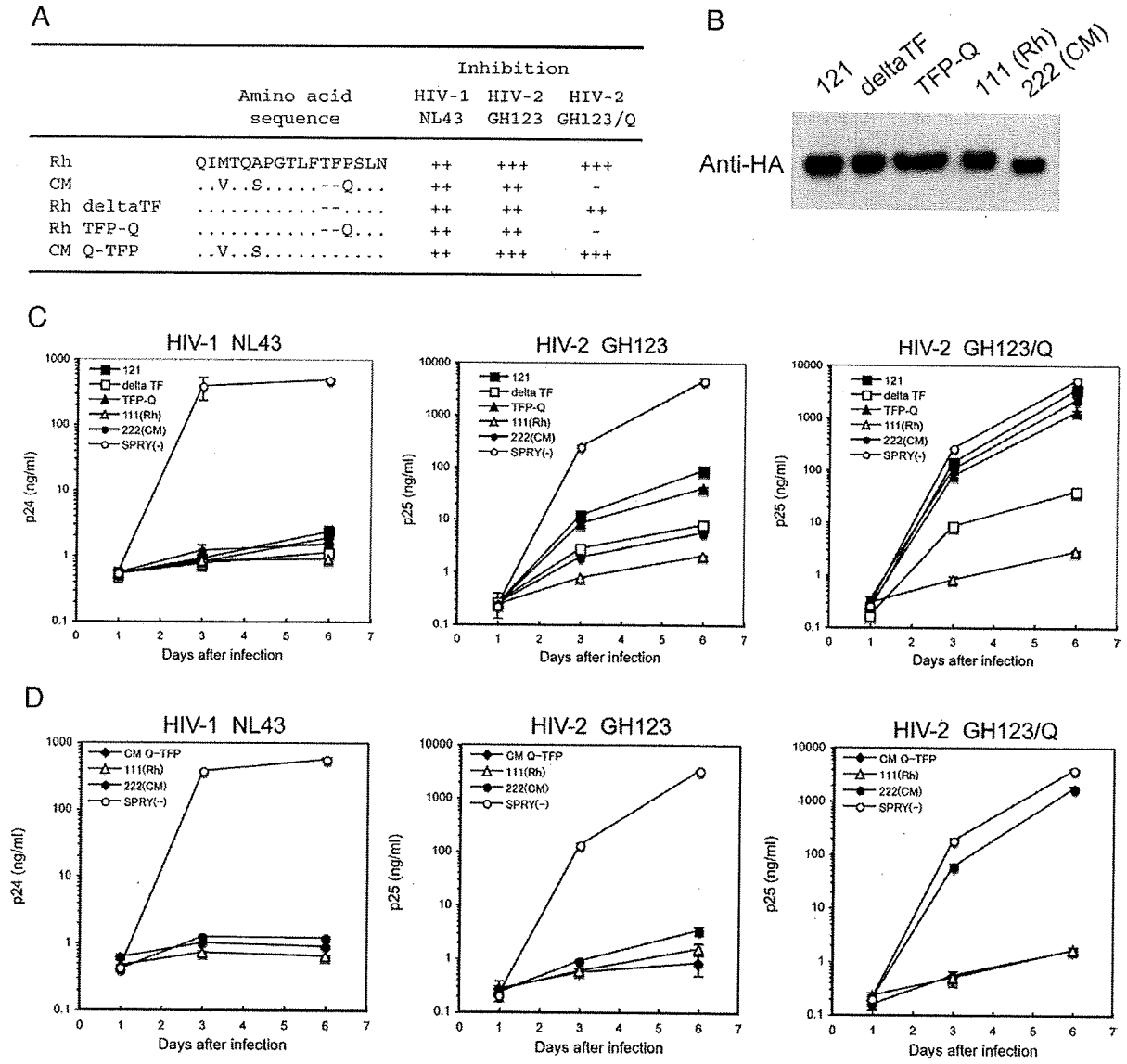

Fig. 5. (A) Mutant Rh deltaTF TRIM5α was generated by deleting 339th-TF-340th and mutant Rh TFP-Q TRIM5α was generated by replacing 339th-TFP-341st with Q by site-directed mutagenesis. Dots denote amino acid identity, dashes a lack of the amino acids residue present only in rhesus monkey (Rh) TRIM5α, and +++, ++, +, and – 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. (B) Twenty-four hours after SeV infection, TRIM5α proteins in lysates of MT4 cells infected with recombinant SeV expressing 121, Rh delta TF, Rh TFP-Q, 111 (Rh), or 222 (CM) TRIM5α were visualized by Western blotting with an antibody against HA-tag. (C) MT4 cells were infected with recombinant SeV expressing 121 (■), Rh delta TF (□), Rh TFP-Q (▲), 111 (Rh) (△), 222 (CM) (●), or CM SPRY(−) (○) TRIM5α. Nine hours after infection, cells were inoculated with HIV-1 NL43, HIV-2 GH123, or HIV-2 GH123/Q viruses. Culture supernatants were respectively assayed for levels of p24 or p25. (D) MT4 cells were infected with recombinant SeV expressing CM Q-TFP (♦), 111 (Rh) (△), 222 (CM) (●), or CM SPRY(−) (○) TRIM5α. Nine hours after infection, cells were inoculated with HIV-1 NL43, HIV-2 GH123, or HIV-2 GH123/Q viruses. Culture supernatants were respectively assayed for levels of p24 or p25.

and their reported ability to grow in these primate peripheral blood mononuclear cells.

Discussion

In the study presented here, we found that rhesus monkey TRIM5α showed anti-HIV-2 activity broader and more potent than that of cynomolgus monkey. We were also able to show that three amino acid residues TFP at the 339th to 341st positions in the V1 were important for the broad HIV-2 restriction activity of rhesus monkey TRIM5α. Previous studies have shown that the V1 of TRIM5α determines species-specific restriction of HIV-1 and SIVmac (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005; Nakayama et al., 2005). Ours is the first study to demonstrate that the V1 of TRIM5α also determines anti-HIV-2 potency. Since MT4 and Hut78 cells express endogenous human TRIM5α, it is possible that endogenous human TRIM5α interfere with exogenous TRIM5α. However, the expression level of SeV-derived TRIM5α is more than 100 times higher than that of endogenous TRIM5α (data

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not shown), and the effect of endogenous TRIM5α is therefore considered to be negligible.

A previous study using a human and rhesus monkey chimeric TRIM5α revealed that a single amino acid substitution from R to P at the 332nd position of human TRIM5α (corresponding to the 334th position in rhesus monkey TRIM5α) confers strong anti-HIV-1 activity to human TRIM5α (Stremlau et al., 2005; Yap et al., 2005). We found that rhesus monkey TRIM5α and chimeric TRIM5α containing baboon V1 strongly restricted HIV-2 GH123/Q, while chimeric TRIM5α containing sooty mangabey V1 did so only slightly, although baboon and sooty mangabey TRIM5α share the SFP motif at the 339th to 341st positions. Rhesus monkey and baboon TRIM5α possess P, whereas human and sooty mangabey TRIM5α possess R at the 334th position. On the other hand, cynomolgus monkey TRIM5α strongly restricted HIV-2 GH123, while chimeric TRIM5α containing pig-tailed monkey V1 did so only slightly. Cynomolgus monkey TRIM5α possesses P, whereas pig-tailed monkey TRIM5α possesses Q at the 334th position. These results indicate the P residue at the 334th position of primate TRIM5α also plays a critical role in the restriction of HIV-2.

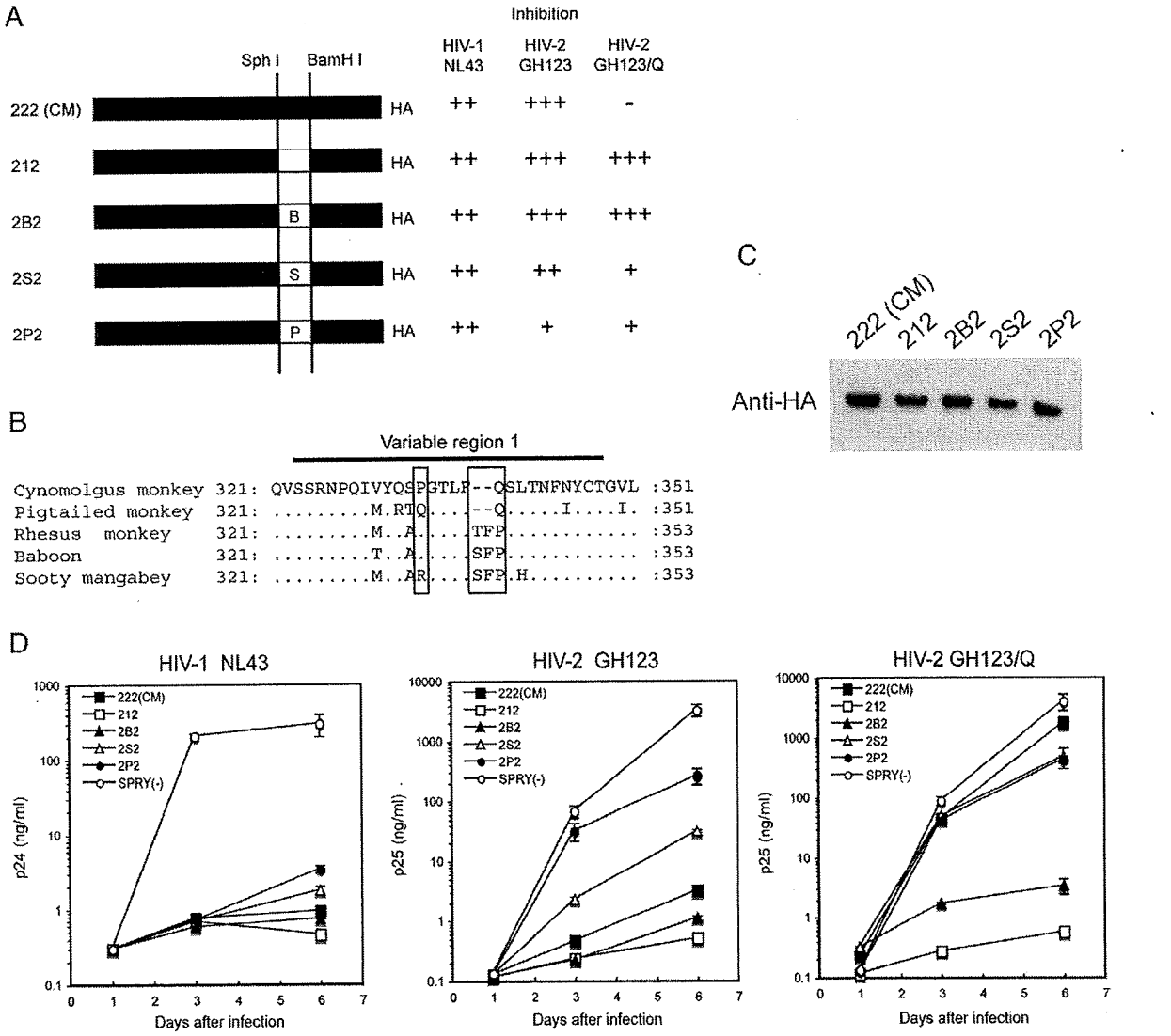

Fig. 6. (A) Schematic representation of chimeric TRIM5α and summary of the results. White and black bars denote rhesus monkey and cynomolgus monkey (CM) sequences, respectively. B, S, and P denote sequence from baboon, sooty mangabey, and pig-tailed monkey, respectively, and +++, ++, +, and − more than 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. (B) Alignment of amino acid sequences of the V1 and franking region within the SPRY domain of cynomolgus monkey, pigtailed monkey, rhesus monkey, baboon, and sooty mangabey TRIM5αs. V1 is indicated by a bar over the sequence. The first box indicates the amino acid residues that were referred to as site 332 in the human TRIM5α. The second box indicates amino acid residues that are important for restricting HIV-2 strains which are resistant to cynomolgus monkey TRIM5α. (C) Twenty-four hours after SeV infection, TRIM5α proteins in lysates of MT4 cells infected with recombinant SeV expressing 222 (CM), 212, 2B2, 2S2, or 2P2 TRIM5α were visualized by Western blotting with an antibody against HA-tag. (D) MT4 cells were infected with recombinant SeV expressing 222 (CM) (■), 212 (□), 2B2 (▲), 2S2 (△), 2P2 (●), or CM SPRY(−) (○) TRIM5α. Nine hours after infection, cells were inoculated with HIV-1 NL43, HIV-2 GH123, or HIV-2 GH123/Q, viruses. Culture supernatants were respectively assayed for levels of p24 or p25.

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HIV-2 is thought to have originated from the SIV from sooty mangabey (SIVsmm) (Hahn et al., 2000). Our results showed that HIV-2 GH123 replication was partially inhibited by chimeric TRIM5α containing sooty mangabey V1, while HIV-2 GH123/Q grew strongly in the presence of the chimeric TRIM5α. This result is consistent with the fact that all SIVsmm sequences in the Los Alamos database possess Q or A at the 119th or 120th position of CA (Song et al., 2007). On the other hand, chimeric TRIM5α containing sooty mangabey V1 has a strong anti-HIV-1 activity, even though it has R at the 334th position as in human TRIM5α. Sooty mangabey TRIM5α, on the other hand, possesses the SFP motif at the 339th to 341st positions. Yap et al. (2005) showed that human TRIM5α carrying the rhesus monkey sequence LFTFPSLT which included the TFP motif instead of the human sequence RYQTFV at the 335th to 340th positions could restrict HIV-1. These results thus indicate that the TFP or SFP motif at the 339th to 341st positions can confer anti-HIV-1 activity as well as the P residue at the 334th position.

In the case of pig-tailed monkey, which could develop an AIDS-like disease by HIV-2 (McClure et al., 2000), anti-HIV-2 activity of chimeric TRIM5α containing pig-tailed monkey V1 was very weak. Furthermore, after we completed this study, it was reported that pig-tailed monkeys lack expression of TRIM5α. Instead, pig-tailed monkeys express TRIM5θ and TRIM5η, novel TRIM5 isoforms lacking anti-HIV-1 activity (Brennan et al., 2007). These findings are probably account for the fact that pig-tailed monkey can be used as an AIDS model.

Previous studies showed that rhesus monkey as well as baboon can be infected with HIV-2 (Dormant et al., 1989; Franchini et al., 1989; Nicol et al., 1989; Franchini et al., 1990; Castro et al., 1991), while the latter can also develop an AIDS-like disease as a result of HIV-2 infection (Barnett et al., 1994; Locher et al., 1998; Locher et al., 2001). In our study, however, rhesus monkey TRIM5α and chimeric TRIM5α containing baboon V1 could strongly inhibit HIV-2 replication. The reason why HIV-2 can replicate in rhesus monkey and baboon, even though the TRIM5α of these monkey species possesses strong anti-HIV-2 activity, is unclear at present. The presence of TRIM5α mRNA in cells of rhesus monkey and baboon cells has been confirmed (Stremlau et al., 2004; Kaiser et al., 2007). It is also known that TRIM5α exhibits a high degree of sequence variation even within species. In rhesus monkey, there is a 339th-TFP-341st to Q polymorphism which diminishes the anti HIV-2 GH123/Q activity of rhesus monkey TRIM5α (Newman et al., 2006) (Fig. 5). It is thus possible that rhesus monkey carrying this polymorphism in the TRIM5α gene could be infected with HIV-2 and that a number of baboons have similar polymorphisms. It would be interesting to investigate the effect of genetic polymorphisms in baboon TRIM5α on its restriction activity against HIV-2.

Materials and methods

Cloning and expression of TRIM5α

Rhesus monkey TRIM5α cDNA was amplified by RT-PCR of mRNA extracted from rhesus monkey kidney LLC-MK2 cells using 5′-GCGGCCGCTACTATGGCTTCTGG-3′ as the forward primer and 5′-GAATTCTCAAGAGCTTGGTGA-3′ as the reverse primer. Amplified products were then cloned into the vector pCR-2.1TOPO (Invitrogen, Carlsbad, CA) and the nucleotide sequence authenticity was verified. For generating rhesus monkey TRIM5α cDNA carrying an HA tag (YPYDVPDYAA) at its C-terminus (Rh-TRIM5α-HA), cloned rhesus monkey TRIM5α cDNA in pCR-2.1TOPO was used as a template for PCR-amplification with a primer (5′-TCAAGCAGCATAATCAGGAACATCATAAGGATAAGAGCTTGGTGAGCACAGAG-3′) containing a nucleotide sequence corresponding to the HA-tag (underline) fused with the C-terminal portion of TRIM5α. The C-terminal portion of TRIM5α fused with the HA-tag (*Bam*HI to *Not*I) and the N-terminal portion of TRIM5α (*Not*I to *Bam*HI) was assembled on a pCEP4 vector (Invitrogen). Construction of cynomolgus monkey TRIM5α carrying an HA tag at the C-terminus (CM-TRIM5α-HA) was described previously (Nakayama et al., 2005).

To generate 121 chimeric TRIM5α, the 188-bp *Sph*I–*Bam*HI fragment of rhesus monkey TRIM5α was replaced with the corresponding 182-bp *Sph*I–*Bam*HI fragment of cynomolgus monkey TRIM5α in the background of Rh-TRIM5α-HA. Conversely, the 182-bp *Sph*I–*Bam*HI fragment of cynomolgus monkey TRIM5α was replaced with the 188-bp *Sph*I–*Bam*HI fragment of rhesus monkey TRIM5α in the background CM-TRIM5α-HA to generate 212 chimeric TRIM5α. To generate 112 chimeric TRIM5α, the C-terminal portion of CM-TRIM5α-HA (*Bam*HI to *Not*I) and the N-terminal portion of rhesus monkey TRIM5α were assembled on a pcDNA3.1 (−) vector (Invitrogen). Conversely, the C-terminal portion of Rh-TRIM5α-HA (*Bam*HI to *Not*I) and the N-terminal portion of cynomolgus monkey TRIM5α (*Not*I to *Bam*HI) were assembled on a pcDNA3.1 (−) vector to generate 221 chimeric TRIM5α. To generate 122 chimeric TRIM5α, the C-terminal portion of CM-TRIM5α-HA (*Sph*I to *Not*I) and the N-terminal portion of rhesus monkey TRIM5α (*Not*I to *Sph*I) were assembled on a pcDNA3.1 (−) vector. Conversely, the C-terminal portion of Rh-TRIM5α-HA (*Sph*I to *Not*I) and the N-terminal portion of cynomolgus monkey TRIM5α (*Not*I to *Sph*I) were assembled on a pcDNA vector to generate 211 chimeric TRIM5α.

Mutant rhesus monkey TRIM5α lacking two amino acid residues TF at the 339th to 340th positions (Rh deltaTF TRIM5α), mutant rhesus monkey TRIM5α in which amino acid residues TFP at the 339th to 341st positions was replaced with a single amino acid Q found in cynomolgus monkey TRIM5α (Rh TFP-Q TRIM5α), and mutant cynomolgus monkey TRIM5α in which a single amino acid Q at the 339th position was replaced with three amino acid residues TFP found in rhesus monkey TRIM5α (CM Q-TFP TRIM5α) were generated by site-directed mutagenesis by PCR-mediated overlap primer exension 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 desired mutations. The resultant two fragments were combined in the subsequent fusion reaction in which the overlapping ends anneal, allowing the 3′ overlap of each strand to serve as a primer for 3′ extension of the complementary strand. The outer primers for generating Rh deltaTF

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TRIM5α were 5′-GCGGCCGCTACTATGGCTTCTGG-3′ and 5′-GAATTCTCAAGAGCTTGGTGA-3′, and the complementary primers were 5′-GGGACATTATTTCCGTCACTCACG-3′ and 5′-CGTGAGTGACGGAAATAATGTCCC-3′. The outer primers were common in all cases of PCR-based mutagenesis of TRIM5α. The complementary primers for Rh TFP-Q TRIM5α were 5′-GGGACATTATTTCAATCACTCACG-3′ and 5′-CGTGAGTGATTGAAATAATGTCCC-3′ (underline; TFP-Q site), and 5′-GGACATTATTTACGTTTCCGTCACTCACG-3′ and 5′-CGTGAGTGACGGAAACGTAAATAATGTCC-3′ (underline; Q-TFP site) for CM Q-TFP TRIM5α.

2B2, 2S2, and 2P2 chimeric TRIM5α, which possessed the 188-bp *Sph*I–*Bam*HI fragment of baboon TRIM5α, 188-bp fragment of sooty mangabey TRIM5α, and 182-bp fragment of pig-tailed monkey TRIM5α, respectively, in the background of CM-TRIM5α-HA were generated by the same method as described above. To obtain *Sph*I–*Bam*HI fragments of baboon and sooty mangabey TRIM5αs, cloned rhesus monkey TRIM5α was used as a template for PCR-amplification with outer primers described above and complementary primers containing a nucleotide sequence corresponding to the 330th to 339th amino acid residues of baboon TRIM5α (5′-CAGATAACGTATCAGGCACCAGGGACATTATTTTCGTTTCCG-3′ and 5′-CGGAAACGAAAATAATGTCCCTGGTGCCTGATACGTTATCTG-3′) and the 334th to 343rd amino acid residues of sooty mangabey TRIM5α (5′-CAGGCACGAGGGACATTATTTTCGTTTCCGTCACACACGAAT-3′ and 5′-ATTCGTGTGTGACGGAAACGAAAATAATGTCCCTCGTGCCTG-3′), respectively. To obtain the *Sph*I–*Bam*HI fragment of pig-tailed TRIM5α, cloned cynomolgus monkey TRIM5α was used as a template for PCR-amplification with outer primers described above and complementary primers containing a nucleotide sequence corresponding to the 330th to 350th amino acid residues of pig-tailed monkey TRIM5α (5′-CAGTCACTCACGAATTTCATTTATTGTACTGGCATCCTGGGC-3′ and 5′-CGTGAGTGACTGAAATAATGTCCCTTGTGTCCGATACATTATCTG-3′). The entire coding sequences of those TRIM5α were then transferred to the *Not*I site of pSeV18+b(+). Recombinant SeVs carrying various TRIM5α were recovered according to a previously described method (Nakayama et al., 2005). The viruses passaged twice in embryonated chicken eggs were used as stock for all experiments.

Viral infection

2×10^5 MT4 or Hut78 cells were infected with SeV expressing each TRIM5α 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 p24 of HIV-1 NL43, 20 ng of p27 of SIVmac239, or 20 ng of p25 of HIV-2 GH123, GH123/Q, or other HIV-2 isolates. The culture supernatants were collected periodically, and the level of p24, p27, or p25 was measured with a RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Western blot analysis

MT4 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 (SDS-PAGE). 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) overnight at 4 °C. Blots were then incubated with peroxidase-conjugated anti-rat IgG (American Qualex, San Clemente, CA), and bound antibodies were visualized with a Chemilumi-One chemiluminescent kit (Nacalai Tesque, Kyoto, Japan).

TRIM5α cDNA sequences

TRIM5α cDNA sequences for rhesus monkey (AY523632), cynomolgus monkey (AB210052), baboon (AY843505), sooty mangabey (AY10303), and pig-tailed monkey (AY899887–AY899893) were obtained from the GeneBank database.

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Original article

A rapid recombination assay of HIV-1 using murine CD52 as a novel biomarker

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Abstract

Biomarkers are commonly used for verification of infection in conjunction with the development of viral vectors or experiments involving virus infection. Leukocyte surface antigens (CDs) are a prime option for biomarkers since they can be easily visualized and analyzed by flow cytometry after indirect fluorescent staining. For analyses of human cells, murine CD24 (Heat Stable Antigen: HSA) and CD90.2 (Thy-1.2) are currently being used. In the study reported here, we attempted to develop a rapid system for measuring retroviral genome recombination efficiency. For this purpose, we looked for an alternative CD molecule which could be used as a marker on a viral vector concurrently with other markers. We found that murine CD52 is suitable for this purpose because of its small gene size, low inhibitory effect on virus production, and measurable level of surface expression. With this novel biomarker, we succeeded in developing a rapid viral recombination measuring system using a flow cytometer.

Keywords: Retrovector; Biomarker; CD52; Recombination; Flow cytometry

1. Introduction

The use of retroviral vectors for gene delivery has become very common in recent years. To determine the efficiency of gene induction and/or to monitor the fate of induced cells, a variety of selectable markers have been incorporated into retroviral vectors. The genes of products that confer resistance to toxic compounds are widely used from the start of vector development, since their stable expression enables positive selection of cells induced by prolonged treatment with antibiotics [1]. Fluorescent proteins such as eGFP and RFP are widely used as markers because of their high stability, minimal toxicity, and non-invasive detection [2].

Cell surface molecules, such as leukocyte surface antigens (CDs), constitute another type of commonly used selectable marker antigens. Staining cells with fluorescent dye-conjugated antibodies and analyzing them with a flow cytometer enables the rapid and quantitative detection of transferred gene expression in the desired target cells while they are still alive. However, most of the genes used as selectable markers are relatively large, leaving limited space in the retroviral vector for other genes of interest. The human hematopoietic cell surface antigen CD24 and its murine homologue, the heat stable antigen (HSA), are two of the few exceptions because of their relatively small gene size (about 0.24 kb) and potential for cell surface expression. Because of these properties, CD24 and HSA are widely used as biomarkers [3,4]. Another surface antigen, CD90.2 (Thy-1.2), is also in common use as a biomarker although its gene size (488 bp) is larger than that of HSA [5].

To monitor retroviral infection and viral genome recombination in cells, several good vector assay systems have been developed [6,7]. Rhodes et al. described an attractive method using two similar vectors with mutated eGFPs and surface markers to measure their infectivity and recombination rate [8]. These systems are based on the principle that only a single

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DNA is formed in an infectious retroviral particle [9]. Although this system promises reasonable and stable results, it requires multiple cell sorting and expansion, so that it takes considerable time and effort to perform. In addition, the vectors carry IRES sequences for marker gene expression, sequences which do not exist in the native retroviral genome. We therefore wanted to modify this recombination assay system since we intended to evaluate HIV-1 genome recombination in a situation more similar to viral physiological conditions and to simplify the experiments. We attempted to generate retrovectors which could express two or more biomarkers at the same time for the development of the recombination assay system. Since it was therefore necessary to find a new biomarker, we explored the database and found that murine CD52 (mCD52), a protein of the CD24/HSA family, might have the characteristics required for our purpose.

2. Materials and methods

2.1. DNA constructs

The replication competent HIV-1 proviral clone pNL4-3 [10] and pMSMBA [11], a derivative of pNL4-3, were used as progenitors for the mutant constructs described below. The HSA gene was amplified from the plasmid pNLrHSAS [12], also a derivative of pNL4-3, the vpr gene of which was replaced with the HSA gene, with a pair of primers (XbaHSAonF: 5′-TCTAGAGCCGCCATGGGCAGAGCG-3′, and EcoRIHSAstpR: 5′-GAATTCTCTAACAGTAGAGATG-3′). pNLrHSAS was digested with NheI, blunt-ended with a Blunting-High kit (Toyobo, Osaka, Japan), and ligated to generate pNLrHSASNh. The amplified fragment was replaced with the XbaI-EcoRI region of pNLrHSASNh to add a Kozak sequence upstream of the ATG codon of the HSA gene to enhance its expression, and the resultant plasmid was named pNLrH. pNLrH was then digested with HpaI and XhoI, and the HpaI-XhoI fragment of pGEMHnGX [13] including the EGFP gene was inserted in the corresponding position to construct pNLrHnG.

The murine CD52 (mCD52) gene coding fragment was generated by synthesizing three oligonucleotide probes (CD52atg-95: 5′-ATGAAGAGCTTCCTCCTCTTCCTCAC TATCATTCTTCTGGTTGTGATTCAGATACAAACAGGAT CCTTGGGACAAGCCACTACGGCCGCTTCTGG-3′, CD52 cmpl170-116: 5′-GGCACCCGCATCGATGATGGATGAGG CCCCACTCTTTAAGGGGGTTTTTTTGGTGGAGGTGCTG TTTTTGTTAGTACCAGAAGCGGCCGTAGTGG-3′, and CD52fwd151-stp: 5′-CCATCATCGATGCGGGTGCCTGCAGTTT CCTCTTCTTTGCCAATACCTTAATGTGCCTCTTCTACC TCAGCTGA-3′) following sequential PCR amplification with two pairs of primers (XbaKozCD52F: 5′-TCTAGAGCCGC CATGAAGAGCTTCCTCCTCTTCC-3′, CD52RevCla: 5′-GG CACCCGCATCGATGATGGATG-3′, CD52FwdCla: 5′-CCAT CATCGATGCGGGTGCCTGC-3′, and CD52RevStp: 5′-TCA GCTGAGGTAGAAGAGGCAC-3′). The 0.23 kb amplified fragment was purified, ligated to the pGEMTeasy vector (Promega, Madison, WI) to construct pGEMmCD52, and verified for its sequence authenticity. pNLrHnG was digested with XbaI and EcoRI, and the XbaI and EcoRI fragment of pGEMmCD52, including the mCD52 gene, was inserted at the corresponding position to construct pNLrCnG. A base substitution mutation was then introduced into the start codon of the eGFP gene of pNLrHnG to eliminate eGFP expression (ATG to TAA) in order to allow for the construction of pNLrHnGΔN. A frame shift mutation of the eGFP gene at the 0.6 kb position from the start codon was introduced as reported elsewhere [8] (mutant pON-H6: one-base substitution and one-base insertion to introduce a stop codon and a frame shift) into pNLrCnG for the construction of pNLrCnGΔC. Furthermore, two-base substitution mutation at the hairpin loop of SL1 (GCGCGC to GTGCAC) was introduced into pNLrCnGΔC to construct SL1MrCnGΔC.

2.2. DNA transfection

293T cells [14] (approximately 3×10^6) were seeded on dishes (diameter 100 mm) the day before transfection with plasmid DNA (total 5 μg) using the calcium phosphate precipitation method [15]. The day after transfection, the supernatant was replaced with fresh medium.

2.3. Virus infection

At 48–72 h post-transfection, the media was centrifuged and the supernatant was used for infection into T-cell lines (MT-4 and M8166).

2.4. RT-PCR assay

Two days after transfection, 293T cells were harvested and total cellular RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). RNAs were treated with RQ1-DNaseI (Promega) for removal of contaminated DNAs. Reverse transcription (RT) reaction using 5 μg of total RNA with Superscript III (Invitrogen) was performed according to the manufacturer's instructions, and one-twentieth of the RT products was used for the PCR template. Two sets of primer pairs were prepared to detect the mCD52 gene (Forward: 5′-TCTAGAGCCGC CATGAAGAGCTTCCTCCTCTTCC-3′; reverse: 5′-TCAGC TGAGGTAGAAGAGGCAC-3′) and the GAPDH gene (forward: 5′-CCACATCGCTCAGACACCAT-3′; reverse: 5′-GGC AACAATATCCACTTTACCAGAGT-3′). Amplified products were subjected to agarose gel electrophoresis and visualized by means of ethidium bromide staining.

2.5. Flow cytometric analysis

Mock-infected cells, an empty vector, and HSA/eGFP/mCD52/Thy-1.2-infected cell populations in growth medium were first centrifuged and washed twice in PBS(−) supplemented with 10% Blocking One solution (Nacalai Tesque Inc., Kyoto, Japan). Aliquots of the cells were then stained with an anti-mCD52 rat monoclonal antibody (MBL Co. Ltd., Nagano, Japan) for 30 min, washed twice, incubated with Allophycocyanin (APC)-labeled anti-rat Ig polyclonal

antibody (BD Biosciences, San Jose, CA) for an additional 30 min, and washed twice. The cells were then stained with directly conjugated anti-murine HSA-Phycoerythrin (PE) antibody and anti-murine Thy-1.2-biotin antibody (both from BD Biosciences) for an additional 30 min, washed twice, and stained with PerCP-Cy5.5-conjugated Streptavidin (BD Biosciences). After antibody labeling, two further washes in PBS(−) were performed, the last together with 1% formaldehyde to fix the cells. Finally, the cells were analyzed on a FACSCalibur (BD Biosciences).

3. Results

3.1. Generation of mCD52 expressing HIV-1 vectors

To construct multi-marker carrying retrovectors, we initially tried to use the existing biomarkers. Within the HIV-1 genome, the vpr, env, and nef coding regions were replaced with HSA, Thy-1.2, and eGFP genes in various combinations. While the HSA gene was well expressed under any conditions, the Thy-1.2 and eGFP genes performed well only when located within the nef coding region (data not shown). Any vector carrying the Thy-1.2 or eGFP gene in the vpr or env region produced little or no viral particles. Hence, we needed to find a novel marker with approximately the same potential as CD24/HSA and that could be used concurrently with them.

Human and murine CD52s (h/mCD52) belong to a group of very small GPI-anchored sialoglycoproteins which include CD24/HSA and with size and protein properties resembling those of CD24. Human CD52 is abundantly expressed on lymphocytes and monocytes, and is also expressed in non-lymphoid tissue in epithelial cells of the distal epididymal and deferent ducts from which it is transferred to the surface of sperm [16]. The gene size of mCD52 is only 222 bp and encodes 74 peptides (Fig. 1A). A computer search found no significant homology of mCD52 to any known molecules except hCD52 at either the DNA or amino acid sequence level [17]. Although it retains a certain homology to hCD52 within the N- and C-terminal signal region, the amino acid sequence of the mature peptide region of mCD52 is significantly different from that of its human homologue [16]. In addition, it has been suggested that the monoclonal antibody BTG-2G, which is the only commercially available anti-mCD52 monoclonal antibody (mAb) [17], can recognize peptides containing KKTPL [18]. This sequence is unique to mCD52 (Fig. 1A) and thus no cross-reaction of the antibody with hCD52 can be expected. These characteristics suggested to us that mCD52 could be a candidate for a novel selectable marker of gene transfer in human cells. We cloned the mCD52 gene by synthesizing and amplifying its DNA primers and inserting it in place of the vpr gene of HIV-1 to construct various retrovectors. Fig. 1B shows representative schematics of the vectors we constructed for the experiments described below. We confirmed expression of the mCD52 gene by means of an RT-PCR assay (Fig. 1C). A transcription of the mCD52 gene was clearly detected in cells transfected with the vectors carrying the mCD52 gene. The production of the viral antigen from cells transfected with vectors carrying the mCD52 insertion was only moderately reduced compared to that from cells transfected with the wild-type or the vectors carrying HSA (data not shown). This indicated that the effect of mCD52 insertion into the viral genome on virus production was nearly negligible.

3.2. Detection of cell surface expression of mCD52 by mAb

We first used a flow cytometer to verify surface expression of the mCD52 protein and its detection with an mAb. 293T cells were then transfected with pNLrCnG, which carries both the mCD52 and eGFP genes as biomarkers. Two plasmids, pNLNh and pNLrHnG, were used for transfection as parallel controls. Unlike pNLrCnG, pNLrHnG carries an HSA gene instead of an mCD52 gene. Forty-eight hours after transfection, the cells were stained with anti-mCD52 mAb, APC-anti-RatIg antibody, and phycoerythrin (PE)-conjugated anti-HSA mAb. The cells were fixed with 1% formaldehyde-containing PBS(−), and analyzed with a flow cytometer. The expressions of eGFP, HSA, and mCD52 were detected through channels FL1, FL2, and FL4, respectively. The results showed good expression and separation of the three marker genes (Fig. 1D), suggesting that these markers could be utilized concurrently within a cell for discrimination of their expression. Double marker positive cells accounted for about 11% (NLrCnG) and 14% (NLrHnG) of total cells.

3.3. Four-color analysis of transduced cells using three surface markers and eGFP

As expression of mCD52 was clearly distinguishable from the expressions of HSA and eGFP, we attempted to identify gene transductions mediated by multiple retrovectors by means of four biomarkers using all fluorescence channels (FL1–4) of a flow cytometer. For this purpose, the vectors pNLrCnGΔC (NLC) and pNLrHnGΔN (NLH) were prepared. NLC carries the mCD52 gene and NLH the HSA gene, and both carry inactivated eGFP genes. In addition, another surface marker gene, murine Thy-1.2, was employed for analysis. The vector pNLΔBgThy [5] (NLT) carries the Thy-1.2 gene in place of the nef gene, features deletion of the Env gene of pNL4-3 and does not carry the eGFP gene. Biotin-conjugated anti-Thy-1.2 mAb and Avidin-PerCP-Cy5.5 were used for staining and labeling Thy-1.2. The order of cell staining is shown in Fig. 2A. 293T cells were transfected with pCG-VSVG and various combinations of retrovectors. Two days post-transfection, the cells and supernatants were harvested, and the cells were stained with the mAbs and analyzed with a flow cytometer (data not shown). As expected, four biomarkers were detected independently and no cross-reaction was observed. The harvested supernatants were then used for the infection assay with MT-4 cells (Fig. 2B). Similar to the finding for 293T, infection of retrovectors resulted in a satisfactory expression and detection of marker genes with a flow cytometer. The bottom row of the panel in Fig. 2B shows

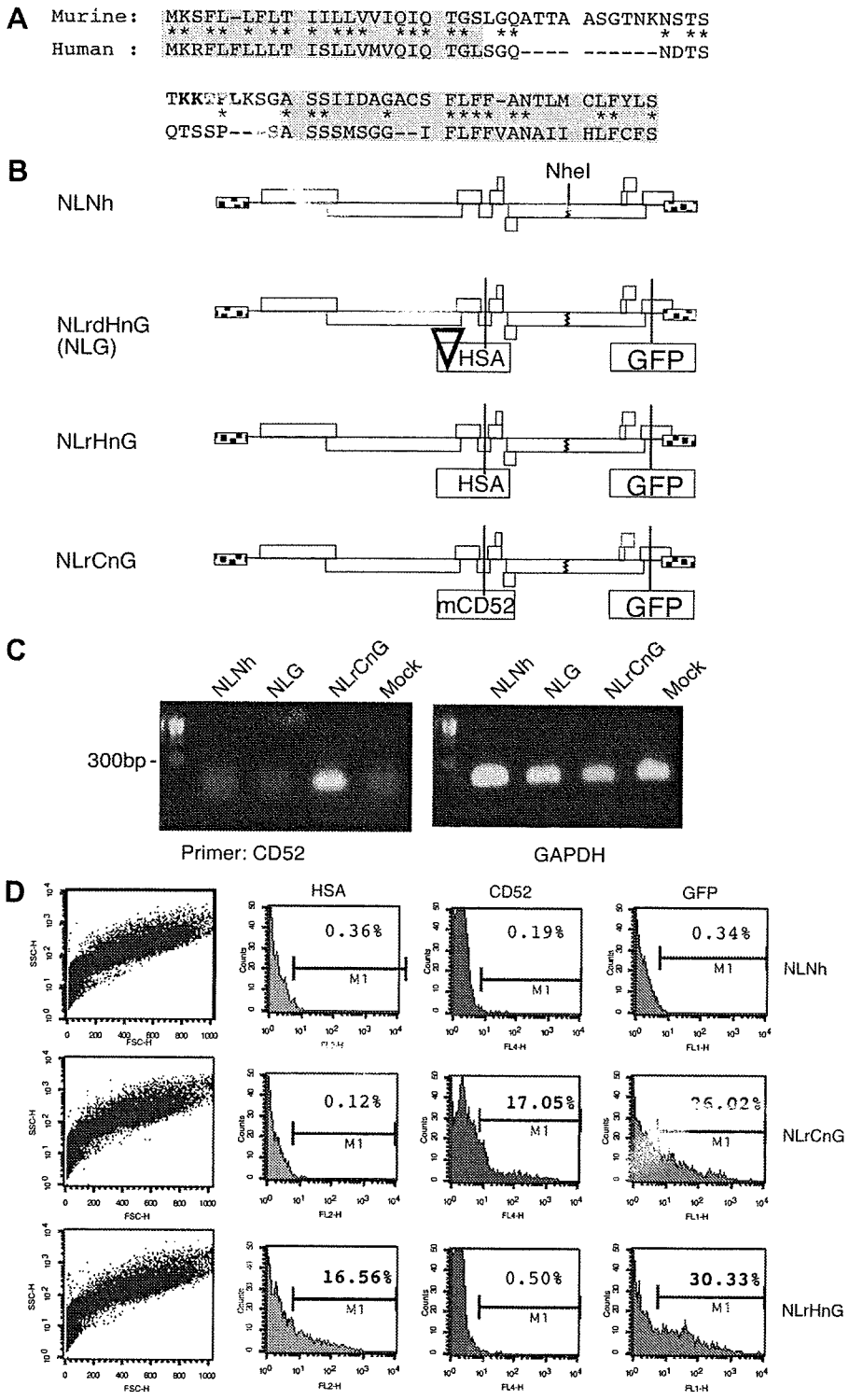

Fig. 1. Construction of murine CD52 expressing HIV-1 vector. A) Comparison of murine and human CD52 protein sequences. N-terminus signal sequences and C-terminus GPI-addition sequences [22] are shaded. The BTG-2G monoclonal antibody recognition sequence [18] are shown in bold. B) Schematic of constructed HIV-1 vectors. Plain boxes represent ORFs of HIV-1 genes and stippled boxes represent LTRs. The env gene in each vector was inactivated by frameshift mutation introduced at the NheI site. The boxes labeled HSA, GFP, and mCD52 represent marker genes inserted in the vectors. The triangle represents the location of mutations introduced to inactivate the gene. C) RT-PCR assays to confirm mCD52 expression. 293T cells were transfected with the plasmids shown in Fig. 1B. Cells were harvested 2 days post transfection and total cellular RNA was extracted. RT products of 5 μg of total cellular RNA were used for PCR amplification. Detection of the expression of the GAPDH gene was used for control. Faint signals for mCD52 in control lanes were non-specific since they also appeared in RT negative controls (data not shown). D) Surface expression of mCD52 molecules caused by transfection. 293T cells were transfected, harvested, and stained with anti-mCD52 Rat monoclonal antibody, APC-conjugated anti-Rat IgG antibody, and PE-conjugated anti-HSA monoclonal antibody. Four charts in one row represent one set of analyses of one sample. Plasmids used for transfection and molecules detected by cytometric analysis are indicated to the left of the rows and above the columns respectively. Percentages of positive cells are shown in each histogram.

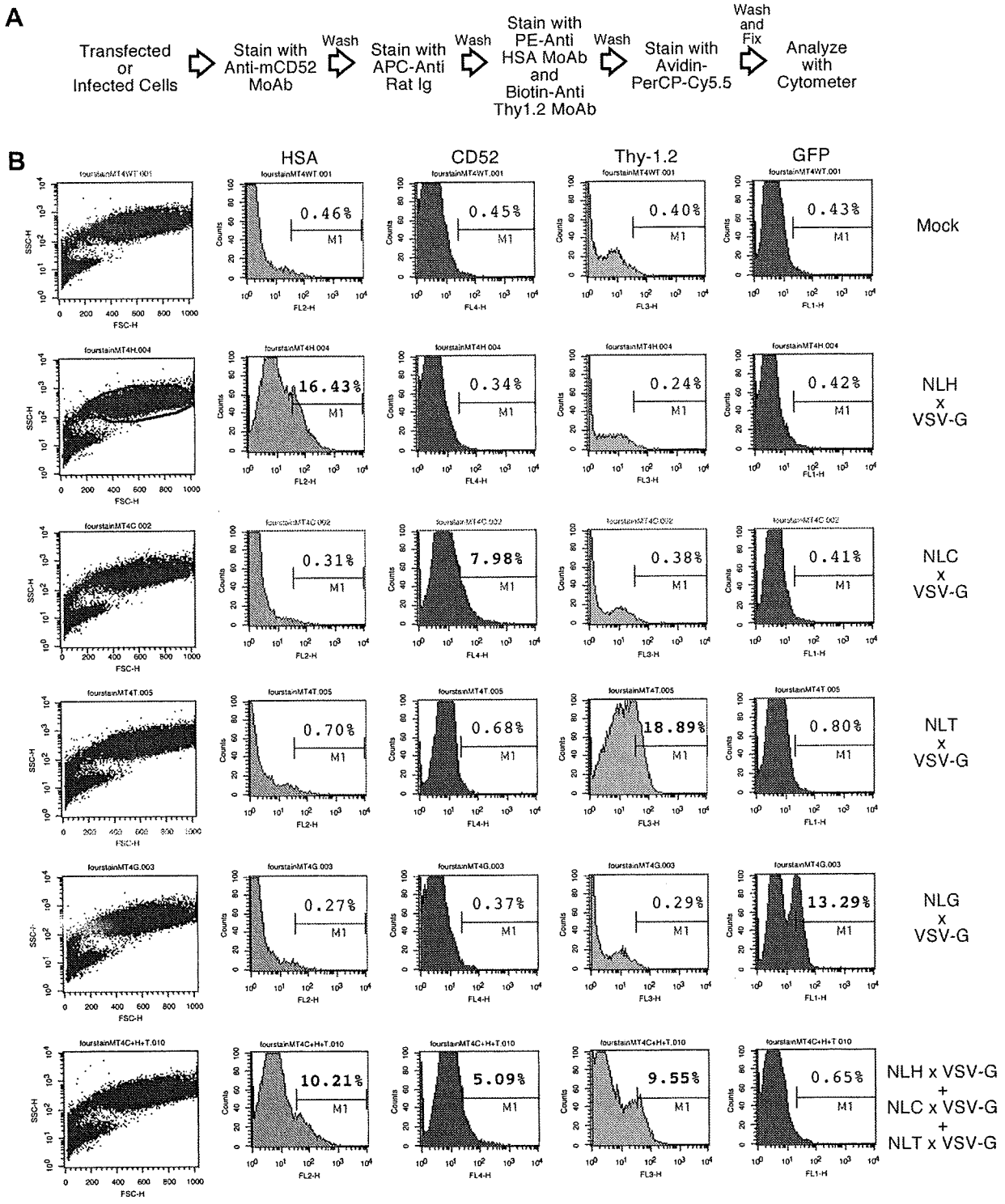

Fig. 2. Simultaneous detection of four biomarkers transduced by retrovectors. A) Flowchart of cell staining of the assay. B) MT-4 cell infection experiment. The supernatants of 293T cells transfected with pCG-VSVG and various retrovectors were collected and used for infection into MT-4. Six charts in one row represent one set of analyses of one sample. The notations are the same as those for Fig. 1D. The "x" between two or three plasmids indicates co-transfection of the plasmids.

that co-infection of the mixture of three vectors resulted in a lack of eGFP expression, thus indicating that no recombination between GFPΔN and GFPΔC from different virus particles had occurred. We therefore proved that these three surface markers could be conveniently leveraged to distinguish the gene induction of different vectors simultaneously, and that their utilization could be expected to have a wide range of applications.

3.4. Construction of HIV-1 genome recombination assay

We then attempted to develop a simple system to monitor retroviral infection and viral genome recombination in cells. The system we designed is shown in Fig. 3. Two similar vectors with the same or different dimerization signals (DLS) were constructed (Fig. 3A) and co-transfected together with or without the VSV-G expression vector (pCG-VSVG) (Fig. 3B). Vector A carries a surface biomarker (Mark-A) and an inactivated eGFP gene with amino-terminal mutation (GFPΔN), while vector B carries another biomarker (Mark-B) and an inactivated eGFP gene with carboxyl-terminal mutation (GFPΔC). After transfection, the released virions can be

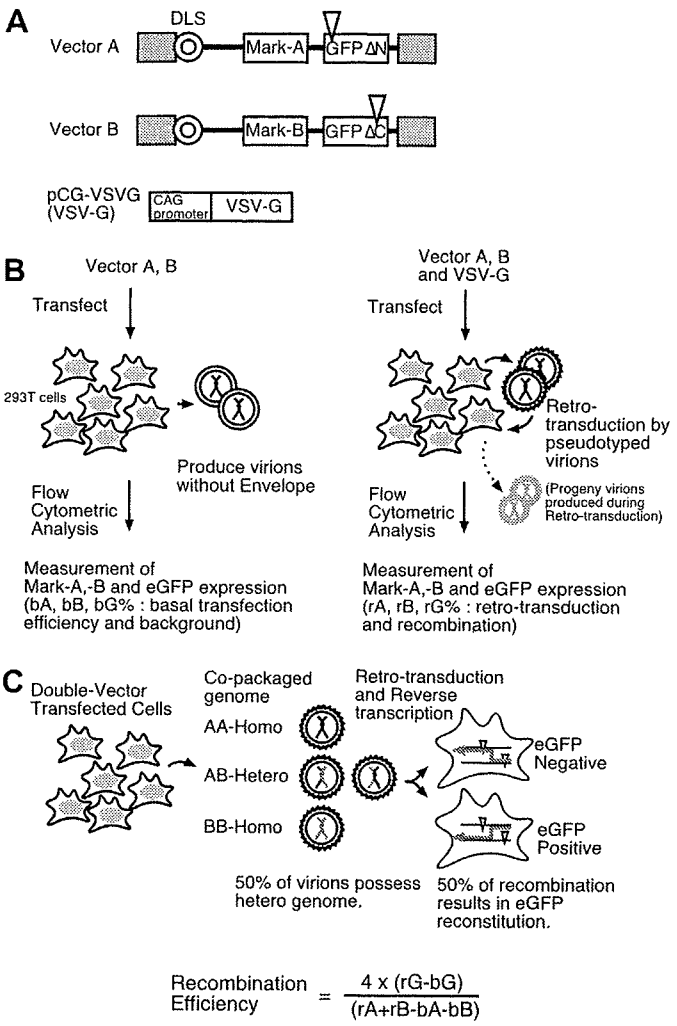

Fig. 3. The system for estimating HIV-1 recombination efficiency using retro-transduction realized by means of pseudotyping. A) Schematics of the vectors planned for the system. Symbols are the same as those for Fig. 1B. Concentric circles represent encapsidation/dimerization signals (E/DLS). B) Experimental design of the system. Without VSV-G, co-transfection of the vectors results in production of non-infectious virions and expression of marker genes (left). With VSV-G pseudotyping, the generated virions infect the cells within the transfected cell culture (retro-transduction), and marker gene expressions are enhanced. C) Estimation of recombination efficiency. Co-transfected cells produce 25% A-A and 25% B-B homo-dimerized genomes, as well as 50% A-B hetero-dimerized genomes containing virions. Fifty % of the opportunity for genome recombination results in reconstitution of the eGFP gene.

expected to co-package the homo- or hetero-dimerized vector genome, while the ratio of homo- to hetero-dimerization should be one-to-one if the genome expression efficiency of the two vectors is similar (Fig. 3C). Without VSV-G, the expression of Mark-A and -B indicates transfection efficiency of the vectors, and no eGFP expression should be observed. With VSV-G expression, pseudotyped virions have been observed to cause retro-transduction to the producer cells [13] and the number of marker genes expressing cells in the transfectant increases with an increase in the occurrence of retro-transduction. Recombination of the two vectors can be assumed to occur only in retro-transduced cells, and is monitored in terms of further restoration and expression of the eGFP gene (Fig. 3C). Transfection and retro-transduction efficiency are measured by expression of Mark-A and -B, while the transduction efficiency is estimated by subtracting marker gene expression ratios of the VSV-G negative sample (bA, bB%) from those of the positive sample (rA, rB%). Finally, the recombination efficiency is estimated by calculating the ratio of eGFP expressing cells (rG−bG%) in vector-transduced cells. If we assume that one of the recombination events always occurs between the ΔN and ΔC mutation of eGFPs during reverse transcription, 50% of the recombination events should result in reconstitution of the eGFP gene. In addition, 50% of the virions from doubly transfected cells possess a hetero-dimerized genome, and thus have the potential to reconstitute eGFP. This means that a ratio of eGFP positive cells of 25% in Mark-A or -B positive cells should be the maximum value for recombination. We therefore adopted this maximum ratio for easy indexing by quadrupling the numeric results (Fig. 3C).

We assessed the efficacy of this system by constructing and testing several vectors derived from HIV-1. We found that the combination of vpr substitution with HSA or mCD52 as surface markers and nef substitution with mutated eGFP genes yielded satisfactory results in terms of virion production, infectivity, and marker expression. We also used this system to verify the recombination between the wild-type retrovectors and those derived from a dimerization initiation site (DIS) mutant of HIV-1 (Fig. 4). DIS is located in DLS and it has been suggested that it performs core functions in viral genome recombination [19]. The vectors NLC and NLH carry the same DIS, whereas pSL1MrCnGΔC (SL1MC) carries a two-base substitution on DIS (Fig. 4A), so that heterogenome dimer formation between NL- and SL1M-vectors can be assumed to be reduced. In this experiment (Fig. 4B), 3–5% of all cells were surface marker positive cells in non-pseudotyped samples (NLH × NLC, NLH × SL1MC), which constitutes evidence of the efficiency of transfection and marker expression. eGFP fluorescence was not detected in these samples, confirming that ΔN or ΔC mutation inactivated the functional eGFP expression. In contrast, HSA/mCD52 expression was dramatically enhanced by about 80% in pCG-VSVG co-transfected samples due to retro-transduction. Efficient reconstitution of eGFP was observed in the homo-DIS sample (20% of total cells) whereas only limited eGFP expression was detected in the hetero-DIS sample (4%), thus indicating diminished occurrence of recombination as also reported elsewhere [19]. Recombination efficiency was

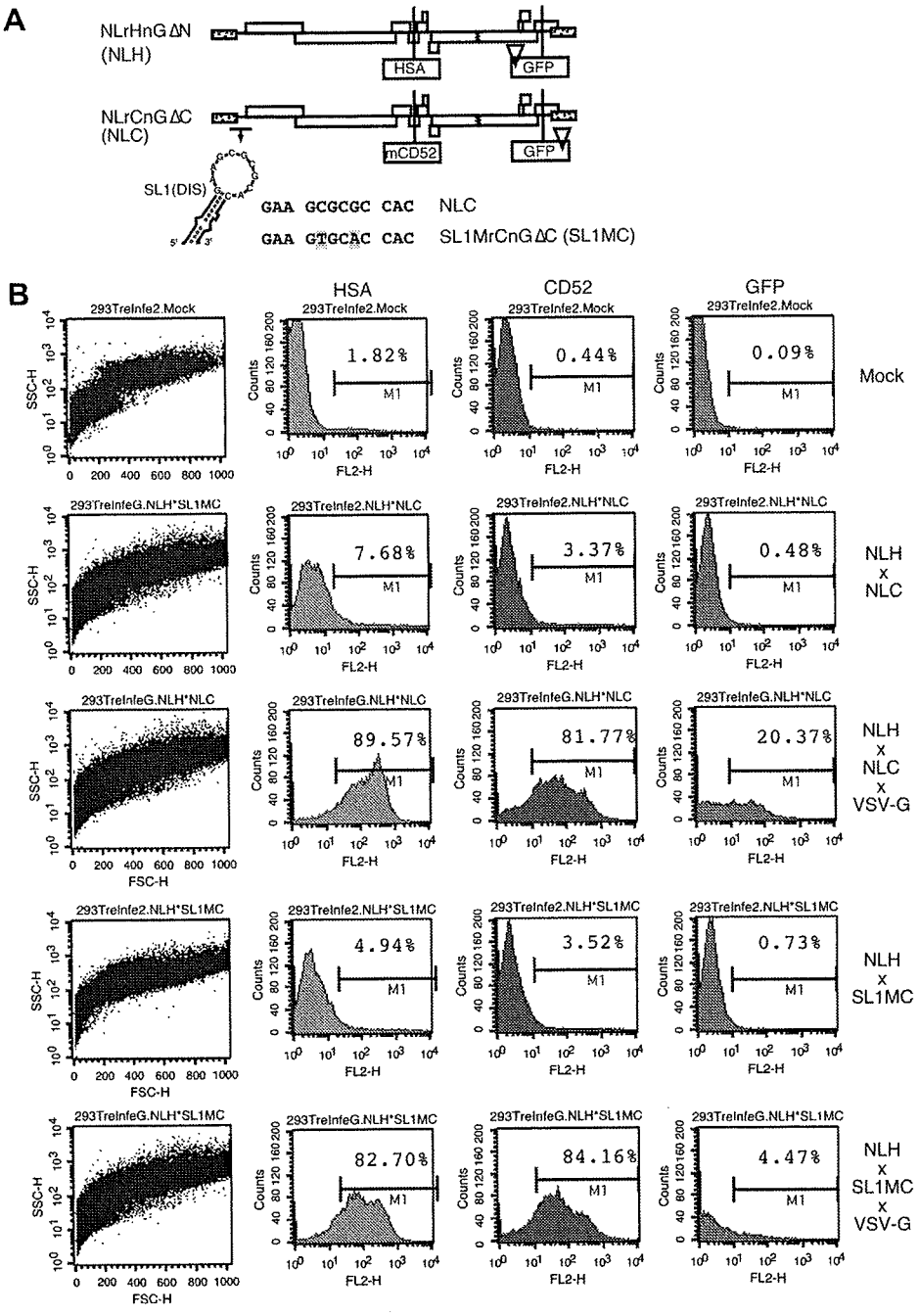

Fig. 4. Detection of HIV-1 recombination. A) Schematic of the vectors actually constructed for the recombination estimation system. Two nucleic acids on DIS of pNLrCnGΔC were substituted for each other (5′-GCGCGC-3′ for 5′-GTGCAC-3′) to construct pSL1MrCnGΔC. B) Genome recombination assay using 293T cells. The notations are the same as those for Fig. 2B.

estimated at 0.5 for NL-NL [4(20.37 − 0.48)/(89.57 + 81.77 − 7.68 − 3.37) = 0.496] and 0.09 for NL-SL1M [4(4.47 − 0.73)/(82.70 + 84.16 − 4.94 − 3.52) = 0.094]. The efficiency of heterogenome dimerization between NL and SL1M mutant estimated with the system devised by us [20] was reduced to about 30% of that of homo-dimerization (data not shown), which suggests there is a parallel interrelation between genome dimerization and recombination. These results clearly showed that our system utilizing retro-transduction is effective and quite practical for evaluation of recombination.

4. Discussion

In this report, we described the practical use of a new reporter gene, mCD52, and the development of a rapid recombination assay system for HIV-1. We focused on the mCD52, which is a protein of the HSA family, since its gene is as small as that of HSA and was assumed to possess similar properties. The results of our study demonstrated that mCD52 can be a useful biomarker since its insertion into the vpr region resulted in its expression from the HIV-1 genome similar to that of HSA.

The recombination assay system we developed is relatively simple, easy and fast. Infection to the transfected cells (=retro-transduction) is the key component of the system, and without it, this system cannot be operated. Retro-transduction is inevitable as far as using the VSV-G protein for pseudotyping, since the VSV-G pseudotyped vector can infect virtually any kind of cell [13]. On the other hand, it is not easy to attain high enough infectivity of the vectors to calculate the recombination rate without VSV-G. We therefore constructed this system with VSV-G by taking advantage of retro-transduction for a quick estimation. For reliability of the calculation, however, the efficiency of retro-transduction is a matter of concern. If the titer of the vectors is too high, multiple infections would occur in a single cell. As the estimate of the recombination rate is based on the assumption of a "single-hit" infection per cell, too high an efficiency of vector production might bias the estimate. In several reports, the recombination frequency of HIV-1 in cultured cells is estimated at about once per 0.9–1 kb of viral genome [6,8]. Our result for the normal recombination rate was 0.5 times per 0.6 kb, so that the estimated frequency is once per 1.2 kb. Since this value represents a lower efficiency than previously reported, it may be the result of multiple infections reflecting a high infection rate (most of the cells in the culture were marker positive as seen in Fig. 4). To prevent biased estimates, a reduction in vector infectivity is needed for this system. In fact, when we reduced the titer of the vector appropriately to minimize "multiple-hit" infection, the calculated recombination ratio was as one event per 0.8 kb, which was very close to the previously published data (data not shown).

There is a possibility that viral genome-derived cDNA and transfected plasmid DNA recombine in retro-transduced cells. We performed an additional experiment to verify this possibility. The 293T cells were cotransfected with the plasmid NLH, pCG-VSVG, and the plasmid carrying only GFPΔC gene. After three-day incubation, we observed no appearance of GFP positive cells in retrotransduced cell culture, whereas a certain number of HSA positive cells appeared (data not shown). This clearly showed that the recombination between the GFPΔN gene derived from viral cDNA and the GFPΔC gene derived from the transfected plasmid was undetectable during retro-transduction. Thus, we believe that plasmid-cDNA recombination is negligible in this system. Like in other established systems [6,8], the recombination rate estimated by our system only reflects that in the nef coding region. By changing the structure of the vectors, it may be possible to further study the recombination rate in regions other than the nef region.

We did not encounter any practical problems such as alterations of cell viability caused by the expression and staining of mCD52. Specific mAb (Campath-1H) treatment of human CD52 has been shown to lead to extensive eradication of CD52 positive cells by complement activation and is thus utilized for bone marrow transplantation therapy [21]. Although our system includes anti-mCD52 mAb treatment, the mAb we used was different from Campath-1H, and the treatment of cells in our study lasts only for a short time prior to cell fixation. In addition, complement components in cell culture media were inactivated by heat inactivation of serum. Although mCD52 seemed to cause no serious defects in the experiment, mCD52 expression may have some deleterious effect under certain conditions such as in *in vivo* experiments. The potential problems associated with wider application of our system thus need to be investigated.

In conclusion, mCD52 constitutes a novel option for a reporter gene which can be leveraged concurrently with other biomarkers. We could demonstrate that mCD52 is a useful marker for transduction by retrovectors, and the utility of this system may be extended to various viral and non-viral gene transfer systems. With this new marker, we developed an easy-to-use HIV-1 recombination assay system, which is expected to be useful for studying and determining the recombination ratios of many viral strains and/or mutants at one and the same time.

Acknowledgements

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TRIM5α-independent anti-human immunodeficiency virus type 1 activity mediated by cyclophilin A in Old World monkey cells

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ABSTRACT

Cyclophilin A (CypA) is a peptidyl-prolyl isomerase that binds to the capsid protein of human immunodeficiency virus type 1 (HIV-1). TRIM5α is an antiretroviral factor influencing species-specific retroviral replication in Old World monkey (OWM) cells. In the study reported here, we investigated the role of CypA in anti-HIV-1 activity of OWM cells. Exogenous expression of CypA inhibited HIV-1 infection in OWM cells but not in human cells when the function of TRIM5α was suppressed by overexpression of dominant negative form of TRIM5α as well as by using RNA interference. This inhibitory action depended upon the interaction of the CypA moiety with HIV-1 capsid and disruption of CypA and capsid interaction by cyclosporine A enhanced the HIV-1 susceptibility of OWM cells even in the absence of functional TRIM5α. These results point to the presence of novel TRIM5α-independent anti-HIV-1 activity mediated by CypA in OWM cells.

Introduction

The hydrophobic pocket of cyclophilin A (CypA) makes direct contact with the proline residue at the 90th position and adjacent residues on the surface-exposed loop between α-helices 4 and 5 (the h4/5 loop) of human immunodeficiency virus type 1 (HIV-1) capsid (CA) (Franke et al., 1994; Gamble et al., 1996; Luban et al., 1993). This interaction can be disrupted experimentally by mutations that alter CA proline 90 or adjacent residues (Franke et al., 1994), by competitive inhibitors of the interaction including cyclosporine A (CsA) (Braaten et al., 1996a; Franke and Luban, 1996; Thali et al., 1994), by CypA knockout by means of gene targeting (Braaten and Luban, 2001), or by CypA knock-down with RNA interference (RNAi) (Sokolskaja et al., 2006; Sokolskaja et al., 2004). Each of these interventions reduces HIV-1 susceptibility in human cells (Braaten et al., 1996a; Braaten and Luban, 2001; Franke and Luban, 1996; Franke et al., 1994; Rosenwirth et al., 1994; Sokolskaja et al., 2006, 2004), with the block occurring early, at the time of reverse transcription (Braaten et al., 1996b). Recent data have led to the hypothesis that, by binding to CA, CypA protects HIV-1 against antiviral restriction activity in human cells (Luban, 2007; Towers, 2007).

In contrast with the situation in human cells, HIV-1 replication in cells from several Old World monkeys (OWM) encounters a block before completion of reverse transcription due to their tripartite motif (TRIM) 5α (Stremlau et al., 2004). TRIM5α is a member of the tripartite motif protein family, which comprises RING, B-box and a coiled-coil domains (Reymond et al., 2001). Rhesus (Rh) and cynomolgus monkey (CM) TRIM5αs inhibit HIV-1 but not simian immunodeficiency virus from macaque (SIVmac), whereas African green monkey (AGM) TRIM5α prevents replication of both HIV-1 and SIVmac (Hatziioannou et al., 2004; Keckesova et al., 2004; Nakayama et al., 2005; Stremlau et al., 2004). Human (Hu) TRIM5α shows very weak antiviral activity against those viruses (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2004, 2005; Yap et al., 2005) but confers strong resistance against N-tropic murine leukemia virus (N-MLV) (Hatziioannou et al., 2004; Perron et al., 2004; Yap et al., 2004). Among several splicing variants of TRIM5, an α isoform carries the SPRY or B30.2 domain that determines virus specificity of this intracellular factor (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2004, 2005; Yap et al., 2005).

CsA treatment has been reported to increase HIV-1 replication in OWM cell lines (Berthoux et al., 2004; Kootstra et al., 2003; Towers et al., 2003). Recently, two groups investigated the role of CypA in OWM TRIM5α antiviral activity and suggested that CypA acts in trans to promote TRIM5α-mediated restriction of HIV-1 (Berthoux et al., 2005; Keckesova et al., 2006). Berthoux et al. reported that HIV-1 susceptibility increased in response to CypA knock-down to the same extent as observed in response to TRIM5 knock-down by RNAi in Rh FRhK4 cells. However, simultaneous knock-down of both CypA and TRIM5 caused minimal additional increase, suggesting that CypA inhibits HIV-1 replication in Rh cells in a TRIM5α-dependent manner. These findings suggest that CypA is required for efficient CA recognition by TRIM5α (Berthoux et al., 2005). Keckesova et al. (2006) also failed to observe additional increase in HIV-1 susceptibility in TRIM5α-knocked-down AGM and Rh cells in response to CsA treatment. Neither of these reports, however, used the overexpression system of CypA protein.

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In contrast to OWM cells, owl monkeys of the New World monkey possess CypA as a fusion protein with TRIM5 (TRIMCyp) (Nisole et al., 2004; Sayah et al., 2004). In a mutagenesis study of TRIMCyp, deletion of the RING domain of TRIMCyp only partially reduced the ability of the TRIMCyp protein to restrict HIV-1, suggesting that overexpression of CypA-like proteins can results in a diminished susceptibility of cells to HIV-1 (Diaz-Griffero et al., 2006). These findings prompted us to test the effect of exogenous expression of CypA in OWM cells, especially in the absence of functional TRIM5α and we were able to show that CypA suppresses HIV-1 infection in OWM cells even in the absence of functional TRIM5α.

Results

Exogenous expression of CypA reduces HIV-1 susceptibility in OWM cells in TRIM5α-independent manner

To examine the effect of exogenously expressed CypA on HIV-1 infectivity in OWM cells, Sendai virus (SeV) vector system was used to express CypA. HIV-1 susceptibility was then tested by using vesicular stomatitis virus G (VSV-G) protein-pseudotyped HIV-1 vector encoding green fluorescence protein (GFP). AGM-TRIM5-specific siRNA transfection was used to reduce TRIM5 levels. Real time PCR analysis revealed that TRIM5 mRNA levels in siRNA transfected cells were reduced to 23.6% of those of control siRNA transfected cells. As shown in Fig. 1, the TRIM5-specific siRNA transfection greatly enhanced HIV-1 susceptibility in AGM CV1 cells, suggesting the critical role of TRIM5α to restrict HIV-1 in CV1 cells. Exogenous expression of CypA apparently reduced HIV-1 infectivity in the absence of TRIM5α expression, suggesting that CypA may restrict HIV-1 in OWM cells in a TRIM5α-independent manner. On the other hand, CypA expression showed no additive effect on HIV-1 restriction in the presence of TRIM5α. This indicates that the amount of endogenous CypA in the presence of TRIM5α in CV1 cells is sufficient to restrict HIV-1.

Since there is one concern that the residual TRIM5α after siRNA knock-down might interact with exogenous CypA, we used SeV expressing CM TRIM5α lacking the SPRY domain (CM-SPRY(-)-SeV) to eliminate function of endogenous TRIM5α. It was reported previously that TRIM5 splicing variant TRIM5γ, which lacks SPRY domain, dominantly interfered with the antiviral activity of TRIM5α (Stremlau et al., 2004). CM-SPRY(-)-SeV had been shown to efficiently interfere with HIV-1 suppression mediated by over-expressed CM, AGM, Rh and human TRIM5αs (Maegawa et al. submitted). In addition, CM-SPRY(-)-SeV was able to introduce dominant negative TRIM5 mutant to virtually all the cells in a dish, and was also able to suppress anti-HIV-1 activity of TRIM5a much more efficiently than siRNA (Maegawa et al. unpublished result). Effects of CM-SPRY(-)-SeV on HIV-1 infection was not observed in human cells (Fig. 2), confirming that SPRY(-) mutant TRIM5 specifically interfere with TRIM5α function. SeV expressing AGM TRIM5α lacking the coiled-coil region (AGM-CC(-)-SeV), was used as a non-interfering control. AGM cell lines Vero and CV1, Rh cell line LLC-MK2 and human cell lines 293T and MT4, were tested for their HIV-1 susceptibility in the presence of exogenous CypA. We also constructed SeV expressing CypA, which carries an HA tag (YPYDVPDYAA) at its C-termini (CypA-HA). Cells were first infected with AGM-CC(-)-SeV or CM-SPRY(-)-SeV, mixed with a SeV expressing CypA or CypA-HA, or with an empty SeV vector parental Z strain, incubated at 37 °C for 16 h, and then challenged with VSV-G-pseudotyped HIV-1 vector encoding GFP. Consistent with our previous finding, CM-SPRY(-)-SeV infection enhanced HIV-1 susceptibility in OWM cells but not in human cells (Fig. 2). As shown in Figs. 2B, D, and F, exogenous expression of CypA in OWM cells dramatically reduced HIV-1 susceptibility when endogenous TRIM5α were interfered with overexpression of the dominant negative form of TRIM5α. In contrast, the exogenous expression of CypA showed only a slight additional enhancement of HIV-1 susceptibility in the presence of endogenous TRIM5α (Figs. 2A, C, E). The exogenous expression of the HA-tagged version of CypA had a similar suppressive effect on HIV-1 infection, even though the effect was slightly weaker than that of the non-tagged version of CypA (Figs. 2B, D, and F). These results are consistent with the findings from the siRNA experiment shown in Fig. 1. In contrast to OWM cell lines, human cell lines were not affected by the exogenous expression of CypA or CypA-HA in HIV-1 susceptibility either in the presence or absence of functional endogenous TRIM5α (Figs. 2G to J). Similar results were observed when we used the empty SeV vector in stead of AGM-CC(-)-SeV (data not shown). We clearly detected expression of exogenous CypA-HA as well as those of endogenous CypA in human and OWM cell lines, although the levels of expression of exogenous CypA were slightly low in human cells than in OWM cells (Fig. 2K). Taken together, these results indicate that the exogenous expression of CypA in OWM cells has a potent antiviral activity in TRIM5α-independent manner.

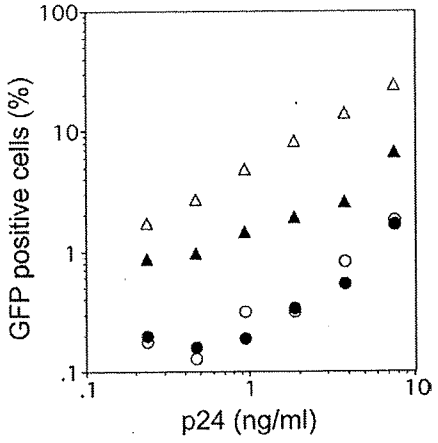

Fig. 1. CV1 cells were transfected with siRNA against AGM TRIM5 (triangles) or SiCONTROL Non-targeting siRNA#2 as a negative control (circles). Two days after transfection, cells were infected with SeV expressing CypA (black triangles or black circles) or empty SeV vector parental Z strain (white triangles and white circles) at a multiplicity of infection (MOI) of 10 plaque forming units (PFUs) per cell and incubated at 37 °C for 16 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were counted with a flowcytometer.

Endogenous CypA also restricts HIV-1 infection in OWM cells in TRIM5α independent manner

CsA treatment disrupts the interaction between CypA and viral capsid and has been reported to increase HIV-1 replication in OWM cells and reduce that in human cells (Berthoux et al., 2004, 2005; Keckesova et al., 2006; Kootstra et al., 2003; Stremlau et al., 2006; Towers et al., 2003). We treated AGM-CC(-)-SeV or CM-SPRY(-)-SeV infected Vero, CV1, LLC-MK2, 293T or MT4 cells with 5 μM of CsA for 1 h and then inoculated them with the VSV-G-pseudotyped HIV-1 vector encoding GFP. As shown in Fig. 3, CsA treatment affected HIV-1 susceptibility in opposite directions in human and OWM cells. CsA treatment enhanced HIV-1 infection in OWM cells (Figs. 3A, C, and E) but reduced HIV-1 infection in human cells in the presence of functional TRIM5α (Figs. 3G and I). These results were consistent with previous findings (Berthoux et al., 2004, 2005; Keckesova et al., 2006; Kootstra et al., 2003; Stremlau et al., 2006; Towers et al., 2003). In the absence of functional TRIM5α, CsA treatment also enhanced the susceptibility of the OWM cells to HIV-1 infection (Figs. 3B, D, and F) and diminished that of the human cells (Figs. 3H and J). These results suggest that endogenous CypA, like exogenous CypA, can also restrict HIV-1 in OWM cells but not in human cells.

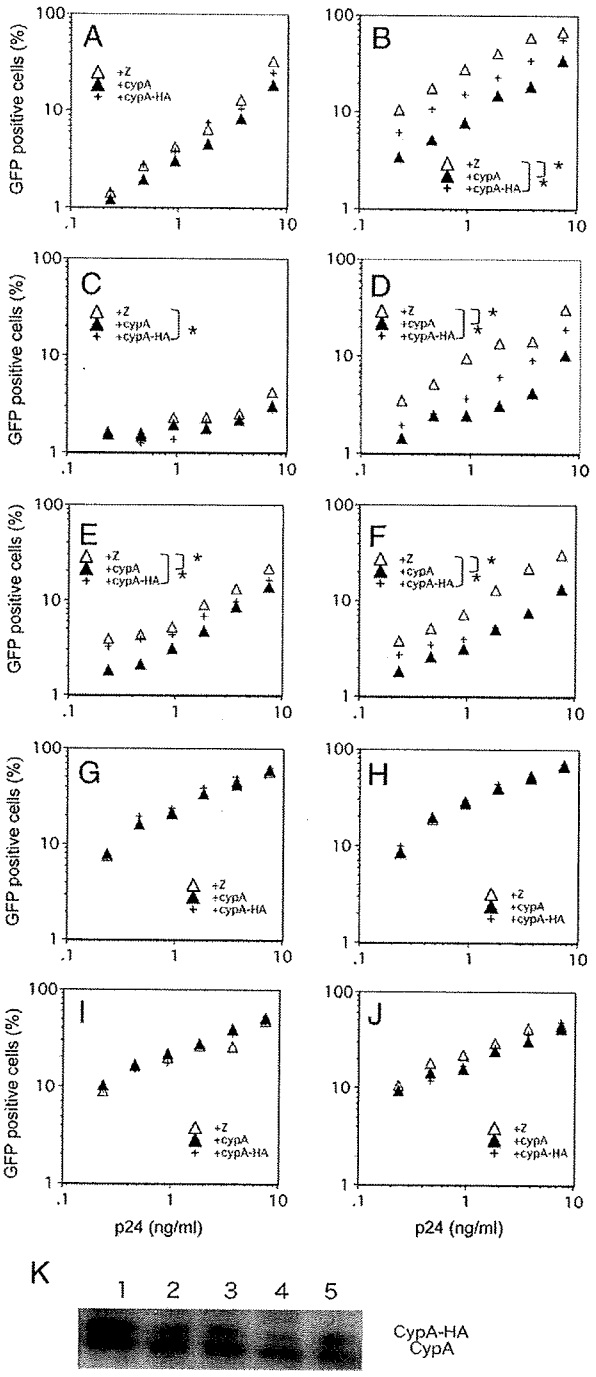

Fig. 2. Vero (A, B), CV1 (C, D), LLC-MK2 (E, F) 293T (G, H) and MT4 (I, J) cells were infected with SeV expressing AGM TRIM5α lacking the coiled-coil domain (A, C, E, G, I) or CM TRIM5α lacking the SPRY domain (B, D, F, H, J) mixed with SeV expressing CypA (black triangles), CypA-HA (crosses) or the empty SeV vector parental Z strain (white triangles) at a MOI of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were counted with a flowcytometer. Asterisks indicate statistically significant differences ($p<0.05$, paired t-test). Representative data of at least three independent experiments is shown. (K) Lysates of CV1 (lane 1), Vero (lane 2), LLC-MK2 (lane 3), 293T (lane 4) and MT4 (lane 5) cells infected with SeV expressing CypA-HA were visualized by western blotting with an antibody against CypA. The lower and upper bands represent endogenous CypA and exogenous CypA-HA, respectively.

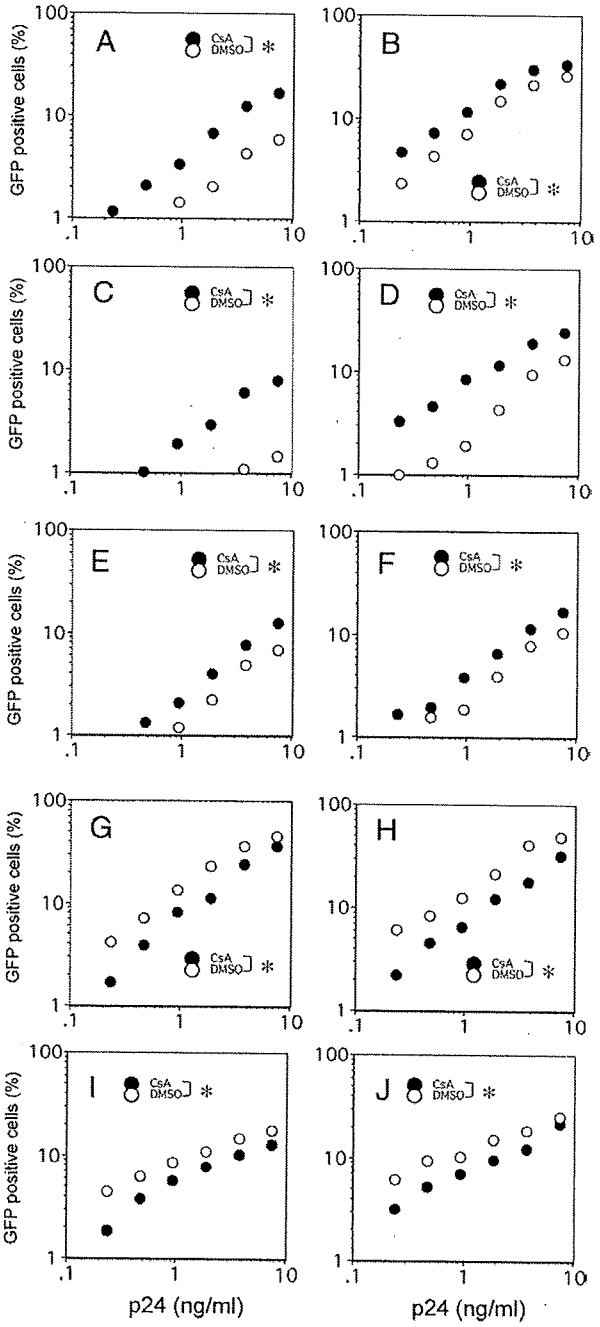

Fig. 3. Vero (A, B), CV1 (C, D), LLC-MK2 (E, F), 293T (G, H) and MT4 (I, J) cells were infected with SeV expressing AGM TRIM5α lacking the coiled-coil domain (A, C, E, G, I) or CM TRIM5α lacking the SPRY domain (B, D, F, H, J) at a MOI of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Cells were treated with 5 μM of Cyclosporin A (black circles) or vehicle control, 0.5% of dimethyl surfoxide (DMSO, white circles), for 1 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP in 5 μM of Cyclosporin A (black circles) or 0.5% of DMSO (white circles) were inoculated for 2 h. After washing out of the inoculum, cells were cultivated for 40 h and GFP positive cells were counted with a flowcytometer. Asterisks indicate statistically significant differences ($p<0.05$, paired t-test). Representative data of at least three independent experiments is shown.

CypA binding to CA is critical for restriction by CypA in OWM cells

The hydrophobic pocket of CypA makes direct contact with HIV-1 CA proline 90 and adjacent residues on the surface-exposed h4/5 loop. Replacement of the h4/5 loop with SIVmac reportedly eliminates CypA