efavirenz and nevirapine levels by rifampicin was much smaller than that by *CYP2B6* 516 TT genotype.

With respect to *CYP3A4*, the analysis was done in only *CYP3A4*-T878C, since there was no variation at the *CYP3A4*-T878C and -C1088T in our study subjects. The results showed that the mean plasma efavirenz concentration at weeks 6 and 12 of ART and 1 month after rifampicin discontinuation were 4.00 ± 0.42, 4.20 ± 0.72 and 3.48 ± 0.34 mg/L, respectively, in patients with homozygous TT genotype and 9.62 ± 6.35, 8.97 ± 6.33 and 3.87 ± 1.69 mg/L, respectively, in those with heterozygous TC genotype. Similarly, the mean plasma nevirapine concentration at weeks 6 and 12 of ART and 1 month after rifampicin discontinuation were 5.85 ± 0.48, 5.50 ± 0.34 and 6.80 ± 0.45 mg/L, respectively, in patients with homozygous TT genotype, and 4.8, 8.69 and 9.12 mg/L, respectively, in one patient with heterozygous mutant TC genotype. Although there was a trend towards higher plasma drug levels in patients with heterozygous mutant TC genotype, appropriate statistical evaluation of this difference was difficult due to small numbers of heterozygous mutant TC.

CD4 T cell counts and HIV-1 viral load among patients with *CYP2B6-G516T* genotypes

The CD4 T cell counts among patients carrying different *CYP2B6* genotypes in efavirenz and nevirapine groups are shown in Figure 2. The number of CD4 T cells in patients with TT, GT and GG genotypes increased in a similar manner at all time points at weeks 12, 24, 36 and 48 of ART compared to the baseline in both efavirenz and nevirapine groups. No significant difference in median CD4 T cell counts of each genotype at different time points was seen in efavirenz group ($p = 0.818$, 0.838, 0.783, 0.753 and 0.587 for baseline, weeks 12, 24, 36 and 48 of ART, respectively), whereas, in nevirapine group, the median CD4 T cell counts of patients with TT genotype seem to be lower than those with the other two genotypes at different time points, although this difference did not reach statistical significance ($p = 0.595$, 0.182, 0.554, 0.573 and 0.494, respectively) (Figure 2a, b).

As shown in Table 2, when the proportion of patients with HIV-1 RNA level <50 copies/mL (log 1.69) were compared among *CYP2B6*-G516T genotypes at week 12 of ART, 88.89% (8/9) of patients with TT genotype in efavirenz group could achieve the HIV-1 RNA levels <50 copies/mL, which were higher than those with GT genotype (77.42%, 24/31) and GG genotype (68%, 17/25), although this difference was not statistically significant ($p = 0.430$). Similarly, in nevirapine group, 100% (2/2) of those with TT genotype, 70.97% (23/31) of those with GT genotype and 60% (15/26) of those with GG genotype could achieve the HIV-1 RNA levels <50 copies/mL, but this difference also did not reach statistical significance ($p = 0.288$) due to small numbers of patients with homozygous TT genotype in this study. At weeks 24, 36 and 48 of ART, nearly all the patients achieved undetectable viral load, since viral load were not detected in 95.38% (62/65), 93.65% (59/63) and 87.9% (54/62), respectively, of efavirenz group and 96.55% (56/58), 94.64% (53/56) and 94.64% (53/56), respectively, of nevirapine group.

Discussion

This is the first report to demonstrate the effects of *CYP2B6*-G516T and *CYP3A4*-T878C polymorphisms on plasma efavirenz and nevirapine concentrations in rifampicin-treated HIV/TB co-infected Thai adults. The results indicated that the wide interindividual variability of efavirenz concentrations is strongly influenced by *CYP2B6*-516TT genotype by the finding of significantly higher plasma efavirenz concentration at weeks 6 and 12 of ART and 1 month after rifampicin discontinuation compared to those with GT or GG genotype. Likewise, it seems to be that this *CYP2B6*-516TT would also influence nevirapine concentrations, although it was less pronounced probably due to the small samples size of homozygous mutant TT in our sample set. The present results were in line with the previous report on efavirenz pharmacokinetics when co-administration with rifampicin in HIV/TB co-infected Indian [26,27] and Ghana patients [28] in that plasma efavirenz was highest in patients with *CYP2B6*-516TT genotype when compared to those with GT or GG genotypes. While the heterozygous TC mutant in *CYP3A4*-T878C in this study seems to have some effects on plasma drug concentrations in patients at weeks 6 and 12 of ART and 1 month after rifampicin discontinuation in both efavirenz and nevirapine groups, further statistical analysis was not done due to the relatively less variation of *CYP3A4* among Thai adults in this study. Further investigation should include a larger sample size with varying genotypes in order to draw a definite conclusion on the effect of *CYP3A4* variations.

In this study, the frequency of *CYP2B6*-G516T among 124 Thai adults was 8.9%, which was close to that of our recent study on 237 HIV-infected Thai adults with different rate of CD4 T cell recovery after ARV treatment (9.7%) (submitted for publication) and slightly lower than what has been reported in Thai children (11%) [22]. Comparing to the other ethnic groups, it was higher than those of Japanese (3.3%) [15] and Caucasian (6%) [14], but lower than that of African-American (20%) [21] or African population (23%) [28,29]. Although the frequencies of *CYP2B6*-G516T were different among populations or ethnicity, the pharmacogenetic studies reported so far in HIV patients

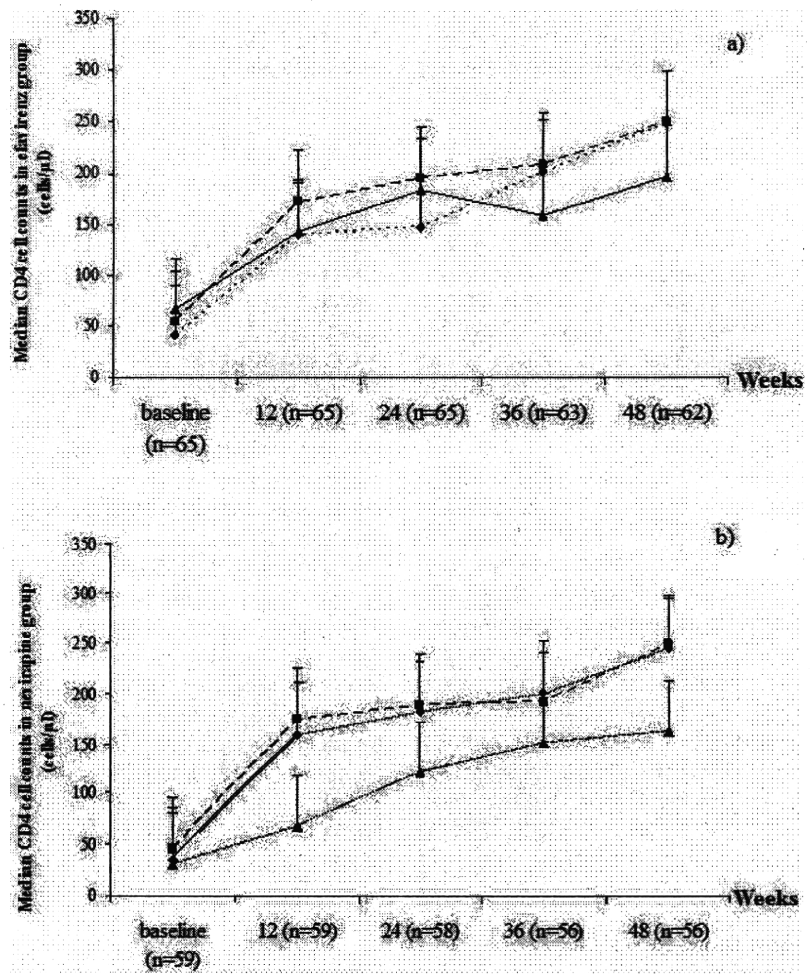

Figure 2 Median CD4 T cell counts among HIV/TB adults with *CYP2B6*-G516T polymorphism at different time points. (Black diamond) GG genotype, (Black square), GT genotype, (Black triangle) TT genotype in efavirenz (a) and nevirapine groups (b) at baseline, 12, 24, 36 and 48 weeks of ART.

Table 2 Number of patients with plasma HIV-1 RNA < 50 copies/ml at week 12 of ART.

	Efavirenz group (N = 65) *CYP2B6*-G516T				Nevirapine group (N = 59) *CYP2B6*-G516T			
	GG n = 25	GT n = 31	TT n = 9	p-value*	GG n = 26	GT n = 31	TT n = 2	p-value**
No. of patients (%)	17 (68)	24 (77.42)	8 (88.89)	0.430	15 (60)	23 (70.97)	2 (100)	0.288

* Chi-square test

** Fisher's exact test

demonstrated that *CYP2B6* 516TT was definitely associated with plasma efavirenz concentration [15,19,21,29,30]. The findings of *CYP2B6* 516TT genotype in the present study support its effect on plasma efavirenz concentration in different ethnic group and gave additional information of this SNP on nevirapine based-ART when co-administered with rifampicin. The recent pharmacogenetic study in HIV patients co-administrated with efavirenz and rifampicin demonstrated that patients carrying TT genotype had significantly higher mean plasma efavirenz but lower oral clearance [28], indicating that rifampicin does not fully reverse the poor metabolizer phenotype and that TT genotype can be used to identify poor metabolizers of efavirenz even

in patients co-administrated with rifampicin. Consistently, the present results also indicated that rifampicin coadministration in HIV/TB infected patients did not significantly alter plasma efavirenz and nevirapine levels in patients with TT genotype ($p > 0.05$). Other possible factors that might affect the plasma drug levels could be excluded since they were carefully controlled.

Although rifampicin can cause the decrease in NNRTI concentrations, the mean plasma efavirenz and nevirapine concentrations in all studied patients with TT, GT and GG genotypes had plasma drug levels above the minimum recommendation (1 mg/L for efavirenz and 3.4 mg/L for nevirapine). One important conclusion from our recent prospective and randomized clinical trial in patients with concurrent HIV/TB receiving rifampicin [24] is that the standard dosage of efavirenz 600 mg or nevirapine 400 mg per day and co-administration with rifampicin was adequate for HIV-1 suppression, however, variation in the plasma drug levels in some patients were found, which might be due to the genetic variations among individuals. Although we reported recently that high body weights of the patients were associated with a low efavirenz C_{12} at weeks 6 and 12 of ART [31], the present results demonstrated that the body weights did not differ among patients with different genotypes of *CYP2B6* G516T polymorphism. The present results thus demonstrated that rifampicin has very small effects on efavirenz and nevirapine plasma drug. The advantage of our present study over previous studies is that plasma efavirenz and nevirapine concentrations during co-administration of rifampicin could be compared with those without rifampicin after completing TB drug treatment.

In general, the high plasma efavirenz and nevirapine levels could lead to the adverse effect such as rash, hepatitis, and neuropsychological toxicity [32,33]. In order to reduce such adverse effects, several studies attempted to test the feasibility of genotype-based dose reduction of efavirenz in African-American [34] and Japanese HIV infected patients [35] and showed that efavirenz dose reduction is feasible and can reduce efavirenz-associated central nervous system symptoms in homozygotes of *CYP2B6*-G516T. Although patients with *CYP2B6*-516TT in our cohort had obviously high plasma efavirenz levels at all time points and certain degree of central nervous system and psychiatric manifestations, they were all well tolerated with the adverse effects. The adverse drug events have not recorded in nevirapine based treatment probably due to the limited number of patients with homozygous TT. Since there were 7 cases who could not complete the study due to side effects [24] it is necessary to determine *CYP2B6* G516T genotypes of these individuals in order to know whether *CYP2B6*-516TT homozygote in Thailand were all well tolerated with the adverse effects of efavirenz and nevirapine.

With respect to possible correlation of the variations in plasma efavirenz and nevirapine levels with the treatment outcome, our results indicated that the patients with *CYP2B6* 516TT genotype had a higher frequency of viral load suppression at week 12 of ART than those with GT and GG genotype. The CD4 T cell counts increased after treatment at all time points which were correlated with HIV-1 viral load reduction. When the effect of different *CYP2B6*-G516T genotypes was analysed, no difference was observed among patients with TT, GT and GG genotypes in both efavirenz and nevirapine groups. Collectively, it is indicated that the efavirenz and nevirapine-based ART co-administered with rifampicin are well correlated with virological and immunological outcomes in patients undergoing treatment for HIV and TB.

In summary, the *CYP2B6* and *CYP3A4* polymorphisms were analysed, for the first time, in HIV/TB co-infected Thai adults receiving efavirenz and nevirapine based-ART co-administered with rifampicin and the results indicated that only 516G>T in *CYP2B6* gene, but not *CYP3A4* gene polymorphism, gave the significant effects on plasma drug levels. Only small effects of rifampicin on efavirenz and nevirapine plasma concentration were observed. However, for further investigation, other SNPs such as *CYP2B6* T983C or TGATC-*CYP2B6* haplotypes which were shown to influence the NNRTI plasma drug levels [23,36,37] should be taken into account and larger sample size with varying genotypes should be included.

Conclusions

CYP2B6-TT genotype had effects on both the plasma efavirenz and nevirapine concentrations in HIV/TB patients when co-administered with rifampicin. The information might be useful for better treatment of patients with HIV or HIV/TB.

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Authors' contributions

SU, SL, WM, NW, TK, SK participated in the study design. SU performed genotyping, CD4 counts and HIV-1 viral load determination, analysed the data and drafted the manuscript. EEN and NW took part in genotyping. SL and WM coordinated the study. TS and SK revised and finalised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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RETROVIROLOGY

RESEARCH Open Access

A single amino acid substitution of the human immunodeficiency virus type 1 capsid protein affects viral sensitivity to TRIM5α

Ayumu Kuroishi[1], Katarzyna Bozek[2], Tatsuo Shioda[1] and Emi E Nakayama*[1]

Abstract

Background: Human immunodeficiency virus type 1 (HIV-1) productively infects only humans and chimpanzees but not Old World monkeys, such as rhesus and cynomolgus (CM) monkeys. To establish a monkey model of HIV-1/AIDS, several HIV-1 derivatives have been constructed. We previously reported that efficient replication of HIV-1 in CM cells was achieved after we replaced the loop between α-helices 6 and 7 (L6/7) of the capsid protein (CA) with that of SIVmac239 in addition to the loop between α-helices 4 and 5 (L4/5) and *vif*. This virus (NL-4/5S6/7SvifS) was supposed to escape from host restriction factors cyclophilin A, CM TRIM5α, and APOBEC3G. However, the replicative capability of NL-4/5S6/7SvifS in human cells was severely impaired.

Results: By long-term cultivation of human CEMss cells infected with NL-4/5S6/7SvifS, we succeeded in rescuing the impaired replicative capability of the virus in human cells. Sequence analysis of the CA region of the adapted virus revealed a G-to-E substitution at the 116th position of the CA (G116E). Introduction of this substitution into the molecular DNA clone of NL-4/5S6/7SvifS indeed improved the virus' replicative capability in human cells. Although the G116E substitution occurred during long-term cultivation of human cells infected with NL-4/5S6/7SvifS, the viruses with G116E unexpectedly became resistant to CM, but not human TRIM5α-mediated restriction. The 3-D model showed that position 116 is located in the 6th helix near L4/5 and L6/7 and is apparently exposed to the protein surface. The amino acid substitution at the 116th position caused a change in the structure of the protein surface because of the replacement of G (which has no side chain) with E (which has a long negatively charged side chain).

Conclusions: We succeeded in rescuing the impaired replicative capability of NL-4/5S6/7SvifS and report a mutation that improved the replicative capability of the virus. Unexpectedly, HIV-1 with this mutation became resistant to CM TRIM5α-mediated restriction.

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 replication of 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]. To establish a monkey model of HIV-1/AIDS, several viruses that are chimeras of HIV-1 and SIVmac (SHIV) have been constructed and tested for replicative capability in simian cells [4,5]. The host range of HIV-1 was limited because of some intrinsic restriction factors in simian cells, such as ApoB mRNA editing catalytic subunit (APOBEC) 3G [6], cyclophilin A (CypA) [7-9], BST-2 (CD317; tetherin) [10,11] and TRIM5α, a member of the tripartite motif (TRIM) family proteins [12]. Rh and CM TRIM5α restrict HIV-1, but not SIVmac [13,14]. A lack of functional TRIM5α expression in pig-tailed monkey enabled Hatziioannou et al. to construct a SHIV strain that differs from HIV-1 only in the *vif* gene and can efficiently replicate in pig-tailed monkeys [15]. Although this virus was designed to escape from monkey APOBEC3G mediated restriction, this virus failed to grow in Rh and CM cells. Kamada et al. attempted to evade the restrictions mediated by CypA in OWM cells by replacing the

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loop between α-helices 4 and 5 (L4/5) of the HIV-1 capsid (CA) with that of SIVmac in addition to *vif* because CypA fails to bind to the L4/5 of SIVmac. However, this was not enough to escape from TRIM5α-mediated restriction [16].

TRIM5α consists of RING, B-box 2, coiled-coil, and SPRY (B30.2) domains [17]. TRIM5α recognizes the multimerized CA of an incoming virus by its α-isoform specific SPRY domain [18-20]. Studies on chimeric TRIM5αs have shown that the determinant of the species-specific restriction against viral infection resides in the variable regions of the SPRY domain [21,22]. On the other hand, we previously identified a single amino acid of the surface-exposed loop between α-helices 6 and 7 (L6/7) of the HIV-2 CA as a determinant of the susceptibility of HIV-2 to CM TRIM5α[23]. On the basis of this finding, we have succeeded in improving simian-tropic HIV-1, which was generated by Kamada et al. [5], by replacing L6/7 of CA with those of SIVmac239 in addition to L4/5 and vif [24]; the new resultant virus has more efficient replication in CM cells. The resultant virus, NL-ScaVR6/7S, showed efficient replicative capability in CM cells; however, the replicative capability of this virus in human cells was severely impaired.

In the present report, we describe our efforts to rescue the impaired replicative capability of NL-ScaVR6/7S after long-term cultivation in human CEMss cells, and we report on the amino acid mutation that improved the replicative capability of this virus.

Materials and methods

Viral adaptation

For viral adaptation in human cells, 100 ng of p24 of NL-ScaVR6/7S [24], renamed in this report as NL-4/5S6/7SvifS, was inoculated into 1 × 10^6 of human T cell line CEMss cells. The infected culture was gradually expanded to keep the cell concentration at 1 × 10^6/mL. The culture supernatants were collected periodically, and p24 levels were measured with an ELISA kit (ZeptoMetrix, Buffalo, NY). Virus in the culture supernatant at day 42 after infection was designated NL-4/5S6/7SvifSd42, and inoculated into fresh CEMss cells. Six days after re-infection, the matrix (MA)-CA region of the integrated provirus was amplified by PCR from the genomic DNA of infected cells and cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) to generate pTopo-MA-CAadp42. Nucleotide sequences of 6 independent clones were determined by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA).

DNA constructions

The HIV-1 derivatives were constructed on a backbone of infectious molecular clone NL4-3 [25]. To introduce a glycine (G)- to-glutamic acid (E) substitution at the 116th position of CA (G116E) into NL-4/5S6/7SvifS, the 0.5 kb SpeI-ApaI fragment, which corresponds to the N-terminus of the CA including the 116th position and L6/7, of pTopo-MA-CAd42 was transferred into NL-4/5S6/7SvifS to generate NL-4/5SG116E6/7SvifS. The G116E substitution was also introduced into NL4-3 and NL-SVR (renamed NL-vifS in this report) by site-directed mutagenesis with the PCR-mediated overlap primer extension method. Resultant constructs were designated NL-G116E and NL-G116EvifS, respectively (Figure 1). To construct the wild type and mutant HIV-1 clones expressing green fluorescence protein (GFP), the 1.3 kb BssHII-ApaI fragment of NL-G116E, NL-4/5S6/7SvifS, or NL-4/5SG116E6/7SvifS, which corresponds to the MA and CA, was transferred to NL-Nhe GFP, in which the *env* gene was interrupted; and the GFP gene was inserted into the *nef* region. Resultant constructs were designated G116E-GFP, 4/5S6/7S-GFP, and 4/5SG116E6/7S-GFP, respectively. To construct the lentivector expressing GFP under the control of cytomegalovirus promoter, we replaced the Eco RI-Apa I fragment corresponding to MA and CA of the pMDLg/p.RRE packaging vector [24,26,27] with that of NL-G116E, and designated the resultant construct as pMDLg/p.RRE-G116E.

Cells and virus propagation

The human kidney adherent 293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human T cell lines CEMss and MT4 were maintained in RPMI 1640 medium supplemented with 10% FBS. Virus stocks were prepared by transfection of 293T cells with HIV-1 NL4-3 and its derivatives using the calcium phosphate co-precipitation method. Viral titers were measured with an ELISA kit.

Sendai viruses (SeV) expressing CM TRIM5α, human TRIM5α, Rh TRIM5α, and CM TRIM5α without the SPRY domain [CM-SPRY (-)] were described previously [18,23,28].

A cell line stably expressing CM or humanTRIM5α was established as described previously [18]. Briefly, a pCEP4 plasmid (Invitrogen) encoding CM or human TRIM5α fused with HA tag in its C-terminus was transfected into TK-ts13 hamster cells. Transfected cells were then cultured in the presence of 0.3 mg/ml of hygromycin B (Gibco) for 14 days to remove untransfected cells. The expression of TRIM5α was confirmed by Western blot analysis of cell lysate with anti-HA antibody (HA High Affinity, Roch).

Viral infections

CEMss or MT4 cells (1 × 10^5) were infected with 20 ng of p24 of NL-4/5SvifS, NL-4/5S6/7SvifS, or NL-4/5SG116E6/7SvifS. The culture supernatants were collected periodically, and p24 levels were measured with an ELISA kit. To analyze the viral sensitivity to TRIM5α, 1 ×

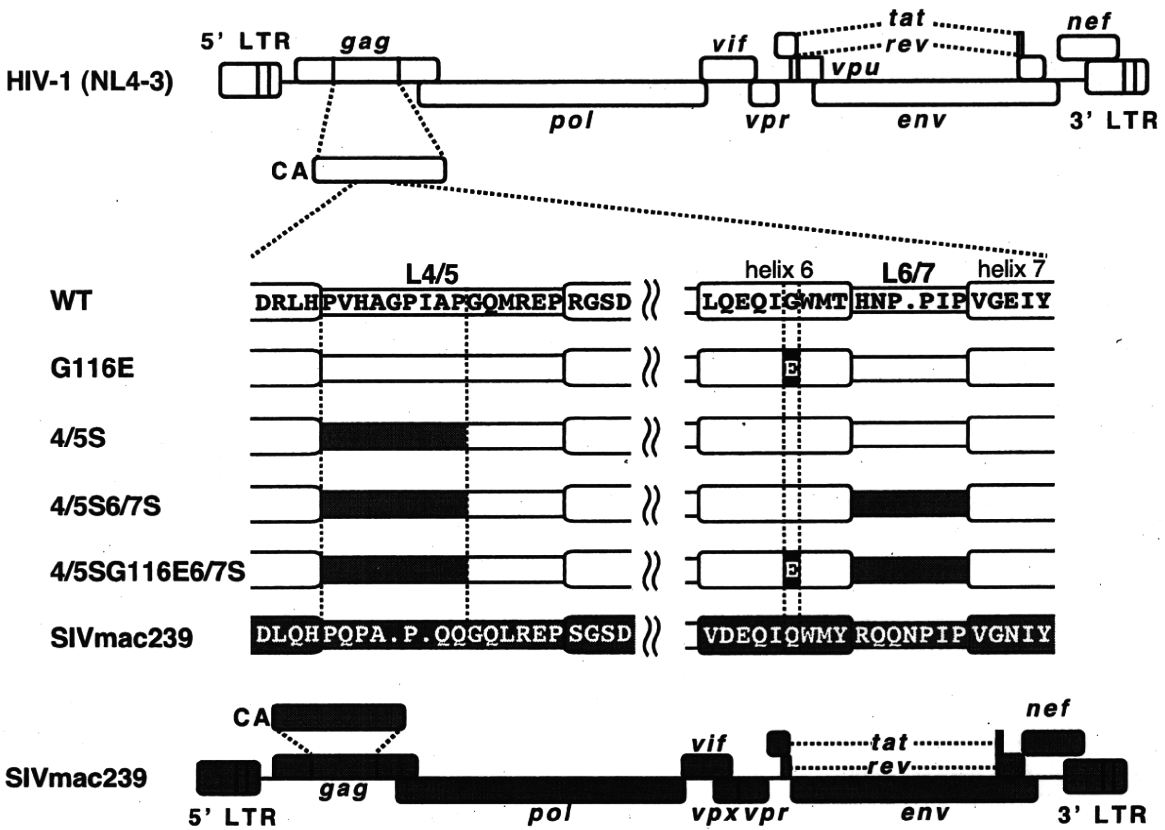

Figure 1 Schematic representation of HIV-1 derivatives. White and gray bars denote HIV-1 (NL4-3) and SIVmac239 sequences, respectively. "E" indicates the amino acid residue at the 116th position of the capsid protein (CA).

10^5 CEMss cells were first 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 hours. Cells were then superinfected with 20 ng of p24 of HIV-1 NL4-3 or its derivatives. The culture supernatants were collected periodically, and the levels of p24 were measured with an ELISA kit.

For the single-round infection assay, CEMss or canine Cf2Th cells were infected with SeV expressing TRIM5α as described above, and super-infected with vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 clones expressing GFP. In case of TK-ts13 hamster cells stably expressing CM, human or CM-SPRY(-) TRIM5α, cells were infected with VSV-G pseudotyped lentivector expressing GFP under the control of cytomegalovirus promoter. Two days after infection, the cells were fixed by formaldehyde, and GFP expressing cells were counted with a flow-cytometer. The percentage of the GFP-positive cells in the presence of TRIM5α was divided by the percentage of GFP-positive cells in the presence of CM-SPRY (-) to define the percent of infection. The differences in percent infection between WT-GFP and G116E-GFP, or 4/5S6/7S-GFP and 4/5SG116E6/7S-GFP were statistically evaluated by using the unpaired t test.

Particle purification and Western blotting

The culture supernatants of 293T cells transfected with plasmids encoding HIV-1 NL4-3 derivatives were clarified by low-speed centrifugation. Nine milliliters of the resultant supernatants were layered onto a 2 mL cushion of 20% sucrose in phosphate buffered saline (PBS) and centrifuged at 35,000 rpm for 2 hours in a Beckman SW41 rotor. After centrifugation, the virion pellets were resuspended in PBS, and p24 antigen concentrations were measured by ELISA. Fifty nanograms of p24 of HIV-1 derivatives were applied to SDS-polyacrylamide gel electrophoresis, and the virion-associated proteins were transferred to a PVDF membrane. CA and CypA proteins were visualized with the anti-p24 antibody (Abcam) and anti-CypA antibody (Affinity BioReagents, Golden, CO), respectively.

Modeling

The structure of the N-terminal domain of the HIV-1 CA protein (PDB number 1GWP) [29] was used as a template for building the domain model with the G116E substitu-

tion. The model was built using Modeller 9v4 [30] and visualized with PyMOL v1.0r2 (The PyMOL Molecular Graphics System, http://pymol.sourceforge.net/).

Results

A virus with SIVmac CA L4/5, L6/7, and vif gained efficient replicative capability after adaptation in human T cell line

We previously reported that in addition to L4/5 of the CA and *vif*, L6/7 of the SIVmac CA is important for the efficient replication of HIV-1 derivatives in CM cells [24]. While introduction of SIVmac L6/7 into an HIV-1 derivative improved viral growth in CM cells, the replicative capability in human cells was greatly attenuated. To gain more insight into the effects of the L6/7 replacement on viral replication, we attempted to rescue the impaired replicative capability by long-term cultivation in human CEMss cells. NL-ScaVR6/7S, a virus with SIVmac L4/5, L6/7, and *vif* renamed NL-4/5S6/7SvifS in the present study, was inoculated into CEMss cells; and culture supernatants were periodically assayed for the levels of p24. Progeny virions were first detectable on day 20 after infection and reached a peak titer on day 42 (Figure 2A). The virus in the culture supernatant on day 42 was designated NL-4/5S6/7SvifSd42 and inoculated into fresh CEMss cells (Figure 2B). This time, the progeny virus was detectable on day 3 and reached a peak on day 20, suggesting that the NL-4/5S6/7SvifSd42 gained certain mutation(s) that overcame the attenuated replicative capability. Therefore, we amplified by PCR and cloned the integrated proviral DNA corresponding to the MA and CA regions in the NL-4/5S6/7SvifSd42-infected CEMss cells on day 6. Nucleotide sequence analysis of the resultant clones revealed that 6 out of 6 independent clones carried a single nucleotide substitution at the 347th position of the CA region, resulting in a G-to-E substitution at the 116th position of the CA (G116E).

Analysis of 95 HIV-1 strains in the Los Alamos HIV sequence databases http://www.hiv.lanl.gov/, including subtypes A to K of group M, revealed that there was no HIV-1 strain carrying glutamic acid at the 116th position of the CA, although this position was occupied with variable amino acid residues (35 strains carried glycine; 36, alanine; 9, threonine; 7, arginine; 6, glutamine; 1 each, isoleucine or aspartic acid).

A single amino acid substitution in CA rescued impaired replicative capability in human cells

To determine whether the single amino acid substitution at the 116th position of the CA improved the replicative capability of NL-4/5S6/7SvifS in human cells, we introduced the G116E mutation into NL-4/5S6/7SvifS. Resultant viruses were designated NL-4/5SG116E6/7SvifS and inoculated into human CEMss or MT4 cells together with their parental viruses to analyze their replicative capability (Figure 3). As described previously [24], NL-4/5S6/7SvifS showed less efficient growth in both CEMss and MT4 human cell lines than did NL-4/5SvifS. NL-4/5SG116E6/7SvifS could grow more efficiently in both human cells than did the parental NL-4/5S6/7SvifS, and its growth was comparable to that of NL-4/5SvifS (Figure 3). These data suggest that the rescued replicative capability of NL-4/5S6/7SvifSd42 in human cells (Figure 2) was the result, at least partly, of the acquisition of the G116E substitution in the CA.

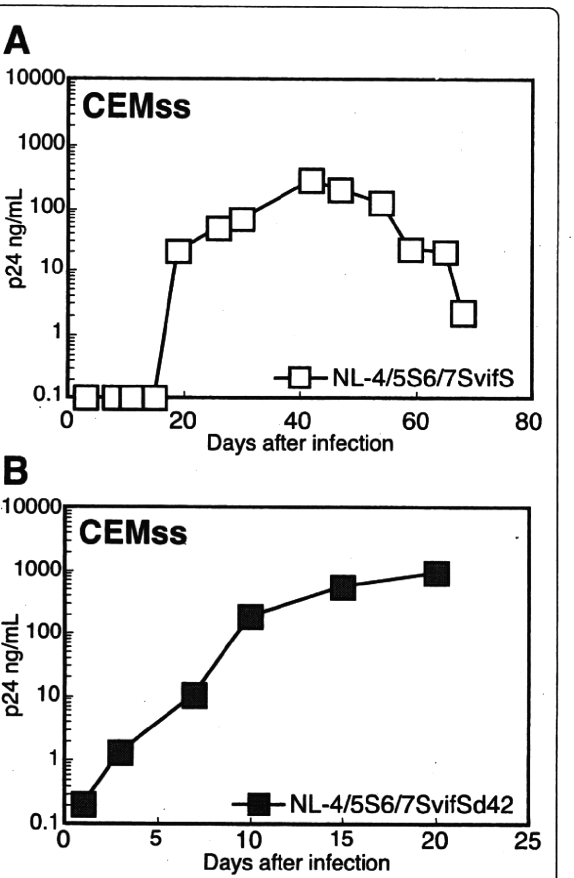

Figure 2 Adaptation of HIV-1 derivatives to human cells. (A) NL-4/5S6/7SvifS, a virus with the SIVmac L4/5, L6/7, and *vif* was inoculated into CEMss cells, and culture supernatants were periodically assayed for the levels of p24. (B) Virus in the culture supernatant on day 42 after infection (NL-4/5S6/7SvifSd42) was inoculated into fresh CEMss cells.

The amino acid residue at the 116th position of the CA affects viral growth in the presence of TRIM5α

We previously reported that NL-4/5S6/7SvifS could grow in CM cells [24], but failed to directly demonstrate that this virus could grow in human cells expressing CM TRIM5α because of its impaired growth capability in human cells. Because the G-to-E substitution at the116th

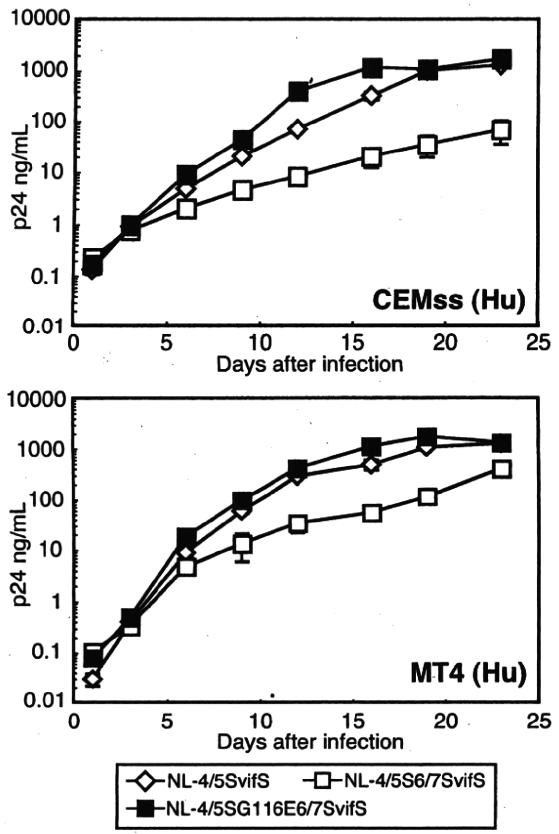

Figure 3 Replication properties of HIV-1 derivatives. Equal amounts of NL-4/5SvifS (white diamonds: virus with SIVmac L4/5 and *vif*), NL-4/5S6/7SvifS (white squares: virus with SIVmac L4/5, L6/7, and *vif*), or NL-4/5SG116E6/7SvifS (black squares: virus with the additional replacement of the 116th amino acid Gly with Glu in NL-4/5S6/7SvifS) were inoculated into human CEMss or MT4 cells, and culture supernatants were collected periodically. The levels of p24 antigen were measured by ELISA. A representative of three independent experiments is shown.

amino acid position rescued the impaired growth capability of NL-4/5S6/7SvifS in human cells, we investigated whether NL-4/5SG116E6/7SvifS could grow in human cells expressing CM TRIM5α (Figure 4A). For TRIM5α expression, we used SeV expressing CM TRIM5α or human TRIM5α. SeV expressing CM-SPRY (-) was used as a TRIM5α-negative control [31]. NL-SVR, a virus with SIVmac *vif* renamed NL-vifS in the present study, did not grow at all in CEMss cells expressing CM TRIM5α. In contrast, NL-4/5SG116E6/7SvifS could grow in CEMss cells expressing CM TRIM5α (Figure 4A), although the viral titers were less than 10% of those in the absence of TRIM5α. Similarly, the human cell-adapted virus NL-4/5S6/7SvifSd42 could also grow in CEMss cells expressing CM TRIM5α (data not shown). To clarify the impact of the single G-to-E substitution in CA on virus growth in the presence of CM TRIM5α, we next introduced a G116E substitution in NL-vifS to generate NL-G116EvifS. We first anticipated that this virus would fail to replicate in CEMss cells expressing CM TRIM5α. Contrary to our expectations, however, this virus grew in the presence of CM TRIM5α to levels similar to those of NL-4/5SG116E6/7SvifS. This result indicates that the single amino acid residue in CA could affect the viral sensitivity to CM TRIM5α mediated restriction. To exclude any possible effect of SIVmac vif in NL-G116EvifS on TRIM5α-mediated restriction, we constructed NL-G116E, a virus with a single amino acid substitution at the 116th position of the CA only (Figure 4B). This virus could also replicate in CEMss cells expressing CM TRIM5α, confirming the importance of the 116th amino acid residue of the CA in TRIM5α-mediated restriction.

With respect to viral sensitivity to human TRIM5α, the growth of both NL-G116EvifS and NL-4/5SG116E6/7SvifS was slightly impaired compared with that of NL-vifS in CEMss cells over-expressing human TRIM5α. The growth of the NL4-3 virus was not affected by human TRIM5α, while that of NL-G116E was slightly suppressed by human TRIM5α. These results suggest that the viruses with G116E substitution were more sensitive to human TRIM5α although the G116E substitution occurred during long-term cultivation of human cells infected with NL-4/5S6/7SvifS. This excludes a possibility that the improved replicative capability of human cell-adapted virus is the result of escape from human TRIM5α-mediated restriction.

A G116E substitution affects viral sensitivity to CM TRIM5α-mediated restriction in a single-round infection assay

The assay described in Figures 3 and 4 investigated the effects of CM TRIM5α on the multi-step growth of the viruses. To evaluate the effects of CM TRIM5α on the early steps of viral infection, we performed a single-round infection assay. The fragment of NL-G116E, NL-4/5S6/7SvifS, or NL-4/5SG116E6/7SvifS corresponding to the MA and CA was transferred to an env-deleted HIV-1 genomic clone, which express GFP after infection. VSV-G pseudotyped wild type and mutant HIV-1 GFP viruses were inoculated into CEMss cells expressing TRIM5α and GFP positive cells were counted 2 days after infection (Figure 5A). Because the replicative capability of NL-4/5S6/7SvifS in human cells was lower than that of the wild type virus as described above, it was highly likely that the infectivity of 4/5S6/7S-GFP would also be lower than those of WT-GFP and G116E-GFP. Therefore, we used higher input doses of 4/5S6/7S-GFP and 4/5SG116E6/7S-GFP than those of WT-GFP and G116E-GFP. Ratios of the GFP-positive percentage of cells expressing CM TRIM5α to those of cells expressing non-functional CM-SPRY(-)-TRIM5α are shown as percent of infection in

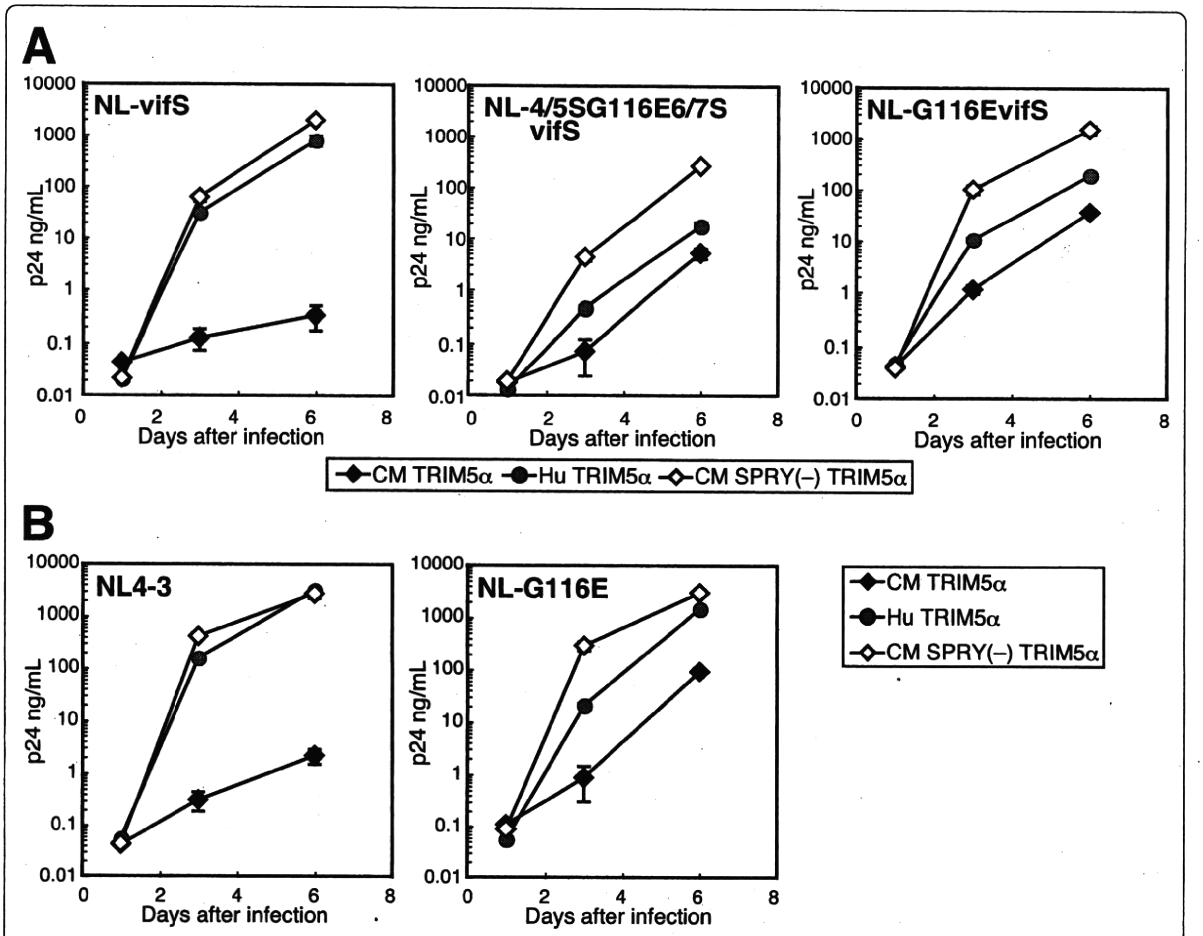

Figure 4 Viral growth in the presence of TRIM5α. CEMss cells were infected with recombinant Sendai virus (SeV) expressing CM (black diamonds), human (gray circles), or CM-SPRY (-) (white diamonds) TRIM5α. Nine hours after infection, cells were superinfected with the indicated HIV-1 derivatives. Culture supernatants were separately assayed for levels of p24. Error bars show actual fluctuations between levels of p24 in duplicate samples. A representative of three independent experiments is shown.

Figure 5B. The percent of infection was relatively constant among the different input doses. Consistent with the results that NL-G116E could replicate in human cells expressing CM TRIM5α (Figure 4B), the GFP-expressing virus with the G116E substitution was more resistant to CM TRIM5α-mediated restriction than the wild type virus, while both viruses were completely restricted by Rh TRIM5α (Figure 5A, Figure 5B left). Similar results were obtained when we used Cf2Th canine cells lacking endogenous TRIM5α expression, although the number of GFP-positive cells was less than that of CEMss cells (data not shown). These results in the single-round infection assay clearly confirmed our results in the live virus replication experiments showing that the G116E substitution conferred resistance against CM-TRIM5α-mediated restriction. While both the GFP-expressing viruses with the 4/5S6/7S (4/5S6/7S-GFP and 4/5SG116E6/7S-GFP) were resistant to CM TRIM5α, an additional effect of the G116E substitution was not observed (Figure 5B, left). To examine the effect of G116E substitution in cells with more physiological levels of TRIM5α expression, we established TK-ts13 hamster cells stably expressing CM or human TRIM5α and inoculated lentivector expressing GFP under the cytomegalovirus promoter into these cells. As shown in Figure 5C and 5D, the GFP expression from the lentivector with the wild type CA was suppressed in TK-ts13 cells expressing CM TRIM5α, although the levels of suppression were less than those in Figure 5B due to lower levels of CM TRIM5α expression. As expected, the lentivector with the G116E substitution showed reduced suppression by CM TRIM5α compared with the wild type CA (Figures 5C and 5D).

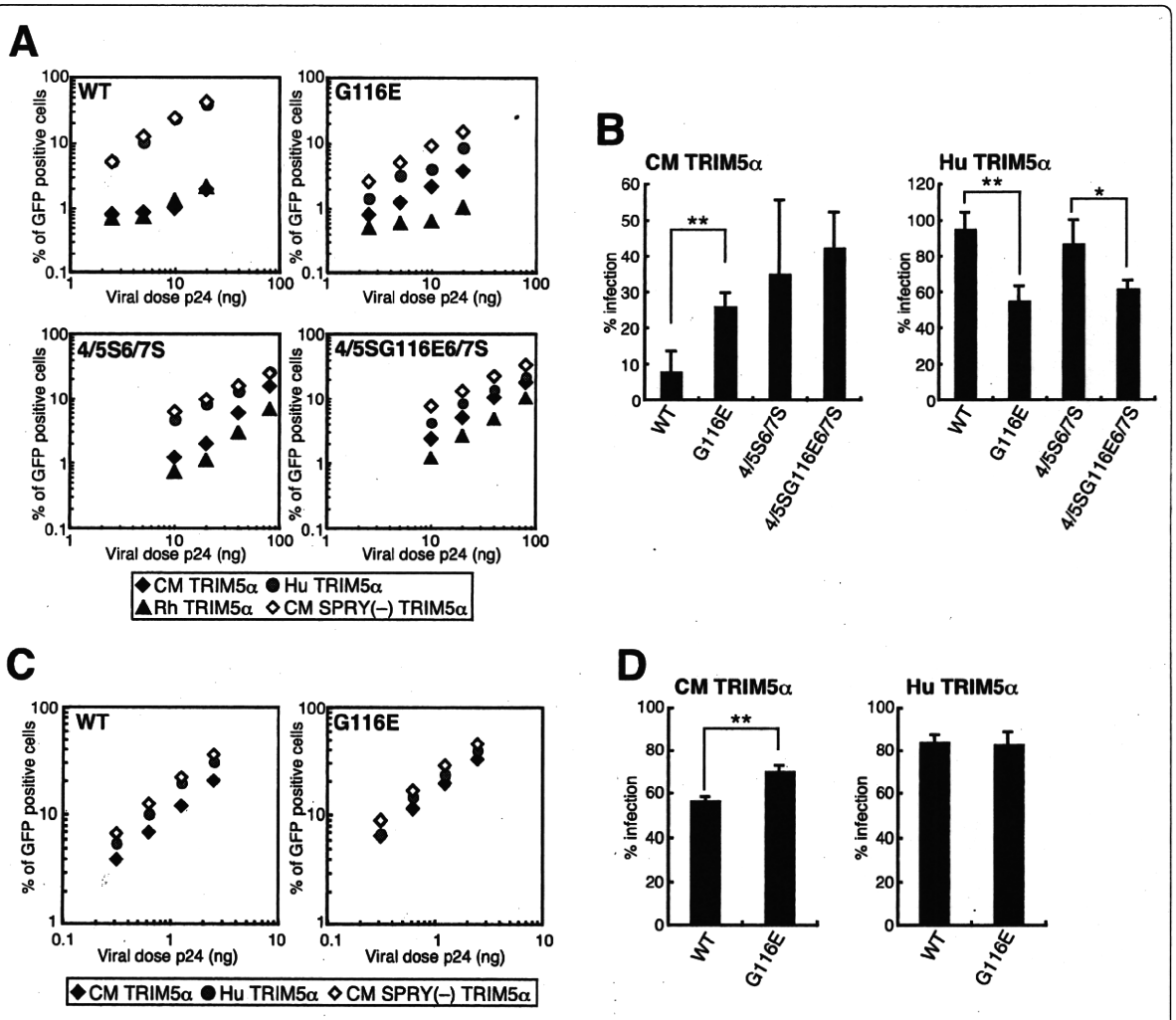

Figure 5 Viral sensitivity to TRIM5α-mediated restriction in a single-round infection assay. CEMss cells were infected with SeVs expressing CM (black diamonds), human (Hu: gray circles), rhesus monkey (Rh: black triangles), or CM-SRPY(-) (white circles) TRIM5α. The cells were then superinfected with serially diluted HIV-1-GFP with the indicated CA. (B) The percentage of the GFP-positive cells in the presence of TRIM5α was divided by the percentage of GFP-positive cells in the presence of CM SPRY (-) TRIM5α to define percent infection. The differences in percent infection between WT-GFP and G116E-GFP, or 4/5S6/7S-GFP and 4/5SG116E6/7S-GFP were statistically evaluated by unpaired *t* test (*: $P < 0.05$, **; $P < 0.01$). The representative results of three independent experiments with similar results are shown. (C) TK-ts13 cells stably expressing CM (black diamonds), human (Hu: gray circles), or CM-SRPY(-) (white circles) TRIM5α were infected with serially diluted lentivector expressing GFP under the control of cytomegalovirus promoter with the indicated CA. (D) The percentage of the GFP-positive cells in the presence of TRIM5α was divided by the percentage of GFP-positive cells in the presence of CM SPRY (-) TRIM5α to define percent infection. The differences in percent infection between the wild type and G116E were statistically evaluated by unpaired *t* test (**; $P < 0.01$). The representative results of three independent experiments with similar results are shown.

On the contrary, the GFP-expressing virus with G116E was more sensitive to humanTRIM5α expressed from the SeV in CEMss cells than the wild type virus (Figure 5B, right). These results again confirmed the results in the live virus replication experiments shown in Figure 4. In the case of TK-ts13, cells stably expressing human TRIM5α in which TRIM5α expression is in more physiological levels; however, the difference in sensitivity to human TRIM5α between the wild type and G116E lentivector was not observed (Figure 5C and 5D). Furthermore, when we used TRIM5α knockout Jurkat cells, we also failed to detect the difference in sensitivity to human TRIM5α between the wild type and G116E virus (data not shown). These results indicated that the effect of G116E substitution is virtually negligible at physiological levels of endogenous human TRIM5α, although this sub-

stitution increases the susceptibility of HIV-1 to human TRIM5α.

A G-to-E substitution at the 116th position did not affect the association between CA and CypA or Gag processing

To clarify whether the 116th amino acid substitution affects the association of CypA with CA, the CypA content in the wild type and mutant virions was evaluated by Western blot analysis. As shown in Figure 6, CypA was detected in virions with HIV-1 L4/5 (lanes 1 to 4, upper panel), but not in those with SIVmac L4/5 (lanes 5 to 7) indicating that the G-to-E substitution at the 116th amino acid position had no effect on CypA binding of HIV-1 CA. When we used anti-p24 antibody (Figure 6, lower panel), p55 Gag precursors and mature p24 CA were detected. The HIV-1 Gag precursor proteins with SIVmac L4/5 and L6/7 were processed nearly normally in the virion, although there were slight differences in the ratios of p24 to p55 among HIV-1 derivatives (Figure 6, bottom). In particular, the virus with SIVmac L4/5 and L6/7 tended to contain increased amount of p55 Gag precursors (lane 6, bottom); however, addition of G116E substitution did not facilitate the cleavage of Gag (lane 7).

Structural model of the capsid protein

To obtain further insight into the effects of the G-to-E single amino acid substitution at the 116th position of the CA on its three-dimensional (3-D) structure, the 3-D model of the N-terminus of the CA was constructed by homology-modeling on the basis of the published crystal structure of the N-terminus of the CA of NL4-3 (PDB number 1GWP) [29] (Figure 7). Position 116 is located in the 6th helix near the L4/5 and L6/7 and is apparently exposed to the surface of the protein (Figure 7 upper panels). The substitution of G to E might be important because in contrast to G, which lacks a side chain, E has a long side chain with a negative charge (Figure 7 lower panels). The mutation can therefore have two possible effects. First, if the residue is located in the interaction site, it can change the local complementarity between CA and TRIM5α. Second, even if the residue is not directly in the binding site, the change in the side chain and polarity can influence the configuration of nearby loops and, thereby, influence a binding site that is located somewhere else on the protein. Notably, the loops being flexible parts of the protein are slightly repositioned in the modeled structure with G116E substitution (Figure 7A and 7B).

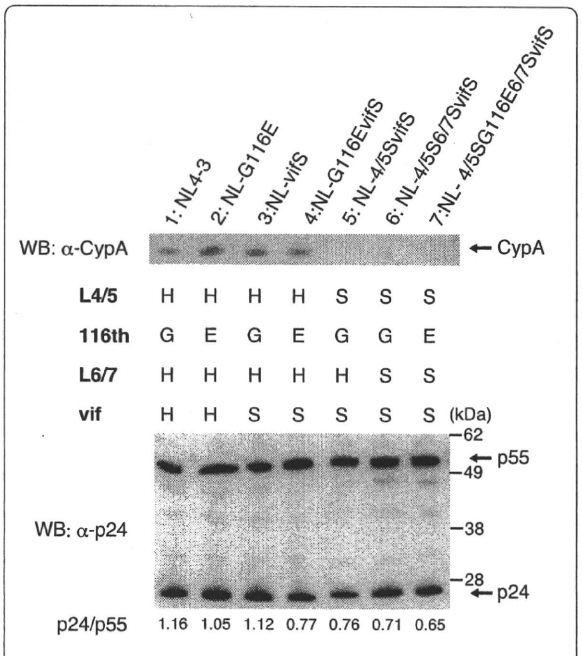

Figure 6 Western blot analysis of the CA and cyclophilin A (CypA) in particles of HIV-1 derivatives. Viral particles of the indicated HIV-1 derivatives were purified by ultracentrifugation through a 20% sucrose cushion. CypA, p24, and p55 proteins were visualized by Western blotting (WB) using anti-CypA and anti-p24 antibody, respectively. "H" and "S" denote the amino acid sequences derived from HIV-1 and SIVmac239, respectively. The ratio of the amount of p24 to that of p55 of each virus is shown at the bottom. A representative of three independent experiments is shown.

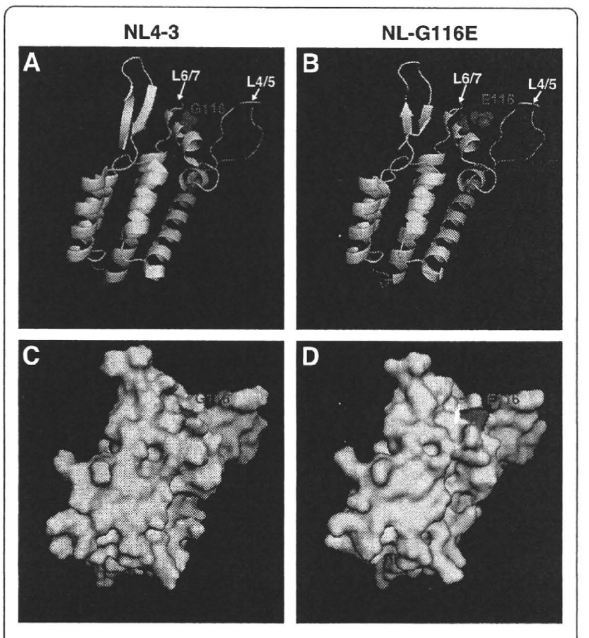

Figure 7 Structural model of the N-terminal domain of HIV-1 CA with G116E substitution. Panel A shows the template structure of the N-terminal domain of the HIV-1 CA; panel B shows the model of the domain structure with the G116E mutation. The ribbons represent the protein backbone; G and E on the 116th position with their side chains are shown in red spheres. Panels C and D show surface views of the template and model structures respectively with the 116th position indicated in red. The loops between α-helices 4 and 5 (L4/5) and 6 and 7 (L6/7) are labeled.

Discussion

By long-term cultivation of human CEMss cells infected with NL-ScaVR6/7S (NL-4/5S6/7SvifS), a simian tropic HIV-1 that could grow efficiently in CM cells but inefficiently in human cells, we succeeded in rescuing the impaired replicative capability of the virus in human cells. Sequence analysis of the MA-CA region of the adapted virus revealed that the there was a G-to-E single amino acid substitution at the 116th position of the CA. Introduction of this substitution into the molecular DNA clone of NL-4/5S6/7SvifS indeed improved the virus' replicative capability in human cells. We thus concluded that the recovered replicative capability in human cells was mainly the result of acquisition of the single amino acid substitution at the 116th position of the CA, although small effects of mutations in regions other than the MA-CA cannot be fully excluded at present.

Although the 116th position of the CA is highly variable among natural HIV-1 strains from subtypes A to K, no virus with E at the 116th position was found in the Los Alamos HIV sequence database 2009 http://www.hiv.lanl.gov/. On the other hand, most HIV-2 and SIVmac strains have glutamine, which has a long side chain similar to E, at this position, and some strains have E. It is possible that the combination of the amino acid residue at the 116th position and L6/7 is important for viral growth. Consistent with this hypothesis, NL-4/5SG116EvifS, a virus with an HIV-1 derived L6/7 and the G116E substitution, showed impaired growth in MT4 cells (data not shown).

The precise reasons for the impaired replicative capability of NL-4/5S6/7SvifS and effect of G116E in human cells remain to be elucidated. Analysis of a series of CA mutants shown in Figures 4 and 5 clearly excluded the possibility that the impaired replicative capability of NL-4/5S6/7SvifS in human cells resulted from an increased sensitivity to human TRIM5α because a virus with the SIVmac L4/5 and L6/7 (4/5S6/7S) showed similar infectivity to the wild-type virus in the presence of human TRIM5α, and a virus with the SIVmac L4/5, L6/7, and G116E substitution (4/5SG116E6/7S) became more sensitive to human TRIM5α (Figure 5B). On the other hand, the virus with the SIVmac L4/5 and L6/7 showed slightly impaired cleavage of p55 Gag precursors, although p24 mature CA proteins were clearly detected (Figure 6). However, the addition of G116E substitution did not facilitate the cleavage of Gag, and a small defect in Gag processing could only partially explain the attenuated growth of NL-4/5S6/7SvifS. Another possibility is that NL-4/5S6/7SvifS was restricted by a certain intrinsic restriction factor that was previously suggested to be present in human cells [13,14], and that the adapted virus could escape from this restriction by G116E substitution, since the G116E was acquired through the adaptation in human cells. It is thus necessary to conduct further analysis to substantiate this unidentified restriction factor.

Although the G116E substitution occurred during long-term cultivation of human cells infected with NL-4/5S6/7SvifS, the viruses with G116E unexpectedly became resistant to CM TRIM5α-mediated restriction (Figures 4 and 5). Replacing the HIV-1 L6/7 (HNPPIP) of the CA with that of SIVmac239 (RQQNPIP) resulted in elongation of the loop by one amino acid, and it is reasonable to assume that the G116E substitution occurred to compensate the structural warp caused by the extended L6/7. This compensatory substitution occurred at the central position of the surface composed of L4/5 and L6/7, a structure considered to be important for TRIM5α binding [24]. The amino acid substitution of G with E at the 116th position caused an important change in the structure of the surface composed of L4/5 and L6/7 because G, which has no side chain, was replaced by E, which has a long, negatively charged side chain as shown in Figure 7. This change in the conformational structure of L4/5 and L6/7 might affect the interaction between the CA and TRIM5α. Alternatively, this single amino acid substitution might influence the configuration of surrounding loops by the changes in the side chain and polarity without directly involving the binding site of TRIM5α.

Conclusion

We succeeded in rescuing the impaired replicative capability of simian tropic HIV-1 NL-4/5S6/7SvifS and unexpectedly identified a single amino acid substitution in the CA that affects viral sensitivity to CM TRIM5α-mediated restriction. This finding will increase our understanding of the detailed molecular interactions between the CA and TRIM5α.

Abbreviations

HIV-1: human immunodeficiency virus type 1; SIVmac: simian immunodeficiency virus isolated form macaque; CM: cynomolgus monkey; Rh: rhesus monkey; SHIV: HIV-1/SIV chimeric virus; CypA: cyclophilin A; TRIM: tripartite motif; CA: capsid; GFP: green fluorescence 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

AK, and EEN performed the in vitro experiments; KB performed computational modeling of CA protein; and AK, TS, KB and EEN wrote the paper.

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Original article

Direct correlation between genome dimerization and recombination efficiency of HIV-1

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Abstract

More than ten subtypes of Human immunodeficiency virus type 1 (HIV-1) have been identified, and many inter-subtype recombinant viruses have been isolated. The genome of HIV-1 is a single-stranded positive sense RNA, and is always found as dimers in virus particles. Frequent recombination between two genomes during reverse transcription is often observed and thus reasonable to assume that genome dimerization controls viral genomic recombination. Recently, several reports indicated *in vitro/in vivo* data to support this idea. In the study reported here, in an attempt to show a comprehensive evidence, we compared the efficiency of various inter-subtype dimerization and recombination and detected a near-complete correlation of the two functions. This suggests that genome dimerization controls recombination and plays an important role in promoting the genetic diversity of HIV-1 in general. We also investigated various inter-subtype hetero-dimerization within HIV-1 virions, and found that the dimer initiation site is a major, but not the sole determinant of dimerization (and recombination) efficiency.

Keywords: HIV; RNA; Packaging; Dimerization; Recombination

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is characterized by a high degree of genetic variability because of its error-prone property of reverse transcriptase (RTase), which allows it to adapt to a wide variety of environments and thus to spread worldwide. Various HIV-1 strains have been classified into many subtypes based on their nucleotide sequence homology. So far, more than ten subtypes have been identified and their worldwide distributions and variations are updated on a daily basis [1,2]. Moreover, many inter-subtype recombinant viruses have been isolated from the boundaries of areas in which distinct subtypes occur and/or from an area in which different subtypes coexist. It has been suggested that these inter-subtype recombinants, known as circulating recombinant forms (CRF), are the result of recombination during reverse transcription (RT) because retroviruses including HIV-1 possess dimerized genomes in their virions.

Genome dimerization of retroviruses has been investigated mainly *in vitro* [3–7], since it was found that synthesized 5′ RNA fragments (several hundred to a thousand bases long) of viral genomes could be dimerized by heating and cooling under suitable buffer conditions. The representative study of hetero-genome dimerization between various subtypes was also performed *in vitro* [8].

Previously, the importance of Stem-loop 1 (SL1) region of HIV-1 dimer linkage structure (DLS) on genome dimerization or recombination was noted by some reports [9–11]. Recent several studies have suggested that the genome recombination frequency of HIV-1 is governed by dimer initiation site (DIS) [12–14], therefore the relationship between genome dimerization and recombination has become a matter of considerable interest. In addition, a series of innovative works which labeled and visualized viral RNA dimerization directly in the

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virion have been published [15,16]. These reports clearly showed the tight hydrogen bonding between two DIS sequences were critical for dimerization.

On the basis of these previous works, we attempted to demonstrate a comprehensive data of hetero-genome dimerization between the various HIV-1 subtypes *in vivo*. Several years ago, we developed a system for assessing the active dimerization signal in the HIV-1 virion without affecting the packaging ability of the genome [17]. We employed this original system to identify the region which is necessary and sufficient for HIV-1 genome dimerization in the virion [18]. As the mutants in this system possess two primary DLS on one genome, it is possible to measure hetero-genome dimerization efficiency between two subtypes by putting two DLS from different subtypes next to each other. We therefore cloned primary DLS from various HIV-1 subtypes and SIVcpz and studied the hetero-genome dimerization efficiencies of different combinations of subtypes and SIVcpz. In parallel, we tried to investigate the hetero-genome recombination frequency of HIV-1 with DLS from various subtypes and mutants. We measured inter-subtype recombination frequency and performed a precise mapping of the determinant for recombination. Finally, we examined the relationship between genome dimerization and recombination by combining the data from the hetero-genome dimerization assay and the recombination assay, and clearly observed a direct correlation between these two functions.

2. Materials and methods

2.1. Constructs

The replication-competent HIV-1 proviral clone pNL4-3 (subtype B) [19] or its *Env* mutant pNLNh [17] was used as a progenitor for the mutant constructs described below. Mutant plasmids were constructed with standard methods. Infectious molecular clones of two subtypes of HIV-1, subtype A/G: p97GH-AG2 [20] and subtype C: pINDIE-C1 [21], were kindly supplied by Drs M. Tatsumi (NIID) and M. Matsuda (Kyoto Univ.). The infectious molecular clone of subtype A/E: p93JP-NH1 [22] was kindly provided by Dr. H. Sato (NIID) and the infectious clone of SIVcpz [23] was generously donated by Dr. A. Adachi (Tokushima Univ.).

Several HIV-1 isolates (subtypes F: BR030, O: MVP5180, G: RU570, and D: ELI) were made available by the NIH AIDS Research and Reference Reagent Program. HIV-1 isolates were inoculated into H9 cells (MVP5180) or human primary mononuclear blood cells (hPBMCs) (other isolates). After several weeks, virus replication was confirmed by examining RTase activity of the supernatants. The infected cells were then harvested and total cellular DNA was extracted and purified.

PCR to amplify the 5′ terminal region of the provirus was performed with viral plasmids or virus-infected cellular DNAs as templates. Primers for each subtype to anneal to the 5′ end of the U3 region (sense) and to the cyclophilin A binding domain in the middle of the CA gene (antisense) were synthesized and used to amplify the plasmids p97GH-AG2, pINDIE-C1, and p93JP-NH1. The cyclophilin A binding domain was well-conserved among various subtypes and virtually all strains had identical amino acid sequences. This enabled us to introduce the SpeI restriction site at the nt. #1510 (in NL4-3 sequences) position of the subtype DNA fragments without changing their amino acid sequences. To make the construction of recombination clones more convenient, several base substitution mutations were introduced into the N-terminal region of the MA gene of pNLNh to create the *Sgr*AI recognition site (pNLAINh), or the *Psp*XI recognition site (pNLXINh). Although the one amino acid substitution (Ser to Thr) at the ninth position of MA in pNLAINh was inevitably introduced as a result of these base substitutions, similar levels of virion production were observed in both cells transfected with pNLAINh or pNLNh (data not shown), suggesting that the effect of the mutations on particle formation was negligible. No amino acid substitution was introduced in MA of NLXINh. Therefore, the primers for each subtype to anneal to the 5′ end of U3 region (sense) and to the N-terminal region of MA gene to introduce *Sgr*AI or *Psp*XI site (antisense) were synthesized and used to amplify the genomic DNAs from cells infected with viral isolates of varying subtypes (D, F, and G for *Sgr*AI; O and SIVcpz for *Psp*XI). The amplified fragments were then subcloned into pGEMTeasy (Promega, Madison, WI) and examined for their sequence authenticity. The U3-MA region of pNLAINh was replaced with the corresponding fragment of each subtype to construct chimeric clones pD-AINh, pF-AINh, and pG-AINh. The U3-MA region of pNLXINh was replaced with the corresponding fragment of each virus to construct chimeric clones pO-XINh and pS-XINh. The L4H fragments (Fig. 1B) were amplified and subcloned by PCR from the various U3-gag subclones by using U5/L junction sense primers and the SL4R antisense primer (5′-GACGCTCTCGCACCCATC-3′). The L4H fragments of various subtypes were isolated and inserted in the *Nhe*I site of pNL4-3 to construct B-Y (Y represents subtype Y), while the *Sal*I–*Hpa*I fragments of B-Y including the *env* region and L4H fragment were isolated and substituted for the corresponding fragments of pX-AINh or pX-XINh (X represents subtype X) to construct various chimeric mutants (X-Y). The amplified regions of the two CRFs, p97GH-AG2 and p93JP-NH1, were assumed to consist exclusively of subtype A and E sequences, respectively. The fragments from these two CRFs are therefore referred to as subtypes A and E in this report.

2.2. DNA transfection

293 T cells [24] (approximately 3×10^6) were seeded on dishes (diameter, 100 mm) the day before transfection with plasmid DNA (5 μg) by means of the calcium phosphate precipitation method [25]. The day after transfection, the supernatant was replaced with fresh medium. For flow-cytometry analysis to examine recombination, two viral vectors (0.1 μg each) were co-transfected along with pCG-VSVG [26] (0.015 μg).

2.3. Isolation of RNA from virions

At 48–72 h post-transfection, virus particles were collected from the media as described elsewhere [27]. The physical

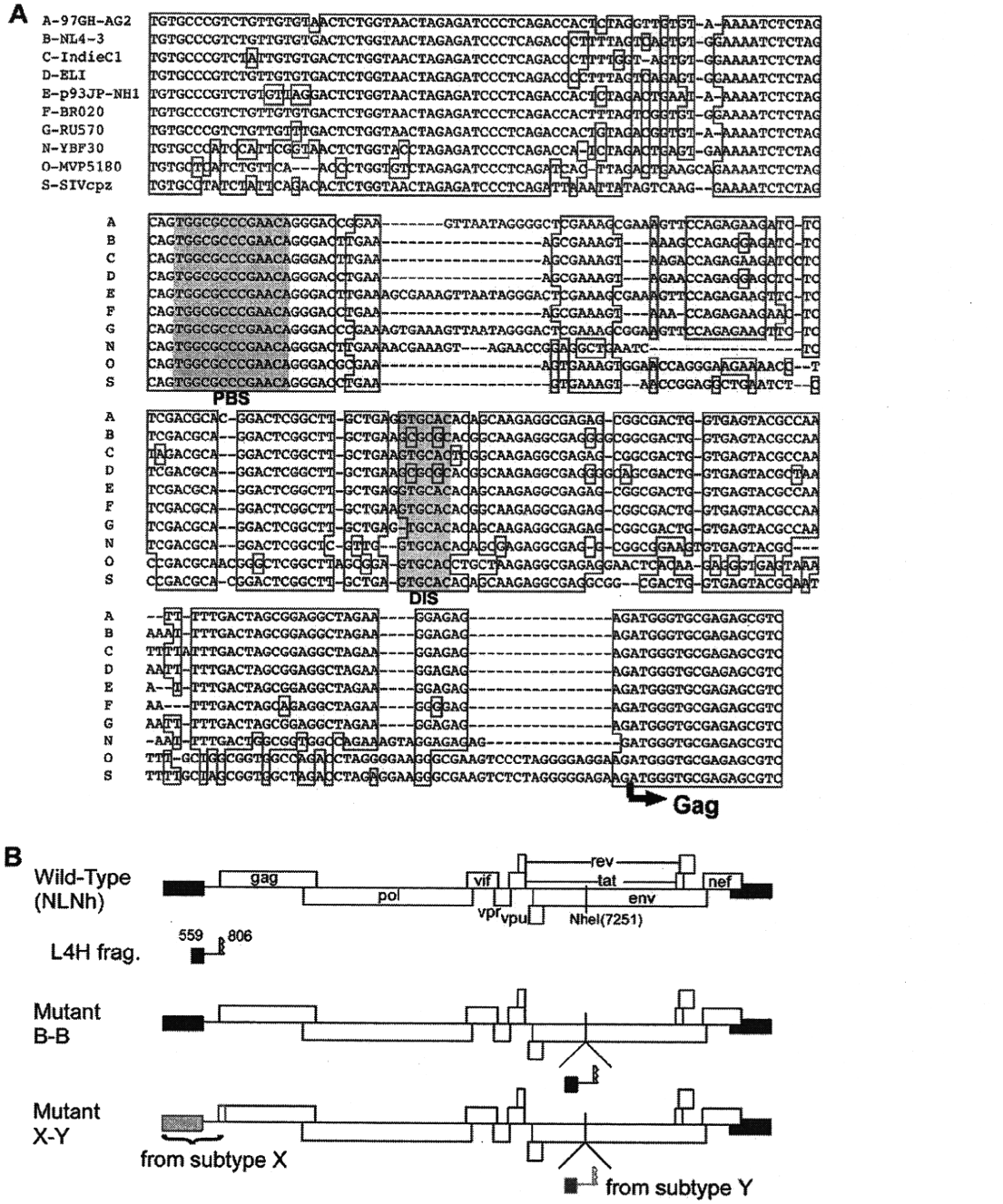

Fig. 1. Viral sequences and constructs used in this study. A) Comparison of the DLS of various HIV-1 subtypes and SIVcpz. Regions sufficient for genome dimerization in virion (fragment L4H) based on previous work are shown. Subtypes A–G were classified into group M. The positions of primer binding site, dimer initiation site, and ATG of gag coding are indicated as PBS, DIS, and Gag, respectively. B) Schematics of constructs. The numbering of the bases follows that of NL4-3.

virus titer was determined with an ELISA assay kit for quantitation of CA-p24 (ZeptoMetrix, Inc., Buffalo, NY). To isolate RNA from particles, virions were disrupted by the addition of 1% sodium dodecyl sulfate (SDS) and treated with proteinase K (300 μg/ml) at room temperature for 60 min, followed by TE-saturated phenol/chloroform extraction, chloroform extraction, and ethanol precipitation.

2.4. Northern blotting analysis

Pelleted RNA was resuspended in T-buffer (10 mM Tris–HCl-pH7.5, 1 mM EDTA, 1% SDS, 100 mM NaCl, and 10% formamide) and the thermostability of dimeric viral RNA was determined by incubating RNA aliquots for 10 min at the prescribed temperatures [28]. RNA electrophoresis on native

agarose gel and northern hybridization analysis were performed as described elsewhere [17]. Plasmid T7pol [28] was used to synthesize a complementary RNA probe for northern hybridization. In experiments designed to assess the conversion of dimers to monomers, relative amounts of both RNA species were quantitated by phosphorimager analysis (Fujifilm Co., Tokyo, Japan) to determine ratios of dimers and monomers. Since the ratio of monomer content to total RNA of virion varied from experiment to experiment, we calculated the "*D* value", which is the index of dimerization efficiency, for the fair validation. The calculation of *D* value was described elsewhere [17]. In short, we made a formula #1 to give an index value of dimer formation efficiency (*D*) of each subtype, to compare the ability of the DLS area of the various subtypes fairly and squarely.

$$D = (X - W)/(B - W) \quad (1)$$

Where *W*, *B*, and *X* represent ratios of monomer content to total RNA of virions produced from wild-type, B-B, and each mutant such as X-Y construct at room temperature, respectively. The *D* value of the wild-type is zero and that of B-B is one. When the combination of subtypes severely affects dimer formation, the monomeric genome content of the virion is reduced and *D* becomes close to zero. Thus, the *D* value was expected to represent the magnitude of the effect of the hetero-dimerization of two subtypes in virions.

2.5. Recombination assay

The HIV-1 recombination assay between two strains was performed as described previously [29]. In short, two similar vectors with the same or different dimerization signals (DLS) were constructed and co-transfected together with or without the VSV-G expression vector. One vector carried an inactivated eGFP gene with N-terminal mutation, while the other carried an inactivated eGFP gene with C-terminal mutation. After transfection, the released virions can be expected to co-package the homo- or hetero-dimerized vector genome, while the ratio of homo- to hetero-dimerization should be 1:1 if the genome expression efficiency of the two vectors is similar. As two vectors also carry surface biomarkers (heat stable antigen (HSA) or mCD52), their expression indicates transfection efficiency of the vectors without VSV-G. With VSV-G expression, pseudotyped virions have been observed to cause retro-transduction to the producer cells [26] 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. The expression of the biomarkers in our study was detected with a flow cytometer.

2.6. Flow cytometric analysis

Mock- and eGFP/HSA/mCD52-expressing 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 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. They were next stained with directly conjugated anti-murine HSA-Phycoerythrin (PE) antibody (BD Biosciences) for an additional 30 min. 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. Hetero-genome dimerization of HIV-1 subtypes

To compare hetero-genome dimerization efficiency of various HIV-1 subtype combinations, we collected infectious proviral plasmids (subtype A, B, C) or virus isolates (other subtypes) of HIV-1. An infectious clone of SIVcpz [23], which is included into HIV-1 group, was also prepared. Virus isolates were infected into cells and total cellular DNA was extracted after confirmation of virus replication. Two regions from each subtype were PCR amplified and subcloned. First, the U3-gag region was amplified to construct chimeric proviral DNA, where the 5′ UTR originated from the various subtypes and the remaining viral genome was derived from NL4-3. The other region was a primary DLS to be inserted in the Env region of a proviral clone, so the vector constructs could be measured for dimerization efficiency. Approximately 250-base-long fragments of the subtypes (Fig. 1A), which contained a minimally sufficient region for functional DLS [18], were PCR amplified and used as DLS in this study. We used the designation "L4H" for this region, and constructed various mutants by exchanging the 5′ LTR-gag region and/or L4H fragment of B-B for corresponding regions of other subtypes to study hetero-dimerization efficiencies (Figs. 1B and 2A).

HIV is divided into three groups, M (major), O (outlier), and N (new) [30], which are highly divergent from each other. In addition, there are some SIVs (e.g. SIVcpz and SIVgor) which are regarded to be included within the HIV-1 lineage [23,31]. To study the relationship between genetic distance and hetero-genome dimerization of HIV-1, we first examined inter-group hetero-genome dimerization efficiency (Fig. 2B) between group M, O, and SIVcpz. The homo-genome dimerization efficiency of group O and SIVcpz showed a similar level to that of group M (Subtype B: NL4-3), proving that the L4H fragment of each group effectively mediated RNA–RNA interactions within the virion. Inter-group dimerization efficiency was 20–40% of that of the homo-group, indicating inefficient hetero-group dimerization. In addition, the efficiency of intra-group M hetero-genome dimerization of some combinations (subtype B-F and B-G) was also not as high as that of homo-dimerization. As the genetic divergence of intra-group virus isolates is much less

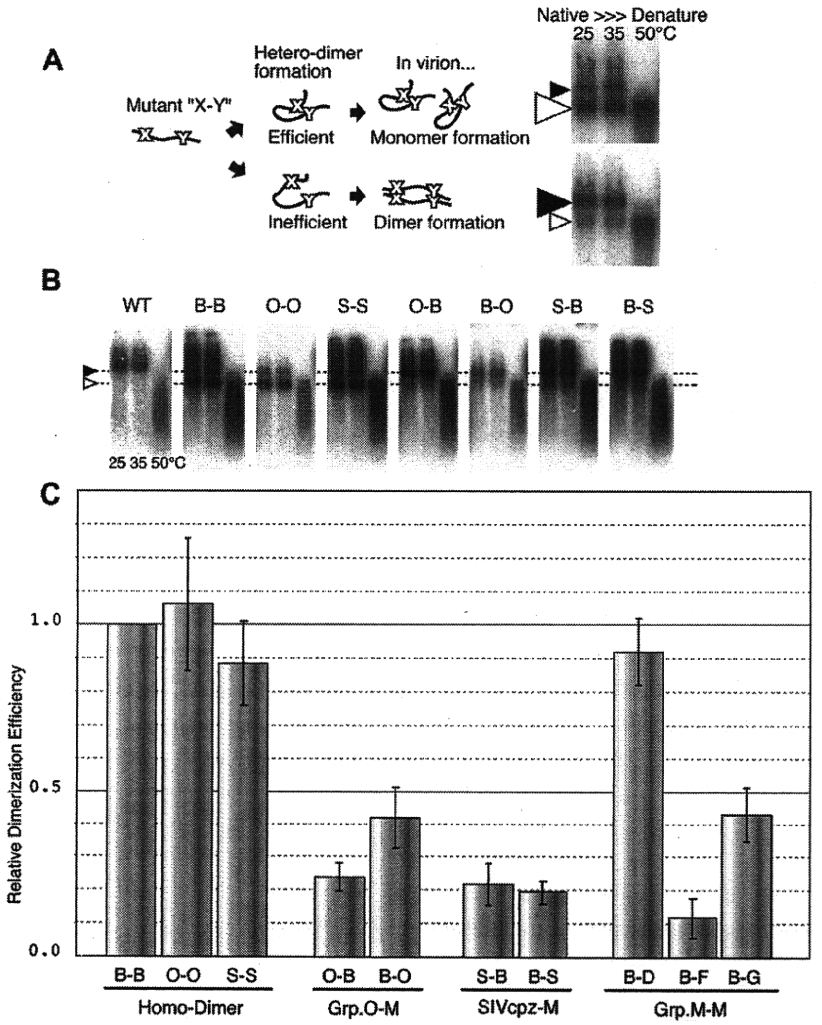

Fig. 2. Inter-group hetero-genome dimerization of HIV-1. A) Schematic representation of hetero-genome dimerization assay. Native northern blots are seen on the right. The black and white arrowheads indicate the dimeric and monomeric viral genome, respectively. The size of the arrowheads indicates the relative strength of the signal. The temperatures of RNA incubation are shown. B) An experimental result from native northern blotting. C) Relative efficiencies of hetero-genome dimerization in various combinations of the groups and the subtypes. The homo-genome dimerization efficiency of subtype B was determined as 1.0 (B-B). Figures show the average of at least three independent experiments. Error bars represent standard errors.

than that of inter-group, our findings suggest that the hetero-genome dimerization does not correlate with the overall genetic distance between virus strains.

3.2. Combination of subtypes that preferentially dimerize each other

To identify certain combinations of subtypes with genomes that dimerize each other efficiently, we examined various combinations of subtypes A, B, and C in group M for hetero-dimerization. We found that the genomes from subtypes A and C hetero-dimerized efficiently, whereas the hetero-dimerization ability of the subtype B genome was relatively low for any combination (Fig. 3). We then explored the region which determines dimerization efficiency by comparing the nucleotide sequences from the various subtypes. As the sequence diversity of U3-5′ UTR region was virtually identical for the three subtypes (Fig. 4A), we focused on the SL1 region in DLS, which a previous report suggested is responsible for genome recombination of HIV-1 [14]. The palindromic sequence on the hairpin loop of SL1, known as DIS (Fig. 4B), of typical subtype B was GCGCGC, whereas those of subtypes A and C were GTGCAC. As it has been suggested that the kissing-loop interaction between two DIS on separate viral genomes is the initial reaction of genome dimerization, coincidence of DIS would be a primary requirement for efficient dimerization. To test whether a difference in DIS affects genome dimerization, we changed the DIS of subtype B to GTGCAC to generate a mutant, SL1M (Fig. 4C). We then examined the difference in hetero-genome dimerization efficiency between SL1M and subtype B by construction of SL1M-B, and observed a dramatic decrease in the dimerization