Effect of viral enzyme inhibitors and fusion defective mutation to retro-transduction

To further test our hypothesis, two more experiments were performed. First, 293T cells were co-transfected with pNLNh and pCG-VSVG in the presence or absence of viral enzyme inhibitors. We used an effective RT inhibitor AZT (Mitsuya et al., 1985) and a protease inhibitor Ritonavir (Markowitz et al., 1995) (Fig. 2). For efficient functioning of AZT, the amount of plasmid DNAs used in AZT experiment was reduced to 0.5 μg in total, and the day after transfection, the medium was replaced with fresh medium with or without the inhibitors. The amounts of virus production were measured 48 h post-transfection. When 0.5 μg of DNA was transfected into 3×10^6 cells, actual amount of CA-p24 of pseudotyped virus was typically 1–2 μg. Virion production was reduced to the same level as that of control in the presence of 5 μM AZT, and the enhancement was recovered as the concentration of the drug was reduced (Fig. 2A). The effect of Ritonavir was similar to that of AZT since 0.417 μM or more Ritonavir severely blocked the enhancement of virion production measured by pr55-Gag amounts (Fig. 2B). For the second experiment, two VSV-G fusion defective mutants, pCGVG-G124E and pCGVG-P127D, were constructed. Both mutants contain one amino acid substitution at the fusion domain of the protein and reportedly lose their membrane fusion activity although their expression level at cell surface remains similar to that of the wild-type (Fredericksen and Whitt, 1995). The mutants were compared to the wild-type VSV-G (WT-G) in terms of viral production enhancement by pseudotyping (Fig. 3A). Both of the mutants showed very similar effects. Unexpectedly, the amount of virus production by the mutants was reduced throughout by approximately half compared to that by the non-pseudotyped virus. Nonetheless, the time course of virus production kinetics was almost identical for the mutants-pseudotyped and non-pseudotyped virus and drastically different from that of WT-G. Some of the data may

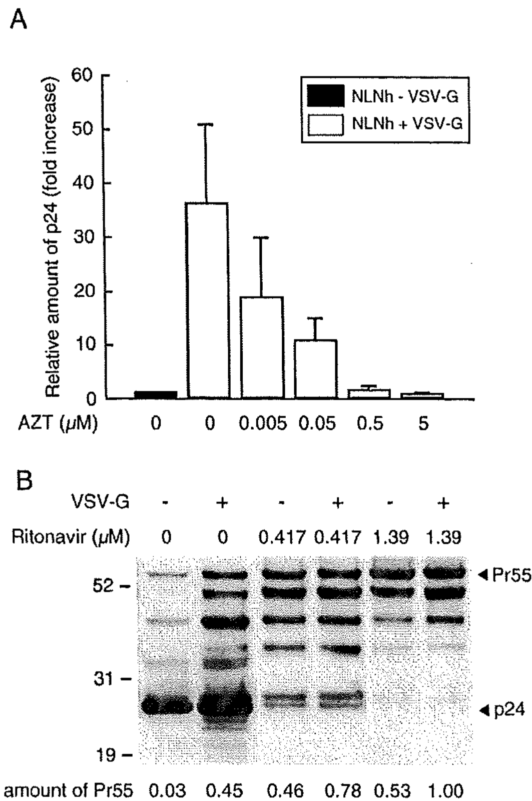

Fig. 2. Effect of viral enzyme inhibitor on enhancement. (A) 293T cells were co-transfected with 0.25 μg of pNLNh and 0.25 μg of either pCG-VSVG or pGEM-5Zf(+) in the absence or presence of 0.005, 0.05, 0.5, and 5 μM AZT. The virus particles derived from the cells were collected at 48 h post-transfection, and CA-p24 antigen was quantified. The value for NLNh without pseudotyping and AZT was set at 1. Results show the averages of three separate experiments. Error bars represent the mean standard error for the difference between experiments. (B) 293T cells were co-transfected with 2.5 μg of pNLNh and 2.5 μg of either pCG-VSVG or pGEM-5Zf(+) in the absence or presence of 0.417 or 1.37 μM Ritonavir. Western blotting of released virion was performed and viral antigen was detected by anti-HIV-1 p24-CA monoclonal antibody. A series of dilution controls was generated (not shown), and the amount of Pr55-Gag were measured. The value for a sample with pseudotyping with 1.37 μM Ritonavir was set at 1. The amount of CA-p24 in the samples was efficiently reduced by the effect of Ritonavir. Results show the representative data of two independent experiments with similar results.

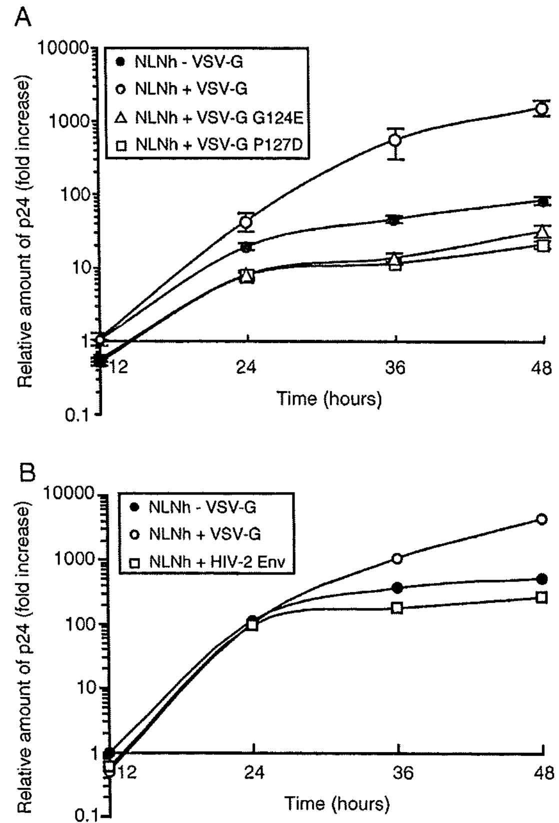

Fig. 3. Effects of fusion-defective VSV-G or HIV-2 Env on enhancement. pNLNh was transfected into 293T cells along with either pCG-VSVG, pCGVG-G124E, pCGVG-P127D, pCGH2Env or pGEM-5Zf(+), and the virus in the culture supernatant was monitored at 12, 24, 36, and 48 h post-transfection by measuring CA-p24 antigen. The values for NLNh without pseudotyping at 12 h was set at 1. (A) Effect of mutation lacking VSV-G fusion. Results show the averages of three separate experiments. Error bars represent the mean standard error of the difference between experiments. (B) Effect of HIV-2 Env pseudotyping. Results show the representative data of two independent experiments with similar results.

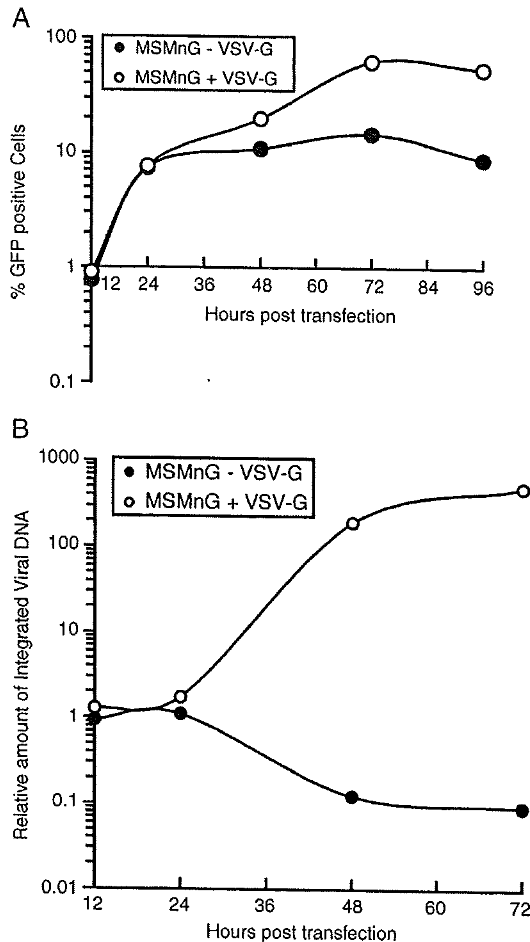

Fig. 4. GFP-virus transfection and viral DNA integration. 293T cells were co-transfected with 0.25 μg of pMSMnG and 0.25 μg of either pCG-VSVG or pGEM-5Zf(+) and were harvested at 12, 24, 48, 72, and 96 h post-transfection. Half of each sample was fixed and subjected for FACS analysis, and total DNA of latter half was extracted for Alu-PCR analysis. (A) GFP expression of transfected cells. Percentages of GFP-positive cells per total cells were indicated. (B) Alu-PCR analysis for integrated viral DNA quantification. The value for the VSV-G(−) sample at 12 h was set at 1. Results show the representative data of two independent experiments with similar results.

be due to reduced virion production in the absence of a suitable envelope. To verify this possibility, we tried pseudotyping of envelope protein (Env) of HIV-2, which is a closely related virus of HIV-1. As the CD4 antigen, the cell surface receptor for HIV-1 and 2 (McClure et al., 1987), is not expressed on the surface of non-lymphocyte cells such as 293T, virion pseudotyped with HIV-2 Env is noninfectious to 293T cells. The data of HIV-2 Env pseudotyping were very similar to that of the fusion defective mutants of VSV-G (Fig. 3B), suggesting that envelope suitability does not affect virion production. HIV-2 Env pseudotyping was confirmed by infectivity assay. The infectivity of it per viral antigen was comparable to that of VSV-G pseudotyped one, suggesting that HIV-2 Env was efficiently incorporated into HIV-1 virion (data not shown). These results are a clear indication that the viral production enhancement by VSV-G pseudotyping was completely eliminated by the blocking of viral infectivity to producer cells and suggest that the enhancement was caused by retro-transduction and subsequent replication of the virus.

The GFP virus experiment and detection of provirus integration

To gain direct evidence of the retro-transduction, we constructed pMSMnG, a derivative of pNL4-3 expressing the green fluorescent protein (GFP) instead of viral nef protein. The 293T cells were transfected with 0.25 μg of pMSMnG along with or without 0.25 μg of pCG-VSVG, and GFP expression and viral DNA integration of the cells were analyzed from 12 h to 96 or 72 h after transfection (Fig. 4). The amount of GFP-positive cells was similar between with and without VSV-G expression until 24 h. After that, that of VSV-G positive sample was increased dramatically and reached nearly to 70% at 72 h whereas that of VSV-G negative ones was only slightly increased to 15% (Fig. 4A). Viral DNA integration was much drastic. It was virtually not observed in VSV-G negative sample throughout the experiment, whereas in VSV-G positive sample, very large amount of viral DNA integration was detected later than 24 h post-transfection (Fig. 4B). These data clearly

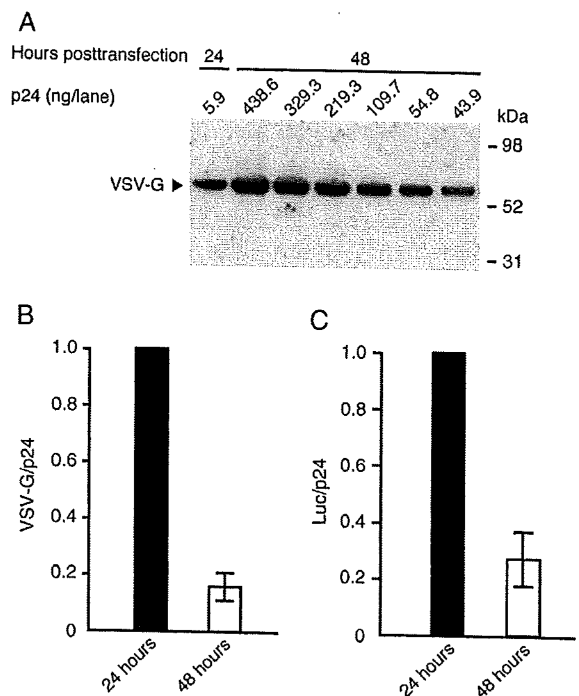

Fig. 5. VSV-G content and infectivity per viral antigen in early and late stages of transfection. Virions derived from 293T cells co-transfected with pNLNh and pCG-VSVG were harvested at 24 and 48 h post-transfection, purified, and lysed. CA-p24 antigen and VSV-G in the lysate were measured by ELISA and by western blot, respectively. (A) Western blot of VSV-G on the virion. The time the virion was harvested and the amount of CA-p24 antigen for each lane are indicated. (B) VSV-G content per viral antigen. Signal intensities of VSV-G on the membrane were quantified as described in Materials and methods. (C) Infectivity of 'early' and 'late' virus. M8166/H1luc cells (10^6) were infected with three serially diluted viruses. At 24 h post-infection, cells were lysed and luciferase activity in the cell lysate was measured. The values for the virus at 24 h were set at 1. Results show the averages of at least three separate experiments. Error bars represent the standard deviation between experiments.

indicated the occurrence of massive retro-transduction and subsequent progenitor production.

Reduction of viral infectivity by retro-transduction

Finally, we examined differences between 'early' and 'late' virus particles. The virion production of the WT-G pseudotyped virus 48 h post-transfection was enhanced up to 20 times compared to that of non-pseudotyped viruses, whereas no such difference was observed at 24 h. This implies that most of the pseudotyped viruses at 48 h can be assumed to have been the result of replication, which might have resulted in some characteristics different from those of the virion produced solely by transfection. To verify this possibility, we harvested the total virus at 24 h ('early') and 48 h ('late') post-transfection and measured the amounts of virus production by CA-p24 ELISA and VSV-G on virus by western blotting (Fig. 5). When we calculated the ratio of VSV-G to CA-p24 at the two time points, we found that the VSV-G content of the 'early' virus was approximately 5 times higher than that of the 'late' virus. This indicates that, under certain conditions, the relative amount of VSV-G of the 'late' virus is drastically reduced compared to that of the 'early' virus particle. To determine the effect of this reduction on virus infectivity, a single-round infection assay using the M8166/H1luc reporter cell line (Nagao et al., 2004) was performed (Fig. 5C). The actual value of infectivity of 'early' virus was typically 1.3×10^5 RLU/s CA-p24 μg. The infectivity of the 'early' virus per CA-p24 was nearly 4 times higher than that of the 'late' virus. This shows that the infectivity of the 'early' and 'late' viruses mostly depends on the amount of VSV-G per virion and therefore could alter dramatically under certain conditions.

Discussion

Retrovirus pseudotyping is one of the most commonly used methods for not only gene delivery, but also virological studies. Since transfection of cells with envelope and *gag/pol* expression vectors makes it easy to generate infectious particles, this method rarely causes concern. However, notable incidents are sometimes observed even in the case of common experiments. In the study reported here, we focused on the enhancement of HIV-1 production by VSV-G protein pseudotyping. In the first set of experiments, we observed up to 20-fold enhancement of viral production by VSV-G pseudotyping, with no enhancement occurring on or before 24 h post-transfection (Fig. 1A). We therefore hypothesized the occurrence of retro-transduction of the virus produced and subsequent virion production. Altered viral antigen profiles in transfected cells with or without pseudotyping indicated the presence of mature viral proteins in the cells with pseudotyping, thus corroborating our hypothesis (Fig. 1B). The experiment using the HIV-1 *pol* mutant also suggested a close correlation between enhancement and viral infectivity (Fig. 1C). In the subsequent experiments, we eliminated the infectivity of the produced virions by viral enzyme inhibitors (Fig. 2), fusion defective mutation in VSV-G, or HIV-2 Env (Fig. 3). Both of the experiments resulted in loss of the enhancement. These data all strongly suggested that the retro-transduction of the produced virus by the transfectant resulted in the release of large quantities of progeny virions.

The virus production was reduced in the virus pseudotyped with VSV-G mutants and HIV-2 Env compared to that in control from 12 h to 48 h post-transfection (Fig. 3). This was possibly due to overexpression of the genes by the CAG promoter since CAG promoter activity is very strong (Niwa et al., 1991) and may take away the transcription/translation factors from other promoters. Reduction of the viral protein was not observed in case of WT-G pseudotyping, probably because the reduction of viral expression offset the enhancement of viral production by WT-G at 12 h, while the enhancement became dominant after that.

The experiment using GFP-virus gained direct evidence for retro-transduction (Fig. 4). From 24 h to 72 h post-transfection, the percentage of GFP-positive cells just doubled in non-pseudotyped sample, which might reflect the period required for maturation of GFP in cells. In contrast, the increase was more than eight times in VSV-G pseudotyped sample. The result of Alu-PCR experiment gave us more solid evidence. The amount of integrated viral DNA was rapidly increased after 24 h post-transfection and reached more than 200-fold higher than the background levels from 24 h to 72 h post-transfection (Fig. 4B). As the viral DNA integration was not detected in non-pseudotyped sample, this result directly demonstrated retro-transduction by pseudotyping during transfection.

The final experiment produced a striking result in terms of alteration of viral envelope incorporation and infectivity (Fig. 5). There was a more than 80% reduction in the quantity of VSV-G per virus at 48 h post-transfection compared to that at 24 h. Moreover, the infectivity of the 'late' virus was reduced in parallel to one-fourth that of the 'early' virus. Throughout the experiments, we used the calcium phosphate method for DNA transfection (Aldovini and Walker, 1990), which is very popular due to its low cost, ease of manipulation, and efficiency. Co-transfection of multiple vectors enables a cell to uptake multiple genes simultaneously, and it is known that, with this method, usually 10–50% of the cells take up the DNA and express the genes thus introduced (Ausubel et al., 1995). In our experiment, we usually noted a transfection efficiency of 20% at most (data not shown). In other words, far more than half the numbers of cells within a dish remained untransfected. The VSV-G pseudotyped virus could equally retro-transduce both transfected and untransfected cells during a transfection experiment. The genome of the pseudotyped virion lacked its original *env* gene and many of the cells were untransfected and thus did not express VSV-G on their surface. As a result, the majority of the cells probably produced progeny virions without envelope proteins. This implies that the reduction of VSV-G per virion observed represents a vast increase in noninfectious particles and a relative decrease in the ratio of pseudotyped virions. As this ratio can vary greatly depending on the time and conditions during transfection, infection experiments using such a mixture of infectious and noninfectious viruses may sometimes produce inconsistent results.

The single-round replication assay involves another issue. To compare the infectivity of mutants to that of the wild-type virus, single-round replication assays of pseudotyped viruses are often performed. If the infectivity of the mutants is reduced, which is very likely, the quantity of noninfectious progeny virions from cells retro-transducted by the mutants pseudotyped with VSV-G would be reduced. As a result, the quantity of infectious virions pseudotyped with the VSV-G contained in the mutants could be much larger than that of the wild-type. In such a case, the infectivity of the mutant per viral antigen would be greatly overestimated and may produce a misleading interpretation of the results.

Construction of a helper-vector system with self-inactivating vector (Miyoshi et al., 1998) eliminates the possibility of reproducing noninfectious viruses, and thus can solve the problems concerned. Nonetheless, retro-transduction of the vector is bound to occur as a result of VSV-G pseudotyping, and retro-transduction of the vector by the vector producing cells might yield somewhat unexpected results such as increase of pseudo-transduction (Liu et al., 1996).

In conclusion, we have demonstrated that retro-transduction of HIV-1 pseudotyped with VSV-G into 293T cells resulted in the enhancement of virus production during transfection. The quantity of infectious viruses pseudotyped by VSV-G to that of the total quantity of viruses is significantly different depending on whether or not the enhancement occurs. In a simple experiment, such as making gene transfer vectors by VSV-G pseudotyping, this issue should not matter much. However, when the harvested virus is used for assays relating to infectivity, there are certain problems that possibly cause misleading of the results. Although VSV-G pseudotyping is very easy and convenient, the conditions under which this procedure is used should be carefully considered.

Materials and methods

Constructs

Replication-competent HIV-1 proviral clone pNL4-3 (Adachi et al., 1986) was digested with *Nhe*I (position 7250), blunted with KOD DNA polymerase, and self-ligated with T4 DNA ligase for the construction of pNLNh. pNLNh thus carries a 4-base insertion mutation within the *env* region, and Env protein expression is abrogated. To construct pNLN-RI, the *Bal*I fragment encoding a part of the *pol* gene (position 2619 to 4551) was removed from pNLNh. EGFP gene was amplified using pCMX-SAH/Y145F (Kallio et al., 1998) (a generous gift from Dr. Takao Masuda, Tokyo medical and dental university) as a template and primers (5′-GGATTTGCTATAAGATGGTGAGCAAGGGCG-3′/5′-CTCGAGTTACTTGTACAGCTC-3′), and a portion of the *env* gene of pNL4-3 was amplified using primers (5′-GTGCTGTTAACTTGCTCAATGCC-3′/5′-CGCCCTTGCTCACCATCTTATAGCAAAATCC-3′). Two fragments were purified, mixed, and amplified again with the second and third primers to generate a fragment including EGFP utilizing ATG codon of *nef* gene as its own start codon and obtaining *Xho*I site just downstream of its stop codon. The fragment was cloned into pGEM-T Easy (Promega) and verified its nucleotide sequence to make pGEMHnGX. The plasmid pMSMBA (McBride and Panganiban, 1996), a derivative of pNL4-3, was digested with *Hpa*I and *Xho*I, and the *Hpa*I–*Xho*I fragment of pGEMHnGX including EGFP gene was inserted in the corresponding position to construct pMSMnG.

pCGVG-G124E and pCGVG-P127D were generated by replacing the 1123-bp *Xba*I–*Acc*65I fragment in pCG-VSVG (a generous gift from Prof. Hideo Iba, Institute of Medical Science, University of Tokyo) with the corresponding fragment carrying the G124E and P127D mutation, respectively. The 5′ part of G124E and P127D fragments were amplified using primer pairs 5′-GCTCTAGAGCCTCTGCTAAC-3′/5′-AGGGAACTCTGGATTCAGC-3′ and 5′-GCTCTAGAGCCTCTGCTAAC-3′/5′-TTTGATCAGGGAAGCCTG, respectively. The 3′ part of the G124E and P127D fragments was amplified using primers 5′-GCTGAATCCAGAGTTCCC-3′/5′-TAGGGTACCATTGATTATGGT-3′ and 5′-CCAGGCTTCCCTGATCAAA-3′/5′-TAGGGTACCATTGATTATGGT-3′, respectively. The total G124E fragment was amplified with primers 5′-GCTCTAGAGCCTCTGCTAAC-3′/5′-TAGGGTACCATTGATTATGGT-3′ (underlined nucleotides represent *Xba*I and *Acc*65I restriction sites) using the 5′ and 3′ parts of G124E as templates. The total P127D fragment was amplified in the same way as described above. The *env* coding region of pGH123 (Shibata et al., 1990), a molecular infectious clone of HIV-2, was amplified with primers (5′-GCCGCCATGTGTGGTAAGAGTCTAC-3′/5′-CTACAAGTCGTAACCATCGTC-3′) to add kozak sequence at 5′ end. The amplified fragment was cloned into pGEM-T Easy to generate pGEMH2env, and the sequence was verified. The HIV-2 *env* expression vector pCGH2env was generated by replacing an *Eco*RI fragment of pCG-VSVG including whole VSV-G fragment with the *Eco*RI fragment of pGEMH2env including the *env* gene.

Cell culturing and transfection

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The cells were transfected with the calcium phosphate method (Aldovini and Walker, 1990) using either pNLNh or pNLN-RI and either pGEM-5Zf(+), pCGH2env, pCG-VSVG, or its derivatives.

Virus purification

At 24 or 48 h after transfection, the culture supernatants of the transfected cells were centrifuged at 1570×g for 20 min at 4 °C, and virus particles in the supernatants were pelleted by ultracentrifugation (151,000×g, 1 h) through a 20% sucrose cushion. The pelleted viruses were then resuspended in growth medium for infection experiment or lysed with lysis buffer (50 mM Tris–HCl—pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS) for western blotting or CA-p24 quantitation. CA-p24 antigen was quantified with an ELISA kit (ZeptoMetrix Corp., Buffalo, NY) according to the manufacturer's instructions.

Virus infection

At 24 and 48 h post-transfection, the resultant virion was purified and used for infection into M8166/H1luc cells (Nagao et al., 2004), which contain integrated reporter DNA carrying HIV-1 long terminal repeat (LTR) and luciferase. Upon infection with HIV-1, HIV-1 LTR is activated along with the expression of viral transactivator Tat, and luciferase expression in cytoplasm is induced. For our study, the cells (1×10^6) were infected at 37 °C with 300 μl of three serially diluted viruses. After 90 min, the cells were washed with PBS, added to 1.5 ml of media and cultured for 40 h at 37 °C. The infected cells were then washed with PBS and lysed with 125 μl of Glo lysis buffer (Promega), and a 50 μl sample of each lysate was assayed for photon emission after the addition of 50 μl of Bright-Glo Reagent (Promega) with a microplate luminometer (Centro LB 960; Berthold Technologies, Bad Wildbad, Germany).

Western blotting analysis

The lysates from the pelleted virus particles and cell lysates were prepared as described previously (Willey et al., 1988), while the proteins were resolved on SDS–4–12% polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes. ECL western blotting detection reagents (Nakalai Tesque, Kyoto, Japan) were used to detect VSV-G or HIV-1 antigen on the membranes. Briefly, the membranes were incubated for 1 h at room temperature with anti-VSV-G polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA), the serum of HIV-1 infected patients, or anti-HIV-1 CA-p24 monoclonal antibody (Advanced Biotechnologies Inc., Columbia, MD) and washed. They were then incubated for another 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-human IgG (Vector Laboratories, Burlingame, CA), washed, and visualized by chemiluminescence with LAS-1000 (Fujifilm, Tokyo, Japan) according to the manufacturer's manual. The relative intensity of each band was quantified by digital image analysis using ImageGauge software (Fujifilm).

Cell cytometry analysis

The 293T cells were harvested, washed twice with PBS (−), and fixed by suspending in 1% formaldehyde–PBS(−). The fixed cells were analyzed by FACScan (Bekton Dickinson, Franklin Lakes, NJ) to measure the expression of GFP.

Genomic DNA preparation

Transfected cells were harvested at 12, 24, 48, and 72 h post-transfection, washed twice with PBS(−), and pelleted. GenElute mammalian genomic DNA miniprep kit (Sigma, St. Louis, MO) was used to extract total DNA of the cells. DNA was digested overnight with *Dpn*I restriction enzyme at 37 °C to eliminate contaminating plasmids which were methylated.

Alu PCR analysis

For integrated proviral DNA quantitation, a modified Alu-PCR method from a recent report (Brussel and Sonigo, 2003) was employed. In the first round of PCR, two outward-facing Alu primers that anneal within the conserved regions of the Alu repeat element were used together with an HIV-1 LTR specific primer (L-M667) to optimize the probability of amplifying an LTR sequence since Alu elements could be present in either orientation relative to the integrated provirus. L-M667 consisted of an HIV-1 LTR-specific sequence fused with a lambda phage-specific tag sequence at the 5′ end (Brussel and Sonigo, 2003). For the second round of PCR (real-time PCR), a lambda-specific primer (Lambda T) (Brussel and Sonigo, 2003) was used as a sense primer to detect only the amplified fragments in the first round of PCR, and a Taqman probe and an anti-sense primer were selected from the set for R/U5 DNA detection in the previous report (Julias et al., 2001). Cycling conditions of the first round of PCR were 94 °C for 3 min followed by 22 cycles of 94 °C for 30 s, 66 °C for 30 s, 70 °C for 10 min, and then 72 °C for 10 min (Ikeda et al., 2004). Equal volume of DNA (0.15 μg) was applied for amplification. Ten-fold serially diluted DNA samples of 72 h post-transfection with VSV-G pseudotyping were employed as standards. Total amount of DNA in each standard was adjusted by adding total DNA of mock-transfected cells. The resultant PCR products were diluted 100-fold and subjected to real-time PCR using ABI7500 (Applied Biosystems, Foster City, CA).

Acknowledgments

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RANTES –28G Delays and *DC-SIGN* –139C Enhances AIDS Progression in HIV Type 1-Infected Japanese Hemophiliacs

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ABSTRACT

The relationships between host immune factors and HIV-1 disease progression are still in dispute. Unlike CCR5Δ32, which has been found to delay disease progression of HIV-1, there still remain several factors whose effect on the clinical course is unconfirmed. To clarify the relationships, we selected seven single-nucleotide polymorphisms (SNPs) out of the previously reported factors, namely, *RANTES* promoter −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* promoter −589T, and *DC-SIGN* promoter −139C/−336C, and examined these in Japanese HIV-1-infected hemophiliacs ($n = 102$). The genotypes were examined by the direct sequencing method, and the distributions of genotype and allelic frequencies were compared between two groups, slow progressors ($n = 54$) who did not develop AIDS more than 10 years after intravenous infection and others (progressors) ($n = 48$). The allelic frequency of *RANTES* −28G was significantly higher in slow progressors (0.185) than in the progressor group (0.074) [$p = 0.023$, OR = 0.35, 95% CI (0.142, 0.880)]. *DC-SIGN* promoter −139C appeared in progressors with significantly higher allelic frequency (0.333) than slow progressors [0.204, $p = 0.040$, OR = 1.95, 95% CI (1.039, 3.677)]. With *RANTES* −403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* −589T, and *DC-SIGN* −336C, no significant difference was observed in allelic frequencies between the two groups. These results suggest that *RANTES* −28G was associated with delayed AIDS progression, while *DC-SIGN* −139C was associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs.

INTRODUCTION

The influence of host immune factor polymorphisms on AIDS progression has continuously been discussed. Previous studies, including multicenter meta-analyses, have almost concluded that CCR5Δ32[1–4] is related to delayed AIDS progression. These studies have mainly focused on sexually transmitted populations and intravenous drug users (IDUs) among whites, though some cohorts included hemophiliacs.

For years, factors other than CCR5, such as CCR2, SDF1, RANTES, and interleukin-4 (IL-4), have been studied. However, none of them has led to a definitive conclusion as to whether they delay AIDS progression in HIV-1-infected individuals or not. It is of note that most of the recent reports[4–8] have analyzed sexually transmitted individuals or IDUs, and, more importantly, most of the studied populations were whites.

In contrast to those subjects, HIV-1-infected hemophiliacs in Japan form a rather homogeneous population. Historically, Japanese hemophiliacs were thought to be infected with HIV-1 between 1982 and 1985 through contaminated blood coagulant, which means the time and mode of infection were virtually identical. We previously reported the relationships between CCR5 promoter polymorphism and the clinical courses in HIV-1-infected Japanese hemophiliacs.[9] In the current study, we tried to clarify further the impact of host immune factor single nucleotide polymorphisms (SNPs) on HIV-1 disease progression in the same population. The SNP sites analyzed were *RANTES* (*CCL5*) promoter −28/−403, *RANTES* intron 1.1

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(*RANTES* In1.1), SDF1 (*CXCL12*) 3′ untranslated region (UTR) position −801, *IL-4* promoter −589, and *DC-SIGN* (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin, also known as *CD209*) promoter −139/−336.

RANTES, the most potent ligand for CCR5, can compete the entry of and thus suppress replication of HIV-1 R5 (macrophage–tropic) strains, which use CCR5 as coreceptor.[10,11] The polymorphism in the promoter region, −28G, was reported to be associated with slower AIDS progression in Japanese HIV-1-infected cases,[12] though it was not supported by studies in other races.[13,14] *RANTES* −403A was initially reported to retard AIDS progression in HIV-1-infected European-Americans.[8] However, it was also reported to increase the rate of HIV-1 disease progression in cooperation with *RANTES* In1.1C, which is in strong linkage disequilibrium with *RANTES* −403A.[13]

SDF-1, the only natural ligand for CXCR4, can prevent T-lymphocyte infection with X4 (T cell tropic) strains of HIV-1, which use CXCR4 as coreceptor, through its direct blockade effect[15,16] and following CXCR4 downregulation.[17–19] The influence of *SDF1* 3′-UTR position −801A (*SDF1* 3′A) on AIDS progression is still in dispute.

IL-4 −589T was once reported to lower viral load and slow the rate of AIDS progression in whites,[7,20] however, this was not supported by other studies.[21,22] Further analyses are needed to confirm this issue.

The SNPs in the *DC-SIGN* promoter region have been reported to affect infectivity of HIV-1,[23] and recently that of *Mycobacterium tuberculosis*[24] and the severity of Dengue disease[25] as well. As for HIV-1 infection, a relationship between *DC-SIGN* −336C and acceleration of the primary parenteral infection has been reported.[23] Since dendritic cells play an important role not only at the initial phase of mucosal infection but in later expansion and reservoir function, we also evaluated whether *DC-SIGN* promoter −139C and −336C, two of the recently identified SNPs, could affect AIDS progression.

MATERIALS AND METHODS

Subjects

Cryopreserved peripheral blood mononuclear cells (PBMCs) collected from 104 HIV-1-positive Japanese hemophiliacs were used. These patients were presumed to be infected with HIV-1 virtually at the same period, between 1982 and 1985, through contaminated unheated blood products, and were enrolled in the study and followed up until 1996 by the Research Committee on Prevention of Developing Illness and Therapy for HIV-1-infected Patients in Japan.[9] The samples analyzed in this study did not overlap those in the previous report.[12]

All the patients were evaluated for their clinical stages according to 1987 CDC criteria.[26] They were divided into two groups: one consisted of 55 patients who did not proceed to AIDS without any treatment until the year 1994 (designated as "slow progressors" and the other included 49 patients who developed AIDS by 1994 ("progressors"). In this context, "slow progressors" were defined as those who did not progress to AIDS 10 years after HIV-1 infection, and "progressors" were defined as those who progressed AIDS within 10 years. None of the patients had been treated with antiretroviral drugs or other drugs, such as interferon-α, glycyrrhizin, or organic germanium compound.

Polymerase chain reaction and sequencing

In the current study, we examined seven SNPs, such as *RANTES* promoter −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* promoter −589T, and *DC-SIGN* promoter −139C/−336C. To genotype the SNPs, we extracted genomic DNA from the cryopreserved PBMC using a DNA extraction kit (Qiagen, Hilden, Germany), amplified the target DNA by polymerase chain reaction (PCR), and did direct sequencing.

For the analysis of *RANTES* promoters, we amplified the target DNA by PCR with primers RA1 (5′-AGAAGGCCTTACAGTGAGA-3′) and RA3 (5′-GCGCAGAGGGCAGTAGCAA-3′).[12] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 49.2°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *RANTES* In1.1, we used 5′-CCTGGTCTTGACCACCACA-3′ and 5′-GCTGACAGGCATGAGTCAGA as forward and reverse primers, respectively.[13] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *SDF1*, 5′-CAGTCAACCTGGGCAAAGCC-3′ was used as the forward primer and 5′-AGCTTTGGTCCTGAGAGTCC-3′ as the reverse primer.[5] Amplification was done with one cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For the *IL-4* promoter 4-1 (5′-GAATTCAATAAAAAACAA-3′) was used as the forward primer and 4-1190 (5′-GAAACAGAGGGGGAAGCA-3′) as the reverse primer.[27] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 49.2°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *DC-SIGN* promoters, we designed a new primer set, PromF 5′-ACCTGACTACCCTAGGCATT-3′ (nt position −499 to −480) and PromR 5′-GGCCACAGCTTTTATTTCCC-3′ (nt position −38 to −57), and used them as forward and reverse primers, respectively. Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min.

PCR was performed with an AmpliTaq Gold PCR kit (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer instruction. All the amplified products were purified with Montage PCR (Millipore Co., Bedford, MA) and then sequenced by the dye terminator method using BigDye v1.1 and ABI 310 (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's instructions. All the sequence reactions except *RANTES* and *IL-4* were done with diluted PCR primers. For the sequencing of *RANTES* −403, RA2F (5′-ACTGATGAGCTCACTCTAGATG-3′)[12] was used as a primer. For the sequencing of the *IL-4* promoter, we designed another set of primers, 5′-GCCAAGGGCTTCCTTATGGGTAA-3′ (nt position −700 to −678) as forward primer and 5′-AATGCAGTCCTCCTGGGGAAAG-3′ (nt position −402 to −423) as reverse one.

Sample analysis

Genotypic distribution and allelic frequency of the SNPs were compared between the two groups, slow progressors and

progressors, with the c2 test or Fischer's exact test. A *p* value less than 0.05 was considered to be statistically significant. To confirm the associations between the SNPs and disease progression, odds ratios and 95% confidence intervals were further calculated by using unconditional logistic regression (SPSS 14.0J Regression Models).

RANTES haploypes were analyzed by an Expectation-Maximization algorithm utilizing Arlequin ver.3.01 (Genetica and Biometry Laboratory, Geneva, Switzerland).

RESULTS

The genotypic distribution and allelic frequency were analyzed for the SNPs, such as *RANTES* promoters −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* −589T, and *DC-SIGN* promoters −139C/−336C, and compared between slow progressors and progressors in Japanese HIV-1-infected hemophiliacs. These results are shown in Tables 1 and 2. As for the SNPs in the *RANTES* promoter and intron, haplotype analysis (−403/−28/In1.1) was also done (Table 3). All the genotypes were in Hardy–Weinberg equilibrium.

Genotype distribution analysis

In the genotypic analysis of *RANTES* −28, there was a weak tendency that C/G and G/G genotypes were more frequent in slow progressors ($p = 0.08$). Besides, the G/G genotype was detected only in slow progressors, though the number was small (three cases).

DC-SIGN −139 T/C and C/C genotypes appeared more frequently in progressors, though the difference was not significant ($p = 0.10$). As for *DC-SIGN* −336, the C/C genotype was not detected in the current study.

RANTES −403, *RANTES* In1.1, *SDF-1* 3′A −801, and *IL-4* −589 showed no significant difference in genotype distribution between the two groups.

Allelic frequency analysis

RANTES promoters. The allelic frequency of *RANTES* −28G was 0.185 in slow progressors. It was significantly higher compared with that of progressors (0.074) [$p = 0.023$, OR = 0.35, 95% CI (0.142, 0.880)]. The allelic frequency of *RANTES* −403A was also higher in slow progressors (0.343) than in progressors (0.271). There was, however, no statistically significant difference [$p = 0.29$, OR = 0.71, 95% CI (0.391, 1.230)].

RANTES In1.1. The allelic frequency of *RANTES* In1.1C was 0.330 in slow progressors. It was higher than that of progressors (0.245), though this difference was not significant [$p = 0.21$, OR = 0.66, 95% CI (0.353, 1.222)].

RANTES haplotype. Four haplotypes, I (ACC), II (ACT), III (AGC), and IV (GCT), were detected in Japanese hemophiliacs (Table 3), and their frequencies were compared between the progressor and slow progressor groups. Haplotype III (A/G/C at RANTES −403/−28/In1.1, respectively) was higher in slow progressors (0.186) than in progressors (0.065), though the difference was not statistically significant ($p = 0.052$).

SDF1 3′-UTR. As for the allelic frequency of *SDF-1* 3′A, there was no significant difference between slow progressors

Table 1. Association between Genotypes of Host Immune Factors and Clinical Outcomes of HIV-1-Infected Japanese Hemophiliacs

Polymorphism		*Genotype distribution (cases)* *Slow progressors*	*Progressors*	*p value (Fisher's exact test)*
RANTES -28	C/C	37	40	
(n = 101)	C/G	14	7	0.08
	G/G	3	0	
RANTES -403	G/G	24	25	
(n = 101)	G/A	23	20	0.48
	A/A	7	3	
RANTES In1.1	T/T	24	25	
(n = 100)	T/C	23	21	0.21
	C/C	6	1	
SDF1 -801	G/G	24	21	
(n = 102)	G/A	24	23	0.96
	A/A	6	4	
IL-4 -589	C/C	5	5	
(n = 100)	C/T	23	22	0.84
	T/T	25	20	
DC-SIGN -139	C/C	3	5	
(n = 102)	C/T	16	22	0.10
	T/T	35	21	
DC-SIGN -336	C/C	0	0	
(n = 101)	C/T	4	4	1.00
	T/T	50	43	

TABLE 2. ASSOCIATION BETWEEN ALLELIC FREQUENCIES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Polymorphism		*Allelic frequency*		*p value*	*Odds ratio (95% CI*
		Slow progressors	*Progressors*		
RANTES -28 (*n* = 101)	C	0.815	0.926	0.023	0.35
	G	0.185	0.074		(0.142, 0.88)
RANTES -403 (*n* = 102)	G	0.657	0.729	0.29	0.71
	A	0.343	0.271		(0.391, 1.230)
RANTES In1.1 (*n* = 100)	T	0.670	0.755	0.21	0.66
	C	0.330	0.245		(0.353, 1.222)
SDF1 -801 (*n* = 102)	G	0.667	0.677	0.88	0.95
	A	0.333	0.323		(0.531, 1.713)
IL-4 -589 (*n* = 100)	C	0.311	0.340	0.76	0.88
	T	0.689	0.660		(0.484, 1.584)
DC-SIGN -139 (*n* = 102)	C	0.204	0.333	0.40	1.95
	T	0.796	0.667		(1.039, 3.677)
DC-SIGN -336 (*n* = 101)	C	0.037	0.043	1.00[a]	1.16
	T	0.963	0.957		(0.281, 4.754)

[a]Fisher's exact test.

(0.333) and progressors (0.323) [$p = 0.88$, OR = 0.95, 95% CI (0.531, 1.713)].

IL-4 promoter. The allelic frequency of *IL-4* −589T was 0.689 in slow progressors and 0.660 in progressors. There was no significant difference between the two groups [$p = 0.76$, OR = 0.88, 95% CI (0.484, 1.584)].

DC-SIGN promoters. The allelic frequency of the *DC-SIGN* promoter −139C was 0.333 in progressors. It was significantly higher than that of slow progressors [0.204, $p = 0.040$, OR = 1.95, 95% CI (1.039, 3.677)]. The allelic frequency of the *DC-SIGN* promoter −336C was 0.037 in slow progressors and 0.043 in progressors, yielding no significant difference [$p = 1.00$, OR = 1.16, 95% CI (0.281, 4.754)].

To validate these univariate associations, we further analyzed all the SNPs chosen in a multivariate manner using an SPSS regression model. The same SNPs were found to be significantly and nonsignificantly associated with clinical outcomes of HIV-1-infected Japanese hemophiliacs (Tables 2 and 4).

DISCUSSION

In the current study, *RANTES* −28G was found to be associated with delayed disease progression in HIV-1-infected Japanese hemophiliacs. This result supports the previous report, in which the −28G mutation increased RANTES expression and secretion, and thus was concluded to retard AIDS progression in Japanese HIV-1-infected individuals including hemo-

TABLE 3. RANTES HAPLOTYPE AND THEIR ASSOCIATIONS WITH CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Haplotype -403/-28/In1.1	*Slow progressors*		*Progressors*		*Overall*	
	No. of alleles	*Frequency (%)*	*No. of alleles*	*Frequency (%)*	*No. of alleles*	*Frequency (%)*
I ACC	13	(12.7)	16	(17.4)	29	(15.0)
II ACT	1	(1.0)	2	(2.2)	3	(1.5)
III AGC	19	(18.6)	6	(6.5)	25	(12.9)
IV GCT	69	(67.7)	68	(73.9)	137	(70.6)
	102		92		194	

TABLE 4. ASSOCIATION BETWEEN ALLELES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS (N = 102)

Allele	*Progressor (cases)*	*Slow progressor (cases)*	*Odds ratio*	*95% CIs*[a]
RANTES -28				
Non-G	41	37	1.000	
G	7	17	0.185	(0.051, 0.635)
RANTES -403				
Non-G	3	7	1.000	
G	45	47	2.371	(0.485, 11.595)
RANTES In1.1				
Non-C	26	25	1.000	
C	22	29	1.489	(0.544, 4.079)
SDF1 -801				
Non-G	4	6	1.000	
G	44	48	2.049	(0.484, 8.682)
IL-4 -589				
Non-C	21	26	1.000	
C	27	28	1.109	(0.468, 2.630)
DC-SIGN -139				
Non-C	21	25	1.000	
C	27	19	3.793	(1.451, 9.916)
DC-SIGN -336				
Non-C	44	50	1.000	
C	4	4	0.488	(0.093, 2.560)

[a]CIs, confidence intervals.

philiacs.[12] While its delaying effect was evaluated only by the decreased CD4 depletion rate in the previous study, a direct relationship between the allelic frequency and clinical outcomes could be observed in the present study. Moreover, *RANTES* −28 G/G homozygotes were found only in the slow progressor group.

RANTES In1.1C was reported to contribute to the rapid progression of AIDS in European-Americans and particularly in African-Americans.[13] In our study, however, the SNP was not found to influence disease progression in Japanese hemophiliacs. Our haplotype analysis showed that *RANTES* −28G, which was found to have AIDS-delaying effect, was always accompanied by *RANTES* In1.1C. The frequency of haplotype III, which contains both mutant alleles −28G and In1.1C, was significantly higher in Japanese hemophiliacs (0.129) than those reported in European-Americans (0.025) and in African-Americans (0.002).[13] In Japanese HIV-1-infected hemophiliacs, therefore, the protective effect of *RANTES* −28G might exceed the detrimental effect of *RANTES* In1.1C.

SDF-1 3′A was first reported to be associated with delayed onset of AIDS,[28] which was followed by conflicting reports,[6,29–31] concluding that *SDF-1* 3′A does not retard HIV-1 disease progression, either early or late in the course of infection. Our result was concordant with those of the reports denying the association, though Modi *et al.*[32] recently reported its protective effect as haplotype.

The polymorphism, *IL-4* promoter region −589T, was reported to be associated with delayed disease progression in HIV-1-infected nonhemophiliac whites.[7] In contrast, *IL-4* −589T was also reported to be associated with X4 strain acquisition,[27] which could lead to AIDS progression. In the current study, no significant difference was observed in allelic frequency of *IL-4* −589T between slow progressors and progressors. These results may be due to the bilateral functions of IL-4 in HIV-1 infection; it may suppress the primary infection of HIV-1 by downregulation of CCR5[33] and promote coreceptor switch by upregulation of CXCR4 as well.[34]

DC-SIGN is known to bind to HIV-1 gp120 and enhance *in trans* infection of HIV-1 from dendritic cells to T cells.[35] Recently, it was reported that an SNP in the promoter region of *DC-SIGN*, −336C, was associated with increased susceptibility to HIV-1 parenteral infection and not to mucosal infection among European-Americans.[23] In our study, however, the influence of *DC-SIGN* −336C on disease progression to AIDS was not observed. The allelic frequency of −336C was too low for statistical evaluation in Japanese hemophiliacs. Unexpectedly, *DC-SIGN* −139C was found to be associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs. These results may be explained by the report[36] that the SNP is located in the vicinity of a candidate binding site of transcription factor AP-1 (activator protein-1) in the *DC-SIGN* promoter region. The nucleotide substitution near the transcription factor-binding site in the promoter region may increase *DC-SIGN* expression level, resulting in the acceleration of AIDS progression.

In conclusion, our results suggest that *RANTES* promoter −28G is associated with delayed AIDS progression and *DC-SIGN* promoter −139C with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs, while *SDF-1* 3′-UTR, *RANTES* −403A, *IL-4* −589T, and *DC-SIGN* −336C do not

influence clinical courses. Further analysis is needed, particularly concerning the relationship among *DC-SIGN* promoter SNPs, modified *DC-SIGN* expression level, and the clinical course of HIV-1 disease.

SEQUENCE DATA

GenBank accession numbers of the sequences reported in this study are as follows: *RANTES* −28 (rs2280788), *RANTES* −403 (rs2107538), *RANTES* In1.1 (rs2280789), *SDF-1* −801 (rs1801157), *IL-4* −589 (rs2243250), *DC-SIGN* −139 (rs2287886), and *DC-SIGN* −336 (rs4804803).

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BRIEF COMMUNICATION

Wild type and *H43Y* variant of human *TRIM5α* show similar anti-human immunodeficiency virus type 1 activity both in vivo and in vitro

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Abstract Polymorphisms in human genes have been shown to affect the rate of disease progression to acquired immune deficiency syndrome in human immunodeficiency virus type 1 (HIV-1)-infected individuals. Recently, tripartite motif 5α (TRIM5α) was identified as a factor that confers resistance to HIV-1 infection in Old World monkey cells. Subsequently, Sawyer et al. (Curr Biol 16:95–100, 2006) reported a single nucleotide polymorphism (H43Y) in the human *TRIM5α* gene and TRIM5α protein with 43Y was found to lose its ability to restrict HIV-1. In the present study, we reevaluated effects of this allele on in vitro anti-HIV-1 activity as well as on HIV-1 disease progression in European and Asian cohorts of HIV-1-infected individuals. Our epidemiological and molecular biological findings clearly indicate H43Y has a very minor effect on anti-HIV-1 activity of TRIM5α, suggesting that this allele is immaterial, at least in HIV-1-infected Europeans and Asians.

Keywords TRIM5α · H43Y · RING domain · Polymorphism · HIV-1 disease progression · Anti-HIV-1 activity

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Human immunodeficiency virus type 1 (HIV-1) has a very narrow host range limited to humans and chimpanzees. In experiments, HIV-1 does not infect Old World monkeys, such as rhesus and cynomolgus monkeys. Recently, the screening of a rhesus monkey cDNA library identified tripartite motif 5 (TRIM5) as a factor that confers resistance to HIV-1 infection (Stremlau et al. 2004). Shortly afterwards, TRIM5α of the African green monkey (AGM), another Old World monkey, was also shown to restrict HIV-1 infection (Hatziioannou et al. 2004; Keckesova et al. 2004; Nakayama et al. 2005), while human TRIM5α reportedly restrict HIV-1 only weakly but potently restrict N-tropic murine leukemia virus (N-MLV; Hatziioannou et al. 2004; Keckesova et al. 2004; Perron et al. 2004; Yap et al. 2004). TRIM5α is composed of two zinc-finger (RING and B-box), coiled-coil, and SPRY (B30.2) domains.

HIV-1 infection in humans is generally characterized by a long-term, chronic disease course gradually progressing to acquired immune deficiency syndrome (AIDS). Polymor-

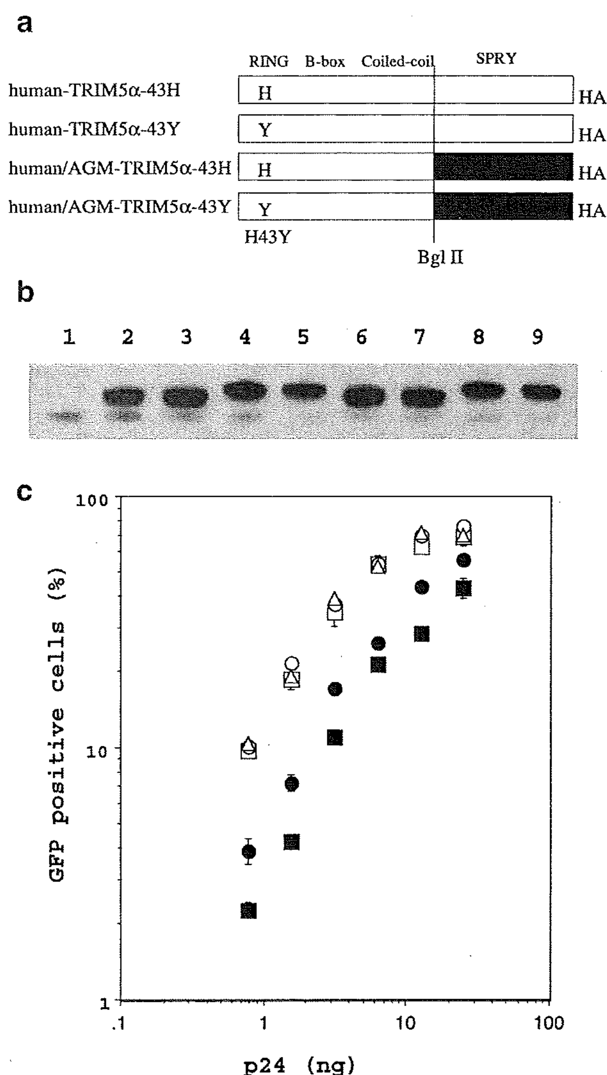

Fig. 1 **a** Schematic representation of TRIM5α fused with HA-tag. Four domains of TRIM5α are shown *at the top*. *Black* and *white bars* denote human and AGM sequences, respectively. A Bgl II site was used to swap SPRY domains between human and AGM TRIM5α. *H* or *Y* denotes the amino acid residue at the 43rd position. **b** Expression levels of HA-tagged TRIM5α proteins. C143 cells were transfected with an empty pCEP4 plasmid (*lane 1*) or pCEP4 carrying human-TRIM5α-43Y (*lanes 2* and *3*), human/AGM-TRIM5α-43Y (*lanes 4* and *5*), human-TRIM5α-43H (*lanes 6* and *7*), human/AGM-TRIM5α-43H (*lanes 8* and *9*) protein and hygromycin-resistant cells were selected for 14 days. One million cells of each transformant were lysed for immunoprecipitation with an anti-HA antibody. Two independent clones for each construct were evaluated for expression levels. **c** C143 cells expressing human-TRIM5α-43H (*open squares*), human-TRIM5α-43Y (*open circles*), human/AGM-TRIM5α-43H (*closed squares*), human/AGM-TRIM5α-43Y (*closed circles*), or cells transfected with an empty vector (*open triangles*) were exposed to the indicated p24 amounts of GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flowcytometry (FACScan, Beckton Dickinson). *Error bars* indicated actual fractures of two independent cell cultures derived from independent clones. Representative data from three independent experiments are shown

phisms in human *CCR5* and other genes reportedly affect the rate of disease progression to AIDS (Kasper et al. 2005). Regarding the human *TRIM5α* gene, Sawyer et al. (2006) reported a common histidine-to-tyrosine polymorphism at the 43rd amino acid residue (H43Y) of human *TRIM5α*. This single nucleotide polymorphism (SNP) locates in the RING domain, and TRIM5α protein with H43Y was found to lose its ability to restrict HIV-1. This SNP was also shown to greatly reduce the ability of TRIM5α to restrict N-MLV. On the other hand, Speelmon et al. (2006) sequenced the *TRIM5α* gene from 110 HIV-1-infected and 96 exposed-seronegative European Americans and found 48 SNPs in their *TRIM5α* genes. However, they did not observe any association between H43Y polymorphism in HIV-1-infected subjects and their set-point viral load after acute infection. Furthermore, they detected no difference in in vitro HIV-1 susceptibility of CD4+ cells between 43Y homozygote and the wild type. Sawyer et al. (2006) and Speelmon et al. (2006) thus came to opposite conclusions; the former suggested that 43Y incapacitates even the modest human TRIM5α resistance to HIV-1 infection, while the latter showed no difference between 43H and 43Y.

After that, two more groups published their observations. Goldschmidt et al. (2006) have published their analysis on Caucasians in Swiss cohort and reported lack of association of H43Y with rapid progression to AIDS in HIV-1-infected individuals. In this report, they showed that HeLa cells stably transduced with human TRIM5α with 43Y do not differ from those with 43H in susceptibility to HIV-1 infection, whereas the 43Y variant failed to restrict N-MLV. Javanbakht et al. (2006) have published their analysis on large number of European Americans and African Americans and reported lack of any significant associations of TRIM5α SNPs with different rate of disease progression in HIV-1-infected individuals, although they found a controversial protective effect of H43Y against HIV-1 transmission only in African Americans but not in European Americans. They showed the 43Y human TRIM5α was less effective in restricting HIV-1 as well as N-MLV infections in vitro. Those reports agreed that 43Y variant failed to restrict N-MLV. However, the effect of H43Y substitution on HIV-1 restriction was not consistent among four reports. To make an addition to this debate, we conducted molecular biological and epidemiological studies in H43Y allele.

To reevaluate the effects of H43Y on in vitro anti-HIV-1 activity of TRIM5α, we first established stable cell lines expressing recombinant TRIM5α proteins. An expression plasmid carrying a hygromycin-resistant gene (pCEP4, Invitrogen) and hemagglutinin (HA)-tagged TRIM5α genes with 43H or 43Y (Fig. 1a) were introduced into CD4-negative human osteosarcoma cell line C143 and hygromycin-resistant cells were selected. Equal levels of HA-tagged TRIM5α expression were detected in those cell lines by

using immunoprecipitation followed by Western blot analysis (Nakayama et al. 2005; Fig. 1b). Serially diluted vesicular stomatitis virus (VSV)-pseudotyped HIV-1 vectors encoding green fluorescent protein (GFP) were then inoculated into the TRIM5α expressing cells, and infected cells were counted by flow-cytometry 40 h after infection. As shown in Fig. 1c, there was no difference in anti-HIV-1 activity among empty vector, human TRIM5α with 43H and that with 43Y, probably because human TRIM5α did not show any anti-HIV-1 effect in C143 cells.

Several recombinant studies of human and monkey TRIM5α revealed that the determinant of the species-specific restriction of HIV-1 lies in the SPRY domain of monkey TRIM5α (Nakayama et al. 2005, 2006; Perez-Caballero et al. 2005; Sawyer et al. 2005; Stremlau et al. 2005; Yap et al. 2005). To enhance the weak anti-HIV-1 activity of human TRIM5α, we introduced H43Y SNP in a chimeric version of TRIM5α, which carried part of the SPRY domain of AGM-TRIM5α and RING, B-box, and coiled-coil domains of human TRIM5α (Fig. 1a). Equal levels of TRIM5α expression were detected (Fig. 1b), and both chimeric TRIM5αs with 43H and 43Y showed potent anti-HIV-1 activity. Although there was a small increase in HIV-1-infected cells in transfectants with 43Y TRIM5α compared with those with 43H, we did not observe any complete loss of anti-HIV-1 activity for this variant.

To evaluate the effect of H43Y on multiple replication of HIV-1, we constructed recombinant Sendai viruses (SeVs) expressing human TRIM5α with 43H or that with 43Y, or their chimeric versions. There were 10^5 cells of human T cell line MT4 infected with recombinant SeV expressing human TRIM5α with 43H or that with 43Y at a multiplicity of infection at 10 plaque forming units per cell. Nine hours after infection, 20 ng of p24 of HIV-1 NL43 strain was challenged, and culture supernatants were periodically assayed for the levels of p24 by enzyme-linked immunosorbent assay (ZeptoMetrix). In this assay, we can observe multiple replications of HIV-1 and weak anti-HIV-1 activity of human TRIM5α can be amplified. As expected, both human TRIM5αs with 43H and 43Y showed weak but apparent anti-HIV-1 activity, although there was a small increase in HIV-1 titer in cells infected with SeV expressing human TRIM5α with 43Y compared with those infected with SeV expressing human TRIM5α with 43H (Fig. 2a). Equal levels of TRIM5α expressions were detected in those SeV-infected cells (Fig. 2b). When we used SeVs expressing human/AGM chimeric TRIM5α, both TRIM5α· with 43H and 43Y completely suppressed HIV-1 replication (Fig. 2c). Again, equal levels of TRIM5α expressions were detected in those SeV infected cells (Fig. 2d).

To exclude the possible effect of endogenous human TRIM5α, we then used TK-ts13 cells, a derivative of baby hamster kidney cell. As HIV-1 cannot complete the late step of its replication in rodent cells, serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated into the cells infected with SeV expressing TRIM5αs. GFP-positive cells were counted 40 h after infection. As shown in Fig. 2c, there was no difference in anti-HIV-1 activity among cynomolgus monkey TRIM5α lacking SPRY domain [CM-SPRY(−)TRIM5α], human TRIM5α with 43H, and that with 43Y. Both chimeric TRIM5αs with 43H and 43Y showed potent anti-HIV-1 activity, and there was no difference in anti-HIV-1 activity between chimeric TRIM5α with 43H and that with 43Y. These results indicate that H43Y exerts only a minor effect on the anti-HIV-1 activity of TRIM5α protein. They are in contrast with those reported by Sawyer et al. (2006), who found the 43Y SNP completely abolished the anti-HIV-1 activity of human TRIM5α. Although the reason for this discrepancy is not clear at present, differences in the expression systems used may be involved.

To evaluate the effects of H43Y polymorphism on anti-HIV-1 activity of human TRIM5α in Asian population, 49 HIV-1-infected Japanese subjects with different rates of disease progression were analyzed. Of the 49 patients, 21 were long-term non-progressors (LTNPs). They are all hemophiliacs and infected through contaminated blood products before 1985, and their CD4 counts were over 200 cells/μl without highly active anti-retroviral therapy until 2001. The LTNPs included ten cases with undetectable viral load without any kinds of anti-retroviral therapy by 2001. The remaining 28 cases were standard progressors (SPs) comprising 15 hemophiliacs, 9 homosexual, and 4 heterosexual cases. The homosexual and heterosexual cases were infected with HIV-1 after 1985. Among 28 SPs, 13 died of AIDS before 1999, and 15 developed AIDS before 1996. RING and B-box region was polymerase chain reaction (PCR)-amplified from genomic DNA by using primer pair forward (5′-TCAGGTCTATCATGACAAGG CAG-3′) and reverse (5′-GGCAGGAGCAGTGGAATA GC-3′). Genotypes of the 43rd position were determined by direct sequencing of the resultant 542-bp PCR product with forward primer. Among 21 LTNPs, one subject was homozygous for 43Y allele, five were heterozygous, and 15 were homozygous for the wild type. Of the 28 SPs, one subject was homozygous for 43Y, five were heterozygous, and 22 were homozygous for the wild type. There was no statistically significant difference in 43Y allele frequencies between LTNPs and SPs (0.167, seven out of 42 chromosomes vs 0.125, seven out of 56 chromosomes $p=0.77$, Yates chi square test). As the number of HIV-1 infected patients studied here was relatively small, we performed statistical simulation with ten times more subject numbers. Nevertheless, difference did not reach statistical significance ($p=0.065$). To exclude possible confounding effect of *CCR2-64I*, which is known as a protective genetic factor

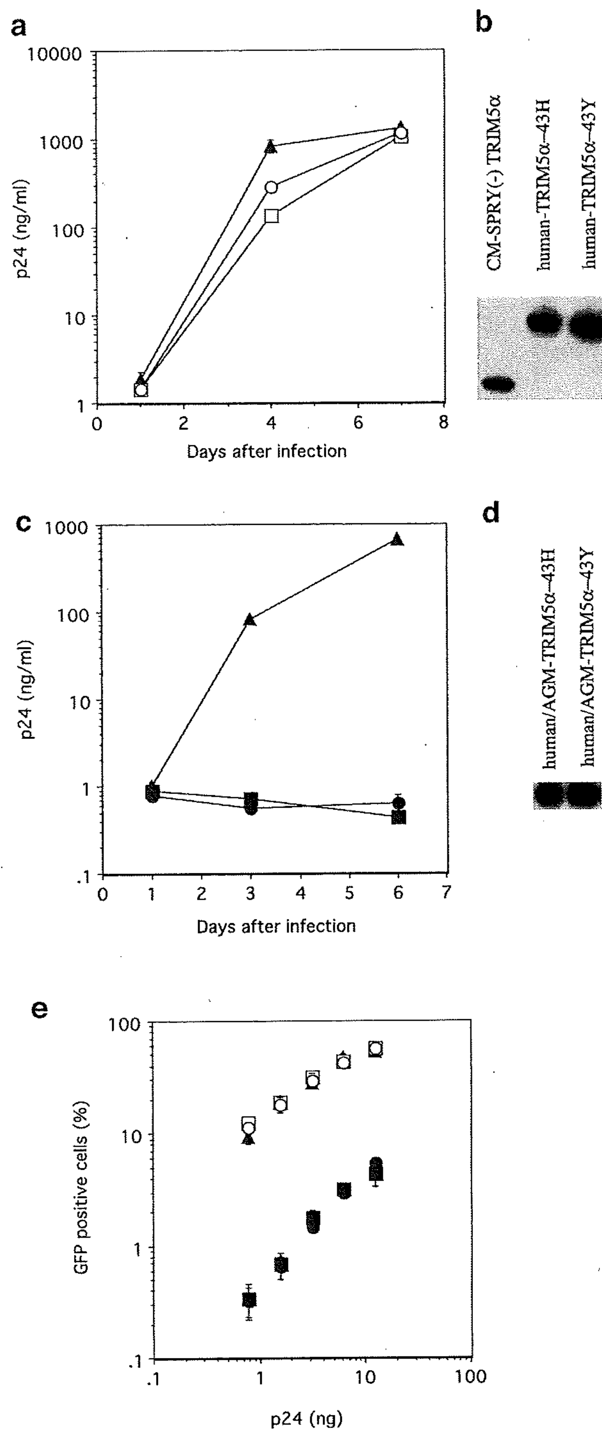

Fig. 2 **a, c** MT4 cells were infected with recombinant SeV expressing human-TRIM5α-43H (*open squares*), human-TRIM5α-43Y (*open circles*), human/AGM-TRIM5α-43H (*closed squares*), human/AGM-TRIM5α-43Y (*closed circles*) or a truncated form of CM-SPRY(−) TRIM5α as a negative control (*closed triangles*). Nine hours after infection, cells were inoculated with 20 ng of p24 of HIV-1 NL43, and culture supernatants were periodically assayed for levels of p24. *Error bars* showed actual fluctuations between measurements of p24 in duplicate samples. Representative data from two independent experiments are shown. **b, d** One million cells of each recombinant SeV-infected cells were lysed for immunoprecipitation with an anti-HA antibody. **e** TK-ts13 cells were infected with SeV expressing human-TRIM5α-43H (*open squares*), human-TRIM5α-43Y (*open circles*), human/AGM-TRIM5α-43H (*closed squares*), human/AGM-TRIM5α-43Y (*closed circles*), or CM-SPRY(−)TRIM5α (*closed triangles*) and then exposed to the indicated p24 amounts of GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flowcytometry. *Error bars* indicated standard deviation of triplicate samples

against AIDS progression, we excluded patients with this allele from the analysis. There was still no statistically significant difference of the 43Y allele frequency between 9 LTNPs and 15 SPs (0.111, two out of 18 chromosomes, vs 0.166, five out of 30 chromosomes, p=0.69, Fisher's exact test). Statistical simulation with ten times more subject numbers again failed to show statistically significant difference (p=0.09)

We also analyzed HIV-1-infected individuals in two well-characterized French cohorts: 45 subjects from the Agence Nationale de Recherches sur le Sida CO16 Long Term Non-Progressors Cohort (ALT) and 62 from the cohort of SPs (IMMUNOCO). The patients in ALT cohort were with no AIDS symptoms at the time of recruit in 1996 without any kinds of treatment, and their CD4 counts were more than 600/μl during last 5 years. The patients in IMMUNOCO cohort were at any stage, with or without treatment, and their CD4 counts were more than 150/μl at entry in 1991 to 1992 (Magierowska et al. 1999). Thirteen of the ALT subjects and 14 of the IMMUNOCO subjects were heterozygous for 43Y. Again, there was no statistically significant difference in the ratio of 43Y heterozygotes between ALT and IMMUNOCO subjects (0.29 vs 0.23, p=0.460, chi square test). The odds ratio was 0.72 with a 95% confidence interval of 0.30–1.73, indicating that H43Y did not exert any strong effect on HIV-1 disease progression in the French subjects. Our findings for Asians and Europeans indicated that the effects of H43Y SNP of the human *TRIM5α* gene on HIV-1 disease progression are minor, if any. Our results are consistent with the previous observation that 43Y does not have a protective effect against HIV-1 replication or disease progression in European Americans (Goldschmidt et al. 2006; Javanbakht et al. 2006; Speelmon et al. 2006).

In conclusion, the results of our epidemiological and molecular biological studies clearly indicate that H43Y SNP in the human *TRIM5α* gene has a minor effect on the anti-HIV-1 activity of TRIM5α. Although we did not evaluate the effects of H43Y on the anti-MLV activity of human TRIM5α, this allele is immaterial, at least in cases with HIV-1 subtype B such as found in Europeans, European Americans, and Japanese hemophiliacs. It might be important to test the restriction capability of human TRIM5α in other subtypes of HIV-1 or HIV-2.

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A Single Amino Acid of the Human Immunodeficiency Virus Type 2 Capsid Affects Its Replication in the Presence of Cynomolgus Monkey and Human TRIM5αs▽

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Human immunodeficiency virus type 2 (HIV-2) strains vary widely in their abilities to grow in Old World monkey (OWM) cells such as those of cynomolgus monkeys (CM). We evaluated eight HIV-2 isolates for their sensitivities to CM TRIM5α, an anti-HIV factor in OWM cells. We found that different HIV-2 isolates showed differences in their sensitivities to CM TRIM5α. Sequence analysis showed that TRIM5α-sensitive viruses had proline at the 120th position of the capsid protein (CA), whereas TRIM5α-resistant viruses had either alanine or glutamine. Mutagenesis studies indicated that the single amino acid at the 120th position indeed affected the sensitivity of the virus to CM TRIM5α.

Human immunodeficiency virus type 1 (HIV-1) is infectious only for humans and chimpanzees. This is due in part to TRIM5α, which blocks infection early after viral entry, before the establishment of a provirus in Old World monkey (OWM) cells. Rhesus monkey and cynomolgus monkey (CM) TRIM5αs inhibit HIV-1 but not simian immunodeficiency virus isolated from macaque (SIVmac), while African green monkey (AGM) TRIM5α prevents replication of HIV-1 and SIVmac (14, 16,

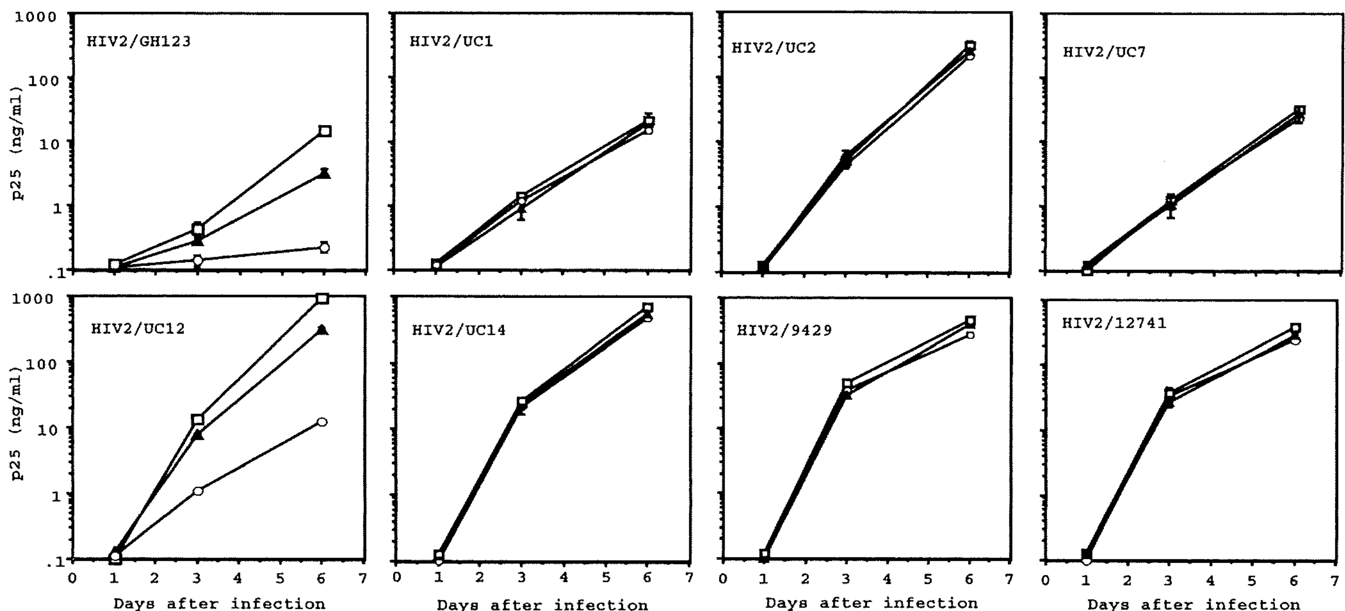

FIG. 1. Hut78 cells (10^5) were infected with CM-TRIM5α-SeV (open circles), Hu-TRIM5α-SeV (filled triangles), or CM-SPRY(−)-SeV (open squares) at a multiplicity of infection of 10 PFU. The CM-SPRY(−)-SeV domain was generated by PCR amplification of the first-296-amino-acid region of CM TRIM5α. Nine hours after infection, cells were inoculated with eight HIV-2 isolates, GH123, UC12, UC1, UC14, UC2, UC7, 9429, and 12741. Culture supernatants were periodically assayed for levels of p25 by using a RETROtek antigen enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY). Error bars show actual fluctuations between measurements of p25 in duplicate samples. A representative of three independent experiments is shown.

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HIV2/GH123/S    1 PVQQTGGGNYIHVPLSPRTLNAWVKLVEDKKFGAEVVPGFQALSEGCTPYDINQMLNCVG  60
HIV2/UC12/S     1 ----VA- --T-----------------E-------------------------------  59
HIV2/UC1/R      1 ----IA- --V-M---------------E-------------------------------  59
HIV2/UC14/R     1 ----IA- --S-L---------------E-------------------------------  59
HIV2/UC2/R      1 ----A-- --V-----------------E-------------------------------  59
HIV2/UC7/R      1 ----A-- --Y-M---------------E------I------------------------  59
HIV2/9429/R     1 ----A-- --V-----------------E-------------------------------  59
HIV2/12741/R    1 ----A-- --V-----------------E-------------------------------  59

HIV2/GH123/S   61 DHQAAMQIIREIINDEAADWDAQHPIPGPLPAGQLRDPRGSDIAGTTSTVEEQIQWMYR|P| 120
HIV2/UC12/S    60 --------------E------V-------------E---------------------F-|-| 119
HIV2/UC1/R     60 --------------E------Q-------------------------------------|A| 119
HIV2/UC14/R    60 E-------------E------Q---S---M-----E-----------------------|A| 119
HIV2/UC2/R     60 -Q------------E-----------------------------------D--------|Q| 119
HIV2/UC7/R     60 -Q------------E-----------------------------------D--------|Q| 119
HIV2/9429/R    60 -Q------------E-----------------------------------D--------|Q| 119
HIV2/12741/R   60 -Q------------E----------V------------------------D--------|Q| 119

HIV2/GH123/S  121 QNPVPVGNIYRRWIQIGLQKCVRMYNPTNILDVKQGPKEPFQSYVDRFYKSLRAEQTDPA 180
HIV2/UC12/S   120 --------------------------------I--------------------------- 179
HIV2/UC1/R    120 --------------------------------I--------------------------- 179
HIV2/UC14/R   120 ---I----------L-----------------I--------------------------- 179
HIV2/UC2/R    120 -----------------------------V---------S----------------A--- 179
HIV2/UC7/R    120 -----------------------------V---------S--N-------------A--- 179
HIV2/9429/R   120 -----------------------------V---------S----------------A--- 179
HIV2/12741/R  120 -----------------------------V---------S--------F-------A--- 179

HIV2/GH123/S  181 VKNWMTQTLLIQNANPDCKLVLKGLGMNPTLEEMLTACQGVGGPGQKARLM 231
HIV2/UC12/S   180 ---------------------------G----------------------- 230
HIV2/UC1/R    180 ----------------------------------------I---------- 230
HIV2/UC14/R   180 ----------------------------------------I---------- 230
HIV2/UC2/R    180 ----------V---------------------------------------- 230
HIV2/UC7/R    180 --------------------------------------------------- 230
HIV2/9429/R   180 --------------------------------------------------- 230
HIV2/12741/R  180 --------------------------------------------------- 230
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B

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H2A UC2       235 SDIAGTTSTVDEQIQWMYR |Q| QNPVPVGNIYRRWIQIGLQK 274
H2A BEN       235 ------------------- |P| -------------------- 274
H2A PEI2      235 ----------E------F- |A| ---I---------------- 274
H2A GH1       236 ----------E-------- |P| -------------------- 275
H2A ISY       235 ----------E---E---- |-| E------------------- 274
H2A D194      235 ------------------- |-| P------------------- 274
H2A ALI       235 ----------E------F- |P| R------------------- 274
H2A MDS       235 -----------------F- |A| -------------------- 274
H2A FG        233 ----------E------F- |P| -------------------- 272
H2A CAM2CG    235 ----------E------F- |A| -------------------- 274
H2A ST        235 ----------E------F- |P| -------S------------ 274
H2A ROD       235 ----------E------F- |P| -------------------- 274
H2B EHO       230 ----------E-------- |P| ---------------L---- 269
H2B UC1       230 ----------E-------- |A| -------------------- 269
H2B D205      230 ----------E-------- |A| ---------------L---- 269
H2B KR020     230 ----------E-------- |P| ---I-----------L---- 269
H2AB 7312A    230 ----------E-------- |G| -S-I-----------L---- 269
H2G ABT96     235 ---------IE-----TH- |-| ---I----X-----XL---- 274
H2U 12034     206 ----------E-------- |P| ---I-----------L---- 245

MAC 239       234 --------S---------- |-| ---I-----------L---- 273
MAC MM142     234 ----------E-------- |-| ---I-----------L---- 273
MAC BK28      234 --------S---------- |-| ---I-----------L---- 273
MAC 1A11      234 --------S---------- |-| ---I-----------L---- 273
MAC PJ5       234 --------S---------- |-| ---I-----------L---- 273
MAC SMM142B   234 ----------E-------- |-| ---I-----------L---- 273
SMM H9        235 ---------X-------X- |-| ---I------X----L---- 274
SMM PBJ-143   235 ---------X-------X- |-| ---I------X----L---- 274
SMM PBJ14-15  235 ------------------- |-| ---I-----------L---- 274
SMM PBJ-6P6   235 ------------------- |-| ---I-----------L---- 274
SMM PBJA      235 ------------------- |-| ---I-----------L---- 274
SMM 17EC1     234 --------S---------- |-| ---I-----------L---- 273
SMM 17EFR     234 --------S---------- |-| ---I-----------L---- 273
SMM F236      235 ------------------- |-| ---I-----------L---- 274
SMM PGM53     235 ----------E-------- |-| ---I-----------L---- 274
SMM SME543    235 ----------E-------- |-| ---I-----------L---- 274
SMM SL92B     235 --------PS----E---- |A| -------D-------L---- 274
MNE MNE027    234 ------------------- |-| ---I-----------L---- 273
MNE MNE-8     234 ------------------- |-| ---I-----------L---- 273
STM STM       236 --------SPE-------- |-| ---I-----------L---- 275
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FIG. 2. (A) Alignments of amino acid sequences of CAs of eight HIV-2 isolates. "/S" after the names of isolates represents sensitivity to CM and Hu TRIM5α, and "/R" indicates resistance to CM and Hu TRIM5α. A dash denotes that the amino acid residue is identical to that of GH123. Blank spaces denote a lack of the amino acid residue that is present only in GH123. The box indicates the amino acid residues that correlate with susceptibility to restriction by CM and Hu TRIM5α. (B) Alignments of partial amino acid sequences of CA of all the HIV-2 and SIV isolates obtained from the Los Alamos database. A dash denotes that the amino acid is identical to that of HIV-2 UC2. X denotes amino acids unidentified due to sequence ambiguity. The box indicates the amino acid residues that correlate with susceptibility to restriction by CM and Hu TRIM5α.

26, 34). Human (Hu) TRIM5α shows very weak antiviral activity against those viruses (30, 32, 34, 35, 38) but strong resistance against N-tropic murine leukemia virus (N-MLV) (14, 31, 37). Among several splicing variants of TRIM5, an α isoform carries the SPRY or B30.2 domain that determines virus specificity of this intracellular factor (26, 27, 30, 32, 34, 35, 38).

HIV-2, simian immunodeficiency virus isolated from sooty mangabey (SIVsmm), and SIVmac have extremely similar genomes (11). SIVmac was resistant to the restriction by rhesus monkey and CM TRIM5αs (26, 34), while HIV-2 isolates GH123 and ROD were shown to be sensitive to those TRIM5αs (26, 39). Since HIV-2 isolates varied considerably in their abilities to grow in OWM cells (3, 4, 7, 22, 23), we studied the effects of CM and Hu TRIM5αs on eight different HIV-2 isolates (UC1, UC2, UC7, UC12, UC14, 9429, 12741, and GH123) (1, 3, 5, 6, 12, 17, 21, 22, 33).

We used Sendai virus (SeV) expressing CM TRIM5α (CM-TRIM5α-SeV) (26) or Hu TRIM5α (Hu-TRIM5α-SeV). We also generated SeV expressing CM TRIM5α without the SPRY domain [CM-SPRY(−)-SeV] as a negative control. There was no variation in TRIM5α expression levels among T-cell-line Hut78 cells infected with those SeVs (data not shown).

Consistent with previous observations (26), HIV-2 isolate GH123 grew to lower titers in CM-TRIM5α-SeV-infected cells and to slightly but significantly lower titers in Hu-TRIM5α-SeV-infected cells than they did in CM-SPRY(−)-SeV-infected cells (Fig. 1). Of the seven newly tested isolates, only UC12 showed a pattern similar to that of GH123 (Fig. 1), while the other six isolates, UC1, UC14, UC2, UC7, 9429, and 12741, grew to almost the same titers in CM-TRIM5α-SeV-, Hu-TRIM5α-SeV-, and CM-SPRY(−)-SeV-infected cells (Fig. 1). These results indicated that HIV-2 isolates GH123 and UC12 are sensitive to CM TRIM5α and moderately sensitive to Hu TRIM5α, while the isolates UC1, UC14, UC2, UC7, 9429, and 12741 are resistant to CM and Hu TRIM5αs.

It has been suggested that viral capsid protein (CA) was the determinant of TRIM5α restriction (13, 28, 29). We therefore