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Retro-transduction by virus pseudotyped with glycoprotein of vesicular stomatitis virus

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Abstract

A virus pseudotyped with glycoprotein of vesicular stomatitis virus (VSV-G) can enter various cell types at a relatively high titer. We observed that the amount of viral antigen from VSV-G pseudotyped human immunodeficiency virus type 1 (HIV-1) producing cells was much higher than that from their non-pseudotyped counterparts. This enhanced viral antigen production was not observed when we used HIV-1 *pol* mutant, viral enzyme inhibitors, HIV Env protein, or VSV-G fusion defective mutants. The transfection experiment using GFP-expressing virus showed time-dependent expansion of GFP-positive cells and viral DNA integration. These results suggested that the increase in viral antigen yield was caused by the release of a progeny virus following retro-transduction by the pseudotyped virus of the cells within the transfected cell culture. The infectivity as well as the amount of VSV-G on virus particles per unit of viral antigen was significantly different before and after the onset of the yield enhancement. This suggests that results of infection assays of the virus pseudotyped with VSV-G may be affected by the occurrence of such enhancement. This means that, while pseudotyping with VSV-G is a simple and effective method, this procedure should be carefully considered when the virus is produced for infectivity assays.

Keywords: VSV-G; HIV-1; Pseudotyping; Viral vector; Retro-transduction

Introduction

Gene delivery using retroviral vectors has become a very popular and indispensable method. To ensure safety of the vectors and to increase their efficiency, envelope pseudotyping of viral vectors lacking their own envelope gene is used in almost all cases. Glycoprotein of vesicular stomatitis. virus (VSV-G) in particular is one of the most frequently used envelope proteins for pseudotyping since it is very stable and promises highly effective and broad-spectrum gene delivery. However, there seems to be very little awareness of its adverse effect, that is, re-infection/retro-transduction. VSV-G interacts with a phospholipid component of the cell surface membrane and mediates viral entry by membrane fusion (Burns et al., 1993; Mastromarino et al., 1987). As viral entry does not seem to require specific protein receptors, VSV-G pseudotyped vectors could infect virtually all kinds of cells. Thus, the VSV-G pseudotyped viral vector has the potential to infect its producer cells to re-generate progenies, which may contain unexpected characteristics. In the study presented here, we examined retro-transduction of VSV-G pseudotyped HIV-1 vectors. Our findings suggest that this problem could confound the interpretation of the experimental results under certain conditions and must therefore be taken into careful consideration.

Results

Enhancement of virion production by VSV-G pseudotyping

We previously noticed that the viral antigen yield of VSV-G pseudotyped HIV-1 by transfection was always much higher than that of the non-pseudotyped virus. We first reconfirmed this phenomenon by using a simplified experimental design. For this purpose, 2.5 μg of HIV-1 proviral plasmid pNLNh, which lacks Env expression, was transfected into 3×10^6 293T cells

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along with 2.5 μg of either pCG-VSVG, which expresses VSV-G under the CAG hybrid promoter (Niwa et al., 1991), or pGEM-5Zf(+) (Promega, Madison, WI) as a control. The quantity of virus production was monitored by measuring CA-p24 antigen in the culture supernatant every 12 h. The amounts of CA-p24 detected in VSV-G pseudotyped virus and in control were almost equal up to 24 h post-transfection. However, CA-p24 production of the former became 10 times higher than that of control at 36 h and finally 20 times higher at 48 h (Fig. 1A). The actual amount of CA-p24 of virus from 3×10^6 of transfected cells with pseudotyping was typically 20–40 μg in total. This enhancement may have been caused by some transactivation effects of VSV-G, or by retro-transduction of the cells within the transfected cell culture, which then leads to production of the progeny virus. The absence of the enhancement at 12 or 24 h post-transfection suggests that transactivation was unlikely since the transactivation effect should become apparent from the onset of protein expression. In subsequent experiments, we therefore examined the possibility of retro-transduction of virus thus produced.

Retro-transduction of pseudotyped virus during transfection

The transfected cells were harvested 48 h post-transfection and viral antigens within the cells were detected by western blotting (Fig. 1B). The viral antigens within cells producing the *env*-lacking virus were mostly large, seemingly uncleaved, and with the precursor protein Gag-Pr55 dominant. On the other hand, the protein profile within the cells producing the VSV-G pseudotyped virus was strikingly different. The majority of viral antigens were mature proteins, such as CA-p24 and MA-p17, while the number of immature proteins was very limited. This observation was in agreement with a hypothesis that the viral antigen within cells producing the pseudotyped virus mainly came from mature virions, which would support the concept of retro-transduction of the released virus.

To examine this possibility, a *pol* mutant pNLN-RI was constructed from pNLNh. The mutant had a large deletion at the RT and IN genes in addition to an Env frameshift, while the expression of functional Env, RT, and IN was eliminated. The VSV-G pseudotyped NLN-RI was therefore not able to complete retro-transduction to produce its progeny. Next, 293T cells were co-transfected with pNLN-RI along with either pCG-VSVG or pGEM-5Zf(+), and the quantity of virus production was monitored by measuring CA-p24. No difference in virus production was observed between VSV-G pseudotyped and non-pseudotyped viruses during 48 h post-transfection (Fig. 1C). This indicates that the enhancement of virus production was impaired by the elimination of RT expression and subsequent inhibition of retro-transduction.

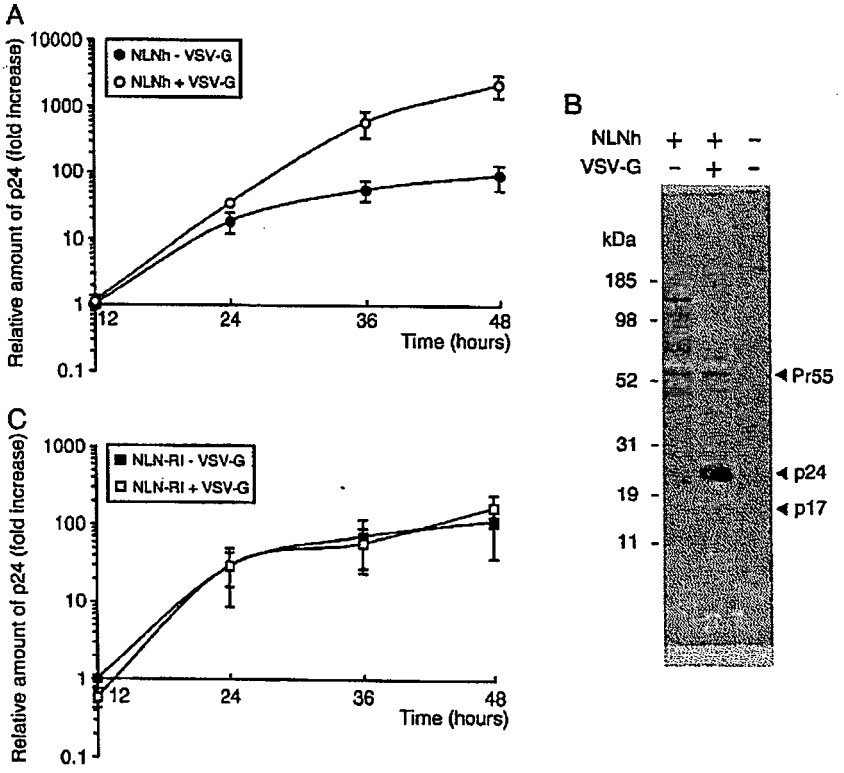

Fig. 1. Enhancement of virus production by pseudotyping. (A) Time course of HIV-1 production during transfection. The plasmid pNLNh was transfected into 293T cells along with either pCG-VSVG or pGEM-5Zf(+), and the quantity of virus production in the culture supernatant was monitored at 12, 24, 36, and 48 h post-transfection by measuring CA-p24 antigen. The value for NLNh without pseudotyping at 12 h 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) Profiles of viral antigen in transfected cells. Cells were harvested at 48 h post-transfection and lysed. Viral antigens within cell lysates were detected by western blotting using serum from HIV-1 infected patients. (C) Similar experiment as in A except for the use of viral *pol* mutant pNLN-RI instead of the wild-type pNLNh.

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

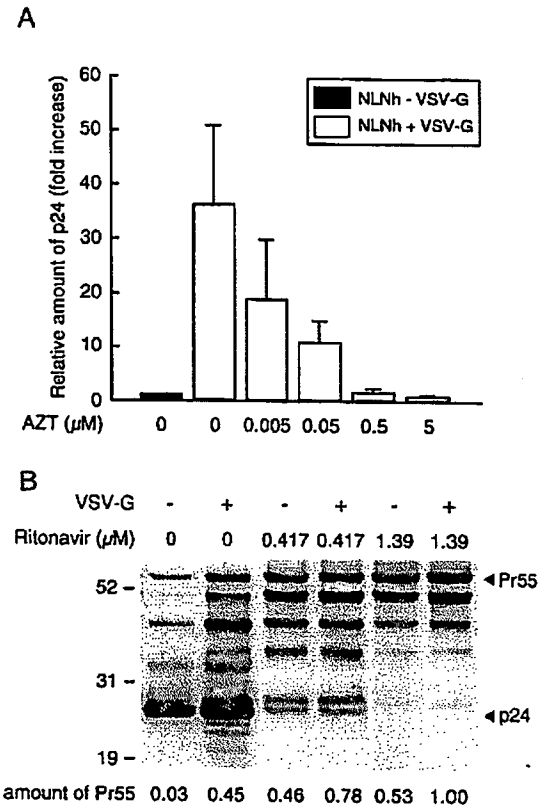

Fig. 2. Effect of viral enzyme inhibitor on enhancement. (A) 293T cells were co-transfected with 0.25 μg of pNLNh and 0.25 μg of either pCG-VSVG or pGEM-5Zf(+) in the absence or presence of 0.005, 0.05, 0.5, and 5 μM AZT. The virus particles derived from the cells were collected at 48 h post-transfection, 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.

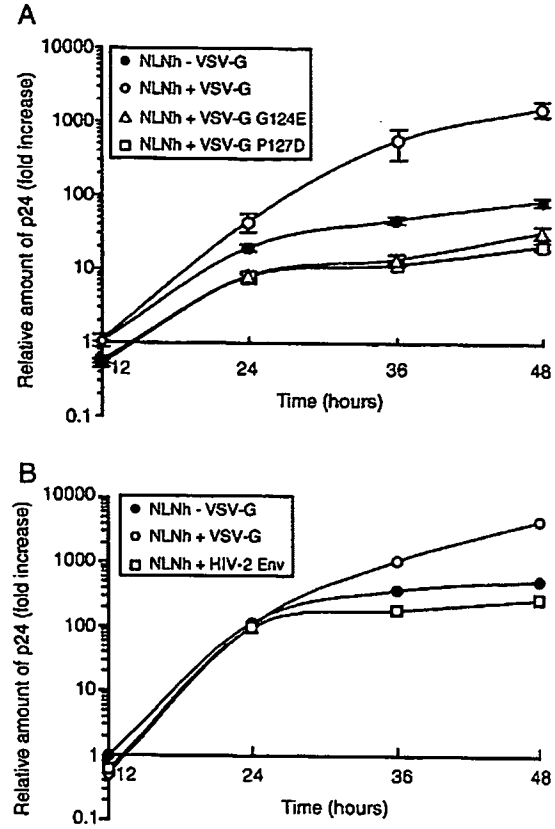

Fig. 3. Effects of fusion-defective VSV-G or HIV-2 Env on enhancement. pNLNh was transfected into 293T cells along with either pCG-VSVG, pCGVG-G124E, pCGVG-P127D, pCGH2Env or pGEM-5Zf(+), and the virus in the culture supernatant was monitored at 12, 24, 36, and 48 h post-transfection by measuring CA-p24 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.

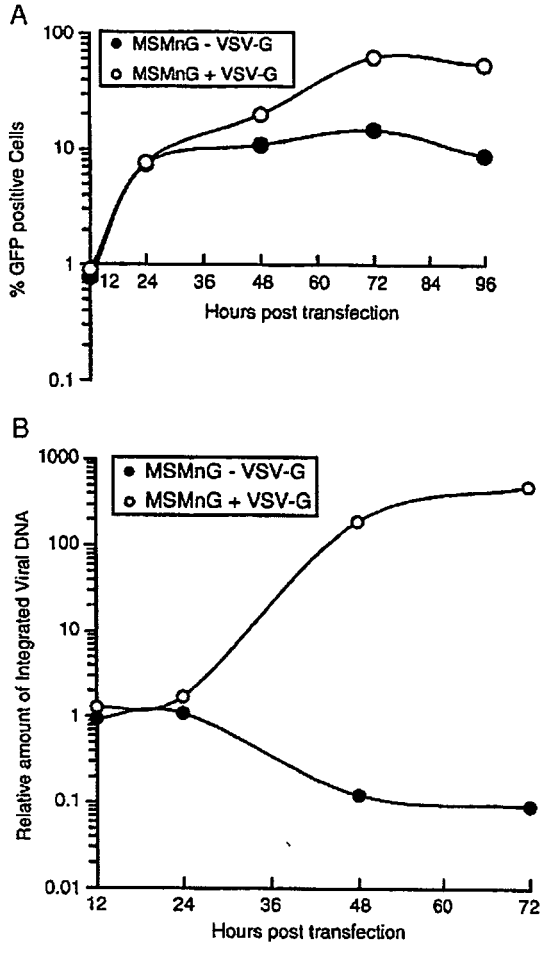

Fig. 4. GFP-virus transfection and viral DNA integration. 293T cells were co-transfected with 0.25 μg of pMSMnG and 0.25 μg of either pCG-VSVG or pGEM-5Zf(+) and were harvested at 12, 24, 48, 72, and 96 h post-transfection. Half of each sample was fixed and subjected for FACS analysis, and total DNA 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

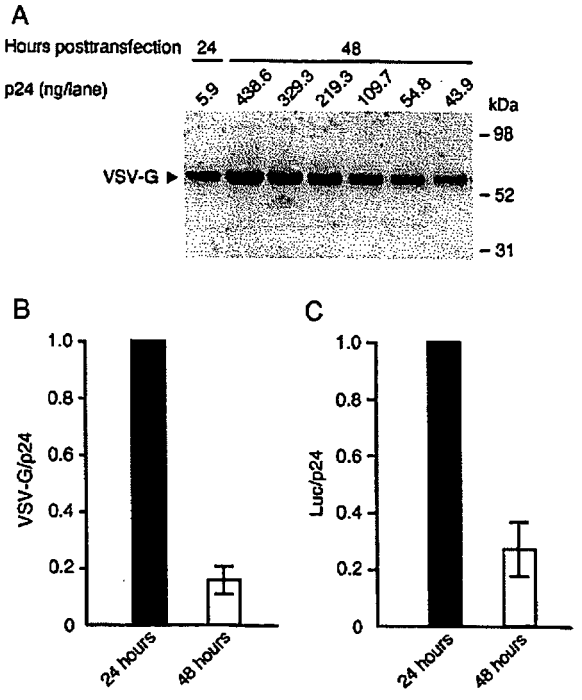

Fig. 5. VSV-G content and infectivity per viral antigen in early and late stages of transfection. Virions derived from 293T cells co-transfected with pNLNh and pCG-VSVG were harvested at 24 and 48 h post-transfection, purified, and lysed. CA-p24 antigen and VSV-G in the lysate were measured by ELISA 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′-GGATTTTGCTATAAGATGGT-GAGCAAGGGCG-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′-AGG-GAACTCTGGATTCAGC-3′ and 5′-GCTCTAGAGCCTCT-GCTAAC-3′/5′-TTTGATCAGGGAAGCCTG, respectively. The 3′ part of the G124E and P127D fragments was amplified using primers 5′-GCTGAATCCAGAGTTCCC-3′/5′-TAGGG-TACCATTGATTATGGT-3′ and 5′-CCAGGCTTC-CCTGATCAAA-3′/5′-TAGGGTACCATTGATTATGGT-3′, respectively. The total G124E fragment was amplified with primers 5′-GCTCTAGAGCCTCTGCTAAC-3′/5′-TAGGG-TACCATTGATTATGGT-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′-GCCGCCATGTGTGG-TAAGAGTCTAC-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).

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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-

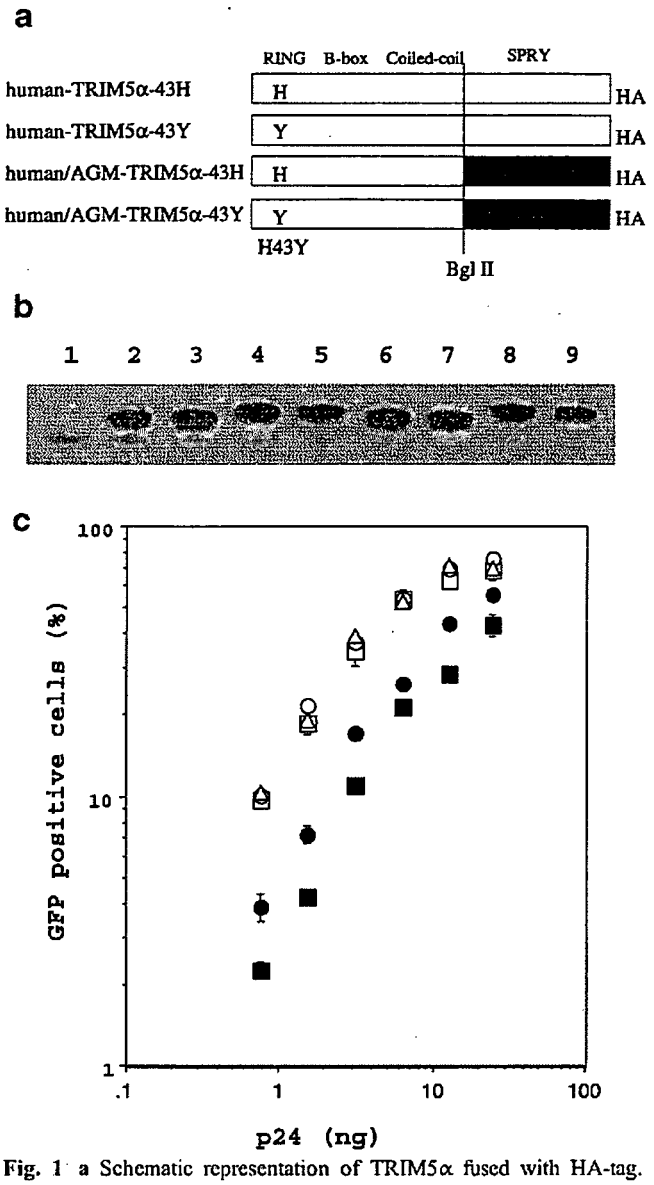


Fig. 1 a Schematic representation of TRIM5α fused with HA-tag. Four domains of TRIM5α are shown *at the top*. *Black* and *white bars* denote human and AGM sequences, respectively. A Bgl II site was used to swap SPRY domains between human and AGM TRIM5α. *H* or *Y* denotes the amino acid residue at the 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

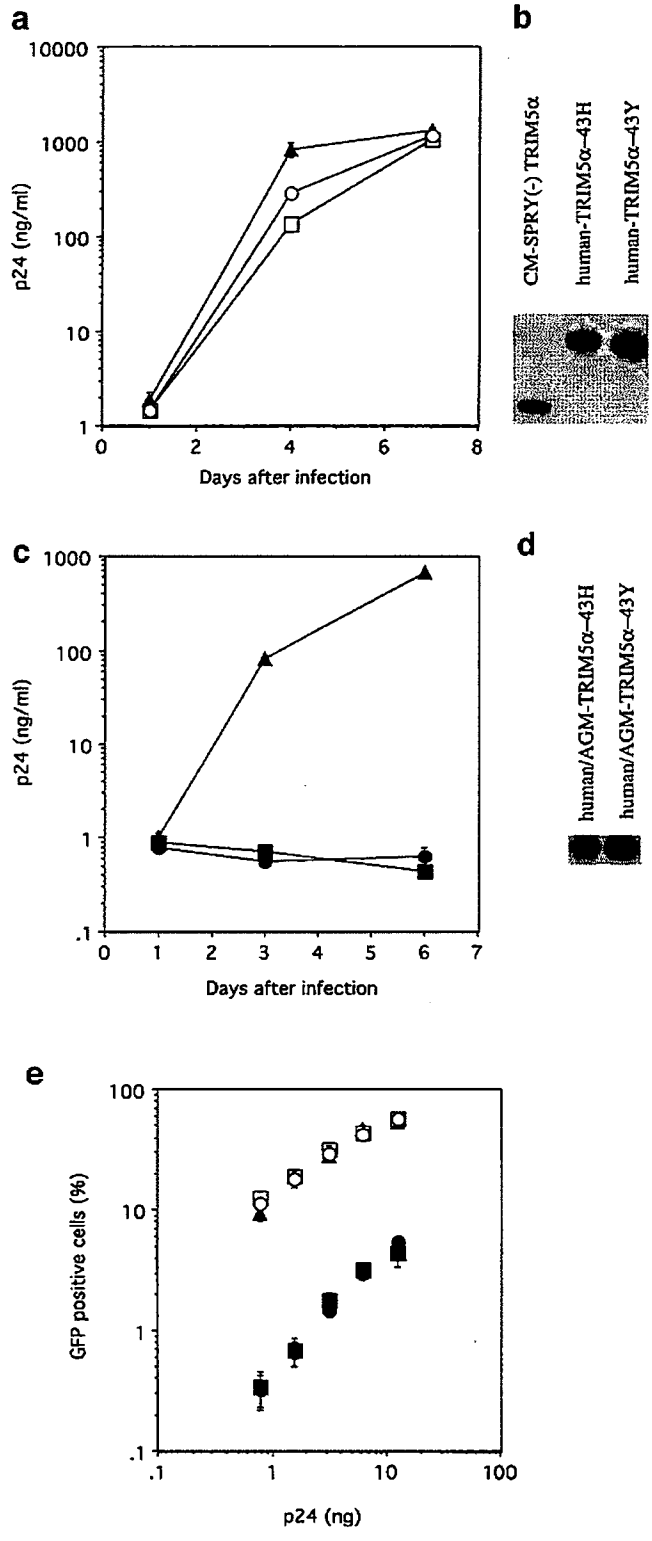
using immunoprecipitation followed by Western blot analysis (Nakayama et al. 2005; Fig. 1b). Serially diluted vesicular stomatitis virus (VSV)-pseudotyped HIV-1 vectors encoding green fluorescent protein (GFP) were then inoculated into the TRIM5α expressing cells, and infected cells were counted by flow-cytometry 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,

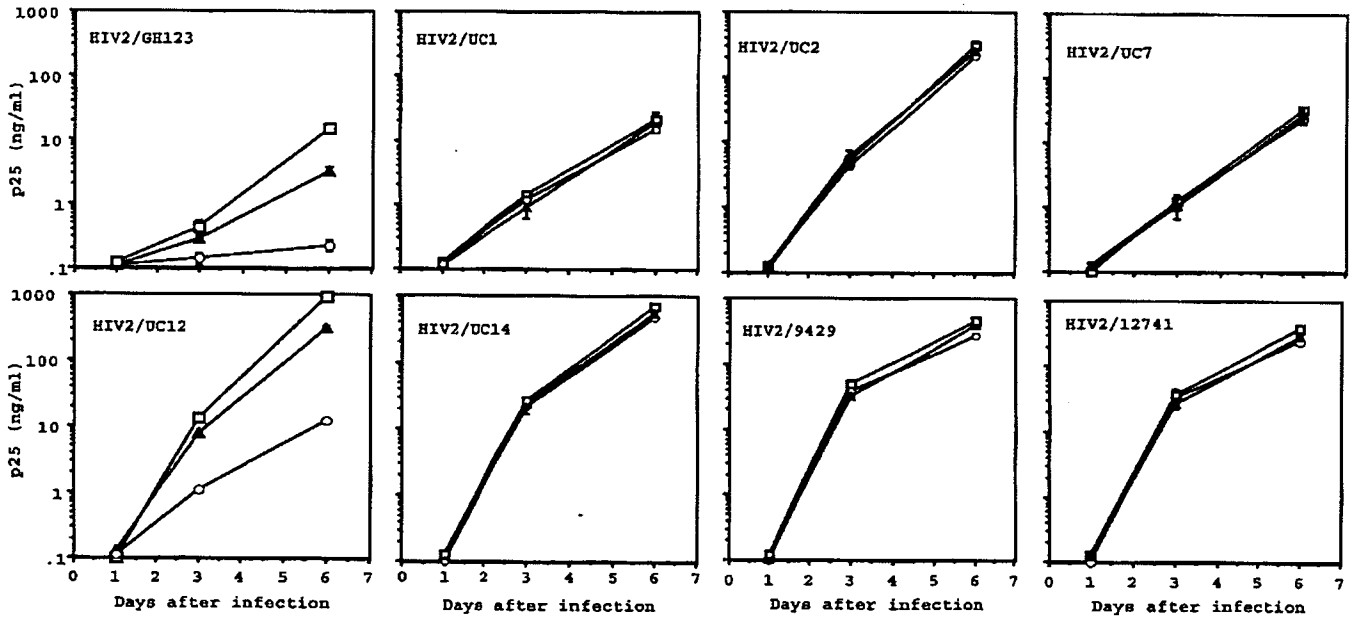

FIG. 1. Hut78 cells (10^5) were infected with CM-TRIM5α-SeV (open circles), Hu-TRIM5α-SeV (filled triangles), or CM-SPRY(−)-SeV (open squares) at a multiplicity of infection of 10 PFU. The CM-SPRY(−)-SeV domain was generated by PCR amplification of the first-296-amino-acid region of CM TRIM5α. Nine hours 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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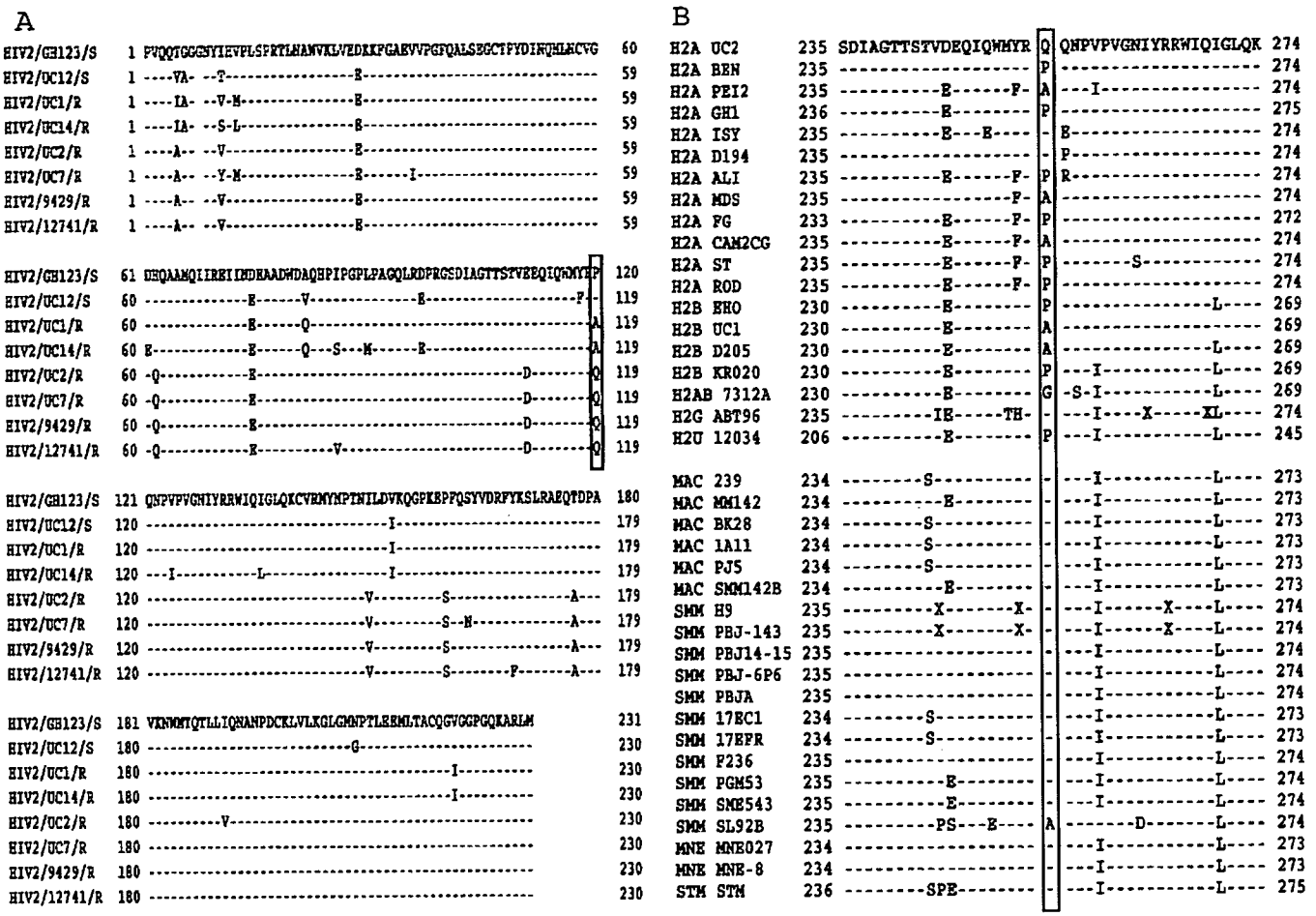

FIG. 2. (A) Alignments of amino acid sequences of CAs of eight HIV-2 isolates. "/S" after the names of isolates represents sensitivity to CM and Hu TRIM5α, and "/R" indicates resistance to CM and Hu TRIM5α. A dash denotes that the amino acid residue is identical to that of GH123. Blank spaces denote 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

A

	Amino acid sequence	Sensitivity to TRIM5α			
		AGM	CM	Hu	SPRY(-)
GH123	WMYRPQNPVP	+++	+++	++	-
GH123/A	----A-----	+++	-	+	-
GH123/Q	----Q-----	+++	-	+	-
SIVmac239	WMYRQQNPIP	+++	+	-	-
SIVmac239/P	----P-----	+++	++	+	-

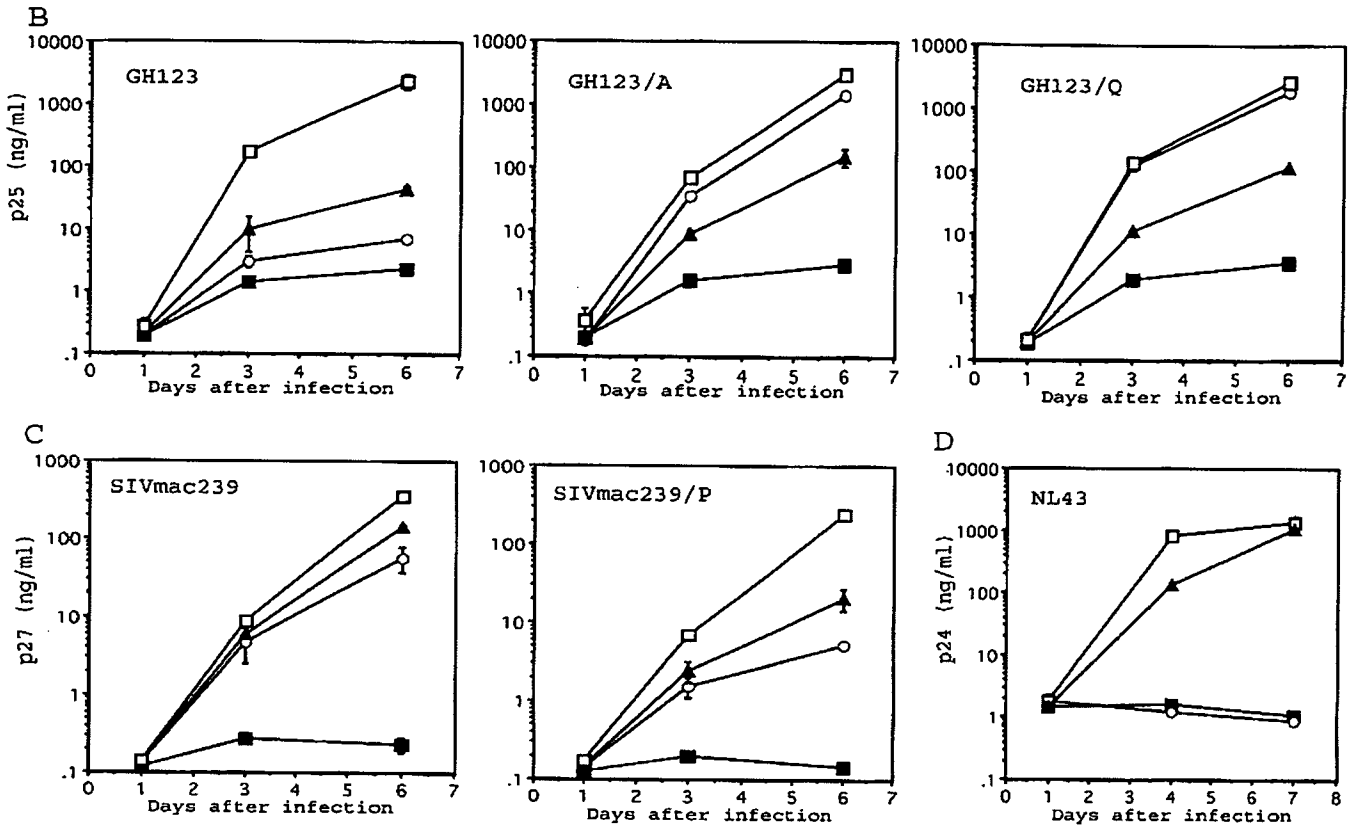

FIG. 3. (A) Mutant GH123/A and GH123/Q viruses generated by changing a single amino acid of proline to alanine or glutamine and mutant SIVmac239/P virus generated by changing a single amino acid of glutamine to proline by site-directed mutagenesis. Dashes indicate the unmutated amino acid residues. +++, ++, +, and − denote more-than-300-fold, 40- to 100-fold, 6- to 40-fold, and less-than-2.5-fold suppression of virus growth, respectively, compared with what was seen for the negative control at day 6 in the presence of the various TRIM5αs indicated. (B to D) MT4 cells were infected with CM-TRIM5α-SeV (open circles), Hu-TRIM5α-SeV (filled triangles), AGM-TRIM5α-SeV (filled squares), or CM-SPRY(−)-SeV (open squares). Nine hours after infection, cells were inoculated with GH123, mutant GH123/A, or GH123/Q viruses (panel B), with SIVmac239 or mutant SIVmac239/P virus (panel C), or with HIV-1 NL43 virus (panel D). Culture supernatants were periodically assayed for levels of virus CA. Error bars show actual fluctuations between measurements of CA in duplicate samples. A representative of three independent experiments is shown.

determined the nucleotide sequences of PCR-amplified DNA fragments corresponding to proviral DNA encoding the CAs of the HIV-2 isolates. Figure 2A shows the deduced amino acid sequences of the HIV-2 CAs. TRIM5α-sensitive GH123 and UC12 had proline at the 120th (GH123) or corresponding 119th (UC12) position, while the other six TRIM5α-resistant isolates showed alanine (UC1 and UC14) or glutamine (UC2, UC7, 9429, and 12741) at the corresponding 119th position. Except for this substitution, there was no substitution observed commonly and specifically for GH123 and UC12.

To determine whether the single amino acid at the 119th or 120th position of the HIV-2 CA affects the virus's susceptibility to CM and Hu TRIM5α, we constructed two mutant GH123 viruses carrying either alanine (GH123/A) or glutamine (GH123/Q) at the 120th position (Fig. 3A). As shown in Fig. 3B, all three viruses grew substantially and to similar titers in CM-SPRY(−)-SeV-infected MT4 cells. In CM-TRIM5α-SeV-infected cells, both GH123/A and GH123/Q grew to titers more than 250 times higher than those of the wild-type GH123. In the Hu-TRIM5α-SeV-infected cells, both mutant viruses grew to titers approximately fourfold higher than those of the wild type (Fig. 3B). In contrast, replication of the three viruses could not be detected in the AGM-TRIM5α-SeV-infected cells (Fig. 3B). Unlike CM TRIM5α, AGM TRIM5α pos-

FIG. 4. Structural model of the N-terminal halves of HIV-2 CAs. The 3-D models of 10 HIV-2 CAs were constructed with the homology-modeling technique using "MOE-Align" and "MOE-Homology" in the Molecular Operating Environment (MOE) as described previously (18, 19). Superimposition of the 10 HIV-2 models showed that the overall 3-D structures of the N-terminal domains of the variants are very similar. N and C indicate the amino termini and carboxyl termini, respectively. The ribbons represent the backbones of HIV-2 CAs, and the seven color-coded α-helices are labeled. P, A, and Q indicate L6/7 with a proline residue (GH123 and UC12 [in red]), an alanine residue (UC1, UC14, and GH123/A [in green]), and a glutamine residue (UC2, UC7, 9429, 12741, and GH123/Q [in blue]), respectively, at the 120th position. L4/5 is labeled with a color scheme the same as that for L6/7. The presence of proline residues conferred a sensitive phenotype; alanine and glutamine conferred phenotypes resistant to restriction by CM TRIM5α. Models from two different angles are shown.

sessed a potent antiviral activity against SIVmac as well as HIV-1 and HIV-2 (26, 27). These results indicate that the single amino acid at the 120th position of the GH123 CA indeed affects susceptibility to the restriction of virus replication by CM TRIM5α.

There is more than 87% amino acid identity in CA between HIV-2 GH123 and SIVmac239. SIVmac239 is resistant to CM TRIM5α (26) and contains glutamine at the 118th position, which corresponds to the 120th position of the GH123 CA. To determine whether this particular amino acid also affects the resistance of SIVmac239 to CM TRIM5α, we constructed a mutant SIVmav239 carrying a proline at the 118th position (SIVmac239/P) (Fig. 3A). As shown in Fig. 3C, both wild-type and mutant SIVmac239 grew substantially and to similar titers in the CM-SPRY(−)-SeV-infected MT4 cells. In CM-TRIM5α-SeV-infected cells, the mutant SIVmac239 grew to titers that were approximately 10 times lower than those of the wild type. The glutamine-to-proline mutation also caused an approximately eightfold decrease in the virus titer in Hu-TRIM5α-SeV-infected cells (Fig. 3C). In contrast, neither virus grew in AGM-TRIM5α-SeV-infected cells (Fig. 3C). These results indicate that the glutamine at the 118th position of the SIVmac239 CA also affects resistance to the CM and Hu TRIM5αs.

Nineteen HIV-2 and 20 SIVsmm or SIVmac CA sequences were listed in the Los Alamos sequence database (Fig. 2B). All SIV isolates except for SMM-SL92B carried glutamine at the position corresponding to the 120th position of GH123 (Fig. 2B). SMM-SL92B, which carries alanine, was most distantly related to all the other HIV-2 and SIV isolates from the phylogenetic analysis (data not shown). These results suggest that all SIV isolates are resistant to CM and Hu TRIM5αs. In contrast, HIV-2 group A isolates showed a mixture of proline, alanine, and glutamine and HIV-2 group B isolates had a mixture of proline and alanine in the CA (Fig. 2B). Other HIV-2 isolates also carried proline and glutamine, with H2AB-7312A, which had glycine, being the only exception (Fig. 2B). These results suggest that different HIV-2 isolates have diverse susceptibilities to CM and Hu TRIM5αs. It is likely that glutamine-to-alanine or glutamine-to-proline substitutions occurred after the proposed zoonotic transfer of virus from monkeys to humans (11).

To obtain structural insight into the mechanisms by which this amino acid change in the CA alters viral susceptibility to restriction by TRIM5α, three-dimensional (3-D) models of HIV-2 CAs were constructed with the homology-modeling technique based on the crystal structure of the HIV-1 CA N-terminal domain (15). These models consist of those with proline (GH123, UC12), alanine (UC1, UC14, GH123/A), and glutamine (UC2, UC7, 9429, 12741, GH123/Q) at the 120th position. The thermodynamically optimized 3-D structures showed that the HIV-2 CA N-terminal domains consist of a packed core structure from which seven α-helices and three loops protrude, which is basically the same conformation as that of their HIV-1 counterparts (15) (Fig. 4).

The 120th amino acid that affects the viral susceptibility to TRIM5α restriction is located in the loop between helices 6 and 7 (L6/7) (Fig. 4, labeled Q, A, P). Especially worth noting is that the amino acid substitution at the 120th position is predicted to induce marked changes in the configuration of L6/7. The loop with the TRIM5α-sensitive proline (GH123 and UC12, Fig. 4, labeled P) is positioned most closely to the loop between helices 4 and 5 (L4/5). We obtained results the same as those described above when we constructed HIV-2 CA models based on the HIV-1 CA structure in solution (data not shown) (8). In HIV-1, L4/5 interacts directly with cyclophilin A (15, 24, 36). It is possible that TRIM5α recognizes the partic-

ular structure formed by two closely aligned L4/5 and L6/7 with proline.

Previously, a single amino acid substitution at the 110th position of N-MLV CA was shown to determine viral susceptibility to Fv1 (20), another type of restriction factor in mice (2), as well as to Hu TRIM5α (14, 31, 37). The fact that the amino acids at analogous positions of both N-MLV and HIV-2 CAs (25, 36) affected sensitivity to Hu and CM TRIM5αs, respectively, suggested that N-MLV and HIV-2 are recognized by TRIM5α in a similar manner. It would be interesting to investigate whether the 120th position of HIV-2 CA affects its sensitivity to TRIM5α of other OWM cells.

Human TRIM5α has no or very little effect on HIV-1 or SIVmac infection (30, 32, 34, 35, 38). Unlike HIV-1 (Fig. 3D), HIV-2 isolates sensitive to CM TRIM5α were slightly more sensitive to Hu TRIM5α than those resistant to CM TRIM5α (Fig. 1 and 3B). As shown in Fig. 4, half of the HIV-2 isolates in the database carried proline, which would be sensitive to Hu TRIM5α. This finding may be one of the reasons why HIV-2 is less pathogenic than HIV-1 (9). Since certain HIV-2 patients with high plasma HIV-2 loads developed AIDS as rapidly as HIV-1 patients (10), examining the effect of sensitivity to Hu TRIM5α of HIV-2 strains in infected individuals on the rate of disease progression merits attention.

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Minimal Region Sufficient for Genome Dimerization in the Human Immunodeficiency Virus Type 1 Virion and Its Potential Roles in the Early Stages of Viral Replication▽

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It has been suggested that the dimer initiation site/dimer linkage sequence (DIS/DLS) region of the human immunodeficiency virus type 1 (HIV-1) RNA genome plays an important role at various stages of the viral life cycle. Recently we found that the duplication of the DIS/DLS region on viral RNA caused the production of partially monomeric RNAs in virions, indicating that this region indeed mediates RNA-RNA interaction. In this report, we followed up on this finding to identify the necessary and sufficient region for RNA dimerization in the virion of HIV-1. The region thus identified was 144 bases in length, extending from the junction of R/U5 and U5/L stem-loops to the end of SL4. The *trans*-acting responsive element, polyadenylation signal, primer binding site, upper stem-loop of U5/L, and SL2 were not needed for the function of this region. The insertion of this region into the ectopic location of the viral genome did not affect the level of virion production by transfection. However, the resultant virions contained monomerized genomes and showed drastic reductions in infectivity. A reduction was observed especially in the reverse transcription process. An attempt to generate a replication-competent virus with monomerized genome was performed by the long-term culture of mutant virus-infected cells. All recovered viruses were wild-type revertants, indicating a fatal defect of the mutation. These results suggest that genome dimerization or DIS/DLS itself also plays an important role in the early stages of virus infection.

The retrovirus genome is a single-stranded, positive-sense RNA. The viral genome always occurs as a dimer in virus particles, and the interaction is noncovalent since heating easily dissociates purified dimeric genomes into monomers. Template strand switching between two genomes during reverse transcription is often observed in the retroviral life cycle (15). It is likely that the presence of two genomes in one virion helps the virus survive by providing an extra template that can be used when one RNA molecule is damaged and/or providing genetic variety for the progeny. However, this may not fully explain why the virion has to carry two identical RNAs in spite of severe space limitation, so the precise nature of retroviral genome dimerization is still unclear.

The identification of *cis*-acting signals for retrovirus genome dimerization was initially attempted in an in vitro assay (10, 11, 21, 34, 36). Synthesized 5′ RNA fragments of a viral genome, with a length of several hundred to a thousand bases, were found to be dimerized by heating and cooling under suitable buffering conditions. The proposed dimer initiation site/dimer linkage sequence (DIS/DLS) region of human immunodeficiency virus type 1 (HIV-1) is located within the untranslated region between the long terminal repeat (LTR) and the *gag* gene (11, 22). As these regions overlap with a packaging signal, however, it was difficult to perform mutational analysis to study the dimerization of the genome within the virion. Therefore, we recently developed a system to assess the dimerization signal operating within the HIV-1 virion without affecting the packaging ability of the genome (41). This system is an application of our previous finding that duplication of the encapsidation/dimerization signal (E/DLS) region on one RNA genome resulted in the appearance of a monomeric genome in the HIV-1 virion (40). We speculated that an additional E/DLS region at the ectopic position binds to the authentic E/DLS region on the same RNA molecules, thus competitively interfering with intermolecular dimer formation (Fig. 1A). Mutational analysis could thus be utilized to map the DIS/DLS precisely on the HIV-1 RNA. By applying this system, part of the 5′ untranscribed region (UTR) of the HIV-1 genome was examined, and separate functional maps for dimerization and encapsidation signals could be created. By comparing these two maps, we concluded that RNA dimer linkage formation must be an essential process in genome packaging, which consists of multiple sequential steps (41). In the study reported here, we employed our original system to identify the region which is necessary and sufficient for HIV-1 genome dimerization in the virion. We also report on our further investigation of the roles of the dimerization signal at various stages of viral replication and discuss the possibility that it may perform functions other than genome packaging in the viral life cycle.

MATERIALS AND METHODS

DNA constructs. The replication-competent HIV-1 proviral clone pNL4-3 (2) and pMSMBA (23), a derivative of pNL4-3, were used as progenitors for the mutant constructs described here. Mutant plasmids were constructed with standard methods. To construct pDDNT4, pDDNU4, and pDDNL4, three pairs of primers were first used for PCR amplification of the HIV-1 leader region by using the plasmid pGEM-MM (40) as a template. The first pair comprised the sense primer TarF (5′-GGTCTCTCTGGTTAGACCAG-3′) and the antisense primer SL4Rs (5′-GACGCTCTCGCACCCATC-3′), the second pair consisted of the sense primer R/U5F (5′-CACTGCTTAAGCCTCAACGATCG-3′) and the antisense primer SL4Rs, and the third pair consisted of the sense primer

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