TABLE 1 (Continued)

Clone designation	CA mutation(s)[b]	Growth potential[c]
L6I-LSDQA	L6I, M94L, R98S, G114Q, Q178A	++++
DLSDQA	E71D, M94L, R98S, G114Q, Q178A	+++
L6I-DLSDQA	L6I, E71D, M94L, R98S, G114Q, Q178A	+++
LSDQAV	M94L, R98S, G114Q, Q178A, P206V	++++
L6I-LSDQAV	L6I, M94L, R98S, G114Q, Q178A, P206V	++++
YQ-LSDQAV	Q50Y, T54Q, M94L, R98S, G114Q, Q178A, P206V	−
DLSDQAV	E71D, M94L, R98S, G114Q, Q178A, P206V	+++
L6I-DLSDQAV	L6I, E71D, M94L, R98S, G114Q, Q178A, P206V	+++
Mutants of the β-hairpin domain		
DdN5	N5 deletion	−
DdN5-Y	N5 deletion, T117Y	−
SDdN5	N5 deletion, R98S	−
SDdN5-Y	N5 deletion, R98S, T117Y	−
DQdN5-Y	N5 deletion, G114Q, T117Y	−
SDQdN5	N5 deletion, R98S, G114Q	−
SDQdN5-Y	N5 deletion, R98S, G114Q, T117Y	−
LSDQdN5	N5 deletion, M94L, R98S, G114Q	−
LSDQdN5-Y	N5 deletion, M94L, R98S, G114Q, T117Y	−
QIG-S	NLQ5-7QIG, R98S	−
QIG-SDQ	NLQ5-7QIG, R98S, G114Q	−
QIG-LSDQ	NLQ5-7QIG, M94L, R98S, G114Q	−
GGN-S	QGQ7-9GGN, R98S	−
GGN-LSDQ	QGQ7-9GGN, M94L, R98S, G114Q	−
GGN-YQ-LSDQ	QGQ7-9GGN, Q50Y, T54Q, M94L, R98S, G114Q	−
L6I-YQ-LSDQ	L6I, Q50Y, T54Q, M94L, R98S, G114Q	−
IL-LSDQA	L6I, Q13L, M94L, R98S, G114Q, Q178A	−
IL-Y-LSDQA	L6I, Q13L, M94L, R98S, G114Q, T117Y, Q178A	−
IN-LSDQA	L6I, Q9N, M94L, R98S, G114Q, Q178A	−
IN-Y-LSDQA	L6I, Q9N, M94L, R98S, G114Q, T117Y, Q178A	−
IY-Y-LSDQA	L6I, M10Y, M94L, R98S, G114Q, T117Y, Q178A	−
INY-LSDQA	L6I, Q9N, M10Y, M94L, R98S, G114Q, Q178A	−
INY-Y-LSDQA	L6I, Q9N, M10Y, M94L, R98S, G114Q, T117Y, Q178A	−
INL-LSDQA	L6I, Q9N, Q13L, M94L, R98S, G114Q, Q178A	−
INL-Y-LSDQA	L6I, Q9N, Q13L, M94L, R98S, G114Q, T117Y, Q178A	−
IYL-LSDQA	L6I, M10Y, Q13L, M94L, R98S, G114Q, Q178A	−
IYL-Y-LSDQA	L6I, M10Y, Q13L, M94L, R98S, G114Q, T117Y, Q178A	−
INYL-LSDQA	L6I, Q9N, M10Y, Q13L, M94L, R98S, G114Q, Q178A	−
INYL-Y-LSDQA	L6I, Q9N, M10Y, Q13L, M94L, R98S, G114Q, T117Y, Q178A	−
Q13L-LSDQ	Q13L, M94L, R98S, G114Q	−
Q13L-Y-LSDQ	Q13L, M94L, R98S, G114Q, T117Y	−
Q13L-LSDQA	Q13L, M94L, R98S, G114Q, Q178A	−
Q13L-Y-LSDQA	Q13L, M94L, R98S, G114Q, T117Y, Q178A	−

[a] MN4Rh-3 CA was constructed by replacing the CypA-binding loop and H6/7L of HIV-1_{NL4-3} CA with the corresponding regions of SIVmac239 CA and by the additional introduction of a Q110D mutation (Fig. 1) (19, 24).

[b] Amino acid number in MN4Rh-3 CA.

[c] Viral growth potential in M1.3S cells. ++++, grows similarly to MN4/LSDQ; +++, grows more efficiently than MN4Rh-3; ++, grows similarly to MN4Rh-3; +, grows more poorly than MN4Rh-3; −, undetectable during the observation period.

clones here, except for MN4Rh-3, exhibited a tendency to have a higher level of resistance to CyM-TRIM5α than to RhM-TRIM5α (Fig. 4). It has been shown that TRIM5α proteins encoded by *TRIM5*^{TFP} are more restrictive to virus infection than are those encoded by *TRIM5*^{Q} (40). The observed tendency for TRIM5α resistance may be due to the difference between RhM-TRIM5α (*TRIM5*^{TFP}) and CyM-TRIM5α (*TRIM5*^{Q}) used in the assay. These results indicate that the enhancement of viral growth in M1.3S cells by CA alterations depends, at least in part, on the increased resistance to TRIM5α.

Virus replication capability in macaque PBMCs with different *TRIM5* alleles reflects the TRIM5α resistance of HIV-1mt clones. We have previously shown that MN4Rh-3 replicates well in *TRIM5α/TRIM5CypA* heterozygous CyM PBMCs/individuals, but its replication was restricted in *TRIM5α* homozygous CyM PBMCs/individuals (19, 24). To confirm the effect of the increased resistance of MN4/LSDQ and MN5/LSDQ against macaque TRIM5α on viral replication, we examined their replication potential relative to that of MN4Rh-3 and MN5Rh-3 in *TRIM5α/TRIM5CypA* heterozygous or *TRIM5α* homozygous macaque PBMCs.

First, we compared viral growth potentials of the clones in CyM PBMCs (Fig. 5A). Growth kinetics of MN4Rh-3 and MN4/LSDQ were similar in *TRIM5α/TRIM5CypA* heterozygous CyM

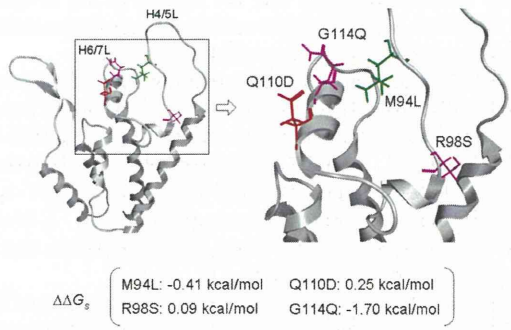

FIG 3 Structural analysis of the HIV-1mt CA NTD. A molecular model of the HIV-1mt CA NTD was constructed by homology modeling and refined as described previously (19). Single-point mutations were generated on the CA model, and ensembles of protein conformations were generated by using the LowMode MD module in MOE (Chemical Computing Group Inc., Quebec, Canada) to calculate average stability by using Boltzmann distribution. The stability scores ($\Delta\Delta G_s$) of the structures refined by energy minimization were obtained through the stability scoring function of the Protein Design application and are indicated below the structural model.

PBMCs. In contrast, the replication efficiency of MN4/LSDQ was markedly enhanced in *TRIM5*α homozygous CyM PBMCs relative to that of MN4Rh-3 (Fig. 5A). Similar results were obtained with RhM PBMCs. MN4Rh-3 exhibited growth kinetics comparable to those of MN4/LSDQ in *TRIM5*α/*TRIM5CypA* heterozygous RhM PBMCs (Fig. 5B). In *TRIM5*α homozygous RhM PBMCs, MN5/LSDQ replicated much more efficiently than MN5Rh-3 (Fig. 5C). CXCR4-tropic HIV-1mt clones (MN4 series) were found to exhibit a higher growth ability than CCR5-tropic HIV-1mt clones (MN5 series) in both M1.3S cells and macaque PBMCs (Fig. 2D and 5B and C) and were therefore used for experiments thereafter. In sum, the replication potential of TRIM5α-resistant HIV-1mt clones (MN4/LSDQ and MN5/LSDQ) markedly increased in *TRIM5*α homozygous PBMCs but was similar to that of TRIM5CypA-resistant/TRIM5α-sensitive clones (MN4Rh-3 and MN5Rh-3) in *TRIM5*α/*TRIM5CypA* heterozygous PBMCs. These results suggest that M94L/R98S/G114Q mutations in MN4Rh-3 CA largely contribute to the acquisition of TRIM5α resistance.

HIV-1 Vpu gains the ability to specifically counteract macaque tetherin by replacing its TM domain with the corresponding region of SIVgsn166 Vpu. Tetherin as well as TRIM5 proteins are important anti-HIV-1 factors in macaque cells (4, 8, 10), but the HIV-1mt clones constructed so far do not display macaque tetherin antagonism due to Vpu derived from HIV-1_{NL4-3}. It has been shown that Vpu from SIVmon/mus/gsn can antagonize macaque tetherin but not human tetherin (26). To confer the ability to counteract macaque tetherin on HIV-1mt clones, we modified the *vpu* gene. The sequence of the cytoplasmic domain of HIV-1 Vpu partially overlaps the 5′-end sequence of Env, and the TM domain of Vpu is a key region for species-specific tetherin antagonism (22). Thus, we constructed Vpu clones that contain SIVmon/mus/gsn TM and HIV-1mt cytoplasmic domains (Fig. 6A). First, RhM tetherin antagonism of various Vpu clones was analyzed by Vpu *trans*-complementation assays for virion release (Fig. 6B). 293T cells were cotransfected with a *vpu*-deficient HIV-1mt clone (MN4Rh-3-ΔU), an RhM tetherin expression vector (pCIneo-RhM tetherin), and various Vpu constructs, and virion production from cells on day 2 posttransfection was measured. While MN4Rh-3-ΔU released progeny virions efficiently upon transfection without RhM tetherin expression, its virion production was significantly inhibited in the presence of RhM tetherin. Although this reduction was not rescued by HIV-1_{NL4-3} Vpu, SIVmon/mus/gsn Vpu restored it to some extent, consistent with a previous report (26). Of the SIV/HIV-1 chimeric Vpu proteins, gsnTM-Vpu appeared to be somewhat better than the others and was therefore used thereafter.

Next, we examined the ability of HIV-1_{NL4-3} Vpu and gsnTM-Vpu to downregulate cell surface CD4 and tetherin (Fig. 6C). MAGI, LLC-MK2, and HEp2 cells were used for analysis of CD4,

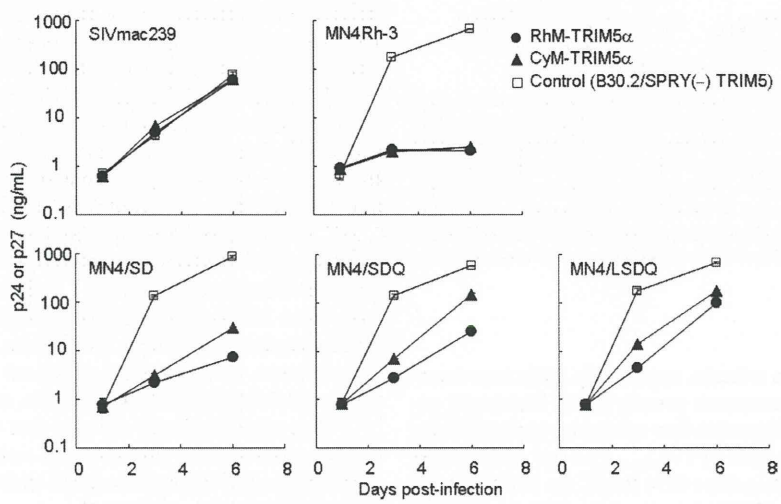

FIG 4 Susceptibility of SIVmac239 and various HIV-1mt clones to macaque TRIM5α. Human MT4 cells (10^5) were infected with recombinant SeV expressing RhM-TRIM5α (*TRIM5*^{TFP}), CyM-TRIM5α (*TRIM5*^{Q}), or B30.2/SPRY(−) TRIM5. Nine hours after infection, cells were superinfected with 20 ng (Gag-p24) of various HIV-1mt clones or 20 ng (Gag-p27) of SIVmac239. Virus replication was monitored by the amount of Gag-p24 from HIV-1mt clones or Gag-p27 from SIVmac239 in the culture supernatants. Error bars show actual fluctuations between duplicate samples. Representative data from two independent experiments are shown.

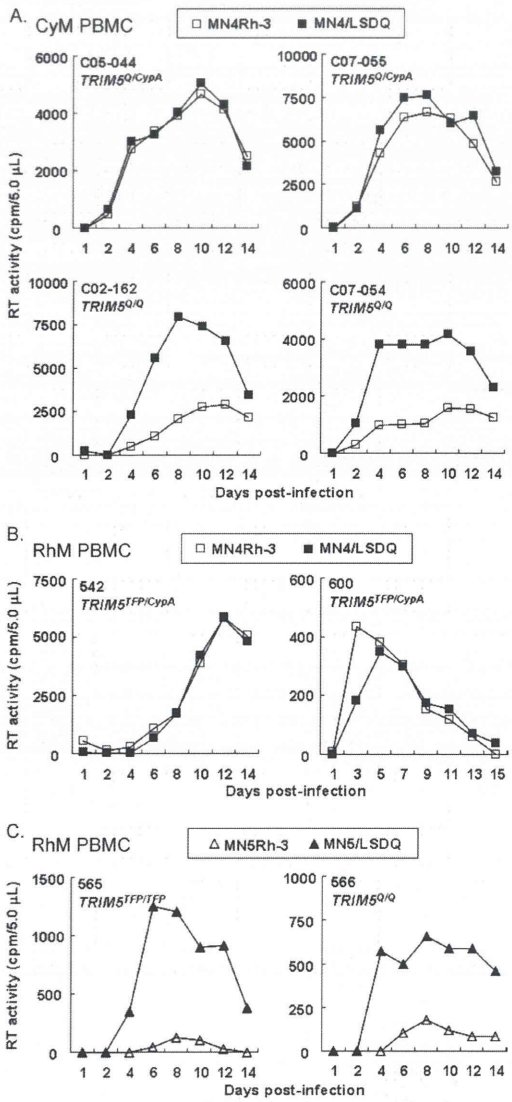

FIG 5 Growth kinetics of HIV-1mt clones with a distinct CA in macaque PBMCs. (A) Infection of PBMCs from four *TRIM5α/TRIM5CypA* heterozygous or *TRIM5α* homozygous CyM individuals. (B and C) Infection of PBMCs from four RhM individuals with different *TRIM5* alleles. For infection, input viruses were prepared from 293T cells transfected with the proviral clones indicated, and equal amounts (2.5×10^6 RT units) were used to spin infect PBMCs (2×10^6 cells). Virus replication was monitored by RT activity released into the culture supernatants. Monkey identification numbers are indicated in each panel.

RhM tetherin, and human tetherin, respectively. Cells were transfected with Vpu-green fluorescent protein (GFP) bicistronic expression plasmids and subjected to flow cytometry analysis on day 2 posttransfection. While both HIV-1_{NL4-3} Vpu and gsnTM-Vpu significantly decreased cell surface CD4 levels, the RhM tetherin level was reduced by gsnTM-Vpu but not by HIV-1_{NL4-3} Vpu. Similar results were obtained for MK.P3(F) cells expressing endogenous CyM tetherin (data not shown). In contrast, HIV-1_{NL4-3} Vpu but not gsnTM-Vpu downmodulated cell surface human tetherin. These results show that the transfer of the SIVgsn166 Vpu TM domain to HIV-1 Vpu is sufficient to confer the ability to specifically antagonize macaque tetherin on viruses.

gsnTM-Vpu in the context of proviral genome functions in macaque cells. To ask if gsnTM-Vpu is functional in the proviral context, we generated an HIV-1mt clone encoding gsnTM-Vpu (MN4/LSDQgtu) (Fig. 1 and 7A). Interestingly, it has been shown that Vpu of HIV-1 composed of HIV-1_{DH12} TM and HIV-1_{NL4-3} cytoplasmic domains counteracts macaque tetherin (22). We thus constructed another HIV-1mt clone, MN4/LSDQdtu, that has chimeric Vpu, as described above (Fig. 7A).

To examine the species-specific tetherin antagonism of these proviral clones, we carried out virion release assays in the presence of RhM or human tetherin (Fig. 7B). Using SIVmac239 Nef as a control antagonist against macaque tetherin (52, 53), the anti-macaque tetherin activities of MN4/LSDQ, MN4/LSDQdtu, and MN4/LSDQgtu were comparatively analyzed. As described above, SIVmac239 Nef exhibited the ability to specifically antagonize macaque tetherin. As expected, virion production of MN4/LSDQ and its *vpu*-deficient clone was similarly restricted in the presence of RhM tetherin, and MN4/LSDQ displayed a higher level of virion production than that of its *vpu*-deficient clone in the presence of human tetherin, indicating its specific antagonism to human tetherin. Also, as expected from a previous report (22), MN4/LSDQdtu showed both RhM and human tetherin antagonism, although its anti-RhM tetherin activity was relatively low. Strikingly, virion production levels of MN4/LSDQgtu in the presence of RhM/human tetherin were similar to those of SIVmac239. This indicates that MN4/LSDQgtu has specifically strong anti-RhM tetherin activity, as is the case for SIVmac239. To see if various Vpu proteins function during viral replication in macaque cells, we determined the growth properties of various HIV-1mt clones carrying distinct Vpu proteins. Although the effect of *vpu* deletion is virologically small, *vpu*-deficient viruses are readily distinguishable from the parental wild-type virus by comparative kinetic analysis of viral growth (22, 29). As shown in Fig. 7C, while MN4/LSDQ and MN4/LSDQdtu exhibited growth kinetics similar to those of their respective *vpu*-deficient clones, *vpu*-deficient MN4/LSDQgtu grew significantly more poorly than its parental virus. Taken together, it can be concluded that MN4/LSDQgtu Vpu but not MN4/LSDQ Vpu functions during viral replication in M1.3S cells. However, the functionality of MN4/LSDQdtu Vpu in macaque cells was not clear in the viral growth kinetics here. Although there are some possible explanations, the relatively low anti-RhM tetherin activity of MN4/LSDQdtu (see the results in Fig. 7B) could account for this observation.

Although the tertiary structure of the HIV-1 Vpu TM domain has been determined by NMR (38), the structure of the TM domain from SIV Vpu has not been solved to date. To investigate how replacement of the Vpu TM domain could lead to changes in TM structure, we constructed structural models of Vpu TM domains of MN4/LSDQ, MN4/LSDQdtu, and MN4/LSDQgtu (Fig. 7D). This modeling study revealed that the types of amino acid residues corresponding to the crucial residues (54) in HIV-1 Vpu for binding with human tetherin are similar between MN4/LSDQ and MN4/LSDQdtu, whereas they are often different in MN4/LSDQgtu. In addition, their steric locations in the helices are also similar between MN4/LSDQ and MN4/LSDQdtu, whereas they are very different in MN4/LSDQgtu. Finally, angles between the

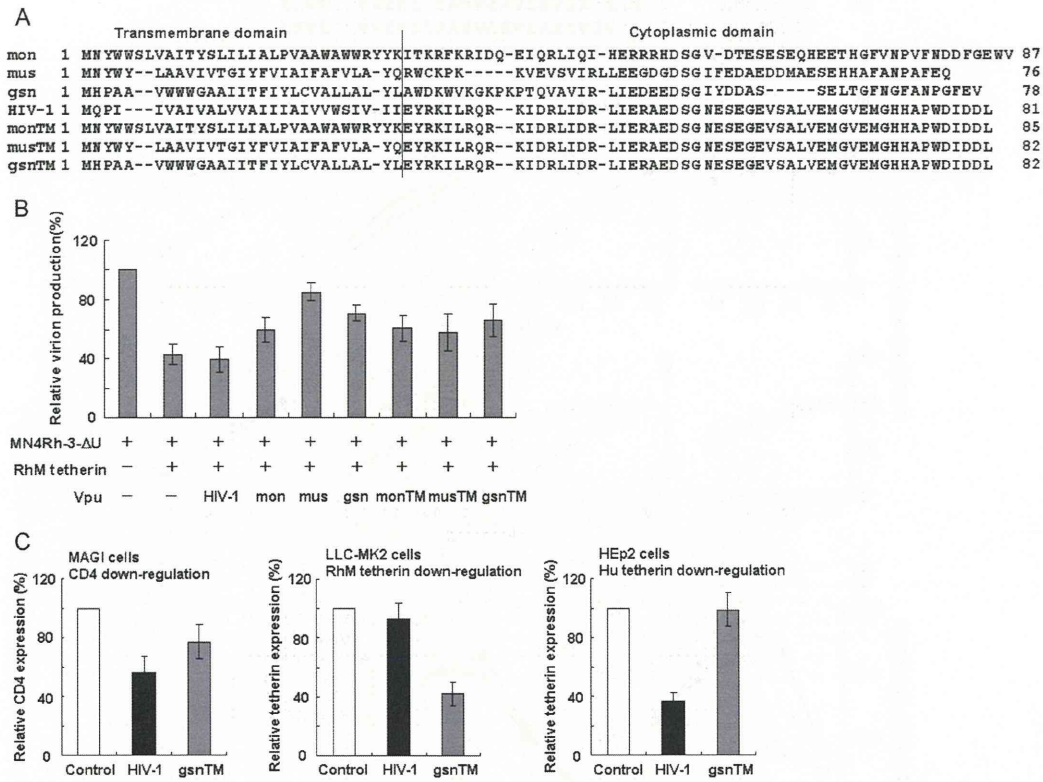

FIG 6 Generation of SIV/HIV-1 chimeric Vpu proteins resistant to macaque tetherin. (A) Amino acid sequences of various Vpu proteins. Alignments of the sequences and the boundary between TM/cytoplasmic domains are shown based on previously reported information (26). mon, SIVmonCML1 (GenBank accession number AY340701); mus, SIVmus1085 (GenBank accession number AY340700); gsn, SIVgsn166 (GenBank accession number AF468659); HIV-1, NL4-3 (32). HIV-1mt clones (MN4 series) have *vpu* genes identical to that of NL4-3. monTM-, musTM-, and gsnTM-Vpu were constructed by fusing each TM domain of SIVmon/mus/gsn Vpu with the cytoplasmic domain of HIV-1_{NL4-3} Vpu. (B) RhM tetherin antagonism by various Vpu proteins. 293T cells were cotransfected with a *vpu*-deficient proviral clone (MN4Rh-3-ΔU), pCIneo-RhM tetherin, and various pSG-VpucFLAG constructs. On day 2 posttransfection, virion production in the culture supernatants was determined by RT assays. Virion production levels relative to that of MN4Rh-3-ΔU in the absence of RhM tetherin were calculated, and mean values of three independent experiments are shown with the standard deviations. (C) Downregulation of cell surface CD4 and tetherin by HIV-1_{NL4-3} Vpu or gsnTM-Vpu. MAGI, LLC-MK2, and HEp2 cells were used to determine the downregulation of CD4, RhM tetherin, and human (Hu) tetherin by Vpu, respectively. Cells were transfected with the pIRES-hrGFP (control), pIRES-HIV-1 Vpu-hrGFP, or pIRES-gsnTM-Vpu-hrGFP construct. On day 2 posttransfection, cells were stained for cell surface CD4 or tetherin and analyzed by two-color flow cytometry. Values presented are CD4 or tetherin fluorescence intensities of GFP-positive cells relative to that of the control. Mean values ± standard deviations of three independent experiments are shown.

central lines of the helices are similar between MN4/LSDQ and MN4/LSDQdtu, whereas they are different in MN4/LSDQgtu. These results suggest the possibility that the structural properties of the tetherin interaction surface of the MN4/LSDQgtu Vpu TM domain are very different from those of the Vpu TM domains of MN4/LSDQ and MN4/LSDQdtu. Further studies are necessary to verify this issue.

RhM APOBEC3-, TRIM5α-, and tetherin-resistant HIV-1mt clone MN4/LSDQgtu replicates comparably to SIVmac239 in RhM PBMCs. Here we constructed distinct HIV-1mt clones with respect to their resistance to RhM TRIM5α and tetherin: TRIM5α- and tetherin-susceptible MN4Rh-3, TRIM5α-resistant but tetherin-susceptible MN4/LSDQ, and TRIM5α- and tetherin-resistant MN4/LSDQgtu. Of note, all these clones are RhM APOBEC3 resistant (see Fig. 1 for their genomes). To investigate the effect of the increased resistance to these macaque restriction factors, various viruses were examined for their growth potential in PBMCs from four *TRIM5α* homozygous RhM individuals. As shown in Fig. 8, SIVmac239, a comparative standard virus in macaque cells, replicated constantly in all PBMC preparations. The growth potentials in the RhM PBMCs of the HIV-1mt clones tested markedly and stably differed. As a likely result of RhM TRIM5α-resistant Gag-CA, MN4/LSDQ replicated much more efficiently than MN4Rh-3. By virtue of RhM tetherin-resistant Vpu, MN4/LSDQgtu grew significantly better than MN4/LSDQ. Essentially the same results for HIV-1mt growth kinetics were obtained in M1.3S cells. The M1.3S cell line and macaque PBMCs always responded similarly to various SIVs/HIVs (our unpublished observations). Moreover, by comparing the peak day of viral growth kinetics and the peak level itself, MN4/LSDQgtu was shown here to have the ability to replicate comparably to SIVmac239 in RhM PBMCs, except for one preparation (from monkey 565) (Fig. 8). The results show that the increased resistance to macaque restriction factors correlates well with the enhanced viral growth potential. In sum, MN4/LSDQgtu, which exhibits resistance to known major restriction factors (APOBEC3, TRIM5, and tetherin

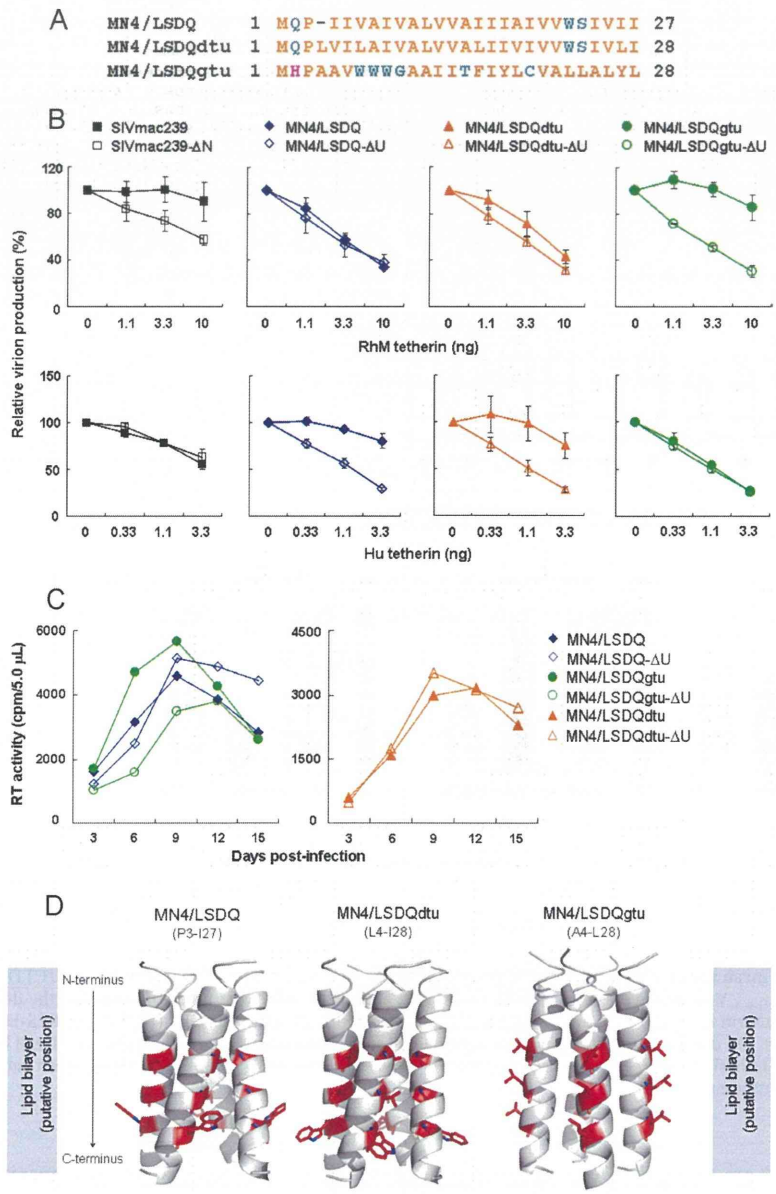

FIG 7 Effects of various Vpu proteins carrying a different TM domain on tetherin antagonism and HIV-1mt replication in macaque cells. (A) Alignment of amino acid sequences of the Vpu TM domain in each HIV-1mt clone. MN4/LSDQ, MN4/LSDQdtu, and MN4/LSDQgtu encode the Vpu TM domain derived from HIV-1_{NL4-3} (32), HIV-1_{DH12} (22), and SIVgsn166 (GenBank accession number AF468659), respectively. (B) Species-specific tetherin antagonism by SIVmac239 and various HIV-1mt clones carrying different Vpu proteins. SIVmac239 (MA239N) and its *nef*-deficient clone (MA239N-ΔN) were used as positive controls for RhM tetherin resistance. 293T cells were cotransfected with proviral clones and the indicated amounts of the pCIneo-RhM tetherin or pCIneo-Human tetherin expression vector. On day 2 posttransfection, virion production was determined by RT activity released into the culture supernatants. Values are presented as RT activity of each sample relative to that of each proviral clone without tetherin expression. Mean values ± standard deviations of three independent experiments are shown. ΔU, *vpu* deficient; Hu, human. (C) Growth kinetics of various HIV-1mt clones and their *vpu*-deficient clones in M1.3S cells. Viruses were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (5×10^5 RT units) were inoculated into M1.3S cells (2×10^5 cells). Virus replication was monitored by RT activity released into the culture supernatants. Representative data from three independent experiments are shown. (D) Structural modeling of Vpu TM domains of MN4/LSDQ, MN4/LSDQdtu, and MN4/LSDQgtu. Predicted models are shown in a ribbon representation. Amino acid residues corresponding to the residues in HIV-1 Vpu crucial for binding with human tetherin (54) are highlighted in a red stick representation. Crucial residues in Vpu TM domains of MN4/LSDQ, MN4/LSDQdtu, and MN4/LSDQgtu are A14/A18/W22, A15/V19/W23, and T15/L19/L23, respectively. TM regions analyzed (see panel A for amino acid sequences) are indicated in parentheses.

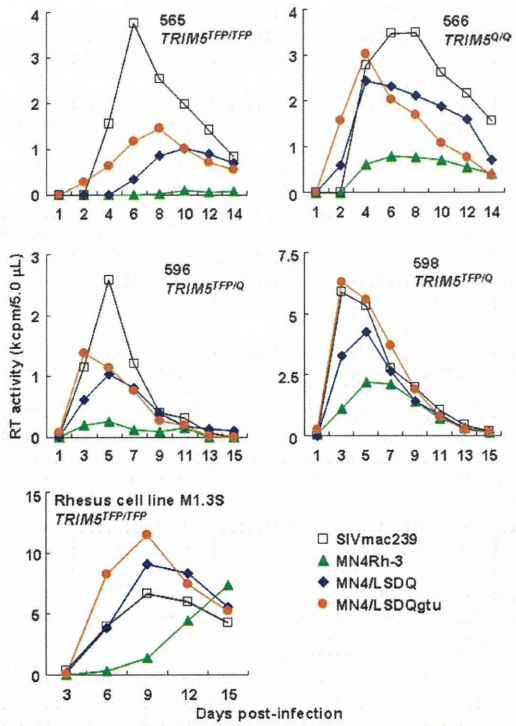

FIG 8 Growth kinetics of SIVmac239 and various HIV-1mt clones in *TRIM5α* homozygous RhM PBMCs. PBMCs were prepared from four RhM individuals with the different *TRIM5* alleles indicated. Viruses were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (2.5×10^6 RT units) were used to spin infect PBMCs (2×10^6 cells). As a control experiment, rhesus M1.3S cells (2×10^5) were infected with equal amounts of viruses (5×10^5 RT units). Virus replication was monitored by RT activity released into the culture supernatants. Monkey identification numbers are indicated in each panel.

proteins), is the best HIV-1mt clone generated so far, replicating with an efficiency similar to that of SIVmac239 in RhM cells.

DISCUSSION

In this study, we generated a novel HIV-1mt clone, designated MN4/LSDQgtu, that exhibits resistance to RhM TRIM5α and tetherin in addition to APOBEC3 proteins (Fig. 1). By sequence homology- and structure-guided CA mutagenesis and by screening the multicycle growth potential of CA mutant viruses in M1.3S cells, we successfully obtained viruses with enhanced replication efficiency in macaque cells as well as increased macaque TRIM5α resistance (Fig. 2 to 5). The transfer of the TM domain of SIVgsn166 Vpu into the corresponding region of HIV-1mt Vpu conferred the ability to specifically counteract macaque tetherin on the virus (Fig. 6 and 7). Furthermore, the increased resistance to both RhM TRIM5α and tetherin contributed to the viral growth enhancement in RhM PBMCs (Fig. 8).

During the preparation of this paper, McCarthy et al. reported several key residues in SIVmac239 CA involved in the interaction with RhM TRIM5α by genetic and structural analysis (55). Interestingly, the HIV-1mt CA amino acid residues identified in this study as being the elements responsible for the increased resistance to RhM TRIM5α (M94L/R98S/Q110D/G114Q) were included in those residues. McCarthy et al. reported that H4/5L and helix 6 of SIVmac239 CA also affect TRIM5α sensitivity (55). In the construction process for our HIV-1mt clones, we found that the CA elements involved in the interaction with RhM TRIM5α are the CypA-binding loop within H4/5L, H6/7L, M94L/R98S within H4/5L, and Q110D/G114Q in helix 6 (18–20, 56; this study). Of the substitutions identified in this study, R98S was the primary residue to increase TRIM5α resistance and improve viral growth in macaque cells. It was also shown that the TRIM5α (*TRIM5*^{TFP})-susceptible SIVsmE543-3 clone acquires an adaptive R97S change in CA (corresponding to R98S in MN4Rh-3 CA) to evade TRIM5α (*TRIM5*^{TFP}) restriction during viral replication in RhM individuals (40). In TRIM5α-sensitive CA, R98S may be a key residue contributing to the evasion of TRIM5α restriction. Together, these results suggest that CA elements critical for recognition by TRIM5α may be conserved among primate lentiviruses. The RhM TRIM5α-resistant HIV-1 CA constructed in this study would be useful to define how TRIM5α recognizes CA. On the other hand, MN4/LSDQ appeared not to evade TRIM5α restriction completely, as SIVmac239 did (Fig. 4). In this regard, since it has been shown that the N-terminal β-hairpin domain in the retroviral CA contributes to circumventing TRIM5α (36, 55, 57), we constructed various HIV-1mt clones carrying mutations in the domain (Table 1). However, except for the L6I substitution, none of the clones were infectious (Table 1). A further CA modification(s) may be necessary for complete evasion of TRIM5α restriction.

Accumulating evidence has shown that tetherin is an important cellular restriction factor that affects the replication, adaptation, and evolution of primate immunodeficiency viruses (4, 26). Its negative effect on viral replication is certainly observed in cultured cell lines and primary cells but is not so evident relative to those of APOBEC3 and TRIM5 proteins (10). Also, in the present study, RhM tetherin-resistant Vpu significantly contributed to viral growth enhancement but not as much as TRIM5α-resistant CA (Fig. 7 and 8). However, tetherin has been suggested to play an important effector role in antiretroviral activity induced by alpha interferon (58–60). Also, it has been shown that the pathogenic revertant virus from nonpathogenic *nef*-deficient virus acquires tetherin antagonism by adaptive mutations in the gp41 subunit of Env (61). Therefore, the ability of HIV-1mt clones to antagonize RhM tetherin may be very important for optimal replication and pathogenesis in RhM individuals. In this regard, it has been described that naturally occurring polymorphisms in RhM tetherin sequences are present (30, 31, 61). Although whether these variations have some appreciable effects on viral replication *in vitro* is undetermined, the relationship between tetherin polymorphisms and the viral replication level *in vivo* (animals)/viral pathogenic activity *in vivo* may be a major issue to address and remains to be extensively analyzed. It would be intriguing to elucidate how the viral accessory protein Vpu *in vitro* is associated with the *in vivo* replicative and pathogenic properties of HIV-1 (22).

We constructed an MN4/LSDQgtu clone resistant to the known major restriction factors (APOBEC3, TRIM5, and tetherin proteins) in RhM cells. The growth potential of MN4/LSDQgtu was similar to that of SIVmac239 in most RhM PBMC preparations (Fig. 8). It was shown previously that the *in vivo* replication of SIV is predictable from the virus susceptibility of PBMCs (62, 63). Also, in a series of our studies, the better our HIV-1mt clones grew in PBMCs, the better they grew in the monkeys (20, 24, 64).

Thus, it is expected that MN4/LSDQgtu will grow much better in RhM individuals, at least in the early infection phase, than the other HIV-1mt clones constructed. As reported previously, the replication of HIV-1 derivatives in infected macaques was eventually controlled, and no disease was induced in the animals (16, 20, 21, 24, 64). It has been suggested that the replication ability of primate lentiviruses in unusual hosts is more severely affected, via an interferon-induced antiviral state mediated by unidentified species-specific factors, than that in natural hosts (23). Moreover, there are the other significant issues to be considered, such as viral coreceptor tropism (CXCR4 versus CCR5), the diversity in viral growth properties (HIV-1 versus SIVmac), and the difference in host immune responses (human versus RhM) (9, 65–67). Most importantly, CCR5-tropic but not CXCR4-tropic clones have been found to be appropriate as input viruses to experimentally infect RhMs for various HIV-1 model studies *in vivo* (65–67). Although MN4/LSDQgtu is a CXCR4-tropic virus, it has clear potential for the establishment of a model system. MN4/LSDQgtu can be changed to a pathogenic CCR5-tropic virus through *in vitro* and *in vivo* approaches, as well documented by previous SHIV studies (68–70). It is also possible to generate entirely new CCR5-tropic HIV-1mt clones other than MN4/LSDQgtu derivatives on the basis of the key findings for Gag-CA and Vpu-TM in this study.

Our study here describes the generation and characterization of a novel HIV-1 derivative minimally chimeric with SIVs. Several infection model systems using distinct viruses and nonhuman primates are now available. It is important to define common and unique characteristics of each virus-host interaction based on the results obtained from various experimental approaches, including SIV/natural host and SIVmac/RhM, SHIV/RhM, and HIV-1mt/RhM infection systems. Such efforts would shed light on a better understanding of HIV-1/human infection and HIV-1 pathogenesis.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan (Research on HIV/AIDS project no. H23-003).

We thank Kazuko Yoshida for editorial assistance.

We declare that no competing interests exist.

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Microbes and Infection 15 (2013) 319–328

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Original article

Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells

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Received 17 December 2012; accepted 24 January 2013
Available online 4 February 2013

Abstract

Fundamental property of viruses is to rapidly adapt themselves under changing conditions of virus replication. Using HIV-1 derivatives that poorly replicate in macaque cells as model viruses, we studied here mechanisms for promoting viral replication in non-natural host cells. We found that the HIV-1s could evolve to grow better in both macaque and human cells by the continuous culture in macaque lymphocyte cell lines. Notably, only several mutations at defined sites of the Pol-integrase and/or the Env-gp120 reproducibly appeared in repeated adaptation experiments and were sufficient to cause the phenotypic change. Meanwhile, no amino acid changes to enhance viral replication in macaque cells were found in interaction sites for the known anti-retroviral proteins. These findings disclose a hitherto unappreciated evolutionary pathway to augment HIV-1 replication in primate cells, where tuning of viral interactions with positive rather than negative factors for replication can play a dominant role.

Keywords: HIV-1; HIV-1mt; Pol-IN; Env-gp120; Adaptive mutation; Macaque cells

1. Introduction

Viruses evolve extremely rapidly under the changing conditions of virus replication. HIV-1 is no exception. HIV-1 possesses high adaptation potential due to the ability to acquire sequence alterations through high mutation rate of reverse transcriptase (RT) and recombination of viral genomes [1,2]. Change of viral properties by genetic alterations leads to resistance to antiviral drugs, escape from host immune system, and adaptation to new hosts upon transmission [3–6]. Experimental approaches that analyze the genomic change and evolution of viruses are commonly used effective measures to know how viruses adapt themselves under a certain selective pressure. Such studies, however, usually have focused on genetic changes in a limited and selected region of viral genomes or sequence variations in a specified mass of virus.

HIV-1 does not replicate in most animal species including rodents and macaques. Inhibition of HIV-1 replication in macaque cells, at least in part, is mediated by host restriction factors such as APOBEC3 proteins, cyclophilin A (CypA), TRIM5α/TRIMCyp (TRIM5 proteins), and tetherin (for review, refer to references [7–10]). Encounters with pathogenic viruses impose selective pressure on restriction factors, and influence their antiviral specificity [11–13]. Even though human cells also have orthologs of these factors, HIV-1 evades their restriction and replicates well in humans. This suggests that both viruses and host cells co-evolve under the mutual selective pressure. Thus, evolution of viruses is determined by adaptation potential of viruses and their interaction with host cells.

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http://dx.doi.org/10.1016/j.micinf.2013.01.005