Saito A, Kawamoto Y, Higashino A, Yoshida T, Ikoma T, Suzaki Y, Ami Y, Shioda T, Nakayama EE, Akari H.	Allele Frequency of Antiretroviral Host Factor TRIMCyp in Wild-caught Cynomolgus Macaques (Macaca fascicularis).	Frontiers in Microbiology	3	314	2012
Bozek K. Nakayama EE, Kono K, Shioda T.	Electrostatic potential of human immunodeficiency virus type 2 and rhesus macaque simian immunodeficiency virus capsid proteins.	Frontiers in Microbiology	3	306	2012

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Nomura T, Yamamoto H, Shiino T, Takahashi N, Nakane T, Iwamoto N, Ishii H, Tsukamoto T, Kawada M, Matsuoka S, Takeda A, Terahara K, Tsunetsugu-Yokota Y, Iwata-Yoshikawa N, Hasegawa H, Sata T, Naruse TK, Kimura A, Matano T	Association of major histocompatibility complex class I haplotypes with disease progression after simian immunodeficiency virus challenge in Burmese rhesus macaques	Journal of Virology	12	6481	2012
Ishii H, Kawada M, Tsukamoto T, Yamamoto H, Matsuoka S, Shiino T, Takeda A, Inoue M, Iida A, Hara H, Shu T, Hasegawa M, Naruse TK, Kimura A, Takiguchi M, Matano T.	Impact of vaccination on cytotoxic T lymphocyte immunodominance and cooperation against simian immunodeficiency virus replication in rhesus macaques	Journal of Virology	2	738	2012

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Original article

Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells

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Abstract

HIV-1 is strictly adapted to humans, and cause disease-inducing persistent infection only in humans. We have generated a series of macaque-tropic HIV-1 (HIV-1mt) to establish non-human primate models for basic and clinical studies. HIV-1mt clones available to date grow poorly in macaque cells relative to SIVmac239. In this study, viral adaptive mutation in macaque cells, G114E in capsid (CA) helix 6 of HIV-1mt, that enhances viral replication was identified. Computer-assisted structural analysis predicted that another Q110D mutation in CA helix 6 would also increase viral growth potential. A new proviral construct MN4Rh-3 carrying CA-Q110D exhibited exquisitely enhanced growth property specifically in macaque cells. Susceptibility of MN4Rh-3 to macaque TRIM5α/TRIMCyp proteins was examined by their expression systems. HIV-1mt clones so far constructed already completely evaded TRIMCyp restriction, and further enhancement of TRIMCyp resistance by Q110D was not observed. In addition, Q110D did not contribute to evasion from TRIM5α restriction. However, the single-cycle infectivity of MN4Rh-3 in macaque cells was enhanced relative to the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells irrelevant to TRIM5 proteins restriction.

Keywords: HIV-1; HIV-1mt; Gag-CA; Macaque cells; Virus growth; Molecular modeling

1. Introduction

Mammalian cells express a variety of host restriction factors to defend themselves against pathogens. Viruses have evolved countermeasures to subvert their restriction and replicate efficiently in cells [1,2]. HIV-1, a causative agent of human AIDS, evades host restriction factors and replicates well in human cells. However, in macaques for experimental use, e.g. cynomolgus macaques (CyMs) and rhesus macaques (RhMs), HIV-1 replication is completely inhibited by host restriction factors present in their cells [3]. Construction of HIV-1 that overcomes species-barrier contributes much to understand the interaction of HIV-1 and its host as well as the establishment of HIV-1-infected macaque models [4,5].

Extensive molecular biological studies on the HIV-1/host interaction conducted to date have revealed main mechanical bases for the narrow host range exhibited by HIV-1. Macaque cells contain potent antiviral factors that effectively restrict or even abolish HIV-1 replication. These include APOBEC3 proteins (APOs), CyclophilinA (CypA), and TRIM5α/TRIMCyp

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(TRIM5 proteins). HIV-1 can indeed counteract human proteins corresponding to these restriction factors. APOs exhibit cytidine deaminase activity, and introduce lethal mutations into HIV-1 genome. HIV-1 Vif is able to neutralize the antiviral activity of human APOs, but not macaque APOs [6–8]. CypA acts on incoming HIV-1 core to regulate infection positively in human cells but negatively in macaque cells [9–11], though amino acid sequences of CypA from human and macaque are identical. Macaque TRIM5α recognizes and interacts with incoming HIV-1 core, and restricts virus infection in a less-defined mechanism [9–11]. Macaque TRIM5α is polymorphic, and has sequence variation in a C-terminal B30.2/SPRY domain important for capsid (CA) binding. Sequence variation in this domain causes modulation of host susceptibility to retrovirus infection [12,13]. Macaque TRIMCyp is a fusion protein resulted from replacement of a B30.2/SPRY domain with CypA. Both CyM and RhM cells express TRIMCyp, but affinity of these proteins to HIV-1 core is different due to amino acid substitutions in Cyp domains. Thus, CyM TRIMCyp restricts HIV-1 replication, but not RhM TRIMCyp [14,15].

Identification of host restriction factors in macaque cells and their target proteins in HIV-1 has prompted us to generate macaque-tropic HIV-1 (HIV-1mt) with a minimal modification of HIV-1 genome. We successfully constructed prototype HIV-1mt, NL-DT5R, by replacing CypA binding region on a loop between helices 4 and 5 (h4/5L) in *gag*-CA and entire *vif* genes with the corresponding regions of pathogenic SIVmac239 (Fig. 1) [16]. But growth potential of NL-DT5R was inferior to that of SIVmac239 both *in vitro* and *in vivo* [16,17]. These results indicated that genetic modifications in NL-DT5R were insufficient to confer the ability on the virus to grow efficiently in macaque cells [16–18]. In an attempt to improve growth potential of NL-DT5R, we adapted NL-DT5R and its R5-tropic version NL-DT562 to a CyM derived lymphocyte cell line HSC-F, and found a number of genetic substitutions in viral genomes of adapted viruses [19]. We introduced these mutations and CA h6/7L from SIVmac239 into NL-DT5R, and the resultant clone was designated MN4-5S (Fig. 1) [19]. MN4-5S exhibited enhanced growth potential in CyM both *in vitro* and *in vivo* compared to NL-DT5R [19]. But growth ability of MN4-5S was still lower than that of SIVmac239.

In this study, to further improve replication potential of HIV-1mt, we adapted MN4-5S in macaque cells and identified an adaptive mutation in CA that enhances growth ability in the cells. *In silico* structural modeling of the adaptive mutation predicted that Q110D mutation on helix 6 in CA (CA-Q110D) would promote viral replication in macaque cells. Indeed, a proviral clone carrying CA-Q110D, designated MN4Rh-3, exhibited marked enhancement of growth potential in macaque cells relative to all the other HIV-1mt clones we have constructed (Fig. 1). CyM TRIM5α/TRIMCyp susceptibility assays revealed that MN4Rh-3 completely evades from TRIMCyp restriction but not TRIM5α restriction as observed for the other HIV-1mt clones. While CA-Q110D contributed to neither endowment of further resistance to TRIMCyp nor evasion from TRIM5α restriction, CA-Q110D did lead to enhanced single-cycle infectivity to a macaque cell line compared with the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells independently of TRIM5 proteins restriction.

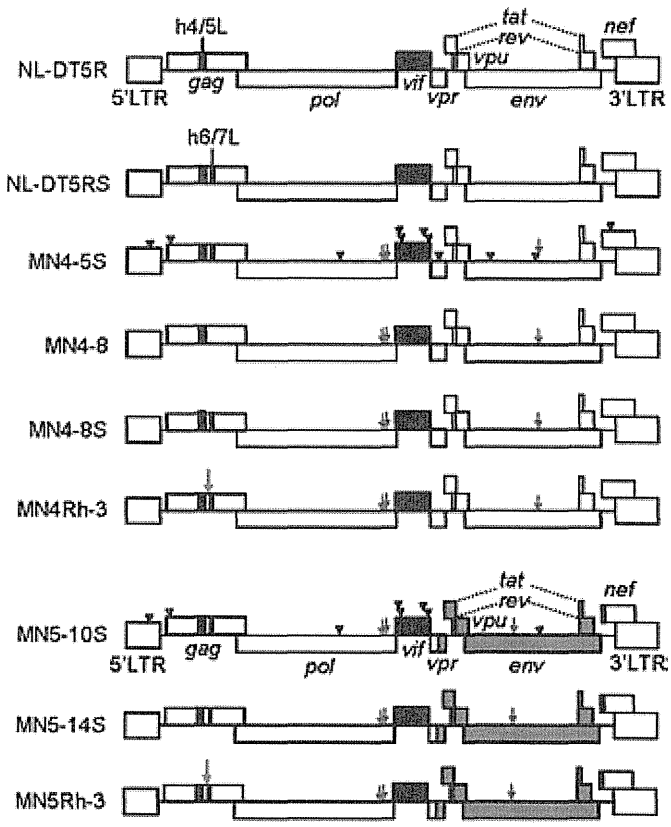


Fig. 1. Proviral genome structure of various HIV-1mt clones used in this study. HIV-1 NL4-3 [26] and SIVmac239 (GenBank: M33262) sequences are indicated by white and black areas, respectively. Gray areas in MN5-10S, MN5-14S and MN5Rh-3 show sequences from NF462 [21]. Blue arrows and black arrowheads show nucleotide substitutions that appeared in viral genomes of NL-DT5R and NL-DT562 during adaptation in HSC-F cells. Among nucleotide substitutions, adaptive mutations that enhance viral growth potential are indicated by blue arrows. Red arrows show the CA-Q110D mutation.

2. Materials and methods

2.1. Plasmid DNA

Construction of NL-DT5R, NL-DT562, NL-DT5RS, and MN4-5S were described previously [16,19–21]. MN4-5S carries all nucleotide substitutions that are present in adapted NL-DT5R and NL-DT562 clones except for mutations in the *env* gene of R5-tropic viruses (MN5-10S, MN5-14S, and MN5Rh-3 in Fig. 1) [19]. MN4-8S contains adaptive (growth-enhancing) mutations in MN4-5S but not the other mutations. MN4Rh-3 was constructed by introduction of the CA-Q110D mutation into MN4-8S. To construct R5-tropic viruses, 3′ halves of viral genomes (*Eco*RI in *vpr* to *Sph*I at the 3′ end of viral genome) of MN4-5S, MN4-8S, and MN4Rh-3 were replaced with the corresponding regions of adapted-NL-DT562,

and were designated MN5-10S, MN5-14S, and MN5Rh-3, respectively. For single-cycle infectivity assays to monitor viral susceptibility to TRIM5 proteins and to determine infectivity for CyM cells, *env*-deficient HIV-1mt variants encoding luciferase gene were constructed. NL-DT5R was cleaved with *Nde*I and *Nhe*I (both sites in *env* gene), blunt ended by T4 DNA polymerase, and resealed by T4 DNA ligase. The resultant clone was designated 5RΔEnv. Luciferase gene was then introduced into *nef* gene of 5RΔEnv as described previously [22], and the resultant clone was designated 5RΔEnv + Luc. A fragment containing the 3′ half genome was cut out from the 5RΔEnv + Luc, and introduced into the corresponding region in HIV-1mt variants (DT5R/4-3, NL-DT5RS, MN4-8, MN4-8S, and MN4Rh-3) to generate 5R/4-3ΔEnv + Luc, 5RSΔEnv + Luc, 4-8ΔEnv + Luc, 4-8SΔEnv + Luc, and 4Rh-3ΔEnv + Luc, respectively.

2.2. Cell culture

A human monolayer cell line 293T [23], a feline kidney cell line CRFK (ATCC CCL-94), and a CyM kidney cell line MK.P3(F) (JCRB 0607) were maintained in Eagles's minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (hiFBS). CRFK cells expressing TRIM5α/TRIMCyp were maintained in MEM containing 10% hiFBS and 400 μg/mL G418 (SIGMA). Macaque lymphocyte cell lines, HSC-F [24] and HSR5.4 [25], were maintained in RPMI-1640 medium containing 10% hiFBS. Recombinant human IL-2 (AbD Serotec) was added to the medium (50 units/mL) for maintenance of HSR5.4 cells. A human lymphocyte cell line MT4/CCR5 (MT4 cells stably expressing CCR5) was maintained in RPMI-1640 medium containing 10% hiFBS and 200 μg/mL hyglomycine (SIGMA).

2.3. Virus replication assays

Virus stocks for infection were prepared from 293T cells transfected with proviral clones as described previously [16,19,26]. Virion-associated reverse transcriptase (RT) activity was measured as described previously [16]. HSC-F cells (10^6) were infected with equal RT units of viruses in the presence of IL-2. For infection of MT4/CCR5 cells (10^6), the spinoculation method [27] was used. Viral growth was monitored by RT activity released into the culture supernatants. We assessed the viral growth potential by the peak day of virus production, and if the viral growth kinetics are similar, by the production level on the peak day.

2.4. Generation and characterization of adapted viral clones

MN4-5S and MN5-10S viruses (Fig. 1) prepared from transfected 293T cells were inoculated into HSR5.4 cells (3×10^6) with an equal amount of viruses (5×10^7 RT units). The cultures were maintained in the presence of IL-2, and HSC-F cells were added on day 34 post-infection. The culture supernatants (collected on day 18 post-cocultivation, the peak day of virus production) were inoculated into fresh HSR5.4 cells, and total DNA was extracted from the cells on day 15 post-infection. Integrated proviruses were amplified from total DNA as two overlapping fragments by the polymerase chain reaction (PCR), and amplified products were cloned into MN5-10S as described previously [16]. Viruses were prepared from 293T cells transfected with the resultant clones, and inoculated into HSR5.4 cells. Only one clone exhibited a rapid growth kinetics compared to MN5-10S, and was designated Ad clone-25. To identify an adaptive mutation that enhances growth potential, each mutation found in the genome of Ad clone-25 was introduced into MN5-14S by site-directed mutagenesis (STRATAGENE). For screening, viruses prepared from transfected 293T cells were inoculated into HSC-F cells, and virus replication was monitored by RT activity released into the culture supernatants.

2.5. Molecular modeling of HIV CA N-terminal domain (NTD)

The crystal structure of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C [28]) was taken from the RCSB Protein Data Bank [29]. The three-dimensional (3-D) models of HIV-1 CA NTD were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Quebec, Canada) as described [30–32]. We obtained 25 intermediate models per one homology modeling in MOE, and selected the 3-D models which were the intermediate models with best scores according to the generalized Born/volume integral methodology [33]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [34] combined with the generalized Born model of aqueous solvation implemented in MOE [35]. Physically unacceptable local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE.

2.6. Single-cycle infectivity assays

To generate CRFK cells expressing CyM TRIMCyp, the cDNA was isolated from HSC-F cells, and expression vector of FLAG-tagged CyM TRIMCyp was constructed as described previously [18]. The sequence of TRIMCyp from HSC-F cells was identical with Mafa TRIMCyp2 (GenBank: FJ609415). CRFK cell lines expressing CyM TRIMCyp were selected by G418 as described previously [18]. Expression and inhibitory effect of the selected cell clones were verified by Western blotting with anti-FLAG antibody (SIGMA) and by infection with vesicular stomatitis virus G protein (VSV-G) pseudotyped 5R/4-3ΔEnv + Luc, respectively. Assays using naïve CRFK, CRFK expressing CyM TRIM5α [18] or CyM TRIMCyp, and MK.P3(F) cells were similarly performed as described previously [36]. VSV-G pseudotyped virus stocks were prepared from 293T cells transfected with individual HIV-1mtΔEnv + Luc clones and pCMV-G (GenBank: AJ318514)

at a molar ratio of 1:1. Naïve CRFK, CRFK expressing TRIM5α/TRIMCyp and MK.P3(F) cells were infected with an equal titer of viruses (to generate approximately 10^7 relative luminescence (RLU) for naïve CRFK cells), and on day 2 post-infection, cells were analyzed for luciferase activity. Assays using recombinant Sendai virus (SeV)-CyM TRIM5α/TRIMCyp expression system were performed as described previously [31].

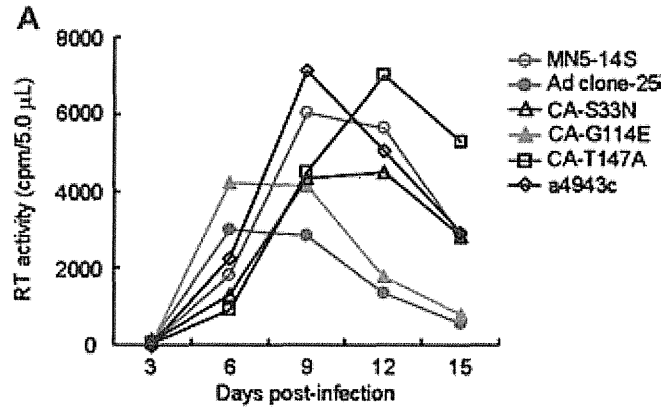
3. Results

3.1. An adaptive mutation G114E on helix 6 in CA (CA-G114E) enhances viral growth potential in macaque cells

An HIV-1mt variant MN4-5S replicated more slowly than SIVmac239 in macaque cells. In order to improve its growth potential, we carried out virus adaptation in a macaque lymphocyte cell line HSR5.4. Virus adaptation was performed by long-term culture of HSR5.4 cells infected with MN4-5S (X4-tropic) or its R5-tropic version MN5-10S (Fig. 1). Construction of proviral clones from adapted viruses was described in Materials and methods. We obtained only one clone (Ad clone-25) with enhanced growth potential from 100 proviral clones constructed and tested. We sequenced the entire genome of Ad clone-25, and found three non-synonymous mutations in CA (S33N, G114E, and T147A in Fig. 2A) and one synonymous mutation in integrase (IN)(a4943c in Fig. 2A). To identify an adaptive mutation that enhances growth potential, each mutation found in Ad clone-25 was introduced into a parental clone MN5-14S (Fig. 1). MN5-14S carries only growth-promoting mutations in MN5-10S, and the two clones exhibit similar growth potential in macaque cells. Viruses were prepared from 293T cells transfected with MN5-14S, Ad clone-25, or clones carrying individual mutations, and inoculated into HSC-F cells (Fig. 2A). Only one clone carrying CA-G114E exhibited similar growth kinetics to that of Ad clone-25 but not the others. This result indicates that CA-G114E is an adaptive mutation enhancing growth potential of HIV-1mt in macaque cells. This mutation is exactly the same as the previously found adaptive mutation, which enhanced growth of NL-4/5S6/7SvifS virus in human CEM-SS cells [37]. NL-4/5S6/7SvifS virus is a prototype HIV-1mt bearing the same CA with that of MN4-5S.

3.2. Molecular modeling of the CA NTD of HIV-1mt variants suggests that CA-G114E and CA-Q110D mutations have a similar positive effect on viral replication

The amino acid at position 114 is located in CA NTD. To obtain structural insights into impacts of the G114E substitution in order to improve growth capability of HIV-1mt variants in macaque cells, we conducted computer-assisted structural study: we constructed 3-D models of CA NTD of three HIV-1mt variants, CA-G114E, CA-G114Q, and MN4-5S, using homology-modeling technique (see Materials and methods). Main chain folds of the three models were indistinguishable, suggesting that 3-D position and type of side chain are critical



Nucleotide change	Region	Amino acid change in the region
g1283a	CA	S33N
g1526a	CA	G114E
a1624g	CA	T147A
a4943c	IN	None

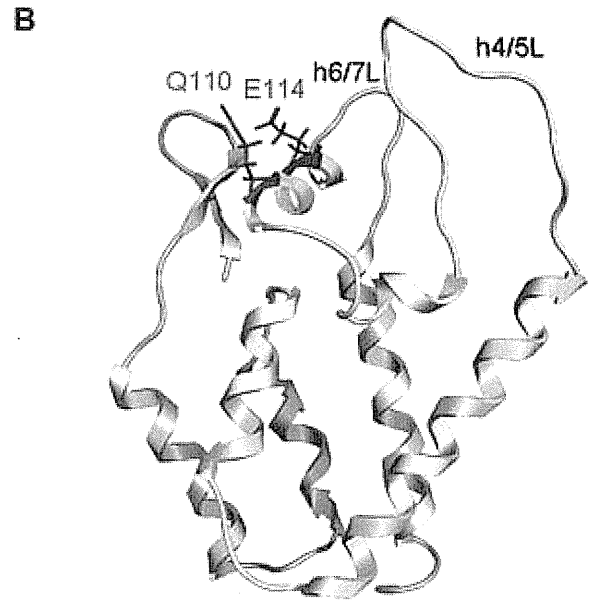


Fig. 2. Mutations in Gag-CA. (A) Identification of an adaptive mutation that enhances viral growth. Nucleotide substitutions found in the genome of Ad clone-25 are indicated at the bottom. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal RT units were inoculated into HSC-F cells. MN5-14S and Ad clone-25 served as controls. Virus replication was monitored by RT activity released into the culture supernatants. (B) 3-D structural models for CA NTD of HIV-1mt variants. Structural models of CA NTD of HIV-1mt variants were constructed by homology-modeling using "MOE-Align" and "MOE-Homology" in MOE as described previously [30–32]. Crystal structure of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C [28]) was used as template for homology modeling. Main chain folds were indistinguishable among the models, and only the model of G114E CA is shown as a representative. Magenta and red sticks: side chains of 110th and 114th amino acid residues, respectively, of the G114E CA NTD.

for the phenotypic change. The modeling study revealed that 114th residue of G114E CA NTD is located on helix 6 in CA NTD such that its side chain protrudes into the exposed surface of CA (Fig. 2B). A charged amino acid residue on a protein surface participates in determining physicochemical properties of interaction surface of the protein and thus influences its structural and functional properties. Therefore, we assumed that the protrusion of a negatively charged side chain from helix 6 into exposed surface could have somehow a positive effect on growth capability of the HIV-1mt variants in macaque cells. In this regard, especially worth noting is that 110th amino acid residue on helix 6 of the HIV-1mt variant CAs was positioned on the same helical face with 114th amino acid residue (Fig. 2B). Therefore, we predicted that substitution of glutamine (Q) at position 110 by acidic amino acid such as aspartic acid (D) and glutamic acid (E) may also have a positive effect on growth capability of the HIV-1mt variants in macaque cells as G114E does. SIVmac239 has aspartic acid and glutamine at the positions 110 and 114, respectively.

3.3. CA-Q110D promotes viral growth more efficiently in macaque cells than CA-G114E mutation but its enhancing effect is species-specific

To confirm our prediction described above, CA-Q110D mutation was introduced into MN5-14S (designated MN5Rh-3), and the growth property in HSC-F cells of MN5Rh-3 and a viral clone carrying G114E (CA-G114E in Fig. 2A) was compared. As shown in Fig. 3A, MN5Rh-3 grew better than CA-G114E, indicating that CA-Q110D further accelerates HIV-1mt replication in macaque cells compared with an adaptive CA-G114E mutation. We next constructed an X4-tropic proviral clone carrying the CA-Q110D (designated MN4Rh-3) (Fig. 1), and compared its growth property with MN5Rh-3 in HSC-F cells (Fig. 3B). MN4Rh-3 was found to exhibit higher growth ability than MN5Rh-3, and was therefore used for infection experiments hereafter.

While CypA and TRIM5α have inhibitory effect on HIV-1 replication in macaque cells, CypA promotes HIV-1 infection in human cells and human TRIM5α only weakly inhibits HIV-1 replication [38–40]. Since the CA-Q110D mutation (acquisition of negatively charged side chain), as predicted by structural modeling, could impact on the interaction of HIV-1 CA and its binding factor(s) by altering physicochemical properties of CA binding surface, it can be speculated that CA-Q110D may promote viral replication specifically in macaque cells. Thus, we analyzed the effect of CA-Q110D on viral growth in macaque and human cells. In this experiment, we used HIV-1mt variants (MN4-8, MN4-8S, and MN4Rh-3) that have distinct CA structures (Fig. 1). Viruses prepared from transfected 293T cells were inoculated into macaque HSC-F and human MT4/CCR5 cells, and examined for growth property (Fig. 3C). The introduction of SIVmac239 CA h6/7L (MN4-8S) resulted in enhanced and reduced viral growth in macaque and human cells, respectively, relative to MN4-8. MN4Rh-3 grew clearly better in macaque cells relative to MN4-8 and MN4-8S, but more poorly in human cells than the other twos. These results demonstrate that the CA-Q110D mutation enhances viral replication in a host cell species-specific manner.

3.4. CA-Q110D does not contribute to evasion from CyM TRIM5 proteins restriction

We predicted that the growth enhancement by CA-Q110D may come from the increased resistance to CyM TRIM5 proteins, and therefore examined the susceptibility of HIV-1mt variants to them by two independent assays.

First, assays were performed in feline kidney CRFK cells expressing TRIM5α or TRIMCyp by using VSV-G pseudotyped viruses encoding the luciferase gene (Fig. 4A–C). The sequence differences between HIV-1mt variants reside only in CA and IN (Figs. 1 and 4). Since adaptive mutations in IN contribute to enhancement of virion production but not early replication phase (manuscript in preparation), only the difference in CA affects the relative single cycle infectivity in this assay. A pseudotyped virus 5R/4-3 carries HIV-1 (NL4-3) CA without any modifications and served as negative control. While 5R and 4-8 have an identical CA structure carrying h4/5L from SIVmac239, 5RS and 4-8S have both h4/5L and h6/7L from SIVmac239 CA. 4Rh-3 carries CA-Q110D mutation in addition to h4/5L and h6/7L from SIVmac239 CA. Viral infectivity was measured by luciferase activity in infected cells and presented as RLU. Naïve CRFK and CRFK cells expressing TRIM5α were infected with an equal amount of viruses generating 10^7 RLU in naïve cells. As shown in Fig. 4B, the infectivity of 5R and 4-8 for cells expressing CyM TRIM5α was similar to that of a negative control 5R/4-3. However, higher infectivity was observed for 5RS and 4-8S relative to 5R and 4-8. These results were consistent with previous reports that h4/5L and h6/7L in HIV-1 CA are a part of determinant for TRIM5α restriction [20,36]. The sensitivity of 4Rh-3 to TRIM5α was similar to that of 5RS and 4-8S. This indicates that CA-Q110D did not contribute to increase the resistance to TRIM5α. It has been reported that CyM TRIMCyp has the ability to restrict HIV-1 replication [15]. To examine the susceptibility of HIV-1mt variants to TRIMCyp, we generated feline CRFK cells expressing TRIMCyp, and the cells were infected with pseudotyped viruses as described above. As shown in Fig. 4C, all the clones tested were more resistant to a similar extent to TRIMCyp than the control 5R/4-3. In agreement with a previous study showing that elimination of alanine at position 88 within h4/5L of HIV-1 CA confers the resistance on the virus to TRIMCyp [15], our results indicate that the replacement of HIV-1 CA h4/5L with that of SIVmac239 is sufficient for HIV-1mt to evade from the TRIMCyp restriction. Second, we performed another susceptibility assay using the recombinant SeV expression system. This system assures a very high expression level of target proteins in cells infected with the recombinant SeV. Therefore, the ability of viruses to completely counteract the restriction effect of TRIM5 proteins could be determined by MT4/SeV-TRIM5 expression system. Human MT4 cells were infected with recombinant SeV expressing CyM TRIM5α, TRIMCyp, or SPRY(−)TRIM5, and then super-infected with HIV-1

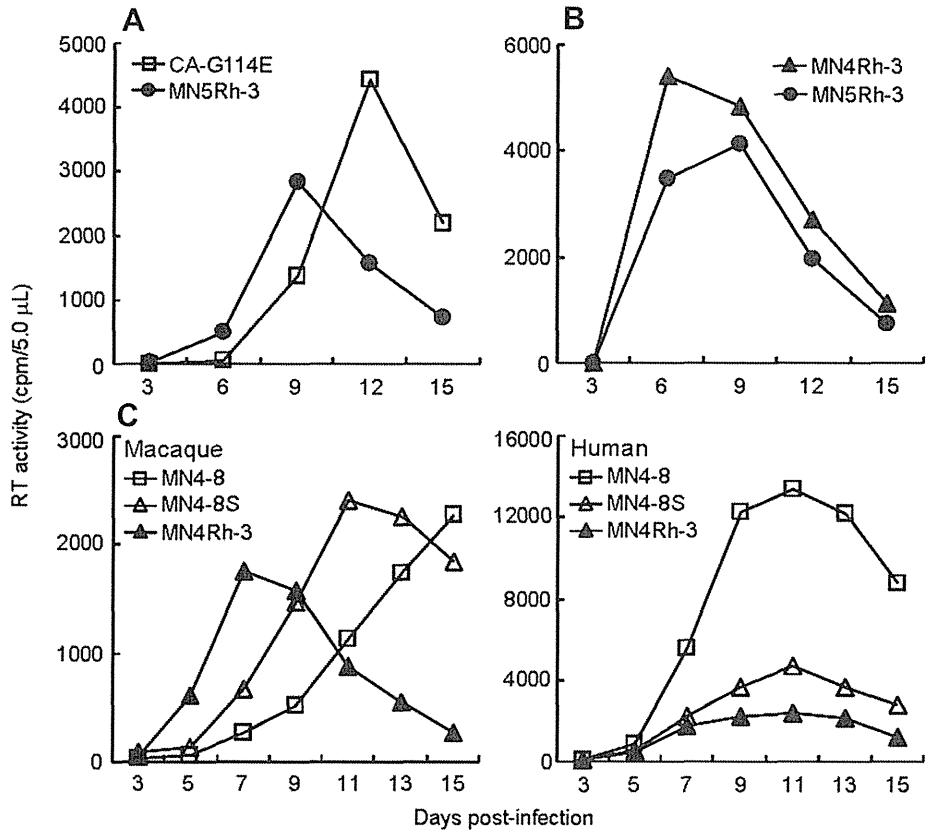


Fig. 3. Effect of CA modification on viral growth in macaque and human lymphocyte cell lines. (A and B) Growth kinetics of HIV-1mt clones carrying CA-G114E or CA-Q110D (MN5Rh-3 and MN4Rh-3) in CyM HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (5×10^5 RT units) were inoculated into HSC-F cells (10^6). Virus replication was monitored by RT activity released into the culture supernatants. (C) Growth kinetics of MN4-8, MN4-8S, and MN4Rh-3 in HSC-F (Macaque) and MT4/CCR5 (Human) cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (10^6 RT units) were inoculated into HSC-F cells (10^6). For spinoculation of MT4/CCR5 cells (10^6), 6×10^5 RT units were used as inocula. Virus replication was monitored by RT activity released into the culture supernatants.

(NL4-3), SIVmac239, or HIV-1mt variants. SPRY(−)TRIM5 which can not bind to viral CA served as control. NL4-3 and SIVmac239 also served as controls for viral replication. As shown in Fig. 4D, NL4-3 replicated in cells expressing SPRY(−)TRIM5, but not in TRIM5α and TRIMCyp expressing cells. SIVmac239 exhibited similar growth kinetics in SPRY(−)TRIM5, TRIM5α and TRIMCyp expressing cells. All HIV-1mt variants replicated in TRIMCyp expressing cells similarly well in SPRY(−)TRIM5 cells. Together with assays in CRFK cells, these results showed that all HIV-1mt variants except for 5R/4-3 completely evade from TRIMCyp restriction. In contrast, the growth of all HIV-1mt variants was inhibited in CyM TRIM5α expressing MT4 cells. These results indicate that HIV-1mt variants do not evade from TRIM5α restriction as effectively as SIVmac239.

Results obtained by our two assay systems with respect to the susceptibility of HIV-1mt variants to CyM TRIM5α were apparently different (Fig. 4B and D), but this difference is most likely to be due to the TRIM5α expression level. In MT4 cells infected with recombinant SeV, TRIM5α is expressed at much higher level than that in transduced CRFK cells, masking the increase of resistance to TRIM5α detectable by the transduced CRFK system (Fig. 4B). Indeed, the growth enhancement of 5RS relative to 5R [20] can be explained by the results in Fig. 4B but not those in Fig. 4D. The apparent discrepancy of the sensitivity depending on TRIM5α expression level was also observed between B-LCL cells and transduced CRFK cells [41]. In sum, we can conclude here that MN4Rh-3 exhibits a partial resistance to TRIM5α insufficient for complete evasion as 5RS and 4-8S do, and that the CA-Q110D mutation is irrelevant to this property.

3.5. CA-Q110D enhances viral infectivity for macaque cells

Results so far showed that CA-Q110D does not contribute to evasion from TRIM5 proteins restriction in rather artificial systems using feline and human cells (Fig. 4). To investigate further how CA-Q110D enhances viral replication, we examined single-cycle viral infectivity in macaque cells. CyM kidney MK.P3(F) cells, which have heterozygote for TRIM5α and TRIMCyp, were infected with various VSV-G pseudoviruses and analyzed for their infectivity as described above. As shown in Fig. 5A, viral infectivity was increased by modification of h4/5L (compare 5R/4-3 and 5R&4-8). Modification of h6/7L in addition to h4/5L further augmented viral infectivity (compare 5R&4-8 and 5RS&4-8S). Introduction of the CA-Q110D mutation into 4-8S clone gave the highest infectivity among the viruses tested (see 4Rh-3). The results in

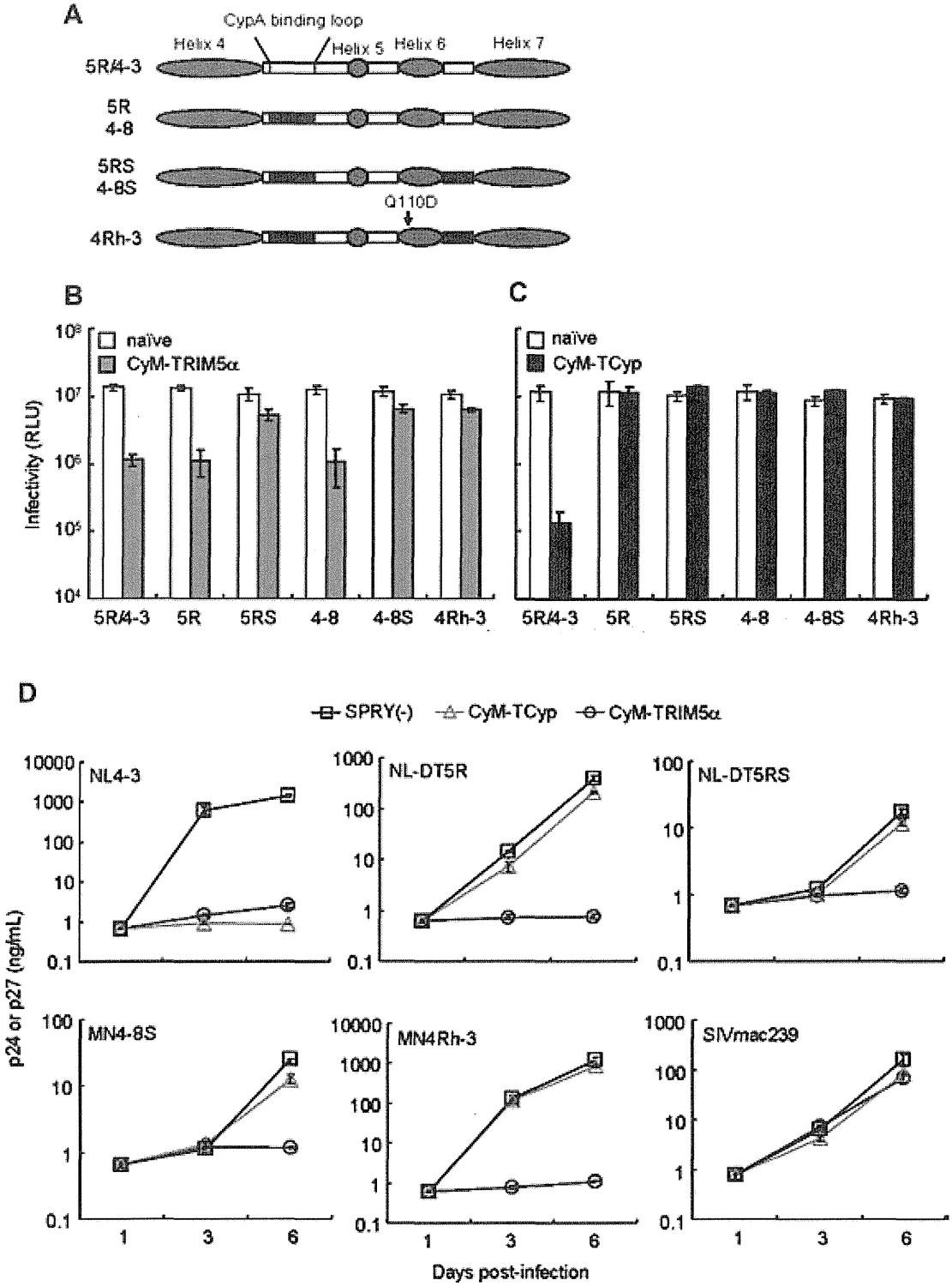


Fig. 4. Effect of CA modification in HIV-1mt variants on viral infectivity. (A) CA structure of viral clones used in TRIM5α/TRIMCyp susceptibility assays. Blue and white areas show helices and loops from HIV-1 NL4-3 CA, respectively. Sequences from SIVmac239 are indicated by black areas. (B and C) Susceptibility of HIV-1mt variants to CyM TRIM5 proteins as examined by CRFK system. Results for CyM TRIM5α (B) and for CyM TRIMCyp (TCyp) (C) are shown. VSV-G pseudotyped viruses were prepared from transfected 293T cells as input samples. Viruses generating 10^7 RLU in CRFK-naïve cells were inoculated into CRFK cells that express CyM TRIM5α or CyM TCyp. On day 2 post-infection, cells were analyzed for luciferase activity by a luminometer. (D) Susceptibility of HIV-1mt variants to CyM TRIM5 proteins as examined by SeV system. Human MT4 cells (10^5) were infected with recombinant SeV expressing CyM TRIM5α, TRIMCyp, or SPRY (−) TRIM5. Nine hours after infection, cells were super-infected with 20 ng (Gag-p24) of HIV-1 NL4-3, various HIV-1mt clones, or 20 ng (Gag-p27) of SIVmac239. Virus replication was monitored by the amount of Gag-p24 from NL4-3 and HIV-1mt clones or Gag-p27 from SIVmac239 in the culture supernatants. Error bars show actual fluctuations between duplicate samples. Data from one representative of three independent experiments are shown.

Fig. 5A show that CA-Q110D uniquely increases viral infectivity in macaque cells not observed in the other experimental systems (Fig. 4), and suggest that some factor(s) in CyM cells other than TRIM5α and TRIMCyp proteins is associated with this enhancement.

As shown in Fig. 5B, MN4Rh-3 displayed slower growth kinetics relative to those of SIVmac239 (note the peak day of virus production), although it grew better than the other HIV-1mt clones in CyM HSC-F cells. Approximately 100-fold more input virus (RT units) compared to SIVmac239 was required for MN4Rh-3 to exhibit similar growth kinetics with SIVmac239 (data not shown). These results have shown that even MN4Rh-3 grows more poorly in macaque cells than a standard SIVmac clone pathogenic for macaque monkeys.

4. Discussion

In this study, we have demonstrated that a single CA mutation (Q110D) greatly promotes HIV-1mt growth in macaque cells (Fig. 3). This enhancing effect was afforded independently of TRIM5 proteins restriction. The virus carrying the CA-Q110D mutation (MN4Rh-3) certainly overcame the anti-viral action of CyM TRIMCyp but not completely CyM TRIM5α. However, the mutation itself (Fig. 1) did not influence anti-TRIMCyp/TRIM5α activity of MN4Rh-3 reported here (Fig. 4). Notably, this mutation exquisitely enhanced viral growth in macaque cells (Fig. 3) by augmenting viral single-cycle infectivity (Fig. 5). The viral growth enhancement reported here is well reproduced in CyM peripheral blood mononuclear cells and in CyMs (manuscript in preparation).

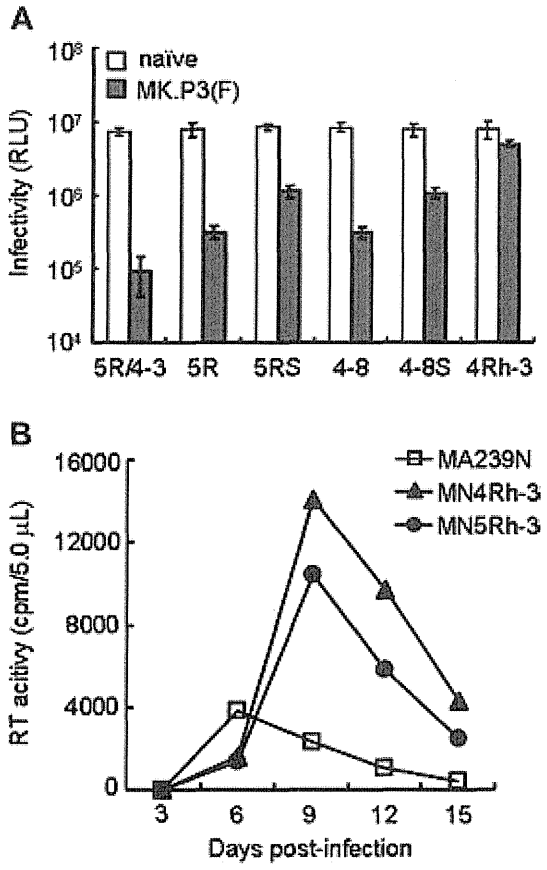
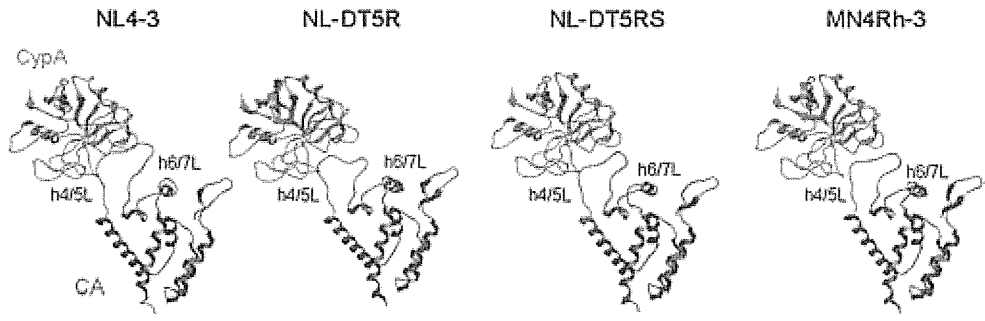


Fig. 5. Replication ability of various viruses in CyM cells. (A) Single-cycle infectivity of various HIV-1mt clones in CyM kidney MK.P3(F) cells. VSV-G pseudotyped viruses indicated were prepared from transfected 293T cells. MK.P3(F) cells were infected with an equal titer of viruses giving 10^7 RLU in CRFK-naïve cells. On day 2 post-infection, cells were analyzed for luciferase activity by a luminometer. (B) Multi-cycle growth kinetics of SIVmac and HIV-1mt viruses in CyM lymphocyte HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (10^4 RT units) were inoculated into HSC-F cells (10^6). Virus replication was monitored by RT activity released into the culture supernatants. MA239N, an infectious clone of SIVmac239 with *nef*-open.

Regarding the mechanism for enhancement of viral growth by CA-Q110D, we initially thought a possibility that CA-Q110D compensates the disadvantage in HIV-1mt genome resulted from replacement of HIV-1 CA h4/5L and h6/7L with those of SIVmac239. However, this is highly unlikely because the enhancing effect is macaque cell-dependent (Fig. 3). Most feasible explanation is that CA-Q110D contributes to evade from a negative factor(s) in macaque cells such as CypA. Because HIV-1mt CA was designed not to bind to CypA, and the interaction between the two molecules was indeed undetectable by monitoring CypA virion-incorporation [18,20], we analyzed the binding by computer-assisted structural modeling. Homology modeling of the CA-CypA complexes was performed based on the crystal structure of HIV-1 CA NTD bound to CypA (PDB code: 1M9C [28]), and the binding energies, E_{bind}, were calculated using MOE as described previously [42,43]. As shown in Fig. 6, HIV-1 (NL4-3) CA was predicted to interact with CypA via its h4/5L (binding energy: −64.4 kcal/mol). The binding energy of CA and CypA was decreased by CA modifications, such as h4/5L replacement (NL-DT5R: −31.0 kcal/mol), h4/5L and h6/7L replacement (NL-DT5RS: −36.1 kcal/mol), and Q110D substitution in addition to h4/5L and h6/7L replacement (MN4Rh-3: −30.1 kcal/mol). Decrease in E_{bind} in NL-DT5R is consistent with the result that the h4/5L region directly interacts with CypA [28]. Notably, the E_{bind} for the NL-DT5RS CA was greater than that of the NL-DT5R and MN4Rh-3 CAs. These results suggest that not only h6/7L replacement but also Q110D substitution can influence structure of CypA binding surface of CA. The Q110D substitution is located on the exposed surface of helix 6 connecting to the h6/7L (Fig. 2B). CA helix 6 has been reported to interact with CypA binding region on h4/5L through hydrogen bonding [44,45]. Thereby it is reasonable that the local electrostatic change on the helix 6 by the Q110D substitution influenced structures of h4/5L via changes in fluctuation and conformation of h6/7L. This in turn could lead to reduction in stability of the MN4Rh-3 CA-CypA complex compared with NL-DT5RS CA-CypA complex, as predicted in Fig. 6. Our computer-assisted structural study suggests that the Q110D substitution can induce electrostatic modulation of the overall CA surface structure including h4/5L and h6/7L. Similar modulation mechanism of binding surface structures via charged amino acid substitution at distant site from the binding surface has been reported for Cyp domain of CyM TRIMCyp [15] and CD4 binding site of HIV-1 gp120 outer



	NL4-3	NL-DT5R	NL-DT5RS	MN4Rh-3
CA	HIV-1 CA	h4/5L from SIVmac239	h4/5L and h6/7L from SIVmac239	h4/5L and h6/7L from SIVmac239 and Q110D
Binding energy (kcal/mol)	-64.447	-31.012	-36.071	-30.051

Fig. 6. Structural models of HIV CA NTD bound to CypA. The model of CA NTD bound to CypA was constructed by homology modeling using the crystal structure of HIV-1 CA NTD and CypA complex (PDB code: 1M9C [28]). The binding energies, E_{bind} (kcal/mol), of the complex were calculated using MOE as described previously [42,43]. The formula $E_{bind} = E_{complex} - (E_{CA} + E_{CypA})$ was used for the E_{bind} calculation, where $E_{complex}$ is the energy of the CA/CypA complex models, E_{CA} is the energy of the CA monomer model, and E_{CypA} is the energy of the CypA monomer model.

domain [46]. Thus, it is not unreasonable to assume that the replication of MN4Rh-3 carrying CA-Q110D is enhanced in macaque cells but reduced in human cells by augmenting its dissociation from CypA (Fig. 6). However, it was found to be difficult to experimentally confirm this structural insight by determining the effect of cyclosporine A or of siRNA against CypA on viral infectivity because interaction between the HIV-1mt CA and CypA was so weak. Alternatively, CA-Q110D may contribute to the alteration of the affinity to unknown anti-CA factor(s) other than CypA and TRIM5 proteins. In this case, it is speculated that the factor(s) might act negatively on HIV-1 replication in macaque cells but positively in human cells, and vice versa. Further study is required to elucidate the mechanism for enhancement of viral growth potential by CA-Q110D.

In conclusion, further modification of the HIV-1mt genome is necessary to overcome unconquered replication block(s) present in macaque cells and obtain viral clones similarly replication-competent in macaque cells and pathogenic for animals with SIVmac (Fig. 5). Considering the genome structure of MN4Rh-3 and the results presented here, major targets for modification now are *gag*-CA (against TRIM5α) and *vpu* (against tetherin). Gag-CA is one of the two principal viral determinants (CA and Vif) for the HIV-1 species-tropism. Construction of HIV-1 CA that evades from TRIM5α restriction is also useful for elucidation of the less-defined CA-TRIM5α interaction and antiviral mechanism of TRIM5α. Tetherin, identified as anti-virion release factor, is antagonized by Vpu [47,48], but macaque tetherin was not counteracted by HIV-1 Vpu [49]. Construction of HIV-1 Vpu that down-modulate macaque tetherin may enhance viral replication *in vivo* as well as *in vitro* [50]. Through these approaches, we may be able to precisely analyze HIV-1 replication and pathogenesis *in vivo* and provide new strategies against HIV-1/AIDS.

Acknowledgments

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References

[1] M.H. Malim, M. Emerman, HIV-1 accessory proteins–ensuring viral survival in a hostile environment, Cell Host Microbe 3 (2008) 388–398.
[2] F. Kirchhoff, Immune evasion and counteraction of restriction factors by HIV-1 and other primate lentiviruses, Cell Host Microbe 8 (2010) 55–67.
[3] R. Shibata, H. Sakai, M. Kawamura, K. Tokunaga, A. Adachi, Early replication block of human immunodeficiency virus type 1 in monkey cells, J. Gen. Virol. 76 (1995) 2723–2730.
[4] M. Nomaguchi, N. Doi, K. Kamada, A. Adachi, Species barrier of HIV-1 and its jumping by virus engineering, Rev. Med. Virol. 18 (2008) 261–275.
[5] M. Nomaguchi, A. Adachi, Virology as biosystematics: towards understanding the viral infection biology, Front. Microbiol. 1 (2010) 2.
[6] R.K. Holmes, M.H. Malim, K.N. Bishop, APOBEC-mediated viral restriction: not simply editing? Trends Biochem. Sci. 32 (2007) 118–128.
[7] H. Huthoff, G.J. Towers, Restriction of retroviral replication by APOBEC3G/F and TRIM5alpha, Trends Microbiol. 16 (2008) 612–619.
[8] K. Strebel, J. Luban, K.T. Jeang, Human cellular restriction factors that target HIV-1 replication, BMC Med. 7 (2009) 48.
[9] J. Luban, Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection, J. Virol. 81 (2007) 1054–1061.
[10] G.J. Towers, The control of viral infection by tripartite motif proteins and cyclophilin A, Retrovirology 4 (2007) 40.
[11] E.E. Nakayama, T. Shioda, Anti-retroviral activity of TRIM5 alpha, Rev. Med. Virol. 20 (2010) 77–92.
[12] R.M. Newman, L. Hall, M. Connole, G.L. Chen, S. Sato, E. Yuste, W. Diehl, E. Hunter, A. Kaur, G.M. Miller, W.E. Johnson, Balancing selection and the evolution of functional polymorphism in old world monkey TRIM5alpha, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 19134–19139.
[13] S.J. Wilson, B.L. Webb, L.M. Ylinen, E. Verschoor, J.L. Heeney, G.J. Towers, Independent evolution of an antiviral TRIMCyp in rhesus macaques, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 3557–3562.

[14] A.J. Price, F. Marzetta, M. Lammers, L.M. Ylinen, T. Schaller, S.J. Wilson, G.J. Towers, L.C. James, Active site remodeling switches HIV specificity of antiretroviral TRIMCyp, Nat. Struct. Mol. Biol. 16 (2009) 1036–1042.

[15] L.M. Ylinen, A.J. Price, J. Rasaiyaah, S. Hué, N.J. Rose, F. Marzetta, L.C. James, G.J. Towers, Conformational adaptation of Asian macaque TRIMCyp directs lineage specific antiviral activity, PLoS Pathog. 6 (2010) e1001062.

[16] K. Kamada, T. Igarashi, M.A. Martin, B. Khamsri, K. Hatcho, T. Yamashita, M. Fujita, T. Uchiyama, A. Adachi, Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16959–16964.

[17] T. Igarashi, R. Iyengar, R.A. Byrum, A. Buckler-White, R.L. Dewar, C.E. Buckler, H.C. Lane, K. Kamada, A. Adachi, M.A. Martin, Human immunodeficiency virus type 1 derivative with 7% simian immunodeficiency virus genetic content is able to establish infections in pig-tailed macaques, J. Virol. 81 (2007) 11549–11552.

[18] K. Kamada, T. Yamashita, K. Hatcho, A. Adachi, M. Nomaguchi, Evasion from CypA- and APOBEC-mediated restrictions is insufficient for HIV-1 to efficiently grow in simian cells, Microbes Infect. 11 (2009) 164–171.

[19] A. Saito, M. Nomaguchi, S. Iijima, A. Kuroishi, T. Yoshida, Y.J. Lee, T. Hayakawa, K. Kono, E.E. Nakayama, T. Shioda, Y. Yasutomi, A. Adachi, T. Matano, H. Akari, Improved capacity of a monkey-tropic HIV-1 derivative to replicate in cynomolgus monkeys with minimal modifications, Microbes Infect. 13 (2011) 58–64.

[20] A. Kuroishi, A. Saito, Y. Shingai, T. Shioda, M. Nomaguchi, A. Adachi, H. Akari, E.E. Nakayama, Modification of a loop sequence between alpha-helices 6 and 7 of virus capsid (CA) protein in a human immunodeficiency virus type 1 (HIV-1) derivative that has simian immunodeficiency virus (SIVmac239) vif and CA alpha-helices 4 and 5 loop improves replication in cynomolgus monkey cells, Retrovirology 6 (2009) 70.

[21] T. Yamashita, N. Doi, A. Adachi, M. Nomaguchi, Growth ability in simian cells of monkey cell-tropic HIV-1 is greatly affected by downstream region of the vif gene, J. Med. Invest. 55 (2008) 236–240.

[22] M. Yamashita, M. Emerman, Capsid is a dominant determinant of retrovirus infectivity in nondividing cells, J. Virol. 78 (2004) 5670–5678.

[23] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 317 (1985) 169–171.

[24] H. Akari, T. Fukumori, S. Iida, A. Adachi, Induction of apoptosis in herpesvirus saimiri-immortalized T lymphocytes by blocking interaction of CD28 with CD80/CD86, Biochem. Biophys. Res. Commun. 263 (1999) 352–356.

[25] N. Doi, S. Fujiwara, A. Adachi, M. Nomaguchi, Growth ability in various macaque cell lines of HIV-1 with simian cell-tropism, J. Med. Invest. 57 (2010) 284–292.

[26] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.

[27] U. O'Doherty, W.J. Swiggard, M.H. Malim, Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding, J. Virol. 74 (2004) 10074–10080.

[28] B.R. Howard, F.F. Vajdos, S. Li, W.I. Sundquist, C.P. Hill, Structural insights into the catalytic mechanism of cyclophilin A, Nat. Struct. Biol. 10 (2003) 475–481.

[29] N. Deshpande, K.J. Addess, W.F. Bluhm, J.C. Merino-Ott, W. Townsend-Merino, Q. Zhang, C. Knezevich, L. Xie, L. Chen, Z. Feng, R.K. Green, J.L. Flippen-Anderson, J. Westbrook, H.M. Berman, P.E. Bourne, The RCSB protein data bank: a redesigned query system and relational database based on the mmCIF schema, Nucleic Acids Res. 33 (Database issue) (2005) D233–D237.

[30] H. Song, E.E. Nakayama, M. Yokoyama, H. Sato, J.A. Levy, T. Shioda, A single amino acid of the human immunodeficiency virus type 2 capsid affects its replication in the presence of cynomolgus monkey and human TRIM5alphas, J. Virol. 81 (2007) 7280–7285.

[31] K. Kono, H. Song, M. Yokoyama, H. Sato, T. Shioda, E.E. Nakayama, Multiple sites in the N-terminal half of simian immunodeficiency virus capsid protein contribute to evasion from rhesus monkey TRIM5α-mediated restriction, Retrovirology 7 (2010) 72.

[32] N. Inagaki, H. Takeuchi, M. Yokoyama, H. Sato, A. Ryo, H. Yamamoto, M. Kawada, T. Matano, A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins, Retrovirology 7 (2010) 90.

[33] P. Labute, The generalized Born/volume integral implicit solvent model: estimation of the free energy of hydration using London dispersion instead of atomic surface area, J. Comput. Chem. 29 (2008) 1693–1698.

[34] J.W. Ponder, D.A. Case, Force fields for protein simulations, Adv. Protein Chem. 66 (2003) 27–85.

[35] A. Onufriev, D. Bashford, D.A. Case, Modification of the generalized Born model suitable for macromolecules, J. Phys. Chem. B 104 (2000) 3712–3720.

[36] T.Y. Lin, M. Emerman, Determinants of cyclophilin A-dependent TRIM5 alpha restriction against HIV-1, Virology 379 (2008) 335–341.

[37] A. Kuroishi, K. Bozek, T. Shioda, E.E. Nakayama, A single amino acid substitution of the human immunodeficiency virus type 1 capsid protein affects viral sensitivity to TRIM5 alpha, Retrovirology 7 (2010) 58.

[38] M. Stremlau, C.M. Owens, M.J. Perron, M. Kiessling, P. Autissier, J. Sodroski, The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in old world monkeys, Nature 427 (2004) 848–853.

[39] Z. Keckesova, L.M. Ylinen, G.J. Towers, Cyclophilin A renders human immunodeficiency virus type 1 sensitive to old world monkey but not human TRIM5 alpha antiviral activity, J. Virol. 80 (2006) 4683–4690.

[40] E. Sokolskaja, L. Berthoux, J. Luban, Cyclophilin A and TRIM5alpha independently regulate human immunodeficiency virus type 1 infectivity in human cells, J. Virol. 80 (2006) 2855–2862.

[41] S.Y. Lim, T. Rogers, T. Chan, J.B. Whitney, J. Kim, J. Sodroski, N.L. Letvin, TRIM5alpha modulates immunodeficiency virus control in rhesus monkeys, PLoS Pathog. 6 (2010) e1000738.

[42] C.O. Onyango, A. Leligdowicz, M. Yokoyama, H. Sato, H. Song, E.E. Nakayama, T. Shioda, T. de Silva, J. Townend, A. Jaye, H. Whittle, S. Rowland-Jones, M. Cotten, HIV-2 capsids distinguish high and low virus load patients in a West African community cohort, Vaccine 28 (2010) B60–B67.

[43] M. Kinomoto, R. Appiah-Opong, J.A. Brandful, M. Yokoyama, N. Nii-Trebi, E. Ugly-Kwame, H. Sato, D. Ofori-Adjei, T. Kurata, F. Barre-Sinoussi, T. Sata, K. Tokunaga, HIV-1 proteases from drug-naive West African patients are differentially less susceptible to protease inhibitors, Clin. Infect. Dis. 41 (2005) 243–251.

[44] R.K. Gitti, B.M. Lee, J. Walker, M.F. Summers, S. Yoo, W.I. Sundquist, Structure of the amino-terminal core domain of the HIV-1 capsid protein, Science 273 (1996) 231–235.

[45] C. Tang, Y. Ndassa, M.F. Summers, Structure of the N-terminal 283-residue fragment of the immature HIV-1 Gag polyprotein, Nat. Struct. Biol. 9 (2002) 537–543.

[46] M. Yokoyama, S. Naganawa, K. Yoshimura, S. Matsushita, H. Sato, Structural dynamics of HIV-1 envelope Gp120 outer domain with V3 loop, PLoS One 7 (2012) e37530.

[47] S.J. Neil, T. Zang, P.D. Bieniasz, Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu, Nature 451 (2008) 425–430.

[48] N. Van Damme, D. Goff, C. Katsura, R.L. Jorgenson, R. Mitchell, M.C. Johnson, E.B. Stephens, J. Guatelli, The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein, Cell Host Microbe 3 (2008) 245–252.

[49] D. Sauter, M. Schindler, A. Specht, W.N. Landford, J. Münch, K.A. Kim, J. Votteler, U. Schubert, F. Bibollet-Ruche, B.F. Keele, J. Takehisa, Y. Ogando, C. Ochsenbauer, J.C. Kappes, A. Ayouba, M. Peeters, G.H. Learn, G. Shaw, P.M. Sharp, P. Bieniasz, B.H. Hahn, T. Hatziioannou, F. Kirchhoff, Tetherin-driven adaptation of Vpu and Nef function and the evolution of pandemic and nonpandemic HIV-1 strains, Cell Host Microbe 6 (2009) 409–421.

[50] M. Shingai, T. Yoshida, M.A. Martin, K. Strebel, Some human immunodeficiency virus type 1 Vpu proteins are able to antagonize macaque BST-2 in vitro and in vivo: Vpu-negative simian-human immunodeficiency viruses are attenuated in vivo, J. Virol. 85 (2011) 9708–9715.

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Allele frequency of antiretroviral host factor TRIMCyp in wild-caught cynomolgus macaques (*Macaca fascicularis*)

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A recent study showed that the frequency of an antiretroviral factor *TRIM5* gene-derived isoform, TRIMCyp, in cynomolgus macaques (*Macaca fascicularis*) varies widely according to the particular habitat examined. However, whether the findings actually reflect the prevalence of TRIMCyp in wild cynomolgus macaques is still uncertain because the previous data were obtained with captive monkeys in breeding and rearing facilities. Here, we characterized the *TRIM5* gene in cynomolgus macaques captured in the wild, and found that the frequency of the TRIMCyp allele was comparable to those in captive monkeys. This suggests that the previous results with captive monkeys do indeed reflect the natural allele frequency and that breeding and rearing facilities may not affect the frequency of *TRIM5* alleles. Interestingly, the prevalence of a minor haplotype of TRIMCyp in wild macaques from the Philippines was significantly lower than in captive ones, suggesting that it is advantageous for wild monkeys to possess the major haplotype of TRIMCyp. Overall, our results add to our understanding of the geographic and genetic prevalence of cynomolgus macaque TRIMCyp.

Keywords: cynomolgus monkey, TRIM5α, TRIMCyp, genetic diversity, host factor

INTRODUCTION

In 2004, TRIM5-Cyclophilin A (CypA) chimeric protein, referred to as TRIMCyp, was first identified in owl monkeys (*Aotus trivirgatus*), which belongs to New World monkeys (NWMs) (Sayah et al., 2004). The discovery of TRIMCyp in owl monkeys explains the novel post-entry restriction of human immunodeficiency virus type 1 (HIV-1), which is uniquely seen in owl monkey-derived cells but not in other NWM-derived cells. Owl monkey TRIMCyp is derived from LINE-1-mediated retrotransposition of CypA cDNA into the region between *TRIM5* exons 7 and 8. On the other hand, the strong post-entry restriction of HIV-1 in Old World monkey (OWM)-derived cells was thought to be dependent on a TRIM5α-mediated mechanism (Stremlau et al., 2004; Nakayama and Shioda, 2010). Interestingly, among OWMs, pig-tailed macaques (*Macaca nemestrina*; hereafter denoted as PMs) uniquely show higher susceptibility to HIV-1 infection when compared with other OWMs (Agy et al., 1992). However, the mechanism underlying this higher susceptibility was unclear. Thereafter, it was found that PMs exclusively have the TRIMCyp genotype, which is a strong genetic determinant of their susceptibility to HIV-1 infection (Liao et al., 2007; Brennan et al., 2008; Virgen et al., 2008). Subsequently, TRIMCyp was also discovered in rhesus macaques (*Macaca mulatta*; hereafter denoted as RMs) and cynomolgus macaques (*Macaca fascicularis*; hereafter denoted as CMs) (Brennan et al., 2008; Newman et al., 2008; Wilson et al., 2008).

TRIMCyp is an alternatively spliced isoform of the *TRIM5* gene in which the PRYSPRY domain of TRIM5α is replaced with a retrotransposed *CypA* gene. Unlike owl monkey TRIMCyp, the *CypA* gene in OWM TRIMCyp, is inserted in the 3′-untranslated region (UTR) of the *TRIM5* gene. The retrotransposition of the CypA sequence is concomitant with a single nucleotide polymorphism (SNP) at the exon 7 splice acceptor site; this leads to skipping of exons 7 and 8 encoding the PRYSPRY domain and splicing to the inserted *CypA* gene (Johnson and Sawyer, 2009). Thus, the presence or absence of the CypA sequence in the 3′ UTR leads to expression of TRIMCyp or TRIM5α (Nakayama and Shioda, 2012).

Current data suggest that PMs exclusively express TRIMCyp and not TRIM5α. In the case of RMs, the frequency of TRIMCyp in Indian RM was approximately 25%, while it was not found in the Chinese RM population (Wilson et al., 2008). In addition, we observed that the frequency of TRIMCyp in Burmese RM was approximately 10% (unpublished data), suggesting a geographical deviation in the frequency of RM TRIMCyp. In the case of CM, we and other groups reported that TRIMCyp is present at higher frequency when compared with RM (De Groot et al., 2011; Dietrich et al., 2011; Saito et al., 2012). Interestingly, we and other groups found a geographical deviation in the frequency of TRIMCyp in CM (Dietrich et al., 2011; Berry et al., 2012; Saito et al., 2012). In particular, we showed that the frequency of TRIMCyp in the Philippine population was higher than that in Indonesian and Malaysian populations. Dietrich et al. also reported that the frequency of TRIMCyp in the Philippine population was higher than that in Indonesia, Indochina, and Mauritian populations (Dietrich et al., 2011). Moreover, they

claimed that the frequency of TRIMCyp in Indonesian CMs was higher than that of Indochina and Mauritian populations. However, all these analyses were performed with captive monkeys in breeding and rearing facilities. Therefore, these results may not reflect the natural gene frequencies. For instance, a small number of animals of a certain genotype introduced into facilities may affect the frequency of TRIMCyp via the founder effect. Furthermore, breeding policies may lead to a deviation of specific genotype. Hence, in order to understand the prevalence of TRIMCyp in CM precisely, it is necessary to analyze the frequency of TRIMCyp in wild CM. Therefore, in the present study, we sought to determine the geographic and genetic diversity of the *TRIM5* gene in wild-caught CM.

MATERIALS AND METHODS

SAMPLE COLLECTION

Blood samples from the wild-caught CMs, which had been cryopreserved for veterinary and microbiological examination as quarantine, were used in this study. These animals had been imported in the 1970's from the Philippines, Malaysia, and Indonesia to Japan as the founders of a breeding colony. These animals were directly sent to Japan without breeding in these countries.

DETERMINATION OF *TRIM5* GENOTYPE

The genotyping of *TRIM5* gene was performed as described previously with slight modifications (Saito et al., 2012). Briefly, the genomic DNA was extracted from frozen blood samples of 88 CMs with a QIAamp DNA Blood Mini kit (*Qiagen, Tokyo, Japan*). The genomic DNA was amplified by PCR using Ex Taq HS (TaKaRa, *Otsu, Japan*) with TC forward (5′-TGA CTC TGT GCT CAC CAA GCT CTT G-3′) and TC reverse (5′-ACC CTA CTA TGC AAT AAA ACA TTA G-3′) primers as described by Wilson et al. (2008). After amplification, PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

SEQUENCING OF THE CypA DOMAIN OF TRIMCyp

Amplified products of the CypA domain from 44 TRIMCyp homozygotes and 21 TRIMCyp/TRIM5α heterozygotes were purified using Wizard SV Gel and PCR Clean-Up System (*Promega, Tokyo, Japan*) and then subjected to direct sequencing using primer pairs of MfasCypA_F (5′-CAA CCC TAC CGT GTT CTT CG-3′) and MfasCypA_R (5′-TCG AGT TGT CCA CAG TCA GC-3′). Sequencing products were analyzed on a 3130xl Genetic Analyzer (*Applied Biosystems, Tokyo, Japan*).

RESULTS

HIGHER FREQUENCY OF TRIMCyp IN A WILD PHILIPPINE POPULATION AS COMPARED TO INDONESIAN AND MALAYSIAN POPULATIONS

We first analyzed the frequency of TRIMCyp in these wild-caught animals. The PCR-based assay performed here was designed to differentiate between the presence and absence of the CypA insertion (**Figure 1A**). The electrophoretic pattern of PCR products is shown in **Figure 1B**. The upper bands indicate TRIMCyp, while the lower bands indicate TRIM5α. A heterozygote is expected to possess both bands. As summarized in **Table 1**, we found that the 35 of the 49 Philippine CMs were homozygous for TRIMCyp, 11 were heterozygous, and 3 were homozygous for TRIM5α. In the case of Malaysian CM, 11 of the 29 animals were homozygous for TRIMCyp, 8 were heterozygous, and 10 were homozygous for TRIM5α. Finally, in the case of Indonesian CMs, none of the 10 animals were homozygous for TRIMCyp, 3 were heterozygous, and 7 were homozygous for TRIM5α. The calculated frequency of TRIMCyp in these populations was 82.7%, 48.3%, and 15.0%, respectively. Statistical analysis revealed that the frequency of TRIMCyp in the Philippine population was significantly higher than that in the Indonesian and Malaysian populations.

DIFFERENCE IN THE HAPLOTYPE FREQUENCY OF TRIMCyp BETWEEN WILD AND CAPTIVE PHILIPPINE POPULATIONS

Others and we have recently demonstrated the presence of several haplotypes in TRIMCyp of captive CM (Dietrich et al., 2011; Saito et al., 2012). Specifically, the major haplotype in which amino acid residues at positions 369 (Cyp66) and 446 (Cyp143) are aspartic acid (D) and lysine (K) is abundant in captive-CM TRIMCyp alleles [denoted as TRIMCyp-major (DK)]. In addition, the minor haplotype encoding asparagine (N) and glutamic acid (E) at positions 369 (Cyp66) and 446 (Cyp143) is also present [denoted as TRIMCyp-minor (NE)].

In this study, we further investigated the haplotypes of TRIMCyp in the wild-caught CM and compared the frequency of each haplotype in these animals with those reared in captivity. We found that although both haplotypes were present in wild-caught CM, the frequency of TRIMCyp-minor (NE) in wild Filipino CM was much less than that in captive Filipino CM (1.2% versus 14.3% of TRIMCyp; $p < 0.01$) (**Table 2**). By contrast, the frequency of TRIMCyp-minor (NE) in wild Malaysian CM was comparable to that in captive Malaysian CM (10.7% versus 11.1% of TRIMCyp; $p > 0.05$). In the case of wild Indonesian CM, all animals analyzed here possess TRIMCyp-major (DK), although the size of samples was too small to determine whether this was significant. Thus, the precise frequency of each haplotype in wild Indonesian CM is unclear.

DISCUSSION

In the present study, we analyzed the incidence of TRIMCyp in wild-caught animals and found that its frequency was comparable to that in captive animals (**Table 1**). Although blood samples from other regions were unavailable, it is reasonable to assume that the equivalence in the frequency of TRIMCyp between captive and wild-caught CM in other regions may have a similar tendency. Interestingly, we also found that the frequency of the TRIMCyp-minor (NE) haplotype in wild CM was lower than that in captive CM in the case of the Filipino population, but not in the case of the Malaysian population (**Table 2**). Although the reason for this discrepancy remains to be elucidated, we speculate that it might be hazardous for wild Filipino CM to possess TRIMCyp-minor (NE), as it may render them susceptible to TRIMCyp-minor (NE)-resistant pathogens present in the Philippines, but not in Malaysia. Based on this hypothesis, wild Filipino CM might be forced to expand TRIMCyp-major (DK) in order to counteract invasions from such pathogens. Conversely, weaker attacks, if any, from these pathogens in the breeding and rearing facilities might allow captive Filipino CM to expand TRIMCyp-minor (NE)

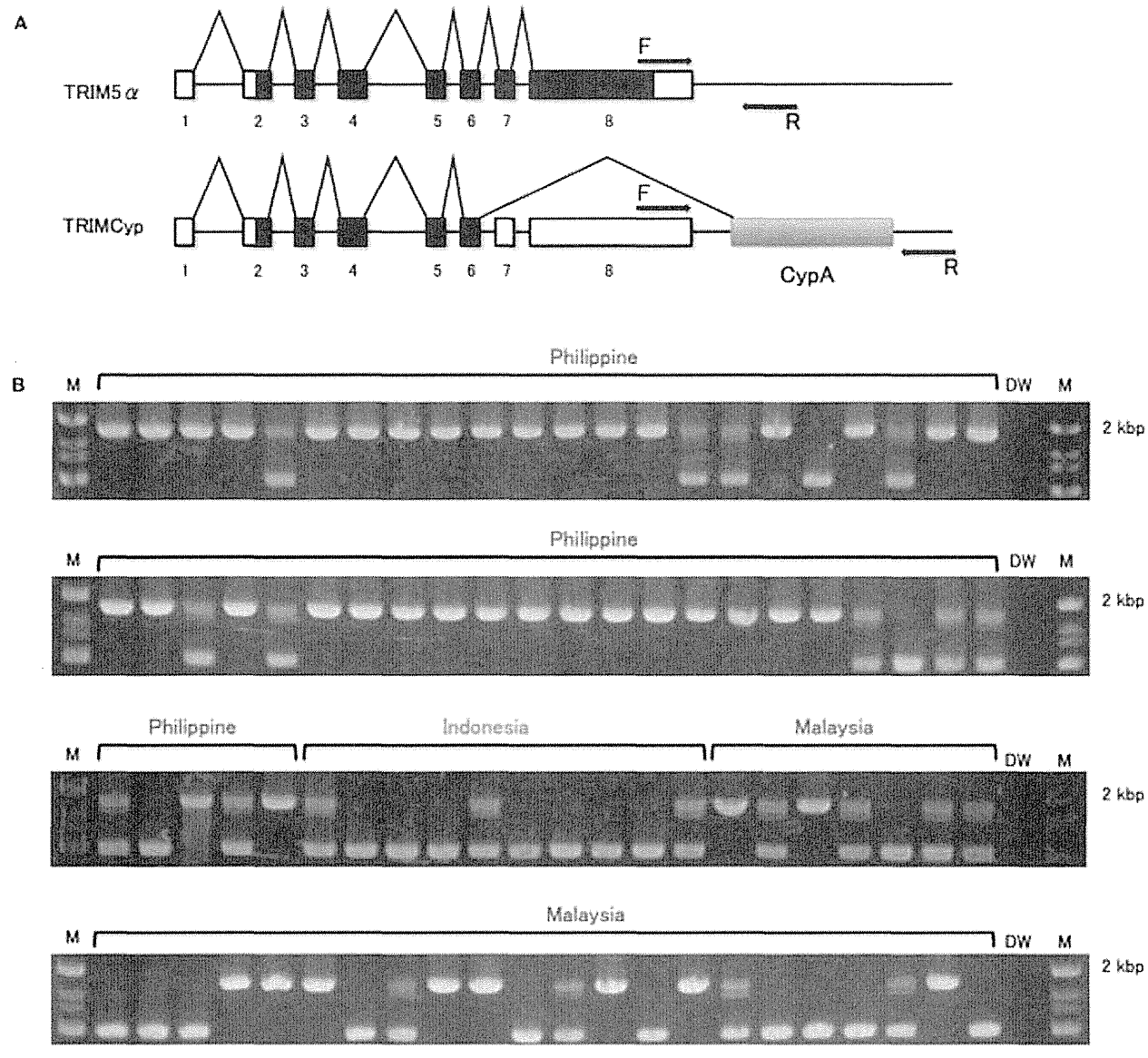

FIGURE 1 | Determination of *CypA* insertion. (A) Diagram indicating splicing of TRIM5α or TRIMCyp. Noncoding and coding exons and CypA sequences are shown in white, black, and shaded white, respectively. F and R denote forward and reverse primers used in this study, respectively. **(B)** The genomic DNA was extracted from whole blood. To test for CypA insertion, the 3′ region of the *TRIM5* gene was amplified by PCR with primers spanning the 3′ UTR and the putative *CypA* insertion. M and DW denote DNA molecular weight standard marker and water control, respectively.

haplotype in their population. Although it might also be hypothesized that the difference in the frequency of these haplotypes between wild-caught and captive animals was a consequence of the founder effect, the fact that more than 100 animals were introduced from wild (wild-caught animals) to breeding and rearing facility (captive animals) by dividing into several times suggests that the difference in the frequency of TRIMCyp haplotype may not be due to founder effect.

Since it is assumed that Filipino CM originated from Indonesian CM stocks (Thierry and Abegg, 2002), the fact that Malaysian and Indonesian CMs also possess TRIMCyp-major (DK) implies that this haplotype arose earlier than the migration of Indonesian CM stocks to the Philippine islands. Probably, TRIMCyp-major (DK) appeared in the ancestor of these CMs for some reason. Since only CM but neither PM nor RM possess TRIMCyp (DK) as one of the TRIMCyp haplotypes, it is reasonable to speculate that some pathogen(s) exerted a strong selection pressure on CM during their evolution. After the appearance of TRIMCyp-major (DK), Malaysian CM continued to maintain TRIMCyp-minor (NE) at a frequency of approximately 10% of total TRIMCyp alleles, while Filipino CM might exclude this haplotype. Alternatively, since Filipino CMs are thought to have originated from a small group of Indonesian CMs (Blancher et al., 2008), the limited prevalence of TRIMCyp-minor (NE) in wild

Table 1 | Frequency of TRIMCyp alleles in wild Philippine, Malaysian, and Indonesian populations.

Country	Origin of sample	#animals	Genotype (# animals)			Allele frequency		Citation
			TRIM5α homozygote	heterozygote	TRIMCyp homozygote	% TRIM5α	% TRIMCyp	
Philippines	Wild-caught	49	3	11	35	17.3	82.7	This study
Philippines	Captive	46	1	10	35	13.0	87.0	Saito et al., 2012
Philippines	Captive	4	0	0	4	0	100	Dietrich et al., 2011
Malaysia	Wild-caught	29	11	8	10	51.7	48.3	This study
Malaysia	Captive	47	11	26	10	51.1	48.9	Saito et al., 2012
Indonesia	Wild-caught	10	7	3	0	85.0	15.0	This study
Indonesia	Captive	33	13	17	3	65.2	34.8	Saito et al., 2012
Indonesia	Captive	18	3	10	5	44.4	55.6	Dietrich et al., 2011

Table 2 | Frequencies of DK and NE haplotypes in TRIMCyps of wild CM.

Country	Origin of sample	#animals	Genotype (# chromosomes)				Frequency		Citation
			TRIM5α/TRIMCyp heterozygote[a]		TRIMCyp homozygote[b]		% DK	% NE	
			DK	NE	DK	NE			
Philippines	Wild-caught	46	10	1	70	0	98.8	1.2	This study
Philippines	Captive	28	6	1	36	6	85.7	14.3	Saito et al., 2012
Malaysia	Wild-caught	18	7	1	18	2	89.3	10.7	This study
Malaysia	Captive	21	14	1	10	2	88.9	11.1	Saito et al., 2012
Indonesia	Wild-caught	3	3	0	0	0	100	0	This study
Indonesia	Captive	15	12	0	4	2	88.9	11.1	Saito et al., 2012

[a] *Haplotypes were determined by direct sequencing of the PCR products.*

[b] *Haplotypes were inferred by the Maximum-Likelihood estimation using the results of direct sequencing of the PCR products.*

Filipino CMs might be due to a founder effect. Unfortunately, we were unable to place a statistically meaningful value on the prevalence of the TRIMCyp-minor (NE) allele in wild Indonesian CM, since the sample size was too small. In the case of Malaysian CM TRIMCyp, the high frequency of the TRIMCyp-major (DK) allele suggests that it is preferable to posses this haplotype in their habitat. From this point of view, it will be of interest to consider why TRIMCyps of PMs and RMs are NE rather than DK type. In particular, the habitats of PM partially overlap with those of CM, except for the Java and Philippine islands (Thierry and Abegg, 2002). As Dietrich et al. proposed (Dietrich et al., 2010), it is likely that TRIMCyp evolved in the common ancestor of Asian macaques since TRIMCyp is present in both the silenus group, which includes PM, and the fascicularis group, which includes RM and CM. Furthermore, Ylinen et al. speculated that although the CypA sequence that has been retrotransposed into the macaque *TRIM5* locus is expected to be identical to the inherent CypA sequence, an arginine-to-histidine substitution at amino acid 69 may have occurred early in a common ancestor of Asian macaques. This may have been advantageous in that it helped to expand the spectrum of antiviral activity (Ylinen et al., 2010). This group further speculated that TRIMCyp (NE) arose in PMs and RMs independently; however, it is possible that TRIMCyp (NE) arose in the common ancestor of Asian macaques, since TRIMCyp (NE) is also present in CMs (**Table 2**). It is reasonable to imagine that the ancestors of PMs and RMs might fix TRIMCyp (NE) in order to protect themselves from invasion by TRIMCyp (NE)-sensitive pathogens. Specifically, the fact that PMs exclusively possess TRIMCyp (NE) instead of TRIM5α or TRIMCyp (DK) implies the importance of maintaining this *TRIM5* genotype in their habitat. Otherwise, the founder or bottleneck effect might affect the prevalence of TRIMCyp haplotypes in these macaque species. As an alternative hypothesis, TRIMCyp-minor (NE) in CM might be a vestige of an introgression between CMs and RMs with TRIMCyp (NE). In any case, future studies should analyze the prevalence of TRIMCyp in wild CMs by using samples from many regions to verify the correlation of genetic prevalence between wild and captive CMs.

More importantly, these two haplotypes in CM TRIMCyp are reported to show different antiviral activity (Ylinen et al., 2010; Dietrich et al., 2011; Saito et al., 2012). We and other groups reported that TRIMCyp-major (DK) suppresses the replication of HIV-1, but not that of HIV-2. Conversely, it was shown that TRIMCyp-minor (NE) suppresses the replication of HIV-2, but not that of HIV-1. Thus, these haplotypes of TRIMCyp present in CM are expected to show different antiviral activity in nature.

It will be of great interest to investigate the pathogens that acted as a selective pressure to alter the prevalence of TRIMCyp haplotypes.

Taken together, we analyzed the geographic and genetic characteristics of TRIMCyp in wild-caught CM for the first time and found (1) a higher frequency of TRIMCyp in the Philippine population as compared to those in other populations; (2) a similar tendency in the frequency of TRIMCyp between wild-caught and captive CM, and (3) a significant difference in the frequency of TRIMCyp-minor (NE) haplotype between captive and wild Filipino CM. These results provide important insights into the prevalence of CM TRIMCyp and increase our understanding of the evolution of antiretroviral host factors in Asian macaques.

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REFERENCES

Agy, M. B., Frumkin, L. R., Corey, L., Coombs, R. W., Wolinsky, S. M., Koehler, J., Morton, W. R., and Katze, M. G. (1992). Infection of *Macaca nemestrina* by human immunodeficiency virus type-1. *Science* 257, 103–106.

Berry, N. J., Marzetta, F., Towers, G. J., and Rose, N. J. (2012). Diversity of TRIM5alpha and TRIMCyp sequences in cynomolgus macaques from different geographical origins. *Immunogenetics* 64, 267–278.

Blancher, A., Bonhomme, M., Crouau-Roy, B., Terao, K., Kitano, T., and Saitou, N. (2008). Mitochondrial DNA sequence phylogeny of 4 populations of the widely distributed cynomolgus macaque (*Macaca fascicularis fascicularis*). *J. Hered.* 99, 254–264.

Brennan, G., Kozyrev, Y., and Hu, S. L. (2008). TRIMCyp expression in Old World primates *Macaca nemestrina* and *Macaca fascicularis*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3569–3574.

De Groot, N. G., Heijmans, C. M., Koopman, G., Verschoor, E. J., Bogers, W. M., and Bontrop, R. E. (2011). TRIM5 allelic polymorphism in macaque species/populations of different geographic origins: its impact on SIV vaccine studies. *Tissue Antigens* 78, 256–262.

Dietrich, E. A., Brennan, G., Ferguson, B., Wiseman, R. W., O'connor, D., and Hu, S. L. (2011). Variable prevalence and functional diversity of the antiretroviral restriction factor TRIMCyp in macaca fascicularis. *J. Virol.* 85, 9956–9963.

Dietrich, E. A., Jones-Engel, L., and Hu, S. L. (2010). Evolution of the antiretroviral restriction factor TRIMCyp in Old World primates. *PLoS ONE* 5:e14019. doi: 10.1371/journal.pone.0014019

Johnson, W. E., and Sawyer, S. L. (2009). Molecular evolution of the antiretroviral TRIM5 gene. *Immunogenetics* 61, 163–176.

Liao, C. H., Kuang, Y. Q., Liu, H. L., Zheng, Y. T., and Su, B. (2007). A novel fusion gene, TRIM5-Cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection. *AIDS* 21 (Suppl. 8), S19–S26.

Nakayama, E. E., and Shioda, T. (2010). Anti-retroviral activity of TRIM5 alpha. *Rev. Med. Virol.* 20, 77–92.

Nakayama, E. E., and Shioda, T. (2012). TRIM5alpha and Species Tropism of HIV/SIV. *Front. Microbiol.* 3:13. doi: 10.3389/fmicb.2012.00013

Newman, R. M., Hall, L., Kirmaier, A., Pozzi, L. A., Pery, E., Farzan, M., O'neil, S. P., and Johnson, W. (2008). Evolution of a TRIM5-CypA splice isoform in old world monkeys. *PLoS Pathog.* 4:e1000003. doi: 10.1371/journal.ppat.1000003

Saito, A., Kono, K., Nomaguchi, M., Yasutomi, Y., Adachi, A., Shioda, T., Akari, H., and Nakayama, E. E. (2012). Geographical, genetic and functional diversity of antiretroviral host factor TRIMCyp in cynomolgus macaque (*Macaca fascicularis*). *J. Gen. Virol.* 93, 594–602.

Sayah, D. M., Sokolskaja, E., Berthoux, L., and Luban, J. (2004). Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 430, 569–573.

Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427, 848–853.

Thierry, B., and Abegg, C. (2002). Macaque evolution and dispersal in insular south-east Asia. *Biol. J. Linn. Soc.* 75, 555–576.

Virgen, C. A., Kratovac, Z., Bieniasz, P. D., and Hatziioannou, T. (2008). Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3563–3568.

Wilson, S. J., Webb, B. L., Ylinen, L. M., Verschoor, E., Heeney, J. L., and Towers, G. J. (2008). Independent evolution of an antiviral TRIMCyp in rhesus macaques. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3557–3562.

Ylinen, L. M., Price, A. J., Rasaiyaah, J., Hue, S., Rose, N. J., Marzetta, F., James, L. C., and Towers, G. J. (2010). Conformational adaptation of Asian macaque TRIMCyp directs lineage specific antiviral activity. *PLoS Pathog.* 6:e1001062. doi: 10.1371/journal.ppat.1001062

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ORIGINAL PAPER

Lineage-specific evolution of T-cell immunoglobulin and mucin domain 1 gene in the primates

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Abstract T-cell immunoglobulin domain and mucin domain containing protein 1 (TIM1), also known as a cellular receptor for hepatitis A virus (HAVCR1) or a molecule induced by ischemic injury in the kidney (KIM1), is involved in the regulation of immune responses. We investigated a natural selection history of *TIM1* by comparative sequencing analysis in 24 different primates. It was found that *TIM1* had become a pseudogene in multiple lineages of the New World monkey. We also investigated T cell lines originated from four different New World monkey species and confirmed that *TIM1* was not expressed at the mRNA level. On the other hand, there were ten amino acid sites in the Ig domain of TIM1 in the other primates, which were suggested to be under positive natural selection. In addition, mucin domain of TIM1 was highly polymorphic in the Old World monkeys, which might be under balanced selection. These data suggested that *TIM1* underwent a lineage-specific evolutionary pathway in the primates.

Keywords Natural selection · Molecular evolution · Pseudogene · TIM1 · Primate

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Introduction

Comparative genomics is a useful tool for understanding the gene function from the view point of evolution. It has recently been reported that genes involved in regulation of immune system may have undergone the control of positive selection (Gibbs et al. 2007; Kosiol et al. 2008). The accelerated evolution may be due to a direct consequence of complex selection pressure exerted by infectious reagents including microbes and viruses (Barreiro and Quintana-Murci 2010). The known cases include genes of defensin family, which play crucial roles in antibacterial activity (Hollox and Armour 2008), and genes of APOBEC family, which are known to function as specific inhibitors against the infection of human immunodeficiency virus-1 (HIV-1) (Sawyer et al. 2004).

We previously performed a comparative genome analysis of primates and reported that genes encoding the immunoglobulin superfamily (IgSF) were classified into 11 functional categories based on the Gene Ontology (GO) database. The IgSF genes in three functional categories, immune system process (GO:0002376), defense response (GO:0006952), and multi-organism process (GO:0051704), had more chance to be under the positive natural selection than the IgSF genes in the other categories (Ohtani et al. 2011). In our previous comparative genome analysis, we focused on the orthologous

IgSF genes that appeared to be functional in all of human, chimpanzee, orangutan, rhesus macaque, and common marmoset. In other words, we excluded several genes of which an ortholog was considered to be non-functional, i.e., deleted gene, grossly rearranged gene, or pseudogene, in any of the five primate species. Such a lineage-specific destruction of IgSF genes, especially those involved in the immune response, may be interesting in view of the natural selection occurred during the evolution of primates. One of the excluded genes in our previous analysis was a gene for T-cell Ig domain and mucin domain containing protein 1 (TIM1), which was suggested to be a pseudogene due to an insertion in the common marmoset, while it should be functional in the other primates. TIM1 tightly linked to immune system, playing an important role in generation and/or maintenance of the balance between T helper 1 (Th1) cells and T helper 2 (Th2) cells, and it is up-regulated in Th2 cells after activation and interacts with its ligand expressed on antigen-presenting cells (de Souza and Kane 2006). *TIM1* can be found in the non-primate mammals including mouse and rat. However, *TIM1* orthologs are not found in the non-mammalian vertebrates such as chiken and zebrafish, implying that it might be involved in the mammalian-specific function. In addition, it was reported that *TIM1* is highly polymorphic in humans, but quite less polymorphic in chimpanzees, especially around the mucin domain (Nakajima et al. 2005). These observations suggested a unique evolutionary feature of *TIM1* in the primates.

In human, *TIM1* located on chromosome 5 at band q33 contains two distinct domains (Ig domain and mucin domain) (Khademi et al. 2004). It is known that TIM1 is a cellular receptor for hepatitis A virus (HAVCR1) in human (Feigelstock et al. 1998). TIM1 is also known to be induced in the kidney by ischemic injury and is called as kidney injury molecule 1 (KIM1) (Ichimura et al. 1998). It has been reported that *TIM1* polymorphisms are associated with various immune-related diseases and infectious diseases, including asthma, allergic rhinitis, atopic dermatitis, multiple sclerosis, type 1 diabetes, rheumatoid arthritis, AIDS, and cerebral malaria (Khademi et al. 2004; Kuchroo et al. 2003; McIntire et al. 2004; Meyers et al. 2005b; Su et al. 2008; Wichukchinda et al. 2010). These data implied that variations in *TIM1* might have been more or less selected during the evolution of humans.

In the present study, we determined nucleotide sequences of exons or equivalent regions of *TIM1* from 24 different primate species, including eight hominoids, six Old World monkeys, nine New World monkeys, and one prosimian, to investigate an evolutionary history of *TIM1*.

Materials and methods

Subjects

DNA samples from 24 primate species including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), western gorilla (*Gorilla gorilla*), Bornean orangutan (*Pongo pygmaeus*), western black-crested gibbon (*Nomascus concolor*), lar gibbon (*Hylobates lar*), siamang (*Symphalangus syndactylus*), rhesus macaque (*Macaca mulatta*), long-tailed macaque (*Macaca fascicularis*), Hamadryas baboon (*Papio hamadryas*), mantled Guereza colobus (*Colobus guereza*), dusky leaf monkey (*Trachypithecus obscurus*), silver leaf monkey (*Trachypithecus cristatus*), Geoffroy's spider monkey (*Ateles geoffroyi*), white-fronted spider monkey (*Ateles belzebuth*), tufted capuchin (*Cebus apella*), common squirrel monkey (*Saimiri sciureus*), white-lipped tamarin (*Saguinus labiatus*), golden-handed tamarin (*Saguinus midas*), cotton-top tamarin (*Saguinus oedipus*), golden lion tamarin (*Leonthopithecus rosalia),* common marmoset (*Callithrix jacchus*), and Sunda slow loris (*Nycticebus coucang*) were the subjects.

Polymerase chain reaction (PCR) and sequencing analysis

Sequence information for homologous regions to the coding regions of human *TIM1* was obtained from 24 primate species by direct sequencing of PCR products from the genomic DNA samples. Primers used for PCR and direct sequencing were designed by referring the human, chimpanzee, rhesus macaque, common marmoset gene sequences, and whole-genome shotgun sequences from prosimians deposited in the UCSC Genome Browser and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Supplemental Table S1). PCR condition was composed of a denaturing step (94 °C for 2 min), 35 cycles of chain reaction (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min), and a final extention step (72 °C for 5 min). The PCR products were then purified and sequenced by the BigDye Terminator cycling system using an ABI3130x automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Editing and assembly of sequences were done by using SEQUENCHER (Gene Codes, Ann Arbor, MI, USA). When sequence variations (heterozygous sequences) in a specific species were detected, the sequences which were more conserved among 24 primate species were considered as ancestral sequences and used for statistical analyses. The *TIM1* sequences determined in this study were deposited in DNA Data Bank of Japan (DDBJ) (Supplemental Table S2).

Expression analysis of *TIM1*

Total RNA was extracted from a human T cell line, Jurkat, and four different T cell lines originated from the New World monkeys, HSF-10 (tufted capuchin), HSQ-115 (common squirrel monkey), HST-3 (white-lipped tamarin) (Akari et al., manuscript in preparation), and HSCj-109 (common marmoset) (Hohjoh et al. 2009), by using RNAiso (TaKaRa Bio Inc., Shiga, Japan). Extracted RNAs (500 ng) were subjected to reverse transcription (RT) by using RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan). Aliquots of RT products were used for the expression analysis of *TIM1*. Primers for PCR were designed in the highly conserved regions of *TIM1* among human and the New World monkeys (Supplemental Table S3). PCR condition was the same as that described in the previous section.

Diversity of TIM1 mucin domain in the Old World monkeys

Nucleotide sequences for the mucin domain from eight samples of rhesus macaques were determined by sequencing of PCR products, which were cloned into pT7Blue Blunt vector and transformed Nova Blue Single Competent cells using the Perfectly Blunt cloning kit (Novagen Inc., Madison, WI). Colony PCR was used to identify positive clones, and at least 20 positive clones from each sample were subsequently sequenced as described previously. We also examined length variations of exon 4 from 16 rhesus macaques and 10 crab-eating macaques by direct sequencing of the PCR products.

Statistical analyses

We used both Bn-Bs program and PAML program as described previously (Ohtani et al. 2011). In brief, the Bn-Bs program was used to investigate the presence of branch-specific positive selection (the branch model). The Bn-Bs program estimates the values of non-synonymous substitution rate (dn) and synonymous substitution rate (ds) based on the modified Nei–Gojobori method, where a phylogenetic tree is given (Zhang et al. 1998). The value of ω, an abbreviation for the value of dn/ds, is a criterion of natural selective pressure acting on the gene, and the modified Nei–Gojobori method has been used for estimating non-synonymous/synonymous substitution rates (Nei and Gojobori 1986). Statistical significance of the difference between dn and ds was examined by *Z*-test (Chatterjee et al. 2009). An ordinary least-squares method was used to estimate branch lengths and variances for *Z*-test. The least-squares method gives estimates for evolutionary distances among the analyzed sequences (Rzhetsky and Nei 1993). The PAML program version 4.7 was used to investigate the presence of site-specific positive selection (the site model). The site model treats ω allowing the variance among codons (Yang 2005; Yang and Nielsen 2000), and the following null and alternative models were implemented in the site model: M0 (null), M1a (nearly neutral), M2a (positive selection) (Wong et al. 2004), M3 (discrete), M7 (beta), and M8 (beta and ω) (Yang and Nielsen 2000). The likelihood ratio tests (LRT) of three pairwise comparisons, i.e., comparisons of M1a vs. M2a, M1a vs. M3, and M7 vs. M8, determined whether particular models would provide a significantly better fit. When the LRT suggested positive selection, the Bayes empirical Bayes (BEB) method was used to detect the sites under the positive selection (Yang et al. 2005). To investigate a possible selection operated on exon 4 region of *TIM1* alleles in rhesus macaques, we calculated Tajima's *D* (Tajima 1989; Tamura et al. 2011).

Results

TIM1 is non-functional in several lineages of New World monkey

TIM1 is a member of *TIM* gene family composing of *TIM1*, *TIM3*, and *TIM4*, in the human genome (Khademi et al. 2004). In the previous comparative genome analysis, we searched for orthologous genes for human *TIM1*, *TIM3*, and *TIM4* in the genome of chimpanzee, orangutan, rhesus macaque, and common marmoset by using the UCSC/MULTIZ alignment program. It was found that there was an insertion of 205 bp in exon 2-equivalent region in the common marmoset gene, which would generate multiple frameshift/nonsense mutations in the coding sequence and/or destroy the splicing junction.

To confirm the presence of deleterious insertion in *TIM1* in the genome of common marmoset and possibly in other primate genomes, we determined nucleotide sequences for exons or equivalent regions of *TIM1* from 24 primate species including human, chimpanzee, orangutan, rhesus macaque, and common marmoset. For this purpose, we designed primers by referring the known *TIM1* sequences (Supplemental Table 1). The sequencing analysis of the genomic gene for *TIM1* revealed the deleterious insertions of 206–212 bp in several New World monkeys, i.e., golden lion tamarin (212 bp), cotton-top tamarin (206 bp), white-lipped tamarin (207 bp), and golden-handed tamarin (210 bp) (Supplemental Figure S1). It was speculated that the insertion had been occurred within a sequence stretch of 13 bp, AGCCTCATCCTAC, corresponding to codons 9–13, because these sequences were repeated and flanked the insertion in the genomes of common marmoset and cotton-top tamarin, and there were a few substitutions in this sequence stretch from the other New World monkeys (Supplemental Figure S1). The inserted sequences belong to the

LINE/L1 (L1PA7) repeat, which contain a poly A stretch at one end in a reverse orientation to the TIM1 coding sequences, and homologous sequences can be found as multiple copies in the marmoset genome. On the other hand, we found nucleotide substitutions in exon 3-equivalent regions, which resulted in termination mutations in three other New World monkeys not carrying the insertion, common squirrel monkey (S84X), tufted capuchin (V22X), and Geoffroy's spider monkey (C36X) (Fig. 1). Among the New World monkey species investigated in this study, only the white-fronted spider monkey appeared to carry a functional gene for TIM1.

To investigate whether *TIM1* was non-functional in the New World monkey lineages, we performed RT-PCR analysis of mRNA expression in T cell lines originated from human, tufted capuchin, squirrel monkey, white-lipped tamarin, and common marmoset. As illustrated in Fig. 2a, two pairs of primers were used in the RT-PCR analysis, where forward primers were designed in exon 3 and junction of exon 5–exon 6, while reverse primers were designed in exon 9 and junction of exon 5–exon 6 (Supplemental Table S3). The *TIM1* expression was confirmed in the human T cell line, but could not be detected in the T cell lines from the New World monkeys carrying either the insertions (common marmoset and white-lipped tamarin) or the nonsense mutations (common squirrel monkey and tufted capuchin) (Fig. 2). No expression of *TIM3* and *TIM4* was observed in the T cell lines from human and the New World monkeys (data not shown).

Positive selection sites of *TIM1* in the primates

In the other primate species than the New World monkey, *TIM1* appeared to be functional, and there were many substitutions. When we calculated the dn and ds values in each primate lineage, it was found that the dn values were higher

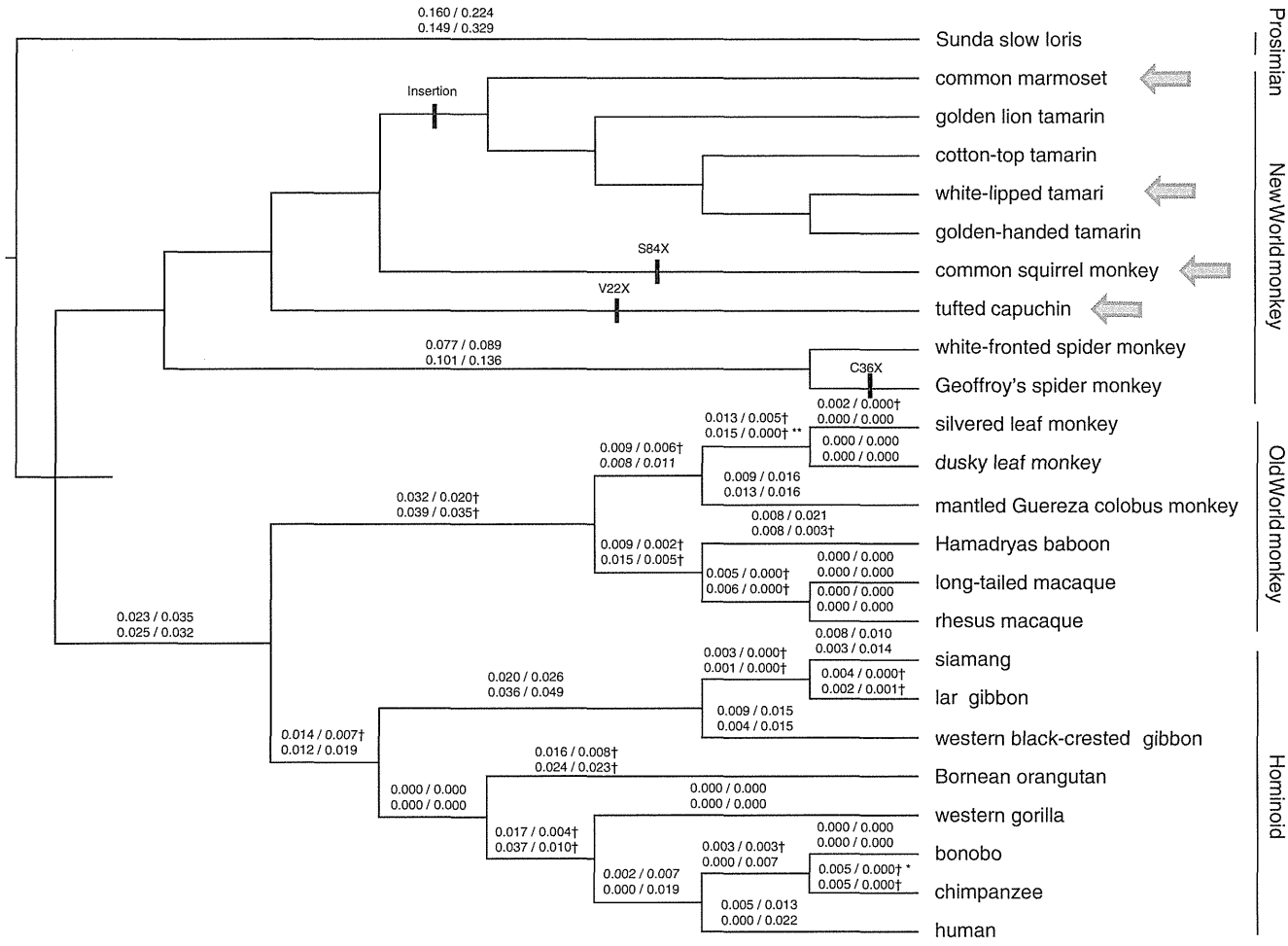

Fig. 1 Phylogenetic trees for TIM1 in the primate evolution. Values above branches indicate estimated values of dn and ds per lineage by using the Bn-Bs program. Upper values are for the entire coding region, while lower values are for the Ig domain. *Daggers* indicate that the dn value was higher than the ds value. *Asterisks* indicate that there is a significant difference between the dn and ds values ($^{**}p<0.01$; $^{*}p<0.05$; *Z*-test). *Vertical lines* indicate that *TIM1* had become pseudogene in the specific lineage. *Arrows* indicate the species for which the mRNA expression of *TIM1* in T cell line was investigated