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Novel mutant human immunodeficiency virus type 1 strains with high degree of resistance to cynomolgus macaque TRIMCyp generated by random mutagenesis

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Old World monkey TRIM5α strongly suppresses human immunodeficiency virus type 1 (HIV-1) replication. A fusion protein comprising cynomolgus macaque (CM) TRIM5 and cyclophilin A (CM TRIMCyp) also potently suppresses HIV-1 replication. However, CM TRIMCyp fails to suppress a mutant HIV-1 that encodes a mutant capsid protein containing a SIVmac239-derived loop between α-helices 4 and 5 (L4/5). There are seven amino acid differences between L4/5 of HIV-1 and SIVmac239. Here, we investigated the minimum numbers of amino acid substitutions that would allow HIV-1 to evade CM TRIMCyp-mediated suppression. We performed random PCR mutagenesis to construct a library of HIV-1 variants containing mutations in L4/5, and then we recovered replication-competent viruses from $CD4^+$ MT4 cells that expressed high levels of CM TRIMCyp. CM TRIMCyp-resistant viruses were obtained after three rounds of selection in MT4 cells expressing CM TRIMCyp and these were found to contain four amino acid substitutions (H87R, A88G, P90D and P93A) in L4/5. We then confirmed that these substitutions were sufficient to confer CM TRIMCyp resistance to HIV-1. In a separate experiment using a similar method, we obtained novel CM TRIM5α-resistant HIV-1 strains after six rounds of selection and rescue. Analysis of these mutants revealed that V86A and G116E mutations in the capsid region conferred partial resistance to CM TRIM5α without substantial fitness cost when propagated in MT4 cells expressing CM TRIM5α. These results confirmed and further extended the previous notion that CM TRIMCyp and CM TRIM5α recognize the HIV-1 capsid in different manners.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) efficiently infects humans and chimpanzees, but not Old World monkeys (OWMs) such as rhesus (Rh) and cynomolgus (CM) monkeys. In contrast, another lentivirus, simian immunodeficiency virus isolated from sooty mangabey (SIVsm), and simian immunodeficiency virus isolated from African green monkey (AGM) replicate in their natural hosts (VandeWoude & Apetrei, 2006). The restricted host range of HIV-1 has greatly hampered its use in

Seven supplementary figures are available with the online Supplementary Material.

animal experiments, thus impeding the development of prophylactic vaccines against HIV-1 infection. In the cells of OWMs, HIV-1 encounters three main restriction factors: tripartite motif 5α (TRIM5α) (Stremlau *et al.*, 2004), apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3 (APOBEC3) (Sheehy *et al.*, 2002), and bone marrow stromal antigen 2 (Neil *et al.*, 2008; Van Damme & Guatelli, 2008). Rh, CM and AGM TRIM5α proteins potently inhibit HIV-1 infection at the early phase before reverse transcription (Nakayama & Shioda, 2010). In these monkey cells, TRIM5α binds to HIV-1 capsid and promotes its rapid, premature disassembly via ubiquitin-dependent (Diaz-Griffero *et al.*, 2006) and -independent (Anderson *et al.*, 2006) processes.

OWMs (macaque monkeys) infected with simian immunodeficiency virus isolated from macaque (SIVmac) have served as important animal models for understanding the pathogenesis of HIV-1 infection. We previously reported that HIV-1 replicated efficiently in CM cells when HIV-1 sequences were replaced with the respective SIVmac239 sequences corresponding to all three of the following: the loop between α-helices 4 and 5 (L4/5) of the capsid protein (CA); the loop between α-helices 6 and 7 (L6/7) of CA; and the *vif* gene (Kuroishi *et al.*, 2009). The resulting virus (NL-4/5S6/7SvifS) was expected to escape from host restriction factors CM TRIM5α and APOBEC3G for two main reasons: first, L4/5 had been reported as a determinant of Rh TRIM5α-mediated restriction (Ylinen *et al.*, 2005); and second, position 119 or 120 in HIV-2 CA L6/7 is a determinant of CM TRIM5α-mediated restriction (Miyamoto *et al.*, 2011; Song *et al.*, 2007). However, the replicative capability of NL-4/5S6/7SvifS in human cells was severely impaired. To increase the growth capability of mutant HIV-1 strains with SIVmac239 sequences, Nomaguchi *et al.* (2013b) performed virus adaptation by long-term culturing in macaque cells, and successfully obtained adapted viruses with enhanced growth ability. Sequence analysis revealed that mutations in *pol*-IN (N222K and V234I) and *env*-gp120 (T110I, F247L and E427K) were responsible for the enhancement of viral replication. Additionally, a Q112D mutation in CA helix 6 yielded increased viral growth potential. This construct was designated MN4Rh-3 (Nomaguchi *et al.*, 2013b).

The *TRIM5* gene-derived allelic variant, TRIMCyp, has been found in at least three species of OWMs, including Rh, pig-tailed monkey and CM. CM TRIMCyp suppresses HIV-1 but not HIV-2 or SIVmac. MN4Rh-3 replicates robustly in CMs homozygous for *TRIMCyp* alleles but not in CMs harbouring only *TRIM5α* alleles (Saito *et al.*, 2013). The MN4Rh-3 virus was created by the inclusion of a net replacement of 11 amino acids in the CA region (Fig. 1). To define the minimum changes required for escape from CM TRIMCyp- or CM TRIM5α-mediated restriction, we performed random PCR-mediated mutagenesis of the sequence encoding the CypA-binding region of the HIV-1 capsid.

RESULTS

Known L4/5 point mutations (previously shown to abolish the interaction between CA and CypA) fail to confer CM TRIMCyp resistance

As reported previously (Saito *et al.*, 2012), HIV-1 NL4-3 encoding the WT CA (Fig. 1) was completely restricted by CM TRIMCyp (Figs 2a and S1, available in the online Supplementary Material). CA/human CypA complex formation is mediated entirely by the contiguous CA loop sequence P85 to P93 (Fig. 1b), L4/5, which is known to bind in the active site of CypA (Yoo *et al.*, 1997). We therefore first tested whether HIV-1 variants encoding a limited number of mutations in CA L4/5 (mutations that are known to abolish the interaction between HIV-1 CA and human CypA) could escape from CM TRIMCyp-mediated restriction. Previous work (Saito *et al.*, 2012) showed that replacement of HIV-1 CA with the L4/5 of SIVmac239 rendered NL-vifS strongly resistant to CM TRIMCyp (4/5S in Fig. 1b). Our present work confirmed this result (Fig. 2a). On the other hand, a modification replacing amino acids 91–93 with a pair of glutamine residues corresponding to the most prominent difference between HIV-1 and SIVmac239 L4/5 [(91–93)QQ in Fig. 1b] was not sufficient to confer CM TRIMCyp resistance (Fig. 2a). The R100S substitution (100S in Fig. 1b), previously shown to be important for Rh TRIM5α resistance of SIVsm (Kirmaier *et al.*, 2010), also did not yield CM TRIMCyp resistance (Fig. 2a). The G89-P90 dipeptide was reported to be the primary CypA recognition motif in HIV-1 CA (Yoo *et al.*, 1997). However, we found that a P90A mutation severely impaired viral growth (data not shown). Previous works also showed that P90AA92E (Qi *et al.*, 2008), H87Q/I91V (Chatterji *et al.*, 2005) and a mutation at residue 88 (Price *et al.*, 2009) abolished interaction between CA and CypA. However, we found that amino acid substitutions at these positions (90A92E, 87Q-88P-91V or 88(–), respectively, in Fig. 1b) failed to confer CM TRIMCyp resistance (Fig. 2a). These results suggest that binding specificity of HIV-1 CA for the CypA domain of CM TRIMCyp is similar, but not identical, to those for the human CypA or for the CypA domain of owl monkey TRIMCyp.

Selection of a random-mutagenized HIV-1 library in MT4 cells expressing high levels of CM TRIMCyp

To define the minimum number of amino acid substitutions of HIV-1 CA sufficient to confer resistance against CM TRIMCyp, we performed PCR-based random mutagenesis to construct a large library of HIV-1 variants encoding mutations in L4/5 (amino acid positions 85 to 93 of CA) (see Methods). The culture supernatant of 293T cells transfected with 10^4 clones of this library was collected as a virus stock. First, we inoculated $CD4^+$ MT4 cells with the virus stock to amplify replication-competent viruses.

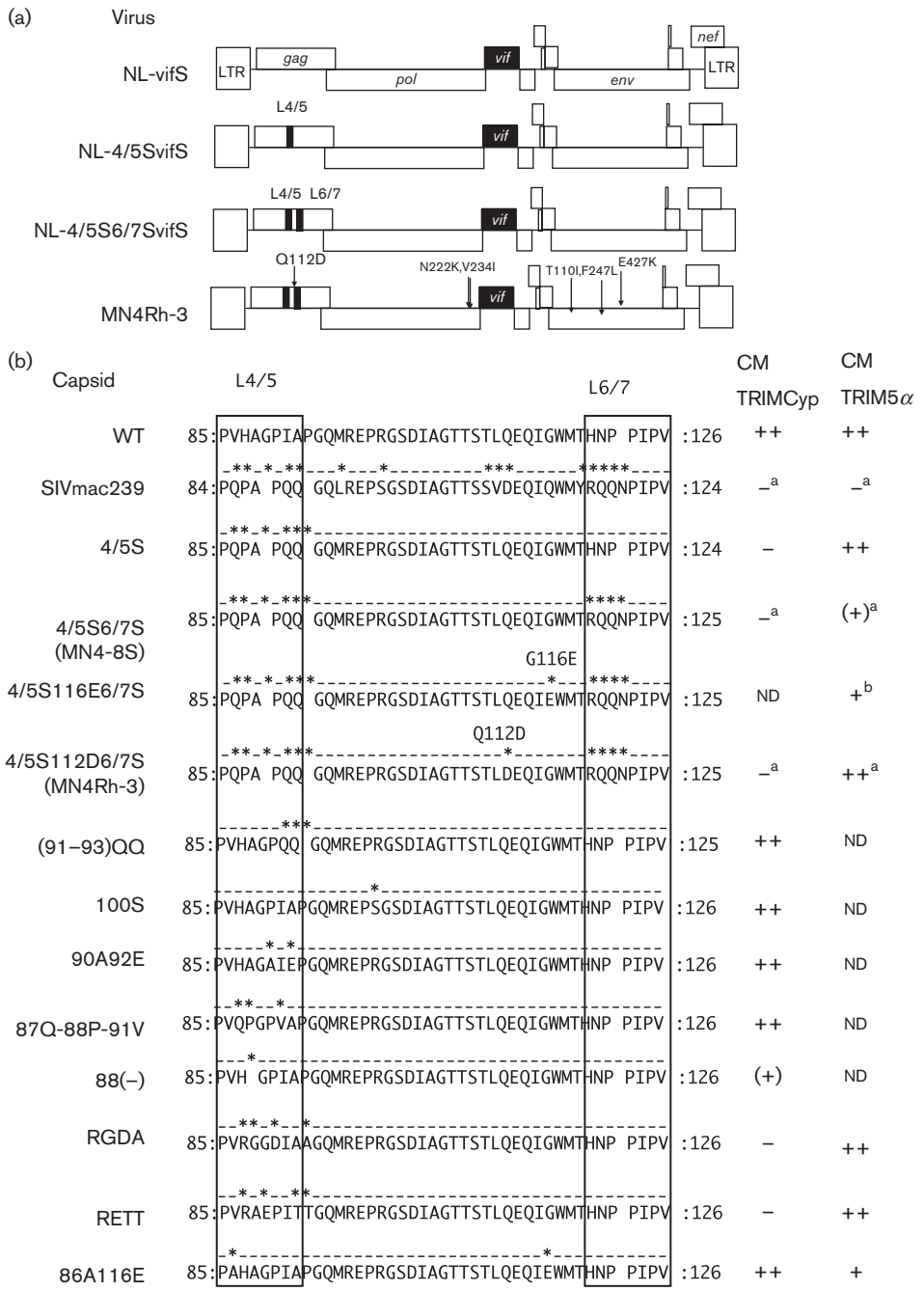

Fig. 1. Schematic representation of HIV-1 derivatives and relevant CA protein sequences. (a) Bars denote ORFs or long terminal repeats (LTRs) of HIV-1 NL4-3 or SIVmac239. White and black rectangles denote HIV-1 NL4-3 and SIVmac239 sequences, respectively. (b) Alignments of partial amino acid sequences of capsid proteins of HIV-1 mutants and SIVmac239. The names of viruses previously used in the referenced reports, are shown in parentheses. [a]Nomaguchi *et al.* (2013b) and [b]Kuroishi *et al.* (2010). A dash denotes the amino acid residue identical to that of NL4-3. An asterisk denotes an amino acid residue different from that of NL4-3. The box indicates the amino acid residues in L4/5 and L6/7. The positions of G116E and Q112D substitutions are indicated. Symbols $++$, $+$ and $-$ denote more than 100-fold, 3- to 100-fold and less than 3-fold suppression of viral growth by CM TRIMCyp or TRIM5α compared with viral growth in the presence of CM-SPRY(–) TRIM5α on day 6. ND, Not determined; $(+)$, virus suppression with poor virus growth even in the absence of functional TRIM5α.

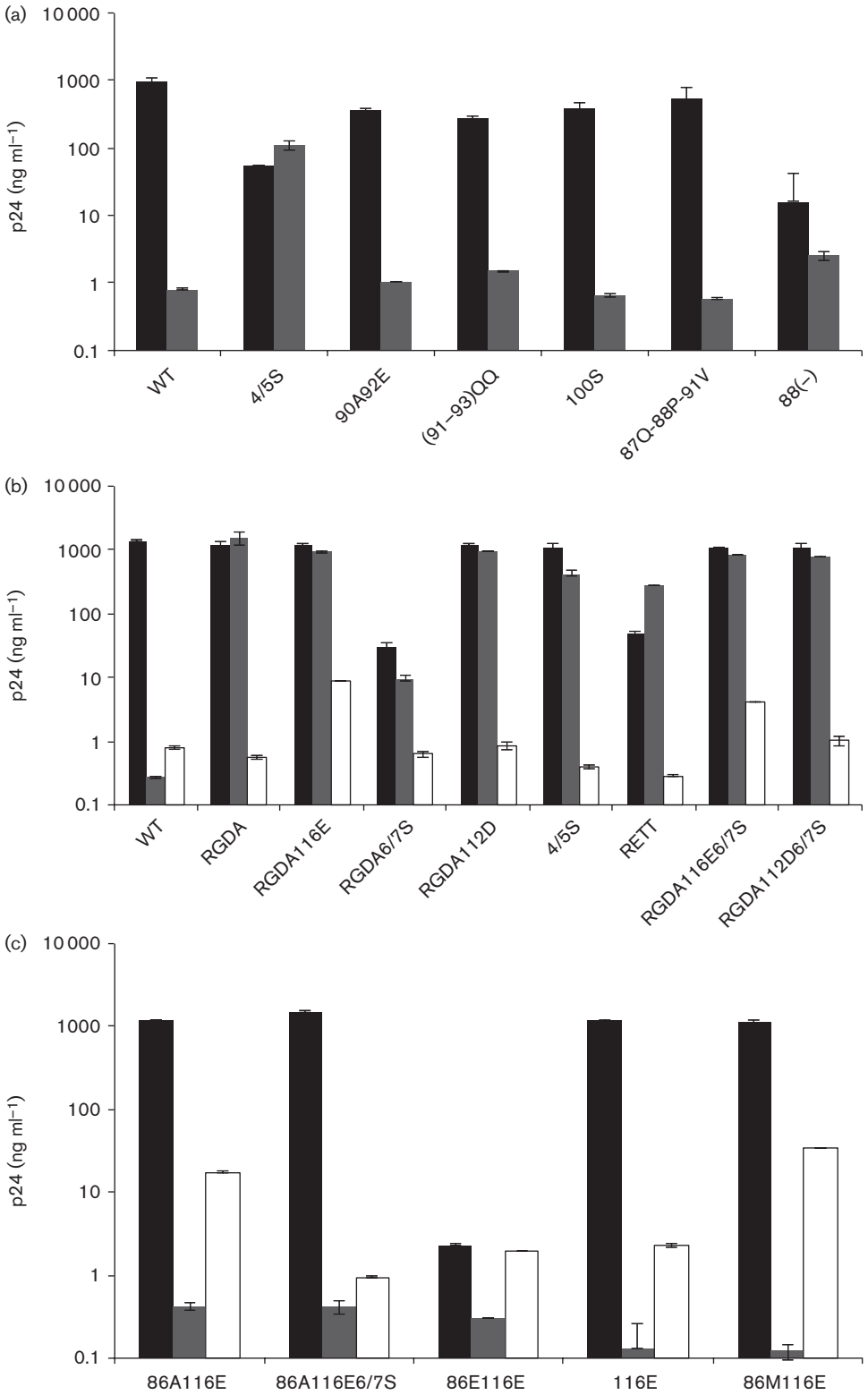

Fig. 2. Growth of HIV-1 mutants in MT4 cells expressing CM-SPRY (–) TRIM5α, CM TRIMCyp or CM TRIM5α. (a) MT4 cells expressing CM-SPRY (–) TRIM5α (black bars) or CM TRIMCyp (grey bars) were superinfected with the indicated HIV-1 derivatives. (b, c) MT4 cells expressing CM-SPRY (–) TRIM5α (black bars), CM TRIMCyp (grey bars) or CM TRIM5α (white bars) were superinfected with the indicated HIV-1 derivatives. Culture supernatants were assayed at day 6 and the levels of p24 antigen were plotted. Error bars show actual fluctuations of p24 in duplicate samples. The results shown are representative of at least two independent experiments.

We then applied a functional screen to isolate adapted virus in MT4 cells expressing CM TRIMCyp. The viruses were rescued by adding fresh MT4 cells without CM TRIMCyp. The selection process was repeated twice more, such that we characterized those viruses obtained after three rounds of selection for CM TRIMCyp resistance (Fig. S2a). Notably, when we subjected parental cloned NL-vifS virus to adaptation in CM TRIMCyp-expressing cells, no virus was recovered from the third round of selection, despite the addition of fresh MT4 cells, in a total interval of 2 months (data not shown).

Sequence analysis of the CA-coding region of the provirus recovered in MT4 cells expressing CM-TRIMCyp revealed the presence of H87R, A88G, P90D and P93A mutations. We obtained a similar but distinct combination of mutations (H87R, G89E, A92T and P93T) from an independent experiment selecting for adaptation to CM TRIMCyp.

H87R/A88G/P90D/P93A and H87R/G89E/A92T/P93T mutations confer complete resistance against CM TRIMCyp

To examine the effect of mutations found in adapted viruses, the respective four mutations in L4/5 were introduced into the parental NL-vifS molecular clone, yielding clones RGDA (encoding H87R, A88G, P90D and P93A) and RETT (encoding H87R, G89E, A92T and P93T). As shown in Figs 2(b) and S3, both viruses were (like 4/5S) strongly resistant to CM TRIMCyp restriction, whereas they were completely sensitive to CM TRIM5α. The growth capability of these viruses was analysed in the absence of TRIM5α-mediated restriction by using MT4 cells infected with a Sendai virus (SeV) encoding a TRIM5 protein lacking the PRYSPRY domain. The results revealed that RGDA grew to higher titres than RETT (Fig. 2b). Therefore we selected H87R/A88G/P90D/P93A mutations for further evaluation.

We had previously succeeded in rescuing the impaired replicative capability of NL-4/5S6/7SvifS; in that work, we reported that the G116E mutation improved the virus's replicative capability (Kuroishi *et al.*, 2010). Notably, HIV-1 with the G116E mutation became partially resistant to CM TRIM5α-mediated restriction (Kuroishi *et al.*, 2010). Therefore, we introduced the G116E mutation into RGDA and tested the resulting clone (designated RGDA116E) for resistance against CM TRIM5α and CM TRIMCyp. We found that inclusion of the additional G116E mutation did not affect virus growth. As expected, RGDA116E was able to grow in MT4 cells expressing CM TRIM5α, confirming that the G116E substitution is important for resistance to CM TRIM5α. The inclusion of the 6/7S mutation in RGDA was detrimental to viral growth; in contrast, inclusion of both the 6/7S and G116E mutations in RGDA was favourable for viral growth (Fig. 2b). These findings are consistent with our previous results (Kuroishi *et al.*, 2010).

Finally, we introduced the Q112D mutation (previously reported as Q110D mutation in MN4Rh-3; Nomaguchi *et al.*, 2013b) (Fig. 1), into RGDA to generate RGDA112D. Similar to the reported data for MN4Rh-3 (Nomaguchi *et al.*, 2013b), the combination of RGDA, L6/7S and Q112D mutations was acceptable for virus growth (Fig. 2b).

V86A/G116E mutations confer partial resistance against CM-TRIM5α

Next, we attempted to obtain CM TRIM5α-resistant viruses from the same library as described above. We inoculated amplified replication-competent virus to MT4 cells infected with recombinant SeV encoding CM TRIM5α. The viruses were rescued by adding fresh MT4 cells. A total of six rounds of selection and rescue were required to obtain CM TRIM5α-resistant virus; the seventh round of selection confirmed the resistance to CM TRIM5α (Fig. S2b). Sequence analysis of the CA-encoding region of the provirus DNA revealed the presence of V86A and G116E mutations. As noted above, we have previously reported that HIV-1 harbouring a G116E mutation was partially resistant to CM TRIM5α-mediated restriction (Kuroishi *et al.*, 2010). The V86A and G116E mutations were introduced into the parental NL-vifS molecular clone, yielding strain 86A116E. When propagated in MT4 cells expressing CM TRIM5α, 86A116E grew to higher p24 levels than obtained with 116E (Figs 2c and S4). These results indicate that the V86A mutation potentiated the CM TRIM5α resistance of the strain carrying the G116E mutation alone, although these double-mutant viruses showed only partial resistance against CM TRIM5α.

Since position 120 in L6/7 of HIV-2 is a determinant of CM TRIM5α sensitivity (Song *et al.*, 2007), we replaced the region encoding L6/7 of 86A116E with that of SIVmac239, generating 86A116E6/7S. However, 86A116E6/7S did not grow in the presence of CM TRIM5α, presumably due to a detrimental effect of the replacement of L6/7 with that of SIVmac239. We also constructed another two strains (designated 86M116E and 86E116E) encoding distinct substitutions at V86. A previous study had shown that V86M CA allows HIV-1 to grow in HeLa-CD4 cells expressing Rh TRIM5α (Pacheco *et al.*, 2010); separate work revealed that the V86E mutant exhibits decreased sensitivity to restriction by the Rh TRIM5α (Soll *et al.*, 2013). When we compared the growth of all three V86 mutants in the presence of CM TRIM5α, 86A116E grew to higher titres in the presence of CM TRIM5α than 86E116E (Fig. 2c). On the other hand, 86M116E exhibited growth similar to that of 86A116E in the presence of CM TRIM5α (Fig. 2c).

To confirm fitness of all the mutant viruses, we used unmodified MT4 cells. Except for viruses carrying 112D substitution, mutant viruses showed similar growth capability in unmodified MT4 cells to those described above (Fig. S5). In contrast, viruses with 112D showed slightly reduced growth capability in unmodified MT4 cells (Fig. S5).

Viral resistance to CM TRIMCyp- and TRIM5α-mediated restriction in a single-round infection assay

The assay system described in Fig. 2 investigated the effect of CM TRIMCyp and CM TRIM5α on the multi-step growth of the viruses. Since TRIM5α restricts HIV-1 infection at the early steps of viral infection, we performed a single-round infection assay by using vesicular stomatis virus glycoprotein (VSV-G)-pseudotyped virus with luciferase gene as a reporter in the *nef* gene. Prior to proceeding with this experiment, we measured reverse transcriptase (RT) activity of each mutant virus to check if mutations hampered the sensitivity of p24 ELISA; we detected no discrepancy between RT and p24 activities among the mutants (Fig. S6). The replacement of L4/5 with that of SIVmac239 and RGDA sequences impaired the infectivity in comparison with that of the WT (Fig. 3). The viruses carrying sequences encoding the CA of 4/5S, 4/5S112D6/7S, RGDA or RGDA112D showed high degrees of resistance to CM TRIMCyp (Fig. 3).

On the other hand, the 86A116E double mutant exhibited only a minor change in infectivity. The mutants carrying sequences encoding the CA of 4/5S, 4/5S112D6/7S, RGDA or RGDA112D showed almost no resistance to CM TRIM5α compared with 86A116E or 86M116E mutants, results that were in good agreement with the growth capabilities in the presence of CM TRIM5α in multi-step experiments (Fig. 2b, c). The virus carrying the G116E mutation alone showed very slight resistance to CM TRIM5α in comparison with virus encoding the WT capsid. The viruses carrying 86A116E or 86M116E mutations showed partial resistance to CM TRIM5α (Fig. 3), confirming that CA position 86 is important for resistance to CM TRIM5α-mediated restriction.

Binding of HIV-1 CA to CM TRIMCyp in virion incorporation assay

To measure the binding capability of HIV-1 CA to the CypA-like domain of CM TRIMCyp, we performed virion incorporation assays. For these assays, 293T cells were co-transfected with a recombinant plasmid encoding the CypA-like domain of CM TRIMCyp fused with the HA tag together with NL-Nh derivatives of viruses encoding WT or mutant (90A92E, RGDA, RETT, 86A116E or 4/5S) capsids. Western blot analysis using antibody against the HA tag showed that HIV-1 virion particles encoding RGDA or RETT mutant capsids showed almost no incorporation of the HA-tagged CypA-like domain of CM TRIMCyp compared with that of the WT and 86A116E (Fig. 4a), although the transfected cells expressed comparable amounts of HA-tagged CypA proteins. These results suggest that capsid with the RGDA or RETT mutations has decreased binding affinity for CM TRIMCyp, and that this loss of affinity for the CypA-like domain of CM TRIMCyp is critical for escape from restriction by CM TRIMCyp.

When we performed an equivalent experiment using 293T cells that had been co-transfected with recombinant plasmid encoding authentic human CypA, the 90A92E and 4/5S capsid mutants showed very low incorporation of authentic CypA (Fig. 4b). In contrast, virions with the 90A92E mutation incorporated large amounts of the CypA-like domain of CM TRIMCyp, whereas virions with the 4/5S mutation incorporated very small amounts of the CypA-like domain of CM TRIMCyp. These results are in good agreement with the observation that 4/5S (and not 90A92E) grew in the presence of CM TRIMCyp (Fig. 2a).

Molecular modelling of the CA N-terminal domain (NTD) of HIV-1 variants

To obtain structural insights into viral resistance to CM TRIMCyp and CM TRIM5α, we constructed three-dimensional models of the CA NTD of four HIV-1 strains with different sensitivities to the antivirals via different sets of amino acid residues on the CA NDT. The modelling revealed that the CA substitutions for the CM TRIMCyp and CM TRIM5α resistance are distributed differently on the NTD: the former are uniformly arranged along the exposed surface on L4/5, whereas the latter are positioned on L4/5 near helix 4 and at helix 6 (Fig. 5a). These results suggest that CM TRIMCyp and CM TRIM5α target distinct surfaces in the CA NTD of HIV-1: L4/5 is critical for CM TRIMCyp-mediated antiviral activity, while other regions containing L4/5 and helix 6 are involved in CM TRIM5α-mediated antiviral activity. These results are consistent with our previous finding of differential antiviral roles of CM TRIMCyp and CM TRIM5α in HIV-1 infection (Saito *et al.*, 2013). In addition, the present results also agree well with our previous findings indicating the critical roles of amino acid residues in L4/5 and helix 6 in CM TRIM5α resistance (Nomaguchi *et al.*, 2013a, 2014). The present study further showed that the main chain folds of the CA NTD of the HIV-1 variants are indistinguishable, although minor changes were noticed in the L4/5 conformation of the CM TRIMCyp-resistant mutants (Fig. 5b). The results suggest that changes in physico-chemical features on the binding surface, rather than surface conformation, are critical for the phenotypic modifications in this study. Such changes might cause alterations in electrostatic attraction (H87R, G89E, P90D and G116E), hydrophobic attraction (V86A), and/or loop dynamics (P93T/A and A88G) for ligand recognition/binding; further study is necessary to address these issues.

Replication of HIV-1 derivatives in monkey peripheral blood mononuclear cells (PBMCs)

Nomaguchi *et al.* (2013b) reported that several mutations at defined sites in the Pol-integrase and/or the Env-gp120 (MN4Rh-3) enhanced growth potential in macaque cells. Therefore, we transferred the CA-encoding regions of

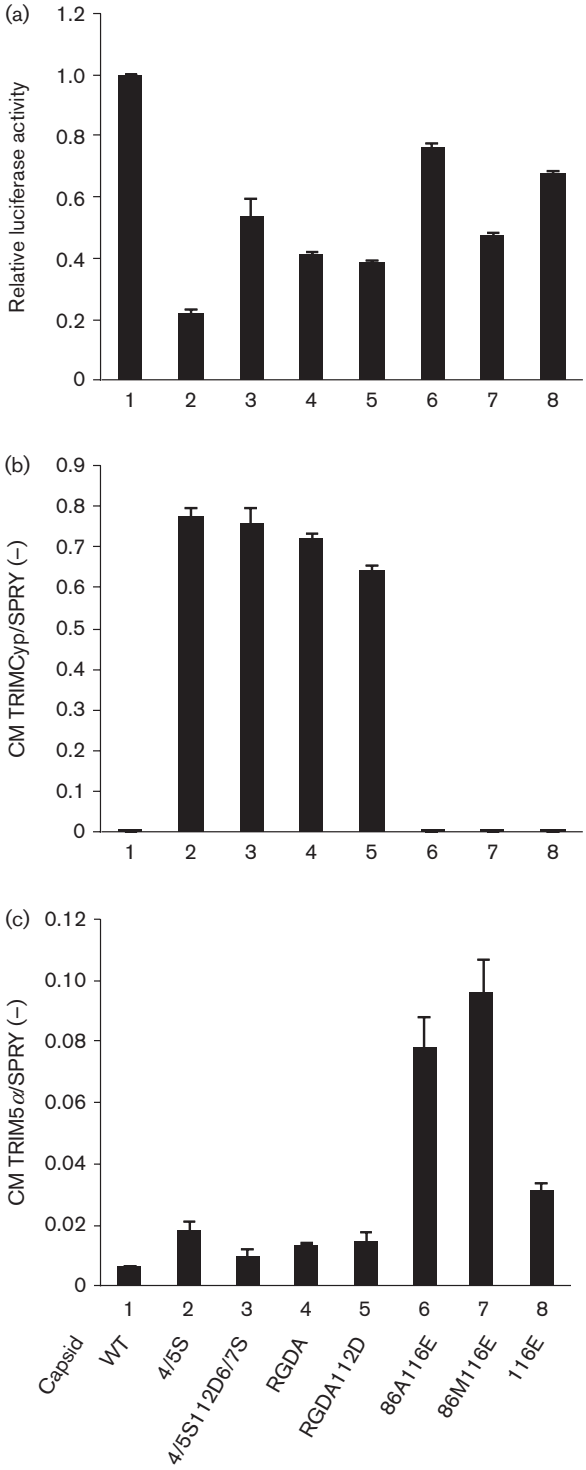

Fig. 3. Viral infectivity (a) and sensitivity to CM TRIMCyp (b) and CM TRIM5α (c) in a single-round infection assay. MT4 cells expressing CM TRIM5Cyp, CM TRIM5α or CM-SPRY (–) TRIM5α were superinfected with VSV-G-pseudotyped HIV-1 derivatives with luciferase reporter gene. The infectivity of a clone was measured by dividing the luciferase activity of that clone in the presence of CM-SPRY (–) TRIM5α by the luciferase activity of the WT NL4-3-Luc-R-E in the presence of CM-SPRY (–) TRIM5α. To quantify the resistance to CM TRIMCyp or CM TRIM5α of a clone, luciferase activity of that clone in the presence of CM TRIMCyp or CM TRIM5α, respectively, was divided by the luciferase activity of that clone in the presence of CM-SPRY (–) TRIM5α. The results shown are representative of at least three independent experiments. Error bars show SD among triplicate samples.

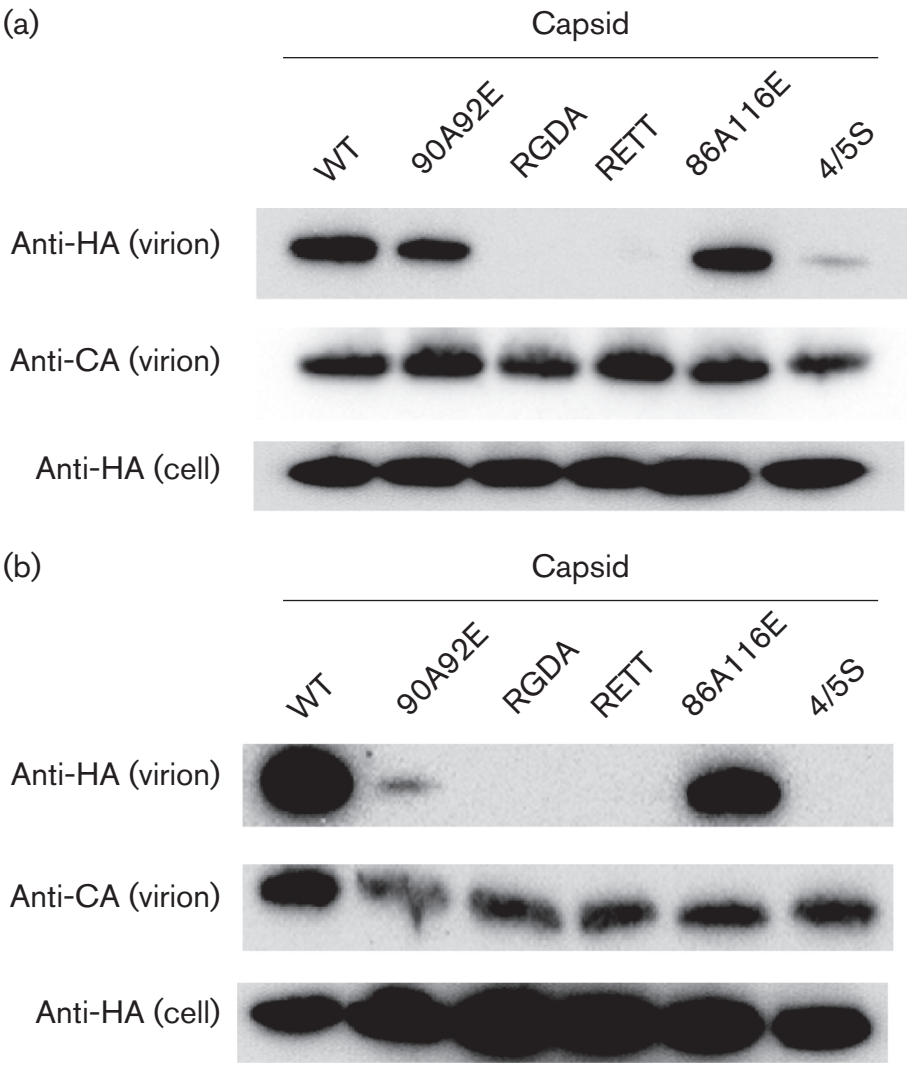

Fig. 4. Characterization by virion incorporation assay of the ability of different capsid mutants of HIV-1 to bind to the CypA-like domain of CM TRIMCyp. 293T cells were co-transfected with NL-Nh (WT) or capsid mutations 90A92E, RGDA, RETT, 86A116E and 4/5S, together with pCEP4 vector encoding the HA-tagged CypA-like domain of CM TRIMCyp (a) or with pCEP4 vector encoding HA-tagged authentic human CypA (b). After normalization for p24 amount, virions were subjected to SDS-PAGE followed by Western blot analysis using anti-HA and anti-p24 antibodies. The cell lysate of transfected 293T cells served as a control for the transfection. This result is representative of at least two independent experiments.

RGDA112D and 86A116E to the MN4Rh-3 backbone, yielding constructs designated RGDA112D-Rh and 86A116E-Rh, respectively. We then confirmed the complete resistance to CM TRIMCyp of MN4Rh-3 and RGDA112D-Rh and partial resistance to CM TRIM5α of 86A116E-Rh in MT4 cells expressing CM TRIMCyp or TRIM5α (Fig. 6a).

To infer the growth capability of HIV-1 derivatives in monkey, CD8-depleted PBMCs isolated from TRIMCyp or TRIM5α homozygous CMs were infected with HIV-1 derivatives *in vitro*. As shown in Fig. 6(b), SIVmac239 replicated very rapidly in PBMCs regardless of the host genotype. MN4Rh-3 and RGDA112D-Rh showed similar growth kinetics in PBMCs from a CM TRIMCyp homozygote, but did not grow in PBMCs from a CM TRIM5α homozygote (Fig. 6b). We evaluated PBMCs from two additional CM TRIMCyp homozygotes and found that PBMCs from all these CM TRIMCyp homozygotes also supported growth of RGDA112D-Rh (Fig. S7). On the other hand, 86A116E-Rh showed growth in PBMCs from a CM TRIM5α homozygote, although the peak titre of this strain was lower than that of SIVmac239 (Fig. 6b). To our knowledge, this result represents the first report of an HIV-1 derivative that can replicate in the PBMCs of a CM TRIM5α homozygote. We evaluated PBMCs from seven additional CM TRIM5α homozygotes and found that PBMCs from two out of the seven CM

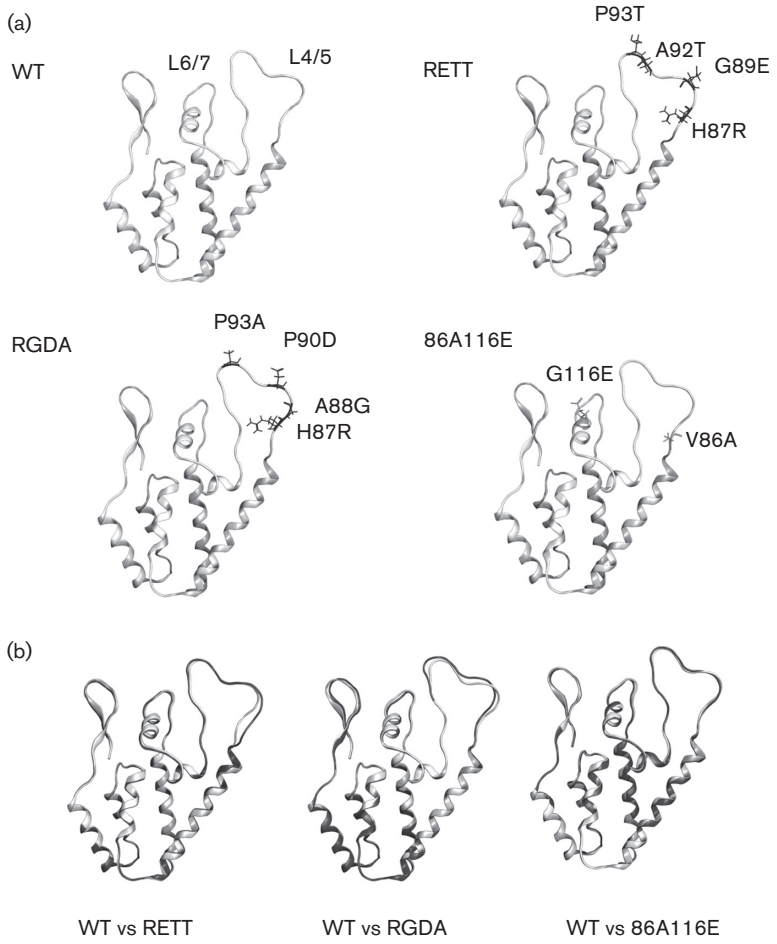

Fig. 5. Structural models for CA NTDs of HIV-1 NL4-3 derivatives. Structural models were constructed by homology-modelling using 'MOE-Align' and 'MOE-Homology' in MOE. The crystal structure of the HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C) was used as the modelling template. (a) CA NTD structural models for NL4-3 (WT, grey) and its derivatives (white) containing amino acid substitutions for CM TRIMCyp resistance (RETT and RGDA) and for CM TRIM5α partial resistance (86A116E) are shown. (b) Superposed structures of CA NTD of NL4-3 and its derivatives.

TRIM5α homozygotes supported growth of 86A116E-Rh (Fig. S7). These results are consistent with the results obtained in MT4 cells expressing CM TRIMCyp and TRIM5α via recombinant SeV, as shown in Fig. 2(b, c), although the reason why PBMCs from five out of eight CM TRIM5α homozygotes failed to support growth of 86A116E-Rh is not clear. As expected, while RGDA112D grew in MT4 cells expressing CM TRIMCyp (Fig. 2b), this strain was not able to grow in PBMCs from a CM TRIMCyp homozygote. Similarly, 86A116E also did not grow in PBMCs from a CM TRIM5α homozygote, although this strain provided resistance against CM TRIM5α in MT4 cells (Fig. 2c).

DISCUSSION

Random mutagenesis of HIV-1 CA L4/5-encoding sequences, followed by selection by CM TRIMCyp, permitted the identification of novel mutations (H87R/A88G/P90D/P93A and H87R/G89E/A92T/P93T) in L4/5 that provided strong resistance to CM TRIMCyp. We also found that the V86AG116E double mutation conferred partial resistance to CM TRIM5α without apparent fitness cost. Position 86 is located at the N-terminal end of L4/5, while the four-mutation combinations (RGDA and RETT) are scattered across the entire L4/5. These results indicate that CM TRIMCyp and CM TRIM5α recognize the HIV-1 capsid in different manners.

In our initial attempt at characterizing the CA–CypA interaction, we tested several HIV-1 derivatives harbouring mutations known to impair CypA binding (Li *et al.*, 2009; Price *et al.*, 2009). However, with the exception 4/5S, none of the selected mutant viruses exhibited replication in the presence of CM TRIMCyp. By using the virion incorporation assay, we found that the capsids with the novel mutations (RGDA or RETT) failed to incorporate

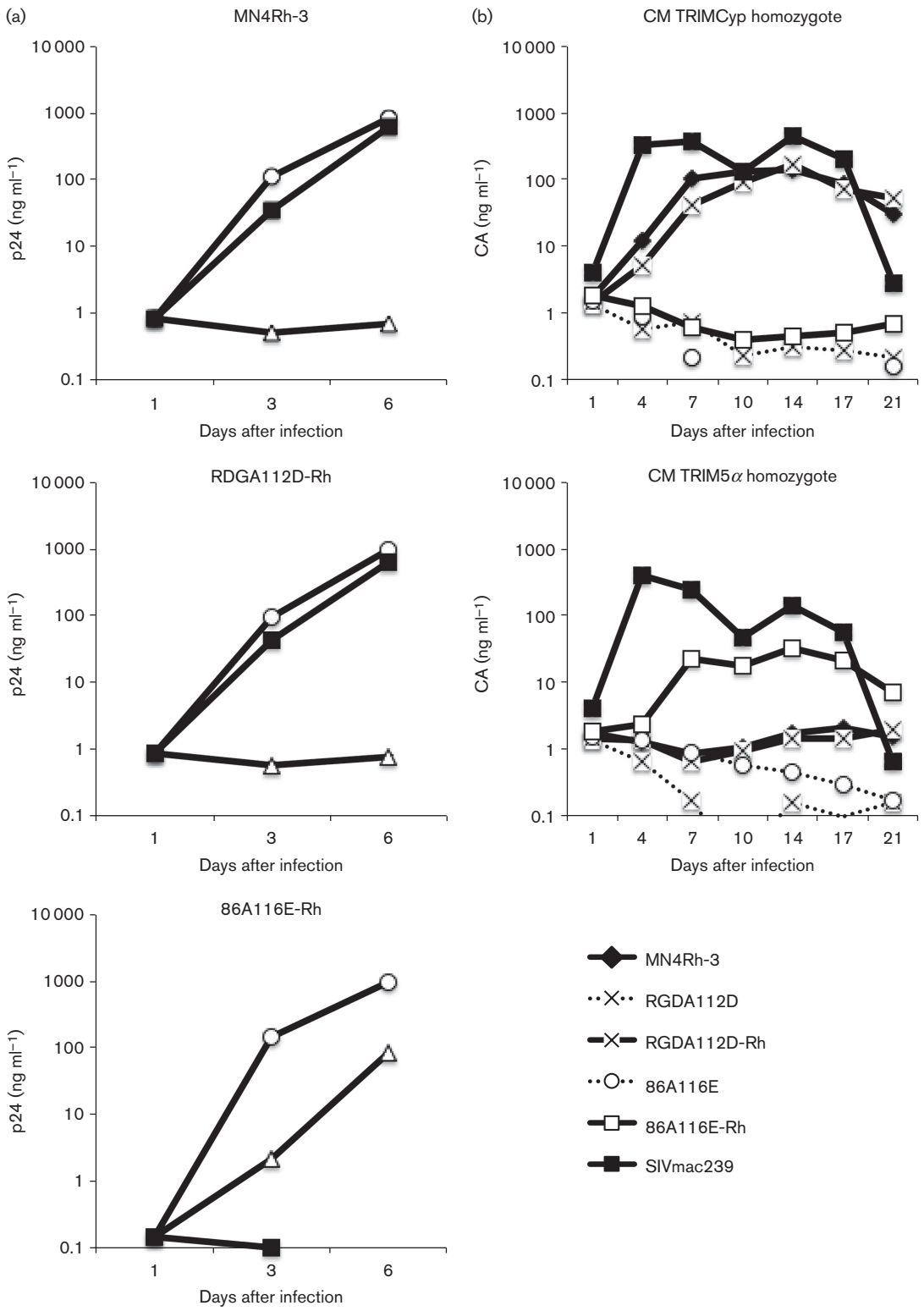

Fig. 6. Replication capabilities of HIV-1 derivatives in CD8-depleted PBMCs from CM. (a) MT4 cells expressing CM TRIM-Cyp (■), CM TRIM5α (Δ), CM-SPRY or (–) TRIM5α (○) were superinfected with the indicated HIV-1 derivatives. The results shown are representative of at least two independent experiments. Error bars, that are hidden behind the symbols due to their small values, show actual fluctuations between measurements of p24 in duplicate samples. (b) CD8-depleted PBMCs from CM TRIMCyp homozygote or CM TRIM5α homozygote were infected with RGDA112D, RGDA112D-Rh, 86A116E or 86A116E-Rh. MN4Rh-3 and SIVmac239 served as controls.

the CypA-like domain of CM TRIMCyp; in contrast, HIV-1 virions with 90A92E mutations still incorporated the CypA-like domain of CM TRIMCyp. We therefore conclude that the loss of binding affinity of capsid to the CypA domain of CM TRIMCyp confers resistance to TRIMCyp-mediated restriction. It would be interesting to see how these CA mutants behave in other contexts, such as stimulation of interferon responses (Rasaiyaah *et al.*, 2013) and interaction with NUP358 (Schaller *et al.*, 2011).

Previous studies showed the importance of capsid binding of authentic CypA for HIV-1 replication in human cells (Braaten *et al.*, 1996). In the present study, we confirmed the importance of this interaction, since all the mutant viruses that are known to lack CypA incorporation showed impaired growth (as shown in Figs 2 and S1). The monkey-tropic HIV-1 MN4Rh-3 strain also showed impaired growth in human cells compared with NL-vifS, a strain that encodes WT capsid. We therefore tried to create (using random mutagenesis) an HIV-1 derivative that can replicate as well as the WT but completely lacks binding affinity for CM TRIMCyp. Contrary to our expectation, the isolated viruses in two independent experiments failed to retain the full capacity for replication, although these viruses completely overcame TRIMCyp-mediated restriction. These results suggest a critical role for CypA binding (and/or a role for the CypA-binding loop) in the context of infection by HIV-1 but not by SIVmac.

It is noteworthy that the G116E substitution was obtained repeatedly in the experiments described here. We previously showed that position 116 is located in the sixth helix, near L4/5 and L6/7, where this residue is apparently exposed to the protein surface. An amino acid substitution at position 116 is expected to cause a change in the structure of the protein surface, given the chemical dissimilarity between G (which has no side chain) and E (which has a long, negatively charged side chain) (Kuroishi *et al.*, 2010). Repeated isolation of this lesion also suggests that this site on the CA tolerates changes and is not essential for viral replication.

The importance of mutation in position 86 was emphasized by the single-round luciferase infection assay. The G116E mutation showed a higher degree of resistance against CM TRIM5α when combined with V86A or V86M (Fig. 3). It was previously reported that V86M CA allows HIV-1 to escape from Rh (Pacheco *et al.*, 2010) and mutant human TRIM5α (Veillette *et al.*, 2013). On the other hand, virus harbouring only the V86E mutation exhibits decreased Rh TRIM5α sensitivity (Soll *et al.*, 2013). In the present study, however, we recovered only the V86A mutant, and not a V86M or V86E mutant. We hypothesize that further rounds of selection may yield V86M, since only one nucleotide substitution is required to convert V to M; we do not expect to recover V86E, given that the V86E mutation showed a detrimental effect on viral growth (Fig. S4).

RGDA112D-Rh, the novel HIV-1 derivative described in this work, showed growth capability similar to that of the previous molecular clone MN4Rh-3 in CD8-depleted CM PBMCs derived from a TRIMCyp homozygote. RGDA112D-Rh bears only five amino acid substitutions in the CA; in contrast, MN4Rh-3 bears 11 substitutions compared with the WT (Fig. 1). We also note here that this work represents the first report (to our knowledge) of an HIV-1 derivative that can replicate in the PBMCs of a CM TRIM5α homozygote. Though the 86A116E mutant showed growth kinetics superior to the other mutants, further improvement will be necessary to obtain an appreciable infection in CM. Random mutagenesis in the whole N-terminal region of the CA may be required, since the region that interacts with TRIM5α is larger than that interacting with TRIMCyp. In the present study, we focused only on the CA-encoding sequence for random mutagenesis and adaptation; other sequences in *pol, env* or other loci were not mutagenized. It also would be interesting to see whether extended intervals of adaptation would yield additional or distinct mutations for escaping the restriction pressure of CM TRIM5α or for compensating for the impaired growth in the presence of CM TRIMCyp. Additionally, Rh and CM TRIM5αs exhibit distinct antiviral activities (Kono *et al.*, 2010); growth of both RGDA and 86A116E were completely suppressed in the presence of Rh TRIM5α. We are currently attempting to recover virus from cells overexpressing Rh TRIM5α.

We evaluated PBMCs from three CM TRIMCyp homozygotes and found that PBMCs from all three CM TRIMCyp homozygotes supported growth of RGDA112D-Rh (Fig. S7). On the other hand, PBMCs from five out of eight CM TRIM5α homozygotes failed to support growth of 86A116E-Rh (Fig. S7). Since SIVmac239 replicated well in all those PBMCs, these results indicated the presence of inter-individual differences in susceptibility to 86A116E-Rh infection among CM TRIM5α homozygotes. It is possible that other host factor(s) that affect HIV-1 replication in CM are involved in this difference. Since RGDA112D-Rh was strongly resistant against CM TRIMCyp, like MN4Rh-3, effects of the other host factor(s) may be neglected. On the other hand, 86A116E-Rh could only partially escape from CM TRIM5α, and effects of the other host factor(s) may become more prominent. Further study is necessary to identify such host factor(s).

METHODS

Cell culture. The human kidney adherent 293T cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated FBS at 37 °C with 5 % CO_2. MT4, a human $CD4^+$T cell line immortalized by human T cell leukemia virus type-1 (Akagi *et al.*, 1985), was maintained in RPMI 1640 medium containing 10 % FBS.

Viruses. NL-vifS possesses the entire *vif* gene of SIVmac239 in the HIV-1 NL4-3 background (NL-SVR in Kamada *et al.*, 2006) (WT). NL-4/5S6/7SvifS encodes CA with the SIVmac239-derived L4/5 and L6/7 in the NL-vifS background (Kuroishi *et al.*, 2009) (4/5S6/7S). NL-G116EvifS encodes a G-to-E substitution at position 116 of the CA in the NL-vifS background (Kuroishi *et al.*, 2010) (116E).

The MN4Rh-3 was reported previously (Nomaguchi *et al.*, 2013b). Amino acid substitutions in the NL-vifS CA shown in Fig. 1 were introduced by site-directed mutagenesis with the PCR-mediated overlap primer extension method. The sequence authenticities of all clones were confirmed by sequencing of the CA-encoding region. Virus stocks were prepared by transfection of 293T cells with HIV-1 NL-vifS and its derivatives using the polyethylenimine (molecular mass 25 000; Polysciences) method. Viral titres were measured using the p24 RetroTek antigen ELISA kit (ZeptoMetrix). Viral RT was quantified with the Reverse Transcriptase Assay, colorimetric (Roche Diagnostics).

Luciferase-expressing viruses. For the 4/5S, MN4Rh-3, RGDA112D, 86A116E, 86M116E and 116E constructs, the respective *Bss*HII–*Apa*I fragment [which encodes matrix (MA) and CA] was subcloned into NL4-3-Luc-R-E-(NIH AIDS Research and Reference Reagent Program) to generate the corresponding luciferase reporter vector. To recover the VSV-G-pseudotyped luciferase-expressing viruses, 293T cells were co-transfected with 12 μg reporter plasmid and 5 μg VSV-G-expressing plasmid (pMD2G) (Dull *et al.*, 1998).

Recombinant Sendai viruses. SeVs encoding CM TRIMCyp, CM TRIM5α and CM TRIM5α without the SPRY domain [CM-SPRY (−)] were described previously (Kono *et al.*, 2008; Nakayama *et al.*, 2005; Song *et al.*, 2007).

Library construction. PCR with degenerate primers was performed using 1 μg NL-vifS plasmid, Ex Taq polymerase (TaKaRa) and (separately) the following primer pairs: (1) M661F (5′-gccagaggagatctctcgacgcagg-3′) and Pst-R (5′-caatctatcccattctgcagcttc-3′) or (2) NL43-ODN (5′-tgggatagattgcatCCaGTgCAtGCaGGgCCtATtGCaCCaggccagatgagagaa-3′) and 2011R (taggggccctgcaattttggctatgtgcccttc-3′). The NL43-ODN primer was designed to introduce random mutations in the L4/5-encoding region of NL4-3, corresponding to amino acid positions 85 to 93 of CA. Specifically, the primer was synthesized so as to introduce random mutations at the nucleotides shown in capital letters in the sequence above. Synthesis was performed so that (at each of those positions) 91 % of the oligonucleotides would have the correct residue, while 3 % would have one of the three other residues. The mutations were confined to the first and second residues of the respective codons in order to minimize the occurrence of silent mutations. The nucleotides underlined in the Pst-R and NL43-ODN sequences are complementary. PCR products obtained from PCR reactions (1) and (2) above were mixed and used as templates for a second round of PCR with the M661F and 2011R primer pair. The resulting PCR products were double-digested with *Bss*HII and *Apa*I, and then ligated into NL-vifS vector that had been subjected to double-digestion with the same enzymes. Recombinant plasmids were recovered by transformation of *Escherichia coli* JM109, yielding 1×10^4 colonies across a total of 12 dishes. We randomly picked 20 of the recombinant colonies and obtained DNA sequences for the CA-encoding region of each clone. We found that approximately two-thirds of the clones harboured mutations in the L4/5-encoding sequences. Among these clones, the number of mutated nucleotides varied from one to six, with a mean of three mutations each. The contents of each plate of the primary library were (separately) pooled and prepped. Twenty micrograms of the random-mutated plasmid library purified from each *E. coli* plate was transfected into 293T cells in 10 cm dishes using polyethylenimine. Three days after transfection, culture supernatants of the 12 plates were collected separately and assessed by ELISA for p24 antigen.

Selection of a random-mutagenized HIV-1 library in MT4 cells expressing CM TRIMCyp or CM TRIM5α. MT4 cells (1.2×10^6) were infected with SeV encoding CM-SPRY (−) TRIM5α at an m.o.i. of 10 p.f.u. per cell and incubated at 37 °C for 9 h. Cells were divided into 12 tubes, and each tube was then infected with a titre of each library equivalent to 100 ng p24. After incubating for 2 h, the cells were washed in 10 % FCS-supplemented RPMI 1640, then resuspended in 10 ml of the same medium and incubated at 37 °C for 4 days. The resulting culture supernatants (the replication-competent virus stocks) were harvested and stored at −80 °C. For the selection of the CM TRIMCyp- or CM TRIM5α-resistant virus, MT4 cells (1×10^6) were infected with SeV encoding CM TRIM5Cyp or CM TRIM5α, and then infected with a titre of each virus stock equivalent to 100 ng p24. Every 3 or 4 days, an aliquot (5 ml) of culture supernatant was collected and replaced with fresh medium. After 2–3 weeks, fresh (not SeV-infected) MT4 cells were added to rescue any existing resistant virus. When culture supernatant had accumulated p24 antigen to levels >100 ng ml^{-1}, we prepared MT4 cells newly infected with recombinant SeV and inoculated these cells with a titre of supernatant equivalent to 100 ng p24 for the next round of selection.

TRIM5 resistance assay. MT4 cells (10^5) were first infected with SeV encoding CM TRIM5Cyp, CM TRIM5α, or CM-SPRY (−) TRIM5α at an m.o.i. of 10 p.f.u. per cell and incubated at 37 °C for 9 h. Cells were then superinfected with a titre corresponding to 20 ng p24 of HIV-1 derivatives. The culture supernatants were collected periodically, and the levels of p24 were measured with an ELISA kit.

Single-round infection assay. MT4 cells were infected with SeV encoding CM TRIM5Cyp, CM TRIM5α or CM-SPRY (−) TRIM5α as described above, and superinfected with VSV-G-pseudotyped HIV-1 clones harbouring a luciferase reporter gene. Two days after infection, the luciferase activities in infected cells were measured by Bright-Glo Luciferase Assay (Promega) according to the manufacturer's instructions. The infectivity of a clone was measured by dividing the luciferase activity of that clone in the presence of CM-SPRY (−) TRIM5α by the luciferase activity of WT NL4-3-Luc-R-E in the presence of CM-SPRY (−) TRIM5α. To ascertain the resistance to CM TRIMCyp or CM TRIM5α of a clone, luciferase activity of that clone in the presence of CM TRIMCyp or CM TRIM5α, respectively, was divided by the luciferase activity of that clone in the presence of CM-SPRY (−) TRIM5α.

Virion incorporation assay and Western blotting. NL-Nh is a mutant of the NL4-3 proviral clone in which an *Nhe*I restriction enzyme cleavage site has been blunted and religated, introducing a frameshift mutation in the *env* gene (Sakuragi *et al.*, 2003). The *Bss*HII–*Apa*I fragments of capsid-encoding regions of 90A92E, RGDA, RETT, 86A116E and 4/5S were subcloned (separately) into the NL-Nh plasmid. Sequences encoding the CypA-like domain of CM TRIMCyp and authentic human CypA were PCR-amplified and cloned (separately) into the pCEP4 mammalian expression vector (Invitrogen). Then 293T cells were co-transfected with plasmids encoding the HA-tagged CypA region of CM TRIMCyp and the aforementioned HIV-1 NL-Nh derivatives. The resulting supernatants were layered onto a 2 ml cushion of 20 % sucrose in PBS and centrifuged at 35 000 r.p.m. for 2 h in a Beckman SW41 rotor to purify the virions. After centrifugation, the virion pellets were resuspended in PBS, and p24 antigen concentrations were measured by ELISA. A titre corresponding to 50 ng p24 of the respective HIV-1 derivative was subjected to SDS-PAGE. Proteins in the gel were then transferred electrically to a PVDF membrane (Immobilon; Millipore). Blots were blocked and probed with anti-HA high-affinity rat monoclonal antibody (Roche Diagnostics) or with anti-p24 antibody (Abcam) overnight at 4 °C. Blots then were incubated with peroxidase-conjugated anti-rat IgG antibody (American Qualex) or anti-mouse IgG antibody (KPL), respectively, and bound antibodies were visualized with a Chemi Lumi-One L chemiluminescent kit (Nacalai Tesque). Equivalent experiments were performed with a plasmid encoding HA-tagged authentic human CypA.

Computational modelling of mutant HIV-1 CA. Molecular models of NTD of HIV-1 CA mutants were constructed by homology

modelling using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) (Chemical Computing Group) and refined as described previously for the modelling of HIV-1 CA NTD (Nomaguchi *et al.*, 2013a, b, 2014). The crystal structure of the HIV-1 CA NTD at a resolution of 2.00 Å [Protein Data Bank (PDB) accession number 1M9C (Howard *et al.*, 2003)] was used as the modelling template. Superimpositions of the structures were done using the Protein Superpose module in MOE.

Propagation of CD8-depleted CM PBMCs and viral infection. The TRIM5 genotypes of individual monkeys were determined previously (Saito *et al.*, 2012). PBMCs from both CM TRIMCyp and CM TRIM5α homozygotes were suspended in RPMI 1640 medium supplemented with 10 % FBS, and the $CD8^+$ cells were removed with a magnetic bead system (Miltenyi Biotec). The CD8-depleted PBMCs were first stimulated for 2 days with 1 μg ml^{-1} PHA-L (Sigma) and then with human IL2 (100 U ml^{-1}) for an additional 2 days. An aliquot of 3×10^5 cells was then inoculated with a titre of virus equivalent to 60 ng p24 (for MN4Rh-3, RGDA112D, RGDA112D-Rh or 86A116E) or with a titre of virus equivalent to 60 ng p27 (for SIVmac239) and incubated at 37 °C in medium containing 100 U ml^{-1} human IL2. The culture supernatants were collected periodically, and the levels of viral antigen were measured using the ELISA kit.

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