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A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins

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Abstract

Background: The Gag capsid (CA) is one of the most conserved proteins in highly-diversified human and simian immunodeficiency viruses (HIV and SIV). Understanding the limitations imposed on amino acid sequences in CA could provide valuable information for vaccine immunogen design or anti-HIV drug development. Here, by comparing two pathogenic SIV strains, SIVmac239 and SIVsmE543-3, we found critical amino acid residues for functional interaction between the N-terminal and the C-terminal domains in CA.

Results: We first examined the impact of Gag residue 205, aspartate (Gag205D) in SIVmac239 and glutamate (Gag205E) in SIVsmE543-3, on viral replication; due to this difference, $\mathrm{Gag}_{206\text{-}216}$ (IINEEAADWDL) epitope-specific cytotoxic T lymphocytes (CTLs) were previously shown to respond to SIVmac239 but not SIVsmE543-3 infection. A mutant SIVmac239, SIVmac239Gag205E, whose Gag205D is replaced with Gag205E showed lower replicative ability. Interestingly, however, SIVmac239Gag205E passaged in macaque T cell culture often resulted in selection of an additional mutation at Gag residue 340, a change from SIVmac239 valine (Gag340V) to SIVsmE543-3 methionine (Gag340M), with recovery of viral fitness. Structural modeling analysis suggested possible intermolecular interaction between the Gag205 residue in the N-terminal domain and Gag340 in the C-terminal in CA hexamers. The Gag205D-to-Gag205E substitution in SIVmac239 resulted in loss of in vitro core stability, which was recovered by additional Gag340V-to-Gag340M substitution. Finally, selection of Gag205E plus Gag340M mutations, but not Gag205E alone was observed in a chronically SIVmac239-infected rhesus macaque eliciting $\mathrm{Gag}_{206\text{-}216}$-specific CTL responses.

Conclusions: These results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. Thus, this study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing insight into immunogen design to limit viral escape options.

Background

One of the characteristics of human immunodeficiency virus (HIV) is to induce persistent viral replication resulting in AIDS progression. HIV has enormous capacity to mutate and escape from host immune recognition, driving genetic diversification of the circulating viruses [1-3]. The Gag capsid (CA), comprising the N-terminal (NTD) and the C-terminal domains (CTD) [4-6], is one of the most conserved proteins in highly-diversified HIVs [7]. Understanding structural constraints in such viral proteins could provide valuable information for immunogen design in AIDS vaccine development.

Virus-specific cytotoxic T-lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infection [7-12]. CTLs exerting strong suppressive pressure on HIV replication select for viral mutations resulting in escape from CTL recognition [13-16]. Escape mutations in viral proteins with structural constraints are often selected with viral fitness costs, possibly facilitating subsequent immune control

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[3,17-23]. Thus, conserved viral proteins such as CA can be a promising antigen for vaccine-based CTL induction toward HIV control.

We previously showed vaccine-based control of a simian immunodeficiency virus mac239 (SIVmac239 [24]) challenge in a group of Burmese rhesus macaques possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [19,25]. $Gag_{206-216}$ (IINEEAADWDL) epitope-specific CTL responses play an important role in this control and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag (CA) with the cost of viral fitness [26]. However, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 [27], that has the same $Gag_{206-216}$ epitope sequence with SIVmac239; $Gag_{206-216}$-specific CTLs did not show responses against SIVsmE543-3 infection due to an aspartate (D)-to-glutamate (E) change, GagD205E, at Gag residue 205 [28].

Thus, the GagD205E substitution in SIVmac239 could result in viral escape from $Gag_{206-216}$-specific CTL recognition. However, in our previous analyses of *90-120-Ia*-positive animals eliciting $Gag_{206-216}$-specific CTL responses for one or two years postchallenge, we observed selection of GagL216S, but not GagD205E mutation in SIVmac239 infection, suggesting a possibility that the GagD205E substitution results in larger reduction of viral replicative ability than GagL216S. In the present study, we first constructed a mutant SIVmac239, SIVmac239Gag205E, with the GagD205E substitution and examined its replication ability in vitro. We found that this amino acid change in the CA NTD results in loss of viral fitness, which can be recovered by an additional amino acid change in the CA CTD. Further analyses presented in vitro and in vivo evidence for a structural constraint in the functional interaction between SIV CA NTD and CTD.

Results

Compensation for loss of viral fitness in SIVmac239Gag205E by additional GagV340M substitution

We first constructed a mutant SIVmac239 molecular clone DNA with a mutation of a D-to-E substitution at the 205th aa in Gag (CA NTD) to obtain the mutant virus, SIVmac239Gag205E (Figure 1). Analysis of viral replication kinetics on HSC-F, a macaque T cell line, revealed delayed peak of the mutant SIVmac239-Gag205E replication, indicating its lower replicative ability compared to the wild-type SIVmac239 (Figure 2).

We further followed up SIVmac239Gag205E replication on HSC-F cells and explored a possibility of viral reversion or additional mutations (Figure 3). No additional gag mutation became dominant on day 10 after SIVmac239Gag205E infection. Interestingly, however, in the second culture after passage of the first culture supernatants on day 10 into uninfected HSC-F cells, an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag (CA CTD), became dominant in two of four sets of experiments; SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. The GagD205E mutation remained dominant, and no other mutations were detected in the CA-coding region even in the second culture.

We then constructed a mutant SIVmac239 molecular clone DNA by introducing the GagV340M mutation into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E340M (Figure 1). This mutant SIV showed similar replication kinetics on HSC-F cells with the wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution (Figure 2). These results imply that SIV CA with Gag205D-340V or Gag205E-340M combination is functional whereas the CA with Gag205E-340V is less functional.

Possible interaction between Gag residues 205 and 340 in SIV CA hexamers

Recovery of viral fitness of SIVmac239Gag205E by the GagV340M substitution suggests a possibility of interaction between Gag residues 205 in the NTD and 340 in the CTD. Modeling of CA monomer structure, however, showed that the Gag 205th residue is located in the helix 4 of CA NTD, while the 340th is in the loop between helices 10 and 11 of CTD, which does not support a possibility of intramolecular contact between Gag residues 205 and 340 (data not shown).

CA molecules are known to form hexamer lattice in mature virions [29-33]. Modeling of CA hexamer structure revealed that the Gag 205th residue in the NTD is located in close proximity to the 340th in the CTD of the adjacent CA molecule (Figure 4). These observations support a possibility of intermolecular interaction between Gag residues 205 and 340 in CA hexamers.

In addition, the 312th residue in the loop between helices 8 and 9 of CTD is located in close proximity to the 205th in the NTD of the adjacent CA molecule. Because SIVmac239 and SIVsmE543-3 have different amino acids at this residue 312, alanine (A) in the former and proline (P) in the latter, we also constructed a mutant SIVmac239 molecular clone DNA by introducing the GagA312P mutation resulting in A-to-P substitution at the 312th aa in Gag into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E312P (Figure 1). Analysis of replication kinetics on HSC-F cells indicated recovery of viral fitness by the additional GagA312P substitution in SIVmac239Gag205E (Figure 2).

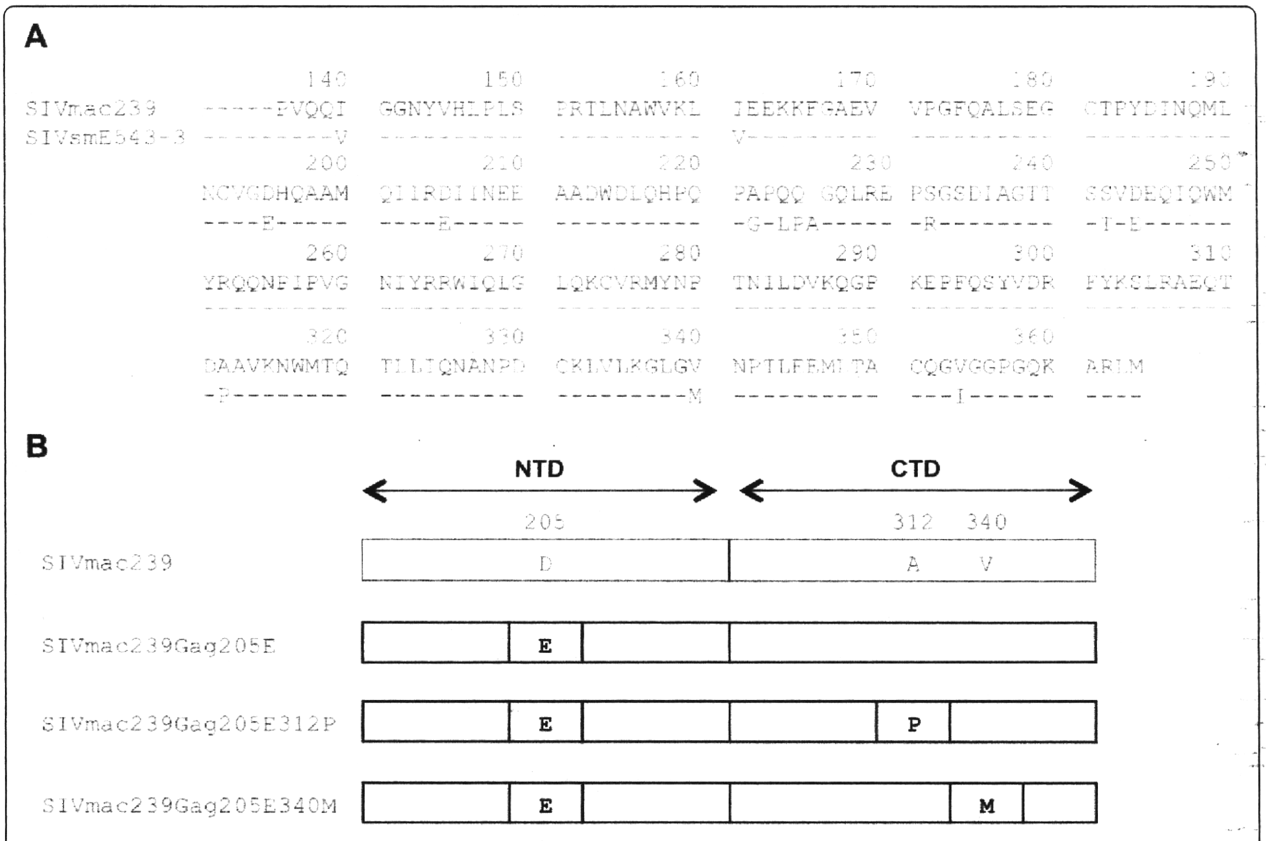

Figure 1 SIV CA amino acid sequences. (A) Comparison of SIVmac239 amino acid sequences in CA, Gag residues 136-364, with SIVsmE543-3 (GenBank accession number U72748). (B) Schema indicating the amino acid substitutions in mutant SIV CA.

Full recovery of viral fitness in SIVmac239Gag205E340M

We then focused on analyzing the possibility of functional interaction between Gag residues 205 in CA NTD and 312/340 in CA CTD. To confirm differences in viral fitness among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M, we compared their replicative ability by viral competition assay (Table 1). The competitions confirmed lower viral fitness of SIVmac239Gag205E compared to wild-type SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag340M. SIVmac239Gag205E312P showed lower viral fitness than SIVmac239, whereas replication ability of SIVmac239Gag205E340M was no less than the wild-type. These results indicate that the GagD205E substitution in SIVmac239 reduced viral fitness, which was recovered partially by an additional GagA312P and fully by an additional GagV340M substitution. The competition between SIVmac239 and SIVmac239Gag205E340M at the ratio of 1:1 resulted in selection of the latter, suggesting that SIV CA with Gag205E-340M combination observed in SIVsmE543-3 may be slightly more functional than that with Gag205D-340V in SIVmac239.

Inhibition of the early phase of SIVmac239Gag205E replication

We examined whether the GagD205E substitution affects the early or late phase of SIVmac239 replication. On LuSIV cells, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, SIVmac239Gag205E312P, or SIVmac239Gag205E340M, indicating suppression of the early phase of SIVmac239GagD205E replication (Figure 5). In contrast, we did not find a significant difference in viral production among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 6). These results indicate that the loss of viral fitness by the GagD205E substitution is mainly due to inhibition of the early phase of viral replication.

Loss of in vitro core stability in SIVmac239Gag205E

If the GagD205E substitution disturbs intermolecular CA interaction for hexamer formation, it may affect SIV core stability. To assess the core stability in vitro [34], concentrated viruses were separated into three fractions by ultracentrifugation under gradient sucrose

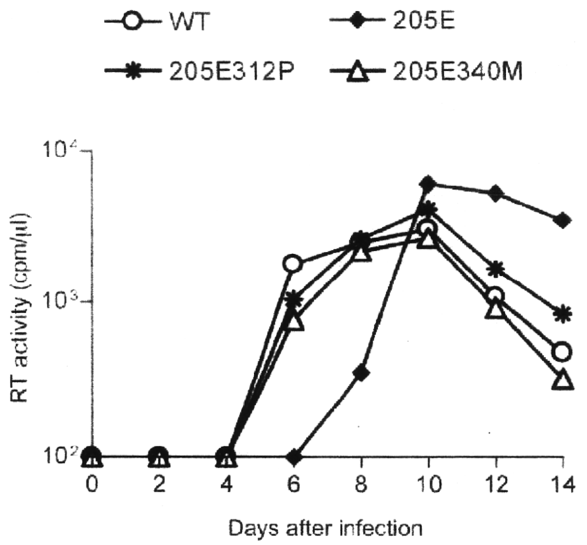

Figure 2 Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (WT, open circles), SIVmac239Gag205E (205E, closed diamonds), SIVmac239Gag205E312P (205E312P, asterisk), or SIVmac239Gag205E340M (205E340M, open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. A representative result from five sets of experiments is shown.

concentrations in the presence of Triton X-100 and each fraction was subjected to Western blot analysis to detect CA p27 proteins (Figure 7). In the absence of Triton X-100, CA proteins were detected in the bottom fraction, whereas those in the presence of 1% Triton X-100 were sensitive to the detergent and detected not in the bottom but only in the top fraction (data not shown). We compared the in vitro viral core stability between SIVmac239 and SIVmac239Gag205E in the presence of 0.6%, 0.9%, and 1.35% Triton X-100, respectively, and found a difference in the presence of 0.6% Triton X-100. Additional experiments revealed that SIVmac239Gag205E core was more sensitive to 0.6% Triton X-100 treatment than SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 7). These results suggest that viral core stability may be reduced by GagD205E substitution but can be recovered by additional GagA312P or GagV340M substitution.

Selection of GagD205E plus GagV340M mutations in a SIVmac239-infected macaque

The GagD205E substitution results in viral escape from Gag$_{206\text{-}216}$-specific CTL recognition. Finally, we examined whether this substitution can be selected in the chronic phase of SIVmac239 infection in *90-120-Ia*-positive macaques eliciting Gag$_{206\text{-}216}$-specific CTL responses using plasma samples obtained in our previous experiments [35,36]. SIVmac239-infected *90-120-Ia*-positive macaques select the GagL216S mutation resulting in viral escape from Gag$_{206\text{-}216}$-specific CTL recognition, but we found selection of both GagD205E and GagV340M mutations in viral genomes in one animal, R01-007 (Table 2). In this animal, GagD205E and GagV340M mutations were undetectable at week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant at week 123 was not detected at week 150. These results present in vivo evidence indicating functional interaction between the Gag 205th residue in NTD and the 340th in CTD of SIV CA.

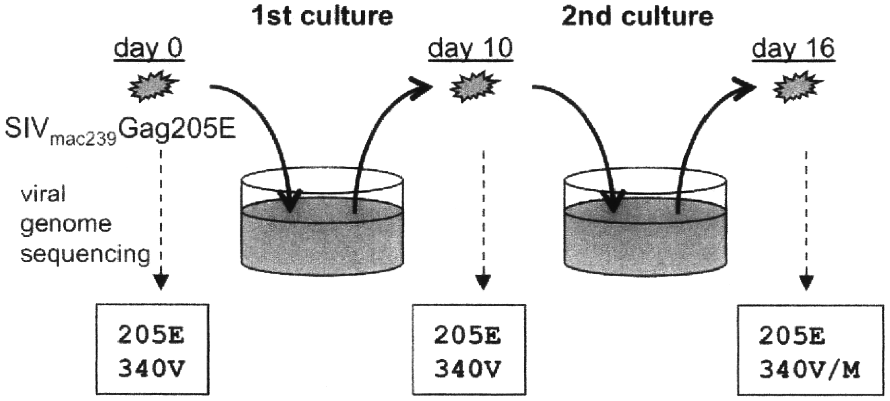

Figure 3 Passage of SIVmac239Gag205E culture supernatants. HSC-F cells were infected with SIVmac239Gag205E. The culture supernatant on day 10 was added to fresh HSC-F cells to start the second culture. Viral RNAs were extracted from the first culture supernatant on day 10 and the second culture supernatant on day 16 after the initial infection and subjected to sequence analyses. Dominant amino acid at the 340th residue remained V on day 10 in all cases but was M on day 16 in two of four sets of experiments (Gag340M was detectable on day 10 in these two sets of experiments). No other amino acid change was observed in the CA-coding region.

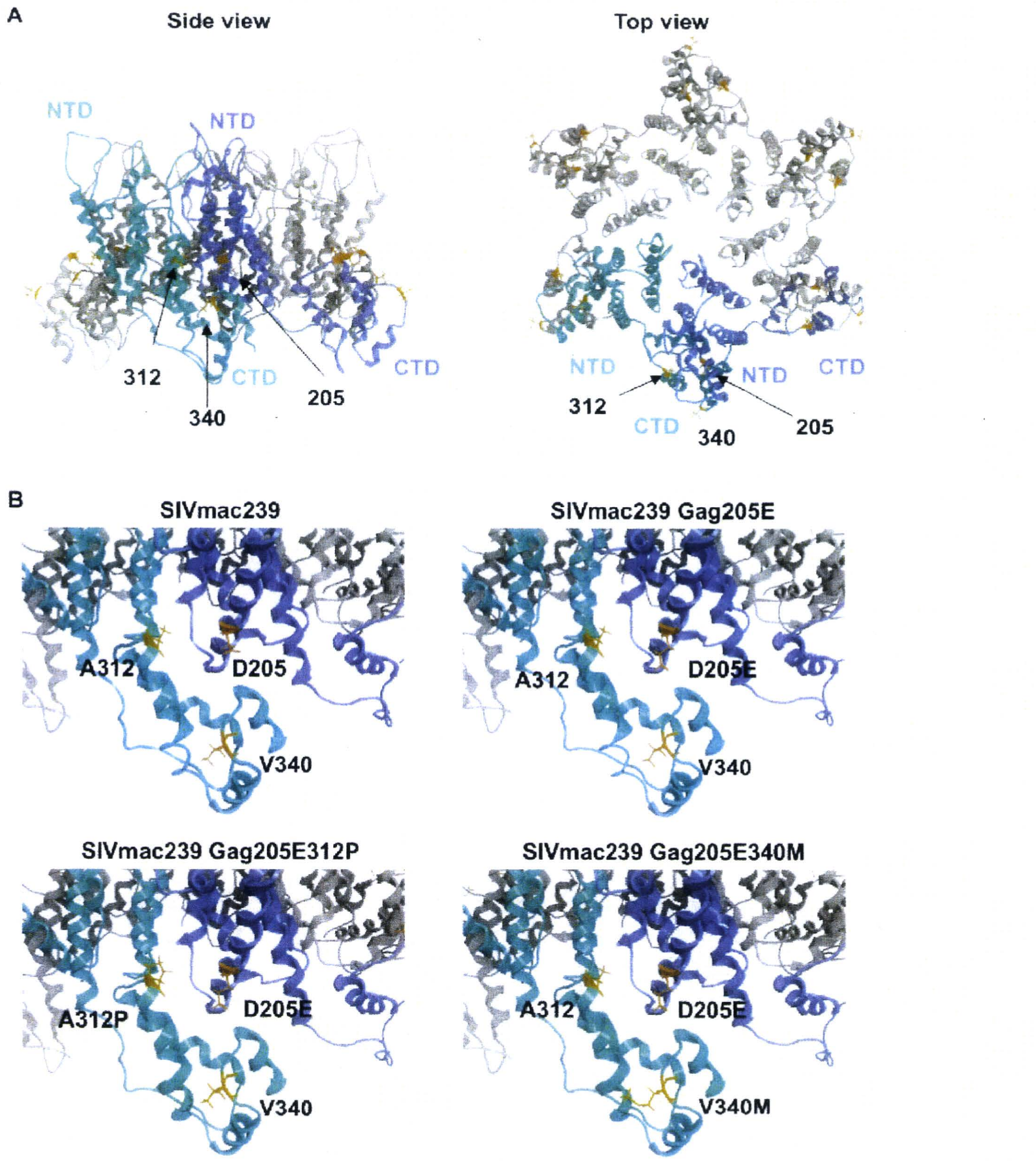

Figure 4 Structural models of SIVmac239 CA hexamer. The hexameric SIVmac239 CA models were constructed by homology-modeling using a crystal structure of the hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47[33]) as a modeling template. "MOE-Align" and "MOE-Homology" in MOE version 2008.1002 were used for the modeling. The side chains of the 205th, 312th, and 340th aa in Gag are shown as orange sticks. (A) Overall structure of SIVmac239 CA hexamer. (B) The hexameric structures near positions 205, 312, and 340 of wild-type and mutant SIVmac239 CAs.

Discussion

The Gag CA which is one of the most conserved proteins in HIV and SIV may be a promising immunogen for CTL-based AIDS vaccines. However, the limitations imposed on amino acid sequences in CA are not fully understood. In the present study, we found that the GagD205E substitution in SIVmac239 CA NTD reduces viral fitness, which is recovered by additional GagA312P or GagV340M substitution in the CTD. SIVmac239-Gag205E passaged in cell culture often resulted in selection of an additional GagV340M mutation. Furthermore, selection of Gag205E plus Gag340M mutations, but not Gag205E alone, was observed in a chronically SIVmac239-infected rhesus macaques. These results provide evidence indicating a functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD,

Table 1 Competition between SIV mutants[a]

SIVs in competition	Ratio of inoc. titers[b]	Exp. no.	Dominant aa sequences[c]			
			day 6		day 18	
SIVmac239 & SIVmac239Gag205E	4:1	#1	205D		205D	
		#2	205D		205D	
	1:1	#1	205D		205D	
		#2	205D		205D	
	1:4	#1	205D		205D	
		#2	205D		205D	
SIVmac239 & SIVmac239Gag205E312P	4:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:4	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
SIVmac239 & SIVmac239Gag205E340M	4:1	#1	205D	340V	205D	340V
		#2	205D	340V	205D	340V
	1:1	#1	205D/E	340V/M	205E	340M
		#2	205D/E	340V/M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
SIVmac239Gag205E & SIVmac239Gag205E312P	4:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:4	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
SIVmac239Gag205E & SIVmac239Gag205E340M	4:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M

[a] HSC-F cells were coinfected with two kinds of SIVs indicated. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants on days 6 and 18 postinfection and then sequenced. Results from two sets of experiments (Exp. #1 and #2) are shown.

[b] The ratio of the dose (RT activity) of the virus indicated at the top to that at the bottom at coinfection.

[c] Dominant amino acid sequences at the positions where mutations were included in the inoculums are shown. 205D/E, D and E were detected equally at the 205th aa in Gag; 340 V/M, V and M were detected equally at the 340th aa in Gag.

presenting a structural constraint for functional interaction between SIV CA NTD and CTD.

HIV and SIV Gag proteins are expressed as unprocessed polyproteins, which are assembled and incorporated into the virions. Concomitant with viral budding, incorporated Gag polyproteins are proteolytically cleaved by viral protease into processed proteins including MA (matrix), CA, and NC (nucleocapsid), participating in mature infectious virion formation [37,38]. Recent structural analyses [31-33,39-41] indicated that CA proteins form hexamer lattice in matured virions; in the mature CA core, the intermolecular NTD-NTD and NTD-CTD interfaces are involved in the formation of CA hexamers, while the intermolecular CTD-CTD interface connects neighboring hexamers. Our modeling analyses did not support a possibility of intramolecular interaction but indicated possible intermolecular interaction between Gag205 in CA NTD and Gag312/340 in CA CTD, which may affect CA hexamer formation during viral maturation. This is consistent with our results in Figure 5 indicating that the GagD205E substitution results in inhibition of the early phase of SIVmac239 replication, which can be overcome by additional GagA312P or GagV340M substitution. This possibility is supported also by our results on viral core stability in vitro, although it remains unclear how much extent the

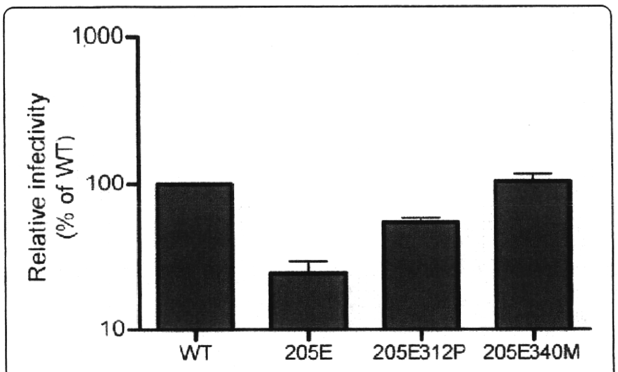

Figure 5 Mutant SIV infection. LuSIV cells were infected with SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340M (205E340M). Luciferase activity was measured 24 hr after infection. Relative infectivity is shown as the ratio (%) of the luciferase activity to that of SIVmac239 (WT). Mean values in three sets of experiments are shown.

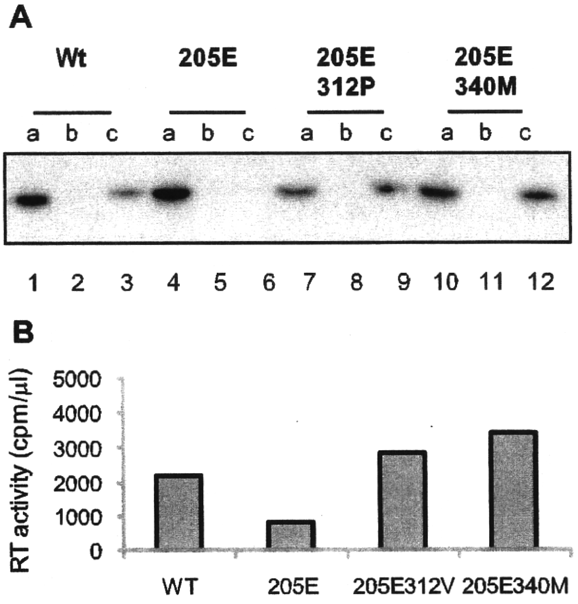

Figure 7 SIV core stability in vitro. Concentrated SIVmac239 (Wt; lanes 1-3), SIVmac239Gag205E (205E; lanes 4-6), SIVmac239Gag205E312P (205E312P; lanes 7-9), or SIVmac239Gag205E340M (205E340 M; lanes 10-12) was separated into three fractions (top [a], middle [b], and bottom [c]) by ultracentrifugation under gradient sucrose concentrations in the presence of 0.6% Triton X-100. Each fraction was subjected to Western blot analysis to detect SIV CA p27 proteins (A). A representative result from three sets of experiments is shown. The bottom (c) fractions were also subjected to RT assay (B).

core stability in vitro can reflect the one in vivo [42]. There has been no report suggesting the influence of the Gag 205 residue on SIV sensitivity to tripartite interaction motif 5α (TRIM5α). A previous report on HIV CA lattice [31,43] indicated a potential interaction between the helix 4 of NTD and the loop connecting helices 10 and 11 of CTD in the adjacent molecule. Our results suggest the possible involvement of Gag205 and Gag340 residues in this intermolecular NTD-CTD interaction in CA hexamers.

The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which looked to be compensated by GagV340M substitution (Figure 4). The modeling can draw a hydrophobic pocket between Gag205 and Gag340 residues in SIVmac239Gag205E340M as well as SIVmac239, but not in SIVmac239Gag205E CA hexamers. Thus, this pocket may be a target candidate for anti-viral drugs.

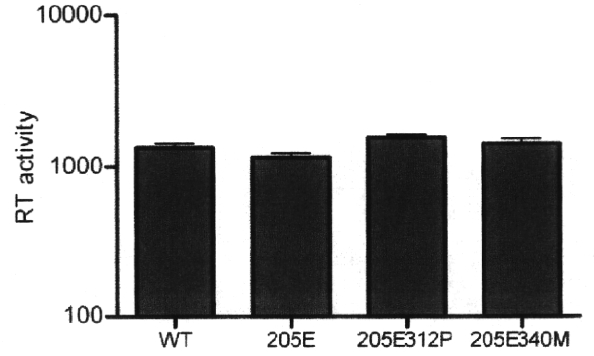

Figure 6 Mutant SIV production. COS-1 cells were transfected with molecular clone DNAs of SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340 M (205E340 M). RT activity of the culture supernatants two days after transfection was measured. Mean values in five sets of experiments are shown.

Both GagL216S and GagD205E mutations can result in escape from $\mathrm{Gag}_{206\text{-}216}$-specific CTL recognition [19,28], but the former is usually selected in SIVmac239-infected *90-120-Ia*-positive macaques probably

Table 2 Viral gag sequences in macaque R01-007 infected with SIVmac239[a]

Wks after challenge	Amino acid sequences[b]		
	at 205th	at 216th	at 340th
123	D	S	V
137	D (E)	S (L)	V (M)
150	E	L	M

[a] Viral RNAs were extracted from plasma obtained from a 90-120-Ia-positive macaque R01-007 at weeks 123, 137, and 150 after SIVmac239 challenge. Viral *gag* fragments were amplified by RT-PCR from viral RNAs and then sequenced. This animal showed efficient $\mathrm{Gag}_{206\text{-}216}$-specific CTL responses and vaccine-based control of a SIVmac239 challenge with rapid selection of the GagL216S escape mutation (at week 5), but accumulated viral mutations in the chronic phase, leading to reappearance of plasma viremia around week 60 after challenge as described previously [19,35].

[b] Dominant amino acid sequences at the 205th, 216th, and 340th aa in Gag are shown. Parentheses indicate the sequences that are not dominant but detectable.

because the latter reduces viral fitness more severely than the former. In this study, we found selection of GagD205E plus GagV340M mutations in the chronic phase of SIVmac239 infection in a *90-120-Ia*-positive macaque. In this animal, the CTL escape GagL216S mutation first selected after SIVmac239 challenge became undetectable and was replaced with the CTL escape GagD205E mutation in combination with GagV340M in the chronic phase. This may imply that the GagD205E plus GagV340M mutations might be more advantageous than the GagL216S mutation for SIVmac239 replication in the presence of $\text{Gag}_{206\text{-}216}$-specific CTL pressure.

We observed the addition of GagV340M mutation but not a Gag205E-to-Gag205D reversion in SIVmac239-Gag205E passage. This may be due to difference in frequencies between purine-to-purine (guanine-to-adenine) change in the former and purine-to-pyrimidine (adenine-to-thymine) change in the latter. The appearance of additional GagV340M mutation in SIVmac239-Gag205E passaged in cell culture as well as the selection of GagD205E plus GagV340M mutations in an animal provides key evidence indicating functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD. The Gag is a promising candidate as a vaccine immunogen for CTL induction, because cumulative studies have indicated the efficacy of Gag-specific CTL responses against HIV and SIV infection [7,25,44,45]. However, viral mutational escape from CTL recognition is a major challenge for AIDS vaccine design. Thus, the information on the structural constraint presented in this study might be helpful for immunogen design in AIDS vaccine development.

Conclusions

Our results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. SIV CA with Gag205D-340V (observed in SIVmac239) or Gag205E-340M combination (observed in SIVsmE543-3) is functional whereas the CA with Gag205E-340V is less functional. Thus, the present study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing valuable information for immunogen design to limit viral escape options.

Methods

Analysis of mutant SIV replication

SIV molecular clone DNAs with gag mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 molecular clone DNA [24]. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs using Lipofectamine LTX PLUS (Invitrogen, Tokyo, Japan). Viral titers were measured by reverse transcription (RT) assay as described previously [46]. For analysis of viral replication kinetics, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) [47] were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. To examine viral infectivity, LuSIV cells, which are derived from CEMx174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, were cultured for 24 hr after viral infection and then lysed in a reporter lysis buffer (Promega Corp., Tokyo, Japan) for measurement of the luciferase activity in a luminometer (GloMax™ 96 Microplate Luminometer, Promega Corp.).

Viral competition assay

HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants harvested every other day were used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 post-coinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire gag region was amplified from the RNA by RT-PCR and sequenced to determine dominant sequences as described previously [19].

Molecular modeling of hexameric SIVmac239 CA

The crystal structures of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C[48]), HIV-1 CA CTD at a resolution of 1.70 Å (PDB code: 1A8O[5]), and hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47 [33]) were taken from the RCSB Protein Data Bank [49]. Three-dimensional (3-D) models of monomeric SIVmac239 CA were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) version 2008.1002 (Chemical Computing Group Inc., Quebec, Canada) as described [50,51]. 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 [52]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [53] combined with the generalized Born model of aqueous solvation implemented in MOE [54]. Physically unacceptable local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE. The structures of hexameric SIVmac239 CA were generated from the monomeric structures by MOE on the basis of the assembly information of hexameric HIV-1 CA crystal structure [33].

Analysis of viral CA core stability in vitro

Detergent treatment of wild-type and mutant SIV particles was performed essentially as described previously [34]. Briefly, viruses from COS-1 cells transfected with viral molecular clone DNAs (normalized by RT activity) were concentrated by ultracentrifugation at 35,000 × rpm for 75 min at 4°C in a SW41 rotor (Beckman Instruments, Tokyo, Japan) through a cushion of 20% sucrose in phosphate buffered saline (PBS). The concentrated viral pellets were suspended in PBS. Sucrose step gradients were prepared in SW55 centrifuge tubes with the 2.0 ml layer of 60% sucrose on the bottom and 2.1 ml layer of 20% sucrose overlaid. Then, 0.1 ml of Triton X-100 in PBS and 0.5 ml of concentrated viruses were overlaid and ultracentrifuged at 35,000 × rpm for 60 min at 4°C in a SW55Ti rotor (Beckman Instruments). Three fractions (top [a], middle [b], and bottom [c]) of 1.1 ml each were collected from the top and subjected to Western blot analysis using plasma from a simian-human immunodeficiency virus 89.6PD-infected rhesus macaque [55] and RT assay.

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Authors' contributions

NI and TM designed the study. NI, HT, and AR performed virological analyses in vitro. MY and HS performed structure modeling analyses. HY and MK examined viral genome sequences. NI and TM analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Contribution of Cyclophilin A to determination of simian immunodeficiency virus tropism: A progress update

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ABSTRACT

An understanding of cellular factors that affect viral replication contributes to elucidation of the mechanism for the determination of viral tropism. Cyclophilin A (CypA), a peptidyl-prolyl *cis-trans* isomerase (PPIase), is an essential host factor for the efficient replication of human immunodeficiency virus type 1 (HIV-1) in human cells. However, its role in simian immunodeficiency virus (SIV) replication has not been determined. In the 2008 US–Japan AIDS panel meeting, I have presented the effect of cyclosporine A (CsA), a PPIase inhibitor, on replication of wild-type SIV. Interestingly, CsA treatment enhanced SIV replication in human cells but abrogated SIV replication in macaque cells, implying a species-specific effect of CsA on SIV replication. After this meeting, analysis using CypA knocked-down human cells indicated that CypA was considered inhibitory for SIV replication. These results suggest possible involvement of CypA in the determination of SIV tropism.

1. Introduction

The conventional innate and adaptive immune systems are very effective at viral infections. However, for retroviral infections, there is another immune system that can recognize at multiple levels, e.g., expression of internal host factors with antiviral activity. This is a component of viral recognition and subsequent restriction that has been called "intrinsic immunity" [1]. Intrinsic immunity can distinguish from innate and adaptive immunity, and it does not need to be induced by viral infections. Viral replication involves many host cell factors, whose species-specific expression contributes to viral tropism. Aside from host factors essential for virus replication, there are host factors that restrict viral replication and provide intrinsic immunity to virus infection. The antiviral effect mediated by these intrinsic restriction factors has been indicated to play an important role in making species-specific barriers against viral infection.

For instance, Fv-1 is known to restrict replication of a murine leukemia virus in mice [2–4]. In addition, tripartite interaction motif 5a (TRIM5a) has recently been found to be responsible for restricting HIV-1 but not simian immunodeficiency virus (SIV) infection in old world monkey (OWM) cells [5–9]. Retrovirus restriction by these host cell factors occurs after viral entry but before integration during the viral replication cycle. The viral determinants for this type of restriction have been mapped to the capsid (CA) protein [2,4,10–12]. Understanding the precise function of these host factors will be important to elucidate the mechanism for determining viral tropism.

CypA is a host cell factor essential for efficient HIV-1 replication in human cells [13–19]. It promotes HIV-1 infection at a post-entry level in the early phase of virus replication [20]. CypA is efficiently incorporated into the virions produced from HIV-1-infected cells through its interaction with viral CA in the context of Gag polyprotein [15,16,18]. Disruption of its incorporation by Gag mutations or by treatment with CsA reduced the infectivity of progeny viruses [14,16,18,21–24]. Furthermore, promotion of HIV-1 replication by post-entry interaction of viral CA with CypA in the target cells has been shown, suggesting the importance of CypA at the site for post-entry step for efficient HIV-1 replication in human cells [17,19,25–27].

In contrast, the effect of CypA on SIV replication has not been determined well. Previous study proposed a possible interaction between CypA and SIV CA [13,28,29]; however, its functional significance remains largely unknown. A more recent our study showed that human CypA exerts an inhibitory effect on *vif*-deleted SIV replication in human Jurkat cells; this effect was negated by SIV Vif, which excluding CypA from SIV virions [29]. This Vif function can be distinguished from its anti-APOBEC3G (apolipoprotein B mRNA-editing enzyme-catalytic subunit 3G) function that has been well established [30].

In my oral presentation at the 2008 US–Japan AIDS panel meeting, I have presented that SIV Vif counteracts human APOBEC3G and CypA-imposed inhibition of SIV in human cells, and the role of CsA, a drug known to inhibit PPIase activity, in wild-type SIV replication. Treatment of target cells with CsA resulted in the enhancement of

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SIV replication in human cells but, surprisingly, abrogated SIV replication in macaque cells, indicating that CypA may exert inhibitory effect on SIV replication in human cells but is required for efficient SIV replication in macaque cells. Comparison between human and macaque CypA revealed no difference in their amino acid sequences. Our results indicate that CypA may contribute to the determination of SIV tropism, implying an unknown host cell factor involved in this contribution.

2. SIV Vif requires functional inactivation of human APOBEC3G (hApo3G) in human cells

Recently, cytidine deaminases were identified as a new class of antiviral factors that target retroviruses such as HIV-1 or SIV [30–32]. Most prominent among those is Apo3G, a host cytidine deaminase with potent antiviral activity whose function is sensitive to the activity of the HIV-1 Vif protein [30]. Unlike Trim5a or Fv1, A3G does not exert its antiviral activity by targeting the incoming viral capsid protein but instead is packaged into virus particles and inhibits virus replication by targeting single-stranded viral cDNA.

The function of Vif is species-specific [32]. Accordingly, human Apo3G (hApo3G) is insensitive to SIVagm Vif while African green monkey Apo3G (agmApo3G) is insensitive to HIV-1 Vif and the determinant of this species specificity depend on amino acid 128 of hApo3G and agmApo3G. However, such species specificity is not absolute. In fact, we showed that SIVagm Vif was able to support the replication of SIVagm virus in the hApo3G-positive human A3.01 T-cell line [33]. Replication of *vif*-defective SIVagm in A3.01 cells was severely restricted and resulted in an accumulation of cytidine deaminase-induced G-to-A mutations in the SIVagm genome [33]. Therefore, it is probable that SIV Vif has evolved to counteract hApo3G restriction and this might contribute zoonotic transmission of SIV.

3. SIV Vif is also required for efficient SIV replication in hA3G-negative human Jurkat T cells

Previous reports showed that human CypA is required for efficient HIV-1 infection but not SIV. There is no known role for CypA in SIV infection in human cells. Recently, our study has shown that replication of *vif*-deleted SIV was still disturbed even in the human Jurkat T-cell line, which lacks APOBEC deaminase activity [29]. This report has further indicated the involvement of Vif in the exclusion of CypA from SIV virions and recovery of replication fitness. These results suggested an inhibitory effect of CypA on *vif*-deleted SIV replication in human cells. This phenomenon can distinguish from the function of SIV Vif against antiviral activity of hApo3G [29] because we used human cells lacking detectable deaminase activity. This observation also raised the possibility that SIV Vif may contribute for zoonotic transmission of SIV from monkey to human.

4. CsA treatment enhanced SIV replication in human T cells

We extended our analysis of SIV replication in another human T-cell line, CEM-SS, also lacking detectable deaminase activity. Similar to the results in Jurkat cells, *vif*-deleted SIVagm incorporated much more CypA into the virion and exhibited less efficient replication compared to the wild-type SIVagm, whereas *vif* deletion in HIV-1 had no effect on CypA incorporation or virus replication in CEM-SS cells. *Vif* deletion also enhanced CypA packaging and abrogated viral replication of SIVmac239 in CEM-SS cells.

Then, we investigated the precise role of CypA in wild-type SIV replication in CEM-SS cells by using CsA and CypA incorporation into virions. Replication of wild-type SIVagm, SIVmac, and HIV-1 in the presence of CsA was compared to virus replicaton in the absence of drug. Consistent with previous reports, CsA treatment inhibited the packaging of CypA into HIV-1 particles and HIV-1 replication in CEM-SS cells (Fig. 1A, left panel). Interestingly, however, CsA treatment enhanced SIVagm and SIVmac replication in CEM-SS cells, although it inhibited the packaging of CypA into SIVagm and SIVmac particles (Fig. 1A, right panel). These results indicate that CypA, essential for efficient HIV-1 replication, inhibits SIV replication in CEM-SS cells. This CsA-mediated enhancement of SIV replication was also observed in another human T-cell line after this meeting.

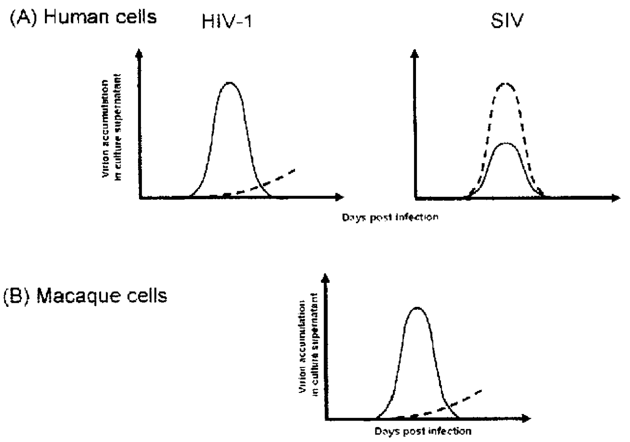

Fig. 1. A schema for the effect of CsA on HIV/SIV replication in human/macaque cells. (A) CsA treatment inhibits HIV-1 replication (left panel) but enhances SIV replication (right panel) in human cells. (B) CsA treatment inhibits SIV replication in macaque cells. The solid line indicates virion accumulation of culture supernatant in the absence of CsA and the broken line indicates that of culture supernatant in the presence of CsA.

5. Treatment of target cells with CsA enhanced the post-entry process in SIV replication in human cells

Recently, CypA in viral target cells has been found to be crucial for the post-entry process of HIV-1 replication in human cells [17,19,25–27], but its role in SIV replication has remained undetermined. We then examined the effect of CsA treatment in target cells on the post-entry process in SIV replication. Viruses were produced from CsA-untreated or CsA-treated CEM-SS cells and infected into CsA-untreated or CsA-treated target CEM-SS cells. Total DNA was prepared from the target CEM-SS cells 24 h after the infection and viral cDNA levels were measured by quantitative PCR analysis. CsA treatment of the producer cells and of the target cells both reduced HIV-1 cDNA synthesis, confirming the requirement of CypA in human producer and target cells for efficient HIV-1 replication as reported previously. In contrast, SIVagm cDNA synthesis was enhanced by CsA treatment of the target cells, although it was diminished by CsA treatment of the producer cells. SIVmac cDNA synthesis was also enhanced by target CypA depletion but inhibited by producer cell-CypA depletion. Thus, SIV produced from CsA-treated CEM-SS cells showed diminished infectivity in CsA-untreated target CEM-SS cells, but the infectivity in CsA-treated target CEM-SS cells was recovered and tended to be higher than that of CsA-untreated CEM-SS-derived SIV in CsA-untreated target cells, explaining the reason for the enhancement of SIV replication in CEM-SS cells by CsA treatment. These results indicate that CypA supports infectious SIV production, suggesting the importance of CypA uptake into the SIV virion for its infectivity, whereas target cell-CypA has inhibitory effect on the post-entry process in SIV replication in human cells.

6. CsA treatment inhibited SIV replication in macaque cells

The above results demonstrated an inhibitory effect of CypA on SIV replication in human cells. We next examined the effect of CsA on SIV replication in macaque cells. We made use of three macaque T-cell lines, cynomolgus macaque-derived HSC-F, rhesus macaque-derived HSR-5.4, and pig-tailed macaque-derived Mn-3942 cells [34]. SIVagm and SIVmac replicated well in all of these cell lines; however, the most efficient replication was observed in HSC-F cells. Remarkably, in contrast to the results obtained in human CEM-SS cells, CsA treatment severely inhibited replication of SIVagm and SIVmac in all of these macaque T-cell lines (Fig. 1B). CypA was incorporated into the virions in the absence of CsA, but its incorporation was inhibited by CsA treatment of these cells. HIV-1 replication was undetectable in these macaque T-cell lines even in the presence of CsA, although the possibility of enhancement of HIV-1 replication by CsA treatment in OWM cell lines has been indicated [35–37]. The inhibitory effect of CsA on SIV replication was also observed in primary peripheral blood mononuclear cells (rhPBMC) from rhesus macaques. These results revealed that in contrast to human cells, CypA is required for efficient SIV replication in macaque cells.

The observation that CypA affects SIV replication negatively in human cells but positively in macaque cells could be due to possible differences in the functional properties of human and macaques CypA. Interestingly, however, sequence analyses of CypA cDNA in human CEM-SS cells and macaque HSC-F, HSR-5.4, and Mn-3942 cells showed no difference in amino acid sequences between human-derived and macaque-derived CypA.

7. Treatment of target cells with CsA inhibited the post-entry process in SIV replication in macaque T cells

Next, we examined the effect of producer cell- and target cell-CypA on SIV replication in macaque cells, respectively. Measurement of viral cDNA levels in target cells infected with SIVagm produced from CsA-untreated or CsA-treated HSC-F cells revealed that CsA treatment of the producer HSC-F cells resulted in the production of SIVagm with lower infectivity, similarly with the results obtained in human CEM-SS cells. SIVmac produced from CsA-treated HSC-F cells also exhibited lower infectivity, indicating the importance of producer cell-CypA for efficient SIV replication in macaque as well as human cells.

More importantly, in contrast to the results obtained in human CEM-SS cells, CsA treatment of the target HSC-F cells resulted in diminished viral cDNA synthesis after SIVagm and SIVmac infection, indicating requirement of the target CypA for the post-viral entry process in SIV replication in HSC-F cells. Therefore, target cell-CypA as well as producer cell-CypA is required for efficient SIV replication in macaque cells.

8. The effect of CypA on SIV replication in human cells: a progress update

To examine whether CypA dysfunction actually contributes to this enhancement of SIV infection in CsA-treated target CEM-SS cells, we established CEM-SS cell lines, CypA-KD, in which CypA expression was stably suppressed by CypA-specific shRNA. Consistent with previous reports, CypA knock-down reduced viral cDNA synthesis after HIV-1 infection (Fig. 2, lower left panel) and inhibited HIV-1 replication as well as CsA-imposed inhibition of HIV-1 replication (Fig. 2, upper left panel). In contrast, the amount of viral cDNA synthesized after SIV infection in CypA-KD cells was only slightly higher than that in CEM-SS cells in the absence of CsA (Fig. 2, lower right panel), while in the presence of CsA, the former was lower than the latter; from these comparisons, it was difficult to define the effect of CypA knock-down on SIV replication (Fig. 2, upper right panel). Importantly, the enhancement of SIV infection by CsA treatment of target cells was reduced by CypA silencing. These results indicate that CypA dysfunction contributed to the enhancement of SIV infection in CsA-treated CEM-SS cells, implying that target cell-CypA has an inhibitory effect on an early step in SIV replication in human cells. These results suggest possible involvement of CypA in the determination of SIV tropism, implying an unknown CsA-sensitive host cell factor involved in this contribution.

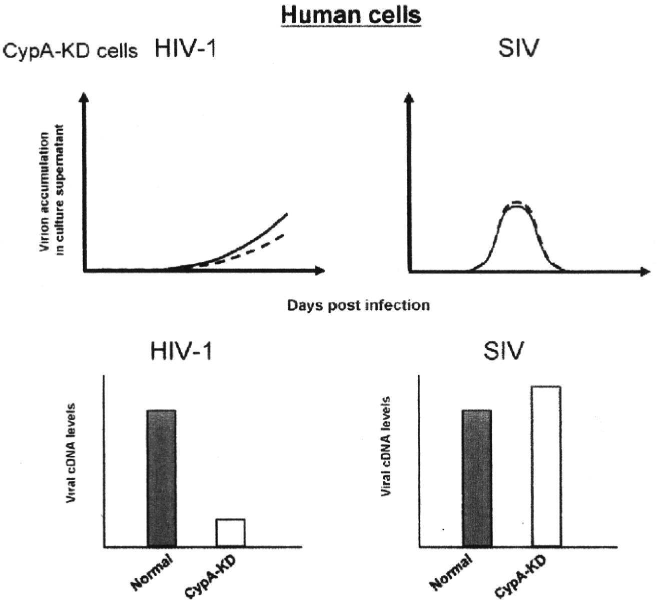

Fig. 2. A schema for the effect of Cyclophilin A on HIV/SIV replication. CypA knock-down reduces viral cDNA synthesis after HIV-1 infection (lower left panel) and inhibits HIV-1 replication (upper left panel). In contrast, the amounts of viral cDNA synthesized after SIV infection are not reduced but rather increased by CypA knock-down in human cells (lower right panel) and overall SIV replication levels are not reduced in CypA knock-down cells (upper right panel). The solid line in upper panels indicates virion accumulation of culture supernatant in the absence of CsA and the broken line indicates that of culture supernatant in the presence of CsA.

9. Discussion

The present study revealed a species-specific effect of CypA on SIV replication. In human T cells, SIV replication was enhanced by CsA treatment, indicating that CypA has an inhibitory effect on SIV replication. However, in macaque T cells, SIV replication was abrogated by CsA treatment, indicating that CypA is required for efficient SIV replication. CsA treatment of producer and target cells indicated that CypA incorporation into virions and the presence of CypA in target cells are both required for efficient SIV replication in macaque cells, like the role of CypA in HIV-1 replication in human cells. These results may provide an additional rationale of using a model of SIV infection in macaques for analysis of HIV-1 infection in humans.

It has been well established that CypA promotes HIV-1 replication after viral entry in the early phase in human cells [14,17,19,20]. CypA is efficiently incorporated into the virion produced from HIV-1-infected cells through interaction with CA in the context of Gag polyprotein [16,18]. Disruption of CypA incorporation into the virion by CsA administration or by Gag mutations resulted in reduction in infectivity of the produced viruses [14,15,21]. Recently, promotion of HIV-1 replication by post-entry interaction of CA with CypA in the target cells has been shown, suggesting the importance of CypA in the target cells for efficient HIV-1 replication [17,19,27]. This suggests involvement of CA–CypA interaction in the determination of retroviral tropism [15,19,23,25,26]. The effect of CypA on SIV replication in human cells has not been determined well, but a recent study has shown, for the first time, that human CypA exerts inhibitory effect on *vif*-deleted SIV replication, which may be recovered by SIV Vif excluding the CypA from the virion [29]. The present study suggests a novel role for CypA in the determination of HIV-1 and SIV tropism, but the effect of CypA on CsA-mediated enhancement of SIV replication in human cells was marginal. Our results imply an unknown host cell factor, which may be involved in this positive/negative effect of CypA on HIV-1/SIV replication. Elucidation of this factor would contribute to understanding the mechanism for species-specific restriction of HIV-1/SIV replication and determination of HIV-1/SIV tropism.

Conflict of interest statement

The author states that they have no conflict of interest.

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