[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).

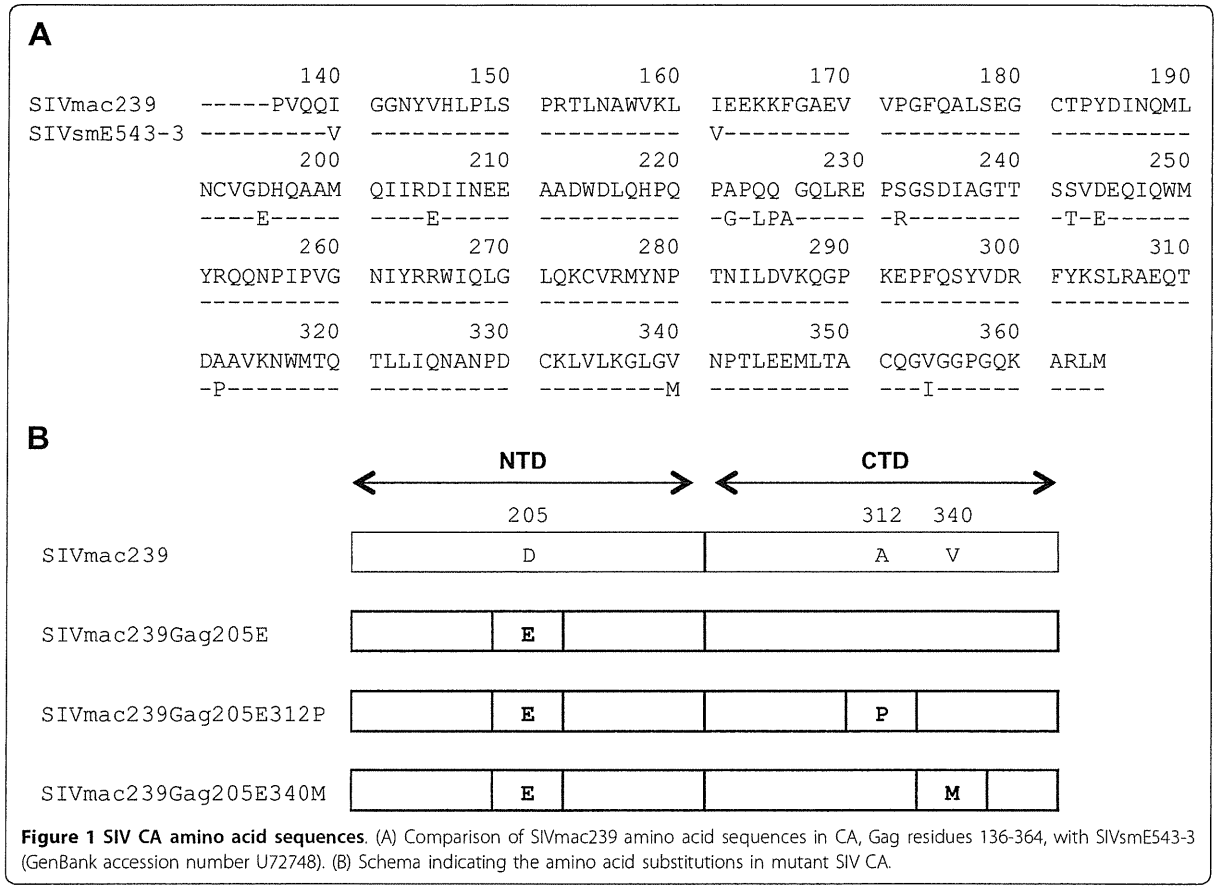

Figure 1 SIV CA amino acid sequences. (A) Comparison of SIVmac239 amino acid sequences in CA, Gag residues 136-364, with SIVsmE543-3 (GenBank accession number U72748). (B) Schema indicating the amino acid substitutions in mutant SIV CA.

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

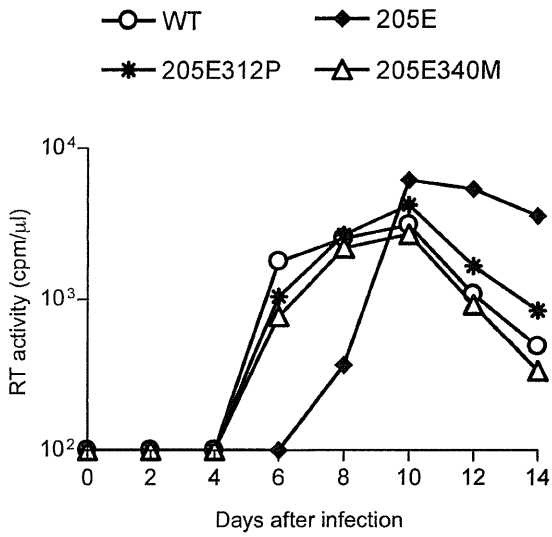

Figure 2 Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (WT, open circles), SIVmac239Gag205E (205E, closed diamonds), SIVmac239Gag205E312P (205E312P, asterisk), or SIVmac239Gag205E340M (205E340M, open triangles). Virus production was monitored by 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.

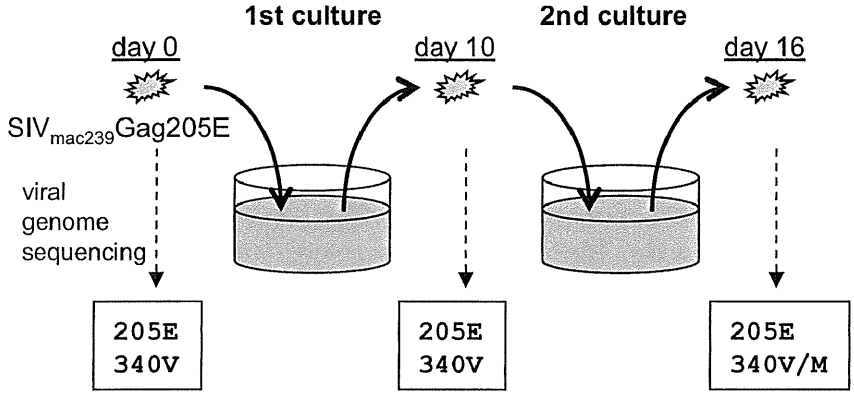

Figure 3 Passage of SIVmac239Gag205E culture supernatants. HSC-F cells were infected with SIVmac239Gag205E. The culture supernatant on day 10 was added to fresh HSC-F cells to start the second culture. Viral RNAs were extracted from the first culture supernatant on day 10 and the second culture supernatant 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.

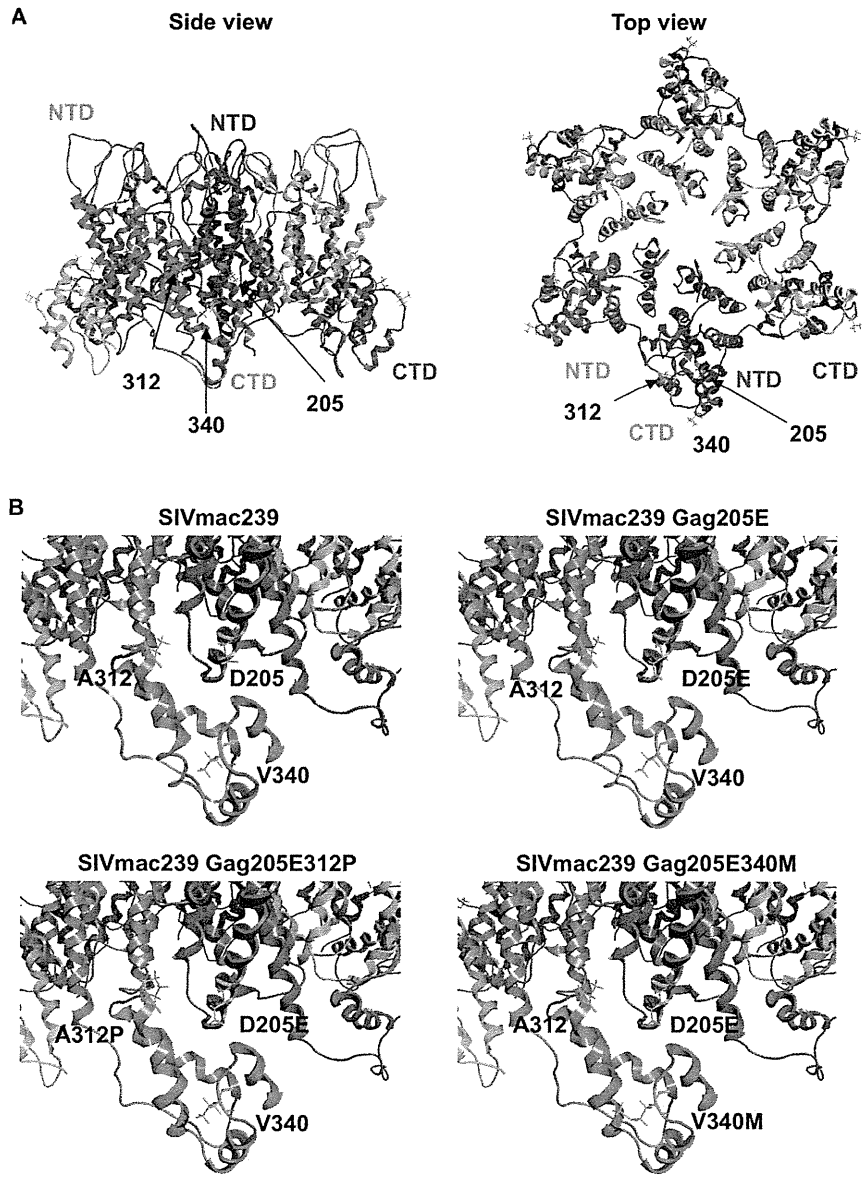

Figure 4 Structural models of SIVmac239 CA hexamer. The hexameric SIVmac239 CA models were constructed by homology-modeling using a crystal structure of the hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47[33]) as a modeling template. "MOE-Align" and "MOE-Homology" in MOE version 2008.1002 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

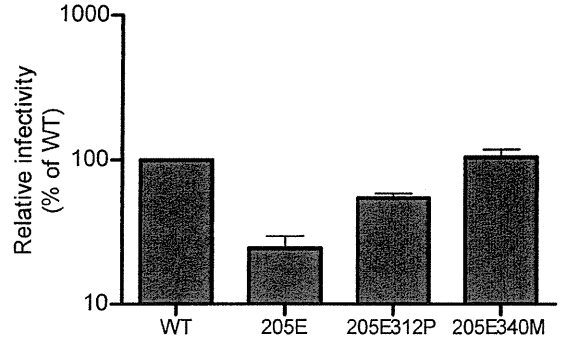

Figure 5 Mutant SIV infection. LuSIV cells were infected with SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340M (205E340M). Luciferase activity was measured 24 hr after infection. Relative infectivity is shown as the ratio (%) of the luciferase activity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

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

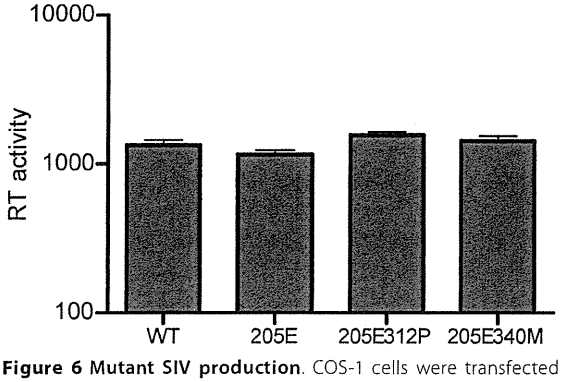

Figure 6 Mutant SIV production. COS-1 cells were transfected with molecular clone DNAs of SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340 M (205E340 M). RT activity of the culture supernatants two days after transfection was measured. Mean values in five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

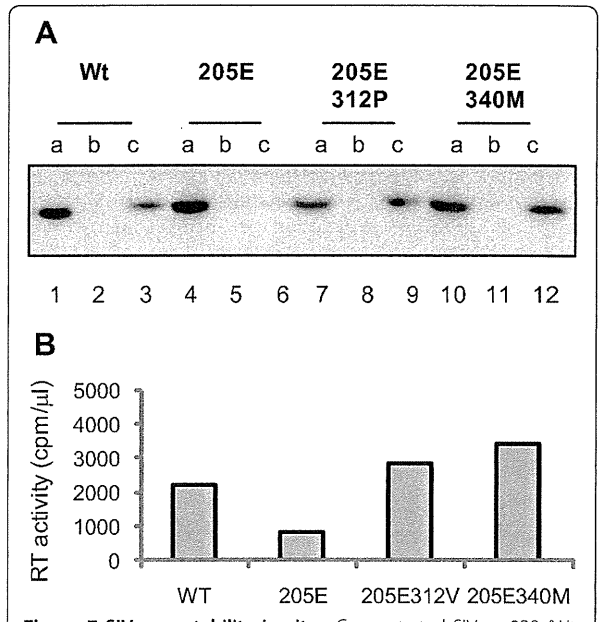

Figure 7 SIV core stability in vitro. Concentrated SIVmac239 (Wt; lanes 1-3), SIVmac239Gag205E (205E; lanes 4-6), SIVmac239Gag205E312P (205E312P; lanes 7-9), or SIVmac239Gag205E340M (205E340 M; lanes 10-12) was separated into three fractions (top [a], middle [b], and bottom [c]) by ultracentrifugation 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).

SIVmac239Gag205E340M as well as SIVmac239, but not in SIVmac239Gag205E CA hexamers. Thus, this pocket may be a target candidate for anti-viral drugs.

Both GagL216S and GagD205E mutations can result in escape from $Gag_{206\text{-}216}$-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 $Gag_{206\text{-}216}$-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 $Gag_{206-216}$-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

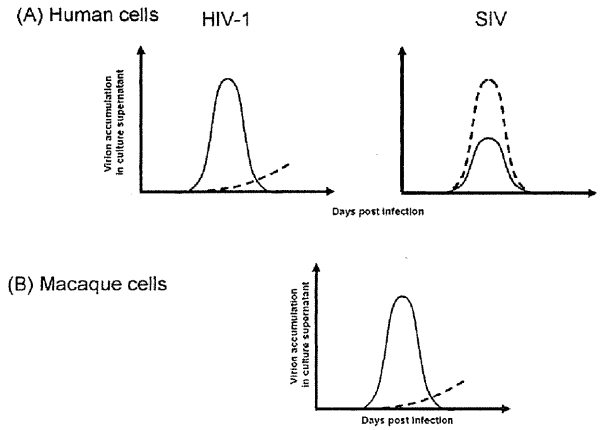

Fig. 1. A schema for the effect of CsA on HIV/SIV replication in human/macaque cells. (A) CsA treatment inhibits HIV-1 replication (left panel) but enhances SIV replication (right panel) in human cells. (B) CsA treatment inhibits SIV replication in macaque cells. The solid line indicates virion accumulation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

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.

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.

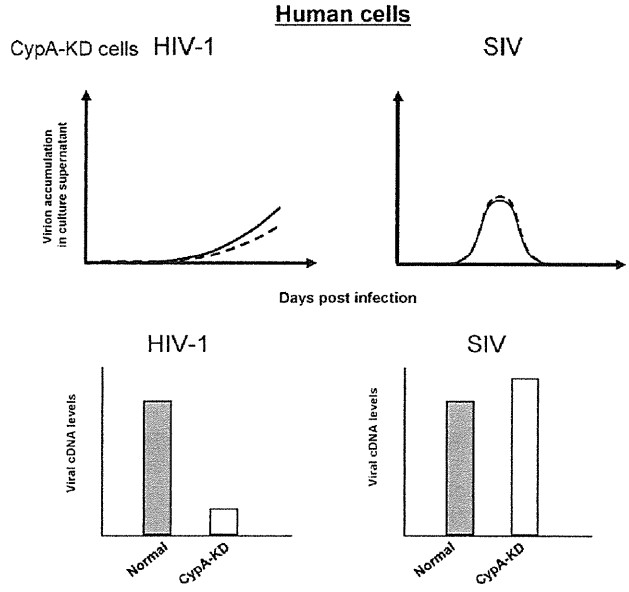

Fig. 2. A schema for the effect of Cyclophilin A on HIV/SIV replication. CypA knock-down reduces viral cDNA synthesis after HIV-1 infection (lower left panel) and inhibits HIV-1 replication (upper left panel). In contrast, the amounts of viral cDNA synthesized after SIV infection are not reduced but 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 determi-

nation 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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Heat Shock Protein 70 Inhibits HIV-1 Vif-mediated Ubiquitination and Degradation of APOBEC3G*

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The cytidine deaminase APOBEC3G, which is incorporated into nascent virus particles, possesses potent antiviral activity and restricts Vif-deficient HIV-1 replication at the reverse transcription step through deamination-dependent and -independent effects. HIV-1 Vif counteracts the antiviral activity of APOBEC3G by inducing APOBEC3G polyubiquitination and its subsequent proteasomal degradation. In this study, we show that overexpression of heat shock protein 70 (HSP70) blocked the degradation of APOBEC3G in the ubiquitin-proteasome pathway by HIV-1 Vif, rendering the viral particles non-infectious. In addition, siRNA targeted knock-down of HSP70 expression enhanced the Vif-mediated degradation of APOBEC3G. A co-immunoprecipitation study revealed that overexpression of HSP70 inhibited APOBEC3G binding to HIV-1 Vif. Thus, we provide evidence for a host protein-mediated suppression of HIV-1 replication in an APOBEC3G-dependent manner.

Human immunodeficiency virus type-1 (HIV-1),[3] the retrovirus that causes AIDS, efficiently replicates within human $CD4^+$ T cells. However, Vif-deficient virions produced by non-permissive cells, including $CD4^+$ T cells and immortalized lines, such as Hut78 or CEM, are non-infections, whereas virions produced in permissive cells, such as SupT1 or CEM-SS, are infectious (1, 2). Previous studies have demonstrated that HIV-1 Vif counteracts the innate antiviral activity of APOBEC3G, a member of the APOBEC family of cytidine deaminase-editing enzymes (3). In the absence of Vif, APOBEC3G induces the deamination of cytidine (C) and its conversion to uridine (U) (4, 5), which can be packaged into budding retroviruses through a direct interaction with the Gag polyprotein (6–11). The C to U conversion in the HIV-1 minus strand leads to a G to A hypermutation, preferentially at CCCA sequences. This motif corresponds to TGGG in the plus-strand sequence, thereby mutating the TGG tryptophan codon to a TAG stop codon and affecting subsequent stages of the viral life cycle (12). Vif predominant mechanism for overcoming the antiviral activity of APOBEC3G is to form an E3 ubiquitin ligase with cullin 5 (Cul5), elongin B (EloB), and elongin C (EloC) and target these proteins for degradation by the ubiquitin-proteasome pathway (13–16). Vif may also inhibit APOBEC3G activity through mechanisms independent of proteasomal degradation (17–19).

Heat shock proteins play critical roles in the life cycle of a variety of RNA and DNA viruses (20–23). For example, heat shock protein 70 (HSP70) is specifically incorporated into HIV-1 virions (24). However, the formation of the P-TEFb/Tat/TAR complex is required to stabilize the CDK9/cyclinT1 heterodimer by HSP70 and HSP90 (25).

To better develop potential novel therapeutic strategies to exploit the APOBEC3G antiviral function, we investigated the role of HSP70 in APOBEC3G function. We found that siRNA against HSP70 significantly reduced the level of APOBEC3G in the presence of HIV-1 Vif, but not in the absence of Vif. In addition, overexpression of HSP70 in 293T cells reduced the Vif-mediated degradation of APOBEC3G by inhibiting APOBEC3G polyubiquitination. This effect is attributed to the impairment of APOBEC3G-Vif binding. Furthermore, overexpression of HSP70 in the presence, but not in the absence, of APOBEC3G clearly suppressed the infectivity of virions in a dose-dependent manner. These results suggest that HSP70 acts as a potential antiviral host factor through interaction with APOBEC3G and may form the basis for new anti-HIV-1 therapies.

EXPERIMENTAL PROCEDURES

Immunoprecipitation—293T cells (5×10^5) were transfected with 1.0 μg of each Vif expression plasmid using Lipofectamine2000 (Invitrogen). At 48 h post-transfection, cells were suspended in a lysis buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Nonidet P-40, and 10% glycerol) and incubated with 5 μl of anti-HSP70 antibody (Santa Cruz Biotechnology) and 30 μl of Dynabeads-protein G (Invitrogen). The beads were

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[3] The abbreviations used are: HIV-1, human immunodeficiency virus type-1; RNP, ribonucleoprotein; HSP, heat shock protein; Ub, ubiquitin.

washed with PBS containing 0.02% Tween 20. The immunocomplex was eluted by boiling with 20 μl of 5× sample buffer and analyzed by SDS-PAGE and Western blot.

Protein Stability Assay—293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and 0.5 μg of a GFP expression plasmid (CS-CDF-CG-PRE), or 1.0 μg of pc-Hu-APOBEC3G-HA, 0.5 μg of CS-CDF-CG-PRE and 0.5 μg of pcDNA-Vif along with either 2.0 μg of pFLAG-HSP70 or an empty plasmid. At 24 h post-transfection, cells were treated with 100 μg/ml of cycloheximide. Cells were harvested, and cell lysates were analyzed by Western blotting with horseradish peroxidase-conjugated anti-HA antibody (Roche Diagnostics) and anti-GFP antibody (Medical & Biological Laboratories). The blots were semi-quantified using ImageJ 1.43u software.

Polyubiquitination Assay—293T cells (3×10^6) were co-transfected with 2.0 μg of pc-Hu-APOBEC3G-HA, 2.0 μg of pVif-V5, 4.0 μg of pFLAG-HSP70, and 2.0 μg of pCMV-Myc-Ubi (26). At 24 h post-transfection, cells were treated with 5 μM MG-132 for 24 h. Cells were suspended in a lysis buffer. Cell lysates were immunoprecipitated using anti-Myc antibody (Cell Signaling) followed by Western blotting with horseradish peroxidase-conjugated anti-HA antibody.

MAGI Assay—MAGI cells were plated in 96-well plates at 1×10^4 cells per well in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The next day, cells were infected with dilutions of the virus in a total volume of 50 μl in the presence of 20 μg/ml DEAE-dextran for 2 h. At 2 days post-infection, cells were fixed with 100 μl of fix solution (1% formaldehyde/0.2% glutaraldehyde in PBS) at room temperature for 5 min and then washed twice with PBS. Cells were incubated with 100 μl of staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg/ml X-Gal) for 50 min at 37 °C. The reaction was stopped by removing the staining solution, and blue cells were counted under a microscope.

Construction of Plasmids—To generate pcDNA-Vif, HIV-1 Vif fragments were amplified from pNL4-3 by PCR with the following primers: forward 5′-GAT ATC ATG GAA AAC AGA TGG CAG GTG ATG-3′ and reverse 5′-CTC GAG CTA GTG TCC ATT CAT TGT ATG CT-3′. The PCR products were inserted into pcDNA3.1 (Invitrogen).

To construct pFLAG-HSP70, whole RNA was isolated from 293T cells with TRIzol (Invitrogen) and amplified by RT-PCR with the following primers: forward 5′-GTT GAA TTC CGC CAA AGC CGC GGC GAT-3′ and reverse 5′-CGC GGA TCC CTA ATC TAC CTC CTC AAT-3′. The products were inserted into pFLAG-CMV2 (Sigma).

To generate pVif-V5, HIV-1 Vif fragments were amplified from pNL4-3. The PCR products were inserted into pENTR using TOPO Cloning kits (Invitrogen) and transferred into pLenti6/V5-DEST (Invitrogen) by LR recombination. This construct contains the β-globin intron sequence of pMDL-g/p-RRE downstream of the CMV promoter. A plasmid construct encoding human APOBEC3G tagged with the influenza hemagglutinin (HA) sequence was a kind gift from Darlene Chen (The Salk Institute for Biological Studies). Vif-defective variants of NL4-3 have been described previously (27).

To generate pCS-U6, the U6 promoter was amplified by PCR. The resulting products were inserted into pCS-CDF-CG-PRE. pCS-U6-shControl or pCS-U6-shHSP70 was constructed by ligating the annealed product of sense oligonucleotide 5′-GAT CCT TCT CCG AAC GTG TCA CGT TTC AAG AGA ACG TGA CAC GTT CGG AGA ATT T-3′ and antisense oligonucleotide 5′-CTA GAA ATT CTC CGA ACG TGT CAC GTT CTC TTG AAA CGT GAC ACG TTC GGA GAA G-3′ or sense oligonucleotide 5′-GAT CCC ACG GCA AGG TGG AGA TCA TTC AAG AGA TGA TCT CCA CCT TGC CGT GTT T-3′ and antisense oligonucleotide 5′-CTA GAA ACA CGG CAA GGT GGA GAT CAT CTC TTG AAT GAT CTC CAC CTT GCC GTG G-3′, respectively, with the BamHI-XbaI fragment from pCS-U6. These plasmids contain a transcriptional termination signal sequence downstream of the shControl and shHSP70 sequences.

Transfection of siRNA—293T cells (3×10^6) were transfected with siRNAs (100 nM) using Lipofectamine2000. Control siRNA (5′-UUC UCC GAA CGU GUC ACG UdTdT-3′) and HSP70-siRNA (5′-CAC GGC AAG GUG GAG AUC AdTdT-3′) were purchased from B-Bridge International.

Preparation of Lentiviral Vectors—293T cells (5×10^5) were cotransfected with the lentiviral vector (1.6 μg), vesicular stomatitis virus G expression vector pMD.G (0.4 μg), *rev* expression vector pRSV-Rev (0.8 μg), and *gag-pol* expression vector pMDLg/pRRE (1.2 μg) using Lipofectamine2000. At 48 h after transfection, culture supernatants were harvested and filtered through 0.45-μm filters. In all experiments, H9 cells (3×10^5) were transduced with equal amounts of the lentivirus vector.

Statistical Analysis—The results are shown as means ± S.D., and statistical analysis was performed using the paired Student's *t* test. A p value of <0.05 was considered significant. At least three replicates were performed for each experiment.

RESULTS

HSP70 Leads to Stabilization of APOBEC3G—HSPs are induced by a variety of stress-related stimuli, including heat, UV radiation, and microbial/viral infections (28). HSPs are involved in the folding and translocation of cellular proteins under normal conditions, whereas under stressful conditions, HSPs bind to proteins and inhibit their misfolding or irreversible aggregation (29). Recent studies revealed that the binding of HSPs to HIV-1 proteins enhances antiviral immunity (30). HSP70 is selectively expressed soon after HIV-1 infection, suggesting that these proteins might be involved in the innate cellular antiviral immune response (31). However, the specific targets of HSPs and their role in the response to HIV infection remain unclear.

HIV-1 Vif targets APOBEC3G for ubiquitination by forming an Skp1-cullin-F-box (SCF)-like complex, which subsequently leads to the degradation of APOBEC3G. To evaluate how HSP70 affects Vif-dependent ubiquitination and degradation, we examined the steady-state level of APOBEC3G in 293T cells co-transfected with FLAG-tagged HSP70 (FLAG-HSP70) and pNL4-3. The expression of HSP70 in 293T cells significantly increased the amount of APOBEC3G in a dose-dependent manner but not the amount of the HIV-1 Gag p24 antigen (Fig. 1*A*, *lanes 1–4* and Fig. 1*B*). Importantly, HSP70 had no effect on

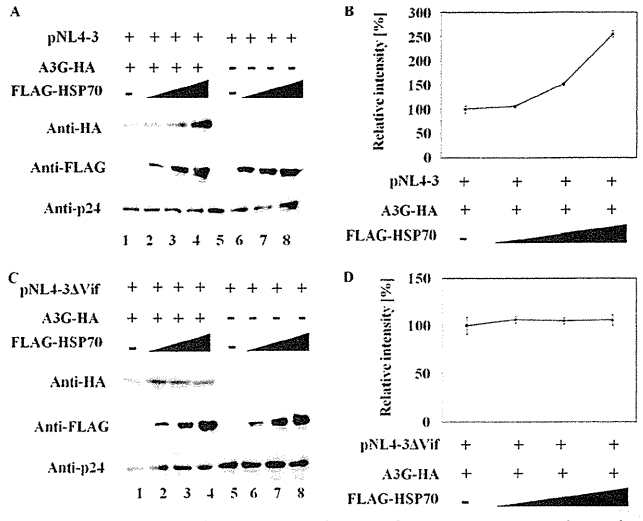

FIGURE 1. **Expression of FLAG-tagged HSP70 blocks APOBEC3G degradation in cells transfected with pNL4-3, but not those transfected with pNL4-3-delta-Vif.** 293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and increasing amounts of pFLAG-HSP70 (0, 0.5, 1.0, or 2.0 μg), adjusted with an empty vector to 2.0 μg of total, along with either 0.1 μg of pNL4-3 (*A*) or 0.1 μg of pNL4-3-delta-Vif (*C*). At 48 h post-transfection, cell lysates were analyzed by Western blotting. The relative intensity of APOBEC3G-HA bands was determined by densitometry (*B* and *D*). Results are representative of three independent experiments, and error bars show the standard deviations of the means.

the expression level of APOBEC3G in 293T cells transfected with Vif-deleted HIV-1 proviral plasmid (Fig. 1, *C*, *lanes 1–4* and *D*). Our results suggest that HSP70 may inhibit the degradation of APOBEC3G by HIV-1 Vif.

HSP70 Blocks HIV-1 Vif-mediated Degradation of APOBEC3G—Next, we investigated whether expression of HSP70 directly blocks APOBEC3G degradation by HIV-1 Vif. 293T cells were co-transfected with pc-Hu-APOBEC3G-HA and pFLAG-HSP70 in the absence or presence of pcDNA-Vif. We found that the steady-state levels of APOBEC3G in the presence of pcDNA-Vif were increased by the expression of HSP70 in a dose-dependent manner (Fig. 2, *A*, *lanes 6–10* and *B*, *right panel*). By contrast, HSP70 did not significantly affect the amount of APOBEC3G expression in the absence of pcDNA-Vif (Fig. 2, *A*, *lanes 1–5* and *B*, *left panel*). These data indicate that the effects of HSP70 on APOBEC3G expression depend on HIV-1 Vif.

Previous studies have reported that microbial HSP70 up-regulates APOBEC3G mRNA (32, 33). To rule out this possibility, pulse-chase experiments were performed using 293T cells that were co-transfected with pc-Hu-APOBEC3G-HA, pVif-V5, pFLAG-HSP70, and a GFP expression plasmid (CS-CDF-CG-PRE). Cycloheximide was used to block protein synthesis. When 293T cells were transfected with pc-Hu-APOBEC3G-HA alone, there was no change in the level of APOBEC3G (Fig. 2, *C*, *lanes 1–4* and *D*, *left panel*). Consistent with previous reports, degradation of APOBEC3G-HA was induced in the presence of HIV-1 Vif (Fig. 2, *C*, *lanes 5–8* and *D*, *middle panel*). In contrast, HSP70 expression significantly suppressed the degradation of APOBEC3G by HIV-1 Vif (Fig. 2, *C*, *lanes 9–12* and *D*, *right panel*). To further evaluate whether HSP70 expression inhibits the ubiquitination of APOBEC3G by HIV-1 Vif, we performed ubiquitination assays. Lysates of cells co-expressing pVif-V5, Myc-tagged ubiquitin (Myc-Ub), pc-Hu-APOBEC3G-HA and either empty plasmid or pFLAG-HSP70 were analyzed for the polyubiquitination of APOBEC3G. We detected the ubiquitination of APOBEC3G as a ladder band (Fig. 2*E*, *lane 2*). The expression of HSP70 resulted in a significant reduction in polyubiquitinated APOBEC3G (Fig. 2*E*, *lane 1*). Thus, the expression of HSP70 causes an increase in the steady-state levels of APOBEC3G by blocking the Vif-mediated ubiquitination and degradation of APOBEC3G.

HSP70 Interacts with Both APOBEC3G and HIV-1 Vif—We performed an immunoprecipitation assay to evaluate the binding between HSP70 and APOBEC3G or HIV-1 Vif (Fig. 3). 293T cells were transfected with pc-Hu-APOBEC3G-HA, pcDNA-Vif, pNL4-3, or pNL4-3-delta-Vif. Cell lysates were precipitated with anti-HSP70 antibody, followed by immunoblotting with anti-ApoC17 or anti-Vif antibody. HSP70 interacted with both APOBEC3G (Fig. 3*A*) and HIV-1 Vif (Fig. 3*B*). These interactions and the intracellular localization of HSP70 and HA-tagged APOBEC3G were confirmed by immunostaining assays (data not shown). To further investigate the role of HSP70 in APOBEC3G-Vif interactions, 293T cells were co-transfected with pc-Hu-APOBEC3G-HA and pVif-V5 along with either an empty plasmid or pFLAG-HSP70 in the presence of a proteasome inhibitor (MG-132). Consistent with previous studies, HIV-1 Vif was bound to APOBEC3G (Fig. 3*C*, *lane 2*). Strikingly, the expression of HSP70 in 293T cells led to the inhibition of APOBEC3G-Vif binding. (Fig. 3*C*, *lane 1*). Because a previous study reported that APOBEC3G binds the N-terminal region of HIV-1 Vif (34), we tested the hypothesis that HSP70 competes with APOBEC3G for binding to the N-terminal region of HIV-1 Vif. We found that FLAG-HSP70 efficiently co-immunoprecipitated with the N-terminal region of Vif (amino acids 1–107) (Fig. 3*D*, *lane 1*). However, the C-terminal region of Vif (amino acids 108–192) exhibited no detectable interaction with FLAG-HSP70 (Fig. 3*D*, *lane 2*). These results suggest that APOBEC3G-Vif binding is reduced by HSP70 through an interaction with the N-terminal region of Vif, resulting in the inhibition of the Vif-mediated ubiquitination and the degradation of APOBEC3G.

Knock-down of HSP70 in 293T Cells Enhances APOBEC3G Degradation by HIV-1 Vif—To further investigate the effect of endogenous HSP70 on the stability of APOBEC3G, we silenced endogenous HSP70 expression by RNA interference. 293T cells were transfected with control siRNA (siCtrl) or HSP70-specific siRNA (siHSP70) for 4 h prior to transfection along with pc-Hu-APOBEC3G-HA and either pNL4-3 or pNL4-3-delta-Vif. At 48 h post-transfection, cells were harvested and subjected to Western blotting. As expected, the level of APOBEC3G in pNL4-3-transfected cells was less stable than that in the pNL4-3-delta-Vif transfected cells (Fig. 4*A*, compare *lane 1* to *lane 3*). Quantification of the relative intensities revealed that transfection with pNL4-3 induced APOBEC3G degradation with a potency ~1.8 times higher than that of pNL4-3-delta-Vif (Fig. 4*B*). Moreover, in the case of transfection with pNL4-3, the level of APOBEC3G, but not the level of HIV-1 Gag, in the siHSP70-transduced cells were lower than in

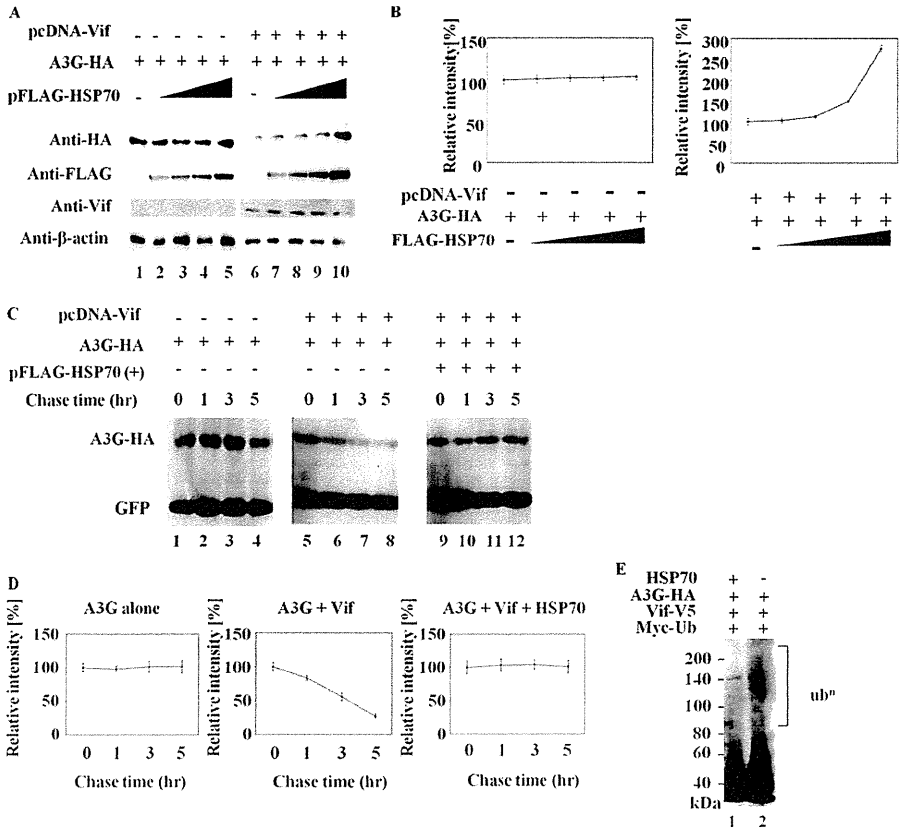

FIGURE 2. **HSP70 expression inhibits Vif-mediated APOBEC3G ubiquitination and degradation.** *A*, 293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and increasing amounts of pFLAG-HSP70 (0, 0.5, 1.0, or 2.0 μg), adjusted to 2.0 μg of total DNA with 0.5 μg of an empty plasmid (pcDNA3.1) or pcDNA-Vif. At 48 h post-transfection, cell lysates were subjected to Western blotting and were then analyzed with the indicated antibody. β-Actin was used as a control for protein levels. *B*, relative intensity of APOBEC3G-HA bands in *A* was determined by densitometry. *C*, 293T cells (5×10^5) were transfected with 1.0 μg of pc-Hu-APOBEC3G-HA alone (*lanes 1–4*); 0.5 μg of pcDNA-Vif and 1.0 μg of pc-Hu-APOBEC3G-HA (*lanes 5–8*); and 0.5 μg of pcDNA-Vif, 1.0 μg of pc-Hu-APOBEC3G-HA and 2.0 μg of pFLAG-HSP70 (*lanes 9–12*). The transfected cells were treated with cycloheximide to block *de novo* protein synthesis. The level of APOBEC3G was detected by immunoblotting after cycloheximide treatment lasting 1, 3, or 5 h. CS-CDF-CG-PRE (0.5 μg), which expresses the green fluorescent protein (GFP), was co-transfected with each plasmid into 293T cells as a control plasmid. *D*, relative intensity of APOBEC3G-HA bands in *C* was determined by densitometry. *E*, 293T cells (3×10^6) were co-transfected with 2.0 μg of pCMV-Myc-Ubi, 2.0 μg of pVif-V5, and 2.0 μg of pc-Hu-APOBEC3G-HA along with 4.0 μg of an empty plasmid or pFLAG-HSP70. At 24 h post-transfection, cells were treated with 5 μM MG132. After 24 h, cell lysates were immunoprecipitated with anti-Myc antibody, followed by immunoblotting analysis with horseradish peroxidase-conjugated anti-HA antibody. Results are representative of three independent experiments, and error bars show the standard deviations of the means.

the siCtrl-transduced cells (Fig. 4*A*, compare *lane 1* to *lane 2*). The amount of APOBEC3G in HSP70 knock-down cells decreased to half the amount in the control cells (Fig. 4*B*). However, in terms of transfection with pNL4-3-delta-Vif, treatment with siHSP70 had no effect on the stability of APOBEC3G (Fig. 4*A*, compare *lane 3* to *lane 4*). These data indicate that depletion of HSP70 facilitates Vif-mediated degradation of APOBEC3G.

HSP70 Suppresses HIV-1 Vif-mediated Degradation of Endogenous APOBEC3G in Non-permissive Cells—Most experiments in this study used permissive cells. To investigate whether our findings have physiologic relevance in non-permissive cells, we used a lentiviral vector encoding FLAG-HSP70 or HIV-1 Vif-V5. In the absence of Vif-V5, there was no significant effect of FLAG-HSP70 on the level of endogenous APOBEC3G in H9 cells (Fig. 5*A*, compare *lane 1* to *lane 2*). When Vif-V5 was expressed in H9 cells, expression of FLAG-HSP70 increased the amount of endogenous APOBEC3G (Fig. 5*A*, compare *lane 3* to *lane 4*). Next, we suppressed the expression of HSP70 using a lentiviral vector to express shHSP70 under the control of the human U6 promoter in H9 cells. APOBEC3G expression in shHSP70-transduced H9 cells was similar to that in shControl-transduced H9 cells (Fig. 5*B*, compare *lane 1* to *lane 2*). The level of endogenous APOBEC3G was lower in H9 cells transduced with shHSP70 than in H9 cells transduced with shControl by expression of Vif-V5 (Fig. 5*B*, compare *lane 3* to *lane 4*). Therefore, HSP70 suppresses Vif-mediated degradation of endogenous APOBEC3G in non-permissive cells.

Expression of HSP70 in the Presence of APOBEC3G Augments APOBEC3G Restriction of HIV-1—To examine whether HSP70 expression influences the function of APOBEC3G, pNL4-3, or pNL4-3-delta-Vif was transfected into 293T cells along with either pFLAG-HSP70 alone or pFLAG-HSP70 and pc-Hu-APOBEC3G-HA. The viral infectivity was measured by MAGI assay. As shown in Fig. 6*A*, expression of FLAG-HSP70 clearly suppressed the infectivity of wild-type HIV-1 in the presence of APOBEC3G in a dose-dependent manner. In the absence of APOBEC3G, FLAG-HSP70 did not affect the infectivity of the wild-type HIV-1. Unexpectedly, HSP70 expression in

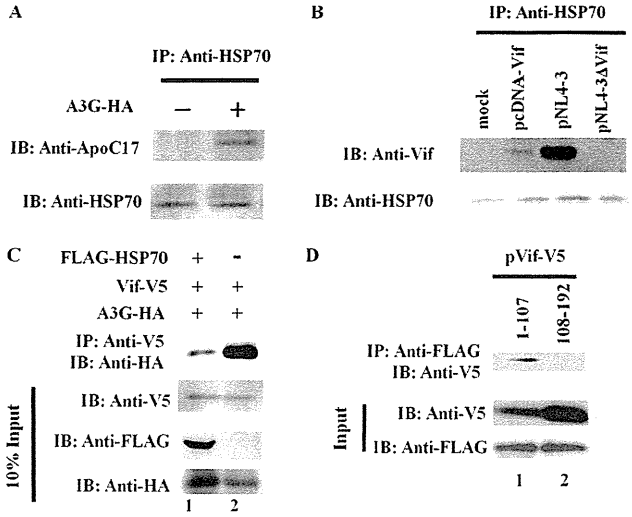

FIGURE 3. **HSP70 interacts with APOBEC3G and HIV-1 Vif.** *A*, 293T cells (5×10^5) were transfected with 1.0 μg of pc-Hu-APOBEC3G-HA. After 48 h, cell lysates were immunoprecipitated with anti-HSP70 antibody, followed by immunoblotting analysis with anti-ApoC17 antibody. *B*, 293T cells (5×10^5) were transfected with 1.0 μg of the indicated plasmids. At 48 h post-transfection, cell lysates were subjected to immunoprecipitation using anti-HSP70 antibody, followed by immunoblotting analysis with anti-Vif antibody. *C*, 293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and 1.0 μg of pVif-V5 together with 2.0 μg of either an empty plasmid or pFLAG-HSP70. At 24 h post-transfection, cells were treated with 5 μM MG132. At 24 h post-treatment, cell lysates were immunoprecipitated with anti-V5 antibody, followed by immunoblotting analysis with horseradish peroxidase-conjugated anti-HA antibody. *D*, 293T cells (5×10^5) were co-transfected with 1.0 μg of pFLAG-HSP70 and either 1.0 μg of pVif-1–107-V5 or pVif-108–192-V5. At 48 h post-transfection, cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting analysis with anti-V5 antibody. Results are representative of three independent experiments.

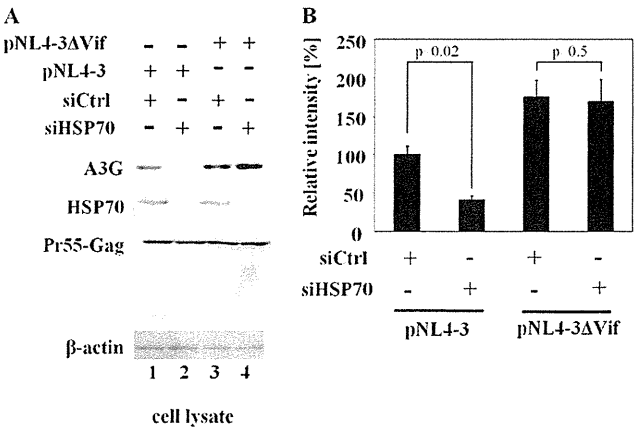

FIGURE 4. **Depletion of HSP70 in 293T cells impairs the stability of APOBEC3G.** *A*, 293T cells (3×10^6) were treated with 100 nM HSP70-siRNA (siHSP70) or 100 nM control-siRNA (siCtrl) for 4 h, prior to co-transfection with 1.0 μg of pc-Hu-APOBEC3G-HA with either 1.0 μg of pNL4-3 or pNL4-3-delta-Vif. At 48 h post-transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. *B*, relative intensity of APOBEC3G bands in *A* was determined by densitometry. Results are representative of three independent experiments, and error bars show the standard deviations of the means.

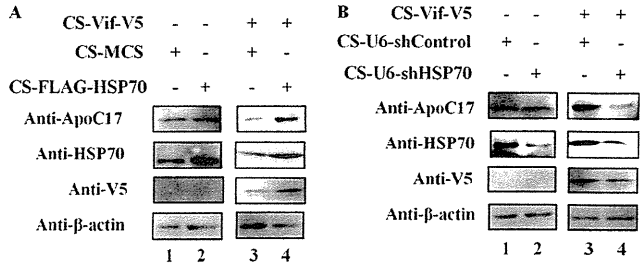

FIGURE 5. **HSP70 affects the level of endogenous APOBEC3G expression in non-permissive T cells expressing HIV-1 Vif.** *A*, H9 cells (3×10^5) were infected with a lentiviral vector encoding an artificial multiple cloning site (MCS) or FLAG-HSP70 in the presence of 8 μg/ml of polybrene. At 48 h after infection, cells were suspended with lysis buffer (*left panel*) or transduced with HIV-1 Vif using a lentivirus vector system (*right panel*). At 48 h post-transduction, cell lysates were analyzed by Western blotting using the indicated antibodies. *B*, H9 cells (3×10^5) were infected with lentivirus-based vectors to express shControl or shHSP70 under the control of the human U6 promoter in the presence of 8 μg/ml of polybrene. At 48 h post-infection, cells were treated as in *A*. Data are representative of three independent experiments.

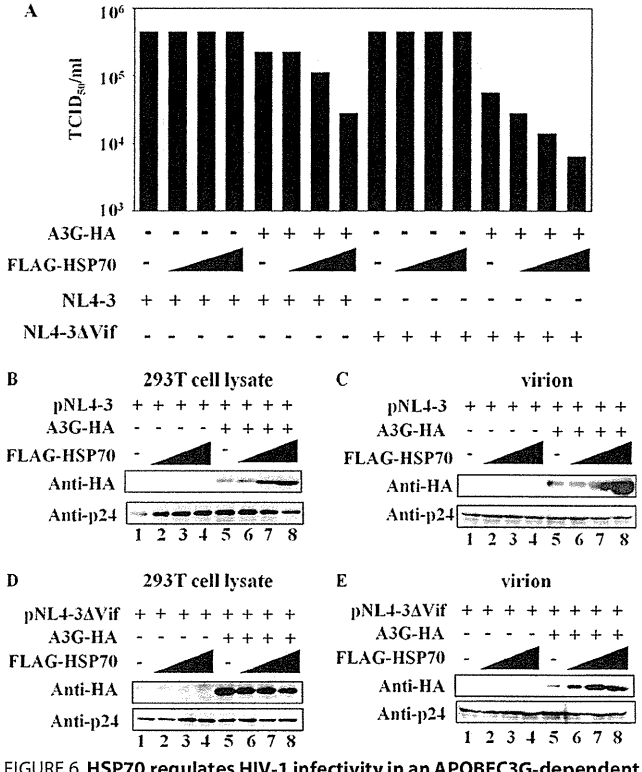

FIGURE 6. **HSP70 regulates HIV-1 infectivity in an APOBEC3G-dependent manner.** *A*, 293T cells (5×10^5) were co-transfected with 0.1 μg of pNL4-3 or pNL4-3-delta-Vif and 1.0 μg of pc-Hu-APOBEC3G-HA alone, pFLAG-HSP70 (0.5, 1.0, or 2.0 μg) alone or 1.0 μg of pc-Hu-APOBEC3G-HA and pFLAG-HSP70 (0.5, 1.0 or 2.0 μg). At 48 h post-transfection, supernatants were harvested, and the amount of each virus was normalized to the equivalent level of p24. MAGI cells (1×10^4) were infected with serially diluting supernatants of each stock of virus, and infected cells were stained with X-Gal 2 days later. 50% tissue culture infective doses (TCID50) is determined by the last virus dilution that is still capable of infecting the cells. *B*, each stock of cell lysate or virus in *A* was subjected to Western blotting and was then analyzed with the indicated antibody. All data are representative of three independent experiments.

APOBEC3G-HA-transfected 293T cells led to a dose-dependent inhibition of the infectivity of Vif-deficient HIV-1 particles (Fig. 6*A*). Moreover, no effect of HSP70 expression on the infectivity of the Vif-deficient HIV-1 particles produced by mock-transfected 293T cells was observed. To further demonstrate whether expression of HSP70 affects virion packaging of APOBEC3G, viral particles produced by 293T cells expressing

HSP70 were analyzed for APOBEC3G expression by Western blotting. We found that expression of HSP70 significantly increased the amount of intracellular and wild type virion-associated APOBEC3G (Fig. 6, *B* and *C*). Interestingly, HSP70 expression enhanced the level of APOBEC3G packaging in Vif-deficient virions, but had no effect on intracellular APOBEC3G and viral release (Fig. 6, *D* and *E*). These results indicate that HSP70 blocks Vif-mediated APOBEC3G degradation and enhances the incorporation of APOBEC3G into both wild type and Vif-deficient virions, which result from inhibition of HIV-1 replication through HSP70 interaction with APOBEC3G.

DISCUSSION

APOBEC3G, which is incorporated into progeny virus particles, restricts the replication of Vif-deficient HIV-1 through cytidine deamination-dependent and independent mechanisms (3–5, 17, 35–41). This restriction can be overcome by HIV-1 Vif, which induces the polyubiquitination of APOBEC3G through recruitment of a ubiquitin E3 ligase complex composed of cullin 5, elongin B, elongin C, and Ring box-1 and facilitates the proteasomal degradation of APOBEC3G (13, 14, 16, 42–45). Thus, mechanistic insights into the quality control of APOBEC3G protein are important for understanding the molecular basis of APOBEC3G-mediated HIV-1 restriction. In this study, we showed that HSP70 suppressed Vif-mediated APOBEC3G degradation. In contrast to our results for HSP70, Pin1 suppresses the HIV restriction activity of APOBEC3G (46). Overexpression of Pin1 reduces the levels of intracellular APOBEC3G. One possibility is that HSP70 regulates Pin1 function, which results in the stimulation of APOBEC3G function, although further analysis is needed to properly address this question.

Pido-Lopez *et al.* (32) have reported that microbial HSP70 up-regulates APOBEC3G mRNA and protein expression in human $CD4^+$ T cells. Our data indicate that in 293T cells, overexpression of human HSP70 in the absence of HIV-1 Vif did not affect the amount of APOBEC3G protein. The stabilization of APOBEC3G is attributed to a reduction in the Vif-dependent polyubiquitination of APOBEC3G (Fig. 2*E*). Whereas we have focused on human HSP70 activity on APOBEC3G stability, it would be interesting to investigate whether human HSP70 can affect the level of endogenous APOBEC3G mRNA.

APOBEC3G associates with ribonucleoprotein (RNP) complexes and is not only dispersed throughout the cytoplasm but is also markedly concentrated in cytoplasmic foci that are identified as mRNA-processing bodies (P bodies) (47). Localization of APOBEC3G in P bodies is not important for its LINE-1 suppression activity (48). However, Y3 and 7SL RNAs, which compose RNP complexes, are required for efficient APOBEC3G packaging (49). Stimulation of cells at 44 °C induces the rapid accumulation of APOBEC3G and many cellular RNA-binding proteins (50). We examined whether HSP70 plays a role in packaging APOBEC3G into virus particles and found that overexpression of HSP70 enhanced APOBEC3G packaging in the absence of Vif (Fig. 6*E*). It is possible that HSP70 interacts with cytoplasmic APOBEC3G, but it remains unclear whether HSP70 induces the accumulation of APOBEC3G in P bodies and increases the association of APOBEC3G with RNP complexes. Further studies will be required to clarify the details of how, where and when HSP70 and APOBEC3G co-localize within cells.

Recently, Nathans *et al.* (51) have identified a small molecule, termed RN-18, that degrades HIV-1 Vif only in the presence of APOBEC3G, resulting in enhanced APOBEC3G abundance and virion incorporation, similar to the function of HSP70. The possibility has been raised that HSP70 may be the target of RN-18. However, HSP70 has no significant effect on HIV-1 Vif expression and leads to the increase of APOBEC3G packaging into virions in a Vif-independent manner. Moreover, RN-18 exhibits a strong dependence on APOBEC3G, whereas HSP70 can interact directly with both HIV-1 Vif and APOBEC3G. Thus, RN-18 probably does not target HSP70. Taken together, the results of the present study suggest that stimulation of innate immunity, such as that mediated by APOBEC3G, may aid in the development of antiviral therapies.

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