Macaque R02-017 inoculated with molecular clones of SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 373T

Wks	aa sequences in Gag		
p-c	**216th**	244th	373rd
1	Mt	**Wt**(mt)	**Wt**(mt)
3	Mt	**Wt**	**Wt**
8	**Wt**	**Wt**	**Wt**

Macaque R05-002 inoculated with molecular clones of SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 373T & SIVmac239 Gag 216S 244E 375A & SIVmac239 Gag 216S 244E 376S

Wks	aa sequences in Gag				
p-c	**216th**	244th	373rd	375th	376th
1	Mt	wt/mt	**Wt**(mt)	**Wt**(mt)	**Wt**
3	Mt	**Wt**	**Wt**	**Wt**	**Wt**
12	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R02-023 inoculated with molecular clones of SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 247L 312V 373T

Wks	aa sequences in Gag				
p-c	**216th**	244th	247th	312th	373rd
1	Mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)
3	Mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)
6	Mt	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R02-022 inoculated with molecular clones of SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 247L 312V 373T & SIVmac239 Gag 172S 216S 244E 375A & SIVmac239 Gag 145A 216S 244E 376S

Wks	aa sequences in Gag								
p-c	145th	172nd	**216th**	244th	247th	312th	373rd	375th	376th
1	**Wt**(mt)	wt/mt	Mt	wt/mt	**Wt**(mt)	**Wt**(mt)	**Wt**	wt/mt	**Wt**(mt)
3	wt/mt	**Wt**	Mt	wt/mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)	wt/mt
14	wt/mt	**Wt**	Mt	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R03-022 inoculated with molecular clones of SIVmac239 Gag 216S 244E 373T & SIVmac239 Gag 216S 244E 247L 312V 373T

Wks	aa sequences in Gag				
p-c	**216th**	**244th**	247th	312th	**373rd**
1	Mt	Mt	**Wt**	**Wt**	Mt
3	Mt	Mt	**Wt**	**Wt**	Mt
20	wt/mt	Mt	**Wt**	**Wt**	Mt
25	**Wt**	Mt	**Wt**	**Wt**	Mt

FIG. 2. Dominant viral genome sequences in competition assay. A *gag* DNA fragment was amplified from plasma RNA by reverse transcription and nested PCR and sequenced as described previously (20). The amino acid sequences at the positions where mutations were included in the inoculums are shown. Q and R groups of SIV mutants are described in the text. **Wt**, only the wild-type sequence was detected; **Wt**(mt), the wild-type sequence was dominant but the mutant was detectable (the mutant/wild-type ratio was less than 1/4); wt/mt, the wild type and the mutant were detected equally; Mt(wt), the mutant was dominant but the wild type was detectable (the wild-type/mutant ratio was less than 1/4); Mt, only the mutant was detected. Other than the residues indicated in this figure, no dominant mutation resulting in an amino acid change was detected in the *gag* region in macaque R02-017, R05-002, R02-023, or R02-022, but macaque R03-022 showed one amino acid change resulting in a GagV375M substitution at weeks 20 and 25. p-c, postchallenge.

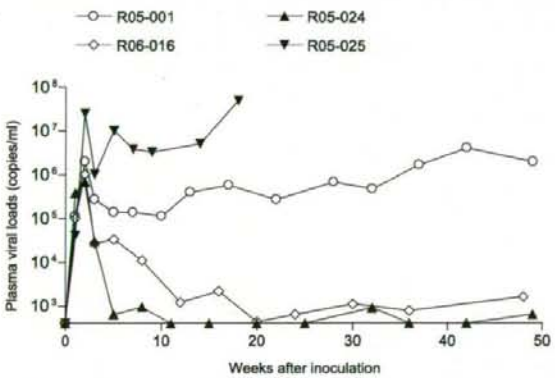

FIG. 3. Plasma viral loads (SIV *gag* RNA copies/ml plasma) in macaques after challenge with SIV carrying five *gag* mutations.

lymphadenopathy conditions and pneumocystis pneumonia. This macaque showed reversion of the GagD244E mutation in a few months, followed by reversion of the GagL216S, GagI247 L, and GagA312V mutations in a year postchallenge, while the GagA373T mutation remained dominant without reversion until euthanasia (Fig. 4). In contrast, macaque R06-016, with lower viral loads, showed no reversion of the five mutations. In the chronic phase, these two macaques showed additional Gag amino acid changes, including GagI140V (I to V at aa 140) and GagV375M (V to methionine [M] at aa 375) that were detected in both. Some of these mutations may contribute to the recovery of viral fitness.

To see the possibility of transmission of the viruses carrying the five gag mutations in the context of the polyclonal, V5-derived SIVs, macaques R05-024 and R05-025 were inoculated with plasma obtained from macaque V5 in the chronic phase of SIVmac239 infection (Fig. 3). For the challenge, plasma was obtained from macaque V5 at weeks 81, 87, 92, 100, and 113

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Macaque R05-001 infected with SIVmac239Gag216S244E247L312V373T

Wks	aa sequences in Gag					
p-c	**216th**	**244th**	**247th**	**312th**	**373rd**	other residues
1	Mt	Mt	Mt	Mt	Mt	
3	Mt	Mt	Mt	Mt	Mt	
10	Mt	**Wt**	Mt	Mt	Mt	I140V
22	wt/mt	**Wt**	Mt	Mt	Mt	I140V
37	wt/mt	**Wt**	Mt	Mt	Mt	V3A, I140V
42	**Wt**(mt)	**Wt**	Mt	Mt	Mt	V3A, (V68L/M), I140V
49	**Wt**	**Wt**	wt/mt	wt/mt	Mt	V3A, (V68L/M), I140V
55	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), I140V, (V340M), D429N
86	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), I140V, D429N
110	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), (I140V), V375M, (D429E)

Macaque R06-016 infected with SIVmac239Gag216S244E247L312V373T

Wks	aa sequences in Gag					
p-c	**216th**	**244th**	**247th**	**312th**	**373rd**	other residues
5	Mt	Mt	Mt	Mt	Mt	
12	Mt	Mt	Mt	Mt	Mt	
30	Mt	Mt	Mt	Mt	Mt	S128P, I140V, V375M
54	Mt	Mt	Mt	Mt	Mt	I140V, V375M

Macaque R05-024 infected with V5-plasma

Wks	aa sequences in Gag										
p-c	3rd	68th	145th	**216th**	**244th**	**247th**	**312th**	**373rd**	390th	404th	other residues
1	Mt(wt)	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	
5	Mt(wt)	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	I257K
49	Mt	Mt	Mt	Mt	Mt	**Wt**	**Wt**	**Wt**	**Wt**	Mt	A222V, I257K, R485K

Macaque R05-025 infected with V5-plasma

Wks	aa sequences in Gag										
p-c	3rd	68th	145th	**216th**	**244th**	**247th**	**312th**	**373rd**	390th	404th	other residues
1	Mt	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	
5	Mt	wt/mt	Mt	Mt	Mt	**Wt**(mt)	**Wt**(mt)	Mt	wt/mt	wt/mt	
7	Mt	wt/mt	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	
14	Mt	wt/mt	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	
18	Mt	**Wt**(mt)	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	

FIG. 4. Dominant viral genome sequences after challenge with SIV carrying five *gag* mutations. The amino acid sequences at the residues where mutations were included in the inoculums and dominant amino acid changes at other residues in *gag* are shown. In the column of other residues, the predominant mutations with detectable wild-type sequence are shown in parentheses. **Wt**, **Wt**(mt), wt/mt, Mt(wt), Mt, and p-c are defined in the Fig. 2 legend.

post-SIVmac239 challenge and 0.2 ml of each was intravenously inoculated into these two macaques. In the challenge SIV plasma, the five *gag* mutations (GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T) and GagV145A were dominant, and additional *gag* mutations were detected in the MA- and NC-coding regions. In macaque R05-024, exhibiting low viral loads, the SIV GagL216S and GagD244E mutations remained dominant, while reversion of the GagI247L, GagA312V, and GagA373T mutations was observed (Fig. 4). Macaque R05-025, exhibiting high viral loads, developed AIDS and was euthanized at week 18 postchallenge. Autopsy revealed lymphoatrophy and cytomegalovirus infection. This macaque showed rapid reversion of the SIV GagI247L and GagA312V mutations but maintained the GagL216S, GagD 244E, and GagA373T mutations until euthanasia.

In samples from these four macaques challenged with SIV mac239Gag216S244E247L312V373T or V5-derived plasma, we examined the virus-specific $CD8^+$ T-cell responses around 3 months postinfection by flow cytometric analysis of antigen-specific gamma interferon induction (data not shown) as described previously (14, 20). Analyses using vesicular stomatitis virus G-pseudotyped SIV-infected cells as a stimulator revealed SIV-specific $CD8^+$ T-cell responses in macaques R05-001, R06-016, and R05-024, but not in macaque R05-025, which may have contributed to the rapid AIDS progression in this animal. Macaque R05-024, exhibiting lower viral loads and rapid selection of a *gag* mutation resulting in an I257K (I to lysine [K] at aa 257) substitution, showed $CD8^+$ T-cell responses specific for the $Gag_{245-269}$ peptide mixture (a mixture of $Gag_{245-260}$, $Gag_{250-265}$, and $Gag_{255-269}$ peptides), suggesting a possibility of this mutation for viral escape from strong CTL pressure. None of these four macaques showed $CD8^+$ T-cell responses specific for the $Gag_{206-225}$ (a mixture of $Gag_{206-220}$ and $Gag_{210-225}$ peptides), $Gag_{206-225}$.216S ($Gag_{206-220}$.216S and $Gag_{210-225}$.216S), $Gag_{232-255}$ ($Gag_{232-246}$, $Gag_{236-250}$, and $Gag_{240-255}$), $Gag_{232-255}$.244E, $Gag_{236-255}$.244E247L, $Gag_{362-385}$ ($Gag_{362-377}$, $Gag_{367-381}$, and $Gag_{371-385}$), or $Gag_{362-385}$.373T peptide mixture, indicating that CTL responses were not involved in the reversion or nonreversion at residue 216, 244, 247, or 373 in these macaques.

The in vivo competition assay in the present study showed loss of viral fitness from the addition of the GagD244E and GagA373T mutations into SIVmac239Gag216S and further loss of viral fitness from additional GagI247L and GagA312V mutations. The reversion of GagD244E in macaque R05-001, GagA373T in macaque R05-024, and GagI247L and GagA312V in macaques R05-024 and R05-025 (Fig. 4) supports this notion. However, reversion was not observed in all the mutations after challenge with SIV carrying the five *gag* mutations. Challenge with SIVmac239Gag216S carrying the single GagL216S mutation has shown its reversion in 3 months (14), whereas the reversion of the GagL216S mutation was

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delayed or not observed after challenge with the SIV carrying five *gag* mutations. This may be due to the predominant selection of the reversion of other mutations or to lower viral replication efficiency in the latter case. Compensatory mutations can also be involved in this delay or nonreversion, but no additional *gag* mutation was observed in the early phase in macaque R06-016. The possibility of a contribution to this delay by GagI140V in macaque R05-001 and GagV145A in macaques R05-024 and R05-025 may be considered, while significant recovery of viral fitness by the latter mutation has not been observed (12).

It has been suggested that a reduction in viral fitness by CTL escape mutations may contribute to HIV/SIV control (19, 20, 28). Pressure by multiple epitope-specific CTLs may result in the selection of HIV/SIV with diminished replicative ability because of accumulating multiple escape mutations. The inefficient viral replication in macaques R02-022 and R03-022 (Fig. 1) and two of four macaques in the second experiment (Fig. 3) may reflect such a lower replicative ability of the mutant SIVs, but conversely, the results of the present study also showed efficient viral replication in macaques R05-001 and R05-025, indicating that the transmission of even such "crippled" HIV/SIV carrying multiple CTL escape mutations can result in persistent viral replication and AIDS progression. It remains unclear what host factors determined the viral replication efficiency in vivo in our study, while macaques with higher viral loads (R02-017, R05-002, R05-001, and R05-025) showed the first reversion earlier than those with lower viral loads (R02-022, R03-022, R06-016, and R05-024), suggesting an association of reversion with viral loads. Earlier reversion may result in the recovery of viral fitness, leading to higher viral loads, or conversely, higher viral loads may accelerate reversion.

Thus, our results suggest that in the transmission of HIV accumulating CTL escape mutations at the cost of viral fitness between MHC-mismatched individuals, even such crippled HIV infection can finally result in AIDS progression. Previous studies on SIVs with single CTL escape mutations showed their rapid reversion, but the present study on SIV with multiple CTL escape mutations indicates that the reversion of all the mutations was not required for the establishment of persistent viral replication or for the onset of disease. Furthermore, it suggests a possibility that CTL escape mutations resulting in viral fitness costs may not always revert rapidly even in the absence of CTL pressure after their transmission into MHC-mismatched hosts and can be transmitted further to other hosts. These results provide an important insight into HIV pathogenicity and evolution in human individuals with divergent MHC polymorphisms.

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Gag-Specific Cytotoxic T-Lymphocyte-Based Control of Primary Simian Immunodeficiency Virus Replication in a Vaccine Trial[▽]

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Gag-specific cytotoxic T lymphocytes (CTLs) exert strong suppressive pressure on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. However, it has remained unclear whether they can actually contain primary viral replication. Recent trials of prophylactic vaccines inducing virus-specific T-cell responses have indicated their potential to confer resistance against primary SIV replication in rhesus macaques, while the immunological determinant for this vaccine-based viral control has not been elucidated thus far. Here we present evidence implicating Gag-specific CTLs as responsible for the vaccine-based primary SIV control. Prophylactic vaccination using a Gag-expressing Sendai virus vector resulted in containment of SIVmac239 challenge in all rhesus macaques possessing the major histocompatibility complex (MHC) haplotype *90-120-Ia*. In contrast, *90-120-Ia*-positive vaccinees failed to contain SIVs carrying multiple *gag* CTL escape mutations that had been selected, at the cost of viral fitness, in SIVmac239-infected *90-120-Ia*-positive macaques. These results show that Gag-specific CTL responses do play a crucial role in the control of wild-type SIVmac239 replication in vaccinees. This study implies the possibility of Gag-specific CTL-based primary HIV containment by prophylactic vaccination, although it also suggests that CTL-based AIDS vaccine efficacy may be abrogated in viral transmission between MHC-matched individuals.

Despite tremendous efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether this approach actually does result in controlling human immunodeficiency virus (HIV) replication remains unknown. Recent trials have shown reductions in postchallenge viral loads by prophylactic vaccination eliciting virus-specific T-cell responses in macaque AIDS models (19, 22, 34), but the first advanced human trial of a T-cell-based vaccine was halted because of a lack of efficacy (5). Hence, it is quite important to determine which T-cell responses are responsible for primary HIV control.

Cytotoxic T-lymphocyte (CTL) responses have been indicated to play an important role in the control of HIV and simian immunodeficiency virus (SIV) infections (2, 9, 10, 17, 23, 29). Above all, the potential of Gag-specific CTL responses to contribute to viral control has been suggested by a cohort study indicating an association of HIV control with the breadth of Gag-specific CTL responses (15). In support of this, a recent in vitro study revealed their ability to rapidly respond to SIV infection (28). However, it has remained unclear whether Gag-specific CTL-based viral containment can be achieved by prophylactic vaccination.

We previously developed a prophylactic AIDS vaccine regimen consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (22, 32). Our trial showed potential for efficiently inducing Gag-specific T-cell responses and containment of SIVmac239 challenge in a group of Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* (22). A follow-up study revealed the reappearance of plasma viremia at >1 year postchallenge in some of these *90-120-Ia*-positive SIV controllers. In these transient controllers, multiple CTL escape mutations were accumulated in the viral *gag* gene, resulting in viremia reappearance and thus suggesting the involvement of $Gag_{206-216}$ (IINEEAADWDL) epitope-specific, $Gag_{241-249}$ (SSVDEQIQW) epitope-specific, and $Gag_{373-380}$ (APVPIPFA) epitope-specific CTLs in sustained viral control (12). Nonetheless, it has remained undetermined whether such Gag-specific CTL responses were responsible for the vaccine-based primary SIV control in *90-120-Ia*-positive vaccinees. In the present study, we challenged the *90-120-Ia*-positive vaccinees with SIVs carrying the *gag* CTL escape mutations to determine the role of Gag-specific CTLs in primary SIVmac239 control.

MATERIALS AND METHODS

Viral competition assay. SIV molecular clone DNAs with *gag* mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 (14) molecular clone DNA. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs, and their titers were

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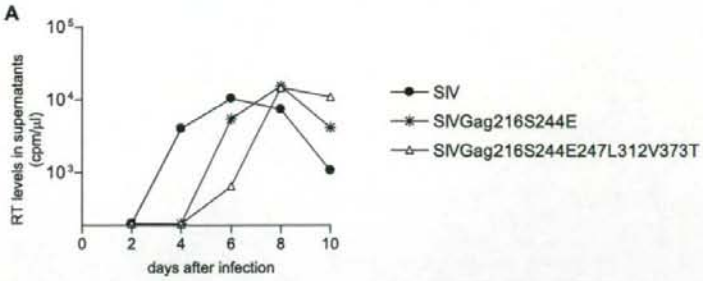

B

Competition between SIVmac239 vs SIVmac239Gag216S244E

inoculated dose ratio 1/1

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt	Wt
Exp 3	Wt (mt)	Wt

Competition between SIVmac239Gag216S244E vs SIVmac239Gag216S244E247L312V373T

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt (mt)	Wt
Exp 3	Wt (mt)	Wt

C

inoculated dose ratio 1/4

	day 6	day 18
SIVmac239	6/13	15/16
SIVmac239Gag216S244E	7/13	1/16

	day 6	day 18
SIVmac239Gag216S244E	7/18	12/16
SIVmac239Gag216S244E247L312V373T	11/18	3/16
*SIVmac239Gag216S244E312V373T		1/16

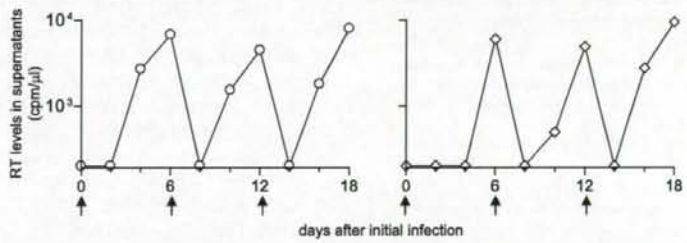

FIG. 1. Replication of mutant SIVs in vitro. (A) Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (closed circles), SIVmac239Gag216S244E (asterisks), or SIVmac239Gag216S244E247L312V373T (open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. Representative results from three sets of experiments are shown. (B) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIV mac239Gag216S244E247L312V373T (right) at a ratio of 1:1. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants at days 6 and 18 postinfection and then sequenced. Dominant amino acid sequences at the 216th and 244th aa (left) or the 247th, 312th, and 373rd aa (right) in Gag in three sets of experiments are shown. Wt, only the wild-type sequence was detected; Wt (mt), the wild type was dominant, but the mutant was detectable (the mutant/wild-type ratio was <1/2). (C) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T (right) at a ratio of 1:4. The amplified *gag* fragments were subcloned into plasmids and sequenced. Frequencies of the indicated SIV clones (number of indicated clone per total number of clones) are shown. Changes in RT levels in the culture supernatants are shown in the bottom panels. The arrows indicate the time points of coinfection (at day 0) and viral passage for the second (at day 6) and the third (at day 12) cultures.

measured by reverse transcription (RT) assay as described previously (25, 33). For analysis of viral replication, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) (1) 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. For competition, HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants were harvested every other day and 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 from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 postcoinfection. 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. Alternatively, it was subcloned into plasmids to determine dominant sequences.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (26). Three animals, R01-007, R02-003, and R02-012, that received a prophylactic DNA prime/SeV-Gag boost vaccine and contained SIVmac239 challenge have been reported previously (22). In the present study, macaques R06-015, R06-035, R06-041, R05-004, R05-027, and R07-005 also received the DNA prime/SeV-Gag boost vaccine. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*- and *nef*-deleted simian-human immunodeficiency virus $SHIV_{MDI4YE}$ molecular clone

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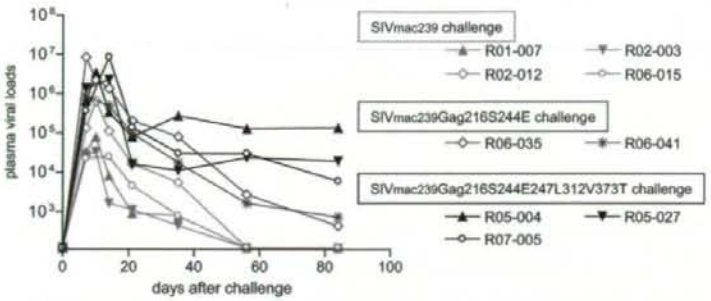

FIG. 2. Plasma viral loads after wild-type or mutant SIV challenge. The *90-120-Ia*-positive vaccinees were challenged with SIVmac239 (red lines), SIVmac239Gag216S244E (blue lines), or SIVmac239Gag216S244E247L312V373T (black lines). Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described before (22). The lower limit of detection is approximately 4×10^2 copies/ml.

DNA (SIVGP1) (31, 32) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime step, animals received a single boost intranasally with 6×10^9 cell infectious units of F-deleted replication-defective SeV-Gag (21, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239, SIVmac239 Gag216S244E, or SIVmac239Gag216S244E247L312V373T. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells (PBMCs). Sequence analysis confirmed the absence of *gag* mutations except for the two or five mutations in the challenge viruses.

Immunostaining of CD4$^+$ T-cell memory subsets. PBMCs were subjected to immunofluorescence staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll protein-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson, Tokyo, Japan). The central memory subset of CD4$^+$ T cells was defined by possession of a CD28$^+$ CD95$^+$ phenotype, as described previously (13, 27).

Measurement of virus-specific CD8$^+$ T-cell responses. We measured virus-specific CD8$^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (13, 22). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and the SIVGP1 DNA. Alternatively, B-lymphoblastoid cell lines were pulsed with 1 to 10 μM peptides for peptide-specific stimulation (11, 12). The 15-mer $Gag_{367\text{-}381}$ peptide was used to detect $Gag_{367\text{-}381}$-specific CTLs, including $Gag_{373\text{-}380}$-specific CTLs. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (Becton Dickinson). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ T-cell frequencies from those after Gag-specific, SIV-specific, or peptide-specific stimulation. Specific T-cell levels of <100 cells per million PBMCs were considered negative.

Statistical analysis. Statistical analysis was performed with Prism software, version 4.03, with significance set at P values of <0.05 (GraphPad Software, Inc., San Diego, CA). Central memory CD4$^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged ($n = 4$) and the mutant SIV-challenged ($n = 5$) macaques ($P = 0.70$ by unpaired two-tailed t test with Welch's correction and $P = 0.73$ by nonparametric Mann-Whitney U test). Ratios of the central memory CD4$^+$ T-cell counts from a few months postchallenge to those prechallenge were log transformed and compared between the two groups by an unpaired two-tailed t test and the Mann-Whitney U test. Gag-specific CD8$^+$ T-cell frequencies postvaccination (prechallenge) or postchallenge were also log transformed and compared between the two groups in the same statistical manner.

RESULTS

Comparison of viral fitness in wild-type and mutant SIVs. We used two mutant SIVs for challenge of the *90-120-Ia*-positive vaccinees. The first, designated SIVmac239Gag216S244E, carries two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag. The second, designated SIVmac239Gag216S244E247L312V373T, carries five *gag* mutations, GagL216S, GagD244E, GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa). In our previous study (12), the former became dominant in the early phase (at approximately 4 months postchallenge) during the period of viral control, and the latter was dominant at viremia reappearance in a transient controller. GagL216S, GagD244E and GagI247L, and GagA373T mutations result in viral escape from recognition by $Gag_{206\text{-}216}$-specific, $Gag_{241\text{-}249}$-specific, and $Gag_{373\text{-}380}$-specific CTLs, respectively, while it remains unclear whether GagA312V was selected for by CTLs.

We first compared viral fitness in wild-type and mutant SIVs. In HSC-F cells (a macaque T-cell line), not only the wild type but also the mutant SIVs were able to replicate, but SIVmac 239Gag216S244E replication was less efficient than that of wild-type SIVmac239, and SIVmac239Gag216S244E247L312 V373T replication was even less efficient (Fig. 1A). In competitions between two SIVs, HSC-F cells were coinfected with both viruses, and viral genome sequences in the culture supernatants were assessed to establish which SIV became predominant. In culture supernatants of HSC-F cells after coinfection with SIVmac239 and SIVmac239Gag216S244E inoculated at a ratio of 1:1, the wild type rapidly became dominant (at day 6) (Fig. 1B). Coinfection at a ratio of 1:4 resulted in equivalence at day 6, but the wild type again dominated by day 18 (Fig. 1C). These results indicate a lower replicative ability of SIVmac239 Gag216S244E than of wild-type SIVmac239. In addition, competition between SIVmac239Gag216S244E and SIVmac239 Gag216S244E247L312V373T showed the lower replicative ability of the latter (Fig. 1B and C).

Challenge of *90-120-Ia*-positive vaccinees with wild-type or mutant SIVs. Next, we challenged *90-120-Ia*-positive macaques

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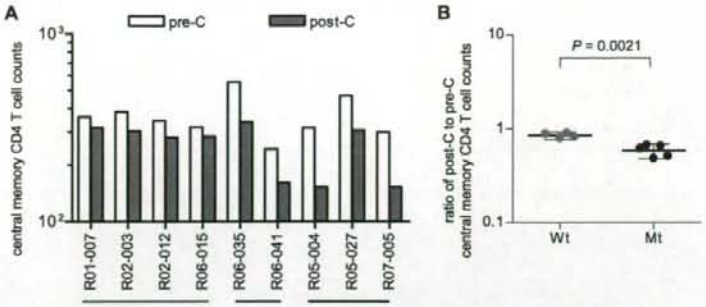

FIG. 3. Changes in central memory $CD4^+$ T-cell counts after wild-type or mutant SIV challenge. (A) Peripheral central memory $CD4^+$ ($CD4^+$ $CD95^+$ $CD28^+$) T-cell counts (/μl) prechallenge (pre-C) and a few months postchallenge (post-C). (B) Statistical comparison of central memory $CD4^+$ T-cell loss between the wild-type SIV-challenged (Wt) and the mutant SIV-challenged (Mt) macaques. The ratios of central memory $CD4^+$ T-cell counts postchallenge to those prechallenge are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidence intervals. The ratios in the mutant group ($n = 5$) were significantly lower than those in the wild-type group ($n = 4$) ($P = 0.0021$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test).

with the mutant SIVs after DNA prime/SeV-Gag vaccination. Remarkably, all three vaccinees (R05-004, R05-027, and R07-005) challenged with SIVmac239Gag216S244E247L312V373T failed to control viral replication and showed high set point plasma viral loads, while all four vaccinees (R01-007, R02-003, R02-012, and R06-015) challenged with wild-type SIVmac239 contained viral replication, with undetectable set point plasma viral loads (Fig. 2). Even the two vaccinees (R06-035 and R06-041) challenged with SIVmac239Gag216S244E failed to contain viral replication, although with lower plasma viral loads, at approximately 10^3 RNA copies/ml at 3 months postchallenge. Central memory $CD4^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged ($n = 4$) and mutant SIV-challenged ($n = 5$) macaques, but ratios of the counts at a few months postchallenge to prechallenge for the latter group were significantly lower than those for the former ($P = 0.0021$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test) (Fig. 3). Thus, *90-120-Ia*-positive vaccinees can contain wild-type SIVmac239 but not SIVmac239Gag216S244E or SIVmac239Gag216S244E247L312V373T challenge.

Viral *gag* sequence analysis confirmed the rapid selection for the GagL216S mutation in all wild-type SIVmac239-challenged macaques, as described previously (22). All of the *gag* mutations in the challenge mutant viruses were maintained during the observation period (Table 1). SIVmac239Gag216S244E247L312V373T-challenged macaques showed no additional dominant *gag* mutations, whereas animals challenged with SIVmac239Gag216S244E rapidly selected viruses with a GagV145A (V to A at the 145th aa) mutation. Recovery of viral fitness by this mutation was not observed, and whether it was selected for by CTLs was unclear in our previous study (12).

Gag-specific CTL responses were induced after SeV-Gag boost in all vaccinees, and there was no significant difference in the levels between the wild-type and mutant challenges ($P = 0.1198$ by unpaired t test and $P = 0.1111$ by Mann-Whitney U test). However, secondary Gag-specific CTL responses were

TABLE 1. Dominant sequences in SIV Gag in macaques after challenge

Macaque	Time (wk) of plasma sample	Amino acid change in Gag at position[a]:								
		140	145	206	216	244	247	312	341	373
R01-007	5				L216S					
R02-003	5				L216S					
R02-012	5				L216S					
R06-015	5			(I206M)	L216S					
R06-035	5				L216S*	D244E*				
	12		V145A		L216S*	D244E*			(N341Y)	
R06-041	5		(V145A)		L216S*	D244E*				
	12		V145A		L216S*	D244E*				
R05-004	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12	(I140V)			L216S*	D244E*	I247L*	A312V*		A373T*
R05-027	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*
R07-005	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*

[a] A fragment containing the entire *gag* region was amplified from plasma RNA by nested RT-PCR and then sequenced. We were unable to amplify the fragment from plasmas obtained at week 12 from the wild-type SIVmac239-challenged macaques with undetectable viremia. Dominant *gag* mutations resulting in amino acid changes are shown. Asterisks indicate the mutations included in the challenge inoculums. Parentheses indicate that both the wild-type and mutant sequences were detected equivalently at that position.

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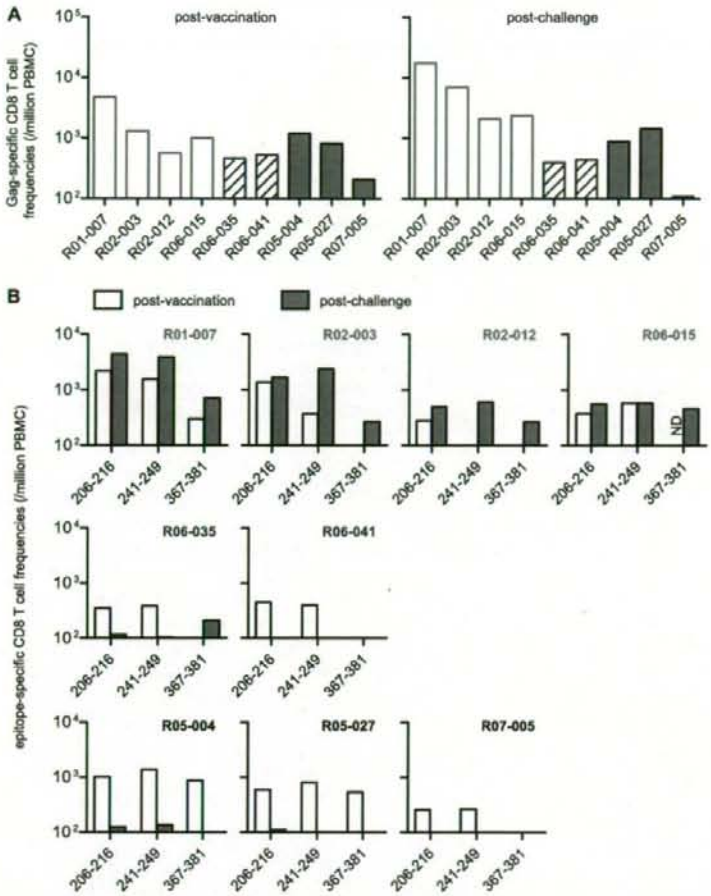

FIG. 4. Gag-specific CD8$^+$ T-cell responses before and after wild-type or mutant SIV challenge. Macaques R01-007, R02-003, R02-012, and R06-015 were challenged with SIVmac239; macaques R06-035 and R06-041 were challenged with SIVmac239Gag216S244E; and macaques R05-004, R05-027, and R07-005 were challenged with SIVmac239Gag216S244E247L312V373T. (A) Gag-specific CD8$^+$ T-cell frequencies at 2 weeks postboost (postvaccination) (left) and 2 weeks postchallenge (right). (B) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CD8$^+$ T-cell frequencies at 2 weeks (all except for R02-012) or 4 weeks (in R02-012) postboost (postvaccination) and 5 weeks (in R01-007, R02-003, R02-012, R06-035, R06-041, and R05-004) or 6 weeks (in R06-015, R05-027, and R07-005) postchallenge. ND, not determined.

less efficient after challenge with mutant SIV than after challenge with wild-type SIV ($P = 0.0095$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test) (Fig. 4A).

SeV-Gag boost induced efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses in all vaccinees and $Gag_{367-381}$-specific CTL responses in some of them (Fig. 4B). Challenge with wild-type SIVmac239 resulted in efficient secondary responses of these three epitope-specific CTLs, whereas SIVmac 239Gag216S244E247L312V373T challenge evoked none of them (Fig. 4B). SIVmac239Gag216S244E challenge did not result in secondary responses of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTLs but did induce $Gag_{367-381}$-specific CTL responses in one case (Fig. 4B). These results indicate that SIVmac239 Gag216S244E evades recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs and that SIVmac239Gag216S244E2 47L312V373T evades recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs.

We next examined Gag-specific and SIV-specific CTL responses after mutant SIV challenge (Fig. 5A). We used an *env*- and *nef*-deleted SHIV molecular clone DNA, SIVGP1, that has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CTLs responding to SIVGP1-transduced cells (referred to as SIV-specific CTLs) as described previously (13, 32). SIV-specific CTL frequencies at week 12 were much higher than those at week 2 for all five macaques challenged with mutant SIVs. In contrast, Gag-specific CTL frequencies at week 12 were lower than those at week 2 for four of five animals; the remaining macaque, R06-035, mounted $Gag_{367-381}$-specific CTL responses. Importantly, in all animals challenged with mutant SIVs, SIV-specific CTL

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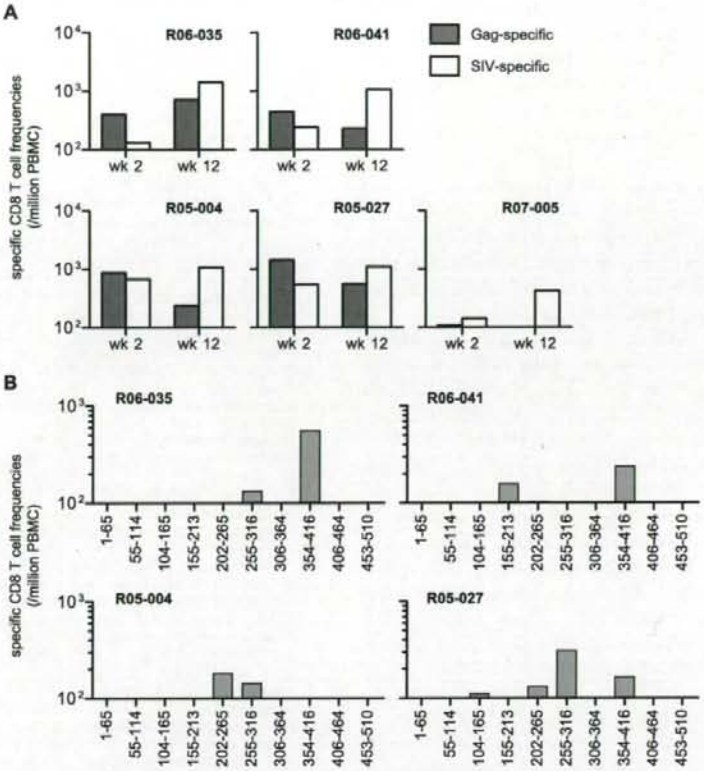

FIG. 5. SIV-specific $CD8^+$ T-cell responses after mutant SIV challenge. (A) Gag-specific (closed boxes) and SIV-specific (open boxes) $CD8^+$ T-cell frequencies at 2 weeks or 12 weeks postchallenge. (B) Frequencies of $CD8^+$ T cells specific for pools of SIV Gag peptides. A panel of 117 overlapping peptides (15 to 17 aa in length and overlapping by 10 to 12 aa) spanning the entire SIV Gag amino acid sequence were divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, 1st to 65th aa in SIV Gag; pool 2, 55th to 114th aa; pool 3, 104th to 165th aa; pool 4, 155th to 213th aa; pool 5, 202nd to 265th aa; pool 6, 255th to 316th aa; pool 7, 306th to 364th aa; pool 8, 354th to 416th aa; pool 9, 406th to 464th aa; and pool 10, 453rd to 510th aa. The pools were used for stimulation to detect peptide pool-specific $CD8^+$ T cells.

frequencies were at marginal levels or lower than Gag-specific CTL frequencies at week 2, but the former became higher than the latter at week 12. These results indicate an induction of CTL responses specific for SIV antigens other than Gag in all five macaques after mutant SIV challenge.

At week 12 after mutant SIV challenge, Gag-specific CTL responses were undetectable in macaque R07-005 but were still detected in the other four macaques. We then analyzed Gag-specific CTL responses in these four macaques by using a panel of overlapping peptides spanning the entire SIV Gag amino acid sequence (Fig. 5B). In both SIVmac239Gag216S244E-challenged animals, R06-035 and R06-041, exhibiting detectable $Gag_{367-381}$-specific CTL responses (data not shown), CTL responses specific for the peptide mixture corresponding to the 354th to 416th aa in SIV Gag were detected at week 12. In addition, we found $Gag_{255-316}$-specific CTL responses in macaque R06-035 and $Gag_{155-213}$-specific CTL responses in macaque R06-041. SIVmac239Gag216S244E247L312V373T-challenged macaques R05-004 and R05-027 showed responses specific for several Gag peptide mixtures, including $Gag_{202-265}$-specific and $Gag_{255-316}$-specific CTL responses. These results indicate an induction of CTL responses specific for Gag epitopes other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes after mutant SIV challenge.

DISCUSSION

In the present study, SIVs carrying multiple *gag* CTL escape mutations showed lower replicative abilities than that of the wild type; nonetheless, the *90-120-Ia*-positive vaccinees were able to contain only the latter. This demonstrates that Gag-specific CTL responses did play a central role in the vaccine-based primary containment of wild-type SIVmac239 replication in *90-120-Ia*-positive macaques.

Elicitation of virus-specific T-cell responses by prophylactic vaccination is believed to be a promising strategy for HIV control (3, 24); whether this approach can actually result in HIV control remains unknown. Recent studies have indicated the possibility of reductions in set point viral loads after SIV challenge by prophylactic vaccination inducing T-cell responses in rhesus macaques (19, 22, 34), yet the immune component crucial for the vaccine-based viral control has not been

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determined. No clear evidence for a contribution of vaccine-induced CTLs to this viral control has been forthcoming to date, although virus-specific CTL responses have been implicated in exerting strong suppressive pressure on HIV/SIV infection (9, 22). Indeed, viral replication persists even in the presence of CTL responses in the natural course of infection; it has thus remained unclear whether HIV/SIV replication can be controlled by vaccine-induced CTLs. The evidence from the present study now strongly implicates Gag-specific CTL responses as responsible for vaccine-based primary SIV control. This offers the possibility of Gag-specific CTL-based HIV containment by prophylactic vaccination and provides insight into the development of CTL-based AIDS vaccines.

The containment of SIVmac239 but failure to contain SIV mac239Gag216S244E in the vaccinees documents a crucial role for $Gag_{206-216}$-specific and/or $Gag_{241-249}$-specific CTL responses in vaccine-based SIVmac239 containment. Furthermore, challenge with SIVmac239Gag216S244E247L312V373T, possessing diminished viral fitness compared to SIVmac239Gag216S244E, tended to result in higher viral loads, indicating the involvement of $Gag_{373-380}$-specific CTL responses in viral control, while more complete viral evasion of $Gag_{241-249}$-specific CTL recognition by addition of the GagI247L mutation may also contribute to the difference between SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T challenge. Taken together, we conclude that these two or three epitope-specific CTL responses are crucial for primary SIVmac239 control in *90-120-Ia*-positive vaccinees. Conversely, this study implies that viral evasion of recognition by two dominant epitope-specific CTLs can result in failure of primary viral containment but may not be sufficient for abrogation of vaccine efficacy. Thus, analysis of CTL-based vaccine efficacy against SIVs carrying single or multiple CTL escape mutations could contribute to an evaluation of its potential for controlling the replication of highly diversified HIVs.

Our results suggest that SIV- but non-Gag-specific CTLs became predominant after mutant SIV challenge. Additionally, CTLs recognizing Gag regions other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes were detected in most cases. These CTL responses may exert suppressive pressure on viral replication but are considered insufficient for controlling replication of the mutant SIVs with lower viral fitness.

Finally, this study also provides evidence indicating a possible abrogation of CTL-based AIDS vaccine efficacy in viral transmission between MHC-I-matched individuals. Indeed, even the mutant SIVs carrying multiple CTL escape mutations were able to replicate persistently in vivo, despite their diminished replicative ability. Transmission of these viruses can result in persistent viral infection and AIDS progression (30). CTL escape mutations resulting in a loss of viral fitness may revert to the wild-type sequence after transmission into MHC-I-mismatched hosts (4, 8, 9, 16, 18, 20), but such reversion does not occur rapidly; alternatively, some may be retained with additional compensatory mutations (6, 7, 30). Thus, there may be a risk of transmission and accumulation of HIV CTL escape variants even among MHC-I-mismatched individuals, resulting in abrogation of CTL-based AIDS vaccine efficacy in a population.

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Determination of a major histocompatibility complex class I restricting simian immunodeficiency virus $Gag_{241-249}$ epitope

Several major histocompatibility complex class I (MHC-I) alleles such as *HLA-B*57* have been shown to be associated with lower viral loads and better prognosis in HIV-1 infections, and MHC-I-restricted epitope-specific effective cytotoxic T lymphocyte (CTL) responses are found to play an important role in this reduction of viral loads [1–3]. Characterization of these effective CTLs could contribute to the development of an effective AIDS vaccine.

We have developed a prophylactic vaccine using a Sendai virus vector expressing simian immunodeficiency virus mac239 (SIVmac239) Gag (SeV-Gag) and have shown its protective efficacy against SIVmac239 challenge in a group of Burmese rhesus macaques (*Macaca mulatta*) sharing an MHC-I haplotype *90-120-Ia* [4]. Involvement of SIVmac239 $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses in this viral control have been indicated [5]. Interestingly, the SIVmac239 $Gag_{241-249}$ epitope is located in a region corresponding to the HLA-B*57-restricted HIV-1 $Gag_{240-249}$ epitope, TW10 (TSTLQEQIAW), and TW10-specific CTL responses have also been indicated to exert strong suppressive pressure on HIV-1 replication resulting in lower viral loads [6,7]. An SIVmac239 $Gag_{241-249}$-specific CTL escape mutation has been shown to result in a loss of viral fitness similarly with a TW10-specific CTL escape mutation [5]. In the present study, for further analysis of SIVmac239 $Gag_{241-249}$-specific CTL function, we have tried to determine the MHC-I that restricts this CTL epitope.

Among eight MHC-I alleles consisting of MHC-I haplotype *90-120-Ia* [4,8], expression of three alleles, *Mamu-A*90120-4*, *Mamu-A*90120-5*, and *Mamu-B*90120-6*, was predominant at RNA levels. We cloned cDNAs of these three alleles and established HLA-A/B/C-negative human 721.221 cell lines [9] expressing these cDNAs, respectively. These cells were pulsed with 10 nmol/l of $Gag_{241-249}$ peptide and used as target cells for the CTL assay using an SIVmac239 $Gag_{241-249}$-specific CTL clone as the effector. Measurement of cytotoxicity in standard ^{51}Cr release assay [5] revealed specific killing of $Gag_{241-249}$-pulsed cells expressing Mamu-A*90120-5, indicating restriction of this CTL epitope by the Mamu-A*90120-5 molecule (Fig. 1a).

Both of the Mamu-A*90120-5-restricted SIVmac239 $Gag_{241-249}$ epitope and the HLA-B*57-restricted HIV-1 TW10 epitope are considered to have the same anchor residues, serine (S) at position 2 and tryptophan (W) at the carboxyl terminus. Comparison of amino acid sequences of antigenic peptide-binding domains (α1 and α2 domains) in Mamu-A*90120-5 with those in HLA-B*5701 revealed limited similarities (154/182 = 84.6%) between these two (Fig. 1b). This might be compatible with previous reports indicating that human and macaque MHC-I molecules with divergent peptide-binding grooves can bind similar or identical peptides [10,11]. MHC-I molecules form a peptide-binding groove including B-pocket and F-pocket that play a key role in determination of the binding peptide motif for its specific binding to the MHC-I. Mamu-A*90120-5 and HLA-B*5701 showed similarity in eight of 11 residues at 7, 9, 24, 25, 34, 45, 63, 66, 67, 70, and 99, which are considered to be anchor residues involved in B-pocket binding and in seven of eight residues at 77, 80, 81, 116, 123, 143, 146, and 147 involved in F-pocket binding [11–13].

In addition, TW10 epitope-specific CTLs, HLA-B*57-restricted HIV-1 $Gag_{147-155}$ [ISW9 (ISPRTLNAW)] epitope-specific CTLs have also been indicated to exert strong selective pressure on HIV-1 [14]. The SIVmac239 $Gag_{149-157}$ amino acid sequence corresponding to the HIV-1 $Gag_{147-155}$ epitope region is LSPRTLNAW, showing a difference at the amino terminus, and CTL responses specific for a peptide including the SIVmac239 $Gag_{149-157}$ amino acid sequence were not induced by SeV-Gag vaccination in Mamu-A*90120-5-positive macaques (data not shown). Interestingly, the SIVmac239 Gag 148th proline (P) and 149th leucine (L) correspond to the HIV-1 Gag 146th P and the 147th L, respectively that have been indicated to be selected in HIV-1-infected humans possessing HLA-B*57. Selection of the former 146th P has been shown to result in escape from ISW9-specific CTL recognition by disturbance in antigen processing [14]. Thus, it is speculated that the SIVmac239 $Gag_{149-157}$-derived peptide may not be presented by Mamu-A*90120-5 even if it has an ability to bind this peptide.

Both SIVmac239 $Gag_{241-249}$-specific CTLs and HIV-1 TW10-specific CTLs have been indicated to exert strong suppressive pressure on SIV/HIV-1 replication and select for a mutation resulting in escape from their recognition at the cost of viral fitness. Thus, this Gag region may be a promising CTL target for viral control, and SIVmac239 infection in Mamu-A*90120-5-positive macaques could be a unique model for examining viral replication in the

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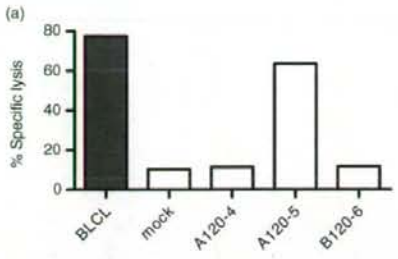

(b)

```
                 * *                    **          *
Mamu-A*90120-5  GSHSMRYFYT SMSRPGRGQP RFFAVGYVDD TQFVRFDSDA
HLA-B*5701      ---------- A-------E- --I-------- ----------

                    *                   * ** *        # #
Mamu-A*90120-5  ESPRMEPRAP WVEQEGPEYW DQETRKAKDT AQNYRVNLRT
HLA-B*5701      A----A---- -I-------- -G---NM-AS --T--E---I

                #                   *                 #
Mamu-A*90120-5  ALRYYNQSEA GSHTIQKMYG CDLGPDGRLL RGYDQSAYDG
HLA-B*5701      ---------- ---I--V--- --V------- --H-------

                  #                   # ##
Mamu-A*90120-5  RDYIALNEDL RSWTAADMAA QNTQRKWEAA GWTEQMRAYL
HLA-B*5701      K--------- S------T-- -I-------- RVA--L----


Mamu-A*90120-5  EGECLEWLRR YLENGKETLQ RA
HLA-B*5701      --L-V----- ---------- --
```

Fig. 1. Mamu-A*90120-5 that restricts the SIV $Gag_{241-249}$ epitope. (a) CTL assay using a $Gag_{241-249}$-specific CTL clone on a B-lymphoblastoid cell line derived from a macaque possessing *90-120-Ia* (BLCL), 721.221 cells (mock), and 721.221 cells expressing Mamu-A*90120-4 (A120-4), Mamu-A*90120-5 (A120-5), and Mamu-B*90120-6 (B120-6), respectively. (b) Amino acid sequences of the Mamu-A*90120-5 α1 and α2 domains in comparison with HLA-B*5701. The anchor residues involved in B and F-pocket binding are indicated by * and #, respectively.

presence of those CTLs targeting this region like TW10-specific CTLs. Finally, we obtained a phycoerythrin-conjugated $Gag_{241-249}$ epitope-Mamu-A*90120-5 tetramer for specific detection of $Gag_{241-249}$-specific CTLs. This could be useful for the analysis of $Gag_{241-249}$-specific CTL responses in Mamu-A*90120-5-positive macaques infected with SIVmac239.

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REVIEW

Host factors involved in resistance to retroviral infection

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ABSTRACT

Viral replication requires the help of host cell factors, whose species specificity may affect viral tropism. On the other hand, there exist host factors that restrict viral replication. The anti-viral system mediated by some of these restriction factors, which is termed intrinsic immunity and is distinguished from conventional innate and adaptive immunity, has been described as playing an important role in making species-specific barriers against viral infection. Here, we describe the current progress in understanding of such restriction factors against retroviral replication, focusing on TRIM5α and APOBEC, whose anti-retroviral effects have recently been recognized. Additionally, we mention cyclophilin A, which is essential for HIV-1 replication in human cells and may affect viral tropism. Understanding of these host factors would contribute to identification of the determinants for viral tropism.

Key words apolipoprotein B mRNA-editing enzyme-catalytic subunit, cyclophilin A, retrovirus, tripartite interaction motif 5 α.

Among host factors exerting inhibitory effects on viral replication, the *Fv-1* and the *Fv-4* loci have been known to confer resistance to Friend virus infection in mice and their restriction mechanisms have been intensively investigated (1–5). The latter, *Fv-4*, corresponds to a defective endogenous provirus with an ecotropic MLV-like *env* gene. Entry of ecotropic MLV into cells expressing *Fv-4* is inhibited by binding of the *Fv-4 env* gene product, Fv-4 Env, to the ecotropic MLV receptor, and this receptor interference has been reported to play a central role in resistance to Friend virus infection (6). Additionally, it has recently been suggested that the Fv-4 Env-mediated dominant negative effect on MLV Env function contributes to efficient resistance in *Fv-4*-expressing mice (7). On the other hand, MLV restriction mediated by the *Fv-1* gene product, Fv-1, occurs after viral entry into the cells but before the integration step during the viral replication cycle (8). The two main alleles of *Fv-1*, *Fv-1*^{n} and *Fv-1*^{b}, confer resistance to replication of B-tropic and N-tropic MLV respectively (9). Although the precise mechanism of Fv-1-mediated restriction remains unclear, comparison of viral genome sequences between B-tropic and N-tropic MLV has indicated the 110th amino acid in Gag CA as the viral determinant for the tropism, suggesting that MLV Gag CA is the target for the host factor involved in this restriction (10, 11).

Ref-1, which shows a similar pattern of inhibition of retroviral replication with Fv-1, is known as a restriction factor in mammalian cells apart from murine cells. The viral determinant for Ref-1-mediated restriction has been

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List of Abbreviations: A, adenine; agmApo3G, African green monkey Apo3G; Ala, alanine; Apo3G, APOBEC3G; APOBEC, apolipoprotein B mRNA-editing enzyme-catalytic subunit; CA, capsid; CypA, cyclophilin A; CsA, cyclosporine A; G, guanine; Gly, glycine; hApo3G, human Apo3G; HIV-1, human immunodeficiency virus type 1; Ile, isoleucine; Lv-1, lentivirus susceptibility factor 1; MLV, murine leukemia virus; OWM, old world monkey; Pro, proline; Ref-1, restriction factor 1; SIVagm, African green monkey simian immunodeficiency virus; SIVmac, simian immunodeficiency virus mac; TRIM5, tripartite interaction motif 5; TRIM5α, tripartite interaction motif 5 α; Vif, virus infectivity factor.

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reported to be at the Gag CA residue, corresponding to the 110th in MLV Gag CA involved in Fv-1-mediated restriction (12). Ref-1 and Fv-1 both show viral restriction post-viral entry in the early phase of the retroviral replication cycle, but the exact point of restriction has been indicated to be different: the former, Ref-1-mediated restriction, occurs at the step prior to reverse transcription, while the latter, Fv-1 mediated restriction, is considered to occur post-reverse transcription (8). Further, restriction of HIV-1 replication post-viral entry has been reported in OWM cells that support efficient replication of SIVmac, and the existence of a restriction factor, Lv-1, responsible for this resistance of non-human primate cells to HIV-1 replication has been suggested (13–15).

TRIM5α: A HOST FACTOR RESTRICTING HIV-1 REPLICATION POST-VIRAL ENTRY IN PRIMATE CELLS

Recently, two independent groups have identified the α-isoform of TRIM5, TRIM5α, as a restriction factor responsible for resistance of monkey cells to HIV-1 infection and shown that restriction of HIV-1 replication by TRIM5α derived from rhesus and owl monkeys but not efficiently from humans (16, 17) (Fig. 1). Subsequent studies have revealed virus-specific restriction activities by TRIM5α and its homologues derived from humans and non-human primates (18–24). For instance, restriction by rhesus monkey TRIM5α is efficient against HIV-1 but inefficient against SIVmac and undetectable against MLV (Fig. 1).

TRIM5α is a trimeric cytoplasmic protein (25, 26) consisting of RING finger, B-box, coiled-coil, and SPRY (B30.2) domains (27, 28). The coiled-coil domain is indispensable for TRIM5α multimerization, and both the coiled-coil and the SPRY domains are required for its binding to the virion core (28, 29).

TRIM5α-midiated restriction of HIV-1 replication is considered to occur after viral entry in the early phase of the viral replication cycle, but its precise mechanism remains unclear and several possibilities have been proposed. First, it has been suggested that binding of TRIM5α to the virion CA after viral entry may accelerate or abrogate the process of its uncoating and disruption, resulting in inhibition of HIV-1 replication (29). Second, involvement of ubiquitin in TRIM5α-mediated restriction has been suggested by recent reports showing that a mutation in its RING finger domain decreases the restriction ability of TRIM5α (16, 30–32) and that recovery from the restriction occurs in the presence of proteosome inhibitors (32, 33), although this is controversial (29, 31, 32). Furthermore, some reports have shown TRIM5α-mediated

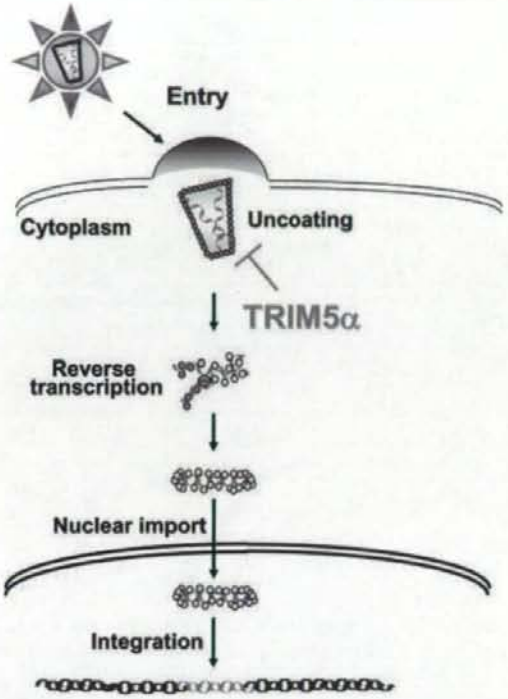

Fig. 1. A schema for TRIM5α-mediated restriction of HIV-1 replication in OWM cells. Recognition of HIV-1 CA by TRIM5α results in restriction of HIV-1 replication at the step after viral entry into the cytoplasm.

inhibition of viral cDNA nuclear import as well as viral cDNA synthesis (34, 35). In addition to restriction at the early phase of retroviral replication cycle, TRIM5α has recently been shown to inhibit virus production by accelerating degradation of viral Gag protein (36).

APOBEC: AN ENZYMATIC HOST FACTOR RESTRICTING RETROVIRAL REPLICATION

HIV-1 replication in primary $CD4^+$ T lymphocytes and monocytes is dependent on the presence of an HIV-1 accessory protein, Vif, which has been reported to work in a host cell-specific manner (37, 38). Vif is required for infectious HIV-1 production from some immortalized human T cell lines such as CEM (termed non-permissive) but not in others such as CEM-SS (termed permissive), and the existence of a restriction factor whose anti-retroviral activity can be abrogated by Vif has been suggested in the case of the non-permissive cells (39–45). Comparison of the non-permissive and permissive cells has

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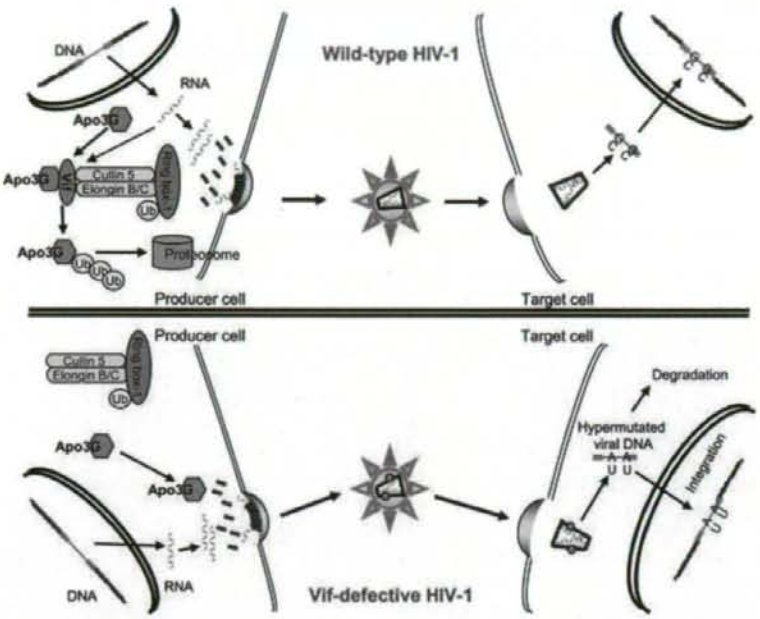

Fig. 2. A putative model for APOBEC3G-mediated restriction of HIV-1 replication and Vif-mediated recovery from the restriction. In the wild-type HIV-1 replication (upper panel), Vif connects hApo3G to an E3 ubiquitin ligase complex including Elongin B/C, Cullin5, and Ring-box-1 to induce polyubiquitylation and proteosomal degradation of hApo3G, resulting in exclusion of hApo3G from the virion with viral genome remaining intact even after viral entry. In contrast, in *vif*-deleted HIV-1 replication (lower panel), hApo3G is incorporated into the virion and its replication ability is abrogated after viral entry into the cells. Thereafter, these C-to-U mutations in the viral minus-strand DNA result in G-to-A mutations in the complementary plus-strand DNA during reverse transcription.

revealed Apo3G, a member of the APOBEC family of cytidine deaminases, to be the restriction factor responsible for inhibition of *vif*-deleted HIV-1 replication in human non-permissive cells (46). Unlike TRIM5α and Fv-1, the target of Apo3G-mediated restriction is not viral CA, but viral single-stranded cDNA synthesized during reverse transcription. It is packaged into virus particles produced from Apo3G-expressing cells and inhibits viral replication after viral entry into the cells (Fig. 2). HIV-1 Vif can inhibit the uptake of Apo3G into the virion by inducing polyubiquitylation and proteosomal degradation of cellular Apo3G, resulting in abrogation of Apo3G-mediated restriction (47) (Fig. 2).

Several mechanisms for Apo3G-mediated restriction against HIV-1 infection have been reported. First, it has been reported that the cytidine deaminase activity of Apo3G can induce hypermutation (a large number of G-to-A substitutions) in proviral DNA during reverse transcription, resulting in failure of infectious HIV-1 production (48–53) (Fig. 2). Second, the possibility of Apo3G-mediated inhibition of tRNA annealing or processing during reverse transcription has been shown (54–56). Additional mechanisms, including inhibition at the step of viral plus-strand cDNA transfer, have also been suggested (56–58).

Restriction of retroviral infection by Apo3G derived from non-human species has also been reported (51, 59–62), and the Vif-Apo3G interaction is considered to be species-specific (51, 63). Indeed, it has been indicated that hApo3G is insensitive to SIVagm Vif while agmApo3G is insensitive to HIV-1 Vif, and that the determinant for this specificity is at the 128th residue in Apo3G (51, 64–67). However, a recent report has shown that SIVagm Vif can support SIVagm replication in an hApo3G-positive human T cell line (A3.01): *vif*-deleted SIVagm replication was severely restricted with accumulation of G-to-A mutations in the viral genome, suggesting ambiguity of species specificity (68).

Restriction of HIV-1 and SIV replication by other members of the APOBEC family has been reported, although it might not be as efficient as Apo3G. Thus, APOBEC proteins are now considered to be a new class of host restriction factors against retroviral replication (61, 69). For instance, human APOBEC3F can inhibit HIV-1 replication in the absence of Vif (59, 60, 70, 71) whereas human APOBEC3B does so even in the presence of

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Vif (59, 72, 73). Association of deaminase activity with Apo3G-mediated restriction of HIV-1 replication has been strongly suspected, but the possibility of involvement of deaminase activity-independent mechanisms in this restriction has also been suggested (57, 58). Indeed, several groups have reported Apo3G and APOBEC3F variants lacking in deaminase activity without loss of restriction activity, as well as variants lacking in restriction activity without loss of deaminase activity (74–77). Additionally, a recent report has suggested a deaminase-independent inhibitory effect of Apo3G on viral DNA synthesis following reverse transcription (78). However, this possibility is controversial (79, 80), and the precise mechanism for Apo3G-mediated restriction of HIV-1 infection remains unclear.

CYCLOPHILIN A: A HOST FACTOR INVOLVED IN RETROVIRAL REPLICATION

CypA, a ubiquitous protein, was first identified as the target of CsA, an immunosuppressive reagent (81). CypA has proline-isomerase activity that catalyzes *cis-trans* isomerization of the Pro residue (82, 83). CsA binding to CypA inhibits this isomerase activity (83). CypA binding to HIV-1 Gag CA has been shown by analysis using the yeast two-hybrid system (84). The Ala-Gly-Pro-Ile residues from the 88th to the 91st in CA are the key portion for its binding to the active site of CypA (85–87). Interestingly, the peptidyl-prolyl bond between the 89th Gly (Gly89) and the 90th Pro (Pro90) exhibits the *trans* conformation, (in contrast to the *cis* conformation usually observed in other known CypA targets) (87, 88), and this Pro90 residue but not other Pro is considered to be critical for the binding of CA to CypA. Thus, it has been suggested that CypA acts as a molecular chaperone without exerting *cis-trans* isomerase activity on HIV-1 CA (87). However, this is still controversial and the possibility of CypA-mediated *cis-trans* isomerization of the Gly89-Pro90 peptidyl-prolyl bond has also been suggested (88).

It has been well established that CypA promotes HIV-1 replication after viral entry in the early phase in human cells (85, 89–95) (Fig. 3). CypA is efficiently incorporated into the virion produced from HIV-1-infected cells through interaction with CA in the context of Gag polyprotein (Fig. 3). Disruption of CypA incorporation

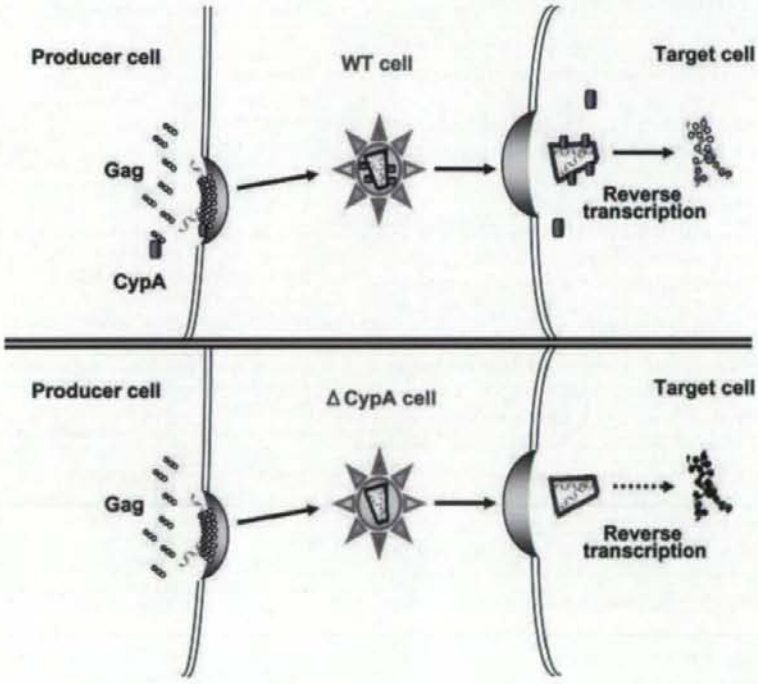

Fig. 3. A putative mechanism for CypA-mediated enhancement of HIV-1 replication. CypA is required for efficient reverse transcription (upper panel), and HIV-1 infection in CypA-deficient human cells shows inefficient reverse transcription (lower panel).

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into the virion by CsA administration or by Gag mutations resulted in reduction in infectivity of the produced viruses (85, 89, 91, 95–98). Several reports have shown that both CA dimerization and CypA multimerization are required for the efficient CA-CypA binding which is critical for HIV-1 infectivity (99, 100). Recently, promotion of HIV-1 replication by post-entry interaction of CA with CypA in target cells has been shown, suggesting the importance of CypA for efficient HIV-1 replication (94, 101, 102).

Only retroviruses with CA capable of binding to CypA exhibit CypA-dependent viral replication (84, 85, 90, 92, 95). This suggests involvement of CA-CypA interaction in the determination of retroviral tropism (14, 16, 17, 34, 98, 101–111). The effect of CypA on SIV replication in human cells has not been clearly determined but, for the first time, a recent study has shown that human CypA exerts an inhibitory effect on *vif*-deleted SIV replication, which may be recovered by SIV Vif excluding the CypA from the virion (112). This Vif function can be distinguished from the anti-hApo3G function of Vif described above.

PERSPECTIVES: HOST FACTORS INVOLVED IN THE POST-ENTRY STEP OF THE EARLY PHASE OF RETROVIRAL REPLICATION

Restriction of HIV-1 replication in non-human primate cells after viral entry occurs at the step prior to reverse transcription, and TRIM5α plays a crucial role in this restriction (13–15, 101, 106, 113–115). Interestingly, CypA-dependency is considered to occur at the same step in the retroviral replication cycle (116).

Several groups have reported modest restriction of HIV-1 replication by human TRIM5α, a restriction which is not altered by disruption of the CA-CypA interaction or by elimination of endogenous CypA (117–119). In contrast, restriction of HIV-1 replication by the TRIM5α derived from OWM (such as rhesus macaques) is abrogated by CsA-mediated or small interfering RNA-mediated inhibition of OWM-derived CypA function, indicating involvement of CypA in TRIM5α-mediated restriction of HIV-1 replication in OWM cells (117, 118, 120). In the owl monkey (a new world monkey), a CypA-TRIM5α-fusion protein has been found and involvement of CypA in the restriction has been suggested (17). Thus, CypA may exert restriction activity against HIV-1 replication in association with TRIM5α in non-human primates but not in humans. Elucidation of the key factors involved in this difference in CypA function between non-human primates and humans may contribute to understanding of the species-specific restriction mechanism against retroviral replication.

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