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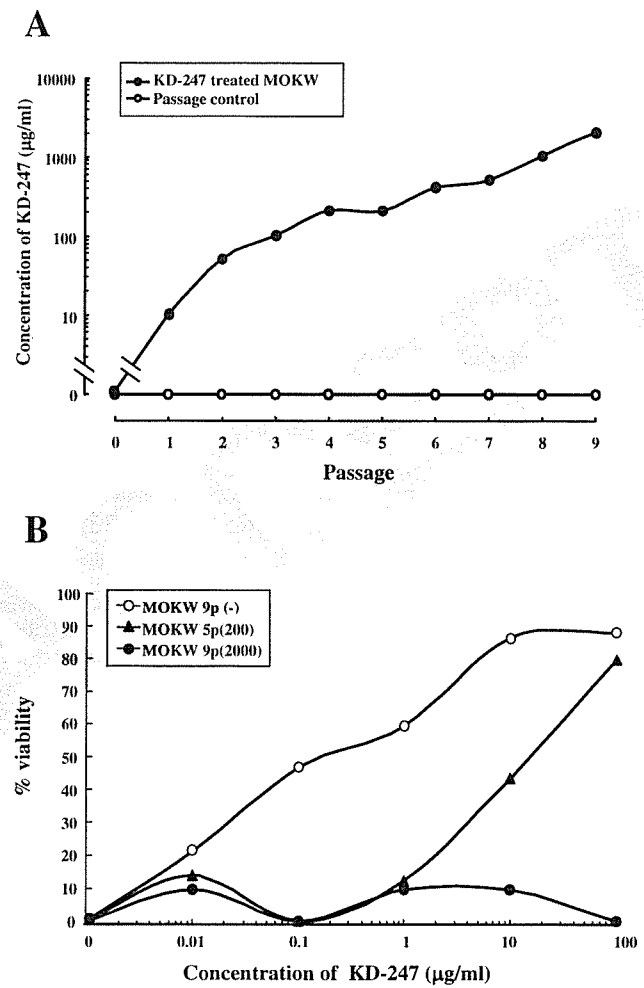
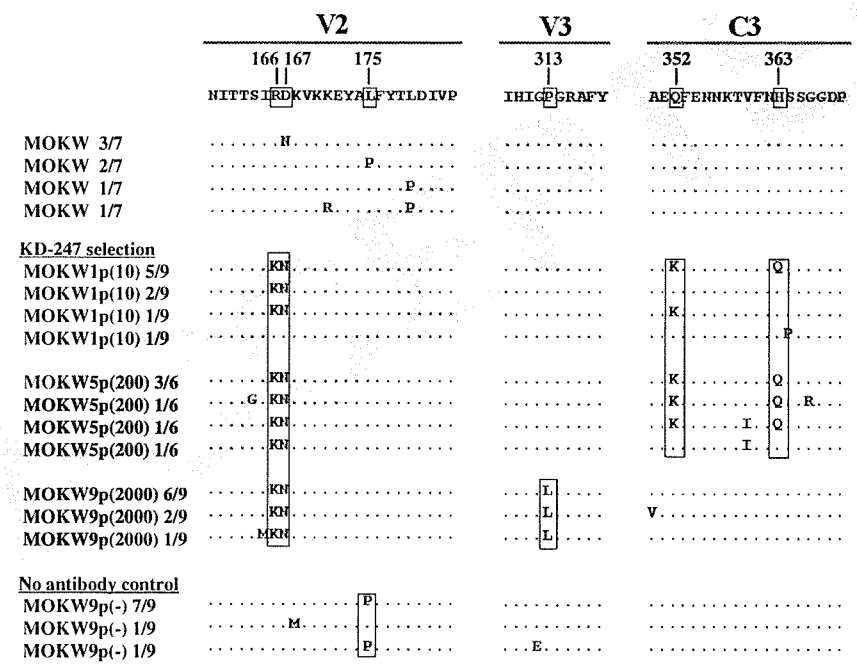
Fig. 1


A
KD-247 treated MOKW
Passage control
Concentration of KD-247 (μg/ml)
10000
1000
100
10
0
0 1 2 3 4 5 6 7 8 9
Passage
B
MOKW 9p (-)
MOKW 5p(200)
MOKW 9p(2000)
% viability
100 90 80 70 60 50 40 30 20 10 0
0 0.01 0.1 1 10 100
Concentration of KD-247 (μg/ml)

305

Fig. 2


V2
V3
C3
166 167
175
313
352
363
NITTSIRDKVKKEYALFYTLDIVP
IHIGPGRAFY
AEQFENNKTVFNHSSGGDP
MOKW 3/7
MOKW 2/7
MOKW 1/7
MOKW 1/7
KD-247 selection
MOKW1p(10) 5/9
MOKW1p(10) 2/9
MOKW1p(10) 1/9
MOKW1p(10) 1/9
MOKW5p(200) 3/6
MOKW5p(200) 1/6
MOKW5p(200) 1/6
MOKW5p(200) 1/6
MOKW9p(2000) 6/9
MOKW9p(2000) 2/9
MOKW9p(2000) 1/9
No antibody control
MOKW9p(-) 7/9
MOKW9p(-) 1/9
MOKW9p(-) 1/9

306

Fig. 3

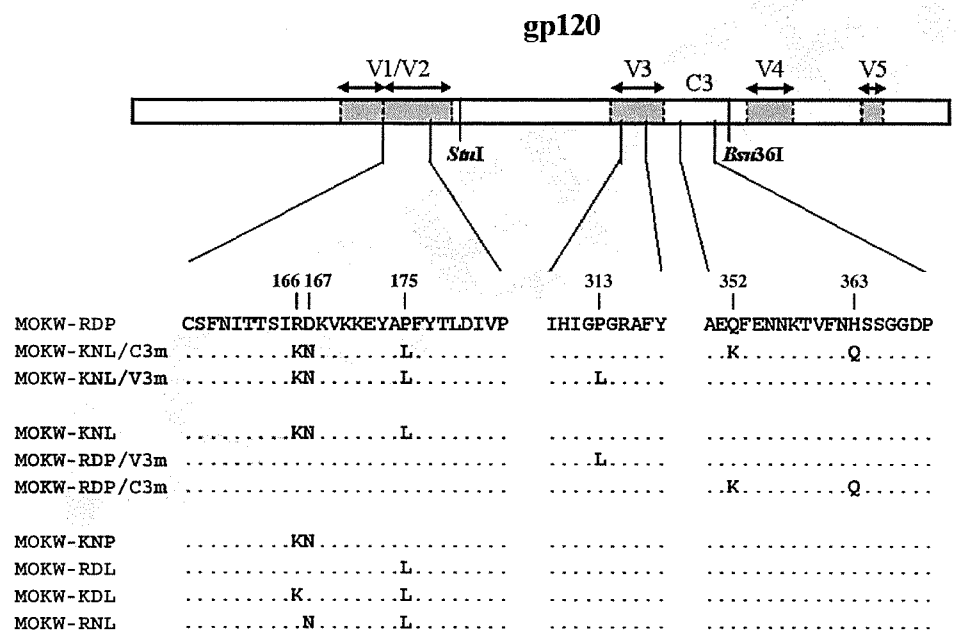
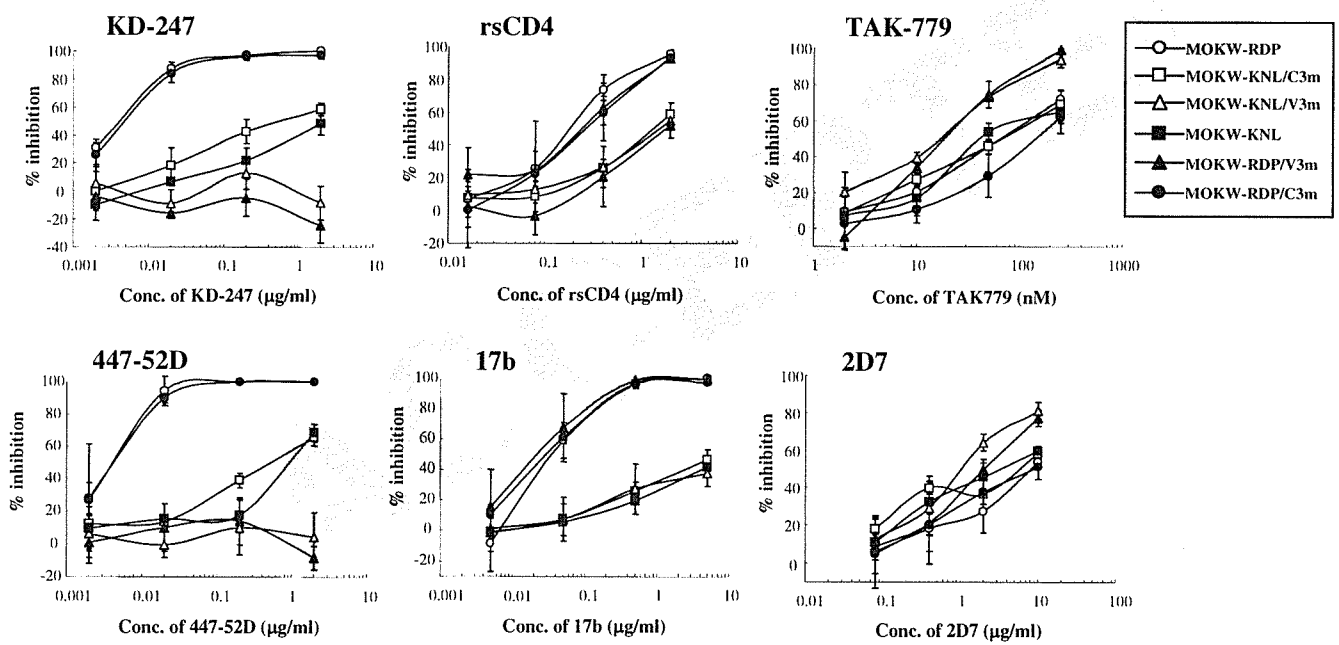
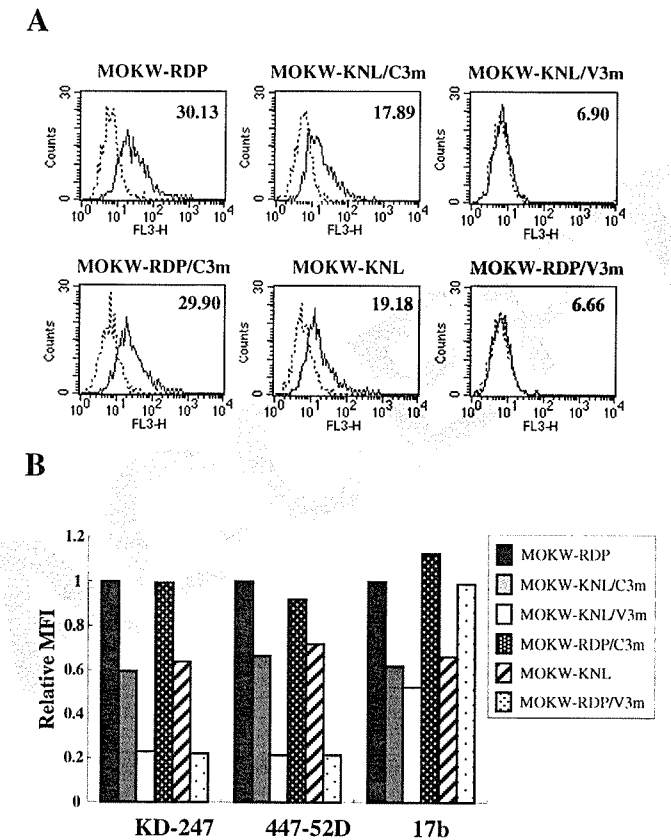

gp120
V1/V2
V3
C3
V4
V5
StuI
Bsm36I
166 167
175
313
352
363
MOKW-RDP CSFNITTSIRDKVKKEYAPFYTLDIVP IHIGPGRAFY AEQFENNKTVFNHSSGGDP
MOKW-KNL/C3m .....KN.....L..... ....K.....Q.....
MOKW-KNL/V3m .....KN.....L..... ....L..... .....
MOKW-KNL .....KN.....L..... ....
MOKW-RDP/V3m ....L..... .....
MOKW-RDP/C3m ....K.....Q.....
MOKW-KNP .....KN..... ....
MOKW-RDL .....L..... ....
MOKW-KDL .....K.....L..... ....
MOKW-RNL .....N.....L..... ....

Fig. 4


KD-247
rsCD4
TAK-779
447-52D
17b
2D7
% inhibition
Conc. of KD-247 (μg/ml)
Conc. of rsCD4 (μg/ml)
Conc. of TAK779 (nM)
Conc. of 447-52D (μg/ml)
Conc. of 17b (μg/ml)
Conc. of 2D7 (μg/ml)
MOKW-RDP
MOKW-KNL/C3m
MOKW-KNL/V3m
MOKW-KNL
MOKW-RDP/V3m
MOKW-RDP/C3m

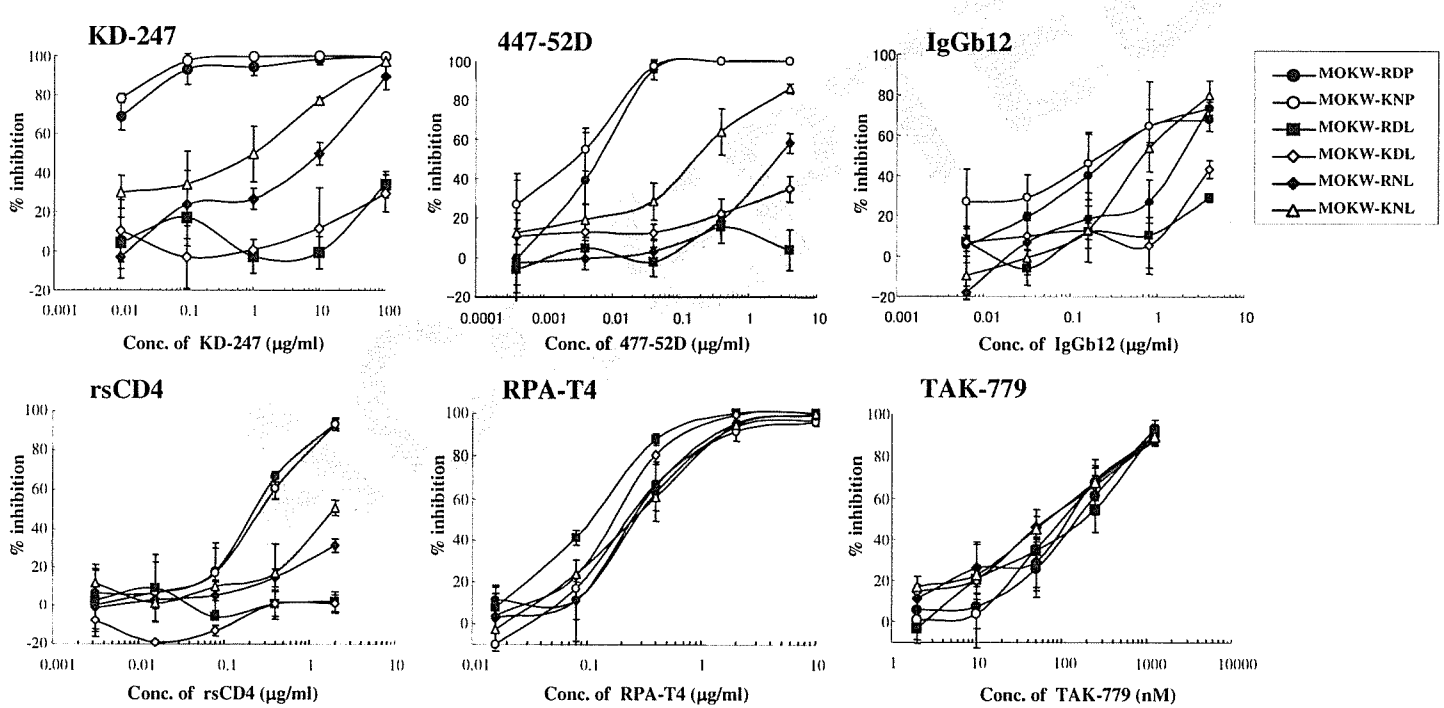
308

Fig. 5


A
MOKW-RDP
30.13
MOKW-KNL/C3m
17.89
MOKW-KNL/V3m
6.90
MOKW-RDP/C3m
29.90
MOKW-KNL
19.18
MOKW-RDP/V3m
6.66
Counts
FL3-H
B
Relative MFI
1.2
1
0.8
0.6
0.4
0.2
0
KD-247
447-52D
17b
MOKW-RDP
MOKW-KNL/C3m
MOKW-KNL/V3m
MOKW-RDP/C3m
MOKW-KNL
MOKW-RDP/V3m

309

Fig. 6


KD-247
% inhibition
Conc. of KD-247 (μg/ml)
447-52D
Conc. of 477-52D (μg/ml)
IgGb12
Conc. of IgGb12 (μg/ml)
MOKW-RDP
MOKW-KNP
MOKW-RDL
MOKW-KDL
MOKW-RNL
MOKW-KNL
rsCD4
Conc. of rsCD4 (μg/ml)
RPA-T4
Conc. of RPA-T4 (μg/ml)
TAK-779
Conc. of TAK-779 (nM)

310

Fig. 7

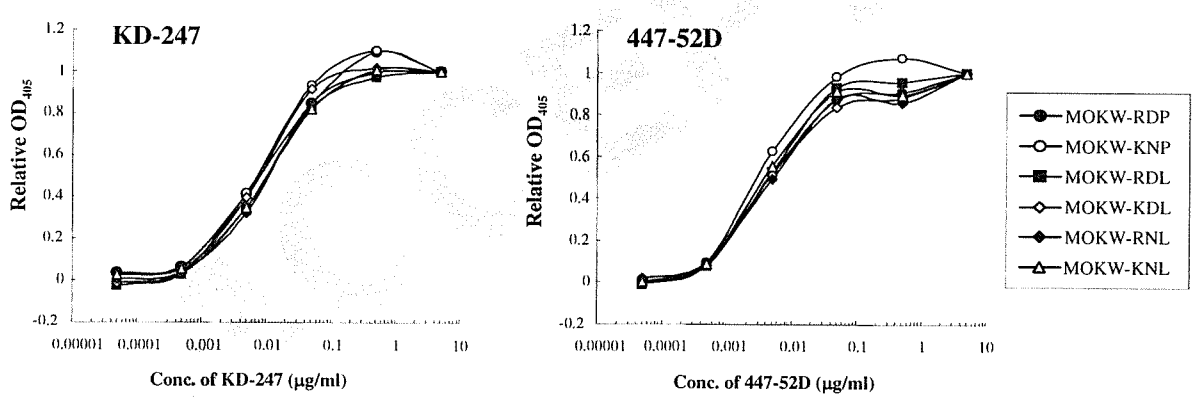
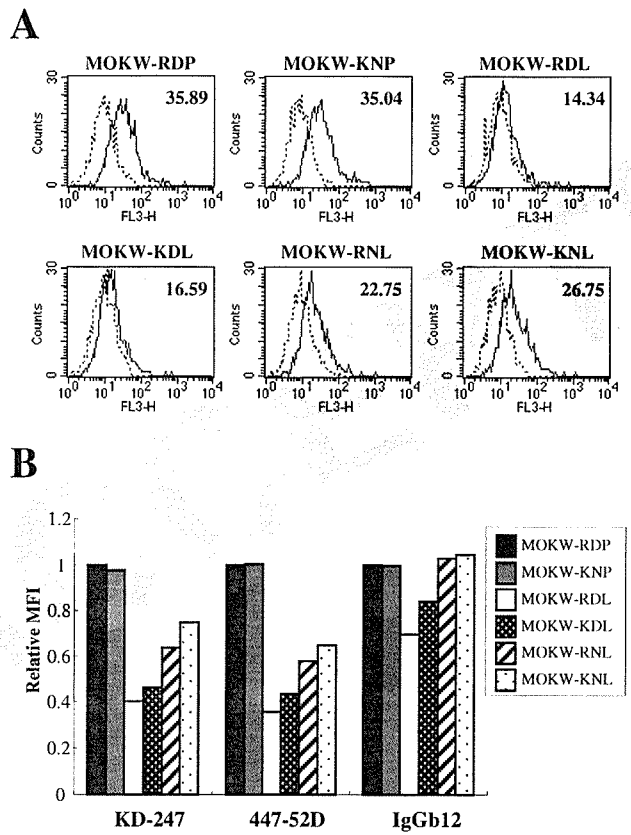
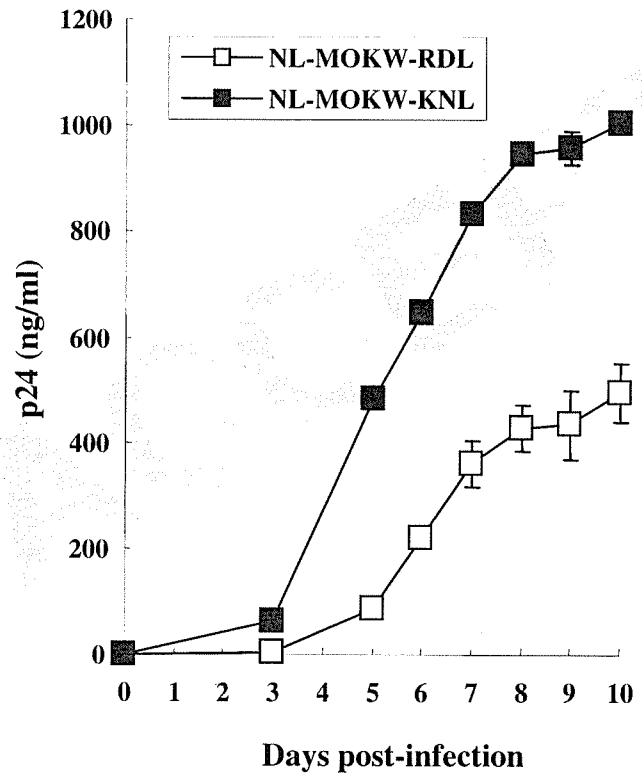

KD-247
Relative OD405
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0.00001 0.0001 0.001 0.01 0.1 1 10
Conc. of KD-247 (μg/ml)
447-52D
Relative OD405
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0.00001 0.0001 0.001 0.01 0.1 1 10
Conc. of 447-52D (μg/ml)
MOKW-RDP
MOKW-KNP
MOKW-RDL
MOKW-KDL
MOKW-RNL
MOKW-KNL

Fig. 8


A
MOKW-RDP
35.89
MOKW-KNP
35.04
MOKW-RDL
14.34
MOKW-KDL
16.59
MOKW-RNL
22.75
MOKW-KNL
26.75
Counts
FL3-H
B
Relative MFI
1.2
1
0.8
0.6
0.4
0.2
0
KD-247
447-52D
IgGb12
MOKW-RDP
MOKW-KNP
MOKW-RDL
MOKW-KDL
MOKW-RNL
MOKW-KNL

312

Fig. 9


NL-MOKW-RDL
NL-MOKW-KNL
p24 (ng/ml)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10
Days post-infection

313

Table 1. Anti-HIV-1 activities of various MAbs and inhibitors toward MOKW pseudoviruses

Class	Compound		IC_{50}[a]					
			MOKW-RDP	MOKW-KNL/C3m	MOKW-KNL/V3m	MOKW-RDP/C3m	MOKW-KNL	MOKW-RDP/V3m
V3 MAbs	**KD-247**	(μg/ml)	0.004 (x1) [b]	0.5 (x125)	>100 (>x25000)	0.005 (x1.3)	2 (x500)	>100 (>x25000)
	447-52D	(μg/ml)	0.004 (x1)	0.5 (x125)	>2 (>x500)	0.004 (x1)	0.8 (x200)	>2 (>x500)
CD4-induced MAb	**17b**	(μg/ml)	0.035 (x1)	>5 (>x143)	>5 (>x143)	0.03 (x0.86)	>5 (>x143)	0.02 (x0.57)
CD4	**rsCD4**	(μg/ml)	0.18 (x1)	1.3 (x7.22)	1.5 (x8.33)	0.24 (x1.33)	1.8 (x10)	0.24 (x1.33)
CCR5 MAb	**2D7**	(μg/ml)	8 (x1)	6.8 (x0.85)	1 (x0.13)	8 (x1)	3.2 (x0.4)	2 (x0.25)
CCR5 small molecule	**TAK-779**	(nM)	63 (x1)	63 (x1)	18 (x0.29)	140 (x2.22)	65 (x1)	18 (x0.29)
CD4 MAb	**RPA-T4**	(μg/ml)	0.4 (x1)	0.26 (x0.65)	0.22 (x0.55)	0.5 (x1.25)	0.22 (x0.55)	0.44 (x1.1)

[a]GHOST-hi5 cells were exposed to 100 $TCID_{50}$ of each MOKW pseudovirus and then cultured in the presence of various concentrations of MAb or inhibitors. The IC_{50} values were determined using the luciferase reporter assay on day 2 of culture. All assays were conducted in triplicate. The values shown are representative of two or three separate experiments.

[b]The values in parentheses are relative IC_{50} values (relative to the IC_{50} value of each compound for MOKW-RDP).

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A Single-Nucleotide Synonymous Mutation in the *gag* Gene Controlling Human Immunodeficiency Virus Type 1 Virion Production▽

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Viral factors as well as host ones play major roles in the disease progression of human immunodeficiency virus type 1 (HIV-1) infection. We have examined cytotoxic T-lymphocyte activity and HIV-1 DNA PCR results of 312 high-risk seronegative drug users in northern Thailand and identified four seronegative cases positive for both assays. Furthermore, we have identified a synonymous mutation in nucleotide position 75 of the *gag* p17 gene (A426G) of HIV-1 that belongs to the CRF01_AE virus circulating in Thailand. The replication-competent HIV-1 clone containing the A426G mutation demonstrated a dramatic reduction of virion production and perturbation of viral morphogenesis without affecting viral protein synthesis in cells.

Indirect evidence of abortive human immunodeficiency virus type 1 (HIV-1) infection in cases of highly exposed persistently seronegative individuals and transient seroconversion have been reported (2, 3, 5, 8, 10, 13, 16, 19–23) wherein host and viral factors are considered to play major roles. The host factors were deemed responsible for the natural resistance to HIV-1 infection; however, the viral factors responsible for the abortive HIV infection process have not been fully understood. For example, Zhu et al. (26) reported remarkable genetic stability of *gag* and *env* sequences of HIV-1 proviral DNA in resting $CD4^+$ T cells obtained from highly exposed persistently seronegative individuals, indicating the involvement of viral factors. Moreover, attenuated HIV-1 isolates obtained from long-term nonprogressors (14) were reported, demonstrating the diminished transmissibility of the virus. These findings indicate that viral factors are responsible for abortive HIV-1 infection. Understanding such mechanisms would be beneficial for the development of novel therapeutic and preventive strategies against HIV-1.

In this study, we focused on the drug user (DU) cohort study conducted in northern Thailand wherein current epidemiological surveys have revealed a dramatic decrease in seroprevalence and incidence rate of HIV-1 infection in spite of unchanged behaviors among DUs (9). A total of 421 cases (HIV-1 seronegative, $n = 320$; seropositive, $n = 101$) in the DU cohort were enrolled with written informed consent from January 1999 to November 2000 and followed up (every 6 months) until November 2003 (Fig. 1A). Eight cases among 320 seronegative DUs had seroconverted, whereas the other 312 cases remained seronegative throughout the study. All enrollment samples were stored, and subsequently diagnostic and PCR tests for HIV-1 were performed in separate laboratories in Thailand to eliminate the possibility of cross-contamination. Duplicate follow-up specimens were also assayed for HIV-1 *gag* p17 by PCR test and sequencing (7, 15); a cytokine enzyme-linked immunospot assay kit (U-CyTech, The Netherlands) (10) was used to detect human gamma interferon responses by using overlapping (20 mer) whole Gag peptides designed from a CRF01_AE clinical isolate. Furthermore, *gag* p17 gene-containing fragments were cloned and sequenced. The nucleotide sequence alignment adjacent to nucleotide position 75 (+426) of the *gag* p17 gene of HIV-1 clones from each seronegative DU subject is shown in Fig. 1C. A mutant molecular clone, G6, containing an A426G mutation was constructed from a replication-competent HIV-1 molecular clone, G5, based on isolate 92TH022 (11).

293 cells were transfected with clones G5 and G6 using Fugene-6 transfection reagent (Roche Diagnostics, Basel, Switzerland) (24). Every 24 h posttransfection, culture supernatant was collected for determination of p24 antigen (HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA]; ZeptoMetrix Co., Buffalo, NY) and HIV-1 RNA level (Am-

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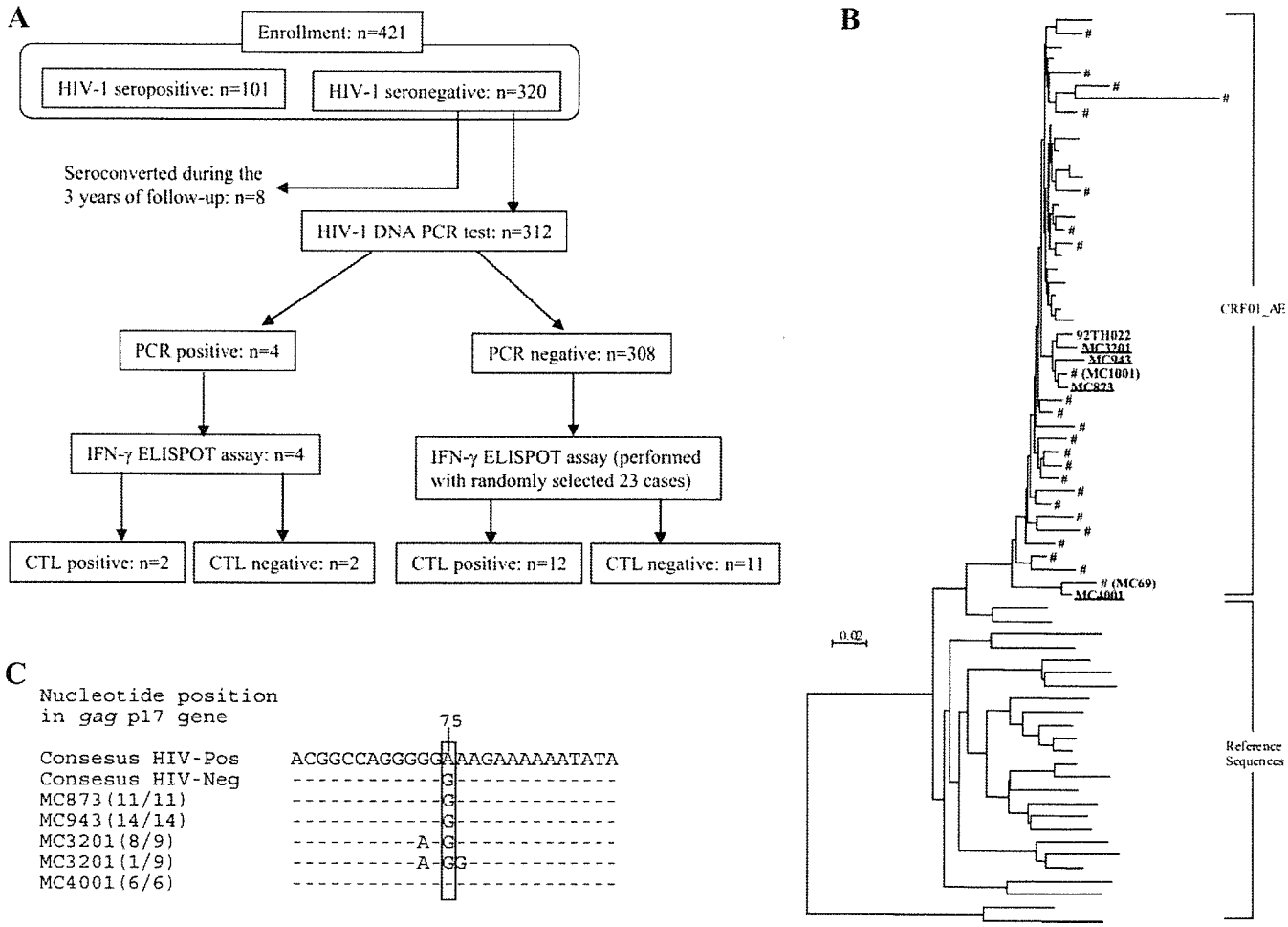

FIG. 1. Results of initial screening and phylogenetic analysis of the HIV-1 *gag* p17 gene. (A) Schematic diagram of this study. Serological studies were carried out with 421 DUs in Chiang Rai, Thailand. HIV-1 DNA PCR analysis was performed with 312 patients who were subsequently followed and who remained seronegative. The human gamma interferon enzyme-linked immunospot assay (IFN-γ ELISPOT) was performed with four seronegative but HIV-1 *gag* gene-positive DU cases and 23 randomly selected seronegative HIV-1 *gag* gene-negative DU cases to determine the anti-HIV-1 cytotoxic T-lymphocyte (CTL) responses. (B) Phylogenetic tree of the *gag* p17 gene. The neighbor-joining method was applied to obtain a phylogenetic tree with nucleotide sequences from HIV-1-seronegative but *gag* gene-positive DUs (underlined) and seropositive DUs infected with CRF01_AE in the same cohort (7) (indicated by #) together with the reference sequences from the Los Alamos National Laboratory HIV, including representatives of all group M clades and CRF01_AE (database available at http://www.hiv.lanl.gov/content/index).The infectious molecular clone G5 was constructed from isolate 92TH022 (11; Codon Usage Database at http://kazusa.or.jp/codon/; GenBank release 151.0) that was obtained in northern Thailand. (C) Sequence alignment of the *gag* p17 gene segment surrounding the nucleotide position 75 (+426). The consensus HIV-1 *gag* p17 sequences of seronegative and seropositive DU cases in the same cohort (Los Alamos National Laboratory HIV database) were obtained from the sequence comparison. The deterministic nucleotide sequence that distinguishes seronegative and seropositive cases was found at nucleotide position 75 (+426) of the *gag* p17 gene (boxed). Pos, seropositive; Neg, seronegative.

plicor HIV-1 Monitor, version 1.5; Roche Diagnostics), and the cells transfected with the clones were assayed for Gag protein expression by Western blotting (24) and transmission electron microscopy (25). Data were analyzed by the StatView program (SAS Institute, Cary, NC). A P value of < 0.05 was considered significant.

Whereas most of the samples (308 cases) were negative for HIV-1 DNA by PCR, we identified four cases (MC873, MC943, MC3201, and MC4001) positive for the HIV-1 *gag* p17 gene (Fig. 1A). Although these four cases appeared healthy without any detectable HIV-1 antigens, antibodies, viral load, or reduction of $CD4^+$ T lymphocytes, two of these four cases were found repeatedly positive for anti-HIV-1 cytotoxic T-lymphocyte responses. These four cases were followed up until November 2003, and no seroconversion was detected (data not shown).

The DNA fragment containing the *gag* p17 gene from these seronegative cases was amplified, cloned, and sequenced for further analyses. We found that the nucleotide sequences obtained from the above-mentioned four seronegative DU cases appeared to belong to the same group of CRF01_AE reference strains (Fig. 1B), suggesting their possible exposure to CRF01_AE which is prevalent in northern Thailand (Los Alamos National Laboratory HIV database available at http://www.hiv.lanl.gov/content/index).We found that the sequences of three out of four seronegative cases conform a tight cluster together with MC1001, obtained from a seropositive DU in our cohort (7), and 92TH022. MC4001 conformed to an isolated

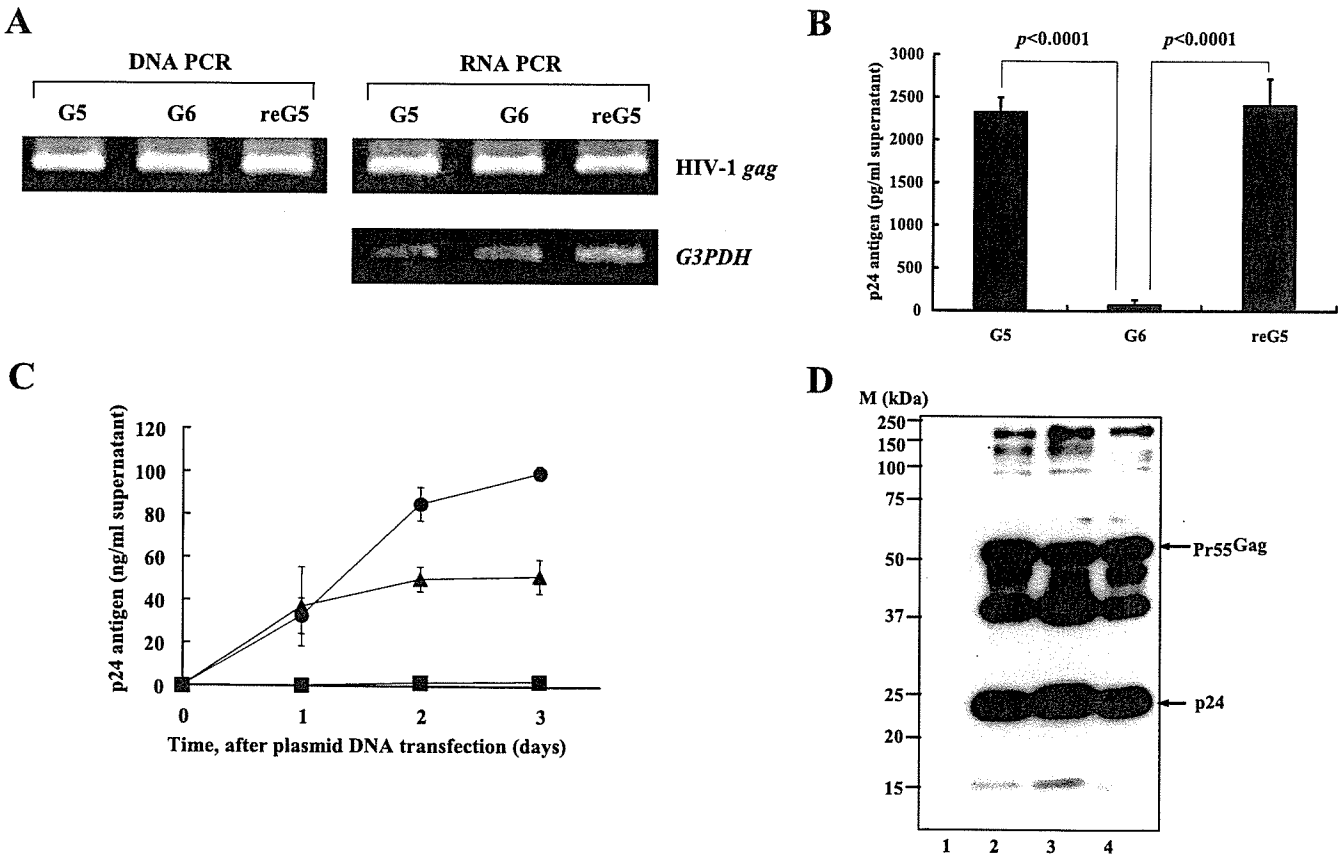

FIG. 2. Viral mRNA expression, viral protein synthesis, and virion production of G5 and G6 HIV-1 clones. (A) Detection of HIV-1 DNA and viral mRNA in cells transfected with the G5, G6 and reG5 clones. HIV-1 DNA and mRNA were detected by DNA PCR and reverse transcription-PCR (RT-PCR) with specific primers previously described (15). *G3PDH* (glyceraldehyde 3-phosphate dehydrogenase) was amplified as an internal control for RT-PCR. (B) Amounts of HIV-1 p24 antigen in the culture supernatants of cells transfected with replication-competent HIV-1 clones G5, G6, and 93JP-NH1 (12). At 24 h posttransfection, culture supernatants were subjected to the HIV-1 p24 antigen ELISA. The experiments were performed in triplicates, and the means ± standard deviations are indicated. The statistical significance was evaluated by a Student's *t* test. (C) Time course of HIV-1 virion production in the culture supernatants of transfected cells. Amounts of HIV-1 p24 antigen in the culture supernatants of cells transfected with replication-competent HIV-1 clones G5 (circle), G6 (square), and 93JP-NH1 (triangle) were measured 1, 2 and 3 days posttransfection. The experiments were performed in triplicates, and the means ± standard deviations are indicated. (D) Western blot detection of viral proteins produced in cells 48 h after transfection with replication-competent HIV-1 clones. Lane 1, negative control (vector plasmid); lane 2, G5; lane 3, G6; lane 4, 93JP-NH1 (positive control). The positions of viral $Pr55^{Gag}$ and p24 are indicated on the right. The positions of protein markers are indicated on the left.

cluster together with MC69, a seropositive DU and long-term nonprogressors living in the same village. To clarify the cause of lack of seroconversion in these DU cases, we inspected the genetic differences between HIV-1 sequences of seropositives and seronegatives in the same local cohort. Whereas the nucleotide at *gag* p17 position 75 (+426) is A in sequences of seropositives, it is G in sequences of seronegatives (Fig. 1C). Moreover, we were not able to find other conserved mutation positions between these four sequences.

We then explored the biological effect of A426G change by creating full-length HIV-1 molecular clones containing A (G5) or G (G6) at nucleotide position +426, based on the parental molecular clone 92TH022 (9,722 bp). Moreover, we also constructed a revertant mutant (reG5) in which the G substitution (G6) was reversed to an A mutation. We detected HIV-1 DNA and similar levels of viral mRNA expression in the transfected cells with the G5, G6, and reG5 clones (Fig. 2A). However, a profound reduction of HIV-1 virion production in the culture supernatant from cells transfected with G6 was observed (Fig. 2B). This also indicates that no undetected mutation was introduced during the construction of the G6 mutant clone. Furthermore, no significant increase in virus production was observed with G6 even after a prolonged period posttransfection, whereas the level of G5 virus production was comparable with that of the parental clone, 93JP-NH1 (Fig. 2C) (12). The levels of intracellular viral protein production as assayed by Western blotting and their stabilities were almost similar in clones G5, G6, and 93JP-NH1 (Fig. 2D), suggesting that the effect of a single synonymous nucleotide substitution at position +426 is not at the level of viral protein synthesis. These experiments were carried out independently in two different laboratories, and essentially the same results were obtained. These findings suggest that the major effect of the A426G nucleotide change might be at the step between viral protein synthesis and virus budding.

Abundant virion production was observed in G5-transfected cells using transmission electron microscopy (Fig. 3A). In contrast, few viral particles were detected in G6-transfected cells

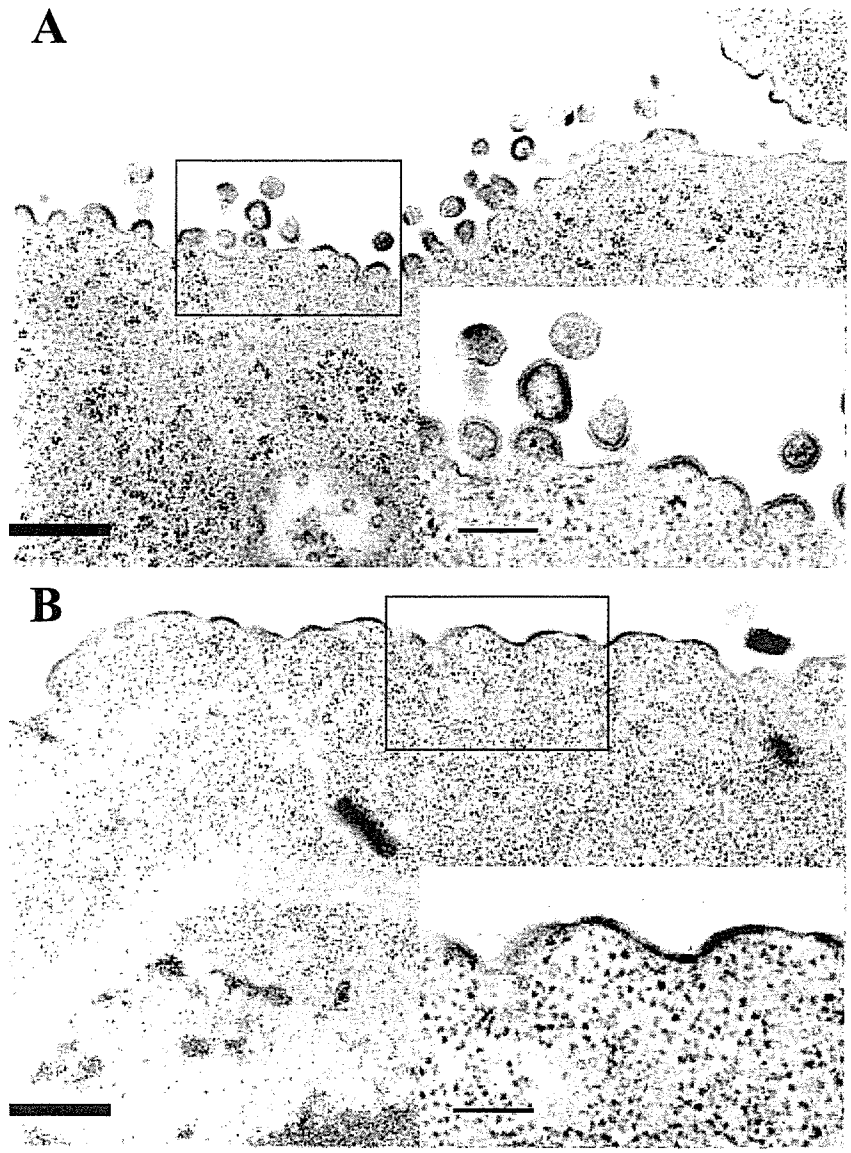

FIG. 3. Transmission electron microscopic examinations of cells transfected with G5 (A) and G6 (B). Note that mature and immature virions were observed in the cell surface of G5-transfected cells, whereas few viral particles were observed in G6-transfected cells. Higher magnifications of the boxes areas are shown as insets. Thick and thin scale bars indicate 0.5 μm and 0.2 μm, respectively.

(Fig. 3B). Although the typical dense patch structure, suggesting the accumulation of viral proteins, was observed, further viral morphogenesis appeared to be blocked in the image shown in Fig. 3B. It is also noted that the curvature of the viral-associated dense patch in the cytoplasmic membrane surface of G6-transfected cells was distinguishable from that of the G5-transfected cells: whereas the former was relatively smooth with less accumulation of high-density material, the latter was characterized by the presence of a rougher surface with more protrusions, eventually leading to virus budding, which suggests that virion morphogenesis was blocked in the membrane-associated area at the early phase of morphogenesis in G6-transfected cells. These findings suggest that a single nucleotide in the *gag* p17 gene (nucleotide position +426) may be involved in viral morphogenesis.

To further clarify the effect of the A426G mutation, we have examined the presence of viral RNA in the culture supernatant. Although viral protein and mRNA expression levels within the cells transfected with G5, G6, and 93JP-NH1 were almost equivalent (Fig. 2A and 4A), virus production in G6 was extremely low. Moreover, in parallel with the reduced virion production, significantly lower copy numbers of viral RNA were found in the supernatant (Fig. 4C). These findings clearly indicate that the A426G mutation in HIV-1 causes a serious defect in virion production.

Our data indicate that a one-nucleotide mutation in *gag* p17 without changing the amino acid is responsible for reduced HIV-1 virion production. Since viral RNA was detected in the supernatant of G6-transfected cells, though to a much lower level, this one-point synonymous mutation cannot completely abolish virus replication. This mutation is clearly different from the one described by Cannon et al. (4), who demonstrated that

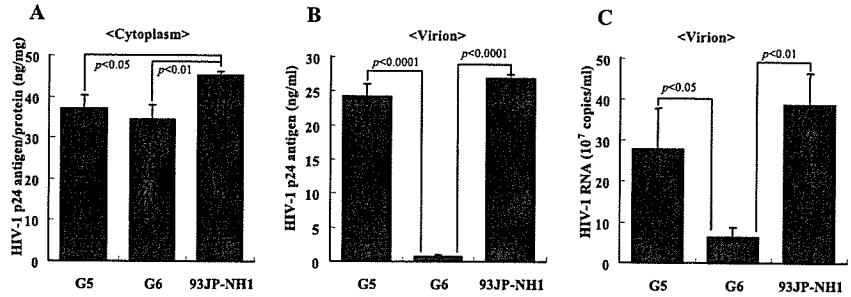

FIG. 4. Comparison of viral p24 (Gag) proteins and virion-associated viral RNA among G5, G6, and 93JP-NH1 clones. (A) Amounts of HIV-1 p24 (Gag) proteins in cells transfected with G5, G6, and 93JP-NH1 viruses. At 24 h posttransfection, whole-cell extracts were prepared and subjected to HIV-1 p24 antigen ELISA. (B) Amounts of virion-associated HIV-1 p24 in the supernatants of cells transfected with G5, G6, and 93JP-NH1 viruses. At 24 h posttransfection, cell culture supernatants were collected, and HIV-1 p24 antigen levels were determined. (C) Amounts of HIV-1 viral RNA in the supernatants of cells transfected with G5, G6, and 93JP-NH1 viruses. At 48 h posttransfection, culture supernatants were collected, and the viral RNA copy numbers were determined by an Amplicor HIV-1 Monitor (version 1.5). In these experiments, 293 cells were transfected with replication-competent full-length HIV-1 clones, G5, G6, or 93JP-NH1 (positive control), and the cells or culture supernatants were collected at indicated time points. All measurements were performed in triplicates, and the means ± standard deviations are indicated.

an amino acid mutation in the *gag* p17 coding sequence resulted in a defect in viral replication.

Findings from electron microscopic examination (Fig. 3) suggested that the G6 virus production was blocked at a step from virion assembly through virus budding in spite of the presence of thick, patchy densities in the cytoplasmic membrane that are indicative of the tethering of viral proteins. Thus, the reduced virion production may be due to diminished protein accumulation to the lipid raft formed at the inner face of the host cell plasma membrane.

HIV-1 virion assembly is initiated by the plasma membrane translocation of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ (6). Formation of virus-like particles by $Pr55^{Gag}$ is a self-assembly process with critical Gag-Gag interactions between multiple domains along Gag precursor, and the assembly of virus-like particles does not necessarily require the participation of viral genomic RNA (1). The interaction between $Pr55^{Gag}$ and viral genomic RNA, as well as dimerization of viral genomic RNA, is considered crucial for HIV-1 morphogenesis (17). However, in spite of our repeated trials, an RNA-protein interaction between the HIV-1 packaging sequence spanning +223/+506 containing A426 (G5) or G426 (G6) and HIV-1 nucleocapsid did not show any difference between G5 and G6 sequences, at least in vitro (data not shown). It is possible that a subtle mutation like A426G may not cause a robust loss in the affinity to the nucleocapsid in the absence of a milieu of cellular and viral proteins. Roldan et al. (18) reported that the viral RNA segment spanning the region +400 to +500, containing A426, is involved in the binding to $Pr55^{Gag}$ and acts as a major packaging sequence, which may explain our finding that the nucleotide A426 in the *gag* p17 gene is crucial for the virion production.

Collectively, although the nucleotide substitution of A to G at position +426 of the *gag* p17 gene appears to contribute to reduced levels of virus production, further studies are crucial to understand the underlying mechanism. These studies should not only decipher a novel regulatory mechanism of viral production but might also indicate a possible target for novel HIV-1 therapy.

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