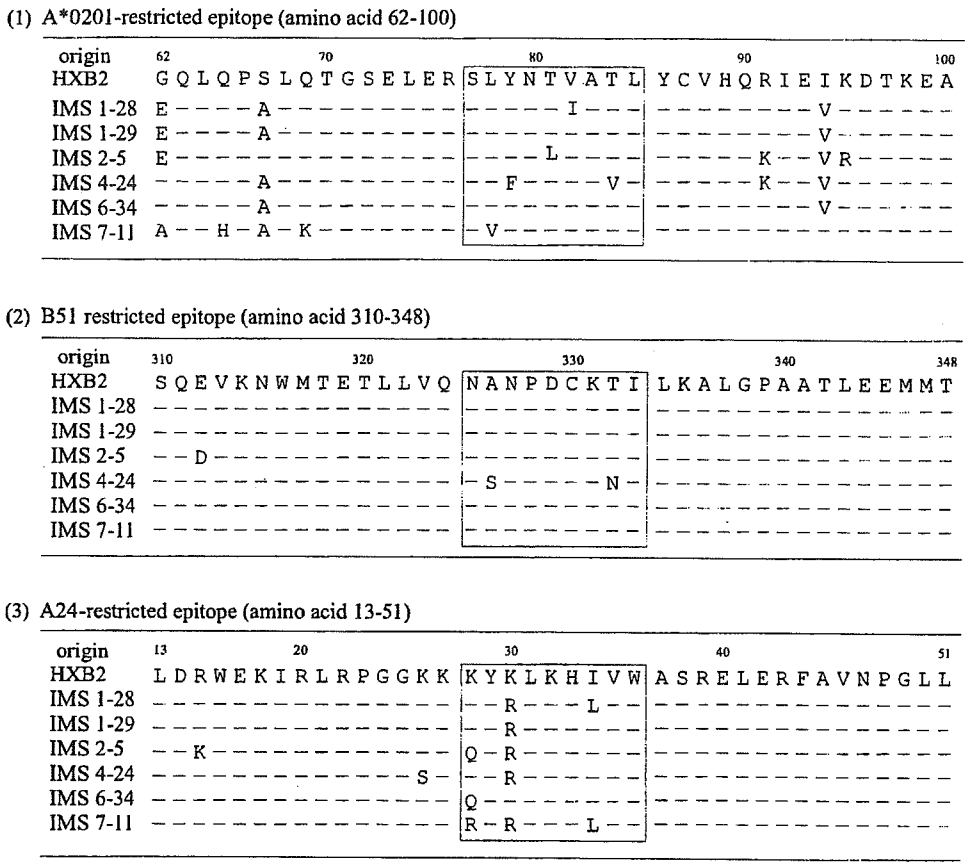

FIG. 2. Sequence variation in three CTL epitopes and their flanking regions. The amino acid sequences of six *gag* clones are shown. The reference sequence is derived from HXB2, and the differences are indicated. The numbering is done according to the HIV sequence database, Los Alamos National Laboratory, Los Alamos, N.Mex. The CTL epitope regions are boxed.

other target cells expressing different variants (Fig. 3e). Interestingly, IMS4-24 with Lys (K)-to-Ser (S) mutation at position 26 outside the epitope region was less well recognized than IMS1-29. We consistently observed this phenomenon in repeated experiments (data not shown).

CTL recognition of exogenously loaded variant peptides. To investigate whether the above findings of escape phenomenon from CTL killing were due to either loss of peptide binding to the MHC class I molecule or to the lack of TCR recognition, we prepared synthetic peptides that represented the variant epitopes and tested them for cross-recognition of the peptides in peptide titration assays by using the same CTL lines or clones that were used in experiments described for Fig. 3. To our surprise, A 0201-restricted CTL lines recognized the peptides of two A 0201-restricted CTL epitope variants, SVYNT VATL and SLENTVAVL, which were not recognized by the CTLs when expressed endogenously. They recognized the SLENTVAVL peptide less efficiently, with an SD_{50} of 100 nM (Fig. 4a). Target cells pulsed with SLYNLVATL peptide representing clone IMS 2-5 were not cross-recognized even at a saturated concentration (10 M) (data not shown).

We also obtained similar discordant results in experiments of A24-restricted CTL epitope variants. A24-restricted 3R mutant-specific CTL lines recognized peptides of three variant s—KYRLKHLVW, RYRLKHLVW, and QYRLKHIVW—that were not recognized by the CTLs when they were expressed endogenously. In fact, the CTLs recognized QYRLKHIVW peptide even better than the 3R mutant peptide but did not cross-recognize the QYKLKHIVW peptide (Fig. 4b).

We tested one B 5101 variant peptide, NSNPDCKNI, in a peptide titration assay. This variant was not cross-recognized by any of the CTL clones even at a high concentration (1 M) (Fig. 4c). The two amino acid mutations in this epitope coincided with two anchor residues to the MHC biding, suggesting that the lack of recognition of this variant was likely due to loss of peptide binding.

Mutations responsible for impairing the epitope processing and presentation. The discrepancies seen above between the CTL recognition of endogenously expressed and exogenously loaded antigen indicate that some mutations have caused the impairment of epitope processing and presentation. To locate specific variations that were responsible for the poor recognition of endogenously expressed HIV-1 *gag* variants, we constructed four different target vectors: an HXB2 *gag* sequence with A 0201-restricted epitope variations (SLENTVAVL [HXB2-3F8V] or SVYNTVATL [HXB2-2V]) and IMS4-24- or IMS7-11-derived *gag* sequence with the wild-type A 0201 epitope sequence (IMS4-24-wild or IMS7-11-wild, respectively). The replacement of the variant epitope region with the

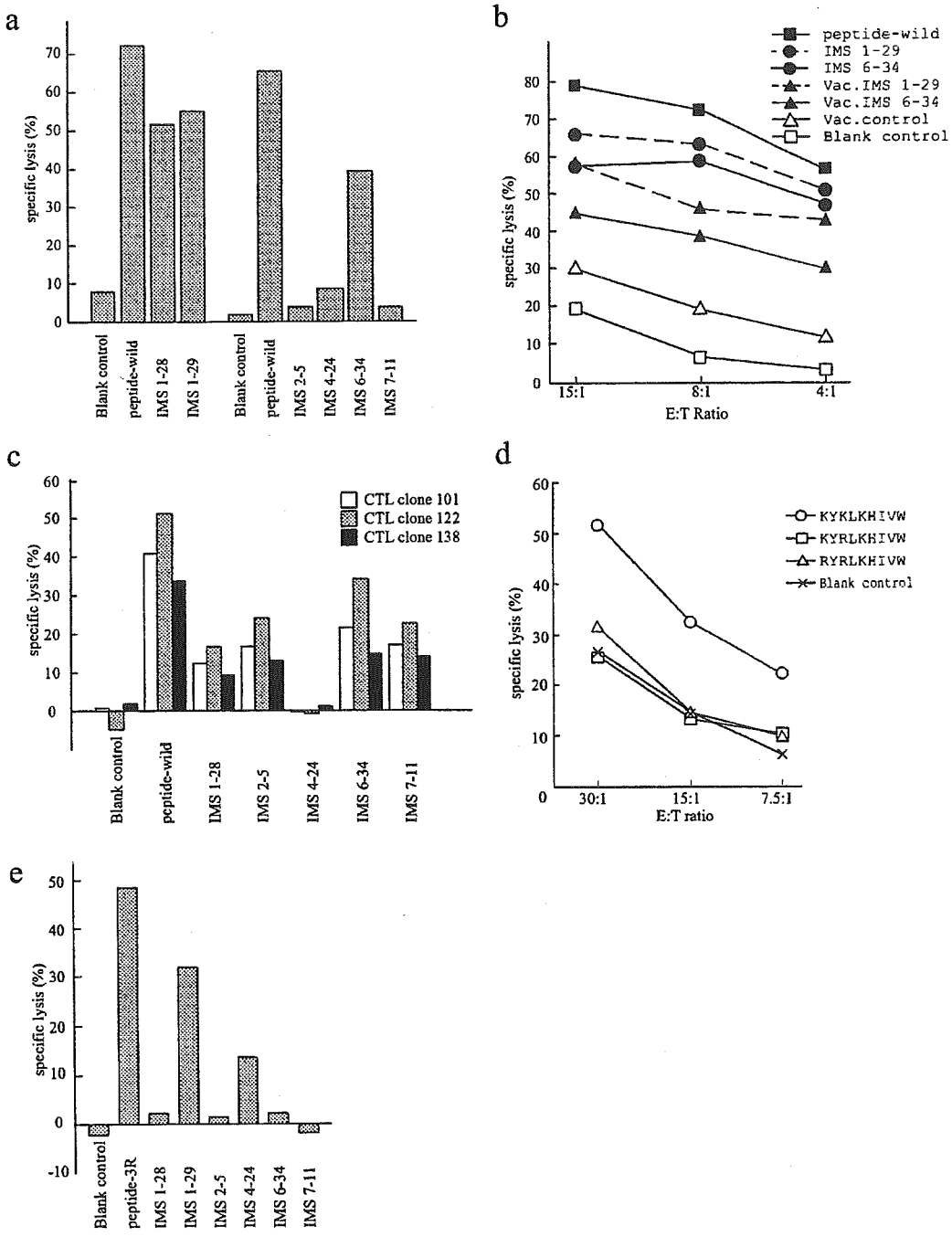

FIG. 3. (a) Specific lysis of A 0201-matched B-LCLs (HLA-A 0201/ and HLA-B 5101/) producing Gag proteins of clinical isolates. Peptide target cells were pulsed with the A 0201 wild-type peptide, SLYNTVATL (10 M). A 0201-restricted SLYNTVATL -specific CTL lines were induced from a single donor (IMS1). The E:T ratio was 10:1. This experiment was repeated, with a different B-LCLs (HLA-A 0201/31 and HLA-B27/ 5101), giving the same pattern of recognition (data not shown). (b) Specific lysis of A 0201-matched B-LCLs (HLA-A 0201/ and HLA-B 5101/) expressing *gag* clones of two clinical isolates with the VSV-G-pseudotyped HIV-1 vector versus recombinant vaccinia viruses. Recombinant vaccinia virus expressing the human CD4 gene was used as a vaccinia virus control (1). The effector and peptide target cells were prepared as described for panel a. (c) Specific lysis of B 5101-matched B-LCLs (HLA-A 0201/ and HLA-B 5101/) producing the Gag proteins of five clones. Three B 5101-restricted NANPDCKTI -specific CTL clones were used as effector cells at an E:T ratio of 2:1 (23). The peptide target was pulsed with the B51 wild-type peptide NANPDCKI (1 M). (d) Specific lysis of A24-matched B-LCLs (HLA-A24/ and HLA-B46/52) pulsed with the peptides KYKLKHIVW, KYRLKHIVW, and RYRLKHIVW at 10 M. A24-restricted, KYKLKHIVW -specific CTL lines were induced from one A24-positive donor. (e) Specific lysis of A24-matched B-LCLs (HLA-A24/ and HLA-B46/52) producing variant Gag proteins. A24-restricted KYRLKHIVW (3R)-specific CTL lines were induced from another A24-positive donor. The peptide target was pulsed with 3R mutant type peptide (10 M). The E:T ratio was 20:1. The lysis of target cells without any peptide pulsing is shown as a blank control.

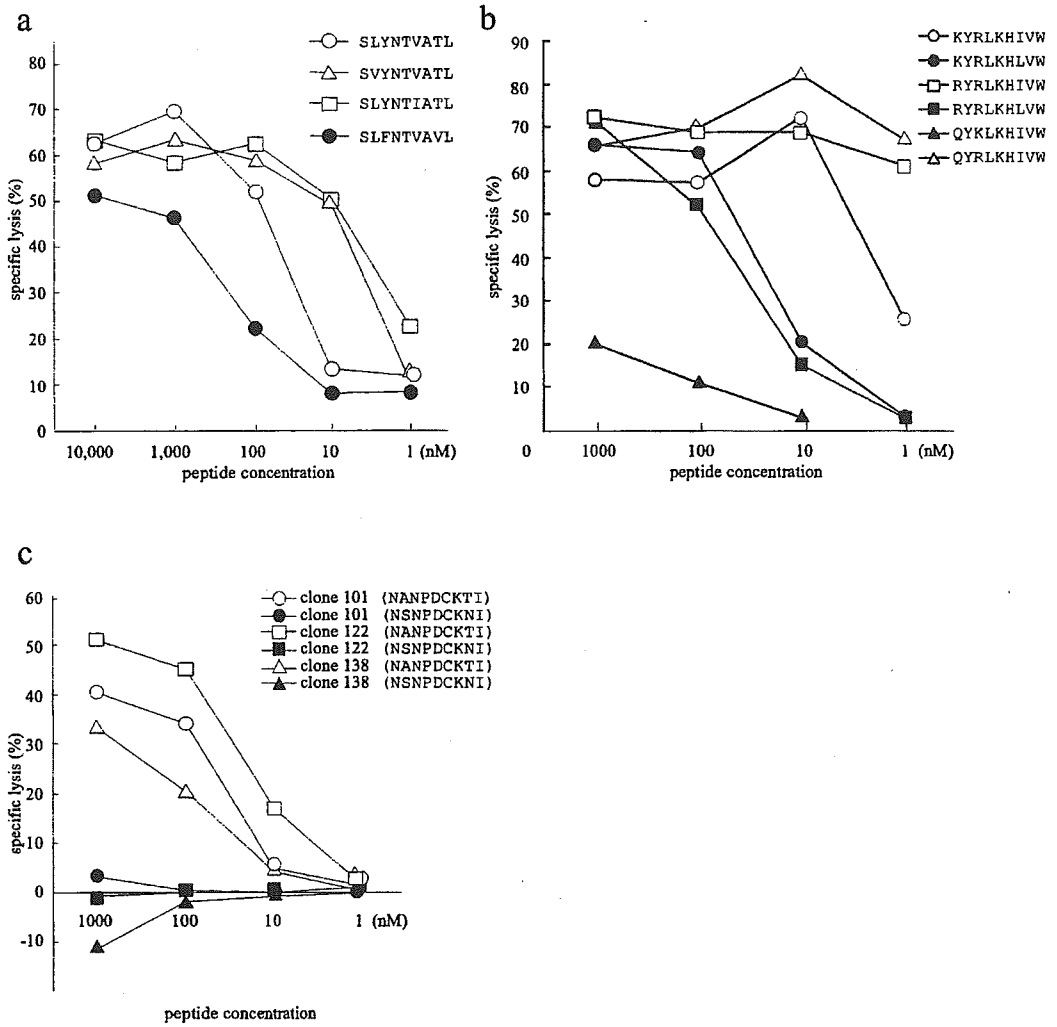

FIG. 4. Peptide titration assays. (a) Specific lysis of A 0201-matched B-LCLs pulsed with A 0201 variant peptides by A 0201-restricted CTLs at an E:T ratio of 20:1. (b) Specific lysis of A24-matched B-LCLs pulsed with 3R and its variant peptides by A24-restricted 3R mutant reactive CTLs at an E:T ratio of 20:1. (c) Specific lysis of B51-matched B-LCLs pulsed with B51 variant peptides by B51-restricted CTL clones at an E:T ratio of 2:1. The same effector and target cells were used as for Fig. 3. The percent lysis of the blank control has been subtracted.

wild-type epitope sequence restored CTL recognition of the escape variants, whereas replacement of the wild-type epitope with the two variant epitopes resulted in no CTL recognition of HXB2 Gag (Fig. 5a). The levels and patterns of Gag protein expression in target cells were analyzed by Western blot experiments (Fig. 5b). The expression levels of p55 Gag precursor and p24 CA did not significantly differ between the mutants and the wild type. The p17 MA band was not clear in HXB2-2V, IMS7-11-wild, and IMS4-24-wild, but the appearance of this band did not correlate with CTL killing. These results indicate that amino acid substitutions within the A 0201-restricted epitope region, rather than those in the flanking regions, have caused the inhibition of CTL recognition in our endogenous expression system.

To further investigate the effect of amino acid substitutions within the A24-restricted epitope on antigen processing and presentation, we introduced various point mutations into the wild-type HXB2 vector, pCTLpac, and tested them for the recognition by A24-restricted 3R mutant-reactive CTL lines. The A24-restricted 3R mutant-specific CTLs did not cross-recognize the wild-type peptide and the wild-type HXB2 vector but did recognize HXB2 with a 3R mutation (HXB2-1R). The substitution of Lys (K) with Arg (R) at position 28 (HXB2-1R3R) did not affect the A24-restricted 3R mutant-specific CTL recognition, but a Lys (K)-to-Gln (Q) substitution at position 28 (HXB2-1Q3R) or an Ile (I)-to-Leu (L) substitution at position 34 (HXB2-3R7L) resulted in the escape from CTL killing (Fig. 5c).

Replication kinetics of HIV-1 mutant viruses. We analyzed the replication kinetics of recombinant viruses carrying mutations that have affected the epitope processing and presentation by infecting H9 or Jurkat cells. All mutants were found to replicate to equivalent levels, suggesting that these mutations do not have a significant influence on HIV-1 replication (Fig. 6).

DISCUSSION

The present study focused on three Gag CTL epitopes restricted by three common HLA alleles in Japanese people (24).

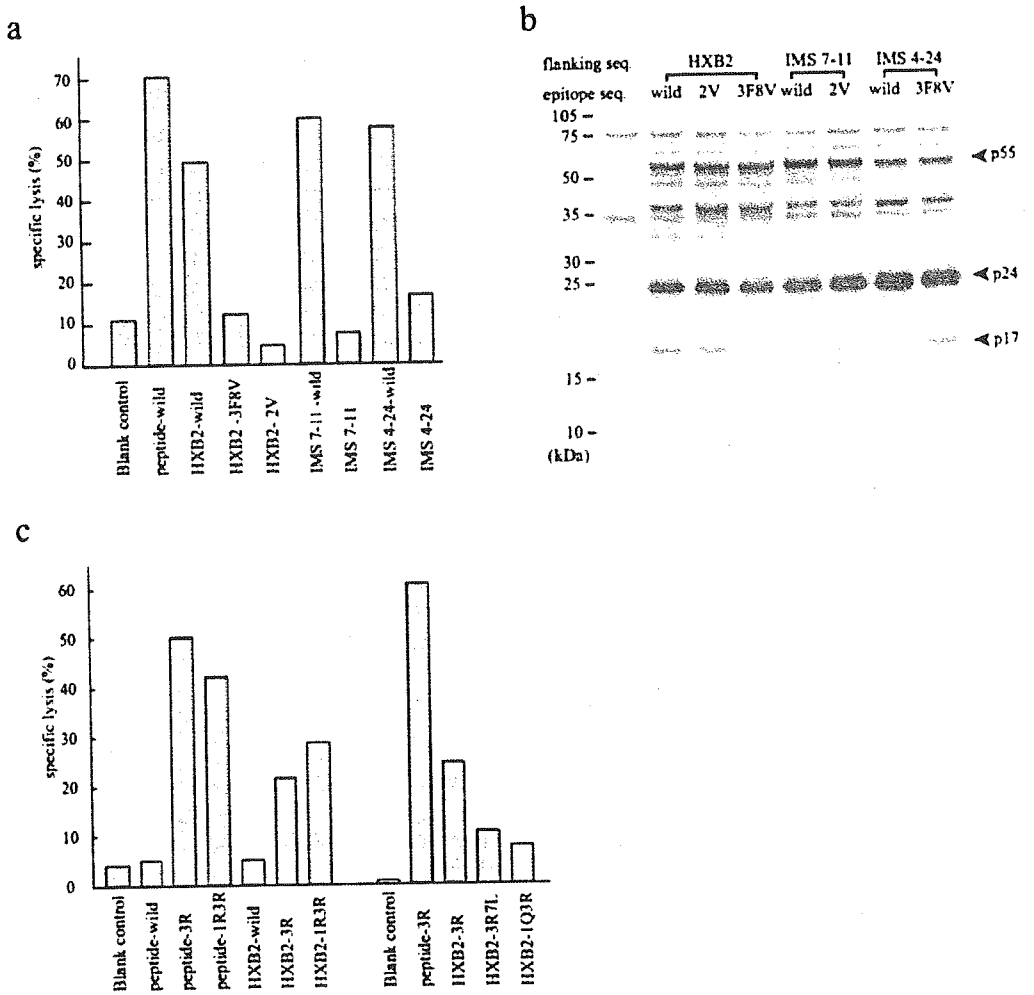

FIG. 5. (a) Specific lysis of A 0201-matched B-LCLs (HLA-A 0201/ and HLA-B 5101/) that endogenously express chimeric *gag* clones bearing the variant CTL epitopes SLENTVAVL and SVYNTVATL in the frame of HXB2 *gag* (HXB2-3F8V and HXB2-2V, respectively) or bearing the wild-type epitope in the frame of IMS7-11 and IMS4-24 Gag (IMS7-11-wild and IMS4-24-wild, respectively). A 0201-restricted SLYNTVATL CTL lines were induced from the same donor as for Fig. 3. Specific lysis of target cells expressing HXB2, IMS7-11, or IMS4-24 *gag* clones and being pulsed with the A 0201 wild-type peptide (10 M) is shown in parallel. The E:T ratio was 20:1. (b) Levels and patterns of HIV-1 protein expression in target cells used in the experiments described for panel a. The Western blot was reacted with the serum from an HIV-1-infected individual. (c) Specific lysis of A24-matched B-LCLs (HLA A24/ and HLA-B46/52 or HLA-A24/26 and HLA-B51/52) that express *gag* clones with various point mutations. Point mutations were inserted into the A24-restricted CTL epitope region in the frame of wild-type HXB2 Gag (HXB2-wild): amino acid substitutions of Lys to Arg at position 30 (HXB2-3R) with Lys to Arg at position 28 (HXB2-1R3R), Ile to Lue at position 34 (HXB2-3R7L), or Lys to Gln at position 28 (HXB2-1Q3R). Peptide target cells were pulsed with either the KYRLKHIVW (3R) or the RYRLKHIVW (1R3R) mutant peptide at 10 M. The effector cells were A24-restricted 3R mutant-specific CTL lines from the same donor as in the Fig. 3e experiment. The E:T ratio was 20:1.

The Gag protein is most commonly targeted by CTL-inducing HIV/AIDS vaccines (15). In our endogenous expression system, three A 0201-restricted epitope variants and one B 5101-restricted epitope variant escaped from the wild-type CTL recognition, and four A24-restricted epitope variants escaped from the A24-restricted 3R mutant-reactive CTL recognition. Intriguingly, two A 0201-restricted variants and three A24-restricted variants escaped from CTL killing when the *gag* clones were expressed endogenously in the target cells by the HIV-1 vector, despite the fact that the synthetic variant peptides were well recognized by the CTLs when loaded onto the MHC class I molecule exogenously. The peptide titration experiments have revealed that the strength of these variant peptides' recognition was almost equivalent to that of the A 0201-restricted wild-type peptide or the A24-restricted 3R mutant peptide. The results were not likely due to differences in the pattern of Gag protein expression, as shown in the Western blot experiments. All target cells were confirmed to express a sufficient level of Gag protein by p24 antigen production. Therefore, we believe that the escape mechanism of these variants resides in the antigen processing and presentation, as has been observed in a mouse model with murine leukemia virus infection (19). The observation of such phenomenon in two epitopes restricted by different alleles implies that this finding is not unique to a particular epitope-MHC pair.

Since all variants investigated here were derived from clinical samples and those mutations did not affect the virus replication, our observations are relevant for discussing what may be going on in HIV-infected individuals. Our results indicate

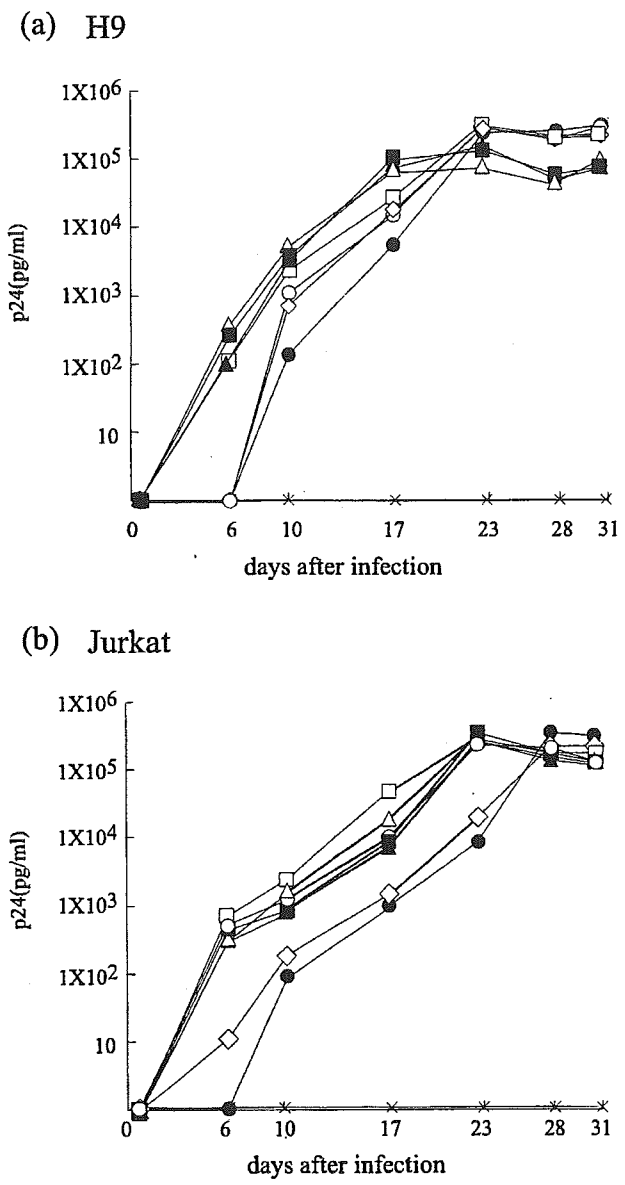

FIG. 6. Replication of HIV-1 clones with mutations that impaired the processing and presentation of A 0201 or A24 CTL epitopes in H9 (a) and Jurkat (b) cells. The kinetics of each recombinant virus replication were monitored as the production of p24 antigen by p24 ELISA. Symbols: ○, wild-type; , A24-3R; ■, A24-K26S 3R; △, A24-3R7L; ▲, A24-1Q3R; ●, A 0201-3F8V; , A 0201-2V; , mock.

that the impaired antigen processing and presentation often occurs in HIV-1 field isolates and thus is one of the major mechanisms that enable HIV-1 to escape from the CTL recognition. To understand further the significance of this escape mechanism, it is important to evaluate an accumulation of such escape variants in infected hosts in a longitudinal study or at a population level. A previous report using a vaccinia virus expression system did not reveal that any mutations in the A 0201-restricted p17 epitope of HIV-1 and its flanking region altered the processing and presentation of its variant epitope (4). However, that study did not investigate A 0201-restricted 2V and 3F8V variants, which we found affected epitope processing and presentation.

Experiments with chimeric genes, as well as point mutations, showed that escapes from epitope processing and presentation were mostly attributable to mutations within the epitope regions rather than its flanking regions. In the present study, we demonstrated that point mutations of Lys (L) to Gln (Q) at position 28 and of Ile to Leu at position 34 drastically impaired the processing and presentation of the A24-restricted CTL epitope. Moreover, the experiment with HXB2 clone carrying IMS 7-11 variant of A 0201-restricted CTL epitope indicates that a substitution of Leu (L) to Val (V) at position 78 was responsible for the impaired processing and presentation of the epitope. These mutations in the epitope region may have induced a proteasome cleavage site within the epitope (19). On the other hand, we observed that the variations in the 15 amino acids up- and downstream of the epitope did not affect CTL recognition. An exception was a Lys (L)-to-Ser (S) substitution (2S) at position 26, which is only two amino acids adjacent to the N terminus of the A24-restricted epitope. However, this 2S substitution did not void the A24-restricted 3R mutant-reactive CTL recognition completely. One possible explanation is that the 2S substitution shifted the optimal proteasome cleavage site, resulting in the generation of a larger peptide, which has a lower affinity to the MHC class I molecule.

We have first attempted to investigate the antigen processing and presentation by the conventional recombinant vaccinia virus method for all variants before we established this VSV-G-pseudotyped HIV-1 vector method. Soon, we realized that preparing recombinant vaccinia viruses was much more laborious and time-consuming. Early experiments of comparing two methods by using the first available recombinant vacinia viruses concluded that the HIV-1 vector method demonstrated CTL killing better than did the recombinant vaccinia virus method (Fig. 3b). In the recombinant vaccinia virus expression system, the massive production of vaccinia virus proteins inevitably takes place, along with the expression of an HIV-1 gene and sometimes causes a high background lysis. The expression manner and the production ratio to non-HIV proteins may also influence antigen processing and presentation (27, 34). Thus, we thought that the antigen processing and presentation in the HIV-1 vector expression system is more physiological than the recombinant vaccinia virus expression system and that continuing vaccinia virus experiments would not be significantly beneficial to address the issue of antigen processing and presentation. Nevertheless, there remains a concern that there might be a potential difference in the antigen processing and presentation between immortalized B cells that were used here and primary CD4 T cells (32, 33). Perhaps it is important to reevaluate the interaction of CTLs and these variants in experiments with variant HIV-1-infected T cells. Our HIV-1 vector carries neither the *nef* gene nor the *vpu* gene, which significantly affect antigen presentation by downregulating MHC class I cell surface expression (5, 13). From this point of view, one might expect that more variants would escape from the CTL recognition in the actual HIV-1 infection than what is shown in our experiments. However, we think that our system is suited to identify a specific association between a certain mutation and the escape from antigen processing and presentation. To prove the existence of this mode of escape mecha-

nism, we may need a new system that can directly detect a trace of specific epitopes that are eluted from MHC class I molecules of HIV-1 antigen-producing cells.

Although the structure analysis of MHC class I molecules and its binding motif has facilitated the prediction of CTL epitopes from the primary amino acid sequence data of HIV-1 (6, 11, 26), it remains difficult to envisage the efficiency of epitope processing and presentation. Enormous diversity realized in HIV-1 field isolates causes a further complexity (7). Our data emphasize the importance of testing HIV-1 variants in an endogenous expression system. Detailed analysis of epitope processing and presentation among HIV-1 field isolates, particularly of non-B subtypes circulating in the vaccine trial fields, is essential, since such information allows us to forecast which virus may elude the immunity elicited by vaccines, thus providing a clue for a rational design for effective HIV/AIDS vaccines.

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A *CCR2-V64I* polymorphism affects stability of CCR2A isoform

Emi E. Nakayama, Yuetsu Tanaka,[a] Yoshiyuki Nagai,[b] Aikichi Iwamoto[c] and Tatsuo Shioda

Objective: A valine to isoleucine substitution at position 64 of CCR2 (*CCR2-64I*) is associated with a delay in progression to AIDS in HIV-1-infected individuals. The aim of the present study is to elucidate the molecular mechanism underlying the effect of this allele.

Design: We analysed the effect of the 64I substitution on levels of expression of CCR2A and CCR2B, two CCR2 isoforms produced by alternative splicing.

Methods: Sendai virus vector was used to express CCR2 molecules.

Results: While CCR2B trafficked well to the cell surface, CCR2A, which differs from CCR2B only by the sequence of its C-terminal cytoplasmic tail, was detected predominantly in the cytoplasm. The level of expression of CCR2A-64I was significantly higher than that of CCR2A without the substitution. On the other hand, the 64I substitution did not affect levels of CCR2B expression. Pulse–chase experiments revealed that the 64I substitution increased the half-life of CCR2A in cells. When co-expressed with CCR5, CCR2A-64I interfered more severely with cell surface expression of CCR5 than did wild-type CCR2A. Furthermore, immunoprecipitation experiments showed that CCR2A co-precipitated with an immature form of CCR5.

Conclusion: These results suggest that CCR2A binds to CCR5 in the cytoplasm and down-modulates its surface expression. We propose that the increased ability of CCR2A-64I to down-modulate CCR5 expression might be a possible cause of a delay in HIV-1 disease progression in patients with this allele.

AIDS 2004, **18**:729–738

Keywords: polymorphism, CCR2-64I, CCR2A, CCR5, stability

Introduction

The chemokine receptor CCR2B has been regarded as a minor HIV-1 coreceptor because only a small number of HIV-1 strains has been shown to use CCR2B as an entry coreceptor [1–3]. Nevertheless, a polymorphism in the CCR2 gene, *CCR2-64I*, has been reported to be associated with delayed disease progression in HIV-1 infected individuals in several Caucasian cohorts [4–8]. This polymorphism, a G-to-A transition at position 190, changes CCR2B codon 64 from valine to isoleucine, introducing a conservative amino acid change into the first transmembrane domain. It was unclear why a single amino acid substitution in a minor coreceptor could affect HIV-1 disease progression, as there was no difference in HIV-1 co-receptor activity between the variant CCR2B-64I and CCR2B without the 64I substitution (CCR2B-64V) [9,10]. Furthermore, these studies also excluded the possibility that CCR2B-64I exerts a dominant-negative effect on the expression and activity of CCR5.

From the Research Institute for Microbial diseases, Osaka University, Osaka, the [a]University of the Ryukyus, Okinawa, [b]Toyama Institute of Health, Toyama, and the [c]Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Correspondence to T. Shioda, Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University. 3-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan.

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It is possible that the *CCR2* polymorphism may be linked to other polymorphisms in genes that influence AIDS progression. The *CCR2* gene is located approximately 15 kb from the 5′ end of the *CCR5* gene, and the *CCR2-64I* allele is indeed linked to a certain *CCR5* promoter haplotype [11]. However, experiments using promoter–reporter fusion constructs showed that the *CCR5* promoter haplotype, which is in a strong linkage disequilibrium with *CCR2-64I*, did not affect transcriptional activity of the *CCR5* promoter [10]. Thus, the mechanism underlying the protective effect of *CCR2-64I* against AIDS progression still remained to be elucidated.

Two alternatively spliced CCR2 isoforms, CCR2A and CCR2B, were reported to be present in freshly isolated human monocyte, THP-1, and MonoMac 6 leukaemia cell lines [12,13]. An open reading frame encoded in the chromosome corresponds to CCR2B, while alternatively spliced transcripts produce CCR2A. The two CCR2 isoforms differ only in their C-terminal cytoplasmic tails (Fig. 1). Therefore, an individual carrying the *CCR2-64I* allele also produces CCR2A molecules with isoleucine at position 64. Although the cytoplasmic tail spans less than one-fifth of the entire CCR2 molecule, this difference caused a drastic alteration in their localization in cells [13]. While CCR2B trafficked well to the cell surface, CCR2A was detected predominantly in the cytoplasm. A progressive truncation study of the C-terminal cytoplasmic tail indicated that a cytoplasmic retention signal(s) was located in the C-terminal cytoplasmic tail [13]. Nevertheless, CCR2A molecules that successfully trafficked to the cell surface could respond to the stimulation of monocyte chemoattractant protein (MCP)-1 in a similar fashion to CCR2B [14].

As none of the previous studies investigated the effect

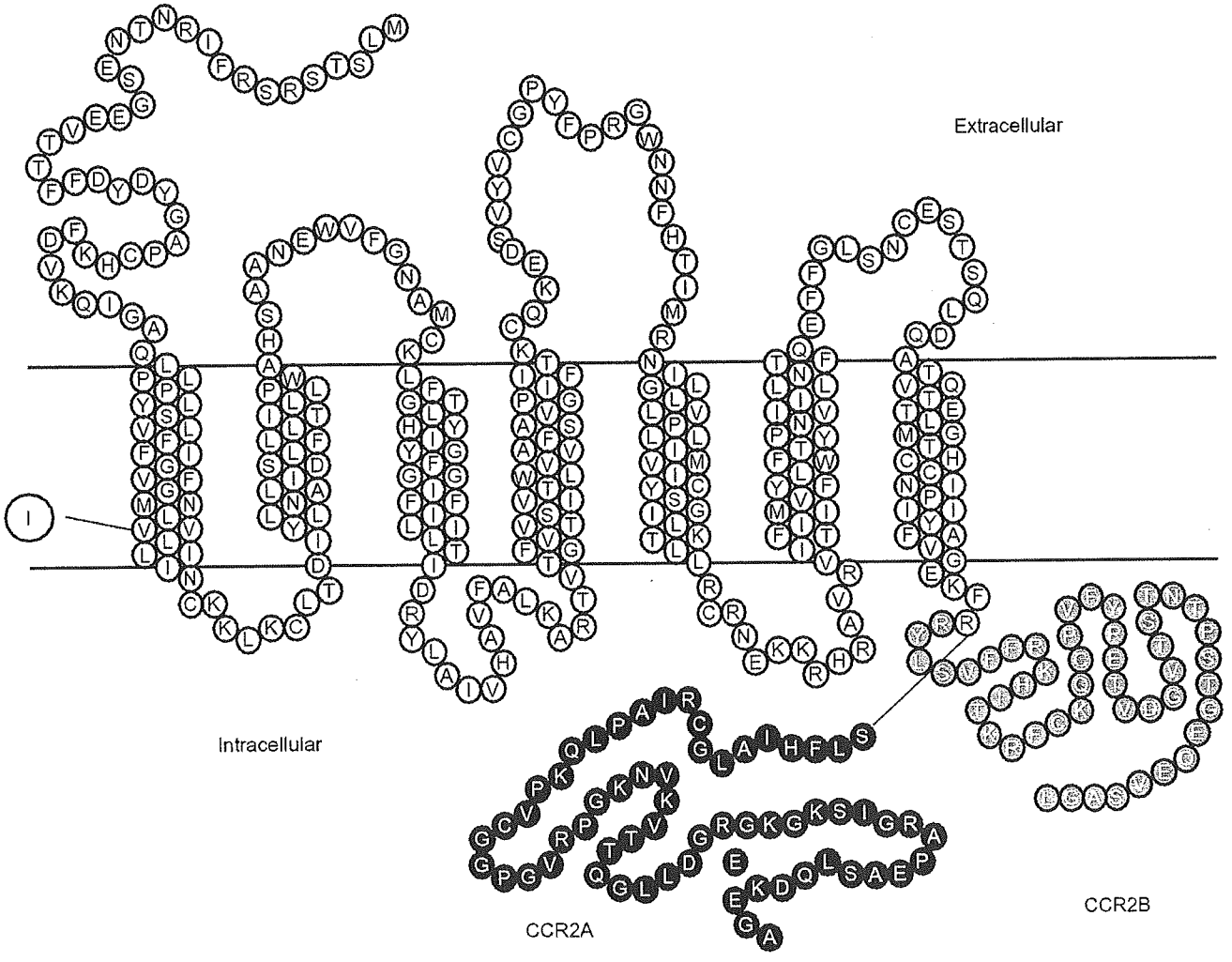

Fig. 1. The structure of the CCR2A and CCR2B molecules. Outlined letters in grey circles denote amino acid residues present in CCR2B. Outlined letters in black circles denote amino acid residues present in CCR2A. A letter I in a large circle denotes a substitution at position 64.

of the 64I substitution on CCR2A molecules, we generated recombinant Sendai viruses (SeV) expressing either CCR2A-64V or CCR2A-64I. Here we show that the 64I substitution indeed affected the stability of CCR2A molecules in cells, and increased the ability of CCR2A to down-modulate the major HIV-1 co-receptor, CCR5.

Materials and methods

Generation of recombinant SeV

THP-1 cells were shown to possess both *CCR2-64V* and *CCR2-64I* alleles by using a standard genotyping method [15]. Therefore, CCR2A-64V, CCR2A-64I, CCR2B-64V, and CCR2B-64I cDNA were obtained by reverse transcription (RT)–PCR from mRNA extracted from THP-1 cells and then inserted to the *Not*I site of pSeV18+b(+). The entire coding regions in the resultant plasmids were verified for sequence authenticity as well as for the presence or absence of the 64I substitution. For generating CCR2A-64V and CCR2A-64I cDNA carrying a c-myc-tag (EQKLI SEEDL) at their C-termini, cloned CCR2A-64V and CCR2A-64I cDNA served as templates for PCR amplification using a primer containing a nucleotide sequence corresponding the c-myc-tag fused with the C-terminal portion of CCR2A. Recombinant SeV carrying CCR2A-64V, CCR2A-64I, CCR2B-64V, CCR2B-64I, or C-myc-tagged versions of CCR2A-64V and CCR2A-64I were recovered according to a previously described method [16]. The wild-type Z strain of SeV served as a control in all the experiments.

Generation of a recombinant vaccinia virus

For generating CCR5 cDNA carrying a HA tag (YPYDVPDYAA) at its C terminus, cloned CCR5 cDNA served as a template for PCR amplification by using a primer containing a haemagglutinin (HA) tag sequence fused with the C-terminal portion of CCR5. The resultant PCR products were then inserted into pNZ68K2-Not. The entire coding region of CCR5-HA was verified for sequence authenticity. A recombinant vaccinia virus (Vac) was recovered from the resultant plasmid according to previously described procedures [17].

Flow cytometric analysis

CV1 monkey kidney cells, U937 monocytic cells and Jurkat T cells were infected with recombinant SeV expressing CCR2A-64V, CCR2A-64I, CCR2B-64V, or CCR2B-64I. Five to 18 h after infection, cells were incubated with MAB150, a mouse monoclonal antibody (MAb) against CCR2 (R & D Systems, Minneapolis, Minnesota, USA). Antibodies bound to cells were detected using fluorescein-5-isothiocyanate (FITC)-conjugated goat antibody directed against mouse IgG (Cappel, Aurora, Ohio, USA). CV1 or H9 cells infected with SeV expressing CCR2A-64V, CCR2A-64I, CCR2B-64V, or CCR2B-64I were superinfected with a recombinant Vac expressing CCR5, CXCR4, or CD4 at 9 h after SeV infection. After incubation for 5 h at 37°C, cells were stained for CCR5 using T227 rat MAb against CCR5 [17] followed by FITC-conjugated goat anti-rat IgG; for CXCR4 using 12G5 mouse MAb (R & D systems) followed by FITC-conjugated goat anti-mouse IgG; or for CD4 using FITC-conjugated anti-human CD4, Leu3a (Becton Dickinson, San Jose, California, USA), and analysed by FACScan (Becton Dickinson).

Immunofluorescence microscopy

CV1 cells expressing CCR2A or CCR2B were fixed and permeabilized before being incubated with MAB150 antibody as described previously [17]. Bound antibodies were then detected using FITC-conjugated goat antibody against mouse IgG. Indirect immunofluorescence was visualized using a Lasersharp2000 Confocal Microscope System (Bio-Rad, Hercules, California, USA). Anti-Calnexin (Stressgen, San Diego, California, USA) or anti-Giantin (CRPinc, Berkeley, California, USA) rabbit polyclonal antibody was used with Cy5-conjugated goat antibody against rabbit IgG (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Chemotaxis assay

Chemotaxis assays were performed according to previously described methods [18]. Briefly, MCP-1 (PeproTech, Rockey Hill, New Jersey, USA) diluted at an indicated concentration of chemotaxis buffer (RPMI 1640 with 0.25% human serum albumin) was added to the bottom chamber of a 5- m pore polycarbonate Transwell culture insert (Costar; Corning, New York, USA). Jurkat cells were infected with a SeV expressing CCR2A-64V or CCR2A-64I and incubated at 37°C for 4 h. Cells were then washed with RPMI1640 and re-suspended in chemotaxis buffer and added to the upper chamber of the insert. Transmigrated cells in 4 h at 37°C were counted using a FACScan.

Pulse-chase analyses of CCR2A and CCR5

CV1 or U937 cells were infected with a SeV expressing CCR2A-64V-myc or CCR2A-64I-myc. Nine hours after infection, cells were labelled with 500 kbq/ml of EXPRE^{35}S^{35}S[^{35}S] protein labelling mix (> 37 Tbq/mmol; PerkinElmer (Boston, Massachusetts, USA) in amino acid-free medium for 30 min. For CCR5 analysis, cells were infected with a recombinant Vac expressing CCR5-HA, incubated at 37°C for 5 h and then labelled. Cells were then washed, fed with fresh medium and incubated for 0, 15, 30, 60, or 120 min at 37°C, chilled on ice, and lysed in lysis buffer (50 mM Tris–HCl pH7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). CCR2A

and CCR5 proteins in the lysates were precipitated with anti-myc mouse MAb (9B11; Cell Signaling, Beverly, Massachusetts, USA) and anti-HA high affinity rat MAb (Roche, Indianapolis, Indiana, USA), respectively, using a Protein G Immunoprecipitation Kit (Roche). Precipitated materials were subjected to SDS–PAGE on a 4–12% NuPAGE Bis–Tris gel (Invitrogen, Groningen, Netherlands), and the amount of radiolabel incorporated was visualized on a BAS Imager (Fujix, Kanagawa, Japan).

Gene reporter fusion assay

A recombinant Vac-based gene activation assay using a -galactosidase gene as a reporter was performed as described previously [19]. Briefly, mouse fibroblast L cells were transfected with -galactosidase reporter plasmid pGINT7 -gal and infected with a recombinant Vac expressing gp160 of an R5 HIV-1 strain SF162. At the same time, CV1 cells were infected with SeV expressing CCR2A-64V or CCR2A-64I and incubated at 37°C for 9 h. Cells were then superinfected with recombinant Vacs expressing T7 RNA polymerase, human CD4, and CCR5, detached by trypsinization, and cultured at 37°C for 5 h. Then, L and CV-1cells were mixed, incubated for 3 h, and -galactosidase activities in the cell lysate were measured by using chlorophenol red- -D-galactopyranoside as substrate.

HIV-1 productive infection

MT4 cells (4×10^5) were infected with SeV expressing CCR2A-64V, CCR2A-64I or parental Z strain of SeV at a multiplicity of infection (MOI) of 40 plaque forming unit (PFU)/cell mixed with SeV expressing CCR5 at an MOI of 10 PFU/cell and incubated at 37°C for 5 h. Cells were then superinfected with 60 ng p24 of an R5 HIV-1 strain SF162. The culture supernatants were collected periodically and p24 levels were measured.

Immunoprecipitation and western blot analysis

CV1 cells were infected with SeV expressing CCR2A-64V-myc or CCR2A-64I-myc, and incubated at 37°C for 9 h. Cells were then superinfected with a Vac expressing CCR5-HA and incubated at 37°C for 5 h and then lysed. CCR2A-64V-myc, CCR2A-64I-myc or CCR5-HA proteins were immunoprecipitated, and subjected to SDS–PAGE as described above. Proteins were then electrophoretically transferred to a PVDF membrane (Immobilon; Millipore, Bedford, Massachusetts, USA). Blots were blocked and probed with the antibodies overnight at 4°C and then incubated with peroxidase-conjugated anti-mouse (Kirkegaard & Perry Laboratories, Gaithersbug, Maryland, USA) or anti-rat IgG (American Qualex, San Clemente, California, USA) and developed using the Immun-Star HRP chemiluminescent kit (Bio-Rad).

Results

Expression of CCR2A and CCR2B

We generated a recombinant SeV expressing either CCR2A-64V or CCR2B-64V. Confocal microscopic observations (Fig. 2a) and flow cytometric analyses (Fig. 2b) confirmed the different subcellular localization of these two CCR2 isoforms. In CCR2B-64V expressing CV1 cells, fluorescent signals of CCR2 were observed mainly on the cell surface. In contrast, CCR2A-64V was localized predominantly to the cytoplasm, although a small portion of CCR2A was observed on the cell surface. In the cytoplasm, signals of an endoplasmic reticulum marker calnexin were only partially co-localized with CCR2A signals (Fig. 2a, left), whereas the majority of signals for the Golgi marker giantin overlapped with those of CCR2A (Fig. 2a, right). These results suggested that most CCR2A molecules were retained in the Golgi.

To assess the effect of the 64I substitution on CCR2A expression, we generated a recombinant SeV expressing CCR2A-64I and compared levels of expression of CCR2A-64I with those of CCR2A-64V. As shown in Fig. 2b, CCR2A-64I showed slightly but significantly higher levels of expression than CCR2A-64V in various cell types, despite the same promoter being used. The mean fluorescence intensity (MFI) of CCR2A-64I and CCR2A-64V was 274 and 140 in CV1, 133 and 40 in U937 monocystic cells, and 29 and 21 in Jurkat T cells. The difference was greater in U937 cells than in Jurkat cells. The difference was also observed at 5, 12, and 18 h after infection of recombinant SeVs (Fig. 2c). Exactly the same result was obtained when recombinant SeV expressing C-myc-tagged versions of CCR2A-64V (CCR2A-64V-myc) and CCR2A-64I (CCR2A-64I-myc) were used (Fig. 2c). In contrast, we failed to detect any difference in the levels of expression between CCR2B-64V and CCR2B-64I (MFI 2698 and 2663, respectively; Fig. 2b), as had been described in the previous reports [9,10]. Northern blot analyses confirmed that there was no difference in the amount of CCR2 mRNA among cells expressing CCR2A-64V, CCR2A-64I, CCR2A-64V-myc, CCR2A-64I-myc, CCR2B-64V and CCR2B-64I (data not shown). These data clearly indicate that the substitution of valine to isoleucine affects levels of cell surface expression of CCR2A, but not of CCR2B.

Chemokine receptor activity of recombinant CCR2A-64V and CCR2A-64I

To determine whether or not CCR2A molecules expressed by a recombinant SeV fully retained chemokine receptor activity, we performed a chemotaxis assay. As shown in Fig. 2d, both cells expressing CCR2A-64V and CCR2A-64I migrate toward MCP-1. However, cells expressing CCR2A-64I migrated

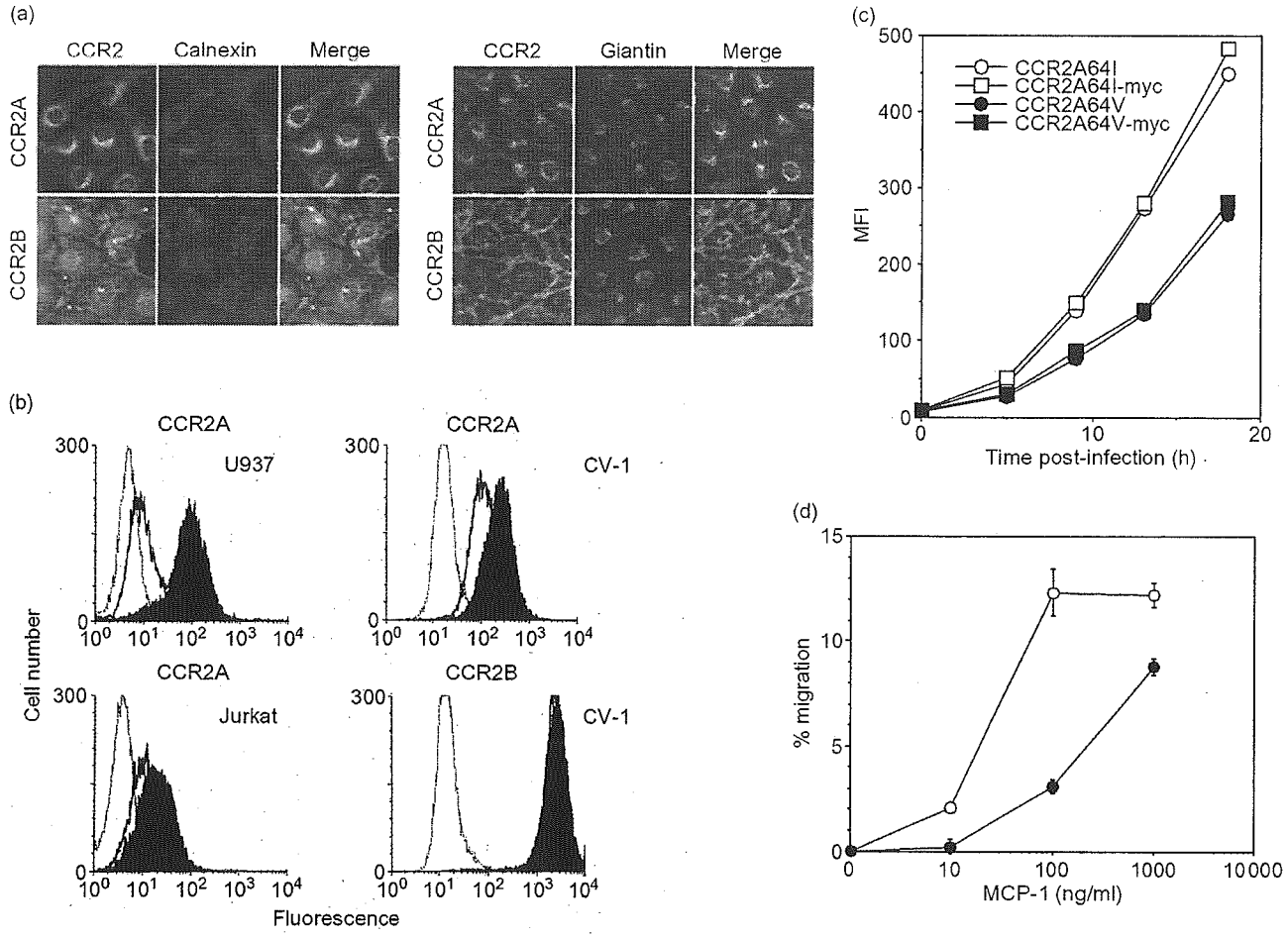

Fig. 2. (a) Subcellular distribution of CCR2A-64V and CCR2B-64V in CV1 cells. SeV vector (SeV) was used to express the CCR2A-64V and CCR2B-64V molecules. Cells were fixed and permeabilized before staining with MAB150 anti-CCR2 mouse MAb followed by FITC-labelled anti-mouse IgG. Cells were then re-stained with anti-calnexin or anti-giantin rabbit polyclonal antibody followed by Cy5-labelled anti-rabbit IgG, and analysed by confocal laser microscopy. (b) Surface expression of CCR2A-64V (green) and CCR2A-64I (red) in U937, CV1 or Jurkat cells. Cells infected with the parental Z strain served as a negative control (black). In lower right panel, green and red indicates CCR2B-64V and CCR2B-64I, respectively. (c) The cell surface expression of CCR2A-64I (open circles), CCR2A-64I-myc (open squares), CCR2A-64V (filled circles), and CCR2A-64V-myc (filled squares) at 5, 9, 12 and 18 h after infection by SeV. MFI indicates mean fluorescence intensity of each sample. (d) Chemokine receptor activity of recombinant CCR2A-64V and CCR2A-64I. Jurkat cells infected with SeV expressing CCR2A-64V (closed circles) or CCR2A-64I (open circles) migrated in response to increasing concentration of MCP-1. Data points are means of triplicate determination with standard deviations.

more efficiently than those expressing CCR2A-64V. These results are in good agreement with the observation that expression of CCR2A-64I is higher than that of CCR2A-64V.

CCR2A-64I is more stable than CCR2A-64V

Differential levels of expression between CCR2A-64V and CCR2A-64I prompted us to compare the rate of degradation of those proteins in pulse–chase experiments. For this purpose, we used recombinant SeV expressing CCR2A-64V-myc or CCR2A-64I-myc. Comparison of immunoprecipitated materials from ^{35}S-labelled CV1 cells expressing CCR2A-64V-myc and CCR2A-64I-myc showed that almost identical levels of CCR2A-64V-myc and CCR2A-64I-myc proteins were synthesized during the 30-min labelling period (t = 0) (Fig. 3a). However, CCR2A-64V-myc proteins appeared to degrade more rapidly than CCR2A-64I-myc proteins. The half-life of CCR2A-64I-myc was approximately 90 min, whereas that of CCR2A-64V-myc was approximately 50 min in CV1 cells (Fig. 3b). More prominent results were obtained when we used U937 cells, as the half-life of CCR2A-64I-myc was approximately 60 min, whereas that of CCR2A-64V-myc was approximately 18 min in U937 cells. This finding is in a good agreement with the observation

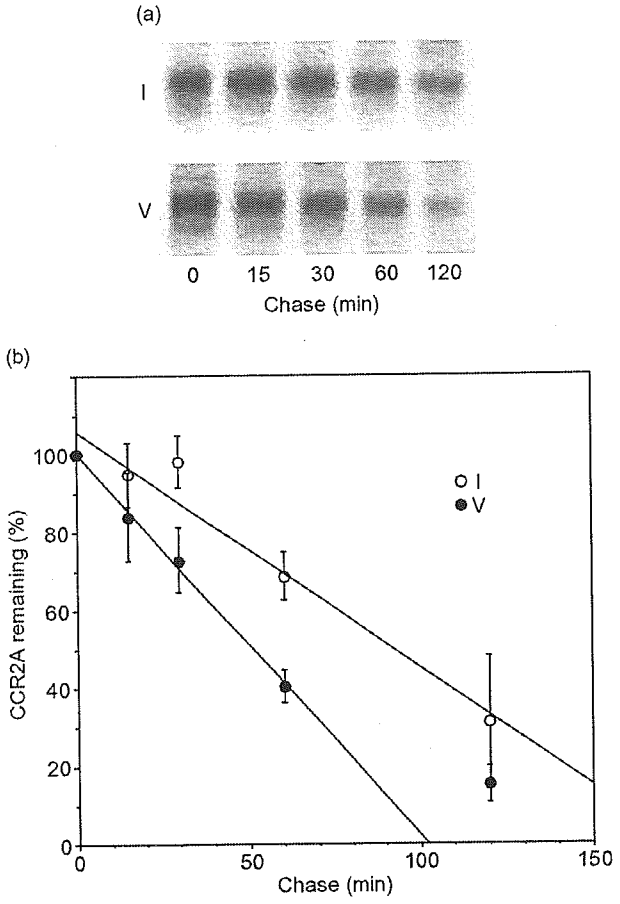

Fig. 3. CCR2A-64I is more stable than CCR2A-64V. CV1 cells were infected with SeV expressing CCR2A-64V-myc and CCR2A-64I-myc for 9 h. Cells were labelled for 30 min and then harvested following the chase time indicated. (a) Representative gels of pulse–chase analysis. (b) Phosphorlmager analysis of the gels shown in (a). Open and closed circles denote cells infected with SeV expressing CCR2A-64V-myc and CCR2A-64I-myc, respectively. Data points are means of four independent experiments with standard deviations.

that the difference in cell surface expression levels between CCR2A-64V and CCR2A-64I was greater in U937 cells than in CV-1 cells (Fig. 2b). These results indicate that higher cell surface expression of CCR2A-64I was due to increased stability of CCR2A-64I. On the other hand, we failed to detect any significant difference in the half-life between CCR2B-64V and CCR2B-64I (data not shown).

CCR5 but not CXCR4 expression was more severely blocked by co-expression of CCR2A-64I than by co-expression of CCR2A-64V

To determine whether or not CCR2A has a dominant-negative effect on the expression of major HIV-1 receptor molecules, we first inoculated SeV expressing CCR2A-64V or CCR2A-64I in CV1 cells and incubated the cells for 9 h at 37°C. The cells were then superinfected with recombinant Vac expressing CCR5, CXCR4, or CD4. Five hours after Vac infection, surface expression of CCR5, CXCR4, or CD4 were examined by flow cytometry. As shown in Fig. 4a, the CCR5 MFI of cells co-infected with parental Z strain of SeV was 391, while that of the cells co-infected with SeV expressing CCR2A-64V was 297, indicating that co-expression of CCR2A-64V significantly reduced levels of CCR5 expression on the cell surface. This dominant-negative effect on CCR5 expression was more prominent when SeV expressing CCR2A-64I were used (MFI, 145) than SeV expressing CCR2A-64V were used. The same results were obtained when we used recombinant SeV expressing CCR2A-64V-myc and CCR2A-64I-myc (MFI, 300 and 179, respectively). Similar results were obtained when CV1 cells were inoculated with Vac expressing CCR5 5 h after infection by SeV expressing CCR2A, as the CCR5 MFI on cells co-infected with Z, SeV expressing CCR2A-64V, and Sev expressing CCR2A-64I, was 299, 205, and 160, respectively. Furthermore, the dominant-negative effect of CCR2A on CCR5 expression was also observed when T cell line H9 was used. The CCR5 MFI on H9 cells co-infected with Z, SeV expressing CCR2A-64V, and SeV expressing CCR2A-64I was 263, 230 and 195, respectively. In contrast, the cell surface expression of CXCR4, another major co-receptor, as well as that of CD4, the main receptor of HIV-1, were not affected by CCR2A-64V or CCR2A-64I (Fig. 4a). In contrast with CCR2A, neither CCR2B-64V nor CCR2B-64I affected the surface expression of CCR5 (Fig. 4b).

HIV-1 coreceptor activity of CCR5 was more dramatically reduced by co-expression of CCR2A-64I than by co-expression of CCR2A-64V

To assess the effect of CCR2A-64I on HIV-1 infection, we examined the ability of cells expressing both CCR2A and CCR5 molecules to support CD4-dependent cell fusion mediated by an HIV-1 envelope protein of the R5 strain SF162. For this purpose, we prepared CV1 cells expressing both CCR5 and CCR2A as described in Fig. 4a, and mixed those cells with mouse L cells expressing HIV-1 envelope protein. As shown in Fig. 5a, the envelope-mediated cell fusion activity of CCR5 was more dramatically reduced by co-expression of CCR2A-64I than by that of CCR2A-64V.

We also inoculated a live SF162 strain of HIV-1 into CD4 positive MT4 cells expressing both CCR5 and CCR2A. As shown in Fig. 5b, MT4 cells expressing CCR5 and CCR2A-64V supported SF162 replication better than those expressing CCR5 and CCR2A-64I.

Co-immunoprecipitation of CCR2A and CCR5

Many seven-transmembrane receptors, including che-

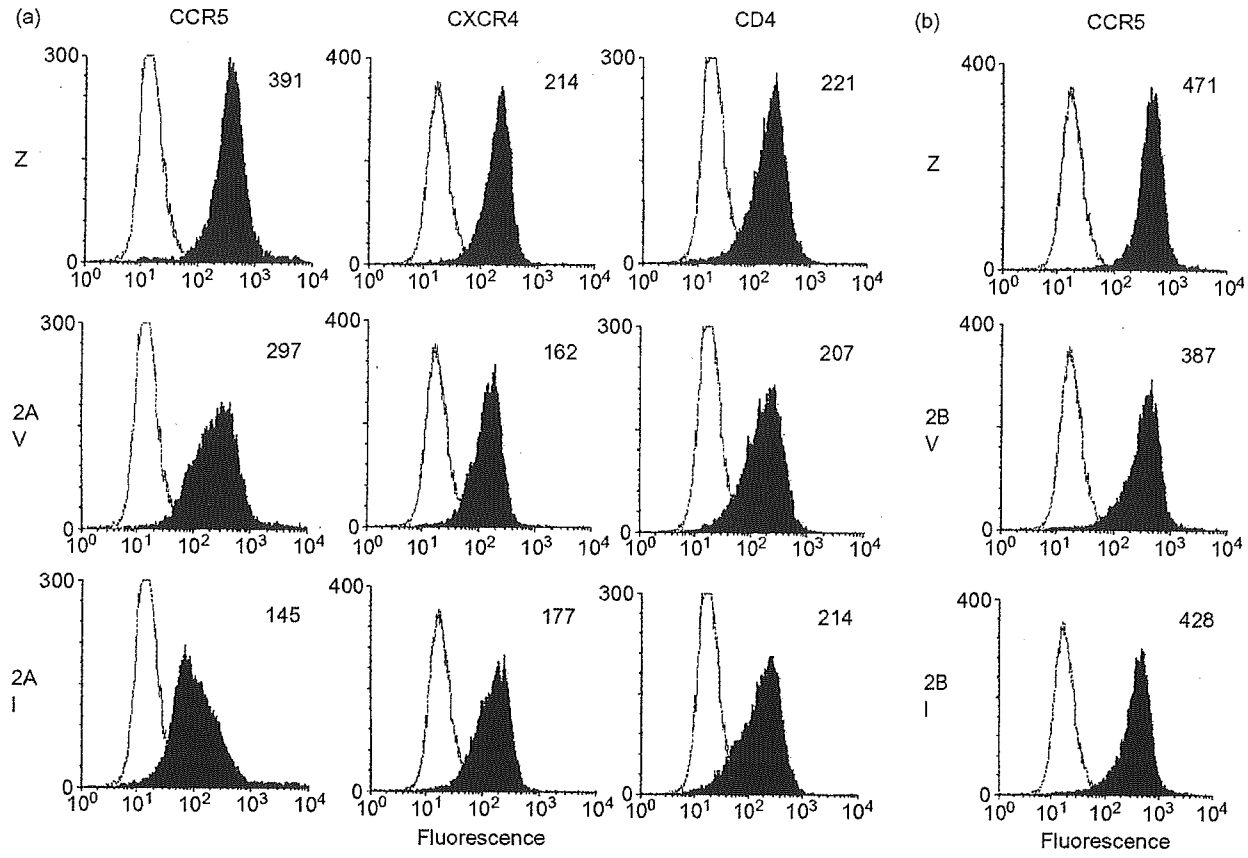

Fig. 4. (a) Effect of CCR2A-64V and CCR2A-64I on HIV-1 corecepter expression. Vac vectors were used to express CCR5, CXCR4 and CD4 in the CV1 cells inoculated with SeV expressing CCR2A-64V or CCR2A-64I. Z denotes the wild-type SeV. Five hours after infection, cells were stained with MAb against CCR5, CXCR4, or CD4. Flow cytometry was used to determine surface expression levels. The number in each panel indicates mean fluorescence intensity. (b) Effect of CCR2B-64V and CCR2B-64I on CCR5 expression.

mokine receptors, have been reported to form homo-oligomers. CCR2A is highly homologous to CCR5 (68% at the amino acid level), and formation of heterodimers between CCR2B and CCR5 was reported previously [20]. The dominant-negative effect of CCR2A on CCR5 expression shown in Figs 4a, 5a and 5b raised the possibility of heterodimer formation between CCR2A and CCR5. To test this hypothesis, we used SeV expressing CCR2A-64V-myc or CCR2A-64I-myc, and Vac expressing HA-tagged version of CCR5 (CCR5-HA). Anti-myc and anti-HA immunoprecipitates from cell lysates were developed in Western blots by using anti-HA or anti-myc antibodies. As expected, CCR5-HA was detected by anti-HA antibody in anti-myc-derived immunoprecipitates from CCR5-HA and CCR2A-64V-myc co-expressed cell lysates as well as from CCR5-HA and CCR2A-64I-myc co-expressed cell lysates. At the same time, CCR2A-64V-myc and CCR2A-64I-myc were detected by anti-myc antibody in anti-HA-derived immunoprecipitates of CCR5-HA and CCR2A-64V-myc co-expressed cell lysates and in that of CCR5-HA and CCR2A-64I-myc co-expressed cell lysates (Fig. 5c). These results clearly indicate that CCR2A formed heterodimers with CCR5.

In CCR5-HA expressing cells, we consistently observed two types of CCR5-HA molecules with different electrophoretic mobility. When we used anti-HA antibody to precipitate CCR5-HA directly, most of the CCR5-HA molecules migrated at approximately 38 kDa. In contrast, most of the CCR5-HA molecules that co-precipitated with CCR2A-64V-myc or CCR2A-64I-myc migrated at 37 kDa. We speculated that the CCR5-HA of 38 kDa represented authentic CCR5 molecules and that of 37 kDa represented immature forms of CCR5. To verify the maturation process of CCR5, we labelled the cells infected with Vac expressing CCR5-HA by [^{35}S]-methionine for 30 min and harvested those cells following chase periods ranging from 15 to 60 min. As shown in Fig. 5d, the 37-kDa CCR5-HA could be detected only after the labelling period (0 min). This result suggests that CCR2A binds to premature forms of CCR5 and

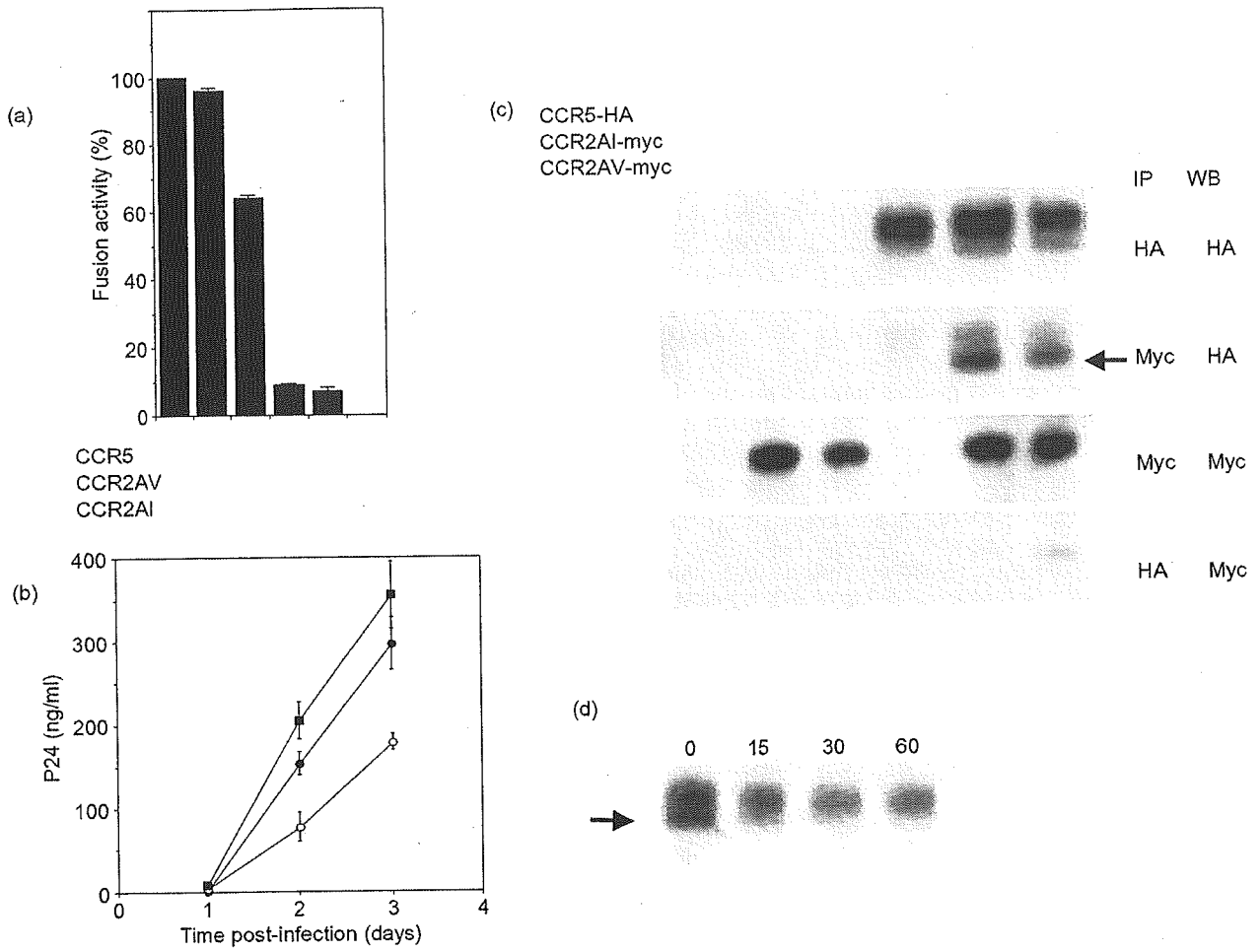

Fig. 5. (a) Coreceptor activity of CCR5 in CCR2A-64V or CCR2A-64I co-expressed cells. SeV vector was used to express CCR2A-64V or CCR2A-64I, and Vac vector was used to express CCR5 as described in Fig. 4. HIV-1 coreceptor activity of each sample was measured using the method described in Materials and methods. The wild-type Vac WR strain was used as a CCR5-negative control, and the wild-type SeV Z strain was used as the CCR2A-negative control. (b) MT4 cells were co-infected with SeV expressing CCR5 and SeV expressing CCR2A-64V (filled circles), CCR2A-64I (open circles), or parental Z strain (filled squares). Five hours after infection, cells were inoculated with an HIV-1 strain SF162. (c) Co-immunopresipitation of CCR2A and CCR5. Recombinant Vac expressing CCR5-HA or parental WR strain (–) was superinfected in CV1 cells infected with SeVs expressing CCR2A-64V-myc, CCR2A-64I-myc, or the parental Z strain (–). Immunoprecipitation and Western blot analysis were performed by using anti-HA or anti-myc antibody. An arrow indicates 37-kDa CCR5-HA molecules. (d) Pulse–chase analysis of CCR5 molecules. A recombinant Vac expressing CCR5-HA was inoculated into CV1 cells. An arrow indicates 37-kDa CCR5-HA molecules.

interferes with the maturation process of CCR5 molecules in cytoplasm.

Discussion

Many independent cohort studies have affirmed the AIDS-delaying effects of the *CCR2-64I* allele [4–8], but the molecular mechanism of this protective effect had not yet been elucidated. In the present study, we demonstrated that a valine to isoleucine substitution at position 64 increased stability of CCR2A but not of CCR2B molecules in cells. When co-expressed with the major HIV-1 co-receptor CCR5, CCR2A-64I more severely interfered with cell surface expression as well as HIV-1 co-receptor activity of CCR5 than CCR2A-64V. Furthermore, CCR2A was shown to co-precipitate with immature form of CCR5. These results suggest that CCR2A binds to CCR5 in the cytoplasm and dominantly interferes with CCR5 maturation and surface expression. On the other hand, the 64I substitution did not affect the level of CCR2B expression, being consistent with results published previously [9,10]. We speculate that increased ability of CCR2A-64I to down modulate CCR5 expression

might be a possible cause of delay in HIV-1 disease progression in patients with this allele. Alternatively, it is also possible that immune cell trafficking and/or signalling might be affected by CCR2A stabilization, leading to a delay in HIV-1 diseases.

Previously, Mellado *et al.* reported that CXCR4 could dimerize with CCR2B-64I variants but not with wild-type CCR2B-64V upon stimulation with SDF-1 and MCP-1. Based on this finding, they proposed that this ability of CCR2B-64I to heterodimerize with CXCR4 may cause a delay in AIDS progression [20]. However, several independent cohort studies have shown that the effects of the *CCR2-64I* allele were more pronounced in earlier stages of disease than in latter stages [5,8,21]. In a Dutch cohort, delay in HIV-1 disease progression was more pronounced before the emergence of X4 variants and was not observed after the emergence of X4 variants in individuals with the CCR2-64I allele [6]. Therefore, it is unlikely that CCR2B-64I/CXCR4 heterodimerization is the main cause of delay in AIDS progression in individuals with CCR2-64I.

Previous studies exploring the oligomerization of chemokine receptors also yielded controversial results. Rodrigues-Frade *et al.* reported that CCR2B forms homodimers upon stimulation by MCP-1 [22]. Other studies, however, have shown that CCR5 [23,24] and CXCR4 [25] can form homodimers without any stimulation by their ligands. Although we did not test whether or not stimulation with MCP-1 and/or RANTES increases hetero-oligomer formation between CCR2A and CCR5, our present results support the latter model that chemokine receptors may form oligomers without stimulation by their ligands.

In addition to AIDS pathogenesis, the CCR2-64I allele was reported to be associated with lower risks of coronary artery calcification [26] and acute rejection in renal transplantation [27]. Our present results shed light onto possible mechanisms of the association of this allele with such diverse human phenotypes. It is now widely accepted that monocyte attachment to cardiovascular wall is the first event implicated in atherogenesis of coronary arteries [28,29]. Since monocytes are known to express both CCR2A and CCR2B [13], an increased stability of CCR2A resulting from the 64I substitution may interfere with the function of CCR2B in monocytes, leading to decreased monocyte invasion to cardiovascular walls. With respect to acute rejection in renal transplantation, CCR5 is known to play an important role in both rejection of renal transplantation [30] and experimental graft-versus-host disease models [31]. Therefore, it is possible that an increased ability of CCR2A-64I to interfere with CCR5 expression can cause a decreased frequency of acute rejection after renal transplantation in recipients with this allele.

Previous studies have failed to show a statistically significant difference in levels of CCR5 expression on stimulated or non-stimulated peripheral blood mononuclear cells between CCR2-64I homozygotes and CCR2-64V homozygotes [9,10,32], although a slight reduction was noted in CCR2-64I homozygotes. In fact, we also failed to observe a statistically significant reduction of CCR5 levels on peripheral CD4 cells of homozygotes of CCR2-64I (data not shown). CCR2 is reported to be expressed on monocytes/macrophages [33], basophils [34,35], B cells [36], NK cells [37], dendritic cells [38,39], and a limited population of T cells [40]. Although we observed very few CCR2 cells in peripheral blood mononuclear cells, Bartoli *et al.* reported that numerous mononuclear cells in tonsil expressed CCR2A [41]. It may be possible that specific cell types expressing both CCR2A and CCR5 in tonsil or lymph nodes play an important role in AIDS pathogenesis and are responsible for the delay in HIV-1 diseases observed in patients with CCR2-64I.

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Original article

Functional analysis of HIV-1 *vif* genes derived from Japanese long-term nonprogressors and progressors for AIDS

Akiko Sakurai [a], Abhay Jere [a], Akiko Yoshida [a], Takeshi Yamada [b], Aikichi Iwamoto [c], Akio Adachi [a], Mikako Fujita [a,*]

[a] *Department of Virology, The University of Tokushima Graduate School of Medicine, 3-18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan*
[b] *Department of Microbiology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan*
[c] *Department of Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan*

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Abstract

We analyzed the function of human immunodeficiency virus type 1 (HIV-1) *vif* gene from Japanese long-term nonprogressors (LTNPRs) and progressors (PRs) for acquired immunodeficiency syndrome (AIDS). We constructed a basic HIV-1 infectious clone, which facilitated the incorporation and evaluation of *vif* from infected individuals. Proviral reporter clones carrying *vif* from six Japanese LTNPRs and seven PRs were then generated and their in vitro growth kinetics were analyzed. The *vif* clones, which could confer infectivity on reporter viruses, were considered active, and the ratio of the active clones to the number of clones examined per individual was determined. For the majority of LTNPRs, there was no correlation between presence or absence of functional *vif* with long-term nonprogression for AIDS. There was one exception in which all the clones examined had inactive *vif*, suggesting a probable association of inactive *vif* with the nonprogression. All PRs with high viral load had a high ratio of active *vif* clones. Our results suggest that the presence of functional *vif* would influence HIV-1 infectivity and disease progression in infected individuals.

Keywords: HIV-1; AIDS; *Vif*; Long-term nonprogressors

1. Introduction

The time between developing acquired immunodeficiency syndrome (AIDS) and first exposure to human immunodeficiency virus type 1 (HIV-1) varies among individuals. Some individuals, termed long-term nonprogressors (LTNPRs), remain free from AIDS related complex (ARC) or AIDS for more than 10 years after infection without any antiretroviral therapy. Viral factors, host factors or both may contribute to this long-term nonprogression for AIDS [1]. HIV-1 *nef* gene has been suggested as one of the viral factors associated with the status of disease progression, as many LTNPRs specifically had gross deletions in *nef* [2–6]. Recently we reported by molecular virological analysis that *nef* is important for AIDS development [7]. In that study, we examined various activities of HIV-1 *nef*; (1) enhancement of viral infectivity, (2) down-regulation of CD4, (3) down-regulation of MHC-I, (4) binding ability with Hck [7]. The results obtained have demonstrated that augmentation of viral infectivity by *nef* is critically associated with AIDS [7].

Earlier studies on simian immunodeficiency virus (SIV) also show the importance of *nef* for simian AIDS [8,9]. In those reports, another accessory gene, *vif*, was found to be responsible for AIDS [8,10]. As for the potential role of HIV-1 *vif* for AIDS induction, few studies have been reported so far [11,12]. In the tissue culture system, Vif is known to confer infectivity on progeny virions in a producer cell-dependent manner, and producer cells are, therefore, divided into permissive and nonpermissive [13–16]. Viruses lacking *vif* fail to replicate in nonpermissive cells such as peripheral blood mononuclear cells (PBMCs), macrophages, and H9 cells. We have reported that various mutant *vif* clones show distinct growth phenotypes in H9 and semi-permissive A3.01

* Corresponding author. Tel.: +81-88-633-9232; fax: +81-88-633-7080.
E-mail address: mfujita@basic.med.tokushima-u.ac.jp (M. Fujita).

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cells [17,18]. Hassaine et al. [11] examined the sequence of *vif* from LTNPRs and found that the amino acid at position 132 of Vif is associated with the viral load in LTNPRs. A recent report by Alexander et al. [12] described that a two-amino-acid insertion in Vif may contribute to the nonprogression of mother and child for AIDS. In this study, proviral reporter clones carrying *vif* from six LTNPRs or seven progressors (PRs) were generated, and their growth kinetics in nonpermissive and semi-permissive cells were examined. The results described here suggest that HIV-1 *vif* is important for the induction of AIDS.

2. Materials and methods

2.1. Study subjects

Samples from six Japanese LTNPRs (p1–p6) and seven PRs (p12–p18) were examined in the present study. The details of all study subjects have been described previously [19,20].

2.2. Cells

Lymphocytic cell lines H9 (ATCC HTB-176) and A3.01 [21] were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum as previously described [21,23]. A monolayer cell line 293T [22] was maintained in Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum as previously described [23].

2.3. Transfection and reverse transcription (RT) assay

For transfection of uncleaved plasmid DNA into lymphocytic H9 and A3.01 cells, the electroporation method was used as previously described [23]. For transfection of adherent 293T cells, the calcium–phosphate coprecipitation technique was used [23]. After electroporation, virus production in the culture supernatants was monitored at intervals by virion-associated reverse transcriptase (RT) activity as previously described [24].

2.4. Western immunoblotting

Cell lysates were prepared as described before [24], and proteins were resolved on sodium dodecyl sulfate–12.5% polyacrylamide gels, followed by electrophoretic transfer to polyvinylidene fluoride membranes (Immobilon-P, Millipore Co., Bedford, MA, USA). The membranes were treated with anti-Vif (NIH AIDS Research and References Reagent Program, catalog no. 2746) and anti-p24 [25] antibodies and visualized using an ECL plus Western blotting detection system (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).

2.5. Construction of basic and pilot clones

The wild-type (wt) infectious molecular clone of HIV-1, designated pNL432 (GenBank Accession no. AF324493), has been described previously [23]. For construction of basic clone pNL-SX, shown in Fig. 1, an appropriate DNA fragment of pNL432 was subcloned into pBluescipt SK(+) (Stratagene, La Jolla, CA, USA), and the mutations were introduced using QuikChange site-directed mutagenesis kit (Stratagene) as follows. *Sma*I and *Xba*I sites were generated at 5′ and 3′ ends of *vif*, respectively. Four methionine codons were changed to one valine and three threonine codons so as not to act as initiation codon. The mutated DNA fragment was cloned back into pNL432 to construct pNL-SX. To construct pilot clones carrying NLVif, NLΔ19Vif and NLΔVif, shown in Fig. 1 (pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif, respectively), wt and mutant *vif* sequences were amplified by polymerase chain reaction (PCR) with *Sma*I at the 5′ and *Xba*I at the 3′ ends. As templates, pNL432 was used for NLVif and NLΔ19Vif, and its frame-shift mutant pNL-Nd [26,27] was used for NLΔVif. Two oligonucleotide primer pairs used were as follows: 5′-TCCCCCGGGATGGAAAACAGATGGCAGGT-3′ (sense) and 5′-GCTCTAGACTAGTGTCCATTCATTGTATG-3′ (antisense) for NLVif and NLΔVif, 5′-TCCCCCGGGATGGAAAACAGATGGCAGGT-3′ (sense) and 5′-GCTCTAGACTATCTGTCCTCTGTCAGTTTC-3′ (antisense) for NLΔ19Vif. The PCR conditions were as follows: 94 °C for 45 s, 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and finally 72 °C for 10 min. The PCR-amplified DNA fragments were inserted into pNL-SX to construct various pilot clones pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif.

2.6. Proviral reporter constructs

Template DNAs from individuals for *vif* amplification in this study have been previously described [20]. Oligonucleotide primers and cycling conditions are essentially the same as described above for NLVif. The amplified DNA fragments were inserted into pNL-SX to obtain various reporter constructs.

3. Results and discussion

3.1. Establishment of an assay system for evaluating vif function

In this study, we analyzed the function of *vif* from individuals infected with HIV-1. As the ability to confer infectivity on virions is the most important function assigned to *vif* so far [13–16], we established a virological assay system based on a molecular clone modified from pNL432 [23]. As shown in Fig. 1A, we constructed a basic clone, designated pNL-SX, carrying *Sma*I and *Xba*I sites downstream of *pol* and upstream of *vpr*. We then amplified, by PCR, the wt *vif* sequence of pNL432 (NLVif), its shorter version of *vif* lacking 3′-57 bp (NLΔ19Vif), and its frame-shift *vif* mutant, which does not express most amino acid sequences of Vif

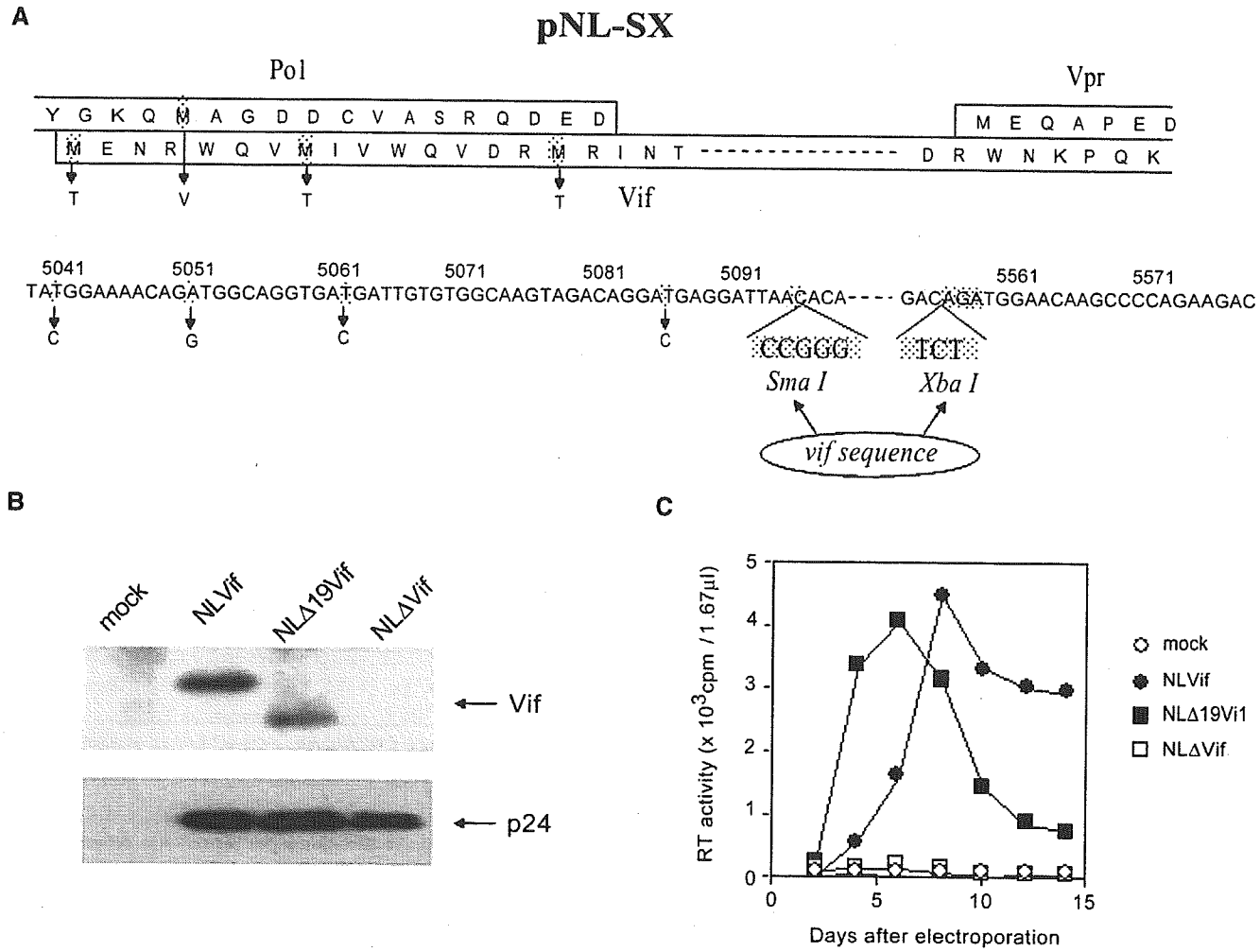

Fig. 1. Construction and characterization of the clones to determine activity of *vif* genes from individuals infected with HIV-1. (A) Schematic representation of the pNL-SX clone used in this study. To construct clone pNL-SX, four methionine (in *pol* and *vif* genes) codons of pNL432 (GenBank Accession no. AF324493) [23] were changed to one valine and three threonine codons so as not to act as initiation codon, and *Sma*I and *Xba*I sites were introduced as indicated. Various *vif* sequences with *Sma*I at 5′ and *Xba*I at 3′ ends were amplified by PCR using pNL432, its *vif* mutants, and DNAs from the individuals as templates. The amplified DNAs were inserted into pNL-SX to generate pilot and reporter clones. Amino acid (Pol, Vif and Vpr) and nucleotide sequences of pNL432 (nucleotide nos. 5040–5579) are shown. (B) Monitoring of Vif expression by Western blotting. 293T cells were transfected with the pilot proviral clones, as previously described [23], and cell lysates were prepared on day 2. Cell lysates were analyzed for the expression of Vif and Gag-p24 proteins by Western immunoblotting using appropriate antibodies. The three sequences inserted into pNL-SX were indicated at the top. NLVif, full-length *vif* sequences of pNL432 [23]; NLΔ19Vif, mutant *vif* sequences of NL432 lacking C-terminal 19 amino acids [28]; NLΔVif, mutant *vif* sequences of pNL432 carrying a frame-shift mutation at the *Nde*I site, which can encode only 28 amino acids of Vif sequence [26]. Mock, pUC19. (C) Growth kinetics in H9 cells of the pilot proviral clones. Cells were electroporated with 10 μg of the proviral clones [23], and RT production in the culture supernatants was determined, as previously described [24]. The same clones were used in (B) and (C).

(NLΔVif), and inserted these sequences into pNL-SX to construct pilot proviral clones pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif. We and others have already reported that the C-terminal 19 amino acids of Vif (174–192) are dispensable without altering its functionality [28,29]. To confirm that pNL-SX is valid for virological assessment, the ability of these pilot clones to express Vif and to confer infectivity on virions was determined. These clones were transfected into 293T cells, and the expression of wt (NLVif) and the short version (NLΔ19Vif) of Vif was monitored by Western blotting. As can be clearly seen in Fig. 1B, wt and short Vif were readily detected. These clones were then electroporated into nonpermissive H9 cells, and RT production in the culture supernatants was monitored to determine their infectivity. As shown in Fig. 1C, viruses carrying wt (NL-SX/NLVif) and short type *vif* (NL-SX/NLΔ19Vif) could efficiently replicate in H9 cells, as expected, in contrast to the virus lacking functional *vif* (NL-SX/NLΔVif). These results indicated that pNL-SX can be used for evaluating the functional ability of *vif* from individuals infected with HIV-1.

3.2. Analysis of vif activity from LTNPRs and PRs

The pNL-SX system described above was used to evaluate the functionality of *vif* derived from six Japanese LTNPRs, who were free from ARC or AIDS for more than 10 years after infection, and from seven PRs [19,20]. *Vif* sequences in

Table 1
Activity of HIV-1 *nef* and *vif* genes derived from LTNPRs and PRs of AIDS

Individual [a]	*nef* [b]	*vif*		
	MAGI infectivity	Clone [c]	Growth [d] in	
			H9	A3.01
p1 LTNPR (<1.0)	m,m	10	–	–
		12-a	+	+
		12-b	+	+
		12-c	+	+
		12-d	+	+
		13	+	+
		14	+	+
			0.86	0.86
p2 LTNPR (<1.0)	nd	1	+	+
		4	+	+
		5-a	+	+
		5-b	+	+
			1.00	1.00
p3 LTNPR (<1.0)	m,–	1	–	–
		3	+*	+*
		4	–	–
		5	+	+
		6	+	+
			0.60	0.60
p4 LTNPR (<1.0)	m,m	1	–	–
		2	–	–
		3	+	+
		4	+	+
		5	+	+*
			0.60	0.60
p5 LTNPR (<1.0)	nd	1	–	–
		3	–	–
		4	–	–
		9	–	–
			0.00	0.00
p6 LTNPR (<1.0)	m,m	c-a	–	–
		c-b	–	–
		g	+	+
		h	+	+
		i	–	–
			0.40	0.40
p12 PR (2.2)	nd	7	+	+
		8-a	–	–
		8-b	–	–
		9	–	–
		10	–	–
			0.20	0.20
p13 PR (2.4)	+	1	+	+
		2-a	+	+
		2-b	+	+
		7	+	+
			1.00	1.00
p14 PR (20)	+,+	3	–	+*
		5	+	+*
		6	+	+
		7	–	–
			0.50	0.75
p15 PR (88)	+,+	1	+	+
		2	+	+
		4	+	+
		5	–	+
		6	+	+
			0.80	1.00
p16 PR (29)	+,+	1	+	+
		2	+	+
		3	+	+
		4	+	+
		5	+	+
			1.00	1.00
p17 PR (21)	+	1	+	+
		3	+	+
		4	+	+
		5	+	+
			1.00	1.00
p18 PR (3.8)	+	1	+	+
		2	+	+
		4	+	+
			1.00	1.00

[a] Identification numbers of individuals, stage of disease progression, and viral RNA level in the plasma ($\times 10^4$ copies per ml), in parentheses, are indicated [19].

[b] Infectivity of the clones based on NL432 carrying *nef* sequences derived from the individuals (one or two clones per person) was determined by MAGI assay [7]. Infectivity of the clones relative to that of the clone carrying NL432 *nef* is shown. +, >60%; –, <30%; m, 30–60%; nd, not done.

[c] (The same number or the same alphabet)-(alphabet) indicates that the clones carry the same *vif* sequences. For example, three and four different *vif* sequences have been identified in p2 and p6, respectively.

[d] Growth ability in H9 and A3.01 cells of viral clones derived from pNL-SX (Fig. 1A) carrying *vif* genes from the individuals. Growth phenotype of the clones similar to that of NL-SX/NLVif and that of NL-SX/NLΔVif (Fig. 2A) is indicated by + and –, respectively. Growth phenotype of the viruses which grew more poorly than NL-SX/NLVif but better than NL-SX/NLΔVif is indicated by +* (Fig. 2A). The ratio of the number of clones with active (+ and +*) *vif* to that of clones examined is indicated on the last line.

these individuals were amplified by PCR and inserted into pNL-SX to construct 53 reporter clones (Table 1). Nonpermissive H9 and semi-permissive A3.01 cells were used to examine the growth ability of all clones generated, as mutant *vif* clones show distinct growth phenotypes in these two cell lines [17,18]. Representative growth kinetics of the clones are shown in Fig. 2A. In these experiments, clones carrying wt (NLVif) and Δ*vif* (NLΔVif) served as positive and negative controls, respectively. Based on the growth phenotypes, various *vif* sequences were considered to be active or inactive. Clones p1-12, p3-3 and p13-1 were thought to be active, while clones p1-10, p3-4, p14-3 and p14-7 were inactive as judged by virus replication in H9 cells (Fig. 2A). In A3.01 cells, clones p1-12, p3-3, p13-1 and p14-3 were active, while p1-10, p3-4, and p14-7 were inactive (Fig. 2A). The functionality of all *vif* sequences from LTNPRs and PRs is summarized in Table 1. Two clones, p14-3 and p15-5, showed