

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 *NotI* 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, Rocky 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- μ 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 EXPRE³⁵S³⁵S^[35S] 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 β -galactosidase gene as a reporter was performed as described previously [19]. Briefly, mouse fibroblast L cells were transfected with β -galactosidase reporter plasmid pGINT7 β -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-1 cells were mixed, incubated for 3 h, and β -galactosidase activities in the cell lysate were measured by using chlorophenol red- β -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, Gaithersburg, Maryland, USA) or anti-rat IgG (American Qualex, San Clemente, California, USA) and developed using the Immuno-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

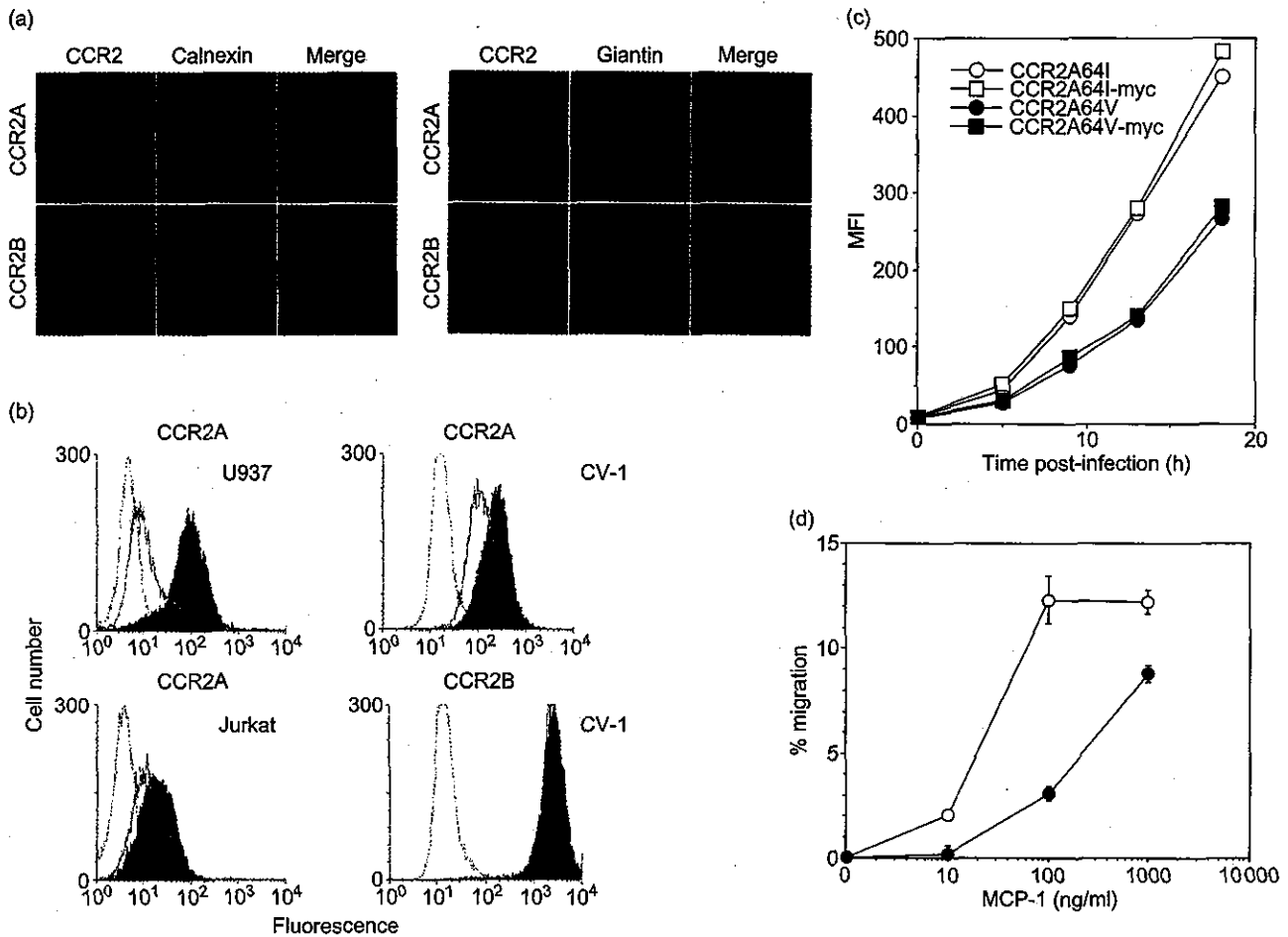


Fig. 2. (a) Subcellular distribution of CCR2A-64V and CCR2B-64V in CV1 cells. SeV vector (SeV) was used to express the CCR2A-64V and CCR2B-64V molecules. Cells were fixed and permeabilized before staining with MAB150 anti-CCR2 mouse MAb followed by FITC-labelled anti-mouse IgG. Cells were then re-stained 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

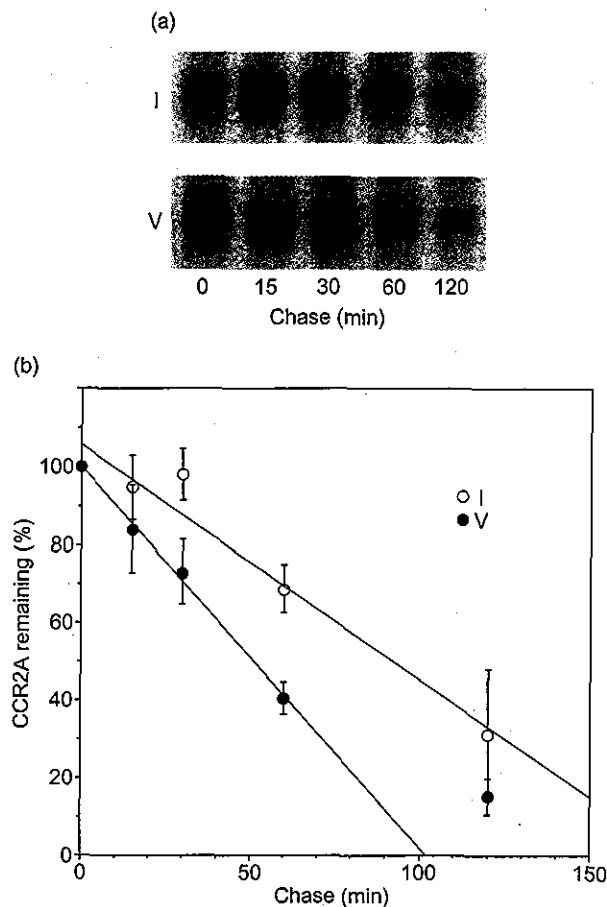


Fig. 3. CCR2A-64I is more stable than CCR2A-64V. CV1 cells were infected with SeV expressing CCR2A-64V-myc and CCR2A-64I-myc for 9 h. Cells were labelled for 30 min and then harvested following the chase time indicated. (a) Representative gels of pulse-chase analysis. (b) Phosphorimager analysis of the 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 incu-

bated 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-

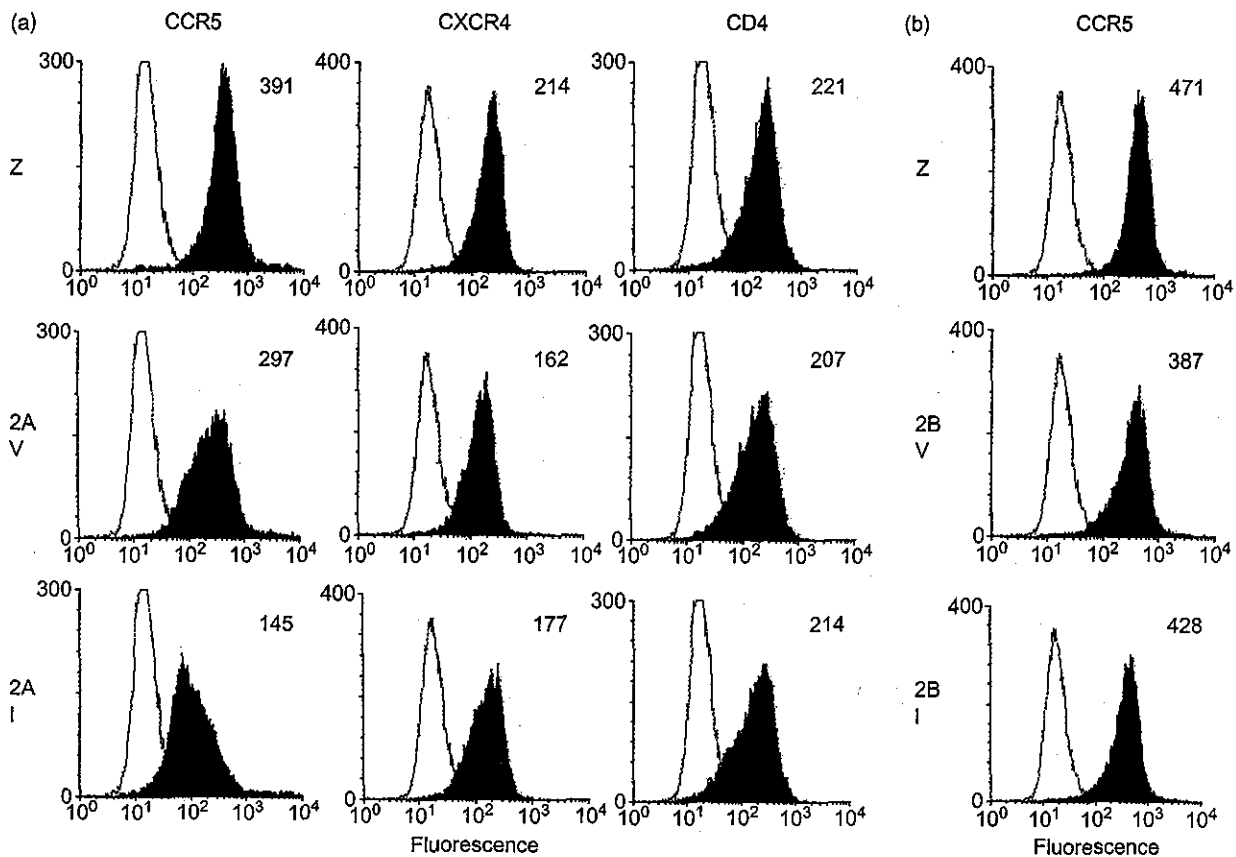


Fig. 4. (a) Effect of CCR2A-64V and CCR2A-64I on HIV-1 coreceptor expression. Vac vectors were used to express CCR5, CXCR4 and CD4 in the CV1 cells inoculated with SeV expressing CCR2A-64V or CCR2A-64I. Z denotes the wild-type SeV. Five hours after infection, cells were stained with MAb against CCR5, 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 homooligomers. 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 [³⁵S]-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

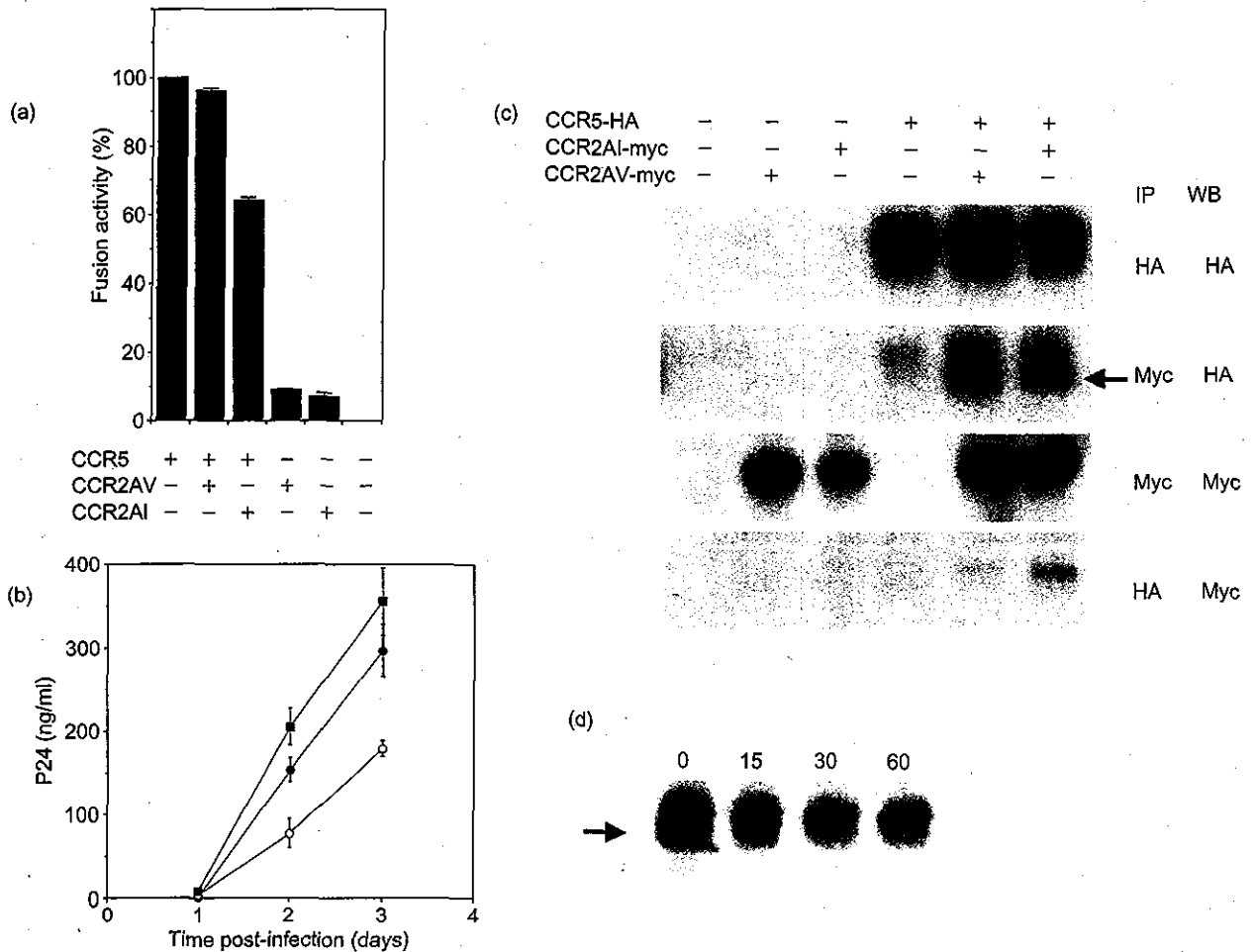


Fig. 5. (a) Coreceptor activity of CCR5 in CCR2A-64V or CCR2A-64I co-expressed cells. SeV vector was used to express CCR2A-64V or CCR2A-64I, and Vac vector was used to express CCR5 as described in Fig. 4. HIV-1 coreceptor activity of each sample was measured using the method described in Materials and 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-immunoprecipitation 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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References

1. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, *et al.* A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 1996, 85:1149-1158.
2. Rucker J, Edinger AL, Sharron M, Samson M, Lee B, Berson JF, *et al.* Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J Virol* 1997, 71:8999-9007.
3. Penton-Rol G, Cota M, Polentarutti N, Luini W, Bernasconi S, Borsatti A, *et al.* Up-regulation of CCR2 chemokine receptor expression and increased susceptibility to the multitropic HIV strain 89.6 in monocytes exposed to glucocorticoid hormones. *J Immunol* 1999, 163:3524-3529.
4. Smith MW, Dean M, Carrington M, Winkler C, Huttley GA, Lomb DA, *et al.* Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study. *Science* 1997, 277:959-965.
5. Kostrikis LG, Huang Y, Moore JP, Wolinsky SM, Zhang L, Guo Y, *et al.* A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation. *Nat Med* 1998, 4:350-353.
6. van Rij RP, de Roda Husman AM, Brouwer M, Goudsmit J, Coutinho RA, Schuitemaker H. Role of CCR2 genotype in the clinical course of syncytium-inducing (SI) or non-SI human immunodeficiency virus type 1 infection and in the time to

- conversion to SI virus variants. *J Infect Dis* 1998, 178: 1806–1811.
7. Ioannidis JP, Rosenberg PS, Goedert JJ, Ashton LJ, Benfield TL, Buchbinder SP, et al. Effects of CCR5-Delta32, CCR2-64I, and SDF-1 3'A alleles on HIV-1 disease progression: An international meta-analysis of individual-patient data. *Ann Intern Med* 2001, 135:782–795.
 8. Mulherin SA, O'Brien TR, Ioannidis JP, Goedert JJ, Buchbinder SP, Coutinho RA, et al. Effects of CCR5-Delta32 and CCR2-64I alleles on HIV-1 disease progression: the protection varies with duration of infection. *AIDS* 2003, 17:377–387.
 9. Lee B, Doranz BJ, Rana S, Yi Y, Mellado M, Frade JM, et al. Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on chemokine receptor function of CCR2b, CCR3, CCR5, and CXCR4. *J Virol* 1998, 72:7450–7458.
 10. Mariani R, Wong S, Mulder LC, Wilkinson DA, Reinhart AL, LaRosa G, et al. CCR2-64I polymorphism is not associated with altered CCR5 expression or coreceptor function. *J Virol* 1999, 73:2450–2459.
 11. Mummidi S, Ahuja SS, Gonzalez E, Anderson SA, Santiago EN, Stephan KT, et al. Genealogy of the CCR5 locus and chemokine system gene variants associated with altered rates of HIV-1 disease progression. *Nat Med* 1998, 4: 786–793.
 12. Charo IF, Myers SJ, Herman A, Franci C, Connolly AJ, Coughlin SR. Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci USA* 1994, 91:2752–2756.
 13. Wong LM, Myers SJ, Tsou CL, Gosling J, Arai H, Charo IF. Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene. Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *J Biol Chem* 1997, 272:1038–1045.
 14. Sanders SK, Crean SM, Boxer PA, Kefner D, LaRosa GJ, Hunt SW, 3rd. Functional differences between monocyte chemotactic protein-1 receptor A and monocyte chemotactic protein-1 receptor B expressed in a Jurkat T cell. *J Immunol* 2000, 165:4877–4883.
 15. Louisirrotchanakul S, Liu H, Roongpisuthipong A, Nakayama EE, Takebe Y, Shioda T, et al. Genetic analysis of HIV-1 discordant couples in Thailand: association of CCR2 64I homozygosity with HIV-1-negative status. *J Acquir Immune Defic Syndr* 2002, 29:314–315.
 16. Kato A, Sakai Y, Shioda T, Kondo T, Nakanishi M, Nagai Y. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* 1996, 1:569–579.
 17. Shioda T, Nakayama EE, Tanaka Y, Xin X, Liu H, Kawana-Tachikawa A, et al. Naturally occurring deletion mutation in the C-terminal cytoplasmic tail of CCR5 affects surface trafficking of CCR5. *J Virol* 2001, 75:3462–3468.
 18. Shioda T, Kato H, Ohnishi Y, Tashiro K, Ikegawa M, Nakayama EE, et al. Anti-HIV-1 and chemotactic activities of human stromal cell-derived factor 1alpha (SDF-1alpha) and SDF-1beta are abolished by CD26/dipeptidyl peptidase IV-mediated cleavage. *Proc Natl Acad Sci USA* 1998, 95:6331–6336.
 19. Nakayama EE, Shioda T, Tatsumi M, Xin X, Yu D, Ohgimoto S, et al. Importance of the N-glycan in the V3 loop of HIV-1 envelope protein for CXCR4- but not CCR5-dependent fusion. *FEBS Lett* 1998, 426:367–372.
 20. Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Martinez AC. Chemokine control of HIV-1 infection. *Nature* 1999, 400:723–724.
 21. Michael NL, Louie LG, Rohrbaugh AL, Schultz KA, Dayhoff DE, Wang CE, et al. The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression. *Nat Med* 1997, 3:1160–1162.
 22. Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Albar JP, Martinez AC, Mellado M. The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc Natl Acad Sci USA* 1999, 96: 3628–3633.
 23. Benkirane M, Jin DY, Chun RF, Koup RA, Jeang KT. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem* 1997, 272:30603–30606.
 24. Issafras H, Angers S, Bulenger S, Blanpain C, Parmentier M, Labbe-Julie C, et al. Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors. *J Biol Chem* 2002, 277: 34666–34673.
 25. Babcock GJ, Farzan M, Sodroski J. Ligand-independent dimerization of CXCR4, a principal HIV-1 coreceptor. *J Biol Chem* 2003, 278:3378–3385.
 26. Valdes AM, Wolfe ML, O'Brien EJ, Spurr NK, Gelfer W, Rut A, et al. Val64Ile polymorphism in the C-C chemokine receptor 2 is associated with reduced coronary artery calcification. *Arterioscler Thromb Vasc Biol* 2002, 22:1924–1928.
 27. Abdi R, Tran TB, Sahagun-Ruiz A, Murphy PM, Brenner BM, Milford EL, et al. Chemokine receptor polymorphism and risk of acute rejection in human renal transplantation. *J Am Soc Nephrol* 2002, 13:754–758.
 28. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993, 362:801–809.
 29. Hanke H, Lenz C, Finking G. The discovery of the pathophysiological aspects of atherosclerosis—a review. *Acta Chir Belg* 2001, 101:162–169.
 30. Segerer S, Cui Y, Eitner F, Goodpaster T, Hudkins KL, Mack M, et al. Expression of chemokines and chemokine receptors during human renal transplant rejection. *Am J Kidney Dis* 2001, 37:518–531.
 31. Murai M, Yoneyama H, Harada A, Yi Z, Vestergaard C, Guo B, et al. Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. *J Clin Invest* 1999, 104:49–57.
 32. Shieh B, Liau YE, Hsieh PS, Yan YP, Wang ST, Li C. Influence of nucleotide polymorphisms in the CCR2 gene and the CCR5 promoter on the expression of cell surface CCR5 and CXCR4. *Int Immunol* 2000, 12:1311–1318.
 33. Fantuzzi L, Borghi P, Ciolli V, Pavlakis G, Belardelli F, Gessani S. Loss of CCR2 expression and functional response to monocyte chemoattractant protein (MCP-1) during the differentiation of human monocytes: role of secreted MCP-1 in the regulation of the chemotactic response. *Blood* 1999, 94:875–883.
 34. Ochensberger B, Tassera L, Bifare D, Rihs S, Dahinden CA. Regulation of cytokine expression and leukotriene formation in human basophils by growth factors, chemokines and chemotactic agonists. *Eur J Immunol* 1999, 29:11–22.
 35. Ikura M, Miyamasu M, Yamaguchi M, Kawasaki H, Matsushima K, Kitaura M, et al. Chemokine receptors in human basophils: inducible expression of functional CXCR4. *J Leukoc Biol* 2001, 70:113–120.
 36. Frade JM, Mellado M, del Real G, Gutierrez-Ramos JC, Lind P, Martinez AC. Characterization of the CCR2 chemokine receptor: functional CCR2 receptor expression in B cells. *J Immunol* 1997, 159:5576–5584.
 37. Polentarutti N, Allavena P, Bianchi G, Giardina G, Basile A, Sozzani S, et al. IL-2-regulated expression of the monocyte chemoattractant protein-1 receptor (CCR2) in human NK cells: characterization of a predominant 3.4-kilobase transcript containing CCR2B and CCR2A sequences. *J Immunol* 1997, 158:2689–2694.
 38. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, et al. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 1998, 28:2760–2769.
 39. Vanbervliet B, Homey B, Durand I, Massacrier C, Ait-Yahia S, de Bouteiller O, et al. Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. *Eur J Immunol* 2002, 32:231–242.
 40. Rabin RL, Park MK, Liao F, Swofford R, Stephany D, Farber JM. Chemokine receptor responses on T cells are achieved through regulation of both receptor expression and signaling. *J Immunol* 1999, 162:3840–3850.
 41. Bartoli C, Civatte M, Pellissier JF, Figarella-Branger D. CCR2A and CCR2B, the two isoforms of the monocyte chemoattractant protein-1 receptor are up-regulated and expressed by different cell subsets in idiopathic inflammatory myopathies. *Acta Neuropathol (Berl)* 2001, 102:385–392.

A specific region of 37 amino acid residues in the SPRY (B30.2) domain of African green monkey TRIM5 α determines species-specific restriction of SIVmac infection.

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Abstract

Human immunodeficiency virus type 1 (HIV-1) efficiently enters cells of Old World monkeys but encounters a block before reverse transcription. This restriction is mediated by a dominant repressive factor. Recently, a member of the tripartite motif (TRIM) family proteins, TRIM5 α , was identified as a blocking factor in a rhesus macaque cDNA library. Among Old World monkey cell lines, an African green monkey kidney cell line CV1 is highly resistant to not only HIV-1 but also simian immunodeficiency virus SIVmac infection. We analyzed TRIM5 α of CV1 cells and HSC-F cells, a T cell line from a cynomolgus monkey, and found that both CV1- and HSC-F-TRIM5 α s could inhibit CD4-dependent HIV-1 infection as well as vesicular stomatitis virus glycoprotein-mediated infection. CV1-TRIM5 α could also inhibit SIVmac infection, whereas HSC-F-TRIM5 α could not. In the SPRY (B30.2) domain of CV1-TRIM5 α , there was a 20-amino acid duplication that was not present in HSC-F-TRIM5 α . A chimeric TRIM5 α containing 37 amino acid residues from CV1-TRIM5 α , which spanned the 20-amino acid duplication, in the background of HSC-F-TRIM5 α gained the ability to inhibit SIVmac infection. Conversely, the reciprocal chimera containing 17 amino acid residues from HSC-F-TRIM5 α in the background of CV1-TRIM5 α lost the ability to restrict SIVmac infection. These findings clearly indicated that a specific region of 37 amino acid residues in the SPRY domain of CV1-TRIM5 α contained a determinant of species-specific restriction of SIVmac.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is thought to have been introduced into the human population from chimpanzees (9) and shows a very narrow host range limited only to humans and chimpanzees. HIV-1 does not experimentally infect Old World monkeys, such as rhesus and cynomolgus monkeys, and fails to replicate in activated CD4 positive T lymphocytes obtained from those monkeys (13, 31). In contrast, simian immunodeficiency virus isolated from a macaque monkey (SIVmac) can replicate well in rhesus (13, 31) and cynomolgus monkeys (2, 3). The restricted host range of HIV-1 has greatly hampered its use in animal experiments and, hence, caused difficulty in developing prophylactic vaccines against HIV-1 infection.

Several studies have suggested that the block of HIV-1 replication in Old World monkey cells occurred at a post-entry step (7, 13, 31) and appeared to result from a failure to initiate reverse transcription (13). The block was still observed when CD4-negative monkey cells were infected with HIV-1 pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) and was overridden by high multiplicity of infection with VSV-G-pseudotyped virus or virus-like particles lacking genomic RNA (5, 10, 16, 19). Importantly, resistance against HIV-1 infection was shown to be dominant in heterokaryons between human and Old World monkey cells, suggesting the presence of inhibitory factor(s) against HIV-1 infection in Old World monkey cells (19). Studies on chimera of HIV-1 and SIVmac have suggested that restriction determinants lie within the HIV-1 P24 capsid protein (CA) (11, 23, 24, 29).

Recently, the screening of a rhesus monkey cDNA library identified tripartite motif 5 α (TRIM5 α), a component of cytoplasmic bodies, as a factor that confers resistance to HIV-1 infection (33). Shortly after, TRIM5 α of African green monkey, another Old

World monkey, was also shown to restrict HIV-1 infection, while human TRIM5 α was reported to restrict N-tropic murine leukemia virus (12, 14, 25, 35).

An African green monkey kidney cell line, CV1, was shown to be highly resistant to SIVmac as well as HIV-1 infection. We analyzed TRIM5 α of CV1 cells and HSC-F cells, a T cell line from a cynomolgus monkey, and report here that the ability of CV1 TRIM5 α to suppress SIVmac infection was determined by a small region composed of 37 amino acid residues in the SPRY (B30.2) domain of CV1 TRIM5 α .

Materials and Methods

Cloning and expression of TRIM5 α .

TRIM5 α cDNA was amplified by RT-PCR from the human T cell line MT4, cynomolgus monkey T cell line HSC-F (2, 3) and African green monkey cell lines CV1 and Vero using 5'-GCGGCCGCTACTATGGCTTCTGG-3' as a forward primer and 5'-GAATTCTCAAGAGCTTGGTGA-3' as a reverse primer. Amplified products were then cloned into the vector pCR-2.1TOPO (Invitrogen) and the nucleotide sequence of 10 clones for each TRIM5 α was determined.

The entire coding regions of selected clones were transferred to pcDNA3.1 (Invitrogen) using NotI and EcoRI sites, which were introduced by primers used in the PCR step. Hamster TK-ts13 cells (4) were transfected with pcDNA3.1 carrying TRIM5 α cDNA and cultured in the presence of 0.75mg/ml G418 (Gibco) for 14 days. The colonies obtained were examined for the expression of TRIM5 α using the TaqMan PCR method according to the manufacturer's instructions (Applied Biosystems). Sequences of the probe and primers used to specifically detect each TRIM5 α were as follows. MT4-TRIM5, forward primer: 5'-AACCTGGAGAAGGAGGAGGAAGAC-3', reverse primer: 5'-CTGGGTCTGCTGCACCAT-3' and probe: 5'-FAM-TCAGTTTCAGAGTTCG-TAMRA-3'. HSC-F-TRIM5, forward primer: 5'-AACCTGGAGAAGGAGAAAGAAGAC-3', reverse primer: 5'-CTGGGTCTGCTGCACCAT-3' and probe: 5'-FAM-TTCGTTTCAGACTTTG-TAMRA-3'. CV1-TRIM5, forward primer: 5'-AACCTGGAGAAGGAGGAAGAAGA-3', reverse primer: 5'-CTGGGTCTGCTGCACCAT-3' and probe:

5'-FAM-TCCGTTTCAGACTTCG-TAMRA-3'. These primers amplify the coiled-coil region of TRIM5 genes. The parental TK-ts13 cells were totally negative for the primate TRIM5 α . Clones expressing each TRIM5 α at comparable levels (4~6 x 10⁷ copies/ μ g total RNA) were used for subsequent experiments.

For generating CV1-TRIM5 α and HSC-F-TRIM5 α cDNAs carrying a HA tag (YPYDVPDYAA) at their C-termini, cloned CV1-TRIM5 α and HSC-F-TRIM5 α cDNAs in pCDNA3.1 were used as templates for PCR-amplification with a primer containing a nucleotide sequence corresponding to the HA-tag fused with the C-terminal portion of TRIM5 α . The C-terminal portion of TRIM5 α fused with the HA-tag (Bam-HI to Not I) and the N-terminal portion of TRIM5 α (Not-I to Bam HI) were assembled on a pCEP4 vector (Invitrogen). The entire coding sequences were then transferred to the Not I site of pSeV18+b(+). Recombinant Sendai viruses (SeVs) carrying CV1-TRIM5 α -tag or HSC-F-TRIM5 α -tag were recovered according to a previously described method (32). The viruses passaged a second time in embryonated chicken eggs were used as stock for all experiments. The wild-type Z strain of SeV served as a control in all the experiments.

To express chimeric TRIM5 α , CV1-TRIM5 α -tag and HSC-F-TRIM5 α -tag cloned into pCEP4 were used. C143 cells were transfected with pCEP4 containing cDNA of CV1-TRIM5 α -tag, HSC-F-TRIM5 α -tag, CV1-60tag, or HSC-F+60tag and cells were cultured in the presence of 0.3mg/ml of hygromycin B (Gibco) for 14 days. The resultant hygromycin B-resistant cells were assayed for the expression of each TRIM5 α protein by immunoprecipitation using anti-HA high affinity antibody (Roche) as described previously (20).

Viruses and HIV-1 lentivirus vector.

Vesicular stomatitis virus glycoprotein (VSV-G)-pseudo typed HIV-1-NL43, SIVmac239, or HIV-2-GH123 was prepared by transfection of 293T cells with a combination of pMD.G (17, 18) and pNL432 (1), pBRmac239(15), or pGH123 (30), respectively. HIV-1 vector expressing green fluorescence protein (GFP) was prepared as described previously (17, 18). Two days after transfection, culture supernatants of 293T cells were collected and assayed for reverse transcriptase activity using RT assay colorimetric (Roche).

Viral infection

Assays for the HIV-1 vector expressing GFP were performed in 24-well plates containing 4×10^4 Tk-ts13-derived target cells. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were enumerated by flowcytometer (FACScan, Becton Dickinson) 40 h after infection. For VSV-psudotyped-HIV-1, SIVmac239, and HIV-2 infection assays in C143 cells, we inoculated viruses containing 1ng of reverse transcriptase. For productive infection assays, 2.5×10^5 MT4 cells were infected with SeV expressing CV1-TRIM5 α -tag, HSC-F-TRIM5 α -tag or the parental Z strain of SeV at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell and incubated at 37 °C for 9 hrs. Cells were then super-infected with 30 ng of p24 of an X4 HIV-1 strain, NL43, or 30 ng of p27 of SIVmac239. The culture supernatants were collected periodically and the level of p24 or p27 was measured.

Real-time PCR to specifically detect the human TRIM5- α isoform.

Total RNA was extracted from 1×10^6 of C143 cells, A172 cells, HeLa cells, MT4 cells, peripheral blood mononuclear cells (PBMC), CD14-positive monocytes, and phytohemagglutinin (PHA-P, $3 \mu\text{g}/\text{ml}$)-stimulated PBMCs. One microgram of the total RNA was used as a template for reverse transcription with High-Capacity cDNA Archive Kit (Applied Biosystems). TaqMan PCR to specifically measure TRIM5 α was performed using a primer/probes set which detects the boundary between exon 7 and 8 of the human TRIM5 α gene (Applied Biosystems, Assay ID:Hs01552552_g1).

Results

Variation in TRIM5 α .

We cloned TRIM5 α cDNA from the human T cell line MT4, cynomologus monkey T cell line HSC-F, and African green monkey kidney cell lines CV1 and Vero. A comparisons of the predicted amino acid sequences of TRIM5 α s is shown in Fig.1A and Fig.1B.

Human TRIM5 α from MT4 cells differed at an amino acid position 249 (G249D) from the previously published sequence (33) and was designated as MT4-TRIM5 α . The cynomologus monkey TRIM5 α from HSC-F (HSC-F-TRIM5 α) was 2 amino acids shorter than the rhesus monkey TRIM5 α (33) and 2 amino acids longer than the human TRIM5 α . All 10 clones derived from Vero cells showed the same sequence as the previously published one (35). On the other hand, we found at least 2 distinct TRIM5 α sequences in CV1 cells. The 2 major TRIM5 α sequences obtained from CV1 were designated CV1-TRIM5 α -type 1 and 2, and 5 amino acids were found to differ between the 2 sequences (I259V, L337S, R351L, G359R, and G438S). Among 10 cDNA clones obtained from CV1, there were 4 type 1 clones and 4 type 2 clones. The remaining 2 clones were most likely chimeric artifacts. Two recently published sequences of TRIM5 α from CV1 cells (AY593973 and AY625002) showed differences at 3 positions, L7V, I259V, and G438S, and have S, L, and R at positions 337, 351, and 359, respectively (14, 35), while both the type 1 and 2 clones had Leucine at the 7th position. All sequences obtained from Vero and CV1 contained a 20-amino acid duplication within the SPRY domain, which was not observed in human MT4 and cynomologus monkey HSC-F (Fig.1B).

The phylogenetic tree of various TRIM5 α sequences showed that cynomolgus and rhesus monkey TRIM5 α s are close to each other, consistent with the fact that those 2 monkeys belong to the genus *Macaca* (Fig. 1C).

African green monkey and cynomolgus monkey TRIM5 α inhibit HIV-1 infection in non-primate cells.

We first asked whether or not each TRIM5 α can inhibit HIV-1 infection in the context of non-primate cells, because human and primate cells express endogenous TRIM5 α that could complicate a functional analysis of TRIM5 α -mediated restriction. The hamster cell line TK-tsl3 was used, because it is very susceptible to a VSV-G pseudotyped, HIV-1-based GFP-expressing lenti-virus vector, HIV-1-GFP. Cell clones stably expressing MT4-, CV1-, and HSC-F-TRIM5 α were selected according to the method described in Materials and Methods. The levels of expression of TRIM5 α were determined by using a real-time PCR and cells expressing comparable amounts of TRIM5 α ($4\sim 6 \times 10^6$ copies/ μ g total RNA) were used for subsequent study. Restriction can be quantified by comparing the percentage of GFP-positive cells with and without TRIM5 α .

As can be seen in Fig.2A, MT4-TRIM5 α had a very weak anti-HIV-1 effect (~ 1.2 -fold), consistent with a previous study (33). In contrast, restriction of HIV-1 was clearly evident over a wide range of initial multiplicities of infection in cells expressing HSC-F-TRIM5 α , CV1-TRIM5 α -type1, and CV1-TRIM5 α -type2 (~ 5 to 10-fold). There was no significant difference between CV1-TRIM5 α -type 1 and 2, therefore we choose CV1-TRIM5 α -type 1 as representative of CV1-TRIM5 α in the subsequent experiments.

We obtained the same results as above when we used a HA-tagged version of TRIM5 α (data not shown).

African green monkey and cynomolgus monkey TRIM5 α inhibit CD4-dependent HIV-1 infection in human cells.

To test the restriction properties of TRIM5 α in CD4-dependent HIV-1 infection, we constructed a recombinant SeV expressing TRIM5 α fused with the HA tag in the C-terminal of HSC-F TRIM5 α or CV1 TRIM5 α (HSC-F TRIM5 α -tag SeV or CV1 TRIM5 α -tag SeV). Human T cell line MT4 cells were first infected with the SeV expressing TRIM5 α -tag (Fig.2B), incubated at 37 °C for 9 hrs, and then infected with an X4-tropic HIV-1 strain NL43. As can be seen in Fig.2C, both HSC-F-TRIM5 α -tag and CV1-TRIM5 α -tag completely inhibited HIV-1 replication, while MT4 cells infected with SeV empty vector fully supported HIV-1 replication.

Distinct patterns of restriction for SIVmac among non-human primate TRIM5 α .

In African green monkey CV1 cells, both HIV-1 and SIVmac239 were restricted, whereas only HIV-1 was restricted in cynomolgus monkey HSC-F cells (Fig.3A). Therefore, we examined whether or not CV1-TRIM5 α also could inhibit the replication of SIVmac. In MT4 cells infected with recombinant SeV expressing HSC-F-TRIM5 α -tag, the replication of SIVmac239 was not suppressed at all (Fig.3B), indicating that the HSC-F-TRIM5 α showed a similar specificity to rhesus monkey TRIM5 α (33). In MT4 cells infected with recombinant SeV expressing CV1-TRIM5 α -tag by contrast, the replication of SIVmac239 was completely

suppressed. These results suggested that the distinct sensitivity of African green monkey and cynomolgus monkey cells to HIV-1 and SIVmac infection was, at least partly, determined by TRIM5 α .

A small region of 37 amino acid residues in the SPRY domain of CV1-TRIM5 α determines SIVmac restriction.

A comparison of the human and non-human primate TRIM5 α sequences showed the presence of a highly variable region in the N-terminal portion of the SPRY domain (Fig. 1A and B). In this region, CV1 and Vero TRIM5 α had a 20-amino acid repetition, which was totally absent in HSC-F-TRIM5 α . These findings prompted us to test whether or not this highly variable region of TRIM5 α determined the species-specific inhibition of SIVmac infection. We constructed chimeric TRIM5 α s from HSC-F-TRIM5 α -tag and CV1-TRIM5 α -tag by using Sph I and Bam HI restriction enzyme digestion (Fig.4A). HSC-F+60tag contained the 242bp fragment of CV1-TRIM5 α in the background of HSC-F-TRIM5 α -tag. The reciprocal chimera, CV1-60tag, contained a 182bp fragment of HSC-F-TRIM5 α in the background of CV1-TRIM5 α -tag. In this fragment, the differences between CV1 and HSC-F TRIM5 α , including the 20-amino acid repetition, were located in a small region of 37 amino acid residues (Fig.1B). Human osteosarcoma C143 cells stably expressing various TRIM5 α s (Fig.4B) were infected with VSV-G-pseudotyped HIV-1 NL43, and levels of P24 in the culture supernatants were assayed periodically. As expected, both chimeric TRIM5 α s as well as parental TRIM5 α s clearly inhibited the replication of HIV-1 NL43 (Fig.4C). When those cells were infected with VSV-G-pseudotyped SIVmac239, the parental CV1-TRIM5 α -tag