第５４回日本ウイルス学会学術集会. 2006. 11, 名古屋.

フィリピンにおける血液媒介感染によるHIV 流行の端緒形成の可能性. 景山誠二、市村宏. 第２０回日本エイズ学会学術集会・総会, 2006, 11, 東京.

アフリカ、ケニアにおけるHIV感染とHPV感染. 第２０回日本エイズ学会学術集会・総会, 2006, 12, 東京.

H. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

該当なし。

2. 実用新案登録

該当なし

別紙４

研究成果の刊行に関する一覧表

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakayama, E. E., Tanaka, Y., Nagai, Y., Iwamoto, A. and Shioda, T.	A CCR2-V64I polymorphism affects stability of CCR2A isoform.	AIDS.	18	729-738	2004
Emi E. Nakayama, Hiroyuki Miyoshi, Yoshiyuki Nagai, and Tatsuo Shioda.	A specific region of 37 amino acid residues in the SPRY (B30.2) domain of African green monkey TRIM5α determines species-specific restriction of SIVmac infection.	J. Virol.	79	8870-8877	2005
Kazuyasu Mori, Chie Sugimoto, Shinji Ohgimoto, Emi E. Nakayama, Tatsuo Shioda, Shigeru Kusagawa, Yutaka Takebe, Munehide Kano, Tetsuro Matano, Takae Yuasa, Masaaki Miyazawa, Yumiko Takahashi, Michio Yasunami, Akinori Kimura, Naoki Yamamoto, Yasuo Suzuki, and Yoshiyuki Nagai.	Influence of glycosylation on the efficacy of an Env-based vaccine against SIVmac239 in a macaque AIDS model.	J. Virol	79	10386-10396	2005

Nuanjun Wichukchinda, Emi E Nakayama, Archawin Rojanawiwat, Panita Pathipvanich, Wattana Auwanit, Suthon Vongsheree, Koya Ariyoshi, Pathom Sawanpanyalert, and Tatsuo Shioda .	Protective Effects of *IL-4 -589T* and *RANTES -28G* on HIV-1 disease progression in infected Thai females.	AIDS	20	189-196	2006
Song, H., Nakayama, E. E., and Shioda, T.	Effects of human interleukin 7 on HIV-1 replication in monocyte-derived human macrophages.	AIDS	20	937-939	2006
Nakayama, EE, Maegawa, H, and Shioda, T.	A dominant-negative effect of cynomolgus monkey tripartite motif protein TRIM5α on anti-simian immunodeficiency virus SIVmac activity of an African green monkey orthologue.	Virology	350	158-163	2006
Sakuragi S, Sakuragi J, Morikawa Y, Shioda T.	Development of a rapid and convenient method for the quantification of HIV-1 budding.	Microbes Infect	8	1875-1881	2006
Shioda T, Nakayama EE.	Human genetic polymorphisms affecting HIV-1 diseases.	Int J Hematol	84	12-17	2006
Song, H, Nakayama, EE, Likanonsakul, S, Wasi, C, Iwamoto A, and Shioda T.	A three-base-deletion polymorphism in the upstream non-coding region of human interleukin 7 (IL-7) gene could enhance levels of IL-7 expression.	International Journal of Imuunogenetics.	34	107-113	2007

Nuanjun Wichukchinda, Archawin Rojanawiwat, Yoshihiro Kitamura, Emi E Nakayama, Panita Pathipvanich, Wattana Auwanit, Pathom Sawanpanyalert, Aikichi Iwamoto, Tatsuo Shioda, and Koya Ariyoshi.	The polymorphisms in *DC-SIGNR* affect the susceptibility to HIV-1 infection.	AIDS. Res. Hum. Retrovir.	In press		
Raphael Lwembe, Washingtone Ochieng, Annie Panikulam, Charles O. Mongoina, Mary Owens, Yusuke Koizumi, Seiji Kageyama, Naohiko Yamamoto, Tatsuo Shioda, Rachel Musoke, Angel D'Agostino, Elijah M. Songok, Hiroshi Ichimura.	ANTI-RETROVIRAL DRUG RESISTANCE-ASSOCIATED MUTATIONS AMONG NON-SUBTYPE B HIV-1-INFECTED KENYAN CHILDREN WITH TREATMENT FAILURE.	J. Med. Virol.	In press		
Masahisa Ohishi, Tatsuo Shioda, and Jun-ichi Sakuragi.	Retro-transduction by virus pseudotyped with glycoprotein of vesicular stomatitis virus.	Virology	In press		
Yusuke KOIZUMI, Seiji KAGEYAMA, Yoshihide FUJIYAMA, Michiko MIYASHITA, Raphael LWEMBE, Keiki OGINO, Tatsuo SHIODA, Hiroshi ICHIMURA.	RANTES -28G delays and DC-SIGN -139C enhances AIDS progression in HIV-1-infected Japanese hemophiliacs.	AIDS Research and Human Retroviruses.	In press		

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Furutsuki, T., Hosoya, N., Kawana-Tachikawa, A., Tomizawa, M., Odawara, T., Goto, M., Kitamura, Y., Nakamura, T., Kelleher, AD., Cooper, DA., Iwamoto, A.	Frequent transmission of cytotoxic-T-lymphocyte escape mutants of human immunodeficiency virus type 1 in the highly HLA-A24-positive Japanese population.	J. Virol.	78	8437-8445	2004
Rojanawiwat, A., Miura, T., Thaisri, H., Pathipvanich, P., Umnajsirisuk, S., Koibuchi, T., Vongheree, S., Iwamoto, A., Ariyoshi, K., and Sawanpanyalert, P.	Frequent detection of Epstein-Barr virus and cytomegalovirus but not JC virus DNA in cerebrospinal fluid samples from human immunodeficiency virus-infected patients in northern Thailand.	J. Clin. Microbiol.	43	3484-3486	2005
Tomonari, A., Takahashi, S., Shimohakamada, Y., Ooi, J., Takasugi, K., Ohno, N., Konuma, T., Uchimaru, K., Tojo, A., Odawara, T., Nakamura, T., Iwamoto, A., and Asano, S.	Unrelated cord blood transplantation for a human immunodeficiency virus-1-seropositive patient with acute lymphoblastic leukemia.	Bone Marrow Transplantation	36	261-262	2005

Shinoe、T., Wanaka, A., Nikaido, T., Kakuta, Y., Masunaga, A., Shimizu, J., Duyckaerts, C.,Imaizumi, K., Iwamoto, A., and Kanawzawa, I.	The pro-apoptotic human BH3-only peptide harakiri is expressed in cryptococcus-infected perivascular macrophages in HIV-1 encephalitis patients.	Neuroscience Letters	393	102-107	2006
Ide, F., Nakamura, T., Tomizawa, M., Kawana-Tachikawa, A., Odawara,T., Hosoya, N., Iwamoto, A.	Peptide-loaded dendritic-cell vaccination followed by treatment interruption for chronic HIV-1 infection: A phase 1 trial.	J. Med. Virol.	78	711-718	2006
Maeda, T., Fujii, T., Matsumura, T., Endo, T., Odawara, T., Itoh, D., Inoue, Y., Ohkubo, T., Iwamoto, A., and Nakamura, T.	AIDS-related cerebral toxoplasmosis with hyperintense foci on T1-weighted MR images: A case report.	Journal of Infection	53	e167-e170	2006
Hoshino, S., Sun, B., Konishi, M., Shimura, M., Segawa, T. Hagiwara, Y., Koyanagi, Y., Iwamoto, A., Mimaya, J., Terunuma, H., Kano, S., and Ishizaka, Y.	Vpr in plasma of HIV-1-positive patients is correlated with the HIV-1 RNA titers.	AIDS Research and Human Retroviruses.	In press		
Takahashi, H., Yotsuyanaggi, H., Yasuda, K., Koibuchi,T., Suzuki, M., Kato, T., Nakamura, T., Iwamoto, A., Nishioka, K., Iino, S., Koike, K., and Itoh, F.	Molecular epidemiology of hepatitis A virus in the Metropolitan Area in Japan.	Gastroenterology	In press		

Wichukchinda, N., Kitamura, Y., Rojanawiwat, A., Nakayama, EE., Song, H., Pathipvanich, P., Auwanit, W., Sawanpanyalert, P., Iwamoto, A., Shioda, T., and Ariyoshi, K.	The polymorphisms in DC-SIGNR affect susceptibility to HIV-1 infection.	AIDS Research and Human Retroviruses.	In press		
Fujii, T, Nakamura T, Iwamoto A.	Pneumocystis pneumonia in patients with HIV infection - Clinical manifestations, laboratory findings and radiological features -.	J Infect Chemother.	In press		
Maeda, T., Oyaizu, N., Endo, T., Odawara, T., Nakamura, T., Iwamoto, A., Fujii, T.	Pneumocystis jiroveci pneumonia in an AIDS patient: Unusual manifestation of multiple nodules with multiloculated cavities.	European Journal of Radiology,	In press		

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Songok EM, Ichimura H, et al.	Active generation and selection for HIV intersubtype A/D recombinant forms in a co-infected patient in Kenya.AIDS.	AIDS Res Hum Retroviruses	20(2)	255-8	2004
Ndembi N, Ichimura H, et al.	Genetic Diversity of HIV Type 1 in Rural Eastern Cameroon.	J Acq Immun Def Synd	37(5)	1641-50	2004
Kita K, Ichimura H. et al.	Genetic Diversity of HIV Type 1 in Likasi, Southeast of Democratic Republic of Congo.	AIDS Res Hum Retroviruses	20(12)	1352-7	2004
Takemura T, Ichimura H. et al.	A novel SIV from black mangabey (Lophocebus aterrimus) in Democratic Republic of Congo.	J Gen Virol	86(pt7)	1967-71	2005
Kurbanov F, Ichimura H. et al.	A New Subtype (Subgenotype) Ac (A3) of Hepatitis B Virus and Recombination between Genotypes A and E in Cameroon.	J Gen Virol	86(pt7)	2047-56	2005
Samoel A, Ichimura H, et al.	HIV-1 subtypes in circulation in Northern Kenya.	AIDS Res Hum Retroviruses	21(9)	810-4	2005
Agdamag DM, Ichimura H, et al.	Rapidly spreading HCV infections from limited sources simulating an AIDS outbreak in the Philippines.	J Med Virol	77(2)	221-6	2005
Ochieng W, Ichimura H, et al.	Tuberculosis and oral Candida species surveillance in HIV infected individuals in Northern Kenya, and the implications on tuberculin skin test screening for DOPT-P.	East Afr Med J	82(12)	609-13	2005
Koizumi Y, Ichimura H, et al.	Emergence of ART-resistance associated primary mutations among drug-naïve HIV-1-infected individuals in rural western Cameroon.	J Acq Immun Def Synd	43(1)	15-22	2006
Kageyama S, Ichimura H, et al.	A Natural inter-genotypic (2b/1b) recombinant of hepatitis C virus in the Philippines.	J Med Virol	78(11)	1423-8	2006

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.

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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

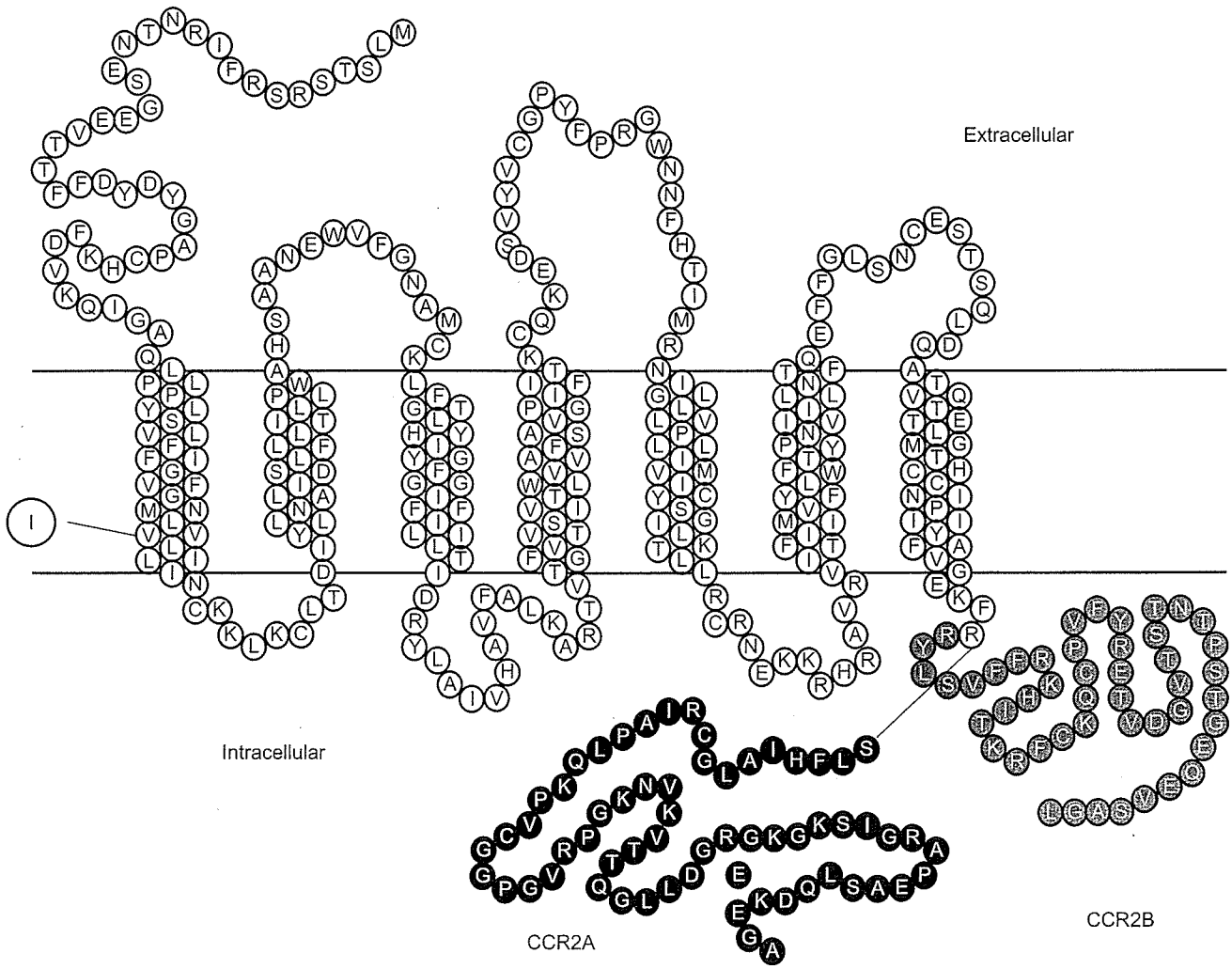

Fig. 1. The structure of the CCR2A and CCR2B molecules. Outlined letters in grey circles denote amino acid residues present in CCR2B. Outlined letters in black circles denote amino acid residues present in CCR2A. A letter I in a large circle denotes a substitution at position 64.

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-μm 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 β-galactosidase gene as a reporter was performed as described previously [19]. Briefly, mouse fibroblast L cells were transfected with β-galactosidase reporter plasmid pGINT7β-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 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, 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

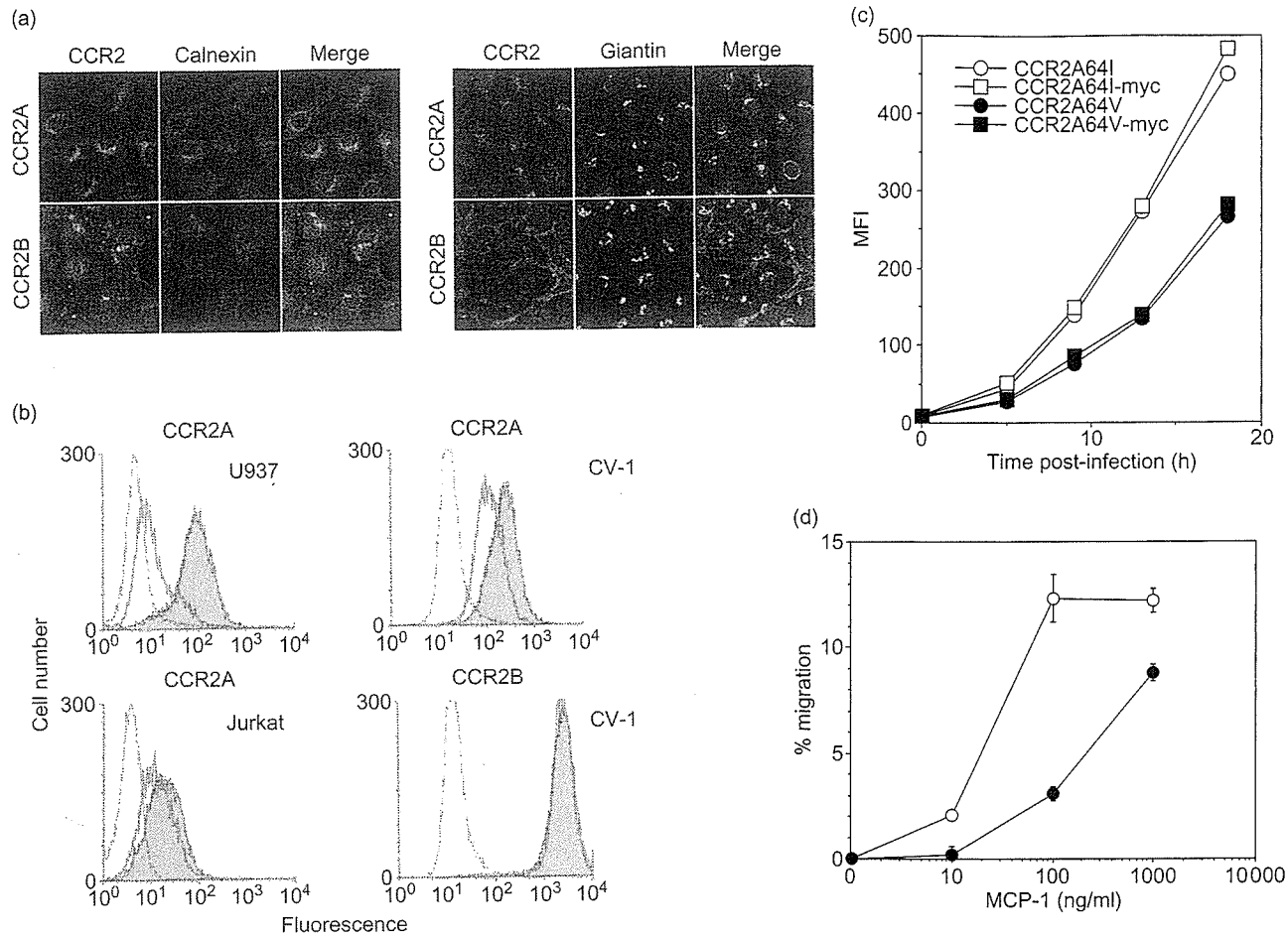

Fig. 2. (a) Subcellular distribution of CCR2A-64V and CCR2B-64V in CV1 cells. SeV vector (SeV) was used to express the CCR2A-64V and CCR2B-64V molecules. Cells were fixed and permeabilized before staining with MAB150 anti-CCR2 mouse MAb followed by FITC-labelled anti-mouse IgG. Cells were then re-stained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

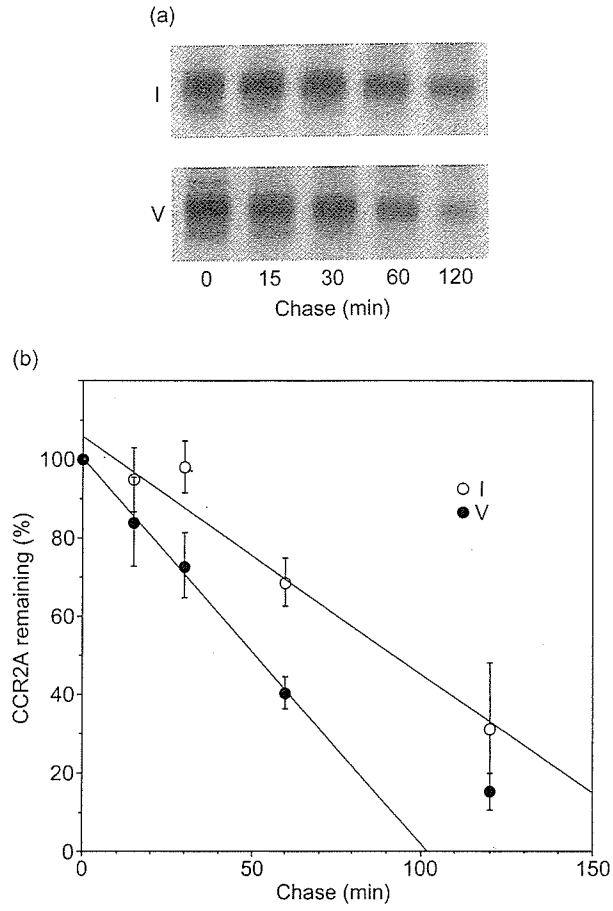

Fig. 3. CCR2A-64I is more stable than CCR2A-64V. CV1 cells were infected with SeV expressing CCR2A-64V-myc and CCR2A-64I-myc for 9 h. Cells were labelled for 30 min and then harvested following the chase time indicated. (a) Representative gels of pulse–chase analysis. (b) Phosphorlmager analysis of the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

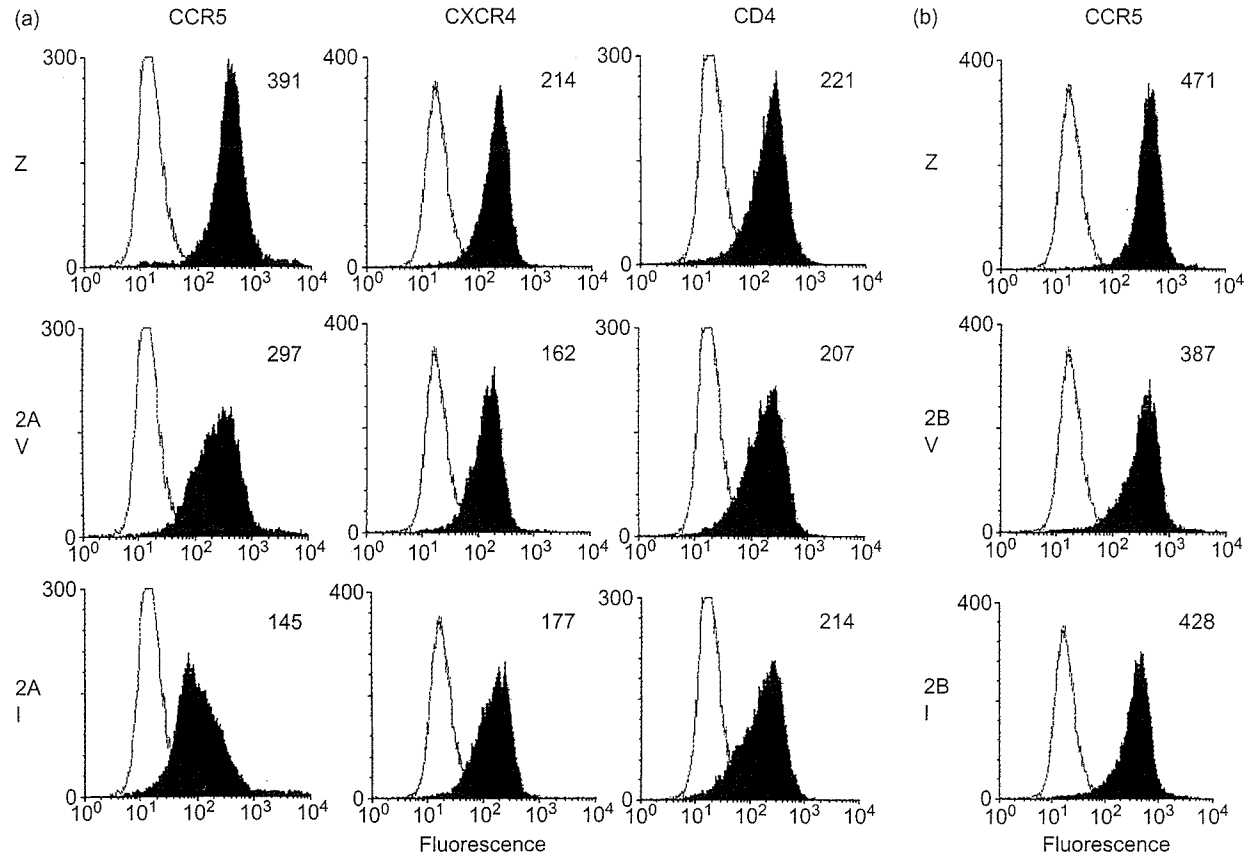

Fig. 4. (a) Effect of CCR2A-64V and CCR2A-64I on HIV-1 corecepter expression. Vac vectors were used to express CCR5, CXCR4 and CD4 in the CV1 cells inoculated with SeV expressing CCR2A-64V or CCR2A-64I. Z denotes the wild-type SeV. Five hours after infection, cells were stained with MAb against CCR5,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

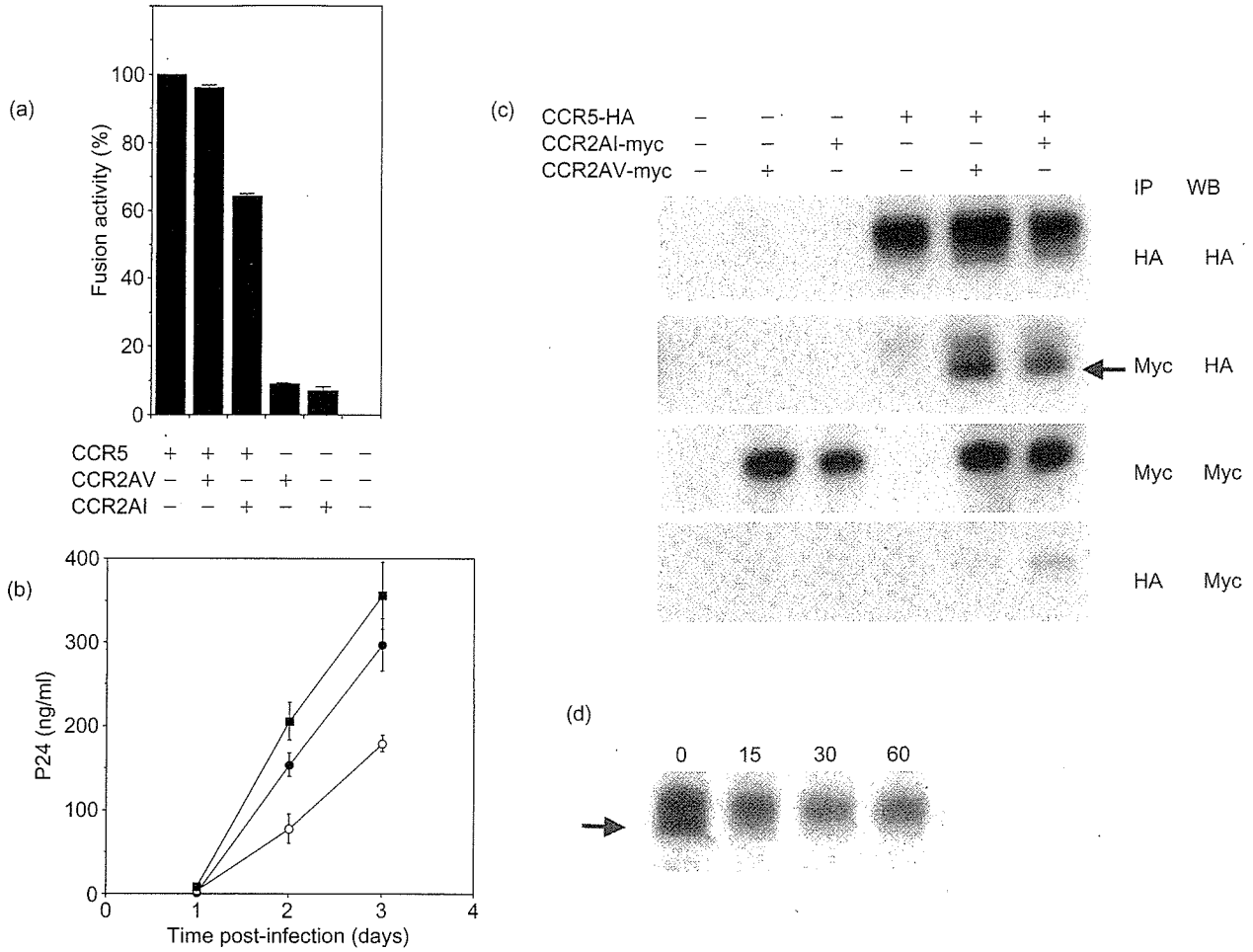

Fig. 5. (a) Coreceptor activity of CCR5 in CCR2A-64V or CCR2A-64I co-expressed cells. SeV vector was used to express CCR2A-64V or CCR2A-64I, and Vac vector was used to express CCR5 as described in Fig. 4. HIV-1 coreceptor activity of each sample was measured using the method described in Materials and 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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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, Kellner 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. Louisirirotchanakul 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 deletional 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 CXCR-4- but not CCR-5-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-Jullie 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, Gefter W, Rut. A, *et al.* **Val641le 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 chemotactic 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, Bifrare 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. Iikura 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 chemotactic 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.

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A Specific Region of 37 Amino Acid Residues in the SPRY (B30.2) Domain of African Green Monkey TRIM5α Determines Species-Specific Restriction of Simian Immunodeficiency Virus SIVmac Infection

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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, TRIM5α, was identified as a blocking factor in a rhesus macaque cDNA library. Among Old World monkey cell lines, the African green monkey kidney cell line CV1 is highly resistant to not only HIV-1 but also simian immunodeficiency virus SIVmac infection. We analyzed TRIM5α of CV1 cells and HSC-F cells, a T-cell line from a cynomolgus monkey, and found that both CV1- and HSC-F-TRIM5αs could inhibit CD4-dependent HIV-1 infection, as well as vesicular stomatitis virus glycoprotein-mediated infection. CV1-TRIM5α could also inhibit SIVmac infection, whereas HSC-F-TRIM5α could not. In the SPRY (B30.2) domain of CV1-TRIM5α, there was a 20-amino-acid duplication that was not present in HSC-F-TRIM5α. A chimeric TRIM5α containing 37 amino acid residues from CV1-TRIM5α, which spanned the 20-amino-acid duplication, in the background of HSC-F-TRIM5α fully gained the ability to inhibit SIVmac infection. Conversely, the mutant CV1-TRIM5α lacking the 20-amino-acid duplication completely 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-TRIM5α contained a determinant of species-specific restriction of SIVmac.

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 these monkeys (13, 31). In contrast, simian immunodeficiency virus (SIV) 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 postentry 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 a high multiplicity of infection (MOI) 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 5α (TRIM5α), a component of cytoplasmic bodies, as a factor that confers resistance to HIV-1 infection (33). Shortly after, TRIM5α of African green monkey, another Old World monkey, was also shown to restrict HIV-1 infection, whereas human TRIM5α was reported to restrict N-tropic murine leukemia virus (12, 14, 25, 36).

An African green monkey kidney cell line, CV1, was shown to be highly resistant to SIVmac infection, as well as to HIV-1 infection. We analyzed TRIM5α of CV1 cells and HSC-F cells, a T-cell line from a cynomolgus monkey, and report here that the ability of CV1-TRIM5α to suppress SIVmac infection was determined by a small region composed of 37 amino acid residues in the SPRY (B30.2) domain of CV1-TRIM5α.

MATERIALS AND METHODS

Cloning and expression of TRIM5α. TRIM5α cDNA was amplified by reverse transcription-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 by using 5′-GCGGCCGCTACTATGGCTTCTGG-3′ as a forward primer and 5′-GAA TTCTCAAGAGCTTGGTGA-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 TRIM5α was determined.

The entire coding regions of selected clones were transferred to pcDNA3.1 (Invitrogen) by 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 TRIM5α cDNA and cultured in the presence of 0.75 mg of

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G418 (Gibco)/ml for 14 days. The colonies obtained were examined for the expression of TRIM5α by using the TaqMan PCR method according to the manufacturer's instructions (Applied Biosystems). Sequences of the probe and primers used to specifically detect each TRIM5α were as follows: MT4-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAGGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCAGTTTCAGAG TTCG-TAMRA-3′); HSC-F-TRIM5, forward primer (5′-AACCTGGAGAAG GAGAAAGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TTCGTTTCAGACTTTG-TAMRA-3′); and CV1-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAAGAAGA-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCCGTTTCAGAC TTCG-TAMRA-3′). These primers amplify the coiled-coil region of TRIM5 genes. Plasmid DNA used for transfection served as a standard to determine the number of copies of TRIM5α transcripts. The parental TK-ts13 cells were totally negative for the primate TRIM5α expression. Clones expressing each TRIM5α at comparable levels (ca. 4 to 6 × 10^7 copies/μg of total RNA) were used for subsequent experiments.

To generate CV1-TRIM5α and HSC-F-TRIM5α cDNAs carrying a hemagglutinin (HA) tag (YPYDVPDYAA) at their C termini, cloned CV1-TRIM5α and HSC-F-TRIM5α 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 TRIM5α. The C-terminal portion of TRIM5α fused with the HA tag (BamHI to NotI) and the N-terminal portion of TRIM5α (NotI to BamHI) were assembled on a pCEP4 vector (Invitrogen). To generate chimeric TRIM5α HSC-F+60tag, the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag was replaced with the corresponding 242-bp SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Conversely, the 242-bp SphI-BamHI fragment of CV1-TRIM5α was replaced with the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag in the background of CV1-TRIM5α-tag to generate CV1-60tag. PCR-based mutagenesis of HSC-F-TRIM5α-tag was performed to generate HSC-delete-tag, which possessed the 5′-proximal 84 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, and HSC-insert-tag, which possessed 3′-proximal 158 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Similarly, CV1-delete-tag, which possessed the 3′-proximal 98 bp of the SphI-BamHI fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, was generated by a PCR-based mutagenesis of CV1-TRIM5α-tag. The entire coding sequences of these TRIM5α-tags were then transferred to the NotI site of pSeV18+b(+). Recombinant Sendai viruses (SeVs) carrying various TRIM5α-tags 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 of the experiments.

To establish human cell lines which constitutively express primate TRIM5αs or their chimeras, human osteosarcoma C143 cells were transfected with pCEP4 containing cDNA of CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, CV1-60tag, or HSC-F+60tag, and cells were cultured in the presence of 0.3 mg of hygromycin B (Gibco)/ml for 14 days.

Immunoprecipitation and Western blot analysis. When we performed Western blot analysis of cells expressing HA-tagged TRIM5α proteins, we consistently observed nonspecific binding of anti-HA antibody to a protein that comigrated with HSC-F-TRIM5α. Therefore, we analyzed the expression of each HA-tagged TRIM5α protein in the hygromycin B-resistant C143 cells or MT4 cells infected with recombinant SeVs by immunoprecipitation, followed by Western blot analysis as described previously (20) to reduce nonspecific background. Briefly, cell lysate was first adsorbed with protein A-agarose before the addition of anti-HA antibody to avoid nonspecific protein binding to protein A-agarose. TRIM5α proteins in the cell lysate were then precipitated with anti-HA high-affinity rat monoclonal antibody (Roche) by using a protein A-immunoprecipitation kit (Roche). Precipitated materials were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen). Proteins in the gel were then electronically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Blots were blocked and probed with anti-HA antibody overnight at 4°C and then incubated with peroxidase-conjugated anti-rat immunoglobulin G (American Qualex) and developed by using the Immun-Star HRP chemiluminescence kit (Bio-Rad). Visualized image was recorded by LAS1000 (Fuji) and quantified by ImageGauge (Fuji). At least three independent experiments were performed, and the means and standard deviations (SD) for the data were calculated.

Viruses and HIV-1 lentivirus vector. 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 a reverse transcriptase colorimetric assay (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 using a flow cytometer (FACScan; Becton Dickinson) 40 h after infection. For VSV-pseudotyped HIV-1, SIVmac239, and HIV-2 infection assays, we inoculated viruses containing 1 ng of reverse transcriptase into 4 × 10^4 C143 cells. For CD4-dependent infection assays, 2.5 × 10^5 MT4 cells were infected with SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain of SeV at a MOI of 10 PFU per cell, followed by incubation at 37°C for 9 h. Cells were then superinfected 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 by using a RETROtek antigen ELISA kit (ZeptoMetrix).

Data deposition. The sequences described here have been deposited in the GenBank database under accession numbers AB210050 to AB210052.

RESULTS

Variation in TRIM5α. We cloned TRIM5α cDNA from the human T-cell line MT4, cynomolgus monkey T-cell line HSC-F, and African green monkey kidney cell lines CV1 and Vero. The predicted amino acid sequences of TRIM5αs are compared in Fig. 1A and B.

Human TRIM5α from MT4 cells differed at an amino acid position 249 (G249D) from the previously published sequence (33) and was designated MT4-TRIM5α. The cynomolgus monkey TRIM5α from HSC-F (HSC-F-TRIM5α) was two amino acids shorter than the rhesus monkey TRIM5α (33) and two amino acids longer than the human TRIM5α. All 10 clones derived from Vero cells had the same sequence as the previously published one (36). On the other hand, we found at least two distinct TRIM5α sequences in CV1 cells. The two major TRIM5α sequences obtained from CV1 were designated CV1-TRIM5α-type1 and CV1-TRIM5α-type2, and five amino acids were found to differ between the two sequences (I259V, L337S, R351L, G359R, and G438S). Among 10 cDNA clones obtained from CV1, there were four type 1 clones and four type 2 clones. The remaining two clones were most likely chimeric artifacts. Two recently published sequences of TRIM5α from CV1 cells (AY593973 and AY625002) showed differences at three positions—L7V, I259V, and G438S—and have S, L, and R at positions 337, 351, and 359, respectively (14, 36), whereas both the type 1 and 2 clones had leucines at the seventh 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 cynomolgus monkey HSC-F (Fig. 1B).

The phylogenetic tree of various TRIM5α sequences showed that cynomolgus and rhesus monkey TRIM5αs are similar to each other, a finding consistent with the fact that these two monkeys belong to the genus *Macaca* (Fig. 1C).

African green monkey and cynomolgus monkey TRIM5α inhibit HIV-1 infection in nonprimate cells. We first sought to determine whether or not each TRIM5α can inhibit HIV-1 infection in the context of nonprimate cells because human and primate cells express endogenous TRIM5α that could complicate a functional analysis of TRIM5α-mediated restriction. The hamster cell line TK-ts13 was used, because it is very susceptible to a VSV-G-pseudotyped, HIV-1-based GFP-expressing lentivirus vector, HIV-1-GFP. Cell clones stably ex-