TABLE 1

Antiviral activity and enzyme inhibition of protease dimerization inhibitors

MT-2 cells (2 × 10^3) were exposed to 100 50% tissue culture infectious dose values of HIV-1_{LAI} and cultured in the presence of various concentrations of each drug, and the IC_{50} values were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All assays were conducted in duplicate, and the data shown represent mean values (±1 S.D.) derived from the results of three independent experiments. The chromogenic substrate Lys-Ala-Arg-Val-Nle-pnitroPhe-Glu-Ala-Nle-amide was used to determine the kinetic parameters. The K_i values were obtained from the IC_{50} values estimated from an inhibitor dose-response curve with the spectroscopic assay using the equation $K_i = (IC_{50} - [E]/2)/(1 + [S]/K_m)$, where [E] and [S] are the PR and substrate concentrations, respectively. The K_i values were measured at four to five substrate concentrations. The measurement was repeated at least three times to produce the average values.

Drug	IC_{50}	K_i
	μM	pM
GRL-0036A	0.005 ± 0.002	29
GRL-06579A	0.0014 ± 0.0008	3.5
GRL-98065	0.0004 ± 0.0001	14
TMC126	0.0003 ± 0.0001	10
DRV	0.0034 ± 0.0005	16
BCV	0.0002 ± 0.0001	6.8
GRL-0026A	0.48 ± 0.04	ND[a]
TPV	0.10 ± 0.04	ND

[a] ND, not determined.

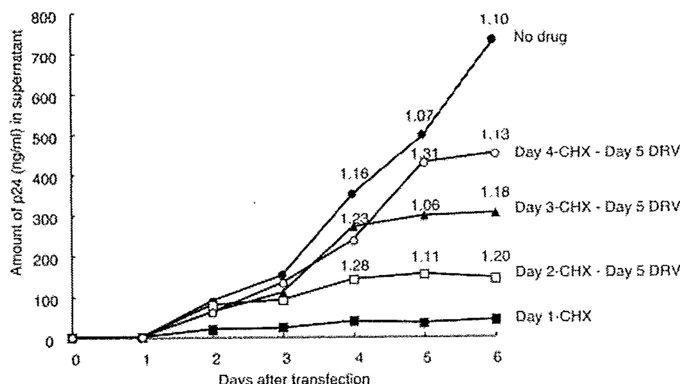

FIGURE 5. **Darunavir does not dissociate once-dimerized protease in cells producing infectious HIV-1 virions.** COS7 cells were co-transfected with two plasmids, pPR_{WT}^{CFP} and pPR_{WT}^{YFP}, exposed to CHX (50 μg/ml) in 24, 48, 72, and 96 h of culture. The cells were exposed to DRV on day 5 of culture. The production of HIV-1 was monitored every 24 h by determining levels of p24 Gag protein produced into culture medium. The values of the $CFP^{A/B}$ ratio were determined at various time points.

administered with ritonavir (38), also blocked protease dimerization (Fig. 4*E*).

We also examined various nucleoside and non-nucleoside reverse transcriptase inhibitors (zidovudine, lamivudine, abacavir, nevirapine, and efavirenz) as well as CCR5 inhibitor aplaviroc (39) for dimerization inhibition. However, none of these anti-HIV-1 agents showed inhibition of dimerization even at relatively higher concentrations of 1–10 μM. Soluble CD4 (5 μg/ml) also failed to inhibit protease dimerization (Fig. 4*G*).

Darunavir Blocks Protease Dimerization as Examined in Dual Luciferase Assay—We also established a dual luciferase assay using the CheckMate™ Mammalian Two-Hybrid System to examine whether DRV blocked protease dimerization in a different assay system. We generated pACT-PR_{WT}, producing PR_{WT}, whose N terminus is connected to the herpes simplex virus VP16 activation domain, and pBIND-PR_{WT}, producing PR_{WT}, whose N terminus is connected to GAL4 DNA-binding domain. In this system, interactions between two different PR_{WT} result in an increase in firefly luciferase expression produced by the pG5*luc* vector. In addition, the pBIND vector expresses *Renilla* luciferase under the control of the SV40 promoter, allowing the user to normalize for the differences in transfection efficiency. Thus, when VP16 and GAL4 closely interact upon protease dimerization, the ratio of the intensity of Fluc over that of Rluc increases, and its decrease indicates the disruption of protease dimerization. As shown in Fig. 4*H*, in the presence of 0.1 and 1 μM of DRV, the relative response ratios significantly decreased, further corroborating that DRV blocks protease dimerization.

Darunavir Does Not Dissociate Once-dimerized Protease in Cells Producing Infectious HIV-1 Virions—Finally, an attempt was made to determine if dimerization inhibitors could dissociate mature protease that had already dimerized. COS7 cells were co-transfected with a pair of plasmids encoding HIV-PR_{WT}^{CFP} and HIV-PR_{WT}^{YFP} and exposed to a protein synthesis inhibitor CHX (50 μg/ml) at 24, 48, 72, and 96 h of culture. The cells were then exposed to DRV on day 5 of culture, the production of HIV-1 was monitored every 24 h by determining levels of p24 Gag protein produced into culture medium, and the values of the $CFP^{A/B}$ ratio were determined at various time points (Fig. 5). When the cells were treated with CHX on day 1 and throughout the rest of the culture period, only a small amount of p24 Gag protein production was seen but no cells emitting fluorescence were observed. When the cells were exposed to CHX on day 2 and beyond, Gag protein production was readily

FIGURE 4. **Inhibition of protease dimerization.** *A*, inhibition of protease dimerization by non-peptidyl and peptidyl compounds. COS7 cells were exposed to each of the agents (1 μM of GRL-0036A, GRL-06579A, GRL-98065, TMC126, DRV, and BCV and 10 μM of P9 and P27) and subsequently co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP}. After 72 h, cultured cells were examined in the FRET-HIV-1 assay system using confocal microscopy Fluoview FV500 confocal laser scanning microscope, and $CFP^{A/B}$ ratios were determined and plotted. The mean of these ratios obtained are shown as *bars*. *B*, distribution of the values of $CFP^{A/B}$ ratio in the presence of 1 μM DRV. 143 cells obtained from 11 independent assays were examined, and $CFP^{A/B}$ ratios determined were plotted. All the assays were conducted in a blinded manner. *C*, dose-responsive dimerization inhibition by DRV. COS7 cells were exposed to various concentrations of DRV, co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP}, and A/B ratios were determined. *D*, dose-responsive dimerization inhibition by various non-peptidyl compounds. COS7 cells were exposed to various concentrations of GRL-0026A, TMC126, and BCV, co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP}, and cultured for 72 h. At the end of the culture, $CFP^{A/B}$ ratio values were determined. *E*, failure of seven clinically available protease inhibitors except DRV and TPV to inhibit the dimerization of PR_{WT}^{CFP} and PR_{WT}^{YFP}. COS7 cells were co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP} in the presence of various anti-HIV-1 protease inhibitors at concentration of 1 μM, and A/B ratios were determined. *F*, failure of a high concentration of four clinically available protease inhibitors to inhibit HIV-1 protease dimerization. COS7 cells were co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP} in the presence of four PIs (saquinavir, amprenavir, nelfinavir, and atazanavir) at a higher concentration of 10 μM, cultured for 72 h, and $CFP^{A/B}$ ratios were determined. Note that all the $CFP^{A/B}$ ratio values were >1.0 except for those of DRV. *G*, various approved anti-HIV-1 agents failed to inhibit HIV-1 protease dimerization. COS7 cells were co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP} in the presence of various nucleoside and non-nucleoside reverse transcriptase inhibitors (zidovudine, lamivudine, abacavir, nevirapine, and efavirenz), CCR5 inhibitor aplaviroc, and soluble CD4, and A/B ratios were determined. *H*, protease dimerization inhibition by DRV on dual luciferase assay. COS7 cells were co-transfected with pACT-PR_{WT}, pBIND-PR_{WT}, and pG5*luc* in the presence or absence of 0.1 or 1.0 μM of DRV, cultured for 48 h, and the intensity of firefly luminescence (*Fluc*) and *Renilla* luminescence (*Rluc*) was measured with TR717 microplate luminometer. DRV was added to the culture medium simultaneously with plasmids to be used. Fluc/Rluc intensity ratios were determined with co-transfection of pACT-PR_{WT}, pBIND-PR_{WT}, and pG5*luc* in the absence of DRV, serving as maximal values.

detected by day 2, but no significant increment in the production of p24 Gag protein was seen on those days subsequent to the addition of CHX. When the cells were exposed to CHX on days 3 and 4, greater amounts of Gag protein were seen (Fig. 5). The $CFP^{A/B}$ ratios determined on days 4 and 5 of culture were all >1.0, signifying that HIV-1 protease had been generated and dimerization had occurred. On day 5, DRV (1 μM) was added to all the cultures described above and the $CFP^{A/B}$ ratios were determined on day 6 of culture. The ratios remained >1.0 in all of the cultured COS7 cells (Fig. 5). These data strongly suggest that DRV does not dissociate mature protease once dimerized within the HIV-1-producing COS7 cells.

DISCUSSION

In the present study, we developed an intermolecular FRET-based HIV-1-expression assay (FRET-HIV-1 expression assay) that employed cyan and yellow fluorescent protein-tagged HIV-1 protease monomers. Using this assay, we identified a group of non-peptidyl small molecule inhibitors of HIV-1 protease dimerization (molecular weight, 547–704). Dimerization of HIV-1 protease subunits is an essential process for the acquisition of proteolytic activity of HIV-1 protease, which plays a critical role in the replication cycle of HIV-1. Hence, the inhibition of dimerization of HIV-1 protease subunits represents a unique target for potential intervention of HIV-1 replication. The strategy to target protease dimerization as a possible anti-HIV-1 modality has been explored (8, 11–13), and certain compounds have been reported as potential protease dimerization inhibitors. However, no direct evidence of dimerization inhibition by such compounds has been documented. The present report represents the first demonstration that non-peptidic small molecule agents can disrupt protease dimerization.

The structural feature that is in common to the four dimerization inhibitors (TMC126 (33), GRL-98065 (36), DRV (24), and BCV (37)) is that all of these agents contain the structure-based designed privileged cyclic ether-derived non-peptidyl P2 ligand, 3(*R*),3α(*S*),6α(*R*)-bistetrahydrofuranylurethane (bis-THF) and a sulfonamide isostere (22, 23). GRL-0036A and GRL-06579A (26) have bis-THF-related ligand instead of bis-THF. Crystallographic data of dimerized protease complexed with three dimerization inhibitors (GRL-98065 (36), TMC126,[3] and DRV (40)) have revealed that bis-THF forms three tight hydrogen bond interactions with Asp-29 and Asp-30, two highly conserved catalytic site amino acids. We also observed that TPV has the ability to disrupt protease dimerization. TPV, which does not possess the bis-THF component, also has interactions with both Asp-29 and Asp-30 through its pyridinesulfonamide group, as shown in crystallographic analysis of a dimerized protease complexed with TPV (41). Thus, the inhibition of protease dimerization is not inherent only to the bis-THF component.

Most of the dimerization inhibitors we examined in this study exerted potent activity against PI-resistant protease in addition to their potent activity to wild-type HIV-1. DRV is potent against $HIV\text{-}1_{NL4\text{-}3}$ variants exposed to and selected for resistance to saquinavir, indinavir, nelfinavir, and ritonavir (24). Crystal structures of HIV-1 protease with a single mutation (D30N, I50V, V82A, I84V, or L90M) complexed with DRV demonstrate that DRV not only binds to the same catalytic active site as it does for wild-type protease but also maintains hydrogen bond interactions with the backbone atoms of Asp-29 and Asp-30 (40, 42). GRL-06579A and GRL-98065 are also potent against multidrug resistant HIV-1 strains, and molecular modeling indicates that for multidrug-resistant clinical isolates, these inhibitors maintain many of the interactions to critical active site residues (26, 36). TPV, which is active against HIV-1 carrying multidrug-resistant protease, also maintains critical hydrogen bond interactions with backbone atoms in the catalytic active site of mutant protease (43).

It is of note that the D30N-carrying HIV-1 variant is infectious and replication-competent (34). Structural studies do not show any hydrogen bond interactions between two monomer proteases mediated through Asp-30, and the FRET-HIV-1 expression assay showed that D30N mutant did not disrupt protease dimerization. This suggests that Asp-30 is not a critical residue for disrupting protease dimerization, and the interaction of these inhibitors with Asp-30 is not linked to the observed dimerization inhibition. However, potential interactions of dimerization inhibitors such as DRV involving Asp-29 could be critical, because D29N and D29A mutations disrupted protease dimer formation (Fig. 2*E*). Our analysis using the FRET-HIV-1 expression assay also revealed that the introduction of T26A and R87K to HIV-1 protease disrupted protease dimerization (the average $CFP^{A/B}$ ratios were all <1.0 (Fig. 2*E*)). If the protease monomer takes a configuration comparable to that in the dimerized protease, it is possible that the hydrogen bonding of the inhibitors with Asp-29, and/or Thr-26 and Arg-87, both of which are in the vicinity of Asp-29 and could be critical for dimerization, could be associated with the disruption of dimerization process through affecting the intermolecular and/or intramolecular hydrogen bond network (Fig. 2, *B–D*). In this regard, Ishima *et al.* (30) have shown that a truncated protease monomer takes a configuration similar to the one in the mature dimerized protease; however, it is unknown whether the untruncated monomer subunit takes a similar mature configuration. Furthermore, it is not known as to what stage of protease maturation (before dimerization) the dimerization inhibitors reported here bind to the monomer subunit in.

Another possible mechanism of the dimerization inhibition by the agents reported here is that they might interact with another dimerization interface formed by an interdigitation of the N- and C-terminal portions of each monomer (residues 1–5 and 95–99 (Fig. 2*A*)). In this regard, when we introduced a Pro-1 to Ala substitution (P1A), Q2A, I3A, T4A, L5A, T96A, L97A, N98A, or F99A into the replication-competent $HIV\text{-}1_{NL4\text{-}3}$, five substitutions (I3A, L5A, T96A, L97A, and F99A) produced the ratios of less than 1.0, strongly suggesting that most of the protease monomer subunit failed to dimerize with each of these five substitutions. These data confirmed the five amino acids at the N terminus and those at the C terminus are critical for protease dimerization (30–32). There are no polar interactions involving Q2A or T4A, so it is not surprising that

[3] Y. Koh, S. Matsumi, D. Das, M. Amano, D. A. Davis, J. Li, S. Leschenko, A. Baldridge, T. Shioda, R. Yarchoan, A. K. Ghosh, and H. Mitsuya, unpublished observation.

these mutations did not affect dimer formation. However, the failure of P1A and N98A to disrupt dimerization does not necessarily indicate that these amino acids are not critical for protease dimerization. It is possible that conversion to a residue other than alanine may disrupt dimerization.

In the present study, DRV failed to dissociate mature protease dimer (Fig. 5). It is of note that mature dimerized protease has as many as 12 hydrogen bonds in the N- and C-terminal regions, which may explain in part why DRV failed to dissociate two subunits of mature protease. These data also suggest that protease dimerization is inhibited before the association of two protease subunits occurs, probably when protease is in the form of nascent Gag-Pol polyprotein. However, the absence of structural data of nascent forms of protease subunit-containing polyprotein makes it difficult to conclusively predict how the dimerization inhibitors inhibit protease dimerization.

It is noteworthy that the D25N substitution, which is known to render HIV-1 protease enzymatically inactive (44), failed to disrupt dimerization (Fig. 2*E*), showing that catalytically inactive subunits are still capable of undergoing dimerization. This observation indicates that the dimerization inhibition is a differing event than the process that confers catalytic activity on two protease monomer subunits.

DRV has a potent activity against a wide spectrum of HIV-1 isolates, including highly multiprotease-inhibitor-resistant HIV-1 variants. The emergence of DRV-resistant HIV-1 seems to be substantially delayed both *in vitro* (45) and clinical settings (46, 47). One can speculate that DRV inhibits protease dimerization, leaving catalytically inert monomers, but if certain monomers escape from DRV and achieve the mature dimer form, DRV again blocks the proteolytic action of mature (wild-type and mutant) protease as a conventional protease inhibitor. This dual anti-HIV-1 function of DRV may explain why DRV is such a highly effective anti-HIV-1 therapeutic and differentiates it from many of the currently available protease inhibitors (46, 47). It is of note that the plasma concentrations of DRV achieved in those receiving DRV and ritonavir remain >2 μg/ml or $\sim$3.66 μM (48). These concentrations substantially exceed the concentration of DRV effectively disrupting protease dimerization (0.1 μM in culture as shown in Fig. 4*C*). Hence, the dimerization inhibition by DRV should be in operation in the clinical settings. Furthermore, DRV could more efficiently disrupt protease dimerization in individuals with HIV-1 infection receiving DRV and ritonavir, because the protease expression levels upon transfection in this study appear to be considerably greater than the protease expression levels *in vivo*, considering that the p24 production levels could be as high as 500–1500 ng/ml by 5 days following transfection of COS7 cells with plasmids used in the FRET-HIV-1 expression assay. The inhibition of HIV-1 protease dimerization by non-peptidyl small molecule agents represents a unique mechanism of HIV-1 intervention, and the dually functional inhibitors reported here might serve as potential candidates as a new class of therapeutic agents for HIV-1 infection and AIDS. The present data should not only help design and examine agents that potentially inhibit HIV-1 protease dimerization but also should give new insights into the process and dynamics of HIV-1 protease dimerization *per se*.

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Polymorphisms in CCR5 chemokine receptor gene in Japan

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Summary

Mutations in the human CC chemokine receptor 5 (*CCR5*) gene may alter the expression or function of the protein product, thereby altering chemokine binding/signalling or human immunodeficiency virus type 1 (HIV-1) infection of the cells that normally express CCR5 protein. We performed a systematic survey of natural sequence variations in an 8.1-kb region of the entire *CCR5* gene as well as *CCR2 V64I* in 50 Japanese subjects and evaluated the effects of those variations on *CCR5* promoter activity. We also analysed *CCR5* promoters and *CCR2 V64I* in 80 more Japanese and 186 Thais. There was no 32-bp deletion observed in Caucasians, but two types of non-synonymous substitutions were found in *CCR5* genes of Japanese. Our results showed several novel characteristics of the *CCR2-CCR5* haplotype structure that were not reported from studies on Caucasians and African-Americans. Specifically, we were able to show that the G allele at position −2852 from the *CCR5* open reading frame in Japanese and Thais is the representative of the CCR5 promoter haplotype that was reported to be associated with rapid progression to acquired immune deficiency syndrome (AIDS) in HIV-1-infected individuals. Furthermore, nearly all non-synonymous polymorphisms in Japanese *CCR5* occurred in haplotypes with elevated promoter activity. We thus hypothesized that there was a certain selective pressure favouring low levels of *CCR5* expression during human evolution.

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Introduction

Human CC chemokine receptor 5 (CCR5) mediates the activation of cells by the CC chemokines macrophage inflammatory protein-1α and -1β (MIP-1α or CCL3, and MIP-1β or CCL4), and regulated on activation normal T cells expressed and secreted (RANTES or CCL5). Identification of CCR5 as an essential co-receptor for the cellular entry of human immunodeficiency virus type 1 (HIV-1) R5 strains (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996), which is preferentially transmitted between individuals (Zhu *et al.*, 1993), has led to many studies on CCR5 and its ligands. Mutations in the *CCR5* gene may alter the expression or function of the protein product, thereby altering chemokine binding/signalling or HIV-1 infection of the cells that normally express the CCR5 protein. Indeed, a 32-base pair (bp) deletion in the *CCR5* coding region (*CCR5Δ32*), which results in a premature termination codon, confers marked resistance to HIV-1 infection in homozygotes (Liu *et al.*, 1996; Samson *et al.*, 1996), and delays progression to AIDS and death by 2–3 years in patients heterozygous for this allele (Dean *et al.*, 1996; Huang *et al.*, 1996). The delayed progression to AIDS and death in individuals heterozygous for *CCR5Δ32* has been attributed to reduced cell surface expression of CCR5 (Wu *et al.*, 1997), which is speculated to result in a slower rate of replication and spread of the virus. Although heterozygosity for *CCR5Δ32* is associated with a small reduction in surface expression of CCR5, cells from individuals with the wild-type *CCR5* genotype showed a wide range in surface expression (Wu *et al.*, 1997), raising the possibility that polymorphisms, other than *CCR5Δ32*, exert significant effects on CCR5 expression. For example, the much more rarely occurring *CCR5 m303A* is a nonsense mutation of the *CCR5* coding region that exerts effects similar to those of *CCR5Δ32* (Quillent *et al.*, 1998). Moreover, Asian-specific *CCR5 893(−)* is a single-nucleotide deletion in the *CCR5* coding region, and the levels of CCR5 expression on the surface of CD4 positive cells are greatly reduced in individuals bearing this allele (Shioda *et al.*, 2001). The *CCR2* mutation, *CCR2 64I*, which is in strong linkage disequilibrium with another mutation *CCR5 −1835T* (*CCR5 927T* in numbering system C, see Materials and Methods for definitions of numbering systems A, B, and C) in the second intron of the *CCR5*

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gene, is also associated with a delay in HIV-1 disease progression (Smith *et al.*, 1997; Kostrikis *et al.*, 1998; Mummidi *et al.*, 1998).

With respect to the promoter region, Martin *et al.* (1998) identified 10 polymorphic nucleotide positions in the 517 bp region of Caucasian *CCR5* promoters, and described 10 haplotypes in this region. Of these, the *P1* haplotype has G, C, and A at the 208th, 627th, and 676th positions, respectively, from the transcription start site of the *CCR5* gene. Although both *CCR2 64I* and *CCR5Δ32* were consistently found in the *CCR5 P1* haplotype, the homozygotes for (*CCR2 64V/CCR5 P1*/lacking *CCR5Δ32*) haplotype exhibited an epidemiological association with rapid progression to AIDS (Martin *et al.*, 1998). McDermott *et al.* (1998) reported that the presence of homozygous for *CCR5 59029G* lacking *CCR5Δ32* and *CCR2 64I* caused a delay by 3.8 years in the progression to AIDS compared to that seen in patients homozygous for *59029 A*. Position 59029 in McDermott's report corresponds to position 303 from the transcription start site. Since different reports have adopted different numbering systems for the *CCR5* gene (Moriuchi *et al.*, 1997; Guignard *et al.*, 1998; Kostrikis *et al.*, 1998; Martin *et al.*, 1998; McDermott *et al.*, 1998; Mummidi *et al.*, 1998), Carrington *et al.* (1999) proposed a new numbering system starting from the translation start codon. Using this numbering system, Mummidi *et al.* (2000) reported six human haplogroups (*HHA, -B, -C, -D, -E, -F* and -G) according to *CCR2 64*, *CCR5Δ32*, and eight polymorphic positions (–2733, –2554, –2459, –2135, –2132, –2086, and –1835) in the 926 bp region of Caucasian and African-American *CCR5* promoters.

It is well known that genetic polymorphisms and haplotype structures can vary among ethnic groups. *CCR5Δ32* is extremely rare in Asian populations (Martinson *et al.*, 1997). To explore the *CCR2-CCR5* haplotype structure in an Asian population, we performed a systematic survey of natural sequence variations in an 8.1-kb region of the entire *CCR5* gene as well as of *CCR2 V64I* in 50 Japanese individuals and evaluated effects of those variations on *CCR5* promoter activity. Our results show several novel characteristics of the *CCR2-CCR5* haplotype structure that were not reported from studies on Caucasians and African-Americans.

Materials and methods

Clinical samples

Blood samples were collected with anticoagulant from randomly selected, non-related 80 non-HIV-1-infected and 50 HIV-1-infected Japanese at the outpatient clinic of the Institute of Medical Science, the University of Tokyo, Tokyo, Japan, after their written informed consent had been obtained. Blood samples were also collected with anticoagulant from randomly selected, non-related 97 non-HIV-1-infected and 89 HIV-1-infected Thai subjects at the Bamrasnaradura Institute, Nonthaburi, Thailand, after their written informed consent had been obtained. Peripheral blood mononuclear cells (PBMC) were obtained from blood with the Ficoll–Histopaque method, and DNA was extracted from the PBMC with a previously described method (Shioda *et al.*, 1994). The six French samples with *CCR5Δ32* (one homozygote and five heterozygotes) used for this study were described previously (Meyer *et al.*, 1997; Magierowska *et al.*, 1999).

Genotyping of CCR5 and CCR2

The 8.1-kb *CCR5* target region was amplified from each of the genomic DNA samples in seven (R5-1 to R5-7) overlapping segments using the amplification primers shown in Table 1. Polymerase chain reaction (PCR) was performed in a 50-μL reaction mixture containing 1 μg of DNA. Thermal cycling was performed with an initial 94 °C for 3 min followed by 40 cycles at 94 °C for 30 s, primer annealing for 30 s and primer extension at 72 °C and a final extension at 72 °C for 7 min. The PCR conditions of annealing temperature and extension time were shown in Table 2. The positions specified in *CCR5* were based on the numbering system proposed by Carrington *et al.* (1999). We defined this system as numbering system A and used this system in the present study. The positions in *CCR2* were based on the sequence with GenBank accession number U95626. McDermott *et al.* (1998) used this system, and we defined this system as numbering system B. The numbering system starting from the transcription start site (Martin *et al.*, 1998) was defined as numbering system C in the present study. Table 3 shows polymorphic positions in those three different numbering systems. Sequencing reactions were performed according to the dideoxy-chain-termination method using the ABI PRISM 377 (Applied Biosystems, Foster City, CA, USA) automated DNA sequencer. The sequences of primers used for sequencing reaction are shown in Table 1. The ABI sequence software (version 2.1.2) was used for lane tracking and first pass base-calling (PerkinElmer, Wellesley, MA, USA). Variant sites identified by both software and visual inspection were scored and entered into a database for subsequent analysis. Each variant site was confirmed by reamplifying and resequencing the variant site from the opposite strand. The linkage disequilibrium coefficient was calculated by using a software (ARLEQUIN version 2.01, Gennetica and Biometry Laboratory, Geneva, Switzerland).

Haplotype determination

Haplotypes were inferred from the samples by using a heuristic algorithm based on population genetic principles (Clark, 1990). Direct molecular haplotyping was used to confirm linkage relationships in compound heterozygotes using allele-specific PCR combined with direct sequencing and limited cloning of the specific products. All inferred haplotypes were confirmed with these molecular haplotyping techniques.

Allele-specific PCR

For this procedure, the amplification refractory mutation system (ARMS) was used (Newton *et al.*, 1989). An

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Table 1. Primer sequences.

Sense primer	5′ to 3′ sequence	Position[a]	Position[b]	Fragment	Antisense primer	5′ to 3′ sequence	Position[a]	Position[b]	Fragment
1. Primers used for PCR amplification and sequencing									
CCR2F	ATGCTGTCCACATCTCGTTC		46106 to 46125	R2-1	CCR2R	GGAAATTATTCCATCCTCGTG		46709 to 46689	R2-1
P61	AGCAAGGAGACAGCAAAGCA	−4796 to −4777	56696 to 56715	R5-1	P63	CCTTGTATTCACTGGTAATCC	−3924 to −3944	57568 to 57548	R5-1
P66	AGAACCAGCAATTGCCACAAACAGA	−4012 to −3988	57480 to 57504	R5-2	P68	GCTTCCGTGACCTTGGCTCTAG	−2941 to −2962	58547 to 58526	R5-2
LK81	AATTTTGCTGTTTGGGGTCTC	−3022 to −3002	58466 to 58486	R5-3	LK83	CTGATTATCTTAAGAGTTGC	−2369 to −2388	59119 to 59100	R5-3
LK84	AAGTCCAGGATCCCCCTCTA	−2443 to −2424	59045 to 59064	R5-4	LK87	CATTCCAAACTGTGACCCTTTCC	−1756 to −1778	59732 to 59710	R5-4
CR53F	TCCAGTGAGAAAAGCCCGTAAATA	−2162 to −2139	59326 to 59349	R5-5	CR53R	TGCCCACAAAACCAAAGATGAACA	142 to 119	61624 to 61601	R5-5
CKR5a +	CAGTTTGCATTCATGGAGGG	−84 to −65	61399 to 61418	R5-6	CKR5a−	CTAAGCCATGTGCACAACTC	1110 to 1091	62592 to 62573	R5-6
CR52F	GGAAATATCTGTGGGCTTGT	1038 to 1057	62520 to 62539	R5-7, IIIa, IIIb	CR52R	CTGCTTATAAAATGCTCTGG	3383 to 3364	64865 to 64846	R5-7
2. Primers used for sequencing									
P62F	TGAACCCCCCTGTCCTGAG	−4425 to −4407	57067 to 57085	R5-1, Ia, Ib	P62R	GCTAGATGGGAAACAAGGTGAGGA	−4295 to −4318	57197 to 57174	R5-1
P67F	GATTCGCTGTCCTTGTTATG	−3533 to −3514	57955 to 57974	R5-2, Ia, Ib	P67R	CCCAGCGATCAAGACACCC	−3436 to −3454	58052 to 58034	R5-2, Ia, Ib
LK82F	AGAACCTGAACTTGACCAT	−2811 to −2793	58677 to 58695	R5-3, Ia, Ib	LK82R	TTTTAACTATGGGCTCACG	−2573 to −2591	58915 to 58897	R5-3, Ia, Ib
LK85	GTGTAGTGGGATGAGCAGAGA	−2198 to −2178	59290 to 59310	R5-4, II a, II b	LK85R	TTTGAAGGAGGGTGGAGTT	−2019 to −2037	59469 to 59451	R5-4
433	ACACCAAGTGCTCATACAAT	−1825 to −1806	59663 to 59682	R5-5	1552R	TAGAGTTAGCCCACAAAGAA	−685 to −704	60801 to 60782	R5-5, IIa, IIb
CR53S1	TTTTCTAGGGGCTTCTCTCA	−1367 to −1348	60121 to 60140	R5-5, IIa, IIb	2046 R	CTGCTAGCTTCCCTGTCCACT	−187 to −207	61296 to 61276	R5-5, IIIa, IIIb
CR53S2	GCAGGCTTCCCGCATTCAAA	−938 to −919	60548 to 60567	R5-5, IIa, IIb	CKR5e−	GCTGCGATTTGCTTCAC	89 to 73	61571 to 61555	R5-5
1770	ACTTGGAGGGGTGAGGTGAG	−486 to −467	61000 to 61019	R5-5, IIa, IIb	CKR5d−	GAAGATTCCAGAGAAGAAGCC	351 to 331	61833 to 61813	R5-6
CKR5b +	GAGCATGACTGACATCTACC	186 to 205	61668 to 61687	R5-6	322	GTGAAGATAAGCCTCACAGCC	716 to 696	62198 to 62178	R5-6
CKR5c +	CTGTGTTTGCGTCTCTCC	467 to 484	61949 to 61966	R5-6, IIIa, IIIb	CR5R1	TGTCTTTTCTCCCCATAGCA	1527 to 1508	63009 to 62990	R5-7
CKR5d +	CAGTAGCTCTAACAGGTTGG	807 to 824	62289 to 62308	R5-6	CR5R4	TCACCACTATAGGGACCCCTT	2578 to 2559	64060 to 64041	R5-7
CR5S1	GCTGATTCTTGAGTTTAGTG	1387 to 1406	62869 to 62888	R5-7					
CR5S2	CATGGGGAGGAAGGACAAGG	1768 to 1787	63250 to 63269	R5-7, IIIa, IIIb, IVa					
CR5S3	GGAGGAGGAGGTTTAGGTCA	2168 to 2188	63650 to 63670	R5-7					
CR5S4	AAGGGGTCCCATAGAGGTGA	2559 to 2578	64041 to 64060	R5-7					
CR5S5	TGAATTTGGGGGATGGCTAA	2954 to 2973	64436 to 64455	R5-7					
3. Primers used for allele-specific PCR amplification									
627UA	TCCTATGGGGTGTCCGAATGT	−4638 to −4618	56854 to 56874	Ia, Ib	627N	GAATAGATCTCTGGTCTGtAA	−2115 −2135	59373 to 59353	Ia
303 N	GAGAGTGGAGAAAAAGGtGG	−2478 to −2459	59010 to 59029	IIa	627M	GAATAGATCTCTGGTCTGtAG	−2115 −2135	59373 to 59353	Ib
303 M	GAGAGTGGAGAAAAAGGtGA	−2478 to −2459	59010 to 59029	IIb	303DA	TAAGAAACTGGGTCAAGCAT	−316 −335	61167 to 61148	IIa, IIb
2398 N	CTGTCTCACAACAACAtCAG	−381 to −362	61105 to 61124	IIIa	2398DA	AACCAGACCCATCCTTTTAC	2226 2207	63708 to 63689	IIIa, IIIb
2398 M	CTGTCTCACAACAACAtCAA	−381 to −362	61105 to 61124	IIIb	5765M	ATGTGCACAATCATATGtGAC	2939 2919	64421 to 64401	IVa
5765UA	GACTTAGAACCAGGCGAGAG	1602 to 1621	63084 to 63103	IVa					

Underline; allele-specific nucleotide.
Lower case t; additional internal (position −3) mismatch that increases specificity of allele-specific PCR.
[a]Position according to Mummidi *et al.* (2000) (numbering system A).
[b]Position in the baseline sequence (GenBank accession number U95626, numbering system B).

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Table 2. Amplification protocol

Fragment	Sense primer	Antisense primer	Annealing temp (C)	Extension time (s)	Length (bp)	Position[a]	Position[b]
R2-1	CCR2F	CCR2R	60	30	604		46106 to 46709
R5-1	P61	P63	60	30	873	−4796 to −3924	56696 to 57568
R5-2	P66	P68	60	30	1072	−4012 to −2941	57480 to 58547
R5-3	LK81	LK83	60	30	654	−3022 to −2369	58466 to 59119
R5-4	LK84	LK87	60	30	688	−2443 to −1756	59045 to 59732
R5-5	CR53F	CR53R	58	120	2303	−2162 to 142	59326 to 61624
R5-6	CKR5a +	CKR5a −	58	60	1194	−84 to 1110	61399 to 62592
R5-7	CR52F	CR52R	56	120	2346	1038 to 3383	62520 to 64865
Ia	627UA	627N	55	120	2520	−4637 to −2115	56854 to 59373
Ib	627UA	627M	55	120	2520	−4637 to −2115	56854 to 59373
IIa	303N	303DA	61	120	2158	−2478 to −318	59010 to 61167
IIb	303M	303DA	61	120	2158	−2478 to −318	59010 to 61167
IIIa	2398N	2398DA	63	120	2607	−382 to 2226	61105 to 63708
IIIb	2398M	2398DA	63	120	2607	−382 to 2226	61105 to 63708
IVa	5765UA	5765M	60	70	1338	1602 to 2939	63084 to 64421

[a]Position according to Mummidi *et al.* (2000) (numbering system A).
[b]Position in the baseline sequence (GenBank accession number U95626, numbering system B).

ARMS primer contained allele-specific nucleotide at its 3′ end. Therefore, we could specifically amplify DNA sequence on the chromosome carrying the allele. Allele-specific products ranged in size from 1.3 to 2.6 kb. Primers used for allele-specific PCR are shown in Table 1. PCR and sequencing were performed in the same manner as described above (Table 1 and Table 2).

PCR-restriction fragment length polymorphism analysis

The region spanning CCR5 −2852 was amplified by PCR with primer pair P67F and LK83 (Table 1). PCR was performed for 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Digestion of the 1164-bp amplified products with Aci I yielded 680- and 464-bp fragments when position −2852 was G.

Promoter assay

Luciferase reporter gene assays were performed as described previously (Liu *et al.*, 1999). Briefly, the promoter region of each haplotype was PCR-amplified and cloned into a pGL3-Basic vector carrying the firefly luciferase gene (Promega, Madison, WI, USA). All constructs were verified for sequence authenticity by sequencing the entire insert, and 5 μg of the resultant constructs was transfected with DMRIE-C (Invitrogen, Carlsbad, CA, USA) into monocytic U937 cells. Transfection efficiency was normalized by cotransfecting 0.2 μg of pRL-TK, which expresses *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter. Cells were harvested 40 h after transfection, and firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Relative luciferase expression was derived with the following equation: (firefly luciferase activity of CCR5 promoter construct/*Renilla* luciferase activity)/(firefly luciferase activity of promoterless vector pGL3-Basic/*Renilla* luciferase activity). Data points are means derived from measurements of two independent clones of each of the constructs.

Results

Sequence polymorphisms in human *CCR5* gene

The human *CCR5* gene, located on chromosome 3, has four exons distributed across 6 kb of genomic DNA (Mummidi *et al.*, 1997). We amplified seven overlapping DNA fragments covering an 8.1-kb genomic region spanning 2031 bp of the upstream non-coding region, the exons and introns in their entirety, and 81 bp of the 3′ untranscribed region of *CCR5* gene. The fragments were obtained from 50 randomly selected Japanese subjects comprising 38 non-HIV-1-infected and 12 HIV-1-infected individuals. Direct sequencing of the PCR fragments allowed us to identify 25 polymorphic positions in this region (Table 3). Among the 25 variable positions, four were insertions or deletions, and 21 were single-nucleotide substitutions. There was one additional C-to-T substitution in the 4-base insertion at position −3887 (Table 3). Except for this tri-allelic site, all the other variations in these samples were di-allelic. No *CCR5Δ32* or *CCR5 m303* was found in these Japanese samples, which confirms previously reported observations (Martinson *et al.*, 1997; Quillent *et al.*, 1998).The genotype frequencies in 50 individuals analysed in this study were consistent with the Hardy–Weinberg equilibrium, suggesting the absence of any tendency towards an excess or deficiency of any particular genotype. Overall, nucleotide diversity was 0.00108, which is the equivalent of approximately one variant in every 926 bp. The sequence diversity in coding-region was lower (0.00012) than in non-coding region (0.00122). In addition to the *CCR5* gene, we analysed the *CCR2* gene of the same 50 individuals for the *CCR2 64I* allele, which has been reported to be associated with delayed HIV-1

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Table 3. Sequence variants identified in *CCR2* and *CCR5* genes and used to define haplotypes

						Allele frequency			
Site[a]	Position[b]	Position[c]	Position[d]	Variant	NCBI SNP ID	Japanese[j]	Thais[k]	Caucasian	African-American
1		46295		G, A (CCR2 V64I)	rs1799864	0.67, 0.33	0.83, 0.17	0.90, 0.10[l]	0.84, 0.16[l]
2	−4358	57134		G, A	rs7637813	0.20, 0.80			
3	−3900	57592		A, C	rs2856757	0.57, 0.43			
4[e]	−3888 ~ −3884	57604		GCTAT, G, GTTAT	rs10577983	0.56, 0.43, 0.01			
5	−3458	58030		G, T	rs2734225	0.57, 0.43			
6	−2852	58636		G, A	rs2227010	0.21, 0.79	0.18, 0.82		
7	−2554	58934	208	G, T	rs2734648	0.57, 0.43			
8	−2459	59029	303	A, G	rs1799987	0.56, 0.44		0.57, 0.43[m]	0.43, 0.57[m]
9	−2135	59353	627	C, T	rs1799988	0.55, 0.45	0.35, 0.65		
10	−2086	59402	676	A, G	rs1800023	0.60, 0.40	0.39, 0.61		
11	−1835	59653	927	C, T	rs3181036	0.66, 0.34	0.83, 0.17	0.90, 0.10[n]	0.80, 0.20[n]
12[f]	−1132 ~ −1130	60356		CAG, C	rs3054375	0.57, 0.43			
13	−1060	60426		C, T	rs2856762	0.99, 0.01			
14	−976	60510		C, T	rs2254089	0.57, 0.43			
15	−651	60835		C, T	rs2856764	0.57, 0.43			
16	−451	61035		C, T		0.97, 0.03			
17	−444	61042		G, A	rs2856765	0.57, 0.43			
18	−362	61124		A, G		0.57, 0.43			
19[g]	−361 ~ −359	61125		CAAC, C		0.57, 0.43			
20[h]	668	62150		G, A	rs1800452	0.95, 0.05			
21[h,i]	893	62375		C, −		0.99, 0.01			
22	1171	62653		C, G		0.98, 0.02			
23	1823	63305		C, T	rs17765882	0.99, 0.01			
24	2077	63559		G, T	rs1800874	0.57, 0.43			
25	2150	63632		G, C		0.98, 0.02			
26	2919	64401		G, T	rs746492	0.56, 0.44			

[a]Site number assigned to a variable character used to define haplotypes in order 5′ to 3′.
[b]Position according to Mummidi *et al.* (2000) (numbering system A).
[c]Position in the baseline sequence (GenBank accession number U95626, numbering system B).
[d]Position according to Martin *et al.* (1998) (numbering system C).
[e]There was one additional C-to-T substitution within the duplication of CTAT at position −3887. Except for this tri-allelic site, all the other variations were di-allelic in these samples.
[f]Insertion of AG.
[g]Duplication of CAA.
[h]Variant in coding region.
[i]Deletion of C.
[j]Allele frequencies of site1,6,9,10 and 11 in 130 Japanese. Allele frequencies of other sites in 50 Japanese.
[k]Allele frequencies of site1,6,9,10 and 11 in 186 Thais.
[l]Allele frequencies reported by Martin *et al.* (1998).
[m]Allele frequencies reported by McDermott *et al.* (1998).
[n]Allele frequencies reported by Mummidi *et al.* (1998).

disease progression (Kostrikis *et al.*, 1998; Smith *et al.*, 1997). The *CCR2* gene was found to be located 14-kb upstream of the *CCR5* gene. The *CCR2* and *CCR5* genotypes of the 50 individuals analysed were sorted and renumbered according to their genotypes and are shown in Fig. 1. Using the method described by Clark *et al.* (1990) we were able to infer all the haplotypes that were subsequently confirmed by allele-specific PCR as well as limited cloning and sequencing of the PCR-amplified fragments. As shown in Fig. 2, we were able to identify 12 independent haplotypes that showed a high degree of linkage disequilibrium of multiple variable sites. *CCR2 64I* was found in *H6*, *H7*, and *H8*, and *CCR5* −1835T in *H6*, *H7*, *H8*, *H9*, and *H10*. As reported previously, *CCR2 64I* is in strong linkage disequilibrium with *CCR5 −1835T* (D′ = 1). *CCR5 P1* (*−2554G*, *−2135C* and *−2086A*, which correspond to 208G, 627C, and 676 A, respectively, in numbering system C) was found in *H6*, *H7*, *H8*, *H9*, *H10* and *H11*. The *−2459A*, which corresponds to 59029 A in numbering system B, was in complete linkage disequilibrium with *CCR5 P1* and found in *H6*, *H7*, *H8*, *H9*, *H10* and *H11*. All the samples showed C in nucleotide position −2132, where T is located in approximately 11.3% of African-American chromosomes (Bamshad *et al.*, 2002).

Twelve haplotypes were subsequently categorized into four major haplotype groups based on the polymorphisms at positions −2135, −2086, and −1835 (Fig. 2). Haplotype group I, comprising *H1*, *H2*, *H3*, *H4*, and *H5*, seems to

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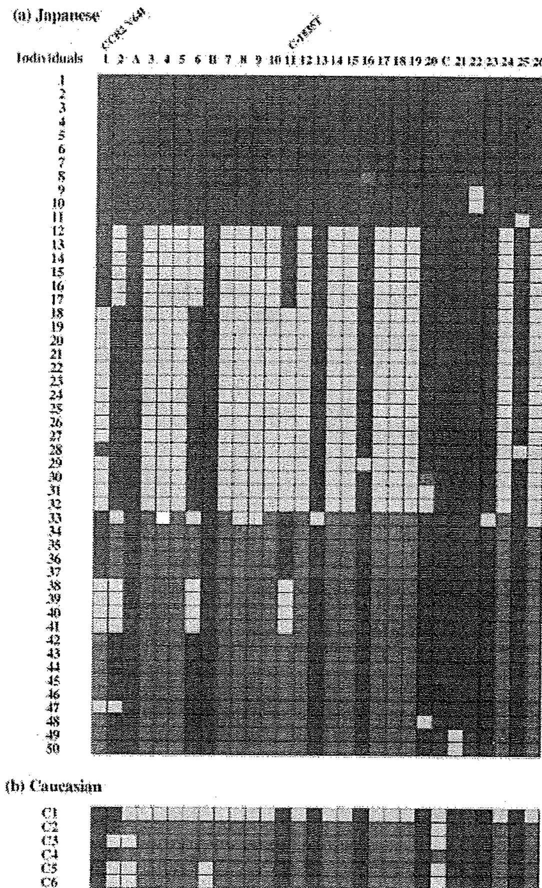

Figure 1. *CCR2* and *CCR5* genotypes of the 50 Japanese (a) and six French (b) subjects at each of the polymorphic sites. Individual samples were sorted and re-numbered according to their genotype, and sample identifiers are shown on the left side of the array. The 26 polymorphic sites observed in Japanese are numbered consecutively across the top. A, B, and C denote polymorphisms specifically observed in the French samples. Positions of *CCR2 V64I* and *CCR5 –1835T* are indicated. Genotypes for each individual were assigned directly based on the fluorescence sequencing trace at each position. At every site, individuals homozygous for the baseline allele (U95626) are shown in red, heterozygotes in yellow and those homozygous for the variant in blue. The heterozygote for the four-base insertion and the additional substitution at position –3887 is shown in white.

correspond to *HHC* according to Mummidi *et al.* (2000). *CCR5 –1835T* was found only in haplotype group II (*H6*, *H7*, *H8*, *H9*, and *H10*), which corresponds to *HHF*. *CCR2 64I* and the Asian-specific disrupting mutation in the *CCR5* open reading frame, *CCR5 893 (–)* (Shioda *et al.*, 2001), was found in haplotype group II. Haplotype group III (*H11*) lacked the *CCR2 64I* but showed sequences identical to the consensus sequence of haplotype group II except for nucleotides at positions *–2852G* and *–1835C*. Haplotype groups III and IV (*H12*) correspond to *HHE* and *HHA*, respectively. None of the single mutations was associated with *CCR2 64I*, except for *CCR5 –1835T*. No *HHB*, *HHD*, *HHG*1* or *HHG*2* was observed in the 100 Japanese chromosome 3. The frequencies of *HHA*, *HHC*, *HHE*, and *HHF* in these chromosomes were similar to those reported for 27 Asians (Bamshad *et al.*, 2002). Mummidi *et al.* (2000) previously pointed out that disease-accelerating genotypes (*CCR5 P1* or *59029 A* lacking *CCR2 64I* and *CCR5Δ32*) are a mixture of haplotypes *HHE*, *HHF*1*, and *HHG*1*. In our study, those disease-accelerating alleles were found in *H9* (*HHF*1*), *H10* (*HHF*1*), and *H11* (*HHE*). On the other hand, the –2852 G allele was found in *H11* (*HHE*), but not in *H9* (*HHF*1*) or *H10* (*HHF*1*). Therefore, the *–2852G* was found to be associated with 90% (19/21) of the disease-accelerating alleles in those Japanese subjects (Fig. 2).

To determine the sequence polymorphisms associated with Caucasian-specific *CCR5Δ32*, we also analysed six French individuals carrying *CCR5Δ32*. The results showed that the nucleotide sequence of the haplotype with *CCR5Δ32* (*H13* in Fig. 2) was very similar to that of the haplotypes containing *CCR2 64I*, *CCR5 –1835T*, or *CCR5 P1*. Phylogenetic analysis of all the haplotypes defined in Fig. 2 showed that there were two major groups (H1-H5 and H6-H13) (Fig. 3), one of which contained all the haplotypes with *CCR2 64I*, *CCR5 –1835T*, or *CCR5 P1*. These data indicate that all the HIV-1 disease-modifying *CCR5* haplotypes were in fact very similar to each other regardless of the direction of their effects on HIV-1 diseases.

Effects on promoter activity by sequence polymorphisms in the regulatory sequences of the CCR5 gene

To compare *CCR5* promoter activity among the three major haplotype groups, I, II, and III, we constructed a series of firefly luciferase reporter fusions containing various lengths of the 5′-non-coding region of *CCR5*, and analysed their promoter activity in monocytic U937 cells. Two distinct promoters for the *CCR5* gene, upstream (Pu) and downstream (Pd), have been identified (Mummidi *et al.*, 1997). Constructs labelled with Pu (Pu-I, Pu-IIa, Pu-IIb, and Pu-III) contained both Pu and Pd, while those labelled with Pd (Pd-I and Pd-III) contained only Pd (Fig. 4). The IIa promoter corresponded to *H6*, *H7*, and *H8*, while its IIb counterpart corresponded to *H9*.

Our results showed that promoters of haplotype groups II and III expressed higher luciferase activity than those of haplotype group I in both constructs (Fig. 4). These results are especially relevant in view of the fact that studies of HIV-1-infected individuals homozygous for a haplotype analogous to haplotype group III (*the CCR5 P1 lacking CCR2 64I and CCR5Δ32*) found that they progressed to AIDS more rapidly than those with other *CCR5* promoter genotypes (Martin *et al.*, 1998; McDermott *et al.*, 1998). One of these studies also reported that a *CCR5* promoter bearing *–2459 A* (*59029 A* in numbering system C), specific for haplotype groups II and III, expressed higher promoter activity than one bearing *–2459G* (McDermott *et al.*, 1998). In addition, Mummidi *et al.* (2000) demonstrated

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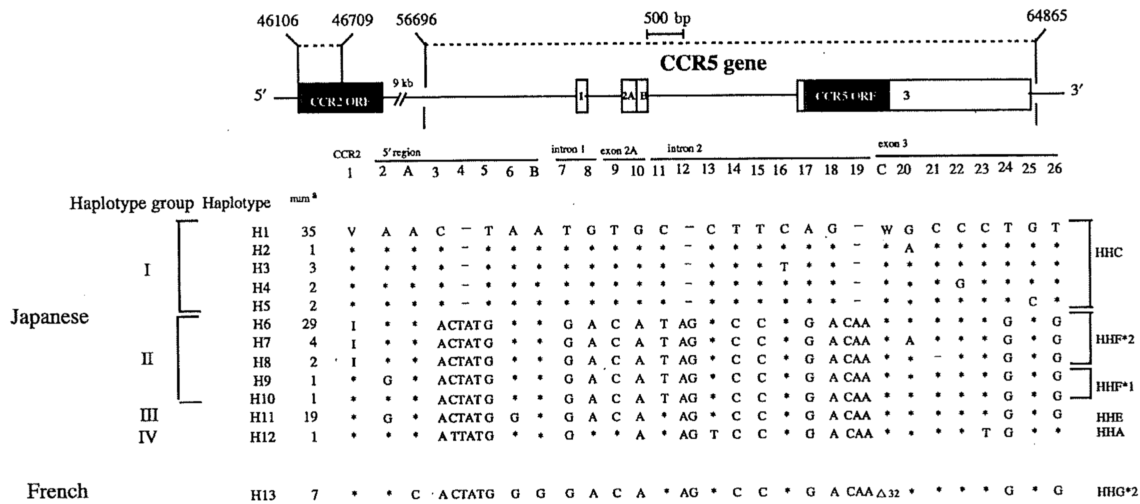

Figure 2. Map of *CCR2, CCR5* and nucleotide polymorphisms. White and black boxes indicate non-coding exons and open reading frames (ORF), respectively. Dotted lines signify the sequenced regions. PCR and sequence screening of eight segments of the *CCR2* and *CCR5* gene identified 29 nucleotide positions with genetic polymorphisms. Twenty-six polymorphic sites observed in Japanese are numbered consecutively across the top. A, B, and C denote polymorphisms specifically observed in the French samples. Combining the 29 polymorphic positions indicated 13 *CCR2-CCR5* haplotype alleles designated H1 through H13, with H1 being exactly the same as the GenBank U95626 sequence. Asterisks indicate nucleotides identical to those of H1; dashes represent deletion sites. W shows *CCR5* coding sequences without 32 bp deletion, while Δ32 indicates 32 bp deletion of the *CCR5* coding region. V and I in position 1 indicate *CCR2 64V* and *CCR2 64I*, respectively. [a]Number in this column represents the actual numbers of haplotypes identified in the 100 sequenced chromosomes. The human haplogroups according to Mummidi *et al.* (2000) are shown on the right.

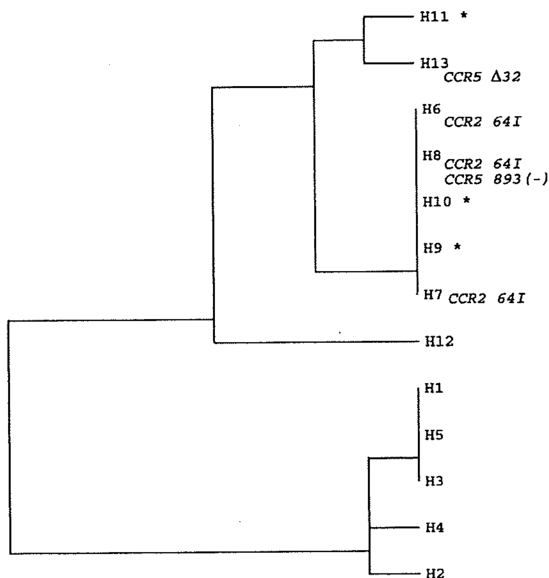

Figure 3. A phylogenetic tree of 13 *CCR2-CCR5* haplotypes defined in Fig. 2. The tree was produced with the UPGMA (unweighted pair-group method with arithmetic averages) method. Haplotypes with *CCR5Δ32, CCR2 64I, CCR5 893(–)* are shown. Asterisks denote haplotypes with *CCR5 P1 lacking CCR5Δ32 and CCR2 64I.*

that the *HHC* (haplotype group I) promoter construct demonstrated lower promoter activity than that of *HHF* (haplotype group II) and of *HHE* (haplotype group III). Our study further identified *–2135C* and *–2086A*, which are linked to *–2459A*, as mutations responsible for elevated promoter activity, since Pd-III was shown to express higher luciferase activity than Pd-I (Fig. 4).

To examine the effects of *CCR5 –1835T* on expression level, we generated two series of constructs with part of the intron containing position –1835 and the splicing acceptor site located immediately upstream of *CCR5* exon 4 (LSA and PdSA in Fig. 4). Again, promoters from haplotype groups II and III expressed higher luciferase activity than the promoter from haplotype group I in both the LSA and the PdSA constructs, and since there were no other differences between haplotype groups II and III, this suggests that *CCR5 –1835T* has no effect on CCR5 expression.

Novel method using PCR-restriction fragment length polymorphism for detecting HIV-1 disease-accelerating haplotype

As described above, in Japanese subjects, *–2852G* was associated with 90% of the disease-accelerating haplotype, this is, *CCR5 P1 lacking CCR5Δ32* and *CCR2 64I,* since no *CCR5Δ32* was identified in these individuals. The remaining 10% were accounted for by individuals with *CCR5 –1835T* without *CCR2 64I.* To confirm the strong linkage disequilibrium between *–2852G* and the disease-accelerating allele observed in 50 Japanese, we established a PCR-RFLP method to detect *–2852G* (see Materials and Methods) and used it to genotype 80 additional (38 HIV-1-infected and 42 non-HIV-1-infected) Japanese. *CCR5 C-2135T, G-2086A,* and *C-1835T* were genotyped by direct-sequencing of PCR product R5-4

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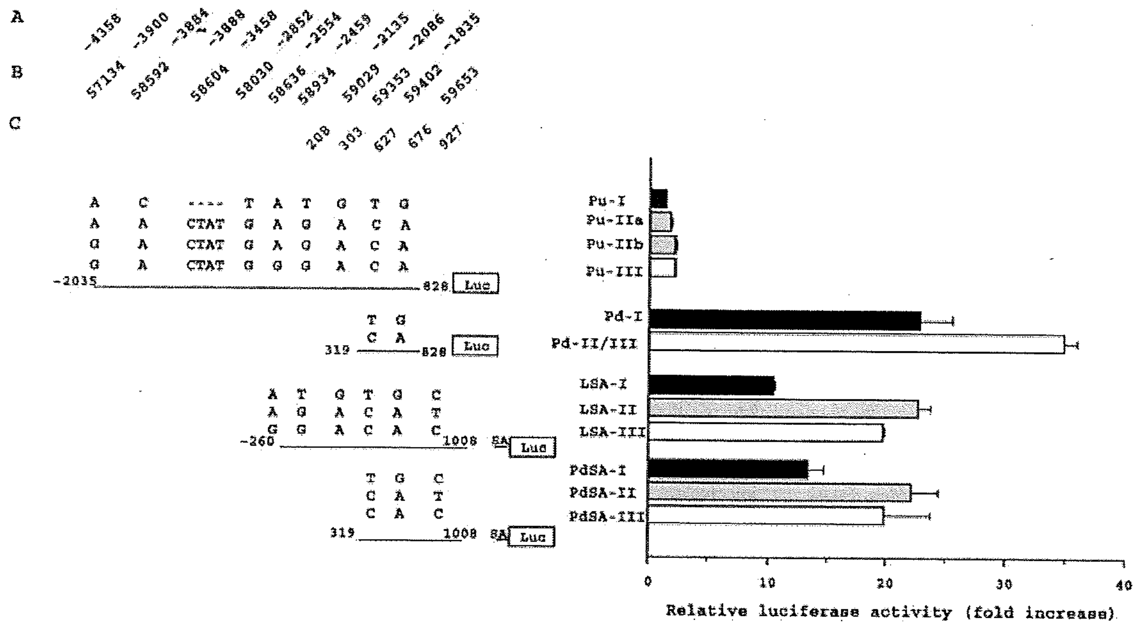

Figure 4. Effect of sequence polymorphism in CCR5 regulatory region on promoter activity. The promoter regions inserted into the pGL3-basic vector are shown by solid lines with the first and last nucleotides enumerated according to numbering system C. SA indicates 0.25 kb of the 5′-non-coding region containing a splice acceptor immediately upstream of the CCR5 open reading frame. Nucleotides at the polymorphic positions are marked. Boxes represent firefly luciferase open reading frames. The relative luciferase activity of each construct is represented by a solid bar (haplotype group I), a grey bar (haplotype group II), and an open bar (haplotype group III). Data shown are representative of five independent experiments with similar results. Error bars indicate fluctuations between measurements of relative luciferase activity in two independent clones of a construct.

Table 4. Linkage disequilibrium between *CCR5 −1835T* and *CCR2 64I* in Japanese and Thai subjects

		CCR2 V64I		
Subjects	*CCR5 C-1835T*	VV	VI	II
Japanese	CC	58	0	0
	CT	2	54	0
	TT	0	1	15
Thais	CC	123	0	0
	CT	0	61	0
	TT	0	0	2

(Table 2), and *CCR2 V64I* was genotyped by PCR-RLFP according to the method described by Smith *et al.* (1997). Frequencies of *CCR2 64I* and *CCR5 −1835T* of 130 Japanese subjects were 33% and 34%, respectively, and were higher than those observed in Caucasians and African-Americans (Table 3). As shown in Table 4 and Table 5, we confirmed the strong linkage disequilibrium between *CCR2 64I* and *CCR5 −1835T* (D′ = 1). We also found the strong linkage disequilibrium between *CCR5 −2852G* and the disease-accelerating haplotype (D′ = 1) since *−2852G* was found in 55 out of 58 (95%) of the disease-accelerating haplotype in 130 Japanese subjects.

In Thai subjects, we previously observed that *CCR2 64I* alleles were 100% associated with *CCR5 −1835T* (unpublished results). We therefore examined whether *−2852G* was associated with the disease-accelerating haplotype in 186 Thai subjects (97 non-HIV-1-infected and 89 HIV-1-infected individuals). Frequencies of *CCR2 64I*, *CCR5 −2852G*, *−2135T*, *−2086 A*, and *−1835T* of 186 Thai subjects were shown in Table 3. As expected, *CCR2 64I* alleles were associated with *CCR5 −1835T* without any exceptions (Table 4). Furthermore, *CCR5 −2852G* was also 100% associated with the disease-accelerating haplotype, *CCR5 P1* lacking *CCR2 64I* and *CCR5Δ32* (Table 5). These results indicated that only one allele (*−2852G*) could be used as a representative of the disease-accelerating haplotype, *CCR5 P1* lacking *CCR2 64I* and *CCR5Δ32* in the Thai population.

Discussion

Polymorphisms in human *CCR5* genes were initially studied for their effects on susceptibility to HIV-1 infection and rate of disease progression to AIDS in HIV-1-infected individuals. Subsequently, these polymorphisms were evaluated for their roles in other human phenotypes such as those accounting for differences in renal transplantation outcomes (Fischereder *et al.*, 2001), myocardial infarction (Gonzalez *et al.*, 2001; Valdes *et al.*, 2002), and autoimmune diseases (Garred *et al.*, 1998; Gomez-Reino *et al.*, 1999; Spagnolo *et al.*, 2005). Our systematic survey of natural sequence variations in an 8.1-kb region of the

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Table 5. Linkage disequilibrium between *CCR5 −2852G* and *CCR5* promoter haplotypes in Japanese and Thai subjects

		CCR2 V64I, CCR5 T-2135C, CCR5 G-2086 A, CCR5 C-1835T genotyping					
Subjects	*CCR5 A-2852G*	*V-P1/V-P1*	*V-P1/I-P1*	*V-P1*/Others	*I-P1/I-P1*	*I-P1*/Others	Others/Others
Japanese	AA	0	1	2	15	37	29
	AG	0	17	20	0	0	0
	GG	9	0	0	0	0	0
Thais	AA	0	0	0	2	46	78
	AG	0	15	39	0	0	0
	GG	6	0	0	0	0	0

V-P1: haplotype group III, *CCR5 P1* haplotype (*−2135C, −2086 A*, and *−1835C*) lacking *CCR2 64I*.
I-P1: haplotype group II, *CCR5 P1* haplotype (*−2135C, −2086 A*, and *−1835C*) with *CCR2 64I*.
Others: haplotype group I (*−2135T, −2086G*, and *−1835C*) and haplotype group IV (*−2135T, −2086 A*, and *−1835C*).

entire *CCR5* gene as well as *CCR2V64I* in 50 Japanese individuals revealed several Asian-specific characteristics of the *CCR2-CCR5* haplotype structure that were not seen in those of Caucasians and African-Americans. In particular, we were able to show that the G allele at position −2852 from the *CCR5* open reading frame may represent the *CCR5* promoter haplotype with a higher promoter activity (*CCR5 P1*) without *CCR2 64I* in a *CCR5Δ32*-negative Thai population. For other ethnic groups, the method for *CCR5* promoter haplotype determination is complicated since single-nucleotide polymorphisms (SNPs) at several different positions need to be determined (Martin *et al.*, 1998; McDermott *et al.*, 1998; Mummidi *et al.*, 2000). It would therefore be useful to be able to analyse only one SNP especially in resource-constrained setting.

Our results for *CCR5* promoter activity also confirmed the previously reported observation that polymorphisms of *CCR5* affect its promoter activity (McDermott *et al.*, 1998; Mummidi *et al.*, 2000). Other studies have shown that the SNP at position −2459 affects the binding of certain factor(s) to the *CCR5* promoter region (Bream *et al.*, 1999; Mummidi *et al.*, 2000). However, our study demonstrated that constructs lacking position −2459 still showed differences in promoter activity and that *−2135C* and *−2086 A* were responsible for elevated promoter activity. Further studies will be needed, however, to identify and clarify the exact molecular mechanisms underlying the effects of those SNPs on *CCR5* promoter activity.

On the other hand, we could not detect any effects on luciferase expression level by *CCR5 −1835T*, which is in strong linkage disequilibrium with disease-delaying *CCR2 64I*. Recently, we reported that *CCR2 64I* substitution increases the stability of CCR2A, which is a splicing variant of CCR2B (Nakayama *et al.*, 2004). CCR2A was shown to bind CCR5 while most of CCR2A molecules are retained in cytoplasm. Therefore, cell surface expression levels of CCR5 co-expressed with CCR2A (64I) were lower than those of CCR5 co-expressed with CCR2A (64 V) (Nakayama *et al.*, 2004). Our results reported here also supported the notion that *CCR2 64I* but not *CCR5 −1835T* is directly responsible for the effects of those SNPs on HIV-1 disease progression (Mummidi *et al.*, 1998).

Phylogenetic analysis of human *CCR5* haplotypes clearly demonstrated that all the protective alleles, *CCR2 64I* (*CCR5 −1835T*), *CCR5Δ32* and *CCR5 893(−)*, belong to haplotype groups with higher promoter activity. These results suggest that a certain selective pressure existed that favoured low levels of CCR5 expression during human evolution. CCR5 has been shown to be involved in several inflammatory diseases (Garred *et al.*, 1998; Gomez-Reino *et al.*, 1999), and *CCR5Δ32* to be associated with a reduction in the risks for patients with multiple sclerosis (Barcellos *et al.*, 2000; Kantor *et al.*, 2003) and asthma (Hall *et al.*, 1999). It is therefore possible that impaired function or low levels of CCR5 expression may be beneficial for the survival of individuals with autoimmune or inflammatory diseases.

It is noteworthy that four of the five *CCR5 G668A* (*R223Q*) alleles were found in the *H7* haplotype, which also contains *CCR2 64I* (Fig. 2). Previously, *CCR5 668 A* was reported to be associated with slower disease progression in HIV-1-infected individuals (Zheng *et al.*, 2002). However, since *CCR5 668 A* is associated with another disease-delaying allele, *CCR2 64I*, an epidemiological study is needed to compare individuals with *CCR5 668 A* with those who carry *CCR2 64I* without *CCR5 668 A* in order to evaluate the effects of *CCR5 668 A* on HIV-1 disease progression.

Our study also suggested that not only the frequency but also the degree of linkage disequilibrium of SNPs varies between Japanese and Thai subjects, both Asian. Among the Japanese, 58 of 260 chromosomes were found to carry *CCR5* −1835T, and 55 of these 58 chromosomes also carried *CCR2 64I*. No haplotype carrying *CCR2 64I – CCR5 −1835C* was found among the 260 Japanese chromosomes. Studies of *CCR5* and *CCR2* polymorphisms of Caucasian individuals revealed that both *CCR2 64I – CCR5 −1835C* and *CCR2 64 V – CCR5 −1835T* did exist in Caucasians (Mummidi *et al.*, 1998). On the other hand, *CCR2 64I* was completely associated with *CCR5 −1835T* in 372 Thai chromosomes. It is likely that the *CCR2 64I* mutation only occurred on a *CCR5 −1835T* bearing chromosome and that the *CCR2 64I – CCR5 −1835C* and *CCR2 64 V – CCR5 −1835T* haplotypes were generated as the results of a recombination event between

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the *CCR2 64I-CCR5 −1835T* and the *CCR2 64 V − CCR5 −1835C* haplotypes. In Thais, however, that kind of recombination never occurred or only at a very low frequency. At the very least, our results presented here point to the need for detailed linkage studies of genetic polymorphisms, such as the HapMap project, in all ethnic groups, since degree of linkage disequilibrium can vary even among Asian groups.

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Letter to the Editor

Host Genetic Analysis of HIV Type 1 Subtype CRF01_AE (E)-Infected Thai Patients with Different Rates of Disease Progression

EDITOR: HIV-1 infection is generally characterized by a long-term, chronic disease course, gradually progressing to AIDS.[1,2] However, there are a few, but strikingly different, variant scenarios. A small fraction of HIV-1-infected individuals remains normal, both clinically and immunologically, for 10 or more years after seroconversion. Conversely, another fraction is featured by extremely rapid disease progression taking place even within 1 year. Determining host factors involved in these different disease courses would be extremely helpful for better understanding and control of HIV infection.

It is known that polymorphisms in human genes involved in viral cell entry and modulation of immune responses play a substantial role in disease progression of European-American and African-Americans infected with HIV-1 subtype B.[3] However, there are few such data from Asian populations. Furthermore, diverse strains of HIV-1 circulate in different geographical areas and the effects of host genetic polymorphisms may differ due to antigenic or other differences between these HIV-1 subtypes. In the present study, we conducted a "proof of principle" study in previously defined and well-characterized HIV-1-infected Thai patients with different rates of disease progression. We show here that host genetic polymorphisms tend to be associated with rates of disease progression in HIV-1-subtype CRF01_AE (E)-infected Thai patients.

EDTA blood was obtained with written informed consent from 20 HIV-1-infected (all with documented CRF01_AE) Thai patients. Immunological and virological features of these patients were extensively studied and reported previously.[4] All 20 patients had similar CD4 cell counts (558–613 cell/μl) at the start of observation. Nine patients were considered as "progressors" (PRs), since they showed a decline of more then 50 CD4 cells per year and lost more than 50% of them during 3–6 year periods of observation. All RPs were symptomatic or had CD4 cell counts less than 200/μl at the end of the follow-up period. The remaining 11 patients were considered as "slow progressors" (SPs), since they showed virtually no CD4 cell decline during observation periods of similar length. We previously reported that SPs retained the immune competence of antibody responses over time.[4]

We genotyped these 20 patients for the following seven polymorphisms: CCR2 64I,[5] SDF-1 3′A,[6] IL4 -589T,[7] RANTES –28G,[8] RANTES –403A,[8] CCR5 m303,[9] and CCR5 P1.[10] Herein, we did not observe any RANTES –28G or CCR5 m303, probably because of a relatively low frequency of those alleles in Thais.[11,12] Table 1 summarizes the other genotype and allele frequencies observed in these 20 patients. The mutant allele frequency of genetic polymorphism was analyzed by Hardy–Weinberg equilibrium and the distribution of each genotype frequency in the PRs and SPs was compared using Fischer's exact test.

CCR2 64I, SDF-1 3'A, and IL-4 –589T were considered to be protective alleles against HIV-1 disease progression.[7,10,11,13] Consistently, we observed a trend toward higher frequencies of those protective alleles in SPs than in PRs. However, none of the differences reached statistical significance, maybe due to the small sample size. CCR5 P1 and RANTES –403A were previously shown to be associated with a more rapid course of HIV-1 disease progression, and the frequencies of those alleles tended to be slightly lower in SPs than in PRs.[5,8,10] In this study, however, the differences did not reach statistical significance (Table 1).

Among genetic polymorphisms studied here, we observed the lowest p value (Table 1, $p = 0.14$) for SDF-1 3′A. SDF1-3′A was elevated in the SRs compared to PRs (0.5 vs. 0.222). Interestingly, the previous study demonstrated that the frequency of this allele in healthy Thais was 0.375,[14] being higher than that of RPs and lower than that of SPs. An international meta-analysis of large numbers of HIV-1-infected European- and African-Americans,[13] however, did not confirm our observation of the potential protective effect of SDF-1 3′A against HIV-1 disease progression in Thai subjects. In contrast, our recent study in infants and another study in adults[15] have shown an apparent protective effect of SDF-1 3′A against HIV-1 transmission in Thais. It is possible that the SDF-1 3′A polymorphism exerts a stronger effect on HIV-1 (CRF01_AE) infection in Thais than in European- and African-Americans with subtype B infections.

The mean number of protective alleles for PRs was lower than that of SPs (mutant allele frequency 2.1 vs. 3.1), and the mean number of accelerating alleles for PRs was higher than that of SPs (mutant allele frequency 1.0 vs. 0.7). Interestingly, 73% (8 out of 11) of SRs compared to 22% (2 out of

TABLE 1. CCR2, SDF1, IL-4, CCR5 PROMOTOR, AND RANTES PROMOTOR GENOTYPE DISTRIBUTION IN HIV-1-INFECTED INDIVIDUALS WITH DIFFERENT RATES OF DISEASE PROGRESSION

Genotype	*Progressors (RPs)* (n = *9*)	*Slow progressors (SPs)* (n = *11*)	p *value*[a]
CCR2 +/+	7	6	
CCR2 +/64I	0	4	
CCR2 64I/64I	2	1	
Frequency of CCR2-64I	0.222	0.273	1
SDF1 +/+	6	3	
SDF1 +/3′A	2	5	
SDF1 3′A/3′A	1	3	
Frequency of SDF1-3′A	0.222	0.5	0.14
IL-4 +/+	2	1	
IL-4 +/−589T	3	3	
IL-4 −589T/−589T	4	7	
Frequency of IL-4 −589T	0.611	0.773	0.45
CCR5 +/+	5	8	
CCR5 +/P1	4	3	
CCR5 P1/P1	0	0	
Frequency of CCR5-P1	0.222	0.136	0.68
RANTES +/+	5	7	
RANTES +/−403A	3	3	
RANTES −403A/−403A	1	1	
Frequency of RANTES −403A	0.278	0.227	1

[a]Fisher's exact test; "+" denotes alleles other than the indicated allele.

9) of PRs carried more than three protective alleles (data not shown, Fisher's exact test, $p = 0.07$). These results suggest that some of the alleles protective against HIV-1 disease progression in European- and African-Americans also modulate HIV-1 disease progression in HIV-1 subtype CRF01_AE (E)-infected Thais, although we tested a small number of patients. In addition, there may be some differences in the repertoire of host genetic polymorphisms that play a role in disease progression in Asian subjects as compared to European or African-American subjects. The other limitation of the present study is our working definition of RPs and SPs without knowing the exact time of infection. Nevertheless, our data are intriguing and suggest that further analysis with at least 500 CRF01_AE-infected Thai subjects, ideally followed from a known time of infection, is indicated to confirm or refute these findings, and to define the usefulness of human genetic polymorphisms in predicting the clinical course of HIV-1 infection in Thais.

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Comparison of anti-viral activity of rhesus monkey and cynomolgus monkey TRIM5αs against human immunodeficiency virus type 2 infection

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Abstract

Human immunodeficiency virus type 2 (HIV-2) strains vary widely in their ability to grow in Old World monkey (OWM) cells. We previously evaluated several HIV-2 isolates for their sensitivity to cynomolgus monkey (CM) TRIM5α, an anti-HIV factor in OWM cells, and found that viruses carrying proline at the 120th position of the capsid protein were sensitive to CM TRIM5α, whereas those with either alanine or glutamine were resistant. In the study presented here, we tested these HIV-2 isolates for their sensitivity to rhesus monkey (Rh) TRIM5α and found that both CM TRIM5α-sensitive and -resistant viruses were restricted by Rh TRIM5α. The variable region 1 of the SPRY domain of Rh TRIM5α appeared to be the determinant of this difference. Furthermore, a mutagenesis study showed that three amino acid residues TFP at the 339th to 341st positions of Rh TRIM5α are important for restricting HIV-2 strains resistant to CM TRIM5α.
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Keywords: TRIM5α; Human immunodeficiency virus; Rhesus monkey; Cynomolgus monkey

Introduction

Human immunodeficiency virus type 1 (HIV-1) has a very narrow host range limited to humans and chimpanzees. Experiments have demonstrated that HIV-1 does not infect Old World monkeys (OWM) such as rhesus and cynomolgus monkeys. Recently, the screening of a rhesus monkey cDNA library identified tripartite motif 5α (TRIM5α) as a factor that confers resistance to HIV-1 infection (Stremlau et al., 2004). Rhesus and cynomolgus monkey TRIM5α restricts HIV-1 infection (Stremlau et al., 2004; Yap et al., 2004; Nakayama et al., 2005), whereas human TRIM5α restricts N-tropic murine leukemia virus (N-MLV) infection (Hatziioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004). African green monkey (AGM) TRIM5α restricts simian immunodeficiency virus isolated from a macaque monkey (SIVmac), human immunodeficiency virus type 2 (HIV-2), and equine infectious anemia virus in addition to HIV-1 infection (Hatziioannou et al., 2004; Keckesova et al., 2004; Nakayama et al., 2005). TRIM5α shares with other splicing variants a common amino-terminal TRIM motif, comprising RING, B-box and coiled-coil domains, and encodes a unique SPRY (B30.2) domain (Reymond et al., 2001). Several recombinant studies of human and rhesus monkey TRIM5α have shown that the determinant of the species specificity lies in the SPRY domain of TRIM5α (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). We also previously demonstrated that 17-amino-acid residues and adjacent AGM-specific 20-amino-acid duplication in the SPRY domain determined species-specific restriction of SIVmac (Nakayama et al., 2005). It is known that the RING and B-box domains are required for restriction and that the coiled-coil domain is required for multimerization (Stremlau et al., 2004; Berthoux et al., 2005; Perez-Caballero et al., 2005; Nakayama et al., 2006). TRIM5α is thought to bind HIV capsid and promote its rapid, premature disassembly in ubiquitin dependent

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and independent manners (Stremlau et al., 2006; Diaz-Griffero et al., 2006; Wu et al., 2006; Anderson et al., 2006). This means that the viral RNA and proteins are exposed to cellular proteins and degraded before nuclear transportation.

HIV-2 has a genome extremely similar to that of SIVmac (Hahn et al., 2000). In contrast with the many reports concerning HIV-1 and SIVmac, there have been only a few on susceptibility of HIV-2 to TRIM5α from various species (Nakayama et al., 2005; Ylinen et al., 2005). We previously evaluated eight HIV-2 isolates for their sensitivity to cynomolgus monkey TRIM5α and found that viruses carrying proline at the 119th or 120th position of the capsid protein (CA) were sensitive to cynomolgus monkey TRIM5α, whereas those with either alanine or glutamine were resistant (Song et al., 2007). In the study presented here, we tested these HIV-2 isolates for their sensitivity to another OWM rhesus monkey TRIM5α and found that both cynomolgus monkey TRIM5α-sensitive and -resistant viruses were restricted by rhesus monkey TRIM5α. We were able to show that three amino acid residues TFP at the 339th to 341st positions of rhesus monkey TRIM5α are important for restriction activity against HIV-2 strains.

Results

Rhesus monkey TRIM5α inhibits both cynomolgus monkey TRIM5α-sensitive and -resistant HIV-2 viruses

We previously reported that HIV-2 isolates carrying proline at the 120th position of CA were sensitive to cynomolgus monkey TRIM5α, whereas those with either alanine or glutamine were resistant. Both cynomolgus and rhesus monkey TRIM5αs are known to restrict HIV-1 but not SIVmac. Predicted amino acid sequences of cynomolgus and rhesus monkey TRIM5α are shown in Fig. 1. Rhesus and cynomolgus monkey TRIM5α share 96.8% of amino acid residues. To test these HIV-2 isolates for their sensitivity to rhesus monkey TRIM5α, we constructed a recombinant Sendai virus (SeV) expressing rhesus monkey TRIM5α fused with the HA tag in the C-terminal. Western blot analysis using an antibody against HA-tag showed that rhesus monkey TRIM5α was expressed at similar levels to those of cynomolgus monkey TRIM5α in recombinant SeV infected human T-cell line MT4 cells (data not shown). Fluorescent microscopic observation confirmed that these TRIM5αs were detected in all the cells infected with recombinant SeVs (data not

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                         RING domain
CM    1 MASGILLNVKEEVTCPICLELLTEPLSLHCGHSFCQACITANHKKSMLYKEGERSCPVCR   60
Rh    1 ............................................................   60
                         B-box2 domain
CM   61 ISYQPENIQPNRHVANIVEKLREVKLSPEEGQKVDHCARHGEKLLLFCQEDSKVICWLCE  120
Rh   61 ............................................................  120
                         Coiled-coil domain
CM  121 RSQEHRGHHTFLMEEVAQEYHVKLQTALEMLRQKQQEAEKLEADIREEKASWKIQIDHDK  180
Rh  121 .........................................................Y..  180

CM  181 TNVLADFEQLREILDREESNELQNLEKEKEDILKSLTKSETKMVQQTQYVRELISDLEHR  240
Rh  181 ...S..........W............E............E.......M.....E....  240

CM  241 LQGSMMELLQGVDGIIKRIENMTLKKPKTFHKNQRRVFRAPDLKGMLDMFRELTDARRYW  300
Rh  241 ......D.....................................................  300
              ▼ Sph I        Variable region 1
CM  301 VDVTLAPNNISHAVIAEDKRQVSSRNPQIVYQSPGTLF--QSLTNFNYCTGVLGSQSITS  358
Rh  301 ......T.....................M..A.....TFP....................  360
              ▼ BamH I       SPRY (B30.2) domain
CM  359 GKHYWEVDVSKKSAWILGVCAGFQSDAMCNIEQNENYQPKYGYWVIGLQEGVKYSVFQDG  418
Rh  361 ............................Y...............................  420

CM  419 SLHTPFAPFIVPLSVIICPDRVGVFVDYEACTVSFFNITNHGFLIYKFSQCSFSKPVFPY  478
Rh  421 .S..........................................................  480

CM  479 LNPRKCTVPMTLCSPSS                                            495
Rh  481 .................                                            497
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Fig. 1. Alignments of amino acid sequences of cynomolgus monkey (CM) and rhesus monkey (Rh) TRIM5αs. The RING, B-box2, coiled-coil and SPRY (B30.2) domains are indicated by labeled bars over the sequence. Variable region 1 is indicated by a broken bar over the sequence. Dots denote the amino acid residues identical to the one of cynomolgus monkey TRIM5α and dashes a lack of the amino acid residue that is present in rhesus monkey TRIM5α. The box marks amino acid residues that are important for rhesus monkey TRIM5α restriction activity against HIV-2 strains (see Results).

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