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Preferential recognition of monomeric CCR5 expressed in cultured cells by the HIV-1 envelope glycoprotein gp120 for the entry of R5 HIV-1

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ABSTRACT

Bimolecular fluorescence complementation (BiFC) and western blot analysis demonstrated that CCR5 exists as constitutive homo-oligomers, which was further enhanced by its antagonists such as maraviroc (MVC) and TAK-779. Staining by monoclonal antibodies recognizing different epitopes of CCR5 revealed that CCR5 oligomer was structurally different from the monomer. To determine which forms of CCR5 are well recognized by CCR5-using HIV-1 for the entry, BiFC-positive and -negative cell fractions in CD4-positive 293T cells were collected by fluorescent-activated cell sorter, and infected with luciferase-reporter HIV-1 pseudotyped with CCR5-using Envs including R5 and R5X4. R5 and dual-R5 HIV-1 substantially infected BiFC-negative fraction rather than BiFC-positive fraction, indicating the preferential recognition of monomeric CCR5 by R5 and dual-R5 Envs. Although CCR5 antagonists enhanced oligomerization of CCR5, MVC-resistant HIV-1 was found to still recognize both MVC-bound and -unbound forms of monomeric CCR5, suggesting the constrained use of monomeric CCR5 by R5 HIV-1.

Introduction

Interaction of the outer envelope (Env) glycoprotein gp120 of human immunodeficiency virus type-1 (HIV-1) with CD4 and one of the coreceptors (either CCR5 or CXCR4) is essential for the entry of HIV-1 (reviewed in (Wilen et al., 2012)). Viruses that exclusively use CCR5 (R5 HIV-1) are transmission variants, and predominant throughout the course of infection. On the other hand, viruses that use CXCR4 emerge at late stage of infection, and are thought to be associated with CD4 depletion and disease progression in half of HIV-1-infected individuals (Connor et al., 1997; Scarlatti et al., 1997). Most of CXCR4-using viruses still use CCR5 (R5X4 HIV-1) while several variants exclusively use CXCR4 (X4 HIV-1).

Previous studies have shown that natural ligands such as macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and RANTES (regulated on activation, normal T-cell expressed and secreted; CCL5) were able to inhibit R5 HIV-1 replication by steric hindrance or internalization of CCR5 (Alkhatib et al., 1997; Cocchi et al., 1995; Oberlin et al., 1996; Scarlatti et al., 1997). The antagonists of CCR5, such as TAK-779 and maraviroc (MVC), also interact with hydrophobic pocket of CCR5 formed by the transmembrane helices, and induce conformational changes in CCR5, thereby blocking entry of R5 HIV-1 (Dragic et al., 2000; Kondru et al., 2008; Maeda et al., 2006; Nishikawa et al., 2005; Seibert et al., 2006; Tsamis et al., 2003). The dimerization of CCR5 induced by anti-CCR5 mAb CCR5-02 was also reported to prevent the entry of R5 HIV-1 (Vila-Coro et al., 2000), suggesting the possible impact of dimerization or oligomerization of CCR5 on HIV-1 susceptibility. Although CCR5 was reported to exist as homo-oligomers without natural ligands (Benkirane et al., 1997; El-Asmar et al., 2005; Hammad et al., 2010; Issafras et al., 2002; Mellado et al., 2001; Sohy et al., 2009), it still remains to be determined whether oligomeric forms of CCR5 are structurally different from the monomeric forms, and affect entry efficiency of R5 HIV-1. It is also unknown whether CCR5 antagonists affect the oligomerization status of CCR5, whereas the CCR5 natural ligands have been shown to induce the dimerization of CCR5 (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000). To address these issues, bimolecular fluorescence complementation (BiFC) assay was applied to detect homo-oligomeric forms of CCR5. Principally, BiFC assay is a non-invasive fluorescent-based technique that allows detection of protein-protein interactions in living cells (reviewed in (Kerppola, 2008)). BiFC assay is based on the association between two non-fluorescent fragments of a fluorescent protein when they are brought in proximity to each other by interaction between

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proteins fused to the fragments. In our present study, by using BiFC, homo-oligomeric forms of CCR5 were detected to some extent without natural ligands, and further enhanced by CCR5 antagonists. In addition, susceptibility of sorted CCR5 oligomers-enriched cell fraction was found to be less susceptible compared to monomer-enriched fraction, indicating the preferential recognition of CCR5 monomer by R5 HIV-1.

Results

Detection of oligomeric forms of CCR5 without ligands

It has been shown that CCR5 exists as homo-oligomer without natural ligands such as CCL3, CCL4, and CCL5 (Benkirane et al., 1997; El-Asmar et al., 2005; Hammad et al., 2010; Issafras et al., 2002; Mellado et al., 2001; Sohy et al., 2009). However, it still remains to be determined whether oligomeric forms of CCR5 are structurally different from CCR5 monomer. To this end, BiFC assay was employed to detect oligomeric forms of CCR5. The CCR5 expression vectors fused to the N- and C-terminal fragments of green fluorescence protein (Kusabira–Green: KG) were constructed, and co-expressed in 293T cells. When the both proteins are expressed and close together, refolded KG protein results in KG signal. This fluorescent signal can be easily detected by flow cytometry or fluorescence microscopy. To analyze the structural differences between monomeric and oligomeric forms of CCR5, the cells were further stained with anti-CCR5 monoclonal antibodies (mAbs) recognizing different epitopes of CCR5 such as N-terminal (clones CTC8, 3A9), second extracellular domain (ECL2) (clones 2D7, 45531), or multiple conformation (clone 45549). As shown in the upper panel of Fig. 1, we were able to detect fluorescent (KG) signal using flow cytometry when both CCR5-KGN and CCR5-KGC were co-expressed in 293T cells, indicating the oligomerization of CCR5 without ligands. We also noticed that proportions of CCR5+KG+ subset were almost equal (24–26%) in all anti-CCR5 mAb clones (CTC8, 3A9, 2D7, and 45531) except the clone 45549. In contrast, CCR5+KG− subset was differentially stained by anti-CCR5 mAbs (Fig. 1, upper panel). The proportions of CCR5+KG− subset were high in the clones 2D7 and CTC8 (62% and 55%, respectively), intermediate in the clone 45531 (43%), and low in the clone 3A9 (26%). These results suggested that monomeric forms of CCR5 were structurally different from the oligomeric forms.

Enhancement of CCR5 oligomerization by CCR5 antagonists

Although natural ligands such as CCL3, CCL4, and CCL5 have been showing to induce oligomerization of CCR5 (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000), it has not been determined how CCR5 antagonists such as TAK-779 or MVC affect the oligomerization of CCR5. Therefore, we also applied BiFC technique to check the effects of CCR5 antagonists on the oligomerization status of CCR5. After co-expressing CCR5-KGN and –KGC in 293T cells in the presence of MVC, the cells were stained with above-mentioned anti-CCR5 mAbs. The proportions of CCR5+KG+ subset were largely increased in all anti-CCR5 mAb clones (Fig. 1, lower panel) compared to those of the same fraction in the absence of ligands (Fig. 1, upper panel). Notably, the proportion of CCR5+KG+ subset was increased in the clone 45549 though its reactivity was quite low in the absence of ligands, confirming that conformational changes of CCR5 were indeed induced by MVC. To verify the enhancement of CCR5 oligomerization by CCR5 antagonists, we then checked whether another CCR5 antagonist TAK-779 enhanced oligomerization of CCR5. The CCR5-KG-expressing 293T cells were stained with 2D7 mAb that was able to equally detect both CCR5+KG− and CCR5+KG+ subsets as shown in Fig. 1, and KG-positive percentages in 2D7-positive population were determined by flow cytometry. We found that TAK-779 also enhanced the oligomerization of CCR5, while a CXCR4 antagonist AMD3100 had no effect (Fig. 2A). In particular, MVC had higher activity to enhance CCR5 oligomerization than TAK-779 in 293T cells. Western blot analysis using 293T cells expressing FLAG-tagged CCR5 with cross-linker indicated that CCR5 largely existed as monomer but also as dimer in the absence of ligands though lesser extent (Fig. 2B). It was also shown that MVC was able to induce expression of not only dimer but also more than dimer forms of CCR5. Notably, the level of CCR5 expression was up-regulated by MVC though the reason was uncertain. Native-PAGE analyses also revealed the similar results (Supplementary Fig. S1). The enhancement by CCR5 antagonists was also observed in different cell types such as HeLa, and NP2 cell lines though both had comparable activities in these cell lines (Supplementary Fig. S2). Dose-escalating study revealed that the concentrations

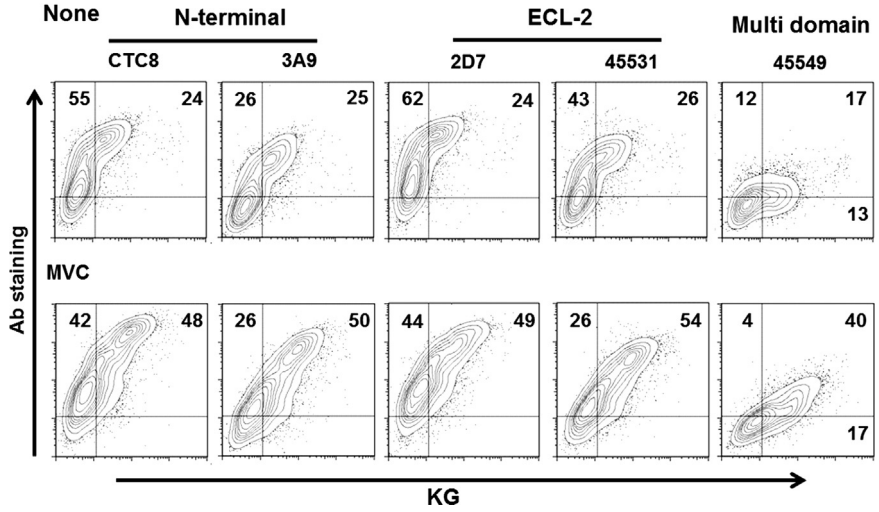

Fig. 1. Flow cytometry analyses of CCR5-KG-expressing 293T cells by anti-CCR5 mAbs in the presence or absence of MVC. The 293T cells were transfected with both CCR5-KG expression vectors, and incubated at 37 °C for 48 h in the absence of ligands (upper panel) or the presence of MVC at 2 μM (lower panel). Anti-CCR5 mAbs recognizing N-terminus (clones CTC8 and 3A9), ECL-2 (clone 2D7, 45531), and multiple domains (clone 45549) were used for the detection of CCR5, and analyzed by flow cytometry. The *y*-, and *x*-axes show the mean fluorescence intensity of CCR5 and KG, respectively. The number of each column shows the percent positive in each region.

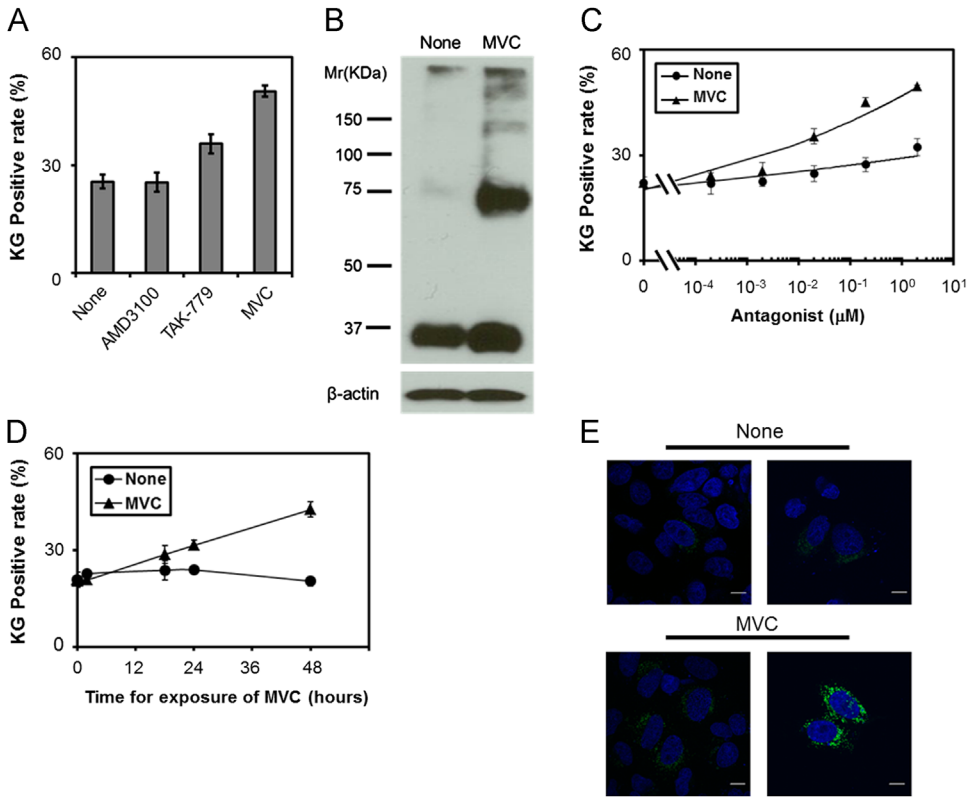

Fig. 2. Enhanced homo-oligomerization of CCR5 by CCR5 antagonists. (A) The 293T cells were transfected with expression vectors of CCR5-KG, and incubated in the presence or absence of AMD3100, TAK-779 or MVC at 2 μM each. The cells were stained with anti-CCR5 mAb 2D7, and analyzed by flow cytometry. The data shown represent the mean values of the percentage of KG-positive in 2D7-positive cell fraction ± standard deviations of three independent experiments. (B) The 293T cells were transfected with pCCR5-FLAG in the presence or absence of MVC. The cells were cross-linked by DSP, lysed, and analyzed by Western blot using anti-FLAG mAb. (C) Transfected cells were treated with increasing concentrations of TAK-779 or MVC ranging from 0.2 nM to 2 μM, and incubated at 37 °C for 48 h. The data shown represent the mean values of percent positive of KG ± standard deviations of three independent experiments. (D) The 293T cells expressing CCR5-KG were incubated at 37 °C for the indicated time of period in the presence of MVC (1 μM). Results are mean values of CCR5-KG positive rates ± standard deviations from experiments performed in triplicate. (E) Transfected HeLa cells with CCR5-KG were incubated in the absence (upper panel) or presence of MVC (lower panels) at 37 °C for 48 h, and fixed with 4% paraformaldehyde. Representative images in the middle sections of the cells are shown. Nuclear staining by DAPI is shown in blue. Scale bars correspond to 10 μm.

for enhanced oligomerization of CCR5 by TAK-779 or MVC were indeed corresponded to the inhibitory concentrations against HIV-1 infection (Fig. 2C). For example, in the case of MVC, the EC_{50} value of inhibitory activity against R5 HIV-1 (JR-FL) was 3.7 ± 1.4 nM (data not shown), while the EC_{50} value of activity to enhance oligomerization of CCR5 was 7.4 ± 5.1 nM, indicating that CCR5 could be oligomerized at enough concentrations for inhibiting R5 HIV-1 infection.

It has been shown that oligomerization of CCR5 was induced shortly after the addition of natural ligands as previously described (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000). However, a time-course experiment showed that more than 24 h were necessary to enhance CCR5 oligomerization by MVC (Fig. 2D). Confocal laser scanning microscopy also showed that the CCR5-KG signals were located not only at the plasma membrane but also in the cytoplasm without ligands, and were further augmented by MVC (Fig. 2E). These results suggested that oligomerization of CCR5 needed de novo synthesis of CCR5 and occurred in the intracellular compartments before expressed on the cell surface.

Infection of KG-positive and -negative cell fractions with R5 HIV-1

Since the structures of oligomeric forms of CCR5 were possibly different from monomeric CCR5 as shown in Fig. 1, we next analyzed the abilities of R5 HIV-1 to recognize monomeric and oligomeric forms of CCR5. To this end, we first stained CD4-positive 293T cells expressing CCR5-KGN and –KGC with anti-CCR5 mAb CTC8, which was able to recognize both monomeric and oligomeric forms of CCR5, and had no neutralizing activity against CCR5-using HIV-1 (data not shown). The KG-positive and -negative subsets having the same CCR5 fluorescent intensity were then collected by fluorescent-activated cell sorter. The mean fluorescence intensities of KG in KG-positive and -negative cell fractions after sorting were 30.2 and 4.1, respectively, while mean fluorescence intensities of CCR5 were comparable (72.3 and 61.1 respectively) (Fig. 3A). The mean fluorescence intensity of CD4 was also confirmed to be comparable in KG-positive and -negative cell fractions (183 and 178, respectively). The sorted each cell fraction was then infected with HIV-1 pseudotyped with various strains of R5 Envs including JR-FL, YU-2, and Ba-L. Since the transfection of CD4-293T cells with KG-expressing vectors was possible to influence the cell condition, each cell fraction was also infected with HIV-1 pseudotyped with vesicular stomatitis virus G protein (VSV-G), which utilizes the ubiquitously expressing molecule(s) although the receptor for VSV-G remains to be confirmed (Coil and Miller, 2004; Schlegel et al., 1983). To normalize the entry efficiency of R5 HIV-1 in each fraction, we divided luciferase activities infected with R5 pseudotyped HIV-1 by those infected with VSV-G pseudotyped HIV-1. The susceptibility of CCR5+KG+ subset to R5 HIV-1 was then compared with that of CCR5+KG− subset. Although each single cell of CCR5+KG− or CCR5+KG+ subset was supposed to have oligomeric and monomeric forms of CCR5 to some extent, respectively, we found that entry efficiencies of R5 HIV-1 in CCR5+KG+ subset were always lower than those in

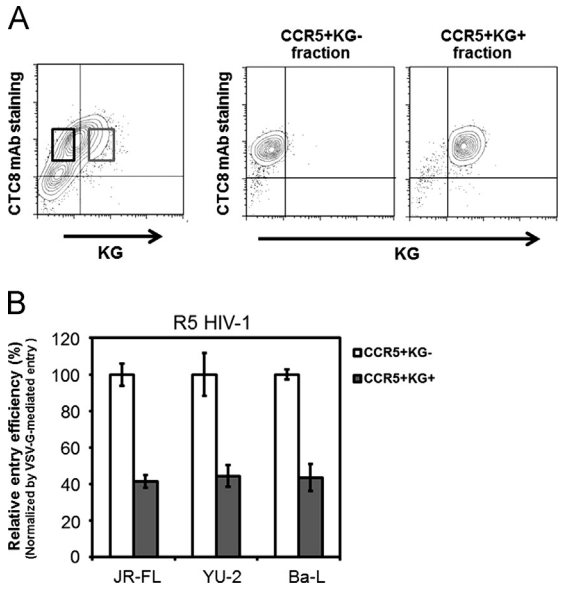

Fig. 3. Sorting of CCR5+KG− and CCR5+KG+ fractions and infection with luciferase-reporter HIV-1 pseudotyped with R5 Envs. (A) CD4-positive 293T cells expressing CCR5-KG were stained with anti-CCR5 mAb CTC8 (shown in the left panel). Representative data of flow cytometric analysis is shown. The KG− (shown in black rectangle) and KG+ (shown in gray rectangle) fractions with the same mean fluorescence intensities of CCR5 were gated, and sorted by fluorescent-activated cell sorter. Each sorted fraction was analyzed using flow cytometry (shown in the right panel). (B) Each sorted fraction was infected with luciferase-reporter HIV-1 pseudotyped with R5 Envs or VSV-G, and luciferase activities of infected cells were determined 24 h post-infection. Entry efficiency of each R5 Env in each fraction was normalized by that of VSV-G. Relative entry efficiency of CCR5+KG+ fraction (shown in gray bar) was expressed as the percentage of that of CCR5+KG− fraction (shown in white bar). The data are expressed as means ± standard deviations in triplicate experiments.

CCR5+KG− subset (Fig. 3B). These results indicated that R5 Envs preferentially recognized monomeric forms of CCR5 rather than its oligomeric forms.

Infection of KG-positive and -negative cell fractions with R5X4 HIV-1

We next checked the susceptibilities of CCR5+KG− and CCR5+KG+ subsets to another CCR5-using HIV-1, R5X4. As we mentioned earlier, there were several phenotypes in the strains of R5X4 HIV-1 such as dual-R5 and dual-X4 (Symons et al., 2011; Toma et al., 2010). We then selected 89.6 as dual-X4, KMT, TIK, and 89.6R308S as dual-R5 as previously described (Maeda et al., 2008). Similar to R5 HIV-1, dual-R5 preferentially infected CCR5+KG− fraction compared to CCR5+KG+ fraction (Fig. 4). Notably, single mutation in 11th position of the V3 loop in 89.6 (89.6R308S), which changed viral phenotype from dual-X4 to dual-R5 (Maeda et al., 2008), also significantly infected CCR5+KG− fraction than CCR5+KG+ fraction. In contrast, wild type 89.6 (dual-X4) comparably infected both CCR5+KG− and CCR5+KG+ fractions. These results indicated that dual-R5 but not dual-X4 HIV-1 also preferentially recognized monomeric CCR5 for the entry.

Infection of CCR5-KG-positive and -negative cell fractions with MVC-resistant HIV-1

Since the CCR5 antagonist MVC strongly enhanced CCR5 oligomerization in 293T cells as shown in Figs. 1 and 2, MVC-resistant HIV-1 seemed to evolve to use oligomeric forms of CCR5 for the entry. In general, MVC-resistant HIV-1s were shown to recognize MVC-bound form of CCR5 to reduce sensitivity to MVC as previously described by others and us (Kuhmann et al., 2004; Maeda et al., 2011; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007; Yuan et al., 2011). We therefore sought to infect CCR5+KG− and CCR5+KG+ fractions with MVC-resistant HIV-1 in the absence or presence of 2 μM MVC, respectively. Similar to general R5 HIV-1s, MVC-resistant HIV-1 also preferentially infected CCR5+KG− fraction in both the absence and presence of MVC compared with CCR5+KG+ fraction (Fig. 5A). We further infected both fractions at various concentrations of MVC ranging from 100 nM to 10 μM in order to check whether MVC-resistant HIV-1 recognizes MVC-bound forms of CCR5. Both fractions were also infected with MVC-sensitive HIV-1 carrying JR-FL Env to check whether sensitivity of general R5 HIV-1 to MVC is different between them. We found that MVC-sensitive HIV-1 had reduced sensitivity to MVC in CCR5+KG− fraction compared with CCR5+KG+ fraction (Fig. 5B), supporting the preferential recognition of CCR5 monomer by CCR5-using Env. We further observed reduced maximal inhibition of MVC-resistant HIV-1 in CCR5+KG− fraction compared with CCR5+KG+ fraction (Fig. 5B). These results indicated that MVC-resistant HIV-1 was likely to use MVC-bound forms of CCR5 monomer though MVC augmented CCR5 oligomerization.

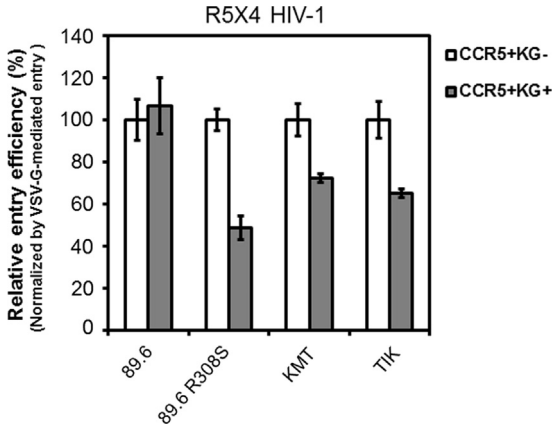

Fig. 4. Infection of CCR5+KG− and CCR5+KG+ fractions with luciferase-reporter HIV-1 pseudotyped with R5X4 Envs. Sorted CCR5+KG− and CCR5+KG+ fractions were infected with luciferase-reporter HIV-1 pseudotyped with R5X4 Envs or VSV-G. The luciferase activities of infected cells were determined 24 h post-infection. Entry efficiency of each R5X4 Env in each fraction was normalized by that of VSV-G. Relative entry efficiency of CCR5+KG+ fraction (shown in gray bar) was expressed as the percentage of that of CCR5+KG− fraction (shown in white bar). The data are expressed as means ± standard deviations in triplicate experiments.

Discussion

HIV-1 coreceptors CCR5 and CXCR4 are members of the seven transmembrane (7-TM) G protein-coupled receptors (GPCRs) superfamily. Recent data have shown that many GPCRs including chemokine receptors function as dimers or higher-order oligomers. To assess the formation of dimerization/oligomerization of GPCRs, fluorescent- or bioluminescent-based techniques have been applied such as BiFC, fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) assay (reviewed in (Vidi et al., 2011)). In the case of BiFC, the expression vectors for BiFC are generally comprised of two fragments of non-functional fluorescent protein split by N- and C-terminus (KGN and KGC of Kusabira–Green: KG in our case). When GPCRs fused to KGN and KGC are brought in close proximity, fluorescent signal can be detected by refolding of the fluorescent protein, KG. It should be noted that KG-signal could be only detected when KGN and KGC are brought together but not the same pairs such as KGN–KGN or KGC–KGC. Nonetheless, we were able to show KG-positive cells in both CCR5-KGN and CCR5-KGC expressing cells

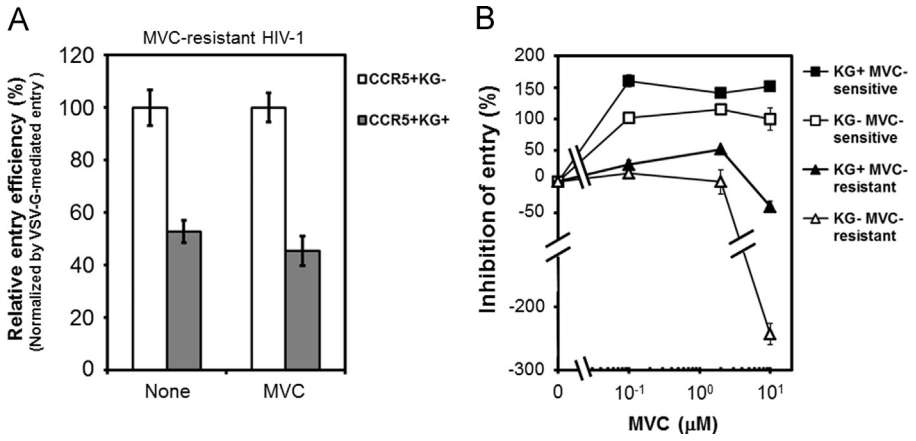

Fig. 5. Infection of CCR5+KG− and CCR5+KG+ fractions with luciferase-reporter HIV-1 pseudotyped with JR-FL and MVC-resistant Envs. (A) Sorted CCR5+KG− and CCR5+KG+ fractions were infected with luciferase-reporter HIV-1 pseudotyped with MVC-resistant Env or VSV-G in the absence or presence of 2 μM MVC. The luciferase activities of infected cells were determined 24 h post-infection. Entry efficiencies of MVC-resistant Env in CCR5+KG− and CCR5+KG+ fractions in the presence or absence of MVC were normalized by those of VSV-G, respectively. Relative entry efficiency of CCR5+KG+ fraction (shown in gray bar) was expressed as the percentage of that of CCR5+KG− fraction (shown in white bar). (B) Percentages of inhibition of MVC-sensitive (JR-FL) and MVC-resistant HIV-1s are expressed as relative values, with that of MVC-sensitive HIV-1 in CCR5+KG− fractions at 10 μM MVC being 100%. The data are expressed as means ± standard deviations in triplicate experiments.

(Fig. 1) without ligands, which were further increased by the CCR5 antagonists but not by the CXCR4 antagonist (Figs. 1 and 2). These results indicated that CCR5 was able to form dimer/oligomers. It is well known that CXCR4 exists as constitutive higher order oligomers without natural ligands (Supplementary Fig. S1) (Babcock et al., 2003; Hamatake et al., 2009; Issafras et al., 2002; Percherancier et al., 2005; Toth et al., 2004; Wu et al., 2010). CCR5 could also exist as dimer or higher order oligomers as recently described (Babcock et al., 2003; Benkirane et al., 1997; Issafras et al., 2002), although to a lesser extent than CXCR4. On the present study, we further showed not only the existence of CCR5 monomer/dimer forms without its ligands but also the enhanced oligomerization by the antagonists (Fig. 2 and Fig. S1). It has been shown that natural ligands for CCR5 such as CCL5 (RANTES) or CCL4 (MIP-1β) have been shown to induce homo-oligomerization of CCR5 (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000), though its physiological role remains to be determined. Similarly, CXCR4's natural ligand SDF-1 also induced homo-dimerization of CXCR4 as previously described (Percherancier et al., 2005; Toth et al., 2004; Vila-Coro et al., 1999). In contrast, oligomerization of chemokine receptors by their antagonists has not been described to date though another GPCR melatonin receptor was reported to form dimer by both agonists and antagonists (Ayoub et al., 2002). The dimerization of melatonin receptor by both agonist and antagonists was explained by the stabilization of its conformations. Interestingly, in the presence of MVC, CCR5+KG+ subset became well detected by the anti-CCR5 mAb recognizing the conformational epitope (clone 45549) (Fig. 1), indicating that conformations of CCR5 induced by MVC might be also structurally stable. In our flow cytometric analyses without addition of ligands, CCR5+KG+ subsets were equally detected by most anti-CCR5 mAbs except the clone 45549 (Fig. 1). Given that several antigenic conformations of CCR5 existed on the cell surface (Berro et al., 2011; Lee et al., 1999), oligomer forms of CCR5 might have the similar antigenic conformations while monomeric forms had different antigenic conformations.

Previous reports have shown that several GPCRs were homo- or hetero-oligomerized in endoplasmic reticulum (ER) (Herrick-Davis et al., 2006; Issafras et al., 2002; Milligan, 2010; Salahpour et al., 2004; Vischer et al., 2011). In our BiFC assay using confocal laser scanning microscopy, CCR5-KG signals were also detected not only at plasma membrane but also in intracellular compartments, both of which were further enhanced by the addition of MVC (Fig. 2E). Time-course experiments also showed that more than 24 h were needed for the enhanced oligomerization of CCR5 by MVC (Fig. 2D). These findings suggested that oligomerization of CCR5 were formed during early biosynthesis and protein maturation in the ER, and that MVC may further enhance CCR5 oligomerization by the binding of intracellular CCR5. Thus, it is possible that MVC could penetrate into the cell membrane and act before the expression of CCR5 on the cell surface. It is of note that the concentration to induce oligomerization of CCR5 was sufficiently low similar to the concentration that is able to inhibit R5 HIV-1 replication (Fig. 2C), indicating the concentrations of MVC, which would be achieved in HIV-1-infected individuals treated with MVC, seems to induce oligomerization of CCR5 to some extent *in vivo*, although the pharmacological and pathological roles of MVC-induced CCR5 oligomerization in primary T cells and macrophages still remains to be determined.

As we mentioned above, it is possible that CCR5 monomer may have multiple forms, whereas the oligomers may have relatively fixed forms. Since R5 HIV-1 is supposed to recognize specific forms of CCR5 (Berro et al., 2011, 2013), we attempted to check which form of CCR5, monomer or oligomer, is used by R5 HIV-1. To this end, CCR5+KG− and CCR5+KG+ subsets expressed in CD4-positive 293T cells were fractionated by fluorescent-activated cell sorter, and infected with pseudotyped R5 HIV-1. The CCR5+KG− and CCR5+KG+ subsets could have relatively lower and higher amount of oligomeric forms, respectively, while both CCR5+KG− and CCR5+KG+ subsets are supposed to have monomeric and oligomeric forms of CCR5 in flow cytometry analysis. Nevertheless, we were able to show that CCR5+KG+fraction was less susceptible to R5 and dual-R5 HIV-1 than CCR5+KG− fraction (Fig. 3 and 4). It is thus likely that R5 and dual-R5 Envs preferentially recognized monomeric forms of CCR5. The dimerization induced by the monoclonal antibody CCR5-02 was previously reported to cause blocking of HIV-1 entry (Vila-Coro et al., 2000). Our present study further clarified that the oligomerization of CCR5 without ligands also affected the susceptibility to R5 and dual-R5 HIV-1.

Although the susceptibility of KG-negative cell fraction to R5 and dual-R5 HIV-1 was significantly high, dual-X4 HIV-1 89.6 equally infected both CCR5+KG− and CCR5+KG+ fraction (Fig. 4). It is therefore possible that dual-X4 lost the preferential recognition of monomeric forms of CCR5, and may commence recognizing homo-oligomeric forms of CCR5. Intriguingly, the single mutation in 89.6 from arginine to serine at 11th position of the V3 loop (89.6R308S), which changed the tropism from dual-X4 to dual-R5 (Maeda et al.,

2008), also reverted to recognize monomeric forms of CCR5. Hence, the single amino acid substitution was sufficient to lose preferential recognition of monomer forms of CCR5 for CCR5-using HIV-1.

As described by others and us, MVC-resistant HIV-1 recognized MVC-bound and -unbound forms of CCR5 (Kuhmann et al., 2004; Maeda et al., 2011; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007; Yuan et al., 2011). Since MVC was found to enhance CCR5 oligomerization in our present study, we then sought to check whether MVC-resistant HIV-1 recognizes MVC-bound forms of CCR5 oligomers. However, similar to R5 HIV-1, we found that MVC-resistant HIV-1 recognized both MVC-bound and -unbound forms of CCR5 monomer (Fig. 5). Since numbers of MVC-bound forms of CCR5 monomer would be dependent on the surface expression levels of CCR5 and cell types, our findings may partly explain why susceptibility to CCR5 antagonists was dependent on the cell types as previously described (Berro et al., 2011). Taken together, it is likely that R5 HIV-1 including MVC-resistant HIV-1 constrained to use monomeric forms of CCR5 for the entry.

In conclusion, we were able to show that oligomeric forms of CCR5 were less susceptible to R5 HIV-1 than the monomeric forms. However, our findings were obtained from the cells expressing high levels of CCR5 *in vitro*. Therefore, it is quite important to understand the role of CCR5 oligomerization in primary T cells and macrophages for HIV-1 entry *in vivo*. The methods to detect native forms of homo- and hetero-oligomerized CCR5 and their susceptibilities to HIV-1 in primary cells should be established and analyzed to elucidate the role of CCR5 oligomerization for HIV-1 infection *in vivo*.

Materials and methods

Cells and culture conditions

The 293T and HeLa cells were maintained in Dulbecco′s modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml of penicillin and 100 μg/ml of streptomycin. A human CD4-expressing glioma cell line (NP2/CD4) was maintained in Eagle′s minimum essential medium (MEM; Gibco BRL) supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Jinno et al., 1998).

Coreceptor antagonists

A CXCR4 antagonist AMD3100 (Schols et al., 1997a, 1997b) and a CCR5 antagonist maraviroc (MVC) (Dorr et al., 2005) were supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. A CCR5 antagonist TAK-779 (Baba et al., 1999) was kindly obtained from Takeda Chemical Industries (Osaka, Japan).

Construction of retrovirus vector and transduction of 293T cells with the CD4 gene

The cDNA encoding human CD4 was obtained by PCR using human lymphocyte cDNA as the template. The primers used were as follows: 5′-CTCGAGTCGCCACCATGAACCGGGGAGTCCCTTTTAGC-3′ and 5′-TCAAATGGGGCTACATGTCTTCTGAAACCG-3′ (underlined are *Xho*I site). The amplified product was cloned into pCR-TOPO (Invitrogen), and the sequence was verified using 3130 Genetic Analyzer (Applied Biosystems). A CCR5 carrying *Xho*I-*Eco*RI fragments was ligated into pMSCVpuro (Clothech) to generate pMSCVpuro-CD4. Retrovirus vector was produced according to the manufacturer′s instructions, and 293T cells were then transduced and selected by puromycin (Sigma-Aldrich). The CD4 expression of the transduced 293T cells was verified by anti-CD4 monoclonal antibody (RPA-T4, eBioscience).

Expression vectors

CCR5 expression vectors for BiFC was constructed using phmKGN-MN and phmKGC-MN (MBL, Japan) according to the manufacture′s instructions. Briefly, human CCR5 gene was amplified using pCR2-CCR5 as a template (Maeda et al., 2000). Primers used were: 5′-CTCGAGGAACAAGATGGATTATCAAGTG-3′ and 5′-GTCTAGATTACT TGTCGTCATCGTCTTTGTAGTCCAAGCCCACAGATA-3′ (underlined are *Xho*I and *Xba*I restriction enzyme sites, respectively). The amplified product was cloned into pCR-TOPO, and the sequence was verified using 3130 Genetic Analyzer. The *Xho*I-*Xba*I fragment carrying CCR5 gene was then ligated into both phmKGN-MN and phmKGC-MN using *Xho*I and *Xba*I sites to generate pCCR5-KGN and pCCR5-KGC respectively. To construct an expression vector of FLAG-tagged CCR5, CCR5 sequence was amplified using primer: 5′-CTCGAGGAACAAGATGGAT-TATCAAGTG-3′ and 5′-GTCTAGATTACTTGTCGTCATCGTCTTTGTAGTC-CAAGCCCACAGATAT-3′ (underlined are the *Xho*I and *Xba*I restriction enzyme sites, respectively). The amplified product was cloned into pCR-TOPO, and the sequence was then verified using a 3130 Genetic Analyzer. The amplified fragment was finally ligated into phmKGC-MN expression vector (In this vector, split fluorescence protein, Kusabira–Green: KG, was replaced with FLAG-tag by digestion of the *Xho*I and *Xba*I restriction enzyme sites). Expression vectors for JR-FL, 89.6, 89.6R308S, KMT and TIK Envs were prepared as previously described (Maeda et al., 2000, 2008). Expression vectors for Ba-L and YU-2 Envs were kindly supplied by K. Yoshimura (National Institute of Infectious diseases, Tokyo). An expression vector for MVC-resistant Env was prepared as previously described (Yuan et al., 2011, 2013).

Production of recombinant luciferase-reporter virus

Recombinant luciferase-reporter virus of pseudotyped with various HIV-1 Envs or VSV-G were produced by transfection of 293T cells using the calcium phosphate method (ProFection Mammalian Transfection System, Promega) as previously described (Maeda et al., 2000, 2008). The cells culture supernatant was collected 48 h post-transfection, filtered with 0.45 μm pore-size, and stocked at −80 °C until use. The p24 Gag in the culture supernatant was measured using HIV-1 p24 Ag ELISA kit (Zeptometrix) according to manufacture′s instructions.

Detection of the CCR5 expression in KG-positive and -negative cell population

The 293T cells were transfected with CCR5-KG expression vectors, pCCR5-KGN and pCCR5-KGC, using calcium phosphate method, and incubated for 48 h or indicated time of period at 37 °C in the presence or absence of 2 μM of AMD3100, TAK-779 or MVC. In a dose-escalating study, transfected cells were treated with various concentrations of MVC (ranging from 0.0002 μM to 2 μM) for 48 h. To detect the CCR5 in CCR5-KG-transfected cells, the cells were first incubated with anti-CCR5 mAbs, 3A9, CTC8, 45531, 45549 (R&D Systems), or 2D7 (BD Pharmingen) for 30 min at 4 °C. The cells were then stained with β-phycoerythrin-conjugated anti-mouse IgG antibody (Jackson Immuno Research). For direct detection of the CCR5 expression in CCR5-KG-transfected cells, the cells were stained with anti-human CCR5 mAb 2D7 conjugated with Alexa Fluor 647 (BioLegend) for 30 min at 4 °C. The cells were analyzed by FACScan or FACSCalibur fluorescent-activated cell sorter (Becton Dickinson).

Detection of monomeric and oligomeric forms of CCR5 by Western blot

The transfected 293T cells with pCCR5-FLAG expression vector were incubated at 37 °C for 48 h with or without MVC. The cells were treated with DSP (dithiobis[succinimidylpropionate]) cross-linker according to the manufacture′s instructions (Thermo Scientific), and solubilized using 1% Brij O10 (Sigma-Aldrich) lysis buffer (1% Brij O10, 20 mM Tris–HCl pH 8.2, 0.15 M NaCl, 5 mM iodoacetamide) including protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were then separated by SDS-PAGE, blotted onto PVDF (polyvinylidene fluoride, Immobilon-P, Millipore) membrane. The membranes were incubated with anti-FLAG mAb (Wako) or anti-β-actin mAb (Sigma-Aldrich) for 90 min, followed by staining with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson Immuno Research). The signals were detected using Chemi-Lumi One (Nacalai Tesque).

Confocal laser scanning microscopy

The HeLa cells were plated to collagen (Atelo Cell)-coated 8-well glass slides (Lab-Tek). The cells were transfected with both pCCR5-KGN and pCCR5-KGC using Lipofectamine 2000 (Invitrogen) according to the manufacture′s instructions. Transfected cells were incubated at 37 °C for 48 h in the presence or absence of 1 μM MVC. The cells were fixed with 4% paraformaldehyde (Wako) for 15 min, and analyzed using LSM-700-ZEN confocal laser scanning microscopy (Carl Zeiss) with a 60X objective lens. The images were processed using LSM Imaging Browser (Carl Zeiss).

Fluorescence-activated cell sorting of CCR5-KG-positive and -negative cell fraction and infection with pseudotyped HIV-1

The CD4-293T cells were transfected with pCCR5-KGN and pCCR5-KGC using calcium phosphate method. After 48 h cultures at 37 °C, cells were stained with anti-CCR5 mAb CTC8, followed by staining with APC-conjugated anti-mouse IgG. The cells were then sorted into CCR5+KG− and CCR5+KG+ fractions with the same expression levels of CCR5 by using FACS AriaII (Becton Dickinson) according to the manufacture′s instructions. Sorted each fraction was then incubated with the same amount (40 ng of p24Ag) of luciferase-reporter HIV-1 pseudotyped with various HIV-1 Envs including R5 (JR-FL, YU-2, Ba-L), R5X4 (89.6 wt, 89.6 R308S (Maeda et al., 2008), KMT, and TIK), MVC-resistant Env (T199K/T275M/V3-M5) (Yuan et al., 2011, 2013) or VSV-G at 37 °C for 30 min to allow adsorption of the virus. The cells were washed to remove unadsorbed virus, seeded into a 96-well plate, and cultured at 37 °C for 24 h. Luciferase activity was measured using a luminometer, Lumat LB 9501/16 (EG&G Berthold, Bad Wildbad). The entry efficiency of HIV-1 infected by HIV-1 Envs in each cell fraction was normalized by the luciferase activity of the same fraction infected by VSV-G.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.12.034.

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