A short amino acid sequence containing tyrosine in the N-terminal region of G protein-coupled receptors is critical for their potential use as co-receptors for human and simian immunodeficiency viruses

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Various G protein-coupled receptors (GPCRs) have the potential to work as co-receptors for human and simian immunodeficiency virus (HIV/SIV). HIV/SIV co-receptors have several tyrosines in their extracellular N-terminal region (NTR) as a common feature. However, the domain structure of the NTR that is critical for GPCRs to have co-receptor activity has not been identified. Comparative studies of different HIV/SIV co-receptors are an effective way to clarify the domain. These studies have been carried out only for the major co-receptors, CCR5 and CXCR4. A chemokine receptor, D6, has been shown to mediate infection of astrocytes with HIV-1. Recently, it was also found that an orphan GPCR, GPR1, and a formyl peptide receptor, FPRL1, work as potent HIV/SIV co-receptors in addition to CCR5 and CXCR4. To elucidate more about the domain of the NTR critical for HIV/SIV co-receptor activity, this study analysed the effects of mutations in the NTR on the co-receptor activity of D6, FPRL1 and GPR1 in addition to CCR5. The results identified a number of tyrosines that are indispensable for the activity of these co-receptors. The number and positions of those tyrosines varied among co-receptors and among HIV-1 strains. Moreover, it was found that a small domain of a few amino acids containing a tyrosine is critical for the co-receptor activity of GPR1. These findings will be useful in elucidating the mechanism that allows GPCRs to have the potential to act as HIV/SIV co-receptors.

INTRODUCTION

G protein-coupled receptors (GPCRs), also know as seven-transmembrane domain receptors, mediate the entry of human immunodeficiency virus type 1 (HIV-1), HIV-2 and simian immunodeficiency viruses (SIVs) into CD4-positive cells as co-receptors. Two chemokine receptors (CKRs), CCR5 and CXCR4, are considered to act as major co-receptors in the establishment of HIV-1 infection in vivo (Bleul et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996).

In addition to CCR5 and CXCR4, some GPCRs such as CCR2b (Doranz et al., 1996), CCR3 (Choe et al., 1996), Apj (Choe et al., 1998), ChemR23 (Samson et al., 1998), CX3CR1 (Combadiere et al., 1998), D6 (Neil et al., 2005) and GPR15 (Farzan et al., 1997) have been shown to be HIV/SIV co-receptors. We also found that four CKRs, CCR8 (Jinno et al., 1998), CXCR1 (Soda et al., 1999), CXCR2 (Soda et al., 1999) and CXCR5/BLR1 (Kanbe et al., 1999), and two orphan GPCRs, GPR1 (Shimizu et al., 1999) and RDC1 (Shimizu et al., 2000), work as co-receptors for several HIV/SIV strains. However, the structural and functional factors that confer the property of HIV/SIV co-receptor on GPCRs have not been elucidated.

The extracellular N-terminal region (NTR) and the second or third extracellular loop (ECL) play important roles in the interaction of CCR5 and CXCR4 with HIV-1 (Doranz et al., 1997, 1999; Picard et al., 1997a; Hill et al., 1998;
Reeves et al., 1998; Misumi et al., 2001; Pontow & Ratner, 2001). Several tyrosine residues are commonly present in the NTRs of HIV/SIV co-receptors. Some amino acids, including these tyrosines, have been shown to be important for the co-receptor activities of CCR5 and CXCR4 (Blanpain et al., 1999; Brelot et al., 2000). Sulfation of these tyrosines in CXCR4 and CCR5 has been demonstrated to enhance co-receptor activity (Farzan et al., 1998, 1999). However, their importance has only been demonstrated for CCR5 and CXCR4. There is no evidence that these tyrosines are critical for the other GPCR to retain co-receptor function for HIV/SIV strains.

Recently, we found that a formyl peptide receptor, FPRL1, works as a co-receptor for HIV/SIV strains. Moreover, HIV-1 strains that use GPR1 or FPRL1 in addition to CCR5 and CXCR4 as co-receptors were readily isolated from peripheral blood lymphocytes (PBLs) of HIV-1-positive subjects (unpublished data). These findings strongly suggest that GPR1 and FPRL1 are potent co-receptors in vivo in addition to CCR5 and CXCR4.

In this study, to elucidate more about the NTR structure critical for HIV/SIV co-receptors, we examined the effects of various amino acid changes in the NTR on the co-receptor activity of CCR5, D6, FPRL1 and GPR1. We found that specific tyrosine residues were dispensable for D6, FPRL1 and GPR1 to retain their co-receptor activity, similar to CCR5. Moreover, only a short amino acid sequence containing a tyrosine constituted the critical domain for the co-receptor activity of GPR1. Our findings will help to clarify the molecular mechanisms of the interaction between co-receptors and HIV/SIV strains.

**METHODS**

**Cells.** The human T-cell line C8166 (Salahuddin et al., 1983) and CCR5-transduced C8166 cells (C8166/CCR5; Soda et al., 1999) were used to propagate HIV-1, HIV-2 and SIV strains. NP-2/CD4 cells were established as described elsewhere (Jinno et al., 1998; Soda et al., 1999). C8166 and C8166/CCR5 cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co.) containing 10% fetal calf serum (FCS). NP-2/CD4 cells expressing CCR5, D6, FPRL1, GPR1 and their mutants were cultured in Eagle’s minimum essential medium (EMEM; Nissui Pharmaceutical Co.) supplemented with 10% FCS.

**Virus strains.** HIV/SIV strains that use CCR5, CXCR4 or both as co-receptors are described as R5-, X4- and R5X4-tropic, respectively. The following strains were used: the R5-tropic HIV-1 strain SF162 (Cheng-Mayer et al., 1990); the X4-tropic HIV-1 strain IIIB (Ratner et al., 1985); three R5X4-tropic HIV-1 strains, GUN-1wt (Takeuchi et al., 1987), GUN-4wt (Shimizu et al., 1994) and GUN-7wt (Shimizu et al., 1994); three HIV-1 variants, GUN-1v (Takeuchi et al., 1987), GUN-4v (Shimizu et al., 1994) and GUN-7v (Shimizu et al., 1994); two HIV-2 strains, CBL23 (Potempa et al., 1997) and ROD/B (Guyader et al., 1987); and the SIV strain mmdGB-1 (Tsujimoto et al., 1988). The culture supernatants of C8166 cells infected with the HIV/SIV strains except for the SF162 strain were harvested as viral stocks. SF162 strain was propagated in C8166/CCR5 cells as described previously (Soda et al., 1999). HCM342 is an R5/X4-tropic primary HIV-1 isolate (Shimizu et al., 2008).

**PCR primers.** Oligonucleotide primers (Proligo) were synthesized to construct mutants of CCR5, D6, FPRL1 and GPR1 as shown in Fig. 1(b). Details of the primers used are provided in Supplementary Table S1, available in JGV Online.

**Mutant construction of co-receptor genes.** CCR5 and GPR1 expression plasmids were constructed as described previously (Shimizu et al., 1999; Soda et al., 1999). The DNA fragments of the entire open reading frames (ORFs) of the D6 and FPRL1 genes were amplified from total RNA of C8166 cells by RT-PCR using D6-specific primers (D6-CN and D6-kr) or FPRL1-specific primers (FPRL1-CN and FPRL1-kr). The ORFs of the D6 and FPRL1 genes were each cloned into the expression plasmid pCXB-bsr (Akagi et al., 2000) and designated pCXB-bsr/D6 and pCXB-bsr/FPRL1, respectively. The amino acid sequences of the cloned D6 and FPRL1 genes were determined and confirmed to be 100% identical to the reported genes (Perez et al., 1992; Nibbs et al., 1997).

Substitution and deletion mutants of the NTR amino acid sequences were constructed for CCR5, D6, FPRL1 and GPR1 by PCR using mutagenic oligonucleotide primers. The PCR product from each primer pair was self-ligated after blunt-end formation using a DNA blunting and ligation kit (Toyobo Biologics). The mutant names, amino acid sequences of their NTRs and mutagenic primers used to produce them are shown in Fig. 1(b). For example, wild-type CCR5 is described as CCR5(YYYY) as there are four tyrosines in its NTR.

**Establishment of NP-2/CD4 cells expressing co-receptor mutants.** NP-2/CD4 cells transfected with pMX-puro plasmids harbouring CCR5, GPR1 or their mutants were selected by cultivation for 2 weeks in medium containing puromycin (10 μg ml⁻¹). The NP-2/CD4 cells transfected with pCXB-bsr plasmids harbouring D6, FPRL1 or their mutants were cultured for 2 weeks in medium containing blastcicidin (10 μg ml⁻¹). Surviving cells were designated NP-2/CD4/ plus the name of the wild-type or mutant co-receptor.

GPCR proteins expressed in these cells were detected by indirect immunofluorescence assay (IFA) using antibodies against CCR5 (clone CTC5; R&D Systems), D6 (polyclonal; Alexis Biochemicals) or FPRL1 (polyclonal; MBL). A rabbit anti-human GPR1 polyclonal antibody raised against a synthetic peptide (aa 1–27) of GPR1 (Jinno-Oue et al., 2005) was also used. The cells were fixed with 1% (v/v) paraformaldehyde (PFA) or acetone.

**Infection assay.** NP-2/CD4 cells expressing CCR5, D6, FPRL1, GPR1 or their mutants were seeded into 24-well culture plates (5 × 10⁴ cells per well) 24 h prior to virus inoculation. The cells were exposed to an HIV/SIV strain at a concentration corresponding to 1 × 10⁵ c.p.m. of reverse transcriptase activity as described previously (Hoshino et al., 1983). After a 2 h exposure, the cells were washed three times with EMEM containing 10% FCS to remove the inoculum and cultured in 500 μl fresh medium at 37 °C under 5% CO₂. The cells were passaged every 2 days. The susceptibility of these cells to HIV/SIV strains was determined by detecting HIV/SIV antigens using IFA (Takeuchi et al., 1987). Sera derived from HIV-1 carriers or from macaques infected with SIV were used as the primary antibody source (Soda et al., 1999).

**NaClO₃ treatment.** Inhibition of tyrosine sulfation in NP-2/CD4 cells expressing HIV/SIV co-receptors was carried out by incubation in sulfate-free EMEM supplemented with 1 or 10 mM NaClO₃ (Sigma) and 10% FCS dialysed against PBS (Farzan et al., 1999). The cells were then exposed to HIV-1 as described above.
RESULTS

Identification of tyrosines in the NTR critical for the co-receptor activity of CCR5

CCR5 has four tyrosines at aa 3, 10, 14 and 15 (Fig. 1a). To determine which tyrosines are critical for the co-receptor activity of CCR5, mutants with tyrosine substitutions in its NTR were constructed as shown in Fig. 1(b).

Wild-type and mutant CCR5 proteins were clearly detected by IFA in NP-2/CD4/CCR5(YYYY), NP-2/CD4/CCR5(AYYY), NP-2/CD4/CCR5(YAYY), NP-2/CD4/CCR5(YYAY) and NP-2/CD4/CCR5(YYYA) cells fixed with PFA (Fig. 2a) and with acetone (data not shown), indicating that they are expressed on the cell surface.

As shown in Fig. 3(a), NP-2/CD4/CCR5(YYYY) cells were susceptible to HIV-1 strains (SF162, GUN-1WT, GUN-4WT and GUN-7wt), HIV-2 strains (ROD/B and CBL23) and SIV strain mndGB-1 as described previously (Soda et al., 1999). Among these four tyrosine mutants, only CCR5(YYYA) completely lost co-receptor activity for the R5-tropic HIV-1 SF162 strain. This tyrosine substitution also markedly reduced the co-receptor activity of CCR5 for HIV-2 and SIV strains as described previously (Rabut et al., 1998; Pontow & Ratner, 2001). The other substitutions in the CCR5(AYYY), CCR5(YAYY) and CCR5(YYAY) mutants had little effect on the co-receptor activity of CCR5 for HIV-1 strain SF162, HIV-2 strains ROD/B and CBL23, and SIV strain mndGB-1.

Unexpectedly, we found that the CCR5(YAYY) and CCR5(YYAY) mutants lost almost all co-receptor activity for R5/X4-tropic HIV-1 strains (GUN-1WT, GUN-4WT and GUN-7wt). It has been reported that the substitution of tyrosine at aa 10 eliminates the co-receptor function of

Fig. 1. (a) Amino acid sequences of the NTRs for the co-receptors CCR5, D6, FPRL1 and GPR1 examined in this study. Tyrosines are indicated in bold. Potential N-glycosylation signals are underlined. (b) Amino acid sequences of the substitution and deletion mutants of CCR5, D6, FPRL1 and GPR1. Amino acids that match those of the wild-type GPCR are indicated by a dot. Deleted amino acids are indicated by a space. Details of the primers used to produce the mutants are given in Supplementary Table S1, available in JGV Online.
Fig. 2. Detection of co-receptor proteins with amino acid substitutions in their NTRs on the surface of NP-2/CD4 cells. NP-2/CD4 cells expressing wild-type or mutant CCR5 (a), D6 (b), FPRL1 (c) or GPR1 (d) were cultured on glass slides and fixed with 1% PFA. Wild-type and mutant proteins expressed on the cell surface were detected by IFA as indicated. Controls were NP-2/CD4 cells transduced with each wild-type GPCR and stained with secondary antibody only.

Fig. 3. Effect of tyrosine substitutions in the NTR on co-receptor activity. The susceptibilities of NP-2/CD4 cells expressing wild-type or mutants of CCR5 (a), D6 (b), FPRL1 (c) or GPR1 (d) to HIV/SIV strains were determined by IFA at day 6 after virus inoculation.
CCR5 for several R5-tropic strains of HIV-1 and SIV (Blanpain et al., 1999). We found that CCR5 function was maintained by the substitution in R5-tropic SF162 but not for R5/X4-tropic strains. These results indicated that tyrosines aa 10 and 14 are also involved in the co-receptor activity of CCR5 for R5- or R5/X4-tropic HIV-1 strains. Thus, recognition of the CCR5 NTR is different for R5- and R5/X4-tropic HIV-1 strains.

**Effects of tyrosine substitutions in the NTR on the co-receptor activity of D6**

D6 was recently identified as a novel HIV-1 co-receptor (Neil et al., 2005). However, the role of tyrosines in its NTR has not been determined. We established NP-2/CD4 cells expressing wild-type or mutant D6 (Fig. 1b).

As shown in Fig. 2(b), D6 proteins were detected in NP-2/CD4/D6(YYYY) cells fixed with PFA by IFA using the polyclonal antibody raised against D6 protein. D6 mutant proteins were also detected in NP-2/CD4/D6(AYYY), NP-2/CD4/D6(DYYY), and NP-2/CD4/D6(YYYA) cells at levels similar to that in NP-2/CD4/D6(YYYY) cells.

As shown in Fig. 3(b), NP-2/CD4/D6(YYYY) cells were susceptible to R5/X4-tropic HIV-1 strains (GUN-1V, GUN-4V, and GUN-7V) and less susceptible to HIV-2 strains (ROD/B and CBL23) and SIV strain mndGB-1. D6(AYYY), D6(YAYY) and D6(YYYA) mutants lost their co-receptor activity. In contrast, the co-receptor activity of NP-2/CD4/D6(YYAY) mutant was clearly maintained. These results indicated that the three tyrosines at aa 23, 24 and 27 in the NTR are indispensable for the co-receptor activity of D6.

Thus, for D6, multiple tyrosines are involved in the co-receptor activity and the epitope recognized by the monoclonal anti-D6 antibody used here is closely related to the NTR conformation critical for the co-receptor activity.

**Co-receptor activity of tyrosine mutants of FPRL1**

FPRL1 is a novel and predominant co-receptor for primary HIV-1 isolates (unpublished data). FPRL1 also contains three tyrosines at aa 12, 17 and 22 in its NTR (Fig. 1a). We investigated the roles of these tyrosines in co-receptor activity by using the tyrosine substitution mutants shown in Fig. 1b).

The levels of FPRL1 protein detected in NP-2/CD4/FPRL1(YYYY), NP-2/CD4/FPRL1(AYYY), NP-2/CD4/FPRL1(YAYY) and NP-2/CD4/FPRL1(YYYA) cells by IFA using a monoclonal anti-FPRL1 antibody raised against a synthetic peptide of the second ECL of FPRL1 were approximately the same, although their detected levels were not higher than the other co-receptors in NP-2/CD4 cells (Fig. 2c). We could not determine the effects of these tyrosine substitutions on the NTR conformation, because the anti-FPRL1 antibody raised against its NTR was not available.

As shown in Fig. 3(c), NP-2/CD4/FPRL1(YYY) cells were susceptible to the cell line-adapted HIV-1 strains (GUN-4v and GUN-7WT). The FPRL1(AYY) and FPRL1(YAY) mutants retained co-receptor activity for strains GUN-4v and GUN-7WT. However, FPRL1(YAY) had markedly reduced co-receptor activity, suggesting that the tyrosine at aa 16 is important for the co-receptor activity of FPRL1.

**Identification of a tyrosine in the NTR critical for co-receptor activity of GPR1**

Expression of GPR1(YYYY), GPR1(AYYY), GPR1(YAYY), GPR1(YYAY), GPR1(YYYA), GPR1(NN) and GPR1(AA) mutants (Fig. 1b) were detected in NP-2/CD4 cells by IFA using the anti-GPR1 antibody raised against its N-terminal 27 aa (Jinno-Oue et al., 2005) (Figs 2 and 4). The expression levels of GPR1(AYYY), GPR1(YAYY) and GPR1(YYAY) were lower than those of GPR1(YYYY) and GPR1(YYYA) (Fig. 2), even when the cells were fixed with acetone (data not shown), suggesting that tyrosine substitutions in the GPR1(YYYY), GPR1(YYYA) and GPR1(YAYY) mutants decreased the antigenicity of the GPR1 epitope recognized by the anti-GPR1 antibody.

As shown in Fig. 3(d), NP-2/CD4/GPR1(YYYY) cells were susceptible to three HIV-1 strains (GUN-1v, GUN-4v and GUN-7v), two HIV-2 strains (ROD/B and CBL23) and SIV strain mndGB-1, as described previously (Shimizu et al., 1999). Mutants GPR1(YAYY), GPR1(YYYA) and GPR1(YYYA) maintained their co-receptor activity. However, the GPR1(YYYY) mutant completely lost its activity. These results indicated that tyrosine at aa 15 is critical for the co-receptor activity of GPR1.

**Determination of regions in the NTR necessary for the co-receptor activity of GPR1**

We constructed deletion mutants of the NTR of GPR1 to map the region critical for its co-receptor activity (Fig. 1b).

As shown in Fig. 4, the GPR1(d1–11) mutant maintained reactivity with the anti-GPR1 antibody. The reactivity of the GPR1(d1–13) mutant with this antibody was much weaker than that of GPR1(d1–11). These results suggested that the region comprising aa 13–14 is closely linked to the epitope for the anti-GPR1 antibody. The mutants with deletions additional to that of GPR1(d1–11), i.e. GPR1(d1–18) and GPR1(d1–20), completely lost their reactivity with this antibody.

As shown in Fig. 5, a deletion of aa 1–11 and a substitution of phenylalanine with methionine at the aa 12 did not appear to affect the co-receptor activity of GPR1. When an additional 2 aa were removed from CCR5(d1–11) and an asparagine at aa 14 was replaced with methionine, i.e. GPR1(d1–13) mutant, its co-receptor activity was completely eliminated, suggesting that aa 13–14 are critical for...
the co-receptor activity of GPR1. GPR1(d1–18) and GPR1(d1–20) mutants had no co-receptor activity as expected.

We deleted a hydrophobic amino acid, leucine, at aa 19 to produce the GPR1(d19) mutant because this deletion may change the conformation of the tyrosine-clustering hydrophilic region (aa 15–22) harbouring the critical tyrosine at aa 15 identified above (Figs 1b and 3d). However, no effect on co-receptor activity was detected (Fig. 5). The GPR1(d19) mutant was clearly detected by the anti-GPR1 antibody (Fig. 4), suggest that leucine at this position is not involved in the co-receptor activity and the epitope of GPR1.

We introduced deletions into the regions proximal to the transmembrane domain of GPR1 (Fig. 1b). The GPR1(d26–28) mutant completely lost its co-receptor activity, whereas GPR1(d36–42) retained it (Fig. 5). Unexpectedly, both of these mutants had decreased reactivity with the anti-GPR1 antibody when the cells were fixed with PFA (Fig. 4) or with acetone (data not shown). These results suggested that these proximal regions of the NTR are also involved in the formation of the structure necessary for the co-receptor activity and the epitope recognized by the anti-GPR1 antibody.

None of these GPR1 mutants were effective as co-receptors for the HIV/SIV strains IIIB, SF162, GUN-4wt and GUN-7wt, which cannot use wild-type GPR1(YYYY) (Figs 3d and 5). The effects of amino acid substitutions and

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**Fig. 4.** Detection of deletion and substitution mutant proteins of GPR1 on the surface of NP-2/CD4 cells. Wild-type and mutant GPR1 expressed on the surface of NP-2/CD4 cells were detected by IFA as indicated. The control was NP-2/CD4 cells expressing wild-type GPR1(YYYY) stained with secondary antibody only.

**Fig. 5.** Co-receptor activity of substitution and deletion mutants of GPR1. The susceptibilities of NP-2/CD4 cells expressing wild-type or mutant GPR1 to HIV/SIV strains were determined by IFA at day 6 after virus inoculation.
deletions in the NTRs of CCR5, D6, FPRL1 and GPR1 on the co-receptor activities are summarized in Fig. 7.

**Effects of tyrosine sulfation in the NTR on co-receptor activity**

Sulfation of tyrosines in the NTR enhances the co-receptor activity of CCR5 and CXCR4 (Farzan et al., 1998, 1999). However, the role of tyrosine sulfation in the co-receptor activity of D6, FPRL1 and GPR1 has not yet been clarified. The Sulfinator method, which is frequently used to predict tyrosine sulfation of proteins (Monigatti et al., 2002), showed that the possibility of tyrosine sulfation in the NTR is high for CCR5 and D6, but low for FPRL1 and GPR1. NP-2/CD4/CCR5(YYYY), NP-2/CD4/D6(YYYY), NP-2/CD4/FPRL1(YYYY) and NP-2/CD4/GPR1(YYYY) cells were incubated in EMEM containing an inhibitor of tyrosine sulfation, NaClO₃, at 1 or 10 mM for 48 h and then inoculated with HIV-1 strains. These concentrations of NaClO₃ have been shown to be sufficient for complete inhibition of tyrosine sulfation of cellular proteins (Farzan et al., 1998, 1999).

As shown in Fig. 6, when NP-2/CD4/CCR5(YYYY) cells were treated with NaClO₃ (10 mM), the susceptibility to HIV-1 strains (GUN-1WT, GUN-7WT and SF162), HIV-2 strains (CBL23 and ROD/B) and SIV strain mndGB-1 was markedly reduced, as described previously (Farzan et al., 1998, 1999). Surprisingly, the susceptibility of NP-2/CD4/D6(YYYY) cells to HIV-2/SIV strains was slightly enhanced by this treatment. We have reported previously that primary HIV-1 isolate HCM342 can efficiently use FPRL1 as a co-receptor (Shimizu et al., 2008). Sulfation inhibitor had little or no effect on the susceptibility of NP-2/CD4/GPR1(YYYY) and NP-2/CD4/FPRL1(YYYY) cells to HIV/SIV strains.

The GPR1 NTR contains an aspartic acid-tyrosine (DY or YD) sequence, which is frequently found as a sulfation signal (Monigatti et al., 2002). We therefore constructed GPR1(NN) and GPR1(AA) mutants to reduce further the possibility of tyrosine sulfation (Fig. 1b). GPR1(NN) and GPR1(AA) maintained co-receptor activity and antigenicity to the anti-GPR1 antibody, even though a partial inhibition of activity was detected (Figs 4 and 5). These results suggested that tyrosine sulfation in the NTR is neither critical nor an enhancing factor for the co-receptor activity of D6, FPRL1 and GPR1.

**Effects of N-glycosylation in the NTR on co-receptor activity**

N-Glycosylation is seen universally in the NTRs of GPCRs. All HIV/SIV co-receptors identified so far also contain an N-glycosylation signal in their NTRs. However, the effects of N-glycosylation of the NTRs in HIV/SIV co-receptor activity have not been determined.

D6, FPRL1 and GPR1 harbour N-glycosylation signals in their NTR (Fig. 1a) whilst CCR5 does not. To clarify the role of these signals in co-receptor activity, we produced mutants GPR1(N14D), D6(N19D) and FPRL1(N4D) in which the N-glycosylation signal was removed by amino acid substitution (Fig. 1b).

Expression of these mutant proteins was detected in NP-2/CD4 cells fixed with PFA (Fig. 2) or acetone (data not shown), indicating that removal of the N-glycosylation

![Fig. 6. Effect of NaClO₃ treatment on the co-receptor activity of GPCRs. NP-2/CD4 cells transduced with wild-type co-receptors CCR5 (a), D6 (b), FPRL1 (c) and GPR1 (d) were pre-treated with 0, 1 or 10 mM NaClO₃ and exposed to HIV/SIV strains. The relative values of the susceptibilities of these cells to HIV/SIV strains were determined by IFA at day 6 after virus inoculation.](image)
signal did not significantly decrease their expression on the cell surface or the reactivity of the epitopes with the antibodies used in this study. All of these mutants maintained their co-receptor activity (Fig. 3b–d), indicating that N-glycosylation in NTRs does not play an important role in either the distribution on the cell surface or the co-receptor activities of D6, FPRL1 and GPR1. The co-receptor activities of all wild-type and mutant GPCRs determined in this study are summarized in Fig. 7.

DISCUSSION

Almost all of the HIV/SIV co-receptors identified so far contain several tyrosines in their NTRs, although the amino acid sequences of the NTRs are highly heterogeneous (Willey et al., 2003). We noticed that, in the GPCR superfamily, molecules with tyrosines in their NTRs are concentrated in the group of CKRs and related receptors (unpublished data). Therefore, amino acid sequences containing tyrosines may be a key factor in elucidating the molecular mechanism of HIV-1 entry into target cells. However, the functional and structural roles of tyrosines and amino acid sequences of NTRs in co-receptor activity have not been clarified sufficiently. Mutational studies have been carried out only for CCR5 and CXCR4.

Recently, we found that GPR1 and FPRL1 work as predominant co-receptors after CCR5 and CXCR4 for primary HIV-1 isolates, even though both are genetically distant from CKRs in the phylogenetic tree of GPCRs (unpublished data). Based on this information, we decided to use D6, FPRL1 and GPR1 in addition to CCR5 to elucidate more about the roles of amino acid sequences of NTRs in the co-receptor activity of GPCRs.

In our analyses of CCR5, we found that two tyrosines at aa 10 and 14 were required for CCR5 to function as a co-receptor of R5/X4-tropic HIV-1 strains in addition to tyrosine at aa 15, which has been reported to be critical for its co-receptor activity (Figs 3a and 7). R5/X4-tropic HIV-1 strains may recognize a larger region of the NTR than R5-tropic strains. Thus, recognition of the NTR amino acid sequence is variable among HIV-1 strains and the different recognition patterns are closely linked to the different co-receptor usages.

D6 was recently reported to be a co-receptor for HIV-1 infection of brain astrocytes (Neil et al., 2005). Unexpectedly, we found that multiple tyrosines were clearly involved in the co-receptor activity of D6 (Figs 2 and 7). In FPRL1, tyrosine at aa 17 was the most important for co-receptor activity. However, with D6, the other two tyrosines were also partially involved in co-receptor
activity. Thus, in D6 and FPRL1, multiple tyrosines contributed to their co-receptor activity. Most of the HIV-1 strains that can use D6 or FPRL1 were R5/X4-tropic. As shown in CCR5, multiple tyrosines are involved in the co-receptor activity for R5/X4-tropic HIV-1 strains (Fig. 3a). These results suggested that R5/X4-tropic HIV-1 strains recognize larger regions of the NTR than R5-tropic strains. Thus, the property of HIV-1 to use more GPCRs as co-receptors may link to the ability to recognize larger and more complex conformations of the NTRs.

GPR1 can mediate infection of brain pericytes and mesangial cells as a co-receptor (Shimizu et al., 1999; Tokizawa et al., 2000). Primary HIV-1 isolates that can use GPR1 as a co-receptor are easily obtained from the PBLS of HIV-1-positive subjects (unpublished data). Moreover, we have reported that a synthetic oligopeptide of the GPR1 NTR efficiently blocks infection of various HIV-1 strains (Jinno-Oue et al., 2005). However, the contribution and the roles of GPR1 in HIV-1 infection in vivo have not been well elucidated. Based on these findings, we thought that further clarification of the roles of the NTR in the co-receptor activity of GPR1 would be informative for further studies of HIV/SIV co-receptors.

We found that only tyrosine at aa 15 was critical for the co-receptor activity of GPR1 (Figs 3d and 7). Unlike the case of CCR5, the other tyrosines were dispensable for GPR1 to work as a co-receptor for R5/X4-tropic HIV-1 strains. These results raised the possibility that only a small region comprising a few amino acids in the NTR may be critical for the co-receptor activity of GPR1. Consequently, we constructed a series of GPR1 mutants to examine this hypothesis.

Unexpectedly, the highly acidic N-terminal region of 12 aa was dispensable for the co-receptor activity of GPR1 (Figs 1b, 5 and 7). However, the adjoining 2 aa, glutamic acid (E) and asparagine (N) at aa 13 and 14, were critical for co-receptor activity. Removal of these 2 aa completely destroyed the epitope recognized by the anti-GPR1 antibody (Fig. 4). It should be noted that these 2 aa are just before the critical tyrosine at aa 15 identified above. These results strongly suggested that the short amino acid sequence glutamic acid-asparagine-tyrosine (ENY, aa 13–15) constitutes the domain critical for interaction with HIV-1.

Next, we investigated the involvement of the other regions of the NTR in co-receptor activity of GPR1. It has been postulated that an electrostatic interaction of acidic regions containing tyrosines in the NTR with basic amino acids of HIV-1 gp120 is important for the co-receptor function of CXCR4 (Blanpain et al., 1999; Brelot et al., 2000). Therefore, we changed two aspartic acids adjoining tyrosines to neutral amino acids, either asparagine or alanine, to make the mutants GPR1(NN) and GPR1(AA) (Fig. 1b). However, these mutants maintained expression on the cell surface as well as their co-receptor activity (Figs 4, 5 and 7), indicating that the acidic region containing tyrosines at aa 17 and 21 does not contribute to the co-receptor activity of GPR1. This conclusion was supported by the fact that these two tyrosines were dispensable for the co-receptor activity (Fig. 3d); moreover, the GPR1(d19) mutant was also expressed on the cell surface and retained co-receptor activity (Figs 1b, 4, 5 and 7).

In the GPR1(d26–28) and GPR1(d36–42) mutants, proximal regions far from the critical tyrosine in NTR were deleted (Fig. 1b). Unexpectedly, these mutants had reduced co-receptor activity (Figs 5 and 7). Therefore, the proximal region deleted in these mutants may contribute to maintaining the structure of the NTR recognized by HIV/SIV strains and the anti-GPR1 antibody. This notion was supported by the fact that expression of GPR1(d26–28) and GPR1(d36–42) was weaker than GPR1(YYYY) when they were detected by the anti-GPR1 antibody (Fig. 4).

Taking all of these results together, we conclude that the structure critical for co-receptor activity of GPR1 is focused on the small domain containing aa 13–15, and that this domain is closely linked to the epitope recognized by the anti-GPR1 antibody used in this study. Moreover, the structure and function of this domain may be maintained by the proximal regions of the NTR.

An N-glycosylation signal was found in the NTRs of D6, FPRL1 and GPR1, but not in CCR5 (Fig. 1a). However, the mutants of co-receptors GPR1(N14D), D6(N19D) and FPRL1(N4D) maintained both their expression on the cell surface and their co-receptor activity (Figs 1b, 2, 3 and 7). Therefore, we conclude that, unlike the case of CXCR4 as reported by Picard et al. (1997b), N-glycosylation of the NTR plays no role in the co-receptor activities of CCR5, D6, FPRL1 or GPR1.

The co-receptor activity of CCR5 was inhibited by NaClO3, an inhibitor of tyrosine sulfation, as reported previously (Farzan et al., 1999) (Fig. 6). However, NaClO3 treatment had no effect on the co-receptor activity of D6 and GPR1. Instead, the susceptibility of NP-2/CD4/D6 cells to HIV-2/SIV strains was enhanced by this treatment; this mechanism requires further investigation. Thus, unlike the cases of CCR5 and CXCR4, tyrosine sulfation in the NTR is neither a critical nor an enhancing factor for the co-receptor activity of D6, FPRL1 and GPR1.

As a common feature, the sequence motif tyrosine-aspartic acid/asparagine/glutamic acid [Y(N/D/E)] or (N/D/E)Y is commonly seen in the NTRs of CCR5, CXCR4, D6, FPRL1 and GPR1. The critical domain of GPR1 identified in this study also contained an aspartic acid-tyrosine (NY) sequence (Fig. 1a). Conformation of the NTRs of GPCRs given by the Y(N/D/E) or (N/D/E)Y sequence motif may be important for GPCRs to act as HIV/SIV co-receptors. The consensus sequence for protein sulfation contains these sequence motifs (Monigatti et al., 2002). However, our results clearly indicated that tyrosine sulfation in the NTR is not a critical factor for the co-receptor activity of GPCRs.
We suggest that unknown cellular function(s) closely linked to these sequence motifs may be necessary for the co-receptor activity of GPCRs.

Thus, GPCRs harbouring tyrosines in the NTR have the potential to be HIV/SIV co-receptors, and their co-receptor activity should be examined. HIV-1 has been expanding its ability to use other GPCRs with tyrosines in the NTR as novel co-receptors, so future studies are likely to identify novel co-receptors. There is no evidence that CCR5 and CXCR4 are the only co-receptors that play important roles in HIV-1 infection in vivo and progression of AIDS. The contributions of additional co-receptors such as D6, FPRL1 and GPR1 in HIV-1 infection in vivo should be elucidated further.

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REFERENCES


