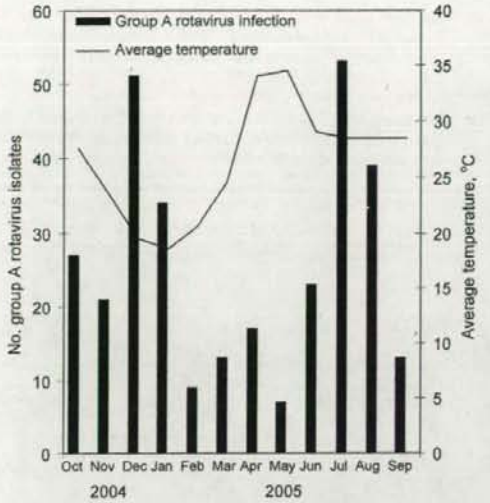

Figure 2. Seasonal pattern of group A rotavirus infection in infants and children with acute gastroenteritis in Dhaka, Bangladesh, October 2004–September 2005.

es. The G1P[8] strains, less common in 2001, became the predominant strains in the following years, but decreased again in 2005–06. Rotaviruses show great genomic diversity, and several studies in different regions of Bangladesh have identified types not targeted by candidate rotavirus vaccines (*11,14*). The frequent genomic reassortment among different rotavirus types was accelerated by mixed infection and generated huge genomic diversity (*13*).

RAV has been associated with gastroenteritis outbreaks in infants and children <5 years of age. However, less is known of the age distribution of rotavirus infection in Bangladesh. In this study, infections were most commonly detected in children <2 years of age.

Common clinical symptoms of RAV-infected patients were dehydration (84%), vomiting (69%), abdominal pain (52%), and fever (31%), which are in agreement with previous published reports (*15*). Number of loose stools per day was increased, with most patients (76%) having loose stools 3–5 times per day. Our study is limited because we could not conduct other tests such as enzyme immunoassay or polyacrylamide gel electrophoresis to confirm rotavirus illness. The incidence of rotavirus gastroenteritis identified by RT-PCR could be an overestimate because healthy controls tested by RT-PCR had a 5%–10% general incidence of rotavirus.

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Mr Dey is a PhD student at The University of Tokyo. His research interest focuses on molecular epidemiology of gastroenteritis viruses in humans.

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Table. Distribution of group A rotavirus G and P genotypes among infants and children with acute gastroenteritis in Dhaka City, Bangladesh, 2004–2005*

Genotype	P[8]	P[4]	P[6]	Mixed*	Nontypeable	Total
G1	33	4	0	1	2	40
G2	11	109	0	9	13	142
G3	8	0	0	1	0	9
G4	51	4	0	2	5	62
G9	36	2	1	2	3	44
Mixed*	10	0	0	0	0	10
Total	149	119	1	15	23	307

*>1 G or P genotype was recognized.

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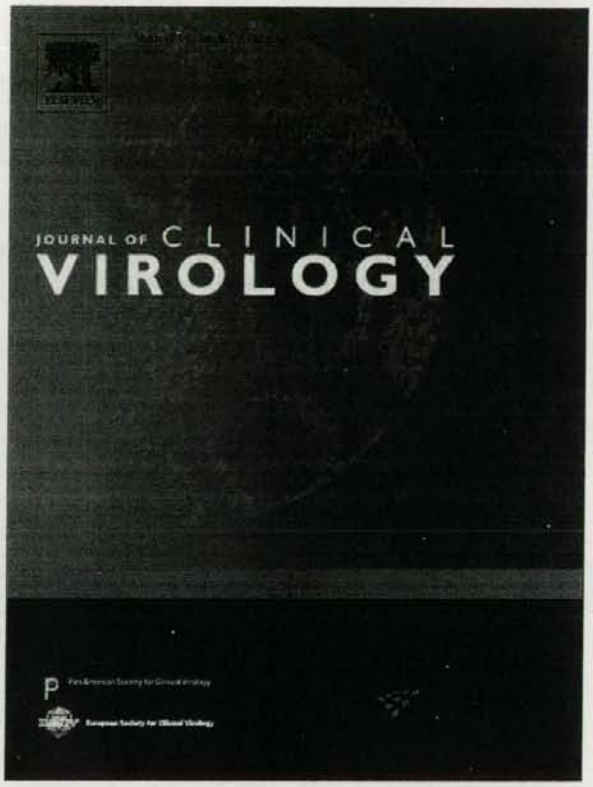

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Short communication

Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis

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ABSTRACT

Background: Norovirus (NoV) infection is thought to be confined to the intestines, whereas many reports suggest antigenemia and viremia occur during rotavirus gastroenteritis.
Objectives: To detect NoV RNA in sera and cerebrospinal fluids (CSF) from NoV-infected children, and to quantify and genetically characterize the NoV found in these compartments.
Study design: Semi-nested PCR was conducted on stool, serum and CSF samples from 56 patients with acute gastroenteritis. Positive samples for NoV were analyzed further by sequencing and real-time PCR.
Results: From 39 patients with NoV RNA in stools, 6 also had NoV RNA in sera and none had NoV RNA in CSF. Genotypes of the NoV in stool and serum from the same patient matched completely. The strains in this study had high homology (98.1–100%) with registered strains in the database. The median viral load in stools of the serum-positive patients was greater than that of the serum-negative patients, but this difference was not statistically significant (9.8×10^6 copies/g versus 1.1×10^9 copies/g ($p = 0.117$)).
Conclusions: NoV RNA appeared in the blood stream in 15% of the patients of NoV gastroenteritis. Although the viral load in stool was not statistically correlated with NoV appearance in serum, genetic analysis indicated that NoV RNA in sera originated from the NoV gastroenteritis.

1. Introduction

Norovirus (NoV), a member of the family *Caliciviridae* in the genus *Norovirus*, is a major cause of non-bacterial acute gastroenteritis all over the world.[1–3] The main symptoms of NoV infection are diarrhea and vomiting, which are usually mild and self-limiting. However, a recent case report demonstrated that a patient suffered from disseminated intravascular coagulation during a NoV outbreak, in association with obtundation, headache and photophobia[4] and Ito et al.[5] reported NoV-associated encephalopathy with altered consciousness. These reports indicate a potential spread of NoV to organs other than the intestines.

Many studies have been conducted seeking evidence of extra-intestinal manifestations of rotavirus infection. These included detection of rotavirus RNA in blood[6,7], CSF[7–9] and throat swabs.[7] Although early works suggested that this was due to unusual rotavirus strains or rare host genetic or immunologic defects in the infected child,[10] recent analysis revealed that rotavirus antigen is commonly detected in sera of immunocompetent children with rotavirus diarrhea (43–67%).[11–13]

Human NoV, unlike rotavirus, is not capable of growing in cell lines and has no animal model available, thus hindering study of systemic spread after intestinal infection. Detection of NoV RNA from specimens other than stools has been limited to one case in which NoV was present in serum and CSF from a previously healthy NoV-infected girl with encephalopathy.[5]

In this study, we sought to detect NoV RNA in blood and CSF from patients with NoV gastroenteritis. Genetic analyses and quantification of NoV RNA were undertaken on positive samples.

2. Materials and methods

2.1. Sample collection

From the diarrheal patients who attended Department of Pediatrics in Teikyo University Hospital, Eijudo clinic, and Red Cross

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Society Wakayama Medical Center from December 2005–2006, 56 cases who needed venepuncture for examination or infusion were recruited into this study. Stool samples were collected from 56 cases only once, while several blood samples were taken from patients who were required additional venepuncture in later course (total = 90). The interval between stool and the first blood sample collection was less than 2 days. CSF was also obtained from two patients who had convulsions. Ethical approvals were obtained from the Ethical Committees and informed consent was given by the guardians of the patients.

2.2. Reverse transcription (RT)-PCR

RT-PCR was performed according to the previously described protocol[14] with primers COG1F/G1-SKR (first round PCR) and G1-SKF/G1-SKR (second round (semi-nested) PCR) for NoV genogroup I (GI), and COG2F/G2-SK (first round PCR) and G2-SKF/G2-SKR (second round PCR) for NoV genogroup II (GII).[15,16] We used known-positive specimen extracts as positive controls, JP 6146 for NoV GI and JP 5235 for NoV GII, respectively. PCR amplicons were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 0.5 μg/ml) for 20 min and visualized under ultraviolet light.

2.3. Sequence analysis

Positive PCR products were subjected to sequencing by Big-Dye terminator cycle sequencing kit and an ABI prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). We used first round PCR products for sequencing if first round PCR gave positive results. If only second round PCR was positive, we used second round PCR products. In both cases, we sequenced with the same primers used in each round on both strands. Sequence analysis was performed using CLUSTAL X software (Version 1.81). Reference NoV strains and accession numbers used in this study were as follows: Norwalk/68/US (M87661), Melksham/89/UK (X81879), SaitamaU201/98/J (AB067542), Arg320/95/AR (AF190817), Lordsdale/93/UK (X86557), Bristol/93/UK (X76716), and Manchester_Sapovirus (X86560).

2.4. Quantification of NoV

We performed real-time PCR to quantify NoV viral RNA in PCR-positive samples, as previously described.[17] Data were corrected by using ROX passive reference as an internal standard and recovery rate of the NoV genome was tested by highly purified NoV plasmid standards containing PCR products of the ORF1–ORF2 junction of the GII strain (Saitama U201, AB039782).[17] Half of the serum samples which were positive by RT-PCR in our laboratory were selected arbitrarily and tested for real-time PCR at the National Institute of Infectious Diseases in Tokyo in order to reconfirm the existence of NoV genes in serum.

2.5. Statistical analysis

SPSS software version 12.0J was used for data analysis. A p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. RT-PCR and patients' characteristics

Among the 56 stool samples collected, 26 were positive for NoV GII by first round PCR and 13 were positive by second round. Among the 90 serum samples collected from the 56 patients, 6 were positive for NoV GII by second round PCR and were confirmed by sequence analysis to be NoV. Neither of the two CSF samples contained NoV RNA even by second round PCR, although stool samples from these patients contained NoV GII RNA by first round PCR. All of the samples tested were negative for NoV GI.

3.2. Nucleotide sequence and phylogenetic analysis of NoV GII

The partial nucleotide sequences (282 bp) of the NoV capsid gene were analyzed by phylogenetic grouping according to the classification schemes described by Kageyama et al.[18] They were grouped into three distinct genotypes; two stool samples in genogroup II genotype 2 (GII/2), four stool samples and one serum sample in GII/3, and 33 stool and five serum samples in GII/4. The genotypes of stool and serum samples collected from the same patients were all matched and the identities of nucleotide sequences between these pairs ranged between 99.2% and 100%. The phylogenetic tree of the six pairs of stool and serum samples together with the reference strains is shown in Fig. 1.

The identities among strains in the same genotypes ranged from 96.8% to 99.6%, and each strain showed high identities (98.1–100%) with previously registered Japanese strains in the DDBJ DNA/GenBank database.

3.3. Quantification of NoV RNA

The median RNA viral load detected in the stool samples from patients with serum-positive and negative for NoV GII was 9.8×10^9 (range 4.2×10^8 to 4.0×10^{10}) and 1.1×10^9 (range 5.6×10^3 to 1.4×10^{11}) copies/g of stool, respectively. This difference was not statistically significant ($p = 0.117$, two-tailed Mann–Whitney U-test). The mean value for each group was 1.4×10^{10} (standard error, 8.1×10^9) and 1.0×10^{10} (standard error, 5.0×10^9) copies/g of stool with no statistical difference ($p = 0.722$, t-test). We also investigated any correlation between RNA viral load in the serum sample and that in the paired stool sample. Patient C was excluded from this analysis because no data by real-time PCR was available. With this small number of pairs ($n = 5$), no linear correlation between the serum and stool viral load was observed ($r = 0.071$, $p = 0.910$).

4. Discussion

Potential extra-intestinal spread is an important issue in understanding the pathogenesis of viral gastroenteritis. In this study, we observed that 15% of the NoV gastroenteritis patients (6/39) had NoV RNA in serum and could not detect NoV RNA in either of two CSF samples.

Our genetic analysis showed a very high homology between strains found in stool and serum, indicating that the viral RNA in blood had originated from the intestinal tract. The high homology between the strains in this study and those deposited in GenBank implied that no unique strains were associated with detection in blood stream.

Although the mean viral load in stool of serum-positive group was greater than that of serum-negative group, there was not statistically significant difference between these two groups. Several studies on rotavirus gastroenteritis showed that the antigen level in blood samples decreased as diarrhea subsided, implying a relationship between the presence of virus in the intestines and in the blood.[11,12] Hence, we may be able to observe a similar relationship in a larger sample sized study and/or samples collected consecutively from the same group.

Recently, experiments with murine NoV in a mouse model showed that infectious virus was detected in peripheral organs such as spleen, liver, and lung after oral inoculation, and that histopathological changes were observed not only in the intestines, but also in the spleen of immunocompetent mice.[19,20] These findings are of

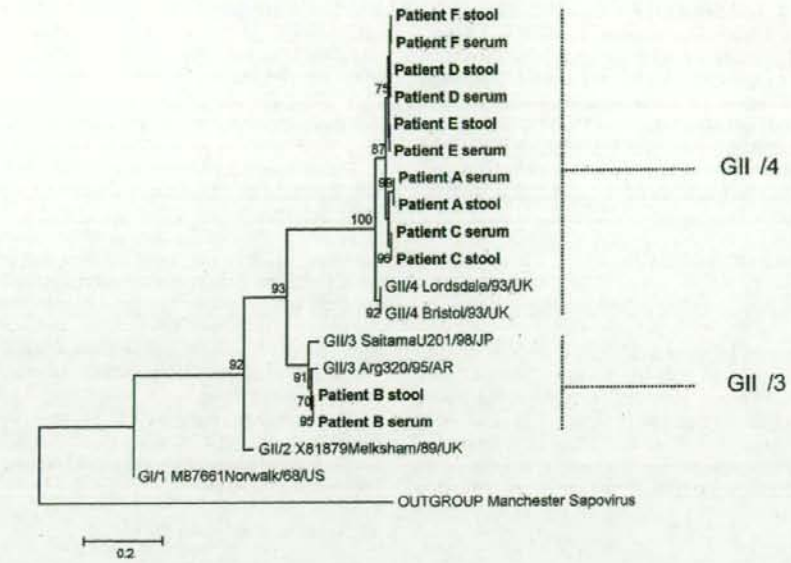

Fig. 1. Phylogenetic tree of NoV GII nucleotide sequences detected in pairs of stool and serum samples. The tree was constructed from partial nucleotide sequences of the capsid region. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Strains detected in this study are highlighted in bold. Percentage bootstrap values above 70% are shown at the branch nodes. The scale indicates nucleotide substitutions per position.

significance in reviewing the current concept of human NoV, which focuses on intestinal infection. The murine model is consistent with our findings that suggest extr-intestinal spread of NoV during NoV gastroenteritis.

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Short communication

Immunochromatography test for rapid detection of norovirus in fecal specimens

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ABSTRACT

An immunochromatography (IC) assay for rapid detection of norovirus (NoV) was evaluated with fecal samples collected from children who suffered from acute gastroenteritis during the winter season of 2007–2008 in Japan. A total of 75 fecal specimens were tested for NoV by the newly developed IC kit and by a gold standard RT-PCR method. The sensitivity, specificity, and agreement of this IC kit were 75.4%, 100%, and 80%, respectively. In addition, phylogenetic analysis revealed that the majority of NoV circulating in Japan during 2007–2008 belonged to the new variant GII/4 2006b genetic cluster. It was demonstrated that the IC kit evaluated in this study could detect these new variant NoV strains, which emerged recently in Japan. Therefore, it is suggested that this NoV IC kit could be used as an alternative method for the screening of NoV in fecal specimens, especially during the season of acute gastroenteritis outbreak.

Viral enteric infections affect millions of people worldwide. The major agents include rotavirus, astrovirus, adenovirus, and enteric caliciviruses (norovirus and sapovirus) (Clark and McKendrick, 2004; Wilhelmi et al., 2003). These viruses are transmitted mainly via fecal–oral route through person-to-person contact or consumption of contaminated food. Recently, norovirus (NoV) has been emerged as significant etiologic agent and is recognized as the major cause of non-bacterial acute gastroenteritis in all age groups. The virus is a member of the family *Caliciviridae*, which is nonenveloped, positive-sense, single-stranded RNA virus (Green, 2007).

Because no readily available cell culture system exists for NoV, characterization and classification of NoV is based on reverse transcription-polymerase chain reaction (RT-PCR), genomic sequencing, and phylogenetic analysis (Kageyama et al., 2004; Zheng et al., 2006). In recent years, extensive molecular epidemiological studies of calicivirus infections in humans have been conducted. These detection techniques have markedly enhanced our understanding of the epidemiology and genetic diversity of NoVs. According to the latest scheme for NoV nomenclature, NoVs have been classified into five separate genogroups (GI to GV) and at least 29 genotypes (Zheng et al., 2006). Several epidemiological studies have clearly indicated that NoV GII/4 has been the most highly prevalent genotype in humans worldwide during the past decade (Buesa et al., 2008; Bull et al., 2006; Hansman et al., 2004; Khamrin et al., 2007; Malasao et al., 2008; Motomura et al., 2008; Reuter et al., 2008; Tu et al., 2007).

Recently, large outbreaks of NoV have occurred in various epidemiological settings in Japan and the main causative agent was the NoV GII/4 genotype (Morioka et al., 2006; Motomura et al., 2008; Okada et al., 2007; Sasaki et al., 2006). When patients are diagnosed with severe diarrhea, rapid virus identification is essential to ensure administration of the appropriate treatment and control. For this reason, a rapid and sensitive diagnostic tool, such as the immunochromatography (IC) test, is required for NoV detection.

Most recently, two reports on the evaluation of IC test (IP-Noro; Immuno Probe Co., Ltd., Saitama, Japan) for rapid detection of NoV directly from stool samples collected in Japan and Vietnam demonstrated the sensitivity, specificity, and overall agreement of the tests at 78.9% and 73.7%, 96.4% and 100%, 92.4% and 95.2%, respectively (Khamrin et al., 2008; Nguyen et al., 2007). However, that commercial IC test was developed by using specific antibodies against NoV GII/3 and GII/4, which previously showed broad reactivity with several NoV VLP genotypes (Shiota et al., 2007). In this report, we have evaluated the efficacy of the newly developed IC kit (Morinaga Milk

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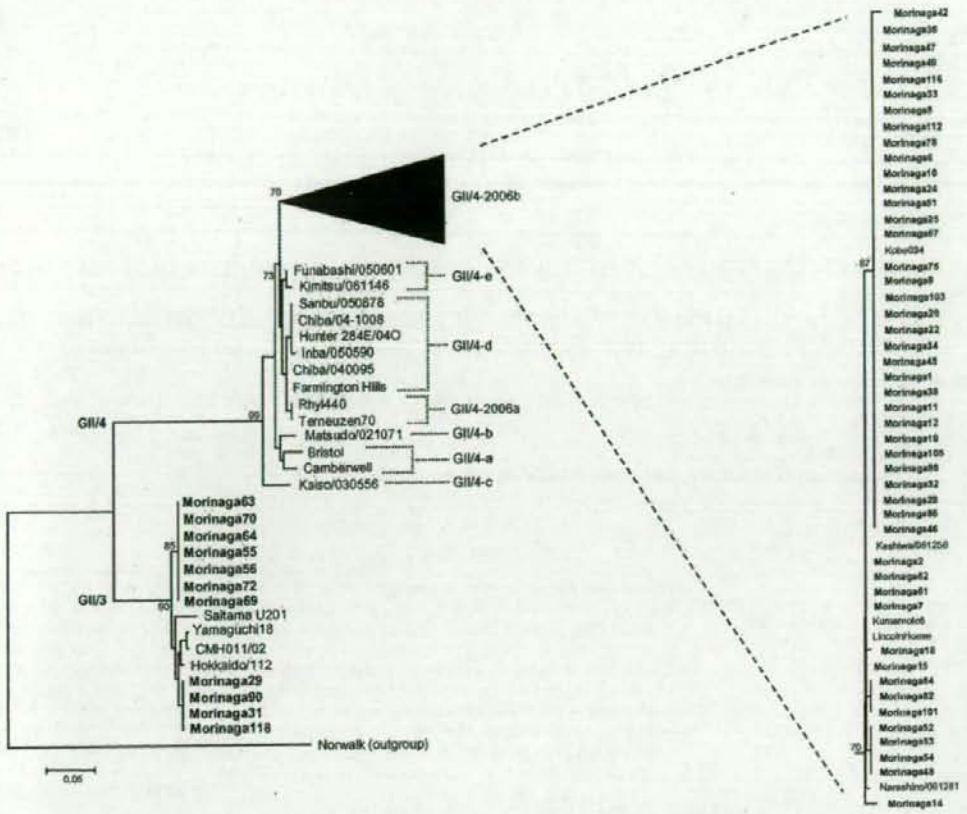

Fig. 1. Phylogenetic analysis of partial capsid sequences of NoV detected in Japan during December 2007 to January 2008. The tree was constructed on the basis of the neighbor-joining method using the MEGA 4 software package, and the numbers on each branch indicate the bootstrap values. The NoV strains detected in the present study are presented in boldface.

Industry Co., LTD.) which was developed for broad detection of several NoV genotypes including GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6.

A total of 75 fecal specimens were tested for rapid detection of NoV by the IC kit. All of these samples were collected from children who suffered from acute gastroenteritis in Shizuoka and Kyoto, Japan. The study period was during the peak season for NoV, winter, from December 2007 to January 2008. To evaluate the sensitivity and specificity of the IC test kit, all 75 fecal samples were tested. This IC test was performed according to the manufacturer's directions. Briefly, 10–20% of stool samples were prepared in tubes containing buffer. Then, stool suspensions were mixed by vortexing for 2 min and centrifuging at 3000 × g for 10 min. This was followed by adding the strip test into the tube which contained the stool mixture. It took only 15 min to obtain the result of the assay.

Table 1
Comparison of NoV detection in stool samples between the newly developed IC kit and the RT-PCR method.

		RT-PCR		Total (%)
		Positive	Negative	
IC	Positive	46	0	46 (61.3)
	Negative	15	14	29 (38.7)
	Total (%)	61 (81.3)	14 (18.7)	75 (100)

For the IC strip test, latex conjugated rabbit polyclonal antibodies against NoV GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 genotypes were coated at the conjugate pad. The test line was also coated with immobilized polyclonal antibodies against NoV GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 genotypes, while the control line was coded with anti-rabbit immunoglobulin.

For the reference test, the RNA genome of NoV was first extracted from 10% to 20% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Germany). The presence of the NoV in fecal specimens was confirmed by RT-PCR using the protocol described previously (Yan et al., 2003). A forward primer, G1-SKF (nt 5342–5261) 5′-CTGCCCGAATTYGTAAATGA-3′, was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACCCARCCATTRTACA-3′ for the amplification of NoV GI. For NoV GII identification, a forward primer, COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGATGAG-3′, was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCATRHCCRTTRTACAT-3′. All of the NoV positive samples were analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

For NoV sequence and phylogenetic analysis, the PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The primers used for amplification

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of the partial capsid genes were also used as sequencing primers. The sequences obtained were compared with those of NoV strains deposited in the GenBank using BLAST searches. The genotypes of NoV were classified using the phylogenetic clustering methods determined previously by Kageyama et al. (2004).

To evaluate sensitivity, specificity, and overall agreement of this newly developed IC kit, a panel of 75 stool samples was tested and the results were compared with those of the RT-PCR gold standard method and sequence analysis. As shown in Table 1, 46 out of 75 (61.3%) samples were positive for NoV by this IC kit, while the RT-PCR standard method showed NoV detection rate of 81.3%; 61 out of 75 samples were positive for NoV. This result indicated 75.4% sensitivity for the IC assay. No false positive for NoV detection was observed for this IC test. Fourteen samples that tested negative for NoV by RT-PCR were also negative by this IC kit, resulting in a specificity of 100%. In addition, by comparing this IC assay and the RT-PCR method, the total agreement of this newly developed IC kit was 80%.

For NoV detection by RT-PCR screening method, a total of 75 fecal specimens were tested and 61 (81.3%) were positive for NoV. Of these, all positive samples were identified as NoV GII genogroup, and only 2 genotypes, GII/3 and GII/4, were identified by sequence and phylogenetic analyses. Fig. 1 shows the phylogenetic analysis of partial NoV capsid sequences detected in this study. It was observed that GII/4 was the most predominant genotype and accounted for the majority (48 out of 61; 78.7%) of NoV detected.

Interestingly, all the NoV GII/4 analyzed in this study belonged to a new variant GII/4 2006b subtype, which differs from NoV subtypes GII/4-a–e, and from 2006a (Okada et al., 2007; Siebenga et al., 2008; Tu et al., 2008). All of the NoV GII/4 strains detected in this study were clustered together in the same branch and showed a close genetic lineage with the NoV 2006b reference strains recently circulating in Japan (Kobe034, Kashiwa/061256, Kumamoto5, and Narashino/061281). NoV GII/3 was detected at a lower prevalence in 11 of 61 samples (18.0%), while genotypes of NoV in 2 samples could not be identified because of insufficient stool samples.

In recent years, molecular techniques have revolutionized the diagnosis of NoV and led to the recognition that NoV is one of the major viruses responsible for non-bacterial gastroenteritis in humans of all age groups (Clark and McKendrick, 2004; Wilhelmi et al., 2003; Green, 2007). The conventional diagnostic methods to detect and identify NoV in stool samples (RT-PCR and sequence analysis) require experience, specialized laboratory equipment, and time. Therefore, a rapid, sensitive, and simple diagnostic tool, such as the IC test, is required, especially during an outbreak of NoV. The advantages of the IC assay are cost effectiveness, speed (15–20 min), and ease of use at the bedside without special laboratory equipment.

Recently, the sensitivity and specificity of the IC assay for detection of NoV in stool samples (IP-Noro; Immuno Probe Co., Ltd., Japan) have been evaluated (Khamrin et al., 2008; Nguyen et al., 2007). Those reports demonstrate that the sensitivity and specificity of the IP-Noro strip test ranged from 73.7% to 78.9% and 96.4% to 100%, respectively. Compared with this newly developed IC kit, the sensitivity and specificity are in good agreement with those previously reported (sensitivity; 75.4% and specificity; 100%). Moreover, this new IC kit was developed for the detection of several NoV genotypes using antibodies against NoV GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 genotypes in the IC strip test.

Several NoV-VLP genotypes were used to test this IC kit to determine reactivity with NoV genotypes. It was found that this IC kit could detect other NoV genotypes of GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 VLPs (data not shown). However, to clarify this point, additional testing with several other NoV genotypes from clinical samples is essential. The limitation of this study is that only a small number of negative samples were included. There were only 14 negative samples, which could affect the specificity test.

The data from phylogenetic analysis clearly demonstrate that this new IC assay can detect the new NoV variant GII/4 2006b strains which have recently begun circulating in Japan. Therefore, this study demonstrates that the new IC kit can be used as an alternative method for detecting NoV in fecal specimens and may be practical for NoV screening during outbreaks of viral gastroenteritis.

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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. However, 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.

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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;

Details of the primers used for the construction of mutants are available with the online version of this paper.

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 GPCRs 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 indispensable 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 and NP-2/CD4 cells transduced with 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 R5/X4-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 R5/X4-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 mndGB-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-CR) or FPRL1-specific primers (FPRL1-CN and FPRL1-CR). The ORFs of the D6 and FPRL1 genes were each cloned into the expression plasmid pCX-bsr (Akagi *et al.*, 2000) and designated pCX-bsr/D6 and pCX-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 $\mu g\ ml^{-1}$; Calbiochem). NP-2/CD4 cells transfected with pCX-bsr plasmids harbouring D6, FPRL1 or their mutants were cultured for 2 weeks in medium containing blasticidin (10 $\mu g\ ml^{-1}$; Calbiochem). 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 (5×10^4 cells per well) 24 h prior to virus inoculation. The cells were exposed to an HIV/SIV strain at a concentration corresponding to 1×10^4 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 % CO_2. 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).

$NaClO_3$ 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 $NaClO_3$ (Sigma) and 10 % FCS dialysed against PBS (Farzan *et al.*, 1999). The cells were then exposed to HIV-1 as described above.

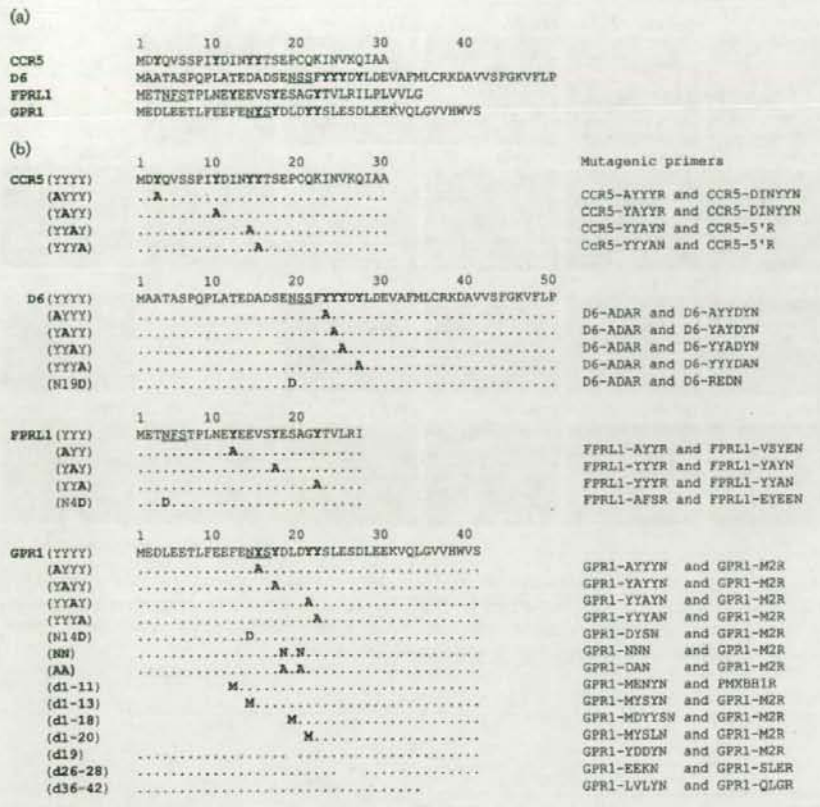

Fig. 1. (a) Amino acid sequences of the NTRs for the co-receptors CCR5, D6, FPRL1 and GPR1 examined in this study. Tyrosines are indicated in bold. Potential *N*-glycosylation signals are underlined. (b) Amino acid sequences of the NTRs of the substitution and deletion mutants of CCR5, D6, FPRL1 and 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.

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

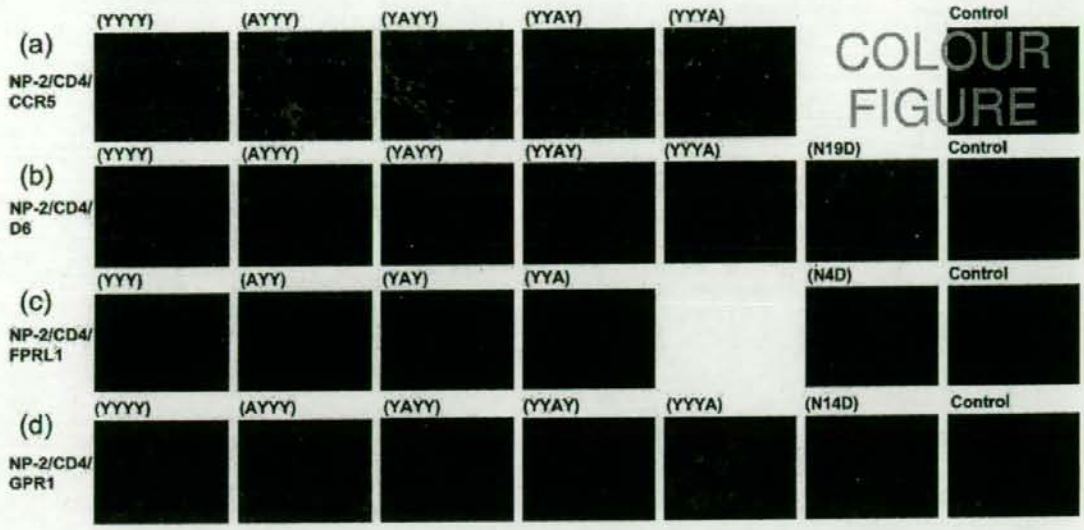

Fig. 2. Detection of co-receptor proteins with amino acid substitutions in their NTRs on the surface of NP-2/CD4 cells. NP-2/CD4 cells expressing wild-type or mutant CCR5 (a), D6 (b), FPRL1 (c) or GPR1 (d) were cultured on glass slides and fixed with 1 % PFA. Wild-type and mutant proteins expressed on 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.

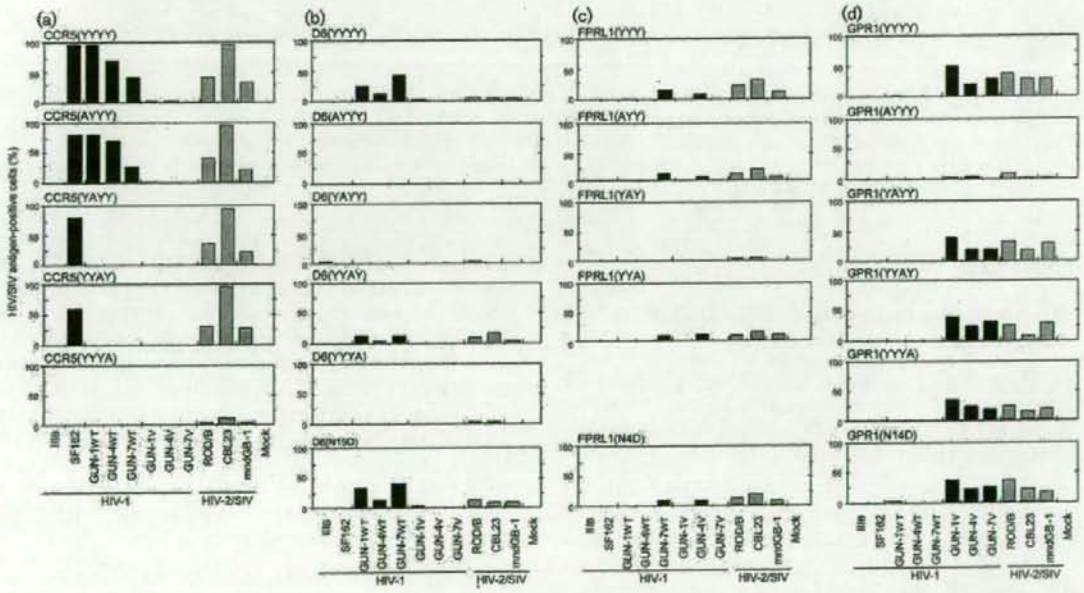

Fig. 3. Effect of tyrosine substitutions in the NTR on co-receptor activity. The susceptibilities of NP-2/CD4 cells expressing wild-type or mutants of CCR5 (a), D6 (b), FPRL1 (c) or GPR1 (d) to HIV/SIV strains were determined by IFA at day 6 after virus inoculation.

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(YAYY), NP-2/CD4/D6(YYAY) 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-1WT, GUN-4WT and GUN-7WT) 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. 1(b).

The levels of FPRL1 protein detected in NP-2/CD4/FPRL1(YYY), NP-2/CD4/FPRL1(AYY), NP-2/CD4/FPRL1(YAY) and NP-2/CD4/FPRL1(YYA) 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 then 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(YYA) 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(AYYY), GPR1(YAYY) and GPR1(YYAY) 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(YYAY) and GPR1(YYYA) maintained their co-receptor activity. However, the GPR1(AYYY) 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(d1–13), 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

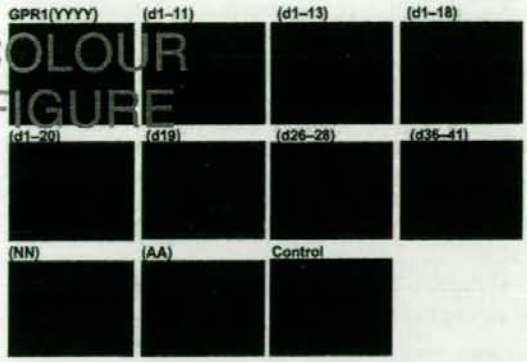

Fig. 4. Detection of deletion and substitution mutant proteins of GPR1 on the surface of NP-2/CD4 cells. Wild-type and mutant GPR1 expressed on the surface of NP-2/CD4 cells were detected by IFA as indicated. The control was NP-2/CD4 cells expressing wild-type GPR1(YYYY) stained with secondary antibody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

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

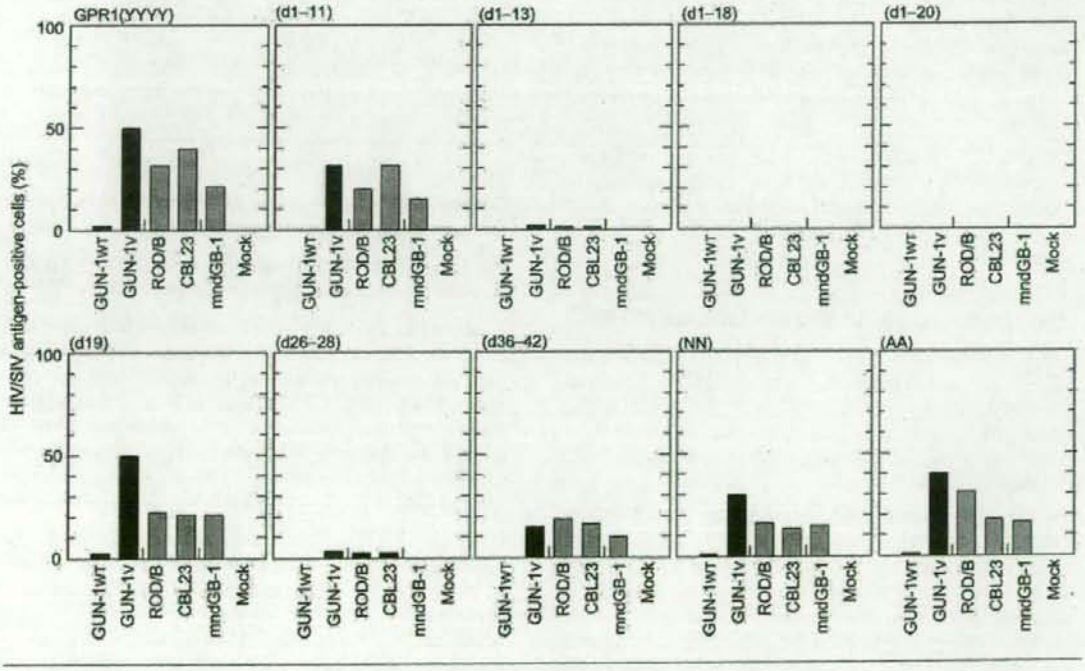

Fig. 5. Co-receptor activity of substitution and deletion mutants of GPR1. The susceptibilities of NP-2/CD4 cells expressing wild-type or mutant GPR1 to HIV/SIV strains were determined by IFA at day 6 after virus inoculation.

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(YYY) and NP-2/CD4/GPR1(YYYY) cells were incubated in EMEM containing an inhibitor of tyrosine sulfation, $NaClO_3$, at 1 or 10 mM for 48 h and then inoculated with HIV-1 strains. These concentrations of $NaClO_3$ 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 $NaClO_3$ (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(YYY) 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.

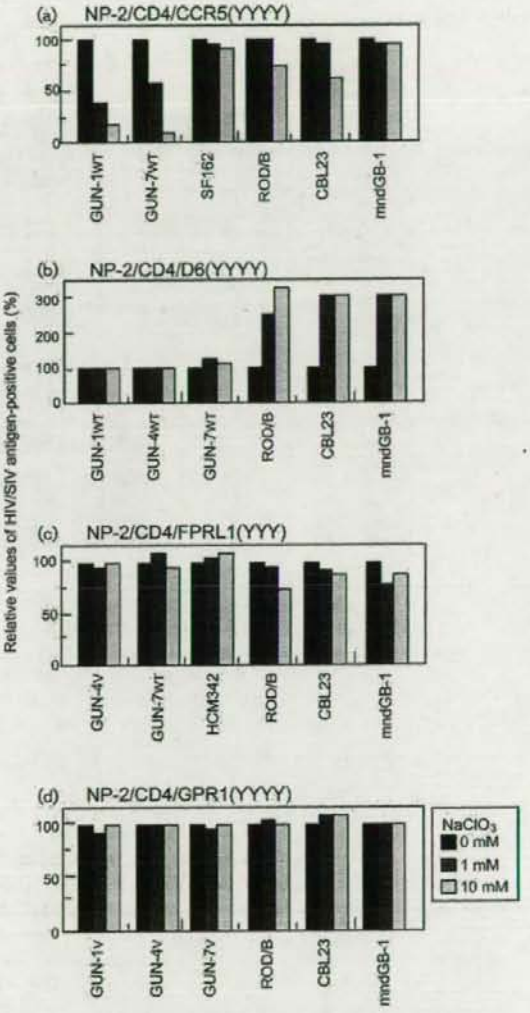

Fig. 6. Effect of $NaClO_3$ treatment on the co-receptor activity of GPCRs. NP-2/CD4 cells transduced with wild-type co-receptors CCR5 (a), D6 (b), FPRL1 (c) and GPR1 (d) were pre-treated with 0, 1 or 10 mM $NaClO_3$ and exposed to HIV/SIV strains. The relative values of the susceptibilities of these cells to HIV/SIV strains were determined by IFA at day 6 after virus inoculation.

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

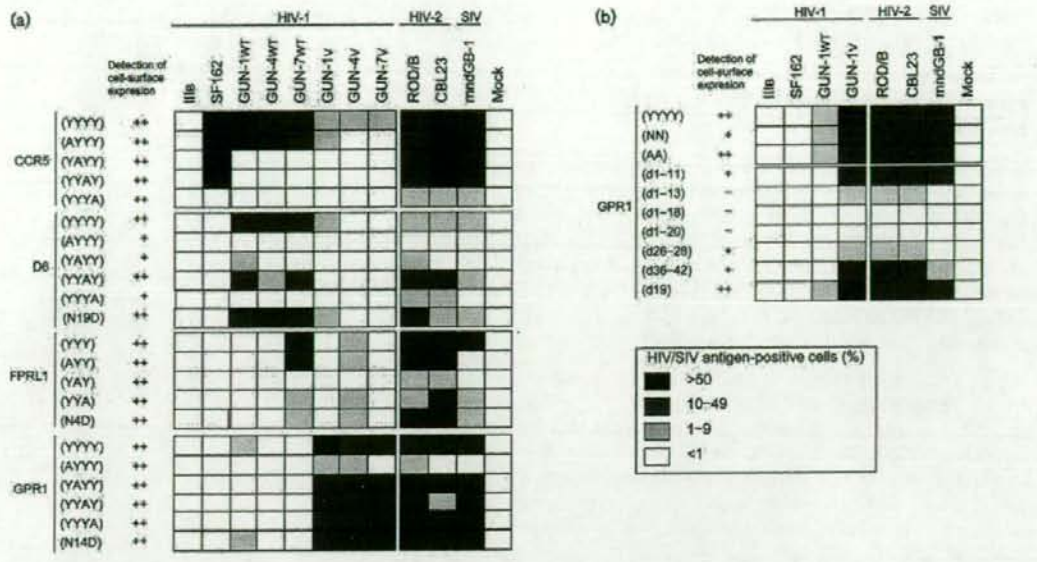

Fig. 7. Summary of the co-receptor activities for CCR5, D6, FPRL1 and GPR1 mutants. (a) Effects of amino acid substitutions of tyrosines or *N*-glycosylation signals in NTRs on the co-receptor activities of CCR5, D6, FPRL1 and GPR1. (b) Effects of amino acid deletions or substitutions in the NTRs on the co-receptor activity of GPR1. Levels of co-receptor activity are shown by the susceptibility of NP-2/CD4 cells expressing these mutants to HIV/SIV strains. HIV- or SIV-related antigen-positive cells were detected by IFA at day 6 after virus inoculation. As shown in Figs 2 and 4, the expression levels of wild-type and mutant GPR1 were determined by IFA: + +, high; +, low; −, negative.

signal did not significantly decreased 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, as 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 3b 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. 3b); 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(d36–41) 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(d36–41) 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 mutant 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 $NaClO_3$, an inhibitor of tyrosine sulfation, as reported previously (Farzan *et al.*, 1999) (Fig. 6). However, $NaClO_3$ 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 of 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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