The Synthetic Peptide Derived from the NH₂-terminal Extracellular Region of an Orphan G Protein-coupled Receptor, GPR1, Preferentially Inhibits Infection of X4 HIV-1*

Received for publication, January 6, 2005, and in revised form, May 19, 2005
Published, JBC Papers in Press, May 26, 2005, DOI 10.1074/jbc.M500195200

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Several G protein-coupled receptors (GPCRs) serve as co-receptors for entry of human immunodeficiency virus type 1 (HIV-1) into target cells. Here we report that a synthetic peptide derived from the NH₂-terminal extracellular region of an orphan GPCR, GPR1 (GPR1 ntP-(1–27); MEDLEETLFEEFENYSYDLDYYSLESC), inhibited infection of not only an HIV-1 variant that uses GPR1 as a co-receptor, but also X4, R5, and R5X4 viruses. Among these HIV-1 strains tested, viruses that can utilize CXC4 as their co-receptors were preferentially inhibited. Inhibition of early steps in X4 virus replication was also detected in the primary human peripheral blood lymphocytes. GPR1 ntP-(1–27) directly interacted with recombinant X4 envelope glycoprotein (rgp120). This interaction was neither inhibited nor enhanced by the soluble CD4 (sCD4) but inhibited by the anti-third variable (V3) loop-specific monoclonal antibody and heparin known to bind to the V3 loop. Although the conformational changes in gp120, including the V3 loop, have been reported to be required for its interaction with a co-receptor after binding of gp120 to CD4, it has also been reported that the V3 loop is already exposed on the surface of virions before interaction with CD4. We found that GPR1 ntP-(1–27) blocked binding of virus to the cells, and this peptide equally bound to rgp120 in the presence or absence of sCD4. Because we detected the binding of GPR1 ntP-(1–27) to the highly purified virions even in the absence of sCD4, GPR1 ntP-(1–27) probably recognized the V3 loop exposed on the virions, and this interaction was responsible for the anti-HIV-1 activity of GPR1 ntP-(1–27).

HIV-1 is the causative agent of AIDS (1, 2). CD4 glycoprotein as a receptor and GPCRs as co-receptors are required for the entry of HIV-1, and the cell tropism of HIV-1 is mainly determined by its ability to use co-receptors expressed on the target cells (3). The major co-receptor for R5 viruses is CCR5, of which ligands are CC chemokines RANTES (regulated on activation normal T-cell expressed and secreted), MIP1α, and MIP1β. On the other hand, the major co-receptor for X4 viruses is CXCR4, of which ligand is the CXC chemokine SDF-1. Dual-tropic viruses (R5X4 viruses) can efficiently utilize both of these co-receptors (4–9).

In addition, primary human brain-derived fibroblast-like cells (BT-cells) are isolated from microvessel segments derived from autopsy of human brain tissue, are positive for CD4 and highly susceptible to HIV-1 variants, but are resistant to R5, X4, and R5X4 HIV-1 strains (10, 11). BT-cells are thought to be originated from smooth muscle cells or pericytes. We have previously shown that the substitution of Pro to Ser at the Gly-Pro-Gly-Arg sequence in the V3 loop of envelope glycoprotein (gp120) is responsible for the BT-cell tropism of the HIV-1 variants (10, 12). We have recently identified orphan GPCRs, GPR1 and RDC1, as co-receptors for the HIV-1 variants in vitro (13, 14). Not only HIV-1 variants, but also several strains of HIV-2 and simian immunodeficiency virus have been shown to utilize GPR1 and RDC1 as their co-receptors (13–15).

During the course of our previous experiments to generate anti-GPR1 antibody (α-GPR1) by immunizing rabbits with a synthetic peptide derived from the NH₂-terminal extracellular region (NH₂-ECR) of GPR1 (GPR1 ntP-(1–27)), we found unexpectedly that not only α-GPR1 but also GPR1 ntP-(1–27) was able to block GPR1-mediated infection of BT-cells with HIV-1 variants. Because it has been demonstrated that the NH₂-ECRs of the co-receptors play an important role in the interaction with gp120 and a subsequent infection steps (16–21), we speculated that GPR1 ntP-(1–27) might inhibit interaction of gp120 (HIV-1 variants) with its co-receptor, GPR1, on target cell membrane.

In the present study, we tested anti-HIV-1 activity of GPR1 ntP-(1–27) by several assays, including focal infectivity assay, HIV-1 p24 Gag detection assay, PCR assay, and syncytium formation assay. These assays all demonstrated an inhibitory activity of GPR1 ntP-(1–27) against HIV-1 infection. Surprisingly, anti-HIV-1 activity of GPR1 ntP-(1–27) was detected not only in HIV-1 variants that utilize GPR1 as a co-receptor, but also in genetically diverse isolates, including X4, R5, and X4R5 viruses, which utilize GPR1 and RDC1 as co-receptors (13–15).
R5X4 viruses. Our findings suggest that synthetic peptides derived from the NH2-ECR of GPR1 are novel candidates for the development of GPCR-based and peptide-based agents to inhibit HIV-1 infection.

EXPERIMENTAL PROCEDURES

Cells—NP-2 cells were derived from a human glioma and kindly provided by Dr. T. Kumanishi (Niigata University, Niigata, Japan). NP-2/CD4 and NP-2/CD4 cells stably expressing human GPCRs (NP-2/CD4/CXCR4, NP-2/CD4/CXCR3, NP-2/CD4/CCR3, and NP-2/CD4/GPR1) as indicator cells for HIV-1 infection were described previously (13–15, 22, 23). NP-2/CD4 and NP-2/CD4/GPCRs cells were maintained in Eagle’s minimum essential medium containing 10% fetal calf serum (FCS). The human T cell line C8166 (24), MOLT-4 clone 8 (25), and MOLT4/IIB (26) were maintained in RPMI 1640 medium supplemented with 10% FCS (RPMI/FCS). Peripheral blood lymphocytes (PBLs) were isolated from the blood of healthy subjects by Ficoll-Paque gradient centrifugation. PBLs were stimulated with phytohemagglutinin prior to HIV-1 infection and cultured in RPMI/FCS and 100 units/ml of recombinant interleukin-2 (Roche Applied Science). All cells were maintained at 37 °C in a humidified, 5% CO2 atmosphere.

HIV-1 Strains—The GUN-1WT strain is a clinical isolate and can infect both T-cell lines and macrophages, but not BT-cells (10, 12, 27). In contrast, the variant of GUN-1WT strain can infect the BT-cells and T-cell lines (10, 12). Similar to the variant of GUN-1WT strain, GUN-1Ser strain, which had been prepared by the ligation and transfection of cloned HIV-1 (GUN-1WT) DNA fragments containing a mutation of proline to serine made by the site-directed mutagenesis at the GPGR clade C (33), is a reverse transcriptase-defective HIV isolates of the GPR1-NT-terminal peptide, the level of p24Gag protein in primary isolates, the level of p24Gag protein in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (38).

PCR Assay to Detect Reverse-transcribed HIV-1 DNA—The virus preparation (IIB strain) was treated with RNase-free DNase I (10 units/ml, Roche Applied Science) for 30 min at 37 °C to remove contaminating RNA or DNA. 100 units/ml of recombinant interferon-β (Roche Applied Science) was added to infected PBLs. The virus was induced with one of the synthetic peptides for 1 h at 37 °C, and inoculated onto MOLT-4 cells (5 × 105) or PBLs (5 × 105) for 2 h at 37 °C. The cells were washed, and fresh medium was added. After incubation for 20 h, the cells were washed and lysed with 10 mM Tris-HCl (pH 8.3) containing 1% EDTA, 0.45% Nonidet P-40 (Sigma), 0.45% Tween 20 (Sigma), and 0.2 mg/ml proteinase K (Sigma). The cell lysates were incubated for 2 h at 52 °C, heated for 10 min at 96 °C to inactivate proteinase K, and used as templates for the subsequent PCR analysis to detect the formation of reverse-transcribed HIV-1 DNA within the cells. PCR was performed with HIV-1 gag-specific primers, SK38 and SK39 (39) (nucleotide sequence: SK38, 5′-AAGGGAGT-GACATAGCAG-3′; SK39, 3′-GGACAAACAGGTTTTCGTC-5′) in a PerkinElmer Life Sciences Cycler under the following conditions: 1 cycle at 95 °C for 1 min, 30 cycles at 94 °C for 1 min, 60 °C for 45 s, 72 °C for 1 min and one cycle at 72 °C for 5 min. The human β-glucuronidase gene primers, KM29 and KM38 (39) (nucleotide sequence: KM29, 5′-GGTTGCG-CAACTACTCCAG-3′; KM38, 5′-TGGTCTCCCTAAACGCTGCT-TG-3′) (40) were used as an internal control. DNA amplification was performed in the following conditions: 1 cycle at 95 °C for 9 min, 30 cycles at 94 °C for 1 min, 60 °C for 45 s, 72 °C for 1 min and one cycle at 72 °C for 5 min. The PCR products were analyzed by electrophoresis through 2% agarose gels containing 0.5 μg/ml ethidium bromide.

HIV-1 Cell Binding Assay—HIV-1 cell binding assay was essentially done according to the protocol described by Valenzuela et al. (41). MOLT-4 cells (6 × 105) were incubated with HIV-1 (IIB strain) (200 ng of p24Gag for 1 h at 37 °C in RPMI/FCS (final volume, 0.5 ml). When indicated, viral preparation was incubated with GPR1ntP-(1–27), GPR1ntP-(Y/A), 0.5β, or heparin, while the cells were incubated without anti-CD4 mAb, NU-TH/I, before binding. After incubation of the cells

| GPCR | Peptide       | Length | Amino acid sequence                  | Molecular weight | Net charge |
|------|---------------|--------|-------------------------------------|------------------|------------|
| GPR1 | GPR1ntP-(1–27) | 27     | MEDLEETLPERFENYSYDLYYESLSC*          | 3375             | 10         |
| CCR4 | CCR4ntP-(1–28) | 28     | MEGISYTVSYTTEGMDSDMKPEC             | 3007             | 6          |
| CCR3 | R3ntP-(1–24)  | 24     | NTTSILDEVTGTTSTIDYDVLCC             | 2766             | 4          |
| R5ntP-(1–20) | 20 | MEDLEETLPEFE             | 2580             | 3          |
| GPR1 | GPR1ntP-(1–13) | 13     | LPERFENYSYDLD                     | 1661             | 7          |
| GPR1 | GPR1ntP-(8–20) | 13     | LPERFENYSYDLD                     | 1684             | 3          |
| GPR1 | GPR1ntP-(15–27) | 13     | YGDLDDYSYELSC                     | 1621             | 3          |
| GPR1 | GPR1ntP-(Y/A) | 27     | MEDLEETLPERFENASADLDVLESC*         | 3010             | 10         |

* As described in the Introduction, the COOH-terminal cysteine residue of GPR1ntP-(1–27) is introduced into the sequence for the purpose of conjugation of the keyhole limpet hemocyanine to use as an immunogen for antibody production.

**All tyrosine residues in GPR1ntP-(1–27) were substituted to alanine (underlined) in GPR1ntP-(Y/A).
with HIV-1, the cells were washed once with PBS containing 5 mM EDTA and twice with RPMI/FCS. Then, the cells were lysed with 150 μl of lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml of aprotinin, 0.5% Triton X-100, and 7 mM 2-mercaptoethanol), and the cell lysates were centrifuged at 1000 × g for 2 min at 4 °C. Then, the concentration of HIV-1 p24Gag in the supernatants was determined by ELISA. The amount of p24Gag in these cell extracts was expressed as the total amount of viral p24Gag associated with the cells as described by Valenzuela et al. (41), because this value showed both viral p24Gag bound to the surface of cells and viral p24Gag that has entered the cells. To detect only HIV-1 that entered MOLT-4 cells, extracellular bound virus was removed. For this, the cells were treated with 2.5 mg/ml of trypsin in PBS containing 5 mM EDTA for 40 s at room temperature after the cells were washed with PBS containing 5 mM EDTA. Then, the cells were washed twice with RPMI/FCS, lysed, and the concentration of intracellular p24Gag was detected by ELISA as described (45), with a few modifications. Wells of a 96-well microtiter plate (NUNC) were coated with GPR1ntP-(1–27) or its fragment or mutant peptide and blocked with blocking buffer B for 1 h at room temperature. The plate was incubated for 1 h at room temperature with washed with PBS, and rinsed with PBS. To develop color, 3,3',5,5'-tetramethylbenzidine solution (Bio-Rad) as a substrate was added for 20 min at room temperature, and 1 N H2SO4 was added to stop the reaction. Then the optical densities (A) at 450 nm were determined.

For detection of interaction of sCD4 with GPR1ntP-(1–27), microtiter wells were coated with GPR1ntP-(1–27) as described above. Then, sCD4 (0.016 μM), which was obtained from the AIDS Reagent Project of the United Kingdom Medical Research Council, was added for 2 h at room temperature. Bound sCD4 was detected by ELISA as described above using antibody against sCD4 and horseradish peroxidase-labeled antibody to sheep IgG (Dako).

To examine the effect of α-GPR1 antibody on binding of GPR1ntP-(1–27) to rgp120, microtiter wells were coated with GPR1ntP-(1–27) as described above. Then, anti-GPR1 antibody or normal rabbit IgG (NRG) was added for 2 h at room temperature prior to addition of rgp120 (0.016 μM), and then bound rgp120 was detected as described above. For detection of binding of GPR1ntP-(1–27) to rgp120-sCD4, rgp120 (0.016 μM) was incubated with sCD4 (0.016 μM) for 1 h at 37 °C before incubation with GPR1ntP-(1–27) and then bound rgp120 was detected as described above. For detection of the binding of GPR1ntP-(1–27) to sCD4 in the presence of heparin (Wako), microtiter wells were coated with either GPR1ntP-(1–27) (1.0 μM) or sCD4 (0.016 μM) as described, and rgp120 (0.016 μM), which had been preincubated with or without heparin (37 °C for 1 h) was added. Bound rgp120 was detected as described above.

To examine the effect of GPR1ntP-(1–27) on binding of V3-specific monoclonal mouse mAb (b) to rgp120, a microtiter plate was coated with rgp120 (0.016 μM) at 4 °C for overnight, blocked, and treated with or without different concentrations of GPR1ntP-(1–27) or XntP-(1–28) (5–100 μM) at room temperature for 2 h. After being washed with PBS, mAb 0.5μg (ascites, 1:50 dilution) was added, and the plate was incubated at room temperature for 2 h, and washed. Captured 0.5μg was detected as described above using a horseradish peroxidase-labeled antibody to mouse IgG (Dako).

RESULTS
Effects of Synthetic Peptides Derived from the NH2-ECR of GPCRs on Cell-free HIV-1 Infection—We have previously established human glioma-derived, new HIV-1 infection indicator cell lines, which are transduced with both CD4 and co-receptors (NP-2/CD4/GPCRs), and showed that infectivity assay using these cell lines were highly reproducible and sensitive (13, 15, 22, 23). As shown in Fig. 1, detection of HIV-1 antigen-positive cells by immunostaining after infection with several HIV-1 strains is dependent on both CD4 and the types of co-receptors expressed on NP-2 cells.

We, therefore, examined the effects of synthetic peptides derived from NH2-ECR of GPCRs (GPR1, CXCR4, CCR3, and CCR5) on the infectivity of several HIV-1 strains by focal infectivity assays with NP-2/CD4/GPCRs, and showed that infectivity assay using these cell lines were highly reproducible and sensitive (13–15, 22, 23). As shown in Fig. 1, detection of HIV-1 antigen-positive cells by immunostaining after infection with several HIV-1 strains is dependent on both CD4 and the types of co-receptors expressed on NP-2 cells.
HIV-1 DNA was inhibited in a dose-dependent manner (Fig. 4, C, lanes 1–5, and D). The inhibitory activity was not again detected when X4ntP-(1–28), R3ntP-(1–24), or R5ntP-(1–20) was used (Fig. 4, C, lanes 6–8 and D). When GPR1ntP-(1–27) was added to MOLT-4 cells only after inoculation of the virus, the formation of HIV-1 DNA was not inhibited (data not shown), suggesting that the reverse transcription of HIV-1 RNA in the host cells was not inhibited by GPR1ntP-(1–27).

We next examined whether GPR1ntP-(1–27) could inhibit HIV-1 infection to primary human PBLs, which are natural targets for HIV-1. The formation of reverse-transcribed HIV-1 DNA in PBLs, prepared from two independent donors, was also clearly inhibited by GPR1ntP-(1–27) in a dose-dependent manner (Fig. 4, E and F). Thus, these findings indicate that GPR1ntP-(1–27) blocks the early step of HIV-1 infection before reverse transcription, such as virus attachment or its entry into target cells.

**Effect of GPR1ntP-(1–27) on Syncytium Formation**—To examine the effect of GPR1ntP-(1–27) on the formation of multinucleated giant cells (syncytia), which are considered to represent cell-to-cell infection, we performed syncytia assays using HIV-1-positive MOLT-4/IIIB cells and HIV-1-negative C8166 cells in the presence of one of the synthetic peptides (Fig. 5). Syncytium formation was clearly inhibited by GPR1ntP-(1–27) but not by the other peptides. IC_{50} of GPR1ntP-(1–27) determined by the syncytia assay was 1.1 µM. Because little effect of 50 µM GPR1ntP-(1–27) on the growth and viability of MOLT-4 cells was observed for up to 4 days (data not shown), the anti-HIV-1 activity of GPR1ntP-(1–27) appears not to be due to its cell toxicity.

**Identification of a Functional Domain and Amino Acids in GPR1ntP-(1–27) Responsible for Its Anti-HIV-1 Activity**—To determine a functional domain in the GPR1ntP-(1–27) peptide more precisely, fragment peptides, overlapped by six amino acids (GPR1ntP-(1–13), GPR1ntP-(8–20), and GPR1ntP-(15–27)) were synthesized (Table I). We examined the effect of these fragment peptides on the infection of NP-2/CD4/GPCR cells with HIV-1 (IIIB, GUN-1Ser, and BaL strains) (Table III). Among a series of the fragment peptides, GPR1ntP-(15–27) inhibited the infection of the IIIB and GUN-1Ser strains but not that of the BaL strain. Although relatively high concentrations of GPR1ntP-(15–27) were required for the infection of HIV-1 infection as compared with GPR1ntP-(1–27), the inhibitory effect of GPR1ntP-(15–27) against HIV-1 was repeatedly detected. This result indicates that the amino acid sequence from 15 to 27 (YSYDLLDYSLESC) is pivotal to inhibit HIV-1 infection by GPR1ntP-(1–27). Because it has been implicated that the tyrosine residues in the NH2-ECR of CCR5 and CCR5 play an important role in the co-receptor activity for HIV-1 infection (16–21), we examined a mutant peptide, in which all tyrosine residues had been substituted with alanine residues (GPR1ntP-(Y/A); Y15A, Y17A, Y22A, and Y23A) on HIV-1 infection. The inhibitory activity of GPR1ntP(Y/A) peptide against HIV-1 infection was completely abrogated, indicating that tyrosine residues play a crucial role for GPR1ntP-(1–27) to inhibit HIV-1 infection.

**Binding of GPR1ntP-(1–27) to Highly Purified HIV-1 Virions**—Because no inhibitory effect of GPR1ntP-(1–27) was detected when cells had been treated with GPR1ntP-(1–27) and washed before HIV-1 inoculation, it is most likely that targets for GPR1ntP-(1–27) are virus particles rather than the host cell molecules. To test this, we performed a GPR1ntP-(1–27)-binding assay using HIV-1 virions highly purified through sucrose density gradient ultracentrifugation. The purified virus (IIIB strain) was incubated with GPR1ntP-(1–27) for 1 h at 37 °C, subjected to sucrose density gradient sedimentation, and then
Anti-HIV-1 Activity of GPR1 NH$_2$-terminal Region Peptide

FIG. 2. Inhibition of cell-free HIV-1 infection by GPR1ntP-(1–27). NP-2/CD4 cells expressing one of the GPCRs were infected with HIV-1 in the presence or absence of synthetic peptides derived from the NH$_2$-ECR of GPCRs, and HIV-1-antigen-positive cells were detected by focal infectivity assay as described under "Experimental Procedures." The target cell and inoculated HIV-1 strain are indicated at the top of graph and within the graph, respectively. The average number of foci in triplicate wells formed in the absence of peptides was counted, and the inhibition (%) was determined by the comparison with the number of foci induced by each HIV-1 strain (IIIB strain) under physiological conditions.

Specific Interaction of GPR1ntP-(1–27) with rgp120 through Its V3 Loop—We next examined whether GPR1ntP-(1–27) binds directly to envelope glycoprotein gp120. Because GPR1ntP-(1–27) efficiently inhibited infection of X4 HIV-1 (IIIB strain), we performed a binding assay using a baculovirus-derived recombinant gp120 (rgp120) (IIIB strain) and GPR1ntP-(1–27). Microtiter plate wells were coated with different concentrations of GPR1ntP-(1–27) (0.015–5 μM) and incubated with rgp120 (0.016 μM), and bound rgp120 was detected by ELISA (Fig. 7A). The binding of rgp120 to GPR1ntP-(1–27) was detected in a concentration-dependent manner, indicating an association of rgp120 with GPR1ntP-(1–27). This binding appears to be specific, because pretreatment of GPR1ntP-(1–27) with α-GPR1, which had been generated by immunization of rabbits with GPR1ntP-(1–27), but not with normal rabbit IgG inhibited binding of rgp120 in a manner dependent on IgG concentrations (Fig. 7B). In contrast to rgp120, no binding of scD4 to GPR1ntP-(1–27) was detected (Fig. 7A).

It is known that the CD4-binding domain (CD4-bd) and the V3 loop within gp120 play an important role for its interaction with target cells at the entry step of virus infection. To determine which region in rgp120 is involved in its binding to GPR1ntP-(1–27), we firstly examined the effect of scD4 on the binding of rgp120 to GPR1ntP-(1–27) (Fig. 7C). When rgp120 had been incubated with scD4 before addition to GPR1ntP-(1–27), scD4 did not affect the binding of rgp120 to GPR1ntP-(1–27), suggesting that the CD4-bd in rgp120 might not be involved in the primary binding site for GPR1ntP-(1–27).

Because it has been reported that the soluble polyanion such as heparin binds to rgp120 at the V3 loop region (48–50), we next examined the effect of heparin on binding of rgp120 to GPR1ntP-(1–27) (Fig. 7D). The rgp120 was incubated with different concentrations of heparin (37 °C for 1 h) before addition to microtiter wells, which had been coated with either GPR1ntP-(1–27) or scD4, and bound rgp120 was detected by ELISA. We found that heparin could inhibit association of

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**TABLE II**

| Strain | Correceptor | IC$_{50}^{a}$ | IC$_{90}^{a}$ |
|--------|-------------|--------------|--------------|
| IIB    | CXCR4       | 0.5          | 4.0          |
| GUN-1ser | CXCR4     | 0.4          | 4.0          |
| GPR1   | CXCR4       | 0.5          | 6.0          |
| GUN-1wt | CXCR4       | 0.8          | 12.0         |
| CCR3   | 0.45        | 3.0          |
| CCR5   | 0.5         | 6.0          |
| GUN-4b | CXCR4       | ND$^{a}$     | ND$^{a}$     |
| CCR5   | 0.6         | 6.5          |
| GUN-14b | CXCR4      | ND$^{a}$     | ND$^{a}$     |
| BaL    | CCR5        | 10.0         | 50.0         |
| SF162  | CCR5        | 5.0          | 33.0         |

$^{a}$ IC$_{50}$ and IC$_{90}$ obtained by foci infectivity assay using NP-2/CD4/GPCR cells in comparison with the number of foci induced by each HIV-1 strain without a peptide. For GUN-4, GUN-14, and SF162 strains, IC$_{50}$ and IC$_{90}$ were determined by measuring the concentrations of p24 in the culture supernatants by ELISA.

$^{b}$ The primary HIV-1 strains isolated from Japanese hemophilia patients.

$^{c}$ ND, not determined.

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the HIV-1 protein and GPR1ntP-(1–27) in each fraction were detected by dot spotting (Fig. 6). The sedimentation of virions with or without GPR1ntP-(1–27) was mainly detected in fractions #8 and #9 (Fig. 6A, the first and second blots), consistent with an expected density for retroviruses (~1.15 g/ml) (43, 44) (Fig. 6B), indicating that the presence of GPR1ntP-(1–27) did not affect the sedimentation of the virions. Sucrose density gradient sedimentation of GPR1ntP-(1–27) without virions revealed that this peptide remained at the top of the gradient (#1 and #2), and it was hardly detectable in the fractions at densities around 1.15 g/ml (#8 and #9) (the third dot blot). In contrast, when GPR1ntP-(1–27) had been mixed with HIV-1 before sedimentation, this peptide was detected not only in the top fractions (#1 and #2) but also in the middle fractions (mainly in #8 and #9) containing viral particles (the fourth dot blot), indicating co-sedimentation of HIV-1 virions and GPR1ntP-(1–27). This was confirmed by measuring the intensity of signal in each dot spot (fractions #5–#11) (Fig. 6B). The interaction of GPR1ntP-(1–27) with the virions is specific for GPR1ntP-(1–27) sequence, because the co-sedimentation of X4ntP-(1–28) with the virions was hardly detectable in the middle fractions (#8 and #9, the fifth blot). Thus, these results indicated that GPR1ntP-(1–27) specifically associates with HIV-1 virions (IIIB strain) under physiological conditions.
Results.

were repeated three times to confirm from duplicate samples. Experiments were performed three times to confirm results.

Even when MOLT-4 cells were treated with NU-TH/I before infection were measured by ELISA. As a control, 5 μg azido-3'-deoxthymidine was added. Data are means obtained from duplicate samples. Experiments were repeated three times to confirm results.

As a control, 5 × 10^6 cells were firstly examined. At this concentration, we confirmed that these anti-HIV-1 reagents inhibited infection of MOLT-4 cells with HIV-1 (IIIB strain) by more than 95% (data not shown). It has been reported that the V3 loop is exposed on the surface of intact virions. In this study, we found that the 27-amino acid-long synthetic peptide derived from the NH2-ECR of GPR1 inhibits HIV-1 infection of human glioma-derived indicator cells (NP-2/CD4/ GPCRs), a T-cell line, and more importantly, primary PBLs, which are natural targets for HIV-1 infection. The inhibitory effect of GPR1ntP-(1–27) was observed not only in GUN-1Ser strain, which utilizes GPR1 as a co-receptor, but also in diverse HIV-1 strains, including X4 (IIIB strain), R5X4 (GUN-1WT, GUN-4, and GUN-14), and R5 (BaL and SF162) (Figs. 2 and 3 and Table I). Among these HIV-1 strains tested, R5 viruses were relatively resistant to GPR1ntP-(1–27) as compared with the other strains. We found that GPR1ntP-(1–27) binds to gp120 (Fig. 7, A and B), and this interaction is inhibited by heparin (Fig. 7D), which has been reported to bind to the V3 loop of gp120 (48–50). Moreover, interaction between anti-V3 loop mAb (0.5β) with gp120 was inhibited by GPR1ntP-(1–27) (Fig. 7E). Be-
cause it has been reported that 0.5/H9252 recognizes 24 amino acids (308–331) of the V3 loop (31) and does not block binding of gp120 to CD4 (54), our data strongly suggested that the V3 loop plays an important role for binding of GPR1ntP-(1–27) to rgp120.

GPR1ntP-(1–27) inhibited binding of 0.5/H9252 to rgp120 by 50% at 25/H9262 M, although the peptide at higher concentrations up to 100/H9262 M showed only a limited decrease in rgp120 binding (Fig. 7E). As for the affinity of 0.5/H9252 for rgp120, an A450 value of binding of GPR1ntP-(1–27) at 0.6/H9262 M to rgp120 was 0.32 (Fig. 7A), whereas almost a similar A450 value was obtained when 0.027/H9262 M of 0.5/H9252 was used (data not shown), suggesting that the affinity of 0.5/H9252 for rgp120 is 22 times as high as that of GPR1ntP-(1–27). Thus, the incubation of 0.5/H9252 with rgp120 may displace GPR1ntP-(1–27) from rgp120 through a competitive

Formation of HIV-1 DNA in MOLT-4 cells was detected by PCR as described above. MOLT-4 cells were infected with heat-inactivated (56 °C, 30 min) HIV-1 (lane 9). Lane 10 is a mock-infected control. PCR amplification was performed without template DNA (lane 11). β-Globin DNA was amplified as a control to confirm the efficiency of the amplification of each sample. D, the intensity of DNA signal in each lane was measured and the relative intensity of gag signal was determined as described above. E, inhibition of HIV-1 infection to primary human PBLs by GPR1ntP-(1–27). Primary PBLs prepared from two independent donors (PBL#1 and PBL#2) were infected with HIV-1 (IIB strain) in the presence or absence of GPR1ntP-(1–27). Formation of reverse-transcribed HIV-1 gag DNA in PBLs was detected by PCR as described above. F, the intensity of DNA band in each lane was measured, and the relative intensity of gag signal was determined as described above. Three independent experiments gave similar results.

| Peptide | Strain | IC50 (μM) |
|---------|--------|-----------|
| GPR1ntP-(1–27) | IIB | 0.5 |
| GPR1ntP-(1–13) | GUN-1Ser | >100 |
| GPR1ntP-(8–20) | BaL | >100 |
| GPR1ntP-(15–27) | | >100 |
| GPR1ntP(Y/A) | | >100 |

* Amino acid sequence of each peptide is shown in Table I.
* NP-2/CD4/CXCR4, NP-2/CD4/GPR1, and NP-2/CD4/CCR5 cells were used for infection with IIB, GUN-1Ser, and BaL strains, respectively.
* IC50 values (μM) of peptides were determined by focal infectivity assay; the number of foci induced by each HIV-1 strain in the presence and absence of a peptide was counted.

It has also been reported that 0.5β recognizes 24 amino acids (308–331) of the V3 loop (31) and does not block binding of gp120 to CD4 (54), our data strongly suggested that the V3 loop plays an important role for binding of GPR1ntP-(1–27) to rgp120.

GPR1ntP-(1–27) inhibited binding of 0.5β to rgp120 by 50% at 25 μM, although the peptide at higher concentrations up to 100 μM showed only a limited decrease in rgp120 binding (Fig. 7E). As for the affinity of 0.5β for rgp120, an A450 value of binding of GPR1ntP-(1–27) at 0.6 μM to rgp120 was 0.32 (Fig. 7A), whereas almost a similar A450 value was obtained when 0.027 μM of 0.5β was used (data not shown), suggesting that the affinity of 0.5β for rgp120 is 22 times as high as that of GPR1ntP-(1–27). Thus, the incubation of 0.5β with rgp120 may displace GPR1ntP-(1–27) from rgp120 through a competitive
density gradient sedimentation (100,000 × g for 2 h). The virus suspension was then subjected to a 20–60% sucrose density gradient sedimentation (100,000 × g for 16 h). Virus-containing fractions were concentrated, resuspended in TNE buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA), and incubated with the GPR1ntP-(1–27) or X4ntP-(1–28) for 1 h at 37 °C. The virus, virus-peptide mixture or peptide (indicated as input) was then subjected to 20–60% sucrose density gradient sedimentation (100,000 × g for 16 h). Fractions (700 μl) were collected from the top of the gradient. The dot blotting was carried out to detect the presence of the HIV-1 p24Gag, GPR1ntP-(1–27), and X4ntP-(1–28) in each fraction using dot blotting was carried out to detect the presence of the HIV-1 p24Gag, GPR1ntP-(1–27), and X4ntP-(1–28) in each fraction using ELISA showed that GPR1ntP-(1–27) binds equally to rgp120 in the presence or absence of sCD4 (Fig. 6), suggesting that GPR1ntP-(1–27) does not bind to the intact virus particles, indicating that the V3 loop is involved in the binding of HIV-1 to MOLT-4 cells (Fig. 6). Because CD4-bd on the surface of HIV-1 virions has not been reported to be exposed as described above, our results suggested that the binding of virus to the cells before interaction with CD4 is mediated by the V3 loop exposed on the surface of virions and that this initial CD4-independent virus binding step is an important target for GPR1ntP-(1–27) to inhibit HIV-1 infection.

Although GPR1 serves as a specific co-receptor for the HIV-1 variants, GPR1ntP-(1–27) blocked infection of diverse HIV-1 strains with different co-receptor usage. It may be explained in two ways. Firstly, the V3 loop is known to have the highest density of positively charged amino acids among all regions of gp120: it contains five to nine basic residues, and their distribution pattern within the V3 loop is well conserved among different strains (51, 55). Because GPR1ntP-(1–27) shows the highest ratio of acidic amino acids (net negative charge is ~10) among the peptides used in this study (Table I), electrostatic interaction might be responsible for GPR1ntP-(1–27) to bind to basic residues within V3 loop, and to inhibit HIV-1 infection. Because the charge of amino acids in the V3 loop of R5 viruses is known to be more acidic than that of X4 or R5X4 viruses (51, 55), the relative resistance of R5 viruses (BaL and SF162) to infection by GPR1ntP-(1–27) might be explained by the electrostatic repulsion between GPR1ntP-(1–27) and the V3 loop of the R5 viruses. Secondly, as the presence of a similarity in the conformation or antigenicity of the V3 loop has been suggested (52, 56), the relative resistance of R5 viruses (BaL and SF162) to infection by GPR1ntP-(1–27) would bind to this conserved conformation. Namely, an mAb, 447–52D, has been reported to bind to the V3 loop exposed on the surface of different HIV-1 strains even in the absence of the linear epitope (52, 56). Thus, recognition of the conserved conformation in the V3 loop by GPR1ntP-(1–27) might be important for its inhibitory activity to diverse HIV-1 strains.

The exact stoichiometry of GPR1ntP-(1–27) binding to target gp120 molecules in HIV-1 virions remains to be determined. It has been reported that gp120 is shed from infected cells and/or from virions soon after budding (57, 58). Schneider et al. (59) reported that virus-free culture fluid contains at least 100-fold more gp120 than virions pelleted from the same volume of culture supernatant. Thus, not only virion-associated gp120 but also free gp120 might also bind to GPR1ntP-(1–27) and affect its anti-HIV-1 activity.

Although GPR1ntP-(15–27) (YSYDLDYYSLESC) binds to gp120 less efficiently than GPR1ntP-(1–27) (Fig. 7F, an inhibitory effect of GPR1ntP-(15–27) is much lower than GPR1ntP-(1–27) (Table III). Thus, there is a discrepancy between the binding ability of gp120 and the anti-HIV-1 activity of GPR1ntP-(15–27). This is probably due to the inefficient binding of GPR1ntP-(15–27) to intact virions, because the conformation of monomer gp120 is different from that of gp120 on the surface of HIV-1 virions. Namely, three gp120 molecules are associated noncovalently with the ectodomain of the gp41 envelope glycoprotein trimer to form oligomers (60).
FIG. 7. Specific interaction of GPR1ntP-(1–27) with rgp120 through its V3 loop. A, binding of rgp120 or sCD4 to GPR1ntP-(1–27). Microtiter wells were coated with different concentrations of GPR1ntP-(1–27) (0.015–5 μM), and incubated with rgp120 (0.016 μM), or sCD4 (0.016 μM). Bound rgp120 and sCD4 were detected using the sera from AIDS patients and anti-sCD4 antibody, respectively, and shown as absorbance values at 450 nm (optical density at 450 nm (A₄₅₀)). Each datum point is the mean of values obtained from triplicate samples, and experiment was repeated on at least three times. B, inhibition of rgp120 binding to GPR1ntP-(1–27) by α-GPR1 antibody. Microtiter wells were coated with GPR1ntP-(1–27) (1.0 μM), treated with α-GPR1 or normal rabbit IgG (NRG), and incubated with rgp120 (0.016 μM). Bound rgp120 was detected as described above. 100 and 0% binding correspond to OD₄₅₀ values of 0.52 and 0.07, respectively. Data are the mean of values obtained from triplicate samples, and experiment was repeated on at least three times. C, effect of sCD4 on the binding of GPR1ntP-(1–27) to rgp120. Microtiter wells were coated with GPR1ntP-(1–27) (0.5 μM), then rgp120 (0.0016 μM), which had been incubated with or without sCD4 (0.016 μM) (37 °C, 1 h) was added, and bound rgp120 was detected as described above. Data are means and standard deviations from triplicate wells. D, inhibition of gp120-GPR1ntP-(1–27) interactions, but not that of gp120-sCD4 by heparin. Microtiter wells were coated with either GPR1ntP-(1–27) (1.0 μM) or sCD4 (0.016 μM), then rgp120 (0.016 μM) which had been incubated with or without heparin (37 °C, 1 h) was added, and bound rgp120 was detected as described above. Data are the mean values from triplicate samples, and experiments were repeated three times to confirm results.
In this study, we showed that the mutant peptide (GPR1ntP-YA) substituting tyrosine to alanine does not inhibit HIV-1 infection (Table III) and has no activity for binding to rgp120 (Fig. 7F). Thus, tyrosine residues in GPR1ntP-(1–27) could contribute for interaction with rgp120 and play a crucial role in inhibition of HIV-1 infection. Farzan et al. (61) have shown that tyrosine residues from NH2-ECR of CCR5 contribute to the co-receptor activity through their sulfation. A post-translational modification resulting in the addition of a negatively charged sulfated tyrosine. Although the sulfation of tyrosine residues in NH2-ECR of GPR1 remains to be elucidated, it is possible that the introduction of sulfated tyrosine residue to GPR1ntP-(1–27) will enhance its anti-HIV-1 activity as a result of an addition of negative charge to GPR1ntP-(1–27). With regard to GPR1ntP-(1–27) analogs, we previously reported that a hexapeptide derived from GPR1ntP-(1–27) (amino acid residues from 17 to 22; YDLDYY) has a weak anti-HIV-1 activity at concentrations around 500 μM (62). The introduction of sulfation to tyrosine residues of this hexapeptide, however, did not significantly improve its inhibitory activity as compared with a non sulfated hexapeptide (62). Because GPR1ntP-(15–27) binds to rgp120 less efficiently than GPR1ntP-(1–27), the amino acid residues of GPR1ntP-(1–27) from 1 to 14 (MEDLEINLFEFEN), which contain no tyrosine but several acidic amino acids, will also be necessary for the strong inhibitory activity of GPR1ntP-(1–27) against HIV-1 infection. Thus, the addition of acidic amino acid residues to sulfated tyrosine may be important to develop GPR1ntP-(1–27) analogs with enhanced anti-HIV-1 activities. This possibility is currently under investigation.

The entry stages, including initial attachment in HIV-1 replication cycle, are effective targets for the development of new antiretroviral therapies, because de novo infection of HIV-1 to humans can be prevented. This has already been shown by the clinical application of several antiretroviral agents. For instance, PRO 542 is a fusion protein of the gp120-binding domain of CD4 with immunoglobulin constant domains, and is expected to block the interaction between gp120 and the CD4 receptor (63); or T20 is a peptide (36 amino acids) that inhibits the HIV-1 fusion step through binding to the viral envelope protein gp41 (64, 65). With a view to the antiviral potency of GPR1ntP-(1–27), the direct use of this peptide as a therapeutic agent will remain uncertain until studies of toxicity and pharmacokinetics are carried out. A critical concern with peptide-based antiviral therapy is its potential to elicit an antibody response to a peptide and make it ineffective (66). Because the amino acid sequence of GPR1ntP-(1–27) is derived from human, the antigenicity of GPR1ntP-(1–27) may be little or not at all in treated patients. The elucidation of the in vitro antiviral mechanism of GPR1ntP-(1–27) shown in this study will disclose valuable insights in the development of a new class of GPCR-based and peptide-based HIV-1 inhibitors.

Acknowledgments—We thank T. Nakamura for excellent technical assistance. We are grateful to Drs. T. Kumanishi (Niigata University, Niigata, Japan) and S. Matsushita (Kumamoto University, Kumamoto, Japan) for kindly providing us with N2-2 gloma cells and an anti-V3 loop mAb. We also thank Dr. H. Holmes (AIDS Reagent Project of the United Kingdom Medical Research Council) for supplying rgp120, sCD4, and anti-sCD4 antibody.

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The Synthetic Peptide Derived from the NH$_2$-terminal Extracellular Region of an Orphan G Protein-coupled Receptor, GPR1, Preferentially Inhibits Infection of X4 HIV-1

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_J. Biol. Chem._ 2005, 280:30924-30934.
doi: 10.1074/jbc.M500195200 originally published online May 26, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500195200

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