The possible involvement of CXCR4 in the inhibition of HIV-1 infection mediated by DP178/gp41

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Abstract The N- (N36/DP107) and C-terminal peptides (C34/DP178) from two α-helical domains of human immunodeficiency virus type 1 (HIV-1) gp41 inhibited HIV infection. A single-round infection using pseudotyped virus clarified that a greater amount of gp41-derived peptides was necessary for the inhibition of R5 virus (ADA) infection than for that of X4 virus (LAI) infection. Furthermore, R5X4 virus (89.6) infection via CCR5 needs more peptides for inhibition than its infection via CXCR4 does. A high sensitivity of X4 virus was partially ascribed to the inhibition of the 12G5 binding to CXCR4 by DP178LAI.

Key words: gp41-derived peptide; R5 virus; X4 virus; R5X4 virus

1. Introduction

The gp120 human immunodeficiency virus type 1 (HIV-1) binds to CD4 receptor as well as to chemokine receptors on target cells [1–3]. Then gp41 undergoes a conformational change that promotes viral and cellular membrane fusion. These conformational changes are thought to be involved in the transition of gp41 from a native (non-fusogenic) to a fusion-active (fusogenic) state.

The ectodomain of the gp41 molecule is directly involved in the membrane fusion process. Recent crystallographic studies on the core of fusion-active gp41 using N36 and C34 peptides showed that they fold into a six-helix bundle, in which three N-terminal helices form an interior, parallel-coiled-coil trimer, while three C-terminal helices pack in the reverse direction into three hydrophobic grooves on the surface of this coiled-coil [4,5]. A number of reports favor the notion that this six-helix structure represents the fusion-active conformation of the gp41 ectodomain core [6,7]. The synthetic peptides with these heptad repeats (N-terminal peptides: N36, DP107; C-terminal peptides: C34, DP178) could inhibit HIV infection and have been proposed to work in a dominant-negative manner [8,9]. Here we found that the R5 or R5X4 virus using CCR5 for entry is more resistant to the inhibition by these gp41-derived peptides than X4 or R5X4 virus using CXCR4 and studied its mechanism.

2. Materials and methods

2.1. Peptide synthesis

T22 derived from polyphemusin II in American horseshoe crab was synthesized as previously described [10]. DP107, DP178, C34, and N36 derived from gp41 of the envelope glycoprotein of the HIV-1 LAI strain and DP178 from the ADA strain were synthesized (Fig. 1) as described [4,9].

2.1. Cell culture

All cells were cultured in 100% humidified air at 37°C with 5% CO_2. A human embryonic kidney cell line, 293T, and human astroglia cell lines (U87) expressing CD4 antigen and the co-receptors CXCR4 (U87.CD4.CXCR4) or CCR5 (U87.CD4.CCR5) were provided by Dr. D. Littman [2]. The CEM-CCR5 (provided by Dr. Maeda, Kumamoto University) and Sup-T1 cells were used for the analysis of co-receptor expression. The establishment of CEM-CCR5 was similar to that of Molt-4#8/CCR5 by Dr. Maeda [11]. The peripheral blood mononuclear cells (PBMCs) were isolated from the buffy-coat, cultured with 5 μg/ml phytohemagglutinin (PHA, Difco, Detroit, MI, USA) for 2 days before they are used for HIV infection or flow-cytometry as described [12].

2.2. Vectors and luciferase assay

The *P. pyralis* luciferase expression vector pNL4-3-Luc-E^-R^-, and the envelope genes of a X4 virus (LAI), R5 virus (ADA), and R5X4 virus (89.6) were co-transfected into 293T cells to produce pseudotyped viruses. The pseudotyped viruses were quantified by a p24 enzyme-linked immunosorbent assay (ABBOTT, Abbott Park, IL, USA), and were used for infection of U87.CD4.CXCR4 or CCR5 cells as described [2,10].

2.3. Preparation of virus stocks and inhibition assay

Virus stocks of HIV-1 SF162 (provided by Dr. Koito, Kumamoto University), HIV-1 ADA (provided from NIH AIDS Research and Reference Reagent Program) and pNL432 (provided from Dr. A. Adachi) [13] were prepared and infected as described [12].

Virus titration and inhibition assays were done in PBMCs as previously described [14]. The 50% inhibition concentration (IC_{50}) of the peptides was calculated to show a 50% reduction of viral p24 antigen expression in the presence of the peptide compared to the positive control.

2.4. Monoclonal antibodies and flow-cytometry

12G5, a monoclonal antibody to CXCR4, was kindly provided by Dr. J.A. Hoxie and 2D7 (anti-CCR5) by LeukoSite, Inc. through the NIH AIDS Research and Reference Reagent Program [15]. Leu3a (anti-CD4) was purchased from Becton Dickinson and Co. (San Jose, CA, USA). Fluorescein-isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin G (Biosource, Menlo Park, CA, USA) was used as second antibody. Surface molecules on reagent-treated Sup-T1 cells or 48 h PHA-stimulated PBMCs were quantitated by a FACScan (Becton Dickinson and Co., Mountain View, CA, USA) using Consort 30 software as described [10].

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DP107 LAI          NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
N36 LAI     SGIVQQQ-----------------------------
DP178 LAI             YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF
DP178 ADA             --G--YT-------------D--A-----------
C34 LAI     WMEWDREINN------------------------
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Fig. 1. Sequences of gp41-derived peptides (N-terminal peptides: DP107LAI, N36LAI; C-terminal peptides: DP178LAI, DP178ADA and C34LAI). These peptides are aligned on the basis of their homology. The dashed line indicates the homology of N36LAI to DP107LAI, or DP178ADA, C34LAI to DP178LAI.

3. Results

3.1. Higher amounts of gp41-derived peptides were necessary for inhibition of R5 virus infection than for that of X4 virus infection

To determine whether the infection of HIV with different tropism was influenced by these gp41-derived peptides (DP107LAI, DP178ADA, DP178LAI, N36LAI and C34LAI), the effects of these peptides on the entry of two pseudotyped viruses with different tropism (X4 pseudotyped virus LAI, R5 pseudotyped virus ADA) were analyzed. U87.CD4.CXCR4 or CCR5 were used as target cells. All gp41-derived peptides inhibited the infection of both the X4 and R5 virus in a dose-dependent manner(data no shown). As shown in Fig. 2, the values of IC_{50}s of all peptides that inhibited the ADA infection of U87.CD4.CCR5 cells were at least 2-fold higher than those of the peptides that inhibited the LAI infection of U87.CD4.CXCR4 cells. The IC_{50} ratios between the inhibition of ADA infection and that of LAI infection by peptides were 3.3 for DP107LAI, 12 for N36LAI, 15 for DP178ADA, 221 for DP178LAI, 2082 for C34LAI, respectively. These results indicate that the R5 virus (pseudotyped virus ADA) is more resistant to the inhibition by both N- and C-peptides than is the X4 virus (pseudotyped virus LAI). Especially, the R5 virus showed much more resistance than the X4 virus to inhibition when the C-peptides (DP178LAI or C34LAI) were used.

It is possible that the high sensitivity of X4 virus to the peptides could be ascribed to the unique feature of U87 cells or pseudotyped viruses. The effects of these peptides were also investigated using PBMCs infected with replicating viruses (pNL43, SF162, and ADA) (Fig. 3). We also found that the R5 virus (SF162 and ADA) is more resistant to the inhibition by both N- and C-peptides than is the X4 virus (pNL43).

3.2. The expression of CXCR4 or CCR5 on target cells

The expression of CXCR4 or CCR5 on U87 cell lines and 48 h PHA-stimulated PBMCs was relatively compared using 12G5 (anti-CXCR4) and 2D7 (anti-CCR5) monoclonal antibodies. The mean fluorescence intensities (MFI) were 13.43 for 12G5 on U87.CD4.CXCR4 and 16.55 for 2D7 on U87.CD4.CCR5, respectively, indicating that the expression levels of CXCR4 and CCR5 on U87 cells are relatively close. The MFI are 159.80 for CXCR4 and 41.38 for CCR5 on PBMCs, respectively, indicating the PBMC expressed a greater amount of CXCR4 than CCR5 following the IL-2 stimulation. Also the expression levels of CD4 on both U87.CD4.CXCR4 and U87.CD4.CCR5 are very close, 76.5 and 73.7 of MFI, respectively.

Therefore, it is less likely that the expression levels of CXCR4 or CCR5 account for the different sensitivities to the peptides.

3.3. The R5X4 virus also needs more peptides for its resistance to CCR5-mediated infection

To determine whether the infection of dual tropic virus was blocked by these gp41-derived peptides, the effects of the peptides on the entry of dual tropic pseudotyped viruses (R5X4 pseudotyped virus 89.6) into U87.CD4.CXCR4 or CCR5 were studied. It should also be noted that luciferase activities of CXCR4 expressing cells were greater than those of CCR5

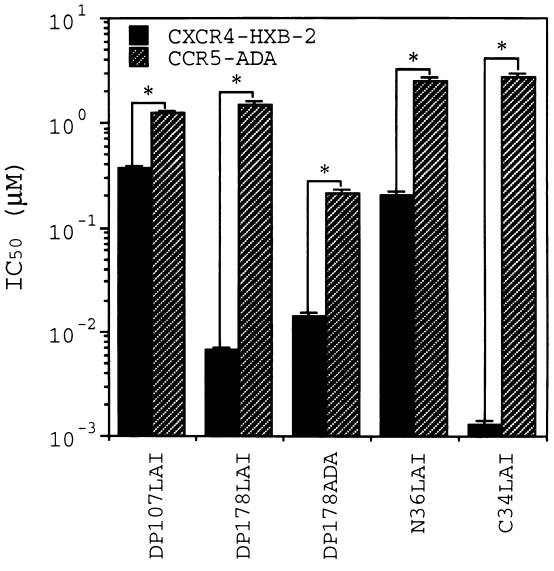

Fig. 2. Effects of gp41-derived peptides on the entry of X4 and R5 pseudotyped viruses. Results are expressed as the means ± S.D. and are representative of three separate experiments. IC_{50} (μM) values are based on the 50% reduction of pseudotyped virus LAI or ADA-induced luciferase activity in U87.CD4.CXCR4 or U87.CD4.CCR5 cells. $^{**}P < 0.01$.

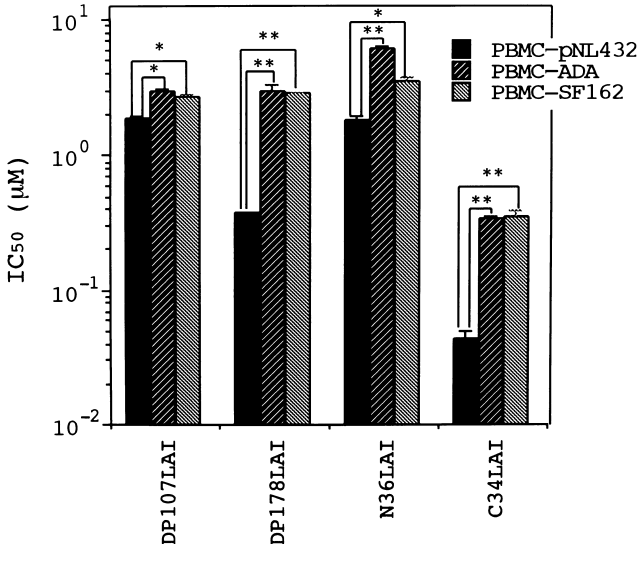

Fig. 3. Effects of gp41-derived peptides on the infection of X4 (pNL432) and R5 viruses (ADA and SF162) in PBMCs. Results are expressed as the means ± S.D. and are representative of three separate experiments. p24 (ng/ml) is 91.5 ± 2.2 for pNL432, 79.1 ± 1.1 for ADA, 77.1 ± 3.1 for SF162 in the absence of peptides. IC_{50} (μM) values are based on the 50% reduction of viral p24 expression in PHA-stimulated PBMCs. $^{**}P < 0.01$; $^{*}P < 0.05$.

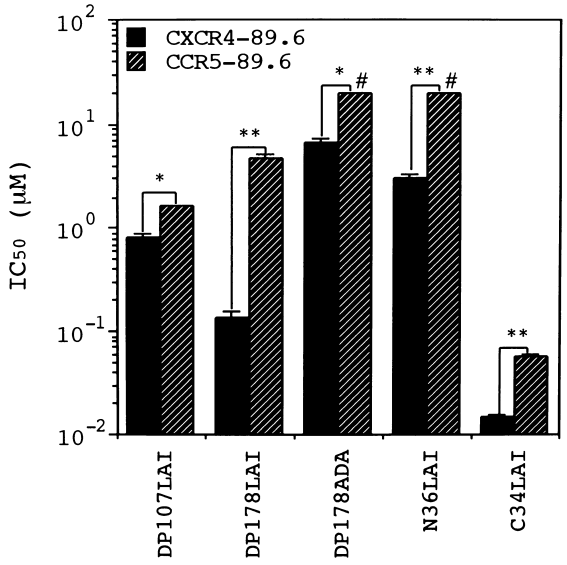

Fig. 4. Effects of gp41-derived peptides on the entry of R5X4 pseudotyped viruses (89.6) into U87.CD4.CXCR4 or U87.CD4.CCR5 cells. The luciferase activity (mv) in the absence of peptide is 673 468.8 ± 30 247 for infection of U87.CD4.CXCR4 and 47 484.35 ± 4128 for infection of U87.CD4.CCR5. Results are expressed as the means ± S.D. and are representative of three separate experiments. IC_{50} (μM) values are based on the 50% reduction of pseudotyped virus 89.6-induced luciferase activity in U87.CD4.CXCR4 or U87.CD4.CCR5 cells. $^{**}P < 0.01$; $^{*}P < 0.05$; #, over 20.

cells. The different IC_{50}s of the two infections could be explained by differences in the efficiencies of infection by the different co-receptor expressing cells. However, the IC_{50} ratios of the infection for U87.CD4.CCR5 and that for U87.CD4.CXCR4 by peptides were variable; 2.0 for DP107LAI, 6.5 for N36LAI, 3.0 for DP178ADA, 33 for DP178LAI, and 3.9 for C34LAI, respectively, indicating that more peptides are needed to inhibit R5X4 infection mediated via CCR5 compared to its infection mediated via CXCR4 (Fig. 4). These results suggested that the efficient infection of the R5X4 virus (pseudotyped virus 89.6) could partly explain the R5 resistance, but the markedly high ratio for DP178LAI must be explained by a different mechanism.

3.4. DP178LAI inhibits X4 or R5X4 virus infection also through interaction with CXCR4

To clarify the reason for the greater resistance of the R5 or R5X4 virus using CCR5 to inhibition by gp41-derived peptides, we investigated whether the peptides could interact with cell surfaces on target cells. We examined the effect of C34LAI and DP178LAI on the binding of an anti-CXCR4 monoclonal antibody (12G5) to CXCR4, and an anti-CCR5 monoclonal antibody (2D7) to CCR5 using flow-cytometry. We found that the incubation of Sup-T1 cells with 10 μM T22 completely inhibited the binding of 12G5 to CXCR4, and incubation of Sup-T1 with 10 μM DP178LAI also inhibited the binding of 12G5 to CXCR4 in a dose-dependent manner, while C34LAI had no effect on the binding of 12G5 (Fig. 5). Other peptides did not show any inhibitory activities. The incubation of CEM-CCR5 with 10 μM of all gp41-derived peptides (DP178LAI, DP107LAI, DP178ADA, N36LAI, and C34LAI) had no effect on the binding of 2D7 to CCR5 (data not shown). These results indicate that DP178LAI inhibits X4 or R5X4 virus infection also through interaction with CXCR4.

4. Discussion

It was recently clarified that a series of monoclonal antibodies derived from patients including 98.6 and 50-69 could recognize fusogenic forms or trimeric forms of gp41 [16–18]. These data indicated the existence of fusogenic forms of gp41 in vivo and the importance of oligomeric forms of gp41 for development of humoral immune response. To further understand the functional role of the putative coiled-coil sequence in ectodomain of gp41 which could arouse conformational antibodies, the N- (N36LAI, DP107LAI), and C-terminal peptides (C34LAI, DP178LAI, and DP178ADA) were synthesized. We found that the R5 virus (pseudotyped virus ADA) is more resistant to the inhibition by both N- and C-peptides than is the X4 virus (pseudotyped virus LAI). The resistance could not be explained by sequence variability, because the IC_{50} of DP178ADA against LAI was lower than that against ADA. The R5X4 virus (pseudotyped virus 89.6) was more resistant to inhibition by N-peptide (N36) and C-peptides (DP178 or C34) when it used CCR5 as co-receptor for entry (Fig. 4). In this case, the pseudotyped virus 89.6 could infect U87.CD4 cells more efficiently via CXCR4 than via CCR5, and differences in the IC_{50} between both infections may be explained by the efficiencies of infection by the different co-receptor expressing cells. However a greater amount of peptides was always necessary for the R5 virus, especially when C-terminal peptides were used.

Recently, DP178 has been reported to be the activator of the human phagocyte *N*-formyl peptide receptor and to bind to the putative cellular receptor protein P62 [19]. Here, we also found that DP178LAI could partially inhibit the binding of CXCR4 on Sup-T1 by 12G5. This may additively influence the sensitivity of X4 virus to inhibition by DP178LAI. Although there is much evidence indicating that gp41-derived peptides exert their inhibitory effect by interfering with the

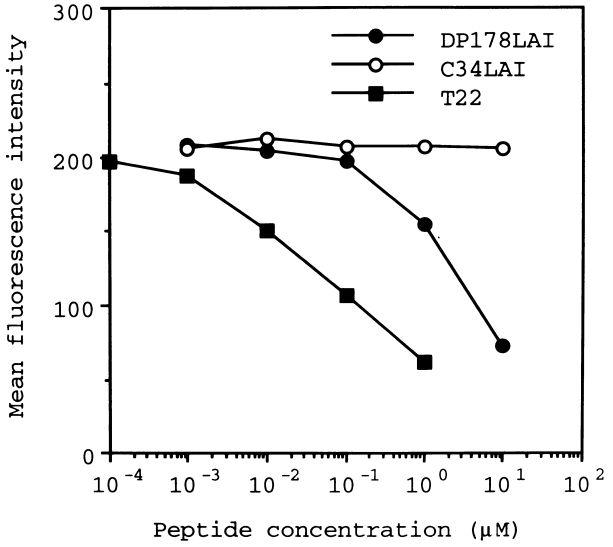

Fig. 5. gp41-derived peptides act as potent inhibitors of chemoattractant receptors. The MFI for 12G5 alone is 206 ± 8. Dose-dependent effect of DP178LAI, C34LAI and T22 on the binding of CXCR4 on Sup-T1 cells by 12G5.

formation of gp41 trimer, our data suggest that DP178LAI may also exert its effect by binding to CXCR4 or hindering the binding of X4 gp120 to CXCR4.

It is not immediately clear why different co-receptor usage would influence susceptibility of a viral isolate to inhibition by gp41-derived peptides. One possible explanation for the resistance of CCR5-using virus to inhibition by gp41-derived peptides is that gp120 binding to CD4 and CCR5 induces conformational changes that hinder the peptide interactions with its counterpart helical domain, while gp120 binding to CD4 and CXCR4 induces a conformation more conducive to the peptide association with its counterpart [6,7,20,21]. Thus, the higher resistance of R5 or X4R5 viruses using CCR5 to gp41-derived peptides' inhibition could be explained by less efficient dissociation of gp120 from gp41 upon interaction with CD4 and CCR5 than with CD4 and CXCR4.

Another possibility is that specific inhibition of X4 virus by tat also partially explains the more sensitivity of X4 virus than R5 virus to inhibition [22].

Furthermore, the resistance of primary viruses against broadly neutralizing monoclonal antibodies has been reported [23]. Our findings here suggested that this resistance of primary isolates could also be observed in gp41-derived peptides, though the mechanisms is not well understood. It should also be examined whether such resistance could also be observed in vivo, because the therapeutic efficacy of these peptides has also been claimed [24].

Note: after submission of this work, we noted that similar findings were reported by Derdeyn et al. [25].

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