Inhibitory role of CXCR4 glycan in CD4-independent X4-tropic human immunodeficiency virus type 1 infection and its abrogation in CD4-dependent infection

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CXCR4 functions as an infection receptor of X4 human immunodeficiency virus type 1 (HIV-1). CXCR4 is glycosylated at the N-terminal extracellular region, which is important for viral envelope (Env) protein binding. We compared the effects of CXCR4 glycan on the CD4-dependent and –independent infections in human cells by X4 viruses. We found that transduction mediated by Env proteins of CD4-independent HIV-1 strains increased up to 5.5-fold in cells expressing unglycosylated CXCR4, suggesting that the CXCR4 glycan inhibits CD4-independent X4 virus infection. Co-expression of CD4 on the target cell surface or pre-incubation of virus particles with soluble CD4 abrogates the glycan-mediated inhibition of X4 virus infection, suggesting that interaction of Env protein with CD4 counteracts the inhibition. These findings indicate that it will be advantageous for X4 HIV-1 to remain CD4-dependent. A structural model that explains the glycan-mediated inhibition is discussed.

Infection by the X4-tropic human immunodeficiency virus type 1 (HIV-1) requires interaction of two cellular surface proteins, CD4 and CXCR4 (Berger et al., 1998; Dimitrov, 1997). CXCR4 is a multi-membrane-spanning protein that possesses an N-glycosylation site in the N-terminal extracellular region (Chabot et al., 2000). Since the glycosylation site locates near the amino acid residues important for HIV-1 entry (Brelet et al., 2000; Chabot & Broder, 2000; Chabot et al., 1999; Picard et al., 1997), the glycan may inhibit X4 virus infection. Consistently, previous studies using mink or canine cells have indicated that the glycan inhibits infections of HIV-1 and HIV-2 X4 viruses (Potempa et al., 1997; Wang et al., 2004). However, it is not known whether the glycan has the same effect in human cells, in which glycan modification or other cellular cofactors necessary for infection might be different from those in non-human cells. Furthermore, studies using human cells failed to observe such inhibitory effects (Brelet et al., 2000; Chabot et al., 2000; Picard et al., 1997; Thordsen et al., 2002). Thus, despite extensive studies, the role of the CXCR4 glycan in X4 virus infection remained to be determined.

As is the case for HIV-1, most simple retroviruses use N-glycosylated multi-membrane-spanning proteins as their receptors (Overbaugh et al., 2001; Sommerfelt, 1999). In contrast to the HIV-1 studies, however, all the studies reproducibly indicated that the N-glycan on the receptors can efficiently suppress simple retrovirus infection (Kubo et al., 2002; Marin et al., 2003; Tailor et al., 2000; Wilson & Eiden, 1991). Entry mechanisms of the simple retroviruses and HIV-1 are similar in that they use multi-membrane-spanning proteins, whereas they differ in that the simple retroviruses need only a single type of receptor. Therefore, we hypothesized that the initial binding of gp120 to CD4 might counteract the glycan-mediated block of X4 virus infection.
To examine this hypothesis, we established human NP2 and U87 cell lines (Soda et al., 1999) that express C-terminally HA-tagged wild-type (wt) CXCR4 or N11A mediated by a murine leukaemia virus vector as reported previously (Kubo et al., 2003). N11A is a CXCR4 mutant lacking the N-glycosylation site by substitution of the asparagine residue by an alanine (Fig. 1a). Western immunoblot analysis using the anti-HA monoclonal antibody showed that N11A migrated faster than wt CXCR4, consistent with the loss of the N-glycan by mutation (Supplementary Figure S1, available with the online version of this paper).

We examined if the lack of the glycan on CXCR4 could affect transduction titres of an HIV-1 vector (Naldini et al., 1996) having CD4-independent mNDK (Dumonceaux et al., 1998) or 8X (Hoffman et al., 1999) HIV-1 gp120. The HIV-1 vector contains the lacZ gene as a marker (Chang et al., 1999; Iwakuma et al., 1999), and transduction titre was estimated by counting blue cells after X-Gal staining of the inoculated cells as reported previously (Kubo et al., 2004). Infection of the NP2 and U87 cells expressing N11A resulted in a moderate but statistically significant increase in the mNDK transduction titres; titres in N11A-expressing cells were about 1.5 and 2.5-fold higher than those in wt-expressing cells (Fig. 1b). Levels of enhancement were greater with 8X vector; titres in N11A-expressing cells were about 2.0 and 5.5-fold higher than those in wt-expressing cells. In contrast, the lack of glycan did not cause significant changes in titres of the VSV-G-pseudotyped vector (Chang et al., 1999).

We analysed levels of cell-surface expression of wt and N11A CXCR4 in NP2 and U87 cells by FACS with an anti-CXCR4 antibody (A80) that recognizes the third extracellular loop of CXCR4 (Tanaka et al., 2001). Fluorescence intensities of N11A-expressing NP2 and U87 cells were about 2 to 10-fold lower than those of wt-expressing cells (Fig. 1c). If wt and N11A were expressed at the same level, N11A could be 15 to 25 times more susceptible to CD4-independent infection than wt in NP2 cells. Similarly, N11A could be 4 to 11 times more susceptible than wt in U87 cells. This indicates that the CXCR4 glycan significantly inhibits CD4-independent infection. However, treatment of wt CXCR4-expressing cells with tunicamycin, an N-glycosylation inhibitor, had severe cytotoxicity, especially in U87 cells, and did not increase 8X vector transduction titre.

We examined whether the CXCR4 glycan could influence X4 virus infection upon CD4-mediated infection. For this purpose, we established NP2 and U87 cell lines that express both CD4 and CXCR4. As was seen with the CD4-negative cell lines, the size of N11A was smaller than that of wt (Supplementary Figure S2, available with the online version of this paper). Similarly, fluorescence intensities of N11A-expressing NP2 and U87 cells detected by FACS analysis were about 2 and 10-fold lower than those of wt-expressing cells in CD4-positive cells, respectively (Fig. 2a and b).

We next measured the effect of CD4 expression on transduction efficiency of various Env-pseudotyped vectors. Transduction titres of the mNDK and 8X vectors in NP2 cells co-expressing CXCR4 and CD4 were about 100-fold higher than in cells expressing CXCR4 alone (Supplementary Figure S3, available with the online version of this paper), suggesting that constitutive exposure of the coreceptor-binding site in these CD4-independent gp120 is partial and that CD4-induced conformational changes result in the full exposure.

We examined whether the CXCR4 glycan could influence X4 virus infection upon CD4-mediated infection. The

Fig. 1. Role of CXCR4 glycan in CD4-independent infection of X4 viruses. (a) Amino acid sequences of the N-terminal extracellular regions of wt CXCR4 and the N11A mutant. Hyphens indicate residues identical to wt CXCR4. The glycosylation site is underlined. The Asn residue was changed to Ala in the N11A mutant. (b) Relative transduction titres of the vectors in human cell lines expressing wt and N11A CXCR4. Relative titres to the titre in cells expressing wt CXCR4 (X4) are indicated. This experiment was repeated three times. Asterisks indicate statistical significance determined by Student’s t-test, P<0.05. (c) Cell-surface expression was analysed with a flow cytometer and the anti-CXCR4 antibody (A80). The black area indicates control cells stained with the A80 antibody. The white area indicates wt CXCR4- and N11A mutant-expressing cells stained with the A80 antibody.
vector solutions were diluted to obtain transduction titres similar to those of CD4-independent vectors in CD4-negative cells (about $5 \times 10^2$ infected cells per ml). Fig. 2(c) shows the relative transduction titres of the various Env-pseudotyped vectors in NP2 and U87 cells co-expressing CD4 and CXCR4. In contrast to the results with cells expressing N11A alone (Fig. 1), we could not detect a statistically significant increase in transduction titre of the vectors carrying the mNDK and the CD4-dependent HXB2 Env proteins in N11A-expressing cells. Similarly, removal of the CXCR4 glycan induced only a moderate increase in transduction titres of the 8X vector. These results showed that the mNDK, 8X and HXB2 viruses infected cells expressing wt CXCR4 as efficiently as cells expressing N11A when the target cells co-expressed CD4. These results suggest that the interaction of gp120 with CD4 counteracts the glycan-mediated inhibition.

To confirm this conclusion, we examined if preincubation of 8X vector particles with soluble CD4 (sCD4) could affect the glycan-mediated inhibition. The preincubation of sCD4 ($20 \mu g/ml$) enhanced transduction efficiency of the 8X vector approximately 3-fold in U87 cells expressing wt CXCR4 (Fig. 2d), as reported previously (Schten et al., 1999). In the absence of sCD4, the transduction titre in cells expressing N11A was about 5.5-fold higher than in cells expressing wt, but in the presence of sCD4, this difference was only 2.5-fold. This result indicates that sCD4 partially cancels the glycan-mediated inhibition of 8X virus infection, supporting the contention that CD4-gp120 interaction counteracts the glycan-mediated inhibition. The sCD4 treatment had lower efficiency to enhance the 8X vector infectivity and to counteract the glycan-mediated inhibition than the surface expression of CD4 on target cells. This may result from the dissociation of Env proteins from virus particles that is induced by the sCD4 treatment (Moore et al., 1990; Hart et al., 1991).

To help understand the molecular mechanisms by which the CXCR4 glycan affects the vector infectivity, we have built a three-dimensional (3D) model of CXCR4 with the carbohydrate moiety at the N terminus (Ponder & Case, 2003) (Fig. 3a). The crystal structure of the bovine rhodopsin [PDB code: 1F88 at 2.80 Å (0.28 nm) resolution] (Palczewski et al., 2000) was used as a template for the homology modelling of CXCR4. The high-mannose carbohydrate structure was used as the N-glycan, so that we could examine minimum effects of steric hindrance against ligand access.
Ramachandran plot, χ plot and energy minimization with AMBER-99 force field showed that the 3D model was physically and thermodynamically favoured and that it also preserves the physico-chemical features of the CXCR4 structure that were reported previously (Huang et al., 2003). These features include high levels of negative electrostatic potential along the top of the extracellular surface region of CXCR4 (Fig. 3a, red residues). The negative amino acids are located on the N-terminal end and extracellular loops (ECL) 1, 2 and 3, and some of them are indicated to play important roles in binding to gp120 (Brelot et al., 1997, 2000; Chabot et al., 1999; Doranz et al., 1999; Kajumo et al., 2000; Zhou et al., 2001). The 3D model suggests that these residues can function as attracting force via Coulombic interactions in the gp120 binding. In addition, the model shows that the glycan at the N terminus protrudes over this negatively charged region, narrowing space for HIV-1 Env protein access.

We found that X4 virus infectivity increased 1.5 to 5.5-fold when the virus infected human cells expressing N11A. Such an increase can be achieved by two mechanisms. First, N11A might have induced a conformation for increased binding to gp120. However, this possibility is unlikely because in silico structural analysis of the unglycosylated CXCR4 was nearly identical to the glycosylated counter-part. Furthermore, it is difficult to explain our results of the CD4-co-expression experiments by this mechanism. Our CXCR4 structural model supports the steric hindrance mechanism by predicting that the glycan will narrow the entry space of gp120 V3 (Fig. 3b). Unfortunately, a binding assay (Kinomoto et al., 2005) did not show higher levels of 8X vector binding to N11A-expressing cells compared with wt-expressing cells (Supplementary Figure S4, available with the online version of this paper), due to the lower sensitivity of the binding assay than the vector transduction assay.

**Fig. 3.** Structural model of glycosylated CXCR4 and a schematic illustration of interactions between gp120 and CXCR4 during CD4-dependent and -independent infections. (a) The 3D structure of CXCR4 was constructed by homology modelling using the crystal structure of bovine rhodopsin. (b) Gp120 core with V3 (cyan, PDB code: 2B4C) was placed near the extracellular loops of the CXCR4 model (white). The purple residue indicates the glycosylated Asn. Red, blue and green residues indicate acidic, basic and uncharged polar amino acids, respectively. (c) Schematic illustration of a model of interactions between gp120 and CXCR4 during CD4-dependent and -independent infections. (i) Before CD4 binding, the V3 can rarely interact with CXCR4 due to low exposure of the CXCR4-binding moiety. (ii) After conformational change of gp120 upon binding to CD4, the V3 tip can reach the binding sites on CXCR4 due to the high level of V3 exposure. (iii) In case of the CD4-independent infection, V3 exposure is constitutive but partial, which increases sensitivity to the steric hindrance of the glycan.
Our findings have implications for the importance of the CD4-dependent infection. CD4-independent variants are attenuated variants that are sensitive to antibody neutralization and that exist in nature only as a minor variant. CD4-dependency seems to have evolved to protect from neutralization antibodies (Bhattcharya et al., 2003; Edwards et al., 2001; Hoffman et al., 1999; Kolchinsky et al., 2001; Puffer et al., 2002; Thomas et al., 2003). Our data suggest that the CD4-dependent infection confers additional selective advantage other than a better defence capability. CD4-dependent viral infection was about 100-fold more efficient than CD4-independent infection. In addition, the glycan-mediated block of infection was counteracted by CD4-dependent infection. These data indicate that the CD4-independent X4 virus gains much better infectivity of human cells by CD4-mediated entry. Thus, CD4-dependent viruses dominate in nature possibly because they have a better defence capability against host immune restriction and better infectivity.

On the basis of our data and those of others, we suggest a model for the interactions between gp120 and CXCR4 upon CD4-mediated and -independent infections. Before the virus binds to CD4, the V3 loop in gp120 is less exposed (Zhu et al., 2006) [Fig. 3c(i)]. Upon viral binding to CD4, massive conformational changes in gp120 induce full exposure of V3 (Huang et al., 2005) [Fig. 3c(iii)] so that glycan-mediated inhibition is less efficient, leading to increase in viral infectivity, as seen in this and other studies (Breil et al., 2000; Chabot et al., 2000; Picard et al., 1997; Thordsen et al., 2002). In the case of CD4-independent infection, however, V3 is constitutively but partially exposed (Edinger et al., 1999; Edwards et al., 2001; Hoffman et al., 2000; Martin et al., 1997), which increases sensitivity to the glycan effect [Fig. 3c(iii)]. This in turn decreases infectivity in cells expressing glycosylated CXCR4 (Potempa et al., 1997; Wang et al., 2004). Further biochemical studies including structure–function analysis of gp120 and receptors will help in our understanding of how the glycan on the infection receptor affect HIV infection.

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