Importance of the *N*-glycan in the V3 loop of HIV-1 envelope protein for CXCR-4- but not CCR-5-dependent fusion

Emi E. Nakayama[a], Tatsuo Shioda[a,b], Masashi Tatsumi[c], Xiaomi Xin[a], Deshan Yu[a], Shinji Ohgimoto[a], Atsushi Kato[a], Yuko Sakai[a], Yukano Ohnishi[a], Yoshiyuki Nagai[a,*]

[a] *Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108, Japan*
[b] *Department of Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan*
[c] *Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan*

Received 6 March 1998

Abstract The V3 region of HIV-1 envelope protein possesses a single *N*-linked sugar chain, which is conserved in most HIV-1 strains. We studied its role in the life cycle of HIV-1 strains with different co-receptor usage. Removal of the glycan appeared to cause a marked reduction of CXCR-4- but not CCR-5-dependent virus entry. A basic amino acid substitution at the 11th position of V3 markedly compensated for the removal of the *N*-glycan. These results indicate that the *N*-glycan plays an important role for CXCR-4-dependent virus entry and that this role is exerted in a particular context of the peptide backbone.

Key words: Human immunodeficiency virus type 1; Third variable loop; *N*-Glycan; Site directed mutagenesis; CXCR-4

1. Introduction

The envelope protein (Env) of human immunodeficiency virus type 1 (HIV-1) consists of the surface gp120 subunit and the transmembrane gp41 subunit, which are non-covalently associated with each other [1–3]. As many as some 30 *N*-asparagine-linked carbohydrate attachment sites were predicted in the Env amino acid sequence and actual attachment of sugar chains appears to modulate the viral biological properties, infectivity and antigenicity [1–9]. However, the functional significance of each of the glycans for viral activities is not fully elucidated.

The infectivity, cellular host range and cytopathogenicity of HIV-1 vary from strain to strain. Primary HIV-1 isolates obtained from individuals at an early asymptomatic stage can replicate in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) and primary macrophages but not in established T cell lines. They are referred to as macrophage tropic (M-tropic) strains. On the other hand, the isolates from about 50% of patients at a late disease stage replicate in established T cell lines but not in macrophages, and are thus termed T-cell-line tropic (T-tropic) strains. Recently, chemokine receptors such as CXCR-4 and CCR-5 were identified as co-receptors that mediate fusion between the viral envelope and the host cell membrane and facilitate the entry of HIV-1 into cells following viral attachment to the major receptor CD4 [10–16]. Differences in cell tropism among HIV-1 strains are now explained by different co-receptor usage. CCR-5 is used by M-tropic strains [10,12–15], whereas CXCR-4 is used by T-tropic strains [11,16]. The third variable (V3) loop in gp120 has been found to be an important determinant for virus cellular host range [17–19], and its basic amino acid substitutions at positions 11 and/or 25 are closely associated with the T-tropic phenotype [20–24].

The V3 loop contains a site for *N*-glycosylation at the 6th position which is conserved in most HIV-1 strains but the significance of this high degree of conservation has been poorly addressed. To our knowledge, it has only been shown that the *N*-glycan in the V3 of some T-tropic strains (HXB2, HXB10, LAI and BRU) may be dispensable for virus infectivity [4–8]. But removal of this *N*-glycan enhanced the virus sensitivity to neutralizing antibodies, suggesting its role in masking the potential antigenicity of V3 peptide [5–8]. Here, we re-evaluated, by site-specific mutagenesis, the significance of this *N*-glycan of HIV-1 strains with various cell tropisms. Contrary to the above reports, our data show that removal of the *N*-glycan from V3 impaired efficient virus replication. This impairment appeared to be associated with a reduced level of CXCR-4-dependent fusion and virus entry. Therefore, our data suggest the importance of the highly conserved V3 *N*-glycan not only for the antigenicity of HIV-1, but also for viral infectivity and cellular host range. However, no such requirement of the glycan has been found for CCR-5-dependent fusion and virus entry characteristic of M-tropic strains. Thus, there appear to be considerable strain-specific differences in the structure-functional relationships in HIV-1-co-receptor interactions.

2. Materials and methods

2.1. Generation and propagation of N-glycosylation mutant viruses

Mutant DNA constructs of infectious molecular clones of HIV-1 strains NL43 [25], SF13 [26] and SF162 [27] were generated by site-directed mutagenesis by the polymerase chain reaction (PCR)-mediated overlap primer extension method [28]. Infectious viruses were recovered by transfection of the mutant proviral DNA into SW480 cells, followed by cocultivation with PBMC as described previously [24]. Virus production was determined by quantitative p24 enzyme-linked immunosorbent assay (ELISA) and 50% tissue culture infectious doses ($TCID_{50}$) after 2 weeks. Infection of cells with wild-type and mutant viruses was performed as described previously [24].

2.2. HIV-1 binding and entry assays

MT4 cells were treated with polybrene for 30 min and then exposed to the wild-type or mutant viruses equivalent to 100 ng of p24 in amount. The amount of virions bound to cells during 1 h at 4°C was quantitated by the p24 ELISA. The cells bound with virions were subsequently incubated at 37°C for 1 h to allow them to enter the cells, and intracellular p24 antigen was quantitated after trypsinizing the cells to digest and eliminate the viruses remaining on the cell surface [29].

*Corresponding author. Fax: (81) (3) 5449-5409.
E-mail: ynagai@ims.u-tokyo.ac.jp

PII S0014-5793(98)00375-5

2.3. Generation of recombinant vaccinia viruses and immunoprecipitation of HIV-1 envelope proteins

The human CCR-5 gene was PCR amplified from the total DNA extracted from PBMC of a normal seronegative donor. A recombinant vaccinia viruses (Vac) expressing CCR-5 or those expressing mutant Env under the control of the vaccinia virus early/late promoter for 7.5 kDa protein were generated using standard techniques as described previously [30,31]. Immunoprecipitation of Env expressed by the recombinant Vac was performed as described previously [32].

2.4. Generation of recombinant Sendai viruses expressing gp120 mutants and CD4 binding assay of the gp120

The gp120 genes of mutant NL43 clones were amplified by PCR and introduced to the plasmid pV(−)SeV18+b(+) which had been designed to generate a full-length Sendai virus (SeV) antigenome RNA with an insertion of a foreign gene at the first locus [33–35]. From the plasmids, recombinant Sendai viruses were recovered according to previously described procedures [33–36]. CD4 binding assay of the gp120 expressed from these recombinant viruses was performed as described previously [34].

2.5. Gene reporter fusion assay

A Vac-based gene activation assay using the β-galactosidase (β-gal) gene as a reporter was performed in L cells, a mouse fibroblast line, as described previously [37]. CCR-5 was expressed by Vac and CXCR-4 by SeV (Hu et al., in press). Data points were means of duplicate assays.

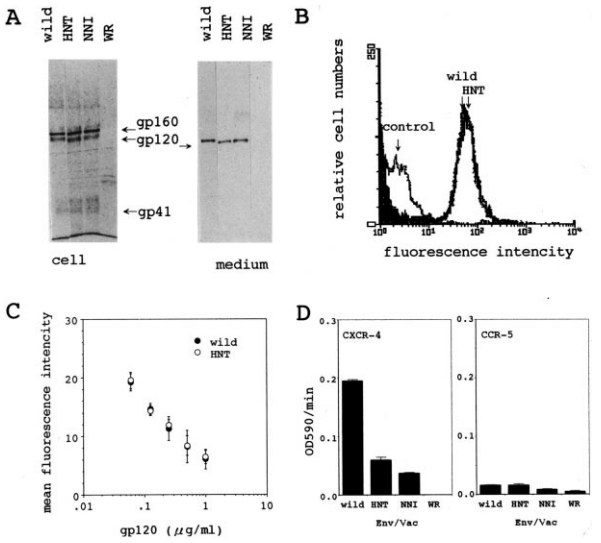

Fig. 1. A: Immunoprecipitation analysis of BHK cells infected with recombinant vaccinia viruses expressing HNT, NNI and wild-type Env proteins of strain NL43. WR indicates infection with the wild-type vaccinia virus strain WR. Infection was initiated with an input multiplicity of 10 plaque-forming units per cell and cells were harvested at 20 h after infection. B: FACS analysis of gp120 binding to CD4 expressing MT4 cells. C: Blocking of anti-CD4 antibody (Leu3a) binding to MT4 cells pretreated with various amounts of wild-type or HNT gp120. Data points are means ± standard deviation of triplicate samples. In experiments B and C, the culture supernatant of CV-1 cells infected with recombinant Sendai virus was used as the source of each gp120 [34]. D: Quantitation of fusion activities of Env proteins of the wild-type, mutant HNT and NNI, expressed from the respective recombinant vaccinia viruses (Env/Vac). Either CXCR-4 or CCR-5 was expressed as a co-receptor.

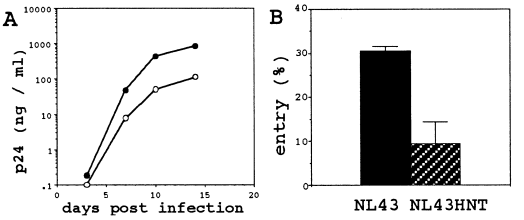

Fig. 2. A: Growth kinetics of the wild-type NL43 (●) and NL43HNT (○) in MT4 cells. Infection was initiated with 50 $TCID_{50}$ per culture of each virus. B: HIV-1 binding and entry assays. MT4 cells were incubated with viruses (100 ng of p24) and the virus entry was determined by the percentage of p24 amount within cells in the total p24 bound to the cell surface as described in Section 2.

3. Results

3.1. The effect of silencing N-glycosylation in the V3 loop on the biochemical and biological properties of the envelope protein of strain NL43

The HIV-1 strain NL43 is a T-tropic strain that has been well characterized. We replaced the canonical *N*-glycosylation motif, Asn-Asn-Thr (NNT), in V3 of its Env with His-Asn-Thr (HNT). We then created recombinant vaccinia viruses expressing the wild-type and mutant (HNT) Env to determine whether or not the mutation affects the levels of expression of gp160 and its processing to gp120 and gp41. BHK cells infected with the respective recombinant vaccinia viruses were labeled with [^{35}S]methionine and then lysed for immunoprecipitation. The gp160, gp120 and gp41 were clearly detected in comparable amounts in the two cell lysates (Fig. 1A). gp120 was detected solely in the culture supernatants again in comparable amounts (Fig. 1A). The HNT gp120 migrated slightly faster than the wild-type gp120. This strongly suggests that the V3 glycosylation site was actually used in the wild-type and indeed silenced by the mutagenesis.

We then compared CD4 binding capacity of the mutant gp120 with that of the wild-type gp120. For this experiment, we produced the gp120 by a SeV vector, which was previously shown to be extremely powerful in producing large quantities of gp120 of NL43 [34]. Here the HNT gp120 was also expressed at a high efficiency by the recombinant SeV. The results clearly show that the HNT gp120 bound to the surface of CD4-positive MT4 cells (Fig. 1B) and further hindered the access of an anti-CD4 antibody to the surface (Fig. 1C), as efficiently as the wild-type gp120 did. Therefore, removal of the *N*-glycan from V3 did not affect the CD4 binding capacity of gp120.

The fusion activities of the wild-type and HNT Env, expressed from the respective recombinant vaccinia viruses, were compared in the context of different co-receptors by a fusion assay using a β-gal gene as a reporter. Immunoprecipitation assays as shown in Fig. 1A were always included to assess comparable levels of expression of the wild-type and mutant glycoproteins in each fusion study. As shown in Fig. 1D, the fusion activity of HNT Env was consistently found to be about 30% of that of the wild-type, when CXCR-4, a co-receptor for T-tropic strains of HIV-1, was used. No CCR-5-dependent fusion was observed for either of the glycoproteins. To rule out the possibility that the impaired fusion activity was due to the amino acid substitution itself rather than the

loss of the *N*-glycan, we generated a second mutant Env, NNI, in which an isoleucine residue was substituted for the third position threonine in the canonical *N*-glycosylation signal. As shown in Fig. 1A, the NNI gp160 was expressed and processed as efficiently as the wild-type or HNT gp160. Similar to HNT gp120, the NNI gp120 migrated a little faster than the wild-type (Fig. 1A), and CXCR-4-dependent fusion activity was also impaired (Fig. 1D). These results strongly suggest that the removal of the *N*-glycan from V3 rather than the specific amino acid substitutions impaired the fusion activity of NL43 Env.

3.2. Effect of the removal of V3 glycan on the replication capability of NL43 virus in MT4 cells

We then generated viruses which contained the above described mutations in the V3, and compared their replication in MT4 cells. Hereafter, the mutant viruses are referred to as

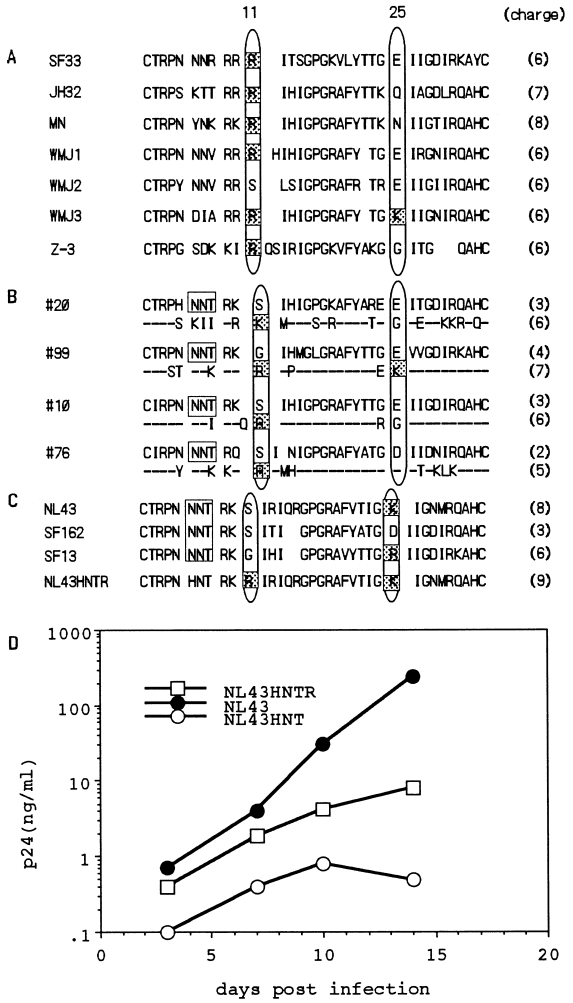

Fig. 3. V3 amino acid sequences of HIV-1 (subtype B) that were previously reported to be devoid of the glycan [38,42–45] (A), determined here or previously [24,46] with sera of four infected individuals (#20, #99, #10 and #76) (B) and of the viruses used in this study (C). Dashes in B indicate sequence identity to the respective top sequences with an *N*-glycan. The *N*-linked glycosylation signal (NNT) is boxed and residues at positions 11 and 25 are surrounded by ellipses. The basic amino acid residues at position 11 and 25 are shaded. D: Replication of NL43HNT (○), NL43HNTR (□) and the wild-type NL43 (●) in MT4 cells.

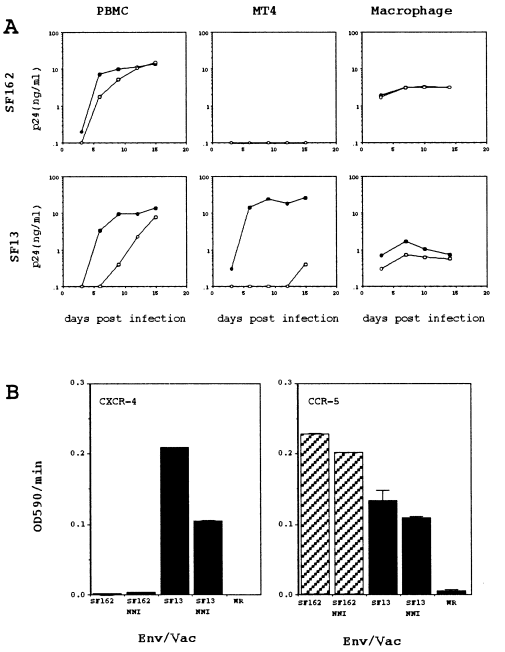

Fig. 4. A: Replication of the wild-type SF13 and SF162 (●) and their glycosylation mutants SF13NNI and SF162NNI (○) in PBMC, MT4 cells and primary macrophages. B: CXCR-4- and CCR-5-dependent fusion activity of the wild-type and mutated envelope glycoproteins expressed from vaccinia viruses (Env/Vac).

NL43HNT and NL43NNI, and the wild-type virus simply as NL43. NL43HNT (Fig. 2A) as well as NL43NNI (not shown) were found to grow more slowly than the parental NL43 virus, when infection was initiated with 50 $TCID_{50}$ per culture. These data suggest that the removal of the *N*-glycan caused significant loss of viral replication capability in MT4 cells. We have to note that when higher input virus doses (e.g. 5000 $TCID_{50}$ per culture) were used, differences between NL43 and NL43HNT or NL43NNI were not well resolved (data not shown), suggesting that the effect of glycan removal would be small in a single round of replication and became appreciable only after numerous rounds of replication. We further compared NL43HNT and NL43 with respect to virus binding to and entry into cells. We found that the amount of p24 bound to cells fluctuated considerably from experiment to experiment, allowing no exact direct comparison. However, the ratios of amount of virus that entered cells to that bound to cells were constant for each virus. The percentages of p24 amounts within cells in the total p24 amounts bound to cell surface indicated consistently that the entry efficiency was lower for NL43HNT than for NL43 (Fig. 2B). These data suggest that NL43HNT was impaired in penetration capability and appeared to be compatible with the reduced fusion activity of HNT Env (Fig. 1D).

3.3. Association of an N-glycan loss in the V3 loop with a basic amino acid substitution at the 11th position in natural isolates

Although our data suggesting the functional importance of V3 *N*-glycan appear to rationalize its conservation in a wide variety of HIV-1 isolates, a small portion of strains were reported to lack the *N*-glycan [21,38]. Fig. 3A shows V3 amino acid sequences lacking the *N*-glycosylation site of HIV-1 sub-

type B strains to which the NL43 strain belongs. In Fig. 3B, V3 sequences obtained from sera of four HIV-1-infected individuals are shown, which reveal the presence of a mixture of V3 sequences with and without the *N*-glycan. A strong correlation was observed between the lack of the *N*-glycan and a basic amino acid substitution at the 11th position (Fig. 3A,B). Inspection of 279 reported sequences of V3 of HIV-1 subtype B [21,39] further revealed that 33% of the sequences carrying a basic amino acid residue at the 11th position lack the *N*-glycan, whereas only 5% of those carrying a non-basic amino acid residue at this position lack the glycan. This difference is statistically significant ($P < 0.001$). On the other hand, there is no such correlation between the loss of *N*-glycan and basic amino acid substitution at the 25th position.

NL43 and its derivatives used in the present study possess a non-basic serine residue (S) at the 11th position. We substituted an arginine residue (R) for the S in the glycosylation-negative NL43HNT mutant, generating NL43HNTR (Fig. 3C). The replication capability in MT4 cells was found to be restored not completely but markedly by this additional mutation (Fig. 3D). These data suggest that a basic amino acid substitution at the 11th position compensated at least partially for the loss of V3 *N*-glycan.

3.4. The effect of N-glycosylation site mutations on HIV-1 strains with different cellular host ranges

HIV-1 displays strain-dependent differences in cellular host range. Since V3 is one of the major determinants for the viral cellular host range [17–19], we also introduced mutations to silence the *N*-glycosylation of V3 of HIV-1 strains with different cell tropisms. The strains used were the M-tropic SF162 and dual tropic SF13. The glycosylation signal NNT in their V3 was replaced with NNI, and the infectious viruses recovered from the respective mutagenized cDNA were named SF162NNI and SF13NNI.

The wild-type and mutant viruses were compared for replication capability in PBMC, human primary macrophages and MT4 cells (Fig. 4A). In PBMC, SF162NNI grew as efficiently as the wild-type SF162, whereas the replication of SF13NNI was retarded compared with SF13. In MT4, SF13 grew well to a high titer, whereas SF13NNI replication was severely impaired. On the other hand, no or only a marginal difference was found in replication capability in primary macrophages between SF162NNI and SF162 or between SF13NNI and SF13. These data suggest that the removal of an *N*-glycan from V3 severely affected the T-tropism but not the M-tropism of HIV-1.

3.5. The co-receptor-dependent fusion activity of the envelope proteins of SF13NNI and SF162NNI

Co-receptor-dependent fusion was studied by Vac-based expression of the Env of SF162, SF13 and their derivatives (Fig. 4B), as described above for the T-tropic strain NL43 Env and its derivatives. As expected, the dual tropic SF13 Env could use both CXCR-4 and CCR-5 in cell fusion, while M-tropic SF162 Env could use CCR-5 but not CXCR-4. CXCR-4-dependent fusion activity of SF13NNI Env was about 50% of that of SF13 Env, whereas CCR-5-dependent fusion activity of SF13NNI Env was nearly the same as that of SF13 Env. No appreciable reduction of CCR-5-dependent fusion activity was observed for SF162NNI Env compared with the parental SF162 Env. These data, together with those presented in Fig. 1D, suggest that the removal of the *N*-glycan from V3 affects the CXCR-4-dependent but not CCR-5-dependent fusion. It appeared that the removal of the glycan converted the dual tropic SF13 to a nearly M-tropic virus (Fig. 4A). Consistent with this notion, SF13NNI became sensitive to β-chemokine MIP-1 β, a natural ligand of CCR-5, while SF13 was resistant to this chemokine possibly because of preservation of the infection route via CXCR-4 (data not shown).

4. Discussion

In the present study, we generated *N*-glycosylation mutants of HIV-1 strains which differ in their cellular host range and demonstrated that the removal of the *N*-glycan from V3 impaired replication capability of a T-tropic HIV-1. The same mutation caused no such impediment for an M-tropic strain. The specificity of co-receptor usage of each mutant Env was confirmed by a Vac-based fusion assay, which demonstrated that the mutation affected CXCR-4-dependent fusion but not CCR-5-dependent fusion. Co-receptor-specific requirement of the *N*-glycan was further supported by similar mutagenesis of a dual tropic strain, as removal of the glycan affected the use of CXCR-4 but not CCR-5.

Previously, the *N*-glycan in V3 of HXB2, HXB10, LAI and BRU strains was reported to be dispensable for their infectivity to T cell lines, although detailed growth kinetics and experimental conditions were not described [4–8]. The experiments appear to have been done by infection initiated with only a single virus dose that was not specified clearly. We have to note that differences between the wild-type and mutant viruses were not observed with higher input virus doses, suggesting that the effect of glycan removal would essentially be small in a single step replication, and become appreciable only after numerous rounds of replication. Even though small to a single replication cycle, the contribution of the glycan would be great enough to in vivo pathogenesis which involves persistence of highly active viral replications for a long period.

It was previously noted that the *N*-glycosylation site in V3 is less conserved among T-tropic isolates than among M-tropic isolates [5]. This is apparently paradoxical if the *N*-glycan is important for CXCR-4- but not CCR-5-dependent entry as proposed in this study. However, inspection of V3 sequences revealed that the loss of the *N*-glycan is closely associated with a basic amino acid substitution at the 11th position. Furthermore, the basic amino acid substitution at the 11th position in NL43HNT partially but significantly restored the infectious capacity. Therefore, the *N*-glycan in V3 seems to be dispensable only in the particular context of the peptide backbone, and plays an important role for CXCR-4-dependent membrane fusion when the 11th position of V3 is occupied by a non-basic residue.

On the other hand, removal of the *N*-glycan from the V3 loop was reported to increase V3-specific antigenicity [5–8]. We could confirm this since the NL43HNT showed increased sensitivity to the V3-specific neutralizing monoclonal antibody 902 (data not shown). Back et al. [5] further raised the view that the *N*-glycan protects the V3 loop from attack by antibodies and hence is dispensable in patients with compromised immunity. However, in view of our present data showing a functional requirement of the glycan, the situation does not appear to be that simple. Their view may be applicable only to

M-tropic strains whose *N*-glycan in V3 does not appear to be functionally important.

The V3 loop was suggested to be involved in the gp120-co-receptor interaction [40,41]. Our present study demonstrated that there is a strain-specific requirement of the V3 glycan for this interaction. It is interesting but difficult to explain why and how the V3 glycan contributes to the infection process dependent on CXCR-4 but not CCR-5. It was previously reported that a subtle charge change in V3 of the T-tropic HIV-1 strain SF2 caused a drastic reduction of viral replicating capability in T cell lines, whereas the corresponding charge change in the M-tropic strain SF162 did not affect macrophage tropism [18]. It is thus possible that the V3 of T-tropic strains should be structurally more stringent for co-receptor recognition than that of M-tropic strains, and therefore the removal of *N*-glycan from the former could make an impact on virus-co-receptor interaction and infectivity.

The *N*-glycan on V3 is supposed to be a complex type and thus negatively charged [1]. A single *N*-linked sugar chain possesses a molecular mass of 2000–3000. These physical and structural properties may somehow be required for V3 to fully interact with CXCR-4. However, the sugar chain may not be directly involved in the interaction, since a basic amino acid substitution at a nearby site partially compensated for the loss of the glycan. It is puzzling if the presence of a negative charge (in the glycan) and a basic amino acid substitution for a neutral nearby residue are both factors leading to upregulation of CXCR-4-dependent fusion. Increase of hydrophilicity is a common feature shared by both. V3 glycosylation and the basic amino acid substitution may thus facilitate V3 exposure for better interaction with CXCR-4. In any case, a basic amino acid substitution at the 11th position appears to be an apt change in vivo for this particular *N*-glycan-negative virus to acquire a sufficiently high infectivity or for the virus to evade clearance due to infectivity reduction caused by spontaneous removal of the glycan.

In summary, our data indicate for the first time that a sugar chain at a specific position of the HIV-1 envelope glycoprotein is required for a specialized function to initiate infection. With respect to the V3 loop of HIV-1, numerous structure-function studies have been done using synthetic peptides without the sugar chain. Such studies may be able to dissect only a part of the natural reaction or may sometimes produce misleading results.

Acknowledgements: The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: monoclonal antibody to gp120 (No 902) from Dr. Bruce Chesebro. pG1T7 β-gal was kindly supplied by Dr. E. Berger. We thank S. Oka, S. Ida, and A. Iwamoto for the supply of patients' blood samples and valuable discussions. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, the Ministry of Health and Welfare, the Science and Technology Agency of the Japanese Government, and by grants from the Human Science Promotion Foundation and the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan.

References

[1] Leonard, C.K., Spellman, M.W., Riddle, L., Harris, R.J., Thomas, J.N. and Gregory, T.J. (1990) J. Biol. Chem. 265, 10373–10382.
[2] Stein, B.S. and Engleman, E.G. (1990) J. Biol. Chem. 265, 2640–2649.
[3] Bernstein, H.B., Tucker, S.P., Hunter, E., Schutzbach, J.S. and Compans, P.W. (1994) J. Virol. 68, 463–468.
[4] Lee, W.-R., Syu, W.-J., Du, B., Matsuda, M., Tan, S., Wolf, A., Essex, M. and Lee, T.-H. (1992) Proc. Natl. Acad. Sci. USA 89, 2213–2217.
[5] Back, N.K.T., Smit, L., Jong, J.-J.D., Keulen, W., Schutten, M., Goudsmit, J. and Tersmette, M. (1994) Virology 199, 431–438.
[6] Schønning, K., Jansson, B., Olofsson, S., Nielsen, J.O. and Hansen, J.-E.S. (1996) Virology 218, 134–140.
[7] Schønning, K., Jansson, B., Olofsson, S. and Hansen, J.-E.S. (1996) J. Gen. Virol. 77, 753–758.
[8] Hansen, J.-E.S., Jansson, B., Gram, G.J., Clausen, H., Nielsen, J.O. and Olofsson, S. (1996) Arch. Virol. 141, 291–300.
[9] Hu, H., Shioda, T., Moriya, C., Xin, X., Hasan, M.K., Miyake, K., Shimada, T. and Nagai, Y. (1996) J. Virol. 70, 7462–7470.
[10] Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M. and Berger, E.A. (1996) Science 272, 1955–1958.
[11] Berson, J.F., Long, D., Doranz, B.J., Rucker, J., Jirik, F.R. and Doms, R.W. (1996) J. Virol. 70, 6288–6295.
[12] Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J. (1996) Cell 85, 1135–1148.
[13] Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Marzio, P.D., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R. and Landau, N.R. (1996) Nature 381, 661–666.
[14] Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G. and Doms, R.W. (1996) Cell 85, 1149–1158.
[15] Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P. and Paxton, W.A. (1996) Nature 381, 667–673.
[16] Feng, Y., Broder, C.C., Kennedy, P.E. and Berger, E.A. (1996) Science 272, 872–877.
[17] Hwang, S.S., Boyle, T.J., Lyerly, H.K. and Cullen, B.R. (1991) Science 253, 71–74.
[18] Shioda, T., Levy, J.A. and Cheng-Mayer, C. (1992) Proc. Natl. Acad. Sci. USA 89, 9434–9438.
[19] Westervelt, P., Trowbridge, D.B., Epstein, L.G., Blumberg, B.M., Li, Y., Hahn, B.H., Shaw, G.M., Price, R.W. and Ratner, L. (1992) J. Virol. 66, 2577–2582.
[20] Chesebro, B., Wehrly, K., Nishio, J. and Perryman, S. (1992) J. Virol. 66, 6547–6554.
[21] Fouchier, R.A.M., Groenink, M., Kootstra, N.A., Tersmette, M., Huisman, H.G., Miedema, F. and Schuitemaker, H. (1992) J. Virol. 66, 3183–3187.
[22] Milich, L., Margolin, B. and Swanstrom, R. (1993) J. Virol. 67, 5623–5634.
[23] Simmonds, P., Zhang, L.Q., McOmish, F., Balfe, P., Ludlam, C.A. and Brown, A.J.L. (1991) J. Virol. 65, 6266–6276.
[24] Shioda, T., Oka, S., Ida, S., Nokihara, K., Toriyoshi, H., Mori, S., Takebe, Y., Kimura, S., Shimada, K. and Nagai, Y. (1994) J. Virol. 68, 7689–7696.
[25] Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A. and Martin, M.A. (1986) J. Virol. 59, 284–291.
[26] Cheng-Mayer, C., Shioda, T. and Levy, J.A. (1991) J. Virol. 65, 6931–6941.
[27] Shioda, T., Levy, J.A. and Cheng-Mayer, C. (1991) Nature 349, 167–169.
[28] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 51–59.
[29] Callebaut, C., Krust, B., Jacotot, E. and Hovanessian, A.G. (1993) Science 262, 2045–2050.
[30] Mackett, M., Smith, G.L., and Moss, B. (1985) in: DNA Cloning, Vol. I (Clover, D.M., Ed.), pp. 191–211, IRL Press, Oxford.
[31] Aoki, N., Shioda, T., Satoh, H. and Shibuta, H. (1991) AIDS 5, 871–875.
[32] Ohnishi, Y., Shioda, T., Nakayama, K., Iwata, S., Gotoh, B., Hamaguchi, M. and Nagai, Y. (1994) J. Virol. 68, 4075–4079.
[33] Hasan, M.K., Kato, A., Shioda, T., Sakai, Y., Yu, D. and Nagai, Y. (1997) J. Gen. Virol. 78, 2813–2820.
[34] Yu, D., Shioda, T., Kato, A., Hasan, M.K., Sakai, Y. and Nagai, Y. (1997) Genes Cells 2, 457–466.

[35] Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T. and Nagai, Y. (1997) EMBO J. 16, 578–587.
[36] Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M. and Nagai, Y. (1996) Genes Cells 1, 569–579.
[37] Nussbaum, O., Broder, C.C. and Berger, E.A. (1994) J. Virol. 68, 5411–5422.
[38] York-Higgins, D., Cheng-Mayer, C., Bauer, D., Levy, J.A. and Dina, D. (1990) J. Virol. 64, 4016–4020.
[39] LaRosa, G.J., Davide, J.P., Weinhold, K., Waterbury, J.A., Profy, A.T., Lewis, J.A., Langlois, A.J., Dreesman, G.R., Boswell, R.N., Shadduck, P., Holley, L.H., Karplus, M., Bolognesi, D.P., Matthews, T.J., Emini, E.A. and Putney, S.D. (1990) Science 249, 932–935.
[40] Cocchi, F., DeVico, A.L., Garzino-Demo, A., Arya, S.K., Gallo, R.C. and Lusso, P. (1995) Science 270, 1811–1815.
[41] Trkola, A., Dragic, T., Arthos, J., Binley, J.M., Olson, W.C., Allaway, G.P., Cheng-Mayer, C., Robinson, J., Maddon, P.J. and Moore, J.P. (1996) Nature 384, 184–187.
[42] Komiyama, N., Hattori, N., Inoue, J., Sakuma, S., Kurimura, T. and Yoshida, M. (1989) AIDS Res. Hum. Retroviruses 5, 411–419.
[43] Gurgo, C., Guo, H.-G., Franchini, G., Aldovini, A., Collalti, E., Farrell, K., Wong-Staal, F., Gallo, R.C. and Reitz Jr., M.S. (1988) Virology 164, 531–536.
[44] Hahn, B.H., Shaw, G.M., Taylor, M.E., Redfield, R.R., Markham, P.D., Salahuddin, S.Z., Wong-Staal, F., Gallo, R.C., Parks, E.S. and Parks, W.P. (1986) Science 232, 1548–1553.
[45] Willey, R.L., Rutledge, R.A., Dias, S., Folks, T., Theodore, T., Buckler, C.E. and Martin, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 5038–5042.
[46] Shioda, T., Oka, S., Xin, X., Liu, H., Harukuni, R., Kurotani, A., Fukushima, M., Hasan, M.K., Shiino, T., Takebe, Y., Iwamoto, A. and Nagai, Y. (1997) J. Virol. 71, 4871–4881.