Chapter 8

Probability of N332 glycan occupancy on HIV-1 gp120 modulates sensitivity to broadly neutralizing antibodies

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Abstract

Objective(s)
The glycan shield of the HIV-1 envelope glycoprotein complex (Env), in particular the glycan at position 332, is frequently targeted by broadly neutralizing antibodies (bNAbs) isolated from HIV-1 infected individuals. We investigated the role of the second amino acid position of the canonical glycosylation motif Asn-X-Ser in HIV-1 evolution and neutralization sensitivity.

Design and Methods
The neutralization sensitivity of viral variants harbouring glycosylation motifs with different probabilities of glycan occupancy were tested for a subset of N332-dependent bNAbs. Furthermore, longitudinal Env sequences of 37 HIV-1 infected individuals were used to analyse the evolution of the N332 glycosylation motif within these individuals. Finally, early Env sequences from 31 historical and 21 contemporaneous seroconverters were compared to analyse this evolution on a population level.

Results
Viral variants with a higher probability of N332 occupancy were more neutralization sensitive for a subset of the tested N332-dependent bNAbs. Furthermore, the longitudinal analyses showed increased probability of glycan occupancy of the N332 site over time, both within patients, and at the population level over the course of 20 years of HIV-1 epidemic.

Conclusions
These observations suggest that modulation of N332 glycan occupancy by the second amino acid position of the canonical glycosylation motif Asn-X-Ser plays a previously unappreciated role in viral escape from immune responses, and should be considered in Env-based vaccine design.
Introduction

HIV-1 neutralization is dependent on the ability of antibodies to recognize their target, the envelope glycoprotein complex (Env). Functional Envs consist of a trimer of heterodimers of the gp120 surface protein non-covalently linked to the gp41 transmembrane protein [1-4]. Glycans that are attached to potential N-linked glycoylation sites (PNGS) on Env compose approximately half of the molecular mass of the external Env domains and are important for protein folding, viral infectivity and, together with variable regions, for shielding of conserved domains from antibody attack [5-7]. Isolation and characterization of broadly neutralizing antibodies (bNAbs) from HIV-1 infected individuals resulted in the identification of at least five different target epitopes on Env (reviewed in [8]). Many bNAbs are glycan-dependent, and in particular the glycan at position N332 (HXB2 numbering) is frequently targeted by bNAbs, constituting a “supersite of vulnerability” on Env [9, 10]. N-linked glycosylation normally occurs at Asn-X-Ser and Asn-X-Thr motifs (NXS/T), where X can be any amino acid except Pro. However, even when these conditions are met, glycan occupancy is not guaranteed. NXS motifs have a 2- to 3-fold lower probability of becoming glycosylated than NXT motifs [11-13], giving rise to a source of heterogeneity in glycan occupancy. Furthermore, in particular in the context of NXS motifs the second position (the “X”) is an important factor in deciding whether a glycan will be attached or not [13]. Previously, we observed that the N332 glycosylation motif in early subtype B Envs from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) was either NLS or NIS, and the NLS motif was associated with the induction of bNAbs [14]. Considering that an NLS motif has a 2-fold lower probability in becoming glycosylated compared to an NIS motif [13] we conducted a more in depth study in the role of the “X” in the N332 NXS motif.

Methods

Neutralization sensitivity of viral variants with different probability of glycosylation

A full-length molecular clone HIV-1LAI was used as the basis for the introduction of env of an early virus isolated from ACS individual ACH19792 that harboured an NLS motif ([15]; termed “wild-type” virus; WT). Mutant viruses with an NIS, NVS or NLT motif, were generated using previously described methods [16]. Virus stocks were generated by transfecting HEK293T cells with 4 μg full-length plasmid, using the lipofectamine method [17]. The neutralization assay using multiple bNAbs was carried out as described previously [18].
Sequences and sequence analysis
Env sequences obtained from ACS participants have been described in earlier studies [14, 19-21]. To analyse the NXS motifs in HIV-1 subtype B infected individuals we used Env sequences from 625 individuals from the Los Alamos Database. Statistical analyses were performed using Graphpad Prism v5.01. Differences in $Pg$ evolution within individuals and population were compared using the Wilcoxon matched paired t-test and the unpaired t-test, respectively, and were considered statistically significant when p values were $\leq 0.05$.

Ethics statement
The Amsterdam Cohort Studies on HIV infection and AIDS (ACS) are conducted in accordance with the ethical principles set out in the declaration of Helsinki, and written consent was obtained prior to data collection. The study was approved by the Academic Medical Center’s Institutional Medical Ethics Committee.

Results
Considering the important role of glycans in escaping NAb responses (reviewed in [8]), we were interested in whether the probability of glycan occupancy at position 332 evolved over time within HIV-1 infected individuals. The probability of glycosylation, termed $Pg$, was defined based on previous studies (NXT where X is not P: $Pg = 1.0$; NLS: $Pg = 0.43$; NVS: $Pg = 0.79$; NIS: $Pg = 0.86$ and no PNGS: $Pg = 0$ [11, 13]). By comparing the first and last available Env sequences from 37 HIV-1-infected ACS participants (median follow-up: 8.3 years), we observed a statistically significant increase in the probability of N332 glycosylation over time ($p = 0.0009$; Fig. 1A). Thus, in 14 of the 37 studied participants the probability of N332 glycosylation was increased, while for the other 23 individuals this probability remained constant. Furthermore, in 10 out of 22 participants harbouring an NLS motif in the first available Env sequence, we observed evolution towards a motif with a higher probability of glycosylation (Fig. 1B), while the reverse was not true for individuals who had an NIS motif at the first time point (not shown).

Next, we were interested in whether the probability of glycosylation at position N332 has changed over the course of the HIV-1 epidemic. We compared early Env sequences (within one year post-seroconversion) from 31 historical and 21 contemporary seroconverters (samples obtained between 1985-1989 and 2003-2006, respectively), all infected with subtype B in Amsterdam. We found a significant increase in the probability of glycosylation of the N332 motif ($p = 0.025$, Fig. 1C). Overall our data suggests that the N332 glycosylation motif evolves towards a higher probability of occupancy, not only within infected individuals but also during the epidemic at the population level, probably as a mechanism to escape from immune responses.
N332 glycan occupancy and neutralization sensitivity

Figure 1: Probability of glycosylation at position N332. (a-c) Mean probability of glycosylation ($P_g$) at N332 (a) for the earliest and last available Env sequence of 37 infected individuals from the ACS (median of 1.6 months and 8.3 months post seroconversion, respectively), (b) over the course of infection for 22 infected individuals from the ACS that had a NLS motif in the earliest sequence, (c) for 31 historical and 21 contemporaneous seroconverters from Amsterdam (earliest sequences only; median of 4.5 and 0.5 months post seroconversion, respectively). The horizontal bars represent the mean probability of glycosylation at position 332. All Env sequences used are from subtype B infected individuals. The $P_g$ for a NXT codon at positions 334-336 was set at 1.0. Differences were considered statistically significant when $p$ values were $\leq 0.05$, represented by asterisks ($*: p < 0.05$, $***: p < 0.001$). (d) Pie chart showing the relative frequencies of different PNGS motifs at positions 332-334 in 625 Env clade B sequences from the Los Alamos Database. NXT334 refers to the presence of a PNGS motif at positions 334-336. (e,f) Neutralization curves of viruses harbouring the Env from ACS individual H19792 [14] containing an NLS (red) motif in comparison with mutants containing NIS (blue) and NVS (green) motifs ((e) PGT126; (f) VRC01).

If the second position of the N332 NXS motif indeed affects the probability of glycan occupancy, then this should also affect neutralization by N332-directed bNAbs. To explore this, we introduced the env of an early virus isolated from ACS individual ACH19792 that
harboured an NLS motif in the full-length molecular clone HIV-1\textsubscript{LAI} ([15]; termed “wild-type” virus; WT). This isolate with low probability of N332 glycosylation ($P_g = 0.43$) was previously shown to be resistant to both 2G12 and PGT126 [14]. In addition, we generated mutant viruses with an NIS ($P_g = 0.86$), NVS ($P_g = 0.79$) or NLT ($P_g = 1.0$) motif. These mutations were chosen based on the enhanced probability of glycosylation and their presence in evolving ACS subtype B isolates and the evolution within ACH19792 (NLS towards NIS). The NIS and NVS mutant viruses were as infectious as the original WT virus, whereas the NLT mutant was not infectious at all (data not shown). This result is consistent with the virtual absence of NXT motifs at position 332 from HIV-1 subtype B Los Alamos sequences (Los Alamos Database; n=625; 1.4\% NXT; 0.5\% NLT; Fig. 1D). The analysis of 625 subtype B sequences from the Los Alamos Database, revealed a high frequency of NXS motifs (84.4\%), of which 92.4\% were NLS or NIS motifs, in concordance with the glycosylation motifs in the ACS samples.

Next we tested neutralization sensitivity of the NLS (WT), NIS and NVS virus variants to N332-dependent bNAbs of the PGT121-family (PGT121-123), the PGT128-family (PGT125-PGT130), the PGT135-family (PGT135-136), and 2G12 (Table 1) [22]. VRC01 was included as a control. We observed that the mutant viruses, with a higher probability of N332 occupancy, were 4.3- (NIS) and 2.6-fold (NVS) more sensitive to PGT121 neutralization, whereas this difference was not observed for PGT122 and PGT123. The mutant viruses were also substantially more sensitive to PGT126 (44- and 48-fold, respectively), PGT128 (39- and 60-fold) and PGT130 (17- and 41-fold; Table 1). All three viruses were resistant to neutralization by PGT125, PGT127, PGT135, PGT136 and 2G12 at the bNAAb concentrations tested here. Subtleties in the fine specificities and dependencies of the individual PGT121- and PGT128-family members are probably responsible for the observed differences between them [23, 24] [Garces \textit{et al.} submitted]. VRC01 neutralization was minimally affected by the mutations. Overall, we found that neutralization by in particular PGT121, PGT126, PGT128 and PGT130 was affected by the second position of the N332 PNGS and, by inference, glycan occupancy at this site. Thus, the virus variants that had a high probability of glycan occupancy were more efficiently neutralized by these bNAAbs.

**Discussion**

Our results highlight a previously unappreciated mechanism by which HIV-1 can modulate its protective glycan shield. The fact that we found the increase in probability of N332 glycan occupancy over time within infected individuals, but also at the population level over the course of 20 years of HIV epidemic suggests that it is a mechanism to escape from neutralization. These results are consistent with studies showing that escape from NABs can be mediated by increasing the total number of PNGS motifs (reviewed in [8]; [19]): both mechanisms increase the density of Env’s glycan shield. Interestingly, we have shown that the presence of an NLS motif, and not an NIS motif, on early Env’s was associated with the
Table 1: Antibody neutralization tested in TZM-bl assay.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>NLS(WT)</th>
<th>NIS</th>
<th>NVS</th>
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<tr>
<td></td>
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<td></td>
<td></td>
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</table>

IC50 values for the N332-dependent bNAb and VRC01, tested against the H19792 NLS(WT), NIS and NVS mutant viruses. N332-dependent bNAb are grouped according to family; PGT121 (PGT121-123), PGT128 (PGT125-128 and PGT130); PGT135 (PGT135-136). The last two columns list the fold increase in neutralization sensitivity of the mutant viruses, NIS and NVS, compared to WT.

development of bNAb, suggesting that glycan occupancy at this position is important for shielding of vulnerable regions [14]. Paradoxically, neutralization escape by increasing glycan density might actually contribute to the presentation of glycan-dependent bNAb epitopes.

The effect of the “X” in the N332 glycosylation motif, Asn-X-Ser, on sensitivity to N332-glycan dependent bNAb is probably not black and white. This residue influences the probability of N332 occupancy and it is possible that some Env molecules on a virion or even within an individual trimer contain an N332 glycan, while others do not. Possibly, this source of heterogeneous glycan occupancy contributes to unusual neutralization curves, as has been proposed for heterogeneous glycan composition [25]. Furthermore, the influence of residue 333 on N332 glycan occupancy is probably dependent on the context of the particular virus isolate. Overall these results suggest an important role for the second position of the N332 glycosylation motif in modulating the sensitivity for bNAb, especially for some of the PGT128 family members. Although the “X” of many of the NXS motifs in Env is conserved (N156: X=C; N197: X=T; N262: X=G; N616: X=K), our findings might be relevant for other NXS PNGS. For example, the X position in the motif for the N611 glycan, which is important for binding of bNAb PGT151 to the gp120-gp41 interface [26, 27], varies substantially between virus isolates, providing a source of glycan heterogeneity.

The observations that glycan-directed antibodies arise frequently and relatively early in infected individuals, are less somatic hypermutated compared to bNAb targeting other
epitopes on Env, and can protect against infection when passively administered, makes them attractive targets for Env-based immunogen design [28-30]. However, our results suggest that the exact sequence of the N332 glycosylation motif should be considered. For example, an immunogen with a low probability of N332 occupancy might be less efficient at inducing N332-glycan directed bNAbs, but more efficient at presenting epitopes that are shielded by the N332 glycan.

Acknowledgements

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Reference List


