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N463 Glycosylation Site on V5 Loop of a Mutant gp120 Regulates the Sensitivity of HIV-1 to Neutralizing Monoclonal Antibodies VRC01/03

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Abstract

Background—HIV-1 gp120/gp41 is heavily modified by n-linked carbohydrates that play important roles either in correct folding or in shielding vulnerable viral protein surfaces from antibody recognition.

Methods—In our previous work, 25 potential N-linked glycosylation sites (PNGS) of a CRF07_BC isolate of HIV-1 were individually mutated, and the resulting effects on infectivity and antibody-mediated neutralization were evaluated. In order to further understand the functional role of these PNGS, we generated double and multiple mutants from selected individual PNGS mutants. The effects were then evaluated by examining infectivity and sensitivity to antibody-mediated neutralization by neutralizing monoclonal antibodies (nMAbs) and serum antibodies from HIV-1 positive donors.

Results—Infectivity results showed that, among the twelve combined PNGS mutants, only 197M.1 (N197D/N301Q) lost infectivity completely, while all others (except for 197M.6) showed reduced viral infectivity. In terms of neutralization sensitivity to known nMAbs, we found that adding N463Q mutation to all the gp120 mutants containing N197D significantly increased neutralization sensitivity to VRC01 and VRC03, suggesting N197 and N463 have a strong synergistic effect in regulating the neutralizing sensitivity of HIV-1 to the anti-CD4bs nMAbs

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VR01/VR03. Structural analysis based on the available structures of gp120 alone and in complex with CD4 and various nMAbs elucidates a molecular rationale for this experimental observation.

Conclusions—The data indicate that N463 plays an important role in regulating the CD4bs MAbs VR01/VR03 sensitivity in the genetic background of N197D mutation of gp120, which should provide valuable information for a better understanding of the interplay between HIV-1 and VR01/03.

Keywords

HIV; N-linked glycosylation site; Pseudovirus; Change of Neutralization Sensitivity; Structural Modeling and Analysis

Introduction

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Induction of an antibody response capable of neutralizing diverse human immunodeficiency virus type 1 (HIV-1) isolates is a critical step for an effective vaccine that can protect against HIV-1 infection. Although vaccines have thus far failed to induce broadly neutralizing antibody responses, it is reported that approximately 1 to 30% of HIV-1-infected subjects eventually develop potent and broadly reactive neutralizing antibodies^{1–3}. Most broadly neutralizing antibodies in chronic HIV-1 sera have been shown to target the region around the CD4 binding site, the CD4 induced site, the glycan-dependent or quaternary epitopes on the gp120, and the conserved elements of V3 region^{4–8}. In recent years, based on new neutralizing antibody screening systems, many potent and broadly MAbs have been isolated^{9–11}.

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The CD4-binding site (CD4bs) on gp120 is essential for viral entry, and is highly conserved compared with other envelope regions. The CD4bs contains the key epitopes recognized by several broadly neutralizing antibodies obtained so far. MAb b12, a CD4bs-MAb, was isolated from a phage display library and can neutralize about 40% of known HIV-1 isolates^{12, 13}. Recently, a battery of anti-CD4bs MAbs, including VR01/VR03¹¹, 3BNC117¹⁴, PGV04¹⁵, and NIH45-46¹⁶ show significantly higher potency and wider breadth compared with b12. VR01 can neutralize ~90% of HIV-1 isolates at a low inhibitory concentration (IC₅₀) and structural studies show that it achieves this neutralization by precisely recognizing the initial site of CD4 attachment on HIV-1 gp120. Mutagenesis studies indicated that VR01 contacts within the gp120 loop D, the CD4 binding loop, and the V5 region were necessary for optimal VR01 neutralization^{17, 18}.

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N-linked glycans are important for processing of gp120 that ultimately influences envelop conformation, oligomerization, receptor binding, membrane fusion and viral entry, infectivity, and antibody neutralization of HIV virus^{19–23}. In our previous work, we individually mutated the 25 PNGS of a CRF07_BC isolate to study the effect on viral infectivity and antibody-mediated neutralization finding that certain N-linked glycosylation sites are critical for virus to infect target cells and protect the virus from neutralizing by monoclonal antibodies²⁴. The partial deglycosylated HIV envelope protein was previously shown to enhance the immunogenicity of some epitopes^{17, 25, 26}. Some studies reported that

combined PNGS elimination at specific sites in clade B HIV virus can increase the neutralizing sensitivity to certain nMAbs^{27, 28}. For example, mutating N186 and N197 simultaneously on gp120 of 3 CRF01_AE clones increased sensitivity to b12, whereas the mutating N186 or N197 individually was not sufficient to increase b12 sensitivity²⁹. Also the combined N460 and N463 mutation on V5 region of 08_B'C viruses resulted in higher VRC01 sensitivity than each single mutation alone³⁰. The conserved PNGS in gp41 were also eliminated individually and in combination to test the viral replication³¹. Clade 07_BC viruses, which have been one of the most predominantly circulated HIV-1 strains in China, have a somewhat different glycan arrangement than the B/AE and other clade viruses, and the elimination of combined PNGS located on different regions of the gp120 of the predominant Clade of HIV viruses in China has not been evaluated before.

In order to further understand the biological functions of the gp120 PNGS of the Clade 07_BC HIV-1 strain predominantly circulating in China, we constructed combined PNGS mutants based on previous single PNGS mutants to evaluate their infectivity and neutralizing sensitivity to nMAbs. We identified certain combined PNGS mutants that become highly sensitive to certain nMAbs, suggesting that the PNGS sites on these mutants likely play a critical role for shielding the virus from being recognized by these nMAbs and targeting/recognition by the host humoral responses.

Methods

Cells and Plasmid

TZM-bl cells, Env-deficient HIV-1 backbone (pSG3^{Env}) were obtained from the US National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (ARRRP), as contributed by John C. Kappes, Xiaoyun Wu, and Tranzyme (Birmingham, AL). The 293FT cells were obtained from Invitrogen (Carlsbad, CA).

MAbs & Positive Serum

MAbs 2F5, 4E10, 2G12 and b12 were obtained from the International AIDS Vaccine Initiative (IAVI, New York, NY); MAbs 2F5 and 4E10 were contributed by Hermann Katinger (Institute of Applied Microbiology, Vienna, Austria) and b12 was contributed by Dennis Burton and Carlos Barbas (The Scripps Research Institute, La Jolla, CA); MAbs VRC01, VRC03, PG9, PG16 were obtained from the ARRRP (NIH).

For use in the study, 20 subtype BC HIV-1 infected positive sera were obtained from chronically HIV-1-infected blood donors in main HIV-1 epidemic regions of China. Individual samples were coded based on the region of China. These samples were collected from Beijing (bj20, bj22), Sichuan (sc59r), Guangdong (gd64), Yunnan (yn99r, yn148r), Guangxi (gx66, gx75, gx76, gx77, gx78, gx79, gx82, gx85, gx87, gx93, gx94, gx95) province and Xinjiang autonomous region (xj16, xj50). Samples were stored at -70 °C and freezing/thawing cycles were avoided. All serum samples were heat-inactivated at 56 °C for 1 h before use.

Elimination of PNGS by Mutagenesis

The motif for an N-linked glycosylation site is Asn-X-Thr/Ser, where X can be any amino acid except proline³². Elimination of PNGS was performed using site-directed mutagenesis, by changing an asparagine (N) to a glutamine (Q) or aspartate (D). The wt *env* gene was inserted into pcDNA 3.1D/V5-His-TOPO (Invitrogen) as a template for mutagenesis. Mutagenesis was performed as described previously²⁴. Standard PCR and cloning procedure were used to obtain the mutant clones. The entire *env* gene of each mutant was sequenced to confirm mutation.

Pseudovirus Preparation, Infectivity, Titration and Neutralization Assays

Pseudoviruses were produced by co-transfection of 293FT cells (>90% confluency in a 25 cm² rectangular canted neck cell culture flask, Corning, USA) with 5.3 µg pSG3 Env plasmid and 2.7 µg Env-expressing plasmids using the Lipofectamine 2000 reagent (Invitrogen). Supernatants were harvested 48 hr after transfection, filtered (0.45-µm pore size), and stored at -80 °C. The concentration of HIV-1 Gag p24 antigen in viral supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (Vironostika HIV-1 antigen micro-ELISA system; bioMérieux, Boxtel, The Netherlands).

A fixed amount of pseudovirus (equivalent to 1.0 ng p24 antigen) was added to TZM-bl cells at 70–80% confluency in a 96-well plate in the presence of 15 µg/ mL DEAE-dextran, in a total volume of 200 µL. 48 hr after infection, the luciferase activity in infected cells was measured using the Bright-Glo™ luciferase assay system (Promega, Madison, WI). Relative infectivity was calculated by dividing the Log10 (RLU of mutant) by Log10 (RLU of wt).

The 50% tissue culture infectious dose (TCID₅₀) of a single infectious pseudovirus batch was determined in TZM-bl cells, as described previously³³.

Neutralization was measured as a reduction in luciferase expression after a single-round infection of TZM-bl cells with pseudoviruses according to previously published method³⁴.

Structural Modeling

The full-length HIV FE gp120 was generated using the homology modeling software Modeller 9.13³⁵. The gp120 pdb structures 4nco, 2ny7, 3ngb, and 3se8 were used as templates for modeling and glycans were modeled from 4nco. The interfacial residues that make up the defined epitope/paratope for the gp120 and protein ligands were calculated using PDBE PISA v1.48 server 'Protein interfaces, surfaces and assemblies' service PISA at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html)³⁶.

Results

Construction of the Combined PNGS Mutants and Viral Infectivity

In the previous study, the asparagine residue in all 25 PNGS on the wild-type gp120/41 of the HIV strain FE were mutated individually to glutamine or aspartate at the following positions: 88 (on C1 of gp120); 133, 142, 156, 160 (on V1); 181 (on V2); 197, 234, 241, 262, 289 (on C2); 301 (on V3); 339, 355 (on C3); 392, 408, 411 (on V4 loop); 442, 448 (on

C4); 463, 466 in V5; 611, 616, 625, 637 (on gp41) (residue positions on gp120/41 are based on HXB2 numbering, Supplemental Fig. 1).

The effects of these individual PNGS mutants on nMAbs-mediated neutralization have been previously examined²⁴. Here, we generated twelve combined PNGS mutants that contain different combinations of the selected PNGS point mutations to evaluate their influence on infectivity and neutralization of the resulting mutant viruses. The twelve combined PNGS mutants constructed in this study were shown in Supplemental Table 1. Eleven of the twelve mutants (except for M46) contain the N197D mutation, and eight of them contain N197D/N463Q mutations. All mutants were confirmed by sequencing.

Among all the combined mutants studied here, only 197M.1 (N197D/N301Q) has completely lost infectivity. Other multiple mutants showed no significant reduction of viral infectivity when compared to the two single point mutants, N197D or N301Q (Fig. 1).

Effect of Combined PNGS Mutations on Neutralization by nMAbs

The non-infectious mutant 197M.1 (N197D/N301Q) was excluded from the further neutralization assay study. All other mutant viruses on Supplemental Table 1, together with some single mutants, were examined for neutralization by nMAbs. In previous work, the single N197D mutant showed an obvious increase in susceptibility to neutralization by b12 (~17-fold increase) and VRC03 (~37-fold increase), as well as a modest 2-fold increase to VRC01 when compared to wild-type²⁴. However, the neutralization result for the eight combined PNGS mutants in this study showed a much more dramatic increase in susceptibility to neutralization by VRC01/VRC03 compared to the single N197D or to wild-type (Table 1, Fig. 2). For example, mutants 197M.3 (N197D/N463Q), 197M.4 (N197D/N463Q/N442Q), 197M.5 (N197D/N463Q/N625Q), 197M.8 (N197D/N463Q/N625Q/N442Q/N339Q/N448) and 197M.9 (N197D/N463Q/N625Q/N442Q/N339Q) showed a 867-fold increase in susceptibility to neutralization by VRC03 when compared with wild-type, whereas mutants 197M.10 (N197D/N463Q/N625Q/N442Q/N339Q/N466Q) and 197M.11 (N197D/N463Q/N625Q/N442Q/N339Q) showed a ~1300-fold increase, and 197M.2 (N197D/N625Q), 197M.6 (N197D/N625Q/N442Q) and 197M.7 (N197D/N625Q/N442Q/N339Q) showed a ~100-fold increase (Table 1, Fig. 2). As for the neutralization by another nMAb VRC01, mutants 197M.3, 197M.4, 197M.5, 197M.8, 197M.9, 197M.10 and 197M.11 showed a ~275–420 fold increase, whereas 197M.2, 197M.6 and 197M.7 had only ~2-fold increase in neutralizing sensitivity compared to wild-type. M46 (N625Q/N463Q) showed no effect on VRC01/VRC03 mediated neutralization. However, all other combined PNGS mutants containing N197D mutation showed little changes of sensitivity to b12 or other nMAbs (PG9, PG16, 2F5, 4E10 and 2G12) when compared to the N197D single point mutant (Table 1, Fig. 2).

The results shown above indicate that whenever a combined mutant of the HIV FE strain (a BC clade virus) contains N197Q together with N463Q, a dramatic increase of the neutralizing sensitivity to VRC01/VRC03 is observed. To investigate whether the combination of N197Q and N463Q mutation has the same effect in other clade viruses to these CD4bs MAbs neutralization, a clade B virus B05 and a clade AE virus GX74.20 were tested. The N197Q mutation alone in the two viruses showed a ~2–4 fold increase in

susceptibility to neutralization by VRC01/VRC03, whereas the N463Q mutation alone showed no effect (Supplemental Fig. 2). The combination of N197Q and N463Q showed a less dramatic, but still significant increase in susceptibility to neutralization by VRC01/VRC03, ranging between 8 to 20 fold increase. For the sensitivity to b12, similar to the behavior of the FE strain mutants, the combined N197Q and N463Q mutations in these two clade viruses showed no obvious changes when compared with the N197Q single mutant (Supplemental Fig. 2).

Effect of PNGS Mutations on Neutralization by Serum Antibodies

Here we used serum collected from 20 subjects infected with subtype BC isolates to test the single and combined PNGS mutants for neutralization phenotype. The ID₅₀ for neutralization is shown in Table 2. Among the single PNGS mutants, N197D had a 2–3-fold ID₅₀ increase in the seven sera of the serum panel; N301Q showed a 2–5-fold ID₅₀ increase in eight of the serum panel; N442Q showed a 2–5-fold ID₅₀ increase in seven of the serum panel; and the N625Q made a 2–4-fold ID₅₀ increase in seven of the serum panel. In addition, the N611Q mutant was neutralized by serum gx66, gx76 with a titer increase of over three-fold compared to the wild-type virus, and the N637Q mutant was neutralized by serum yn148r with a five-fold titer increase compared to the wild-type virus. A few PNGS mutations also decrease the neutralizing sensitivity to HIV-1 positive serum. The N197D mutation resulted a 2-fold reduction in serum xj50 neutralization, this neutralization reduction also happened in the N611Q mutation to serum yn99r, N289D to serum bj22 and gx75, N448Q to serum sc59r and N625Q to serum yn99r. These data suggest that the presence of the PNGS in the C2 (N197), V3 (N301), C4 (N442) regions and gp41 (N625) protect FE from antibody-mediated neutralization by the serum panel.

Interestingly, compared with the single point mutants, most of the combined PNGS mutants displayed higher neutralizing sensitivity to the serum. All the eleven combined PNGS mutants (except for M46) showed a 2–34-fold ID₅₀ increase in 7–15 of the serum panels, with the mutant 197M.11 (N197D/N463Q/N625Q/N442Q/N339Q) showing 2–34 fold increase in 15 tested serum samples compare to the respective single PNGS mutants and wild-type virus. 197M.11 also showed a ~9-fold and ~4-fold increase in neutralization sensitivity to serum gx66 and gx76, compared to the other mutants (Table 2). Another interesting observation is that, while some the single PNGS mutants showed no effect on neutralizing sensitivity by serum, combining such single PNGS mutants somehow displayed increased neutralizing sensitivity to serum antibodies, as shown by the sensitivity increase of mutants 197M.6 (N197D/N625Q/N442Q) and 197M.7 (N197D/N625Q/N442Q/N339Q) to serum sc59R, and mutants 197M.8 (N197D/N463Q/N625Q/N442Q/N339Q/N448), 197M.9 (N197D/N463Q/N625Q/N442Q/N339Q) and 197M.10 (N197D/N463Q/N625Q/N442Q/N339Q/N466Q) to serum gx93 (Table 2).

Structural Modeling and Rationalization

In order to understand the molecular basis for the observed increase of sensitivity to VRC01/VRC03 when N463Q mutation is added on top of N197D, we analyzed the available structural information for gp120 structures alone and its complexes with CD4 and various nMAbs^{18, 30, 37–40}. In doing so, the antibody bound gp120 structures available to date were

aligned to the FE gp120 model, and the structural basis by which specific glycosylation positions influence neutralization were examined. Of the 25 potential PNGS sites, both N197 and N463 map to the CD4 binding “face” of gp120 (Fig. 3A, 3B). However, N197 and N463 are not directly on top of the CD4 binding interface, neither N197 nor N463 appears to occlude CD4 binding (Fig. 3A, 3B), suggesting that they should not directly interfere with or participate in CD4 binding for infection. This is consistent with the observed viral infectivity for the mutants containing N197/N463 residues (Fig. 1). As for the antibody neutralization, nMAb b12 relies solely on heavy chain contacts that partially overlap with the CD4 binding site⁴⁰, and unlike the VRC antibodies, neutralization of HIV FE by b12 is not as dramatically affected by FE gp120 N197D/N463Q mutation (Fig. 2, Table 1). Based on the structural modeling, N197 and N463 of FE gp120 should not present steric hindrance to b12 antibody binding to the CD4 binding site (Fig. 3C, 3D). Compared to the binding interfaces for b12 and CD4, the N197 and N463 residues of FE gp120 are located directly adjacent to or within the VRC01 epitope/paratope (Fig. 3E, 3F), and these two residues are located within the VRC03 epitope/paratope (Fig. 3G, 3H). The degree of steric occlusion on the structural model appears to be directly related to the level of our observed sensitivity changes of neutralization by VRC01 and VRC03, where VRC03 binding to gp120 should be more hindered by glycosylation at N197/N463 compared to VRC01 binding, which is consistent with the observation that mutation at N197/N463 has even more marked increase of neutralizing sensitivity to VRC03 than VRC01. Therefore, the effects of FE gp120 glycosylation at N197 and N463 on neutralization sensitivity can be directly correlated to the structural properties that rationalize nMAb recognition and binding.

Discussion

Among the twelve combined PNGS mutants of the HIV-1 gp120/gp41, eleven of them had no significant loss of infectivity, suggesting that these PNGS are not important for gp120/gp41 folding, maturation, viral assembly, and cell entry during infection. Surprisingly, one of the twelve mutants, the double mutant 197M.1 (N197D and N301Q) resulted in a complete loss of viral infectivity, which is rather surprising considering that as all the other combined mutants contain the N197D mutation had infectivity, and some of them having additional 3 to 7 point mutations on top of N197D. Our previous study indicated that N197D or N301Q mutation alone did not have severe impact on the infectivity²⁴, which is also confirmed here (Fig. 1). However, the combination of the two point mutations proved to be deadly for the virus, which suggests that such double mutant somehow abolish certain essential function(s) required for viral infection.

For the eleven combined PNGS mutants that showed viral infectivity, we examined their sensitivity to seven broadly neutralizing MAbs^{41, 42}. The neutralization ability of CD4bs MAbs VRC03 showed a marked increase for combined PNGS mutants that contained the both N197D and N463Q mutations, with a remarkable ~800–1300 fold increase over wt FE. These mutants also showed a ~300–400 fold increase in sensitivity to VRC01. Compared with the combined PNGS mutants, only the N197D and the N442Q among the single mutants showed relatively modest increase in neutralizing sensitivity to VRC03, with N197D showing 37-fold increase and N442Q showing 3-fold to VRC03. N197D also showed a 2-fold increase sensitivity to VRC01. However, N463Q single mutant, together

with most other single mutants alone, showed little effect on neutralization by VRC01/VRC03, b12 or other nMAbs (Table 1).

Two pairs of combined mutants 197M.2/197M.5, and 197M.7/197M.9 (Supplemental Table 1), which differ only in containing N463Q mutation or not, showed significant difference in VRC01/VRC03 sensitivity, with the mutants containing N463Q displaying much higher VRC01/03 sensitivity (Fig. 2, Table 1). In fact, all the seven mutants that contain both N197D and N463Q in them showed the same dramatic increase of sensitivity to VRC01/VRC03, whereas those combined mutants with N197D but without N463Q (mutants 197M.2, 197M.6, and 197M.7) showed little effect (Supplemental Table 1 & Table 1).

Interestingly, further mutations of the PNGS up to five sites on top of the N197D/N463Q mutation appear to have very minor additional effect on the sensitivity level to the two VRC nMAbs. Thus, N463Q mutation seems to play an important role in determining the neutralizing sensitivity to nMAbs VRC01/VRC03 in the N197D mutant background. In other words, even though the effect of N463Q mutation alone did little in altering the sensitivity to nMAbs VRC01/VRC03 (with ~1.5 fold increase), combining N463Q mutation with N197D has an unexpected synergistic effect in increasing the sensitivity to VRC01/VRC03 by a remarkable 300–1300 fold (Fig. 2, Table 1), and a less pronounced, but still significant change was also observed in clade B/AE virus in our experiment (Supplemental Fig. 2). The fact that neither single mutant of N197 and N463 exhibits a drastic change in neutralization sensitivity may be a bit confusing/confounding but in considering the structural properties of the N-linked sugars (being rather bulky and unstructured), it seems not surprising that changes neutralization sensitivity do not appear until both N197 and N463 are mutated. Firstly, N197 and N463 are both located on flexible loops within gp120 and so their positioning within gp120 may be quite dynamic. This property has direct implications when considering interactions with CD4bs antibodies. Secondly, and in addition to the structural properties of N197/463, the structural properties of the sugars (mainly mannose 5 or 9 with regards to gp120) linked to glycosylated N residues should be taken into account. Such synergistic effect could be the result of the absence of glycosylation on the PNGS mutant, as suggested by the structural modeling. Alternatively, the PNGS mutation could also affect the Env conformation to increase the sensitivity to some of the nMAbs.

Very interestingly, the remarkable increase of sensitivity of mutants containing N463Q/N197D was only observed for nMAbs VRC01/VRC03, and not for other tested nMAbs (Table 1). This is rather intriguing especially considering b12 belongs to the class of CD4bs nMAbs as VCR01/03. The results further confirm that b12 neutralizes HIV-1 with mechanisms that are not identical to those used by VCR01/03, despite the fact that they all bind to the general area of CD4bs^{15, 17, 18, 39, 43–45}.

The neutralization phenotype of combined mutants using the serum antibodies showed a much less profound impact on sensitivity to the serum antibody than to the nMAbs, with ~20 fold as the highest increase of sensitivity for mutant 10 and 11 (197M.10 and 197M.11). Another interesting observation is that, while the combined mutants containing N197D/N463Q did not show continued increase of neutralizing sensitivity to nMAbs with additional PNGS mutations, these combined mutants displayed a general trend of cumulative

mutational effect on the increase of neutralizing sensitivity to the serum antibodies (Table 2). These combined mutants also showed a broader sensitivity increase to HIV-1 positive serum than the single PNGS mutants (Table 2). However, there appears to be a general trend that the observed cumulative effect to the serum antibodies become stronger when the combined mutants contain at least the N197D and N463Q mutations, which further suggests that the neutralizing antibodies in HIV-1 positive serum targeting the CD4bs and the conserved elements of V3 region may play a dominant role in neutralizing the virus for those patient serum in our assays.

Through analyzing HIV sequences, we found that only ~30% of 4,829 sequences of HIV Env retrieved from LANL HIV Databases have N463 site. ~70% of them have Asn-X-Thr/Ser PNGS motif and others lost it by amino acid substitution at Thr/Ser. Thus, the glycosylation at N463 is present in small part of HIV isolates and they maybe involved in viral evolution to escape from neutralization.

In conclusion, our results indicate that N463 plays an important role in regulating the CD4bs MAbs VRC01/VRC03 sensitivity in the genetic background of N197D mutation of gp120. We also found that combined PNGS mutations that include N197D/N463Q mutations of gp120 made the virus more readily to be neutralized by serum polyclonal antibodies from patients. We have provided a structural basis to rationalize the observed synergistic effects of this combined mutations through molecular modeling. In the future, it remains to be examined regarding the issue of whether the modified gp120 Env bearing such combined mutations can induce a stronger immune response as a more potent vaccine candidate source.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV	human immunodeficiency virus
Env	envelope glycoprotein
PNGS	potential N-linked glycosylation site
CRFs	The circulating recombinant forms
TCID₅₀	the 50% tissue culture infectious dose
nMAb	neutralizing monoclonal antibody
MPER	membrane proximal external region.

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Figure 1. Infectivity of the wt HIV strain and the PNGS mutants (see Supplemental Table 1 for the listed mutants)

Infectivity is shown as relative luminescence units (RLU) in a logarithmic scale. Relative infectivity was calculated by dividing the Log₁₀ (RLU of mutant) by Log₁₀ (RLU of wt). The data represent the means of three independent experiments, and the error bars indicate the standard deviations from the means.

Figure 2. Fold difference of neutralizing sensitivity of PNGS mutants to the three available Anti-CD4bs neutralizing nMAbs

Fold difference was calculated by dividing the mean IC₅₀ (wt) by mean IC₅₀ (mutant), and was expressed in logarithm. The mean IC₅₀ of three independent experiments was shown in Table 1.

Figure 3. Structural basis for effects of N197D/N463Q mutations of HIV FE gp120 on neutralization of by different CD4bs nMAbs

Shown is a FE gp120 structural model aligned to the structures of gp120 binding to CD4, b12, VRC01, or VRC03. (Top panels) The respective gp120 epitopes (red) are mapped with respect to the N197 and N463 glycans (green). (Bottom panels) The CD4, b12, VRC01, and VRC03 paratopes (magenta) for binding FE gp120 are shown with respect to N197 and N463 glycans of FE gp120. Combined mutations of N197D and N463Q did not affect viral infectivity and minimally affect b12-mediated neutralization. In contrast, the N197D and N463Q drastically increased neutralization sensitivity to VRC01 and VRC03.

Table 1

Neutralization of Env glycosylation mutants by nMAbs

Env	Region	nMAbs									
		2F5	4E10	b12	VRC03	VRC01	PG9	PG16	3869		
FE(WT)	-	2.224 (100)	2.986 (100)	0.088 (100)	2.601 (100)	5.500 (100)	1.415 (100)	7.600 (100)	>25 (100)		
N197D	V1/V2	0.396 (562)	0.477 (626)	0.005 (1760)	0.070 (3716)	2.355 (234)	16.123 (9)	>25 (<30)	5.540 (>451)		
N301Q	V3	0.210 (1059)	0.585 (510)	0.040 (220)	1.235 (211)	2.330 (236)	0.875 (162)	14.884 (51)	ND		
N339Q	C3	1.127 (197)	1.579 (189)	0.083 (106)	1.799 (145)	4.899 (112)	1.664 (85)	14.644 (52)	ND		
N442Q	C4	0.781 (285)	0.934 (320)	0.045 (196)	0.791 (329)	4.120 (133)	1.650 (86)	16.111 (47)	ND		
N448Q	C4	0.635 (350)	0.694 (430)	0.057 (154)	2.917 (89)	4.987 (110)	0.878 (161)	13.386 (57)	ND		
N463Q	V5	0.763 (291)	0.895 (334)	0.056 (157)	2.170 (120)	3.450 (159)	2.544 (56)	16.333 (47)	ND		
N466Q	V5	1.339 (166)	1.397 (214)	0.087 (101)	2.139 (122)	3.204 (172)	3.070 (46)	16.378 (46)	ND		
N611Q	gp41	1.836 (121)	0.704 (424)	0.116 (76)	3.103 (84)	5.431 (101)	1.797 (79)	15.830 (48)	ND		
N625Q	gp41	0.400 (556)	0.577 (518)	0.041 (215)	3.329 (78)	5.398 (102)	1.069 (132)	16.450 (46)	ND		
M46	combination	0.435 (511)	0.628 (475)	0.149 (59)	1.734 (150)	3.683 (149)	2.160 (66)	16.560 (46)	ND		
197M.2	combination	0.237 (938)	0.329 (908)	0.008 (1100)	0.031 (8390)	2.685 (205)	12.694 (11)	>25 (<30)	ND		
197M.3	combination	0.363 (613)	0.389 (768)	0.004 (2200)	0.003 (86700)	0.020 (27500)	16.105 (9)	>25 (<30)	ND		
197M.4	combination	0.486 (458)	0.706 (423)	0.004 (2200)	0.003 (86700)	0.018 (30556)	15.755 (9)	>25 (<30)	ND		
197M.5	combination	0.372 (598)	0.585 (510)	0.005 (1760)	0.003 (86700)	0.020 (27500)	16.360 (9)	>25 (<30)	ND		
197M.6	combination	0.284 (783)	0.523 (571)	0.006 (1467)	0.043 (6049)	2.043 (269)	16.230 (9)	>25 (<30)	ND		
197M.7	combination	0.253 (879)	0.399 (748)	0.005 (1760)	0.023 (11309)	1.564 (352)	16.378 (9)	>25 (<30)	ND		
197M.8	combination	0.284 (783)	0.463 (645)	0.004 (2200)	0.003 (86700)	0.017 (32353)	15.611 (9)	>25 (<30)	ND		
197M.9	combination	0.457 (487)	0.700 (427)	0.005 (1760)	0.003 (86700)	0.014 (39286)	16.580 (9)	>25 (<30)	ND		
197M.10	combination	0.464 (479)	0.886 (337)	0.004 (2200)	0.002 (130050)	0.017 (32353)	16.177 (9)	>25 (<30)	ND		
197M.11	combination	0.435 (511)	0.536 (557)	0.003 (2933)	0.002 (130050)	0.013 (42308)	16.325 (9)	>25 (<30)	ND		

Both IC₅₀(μg/mL) and Percentage of neutralization sensitivity relative to WT (%) were showed in each box.Percentage of neutralization sensitivity relative to WT (%) = IC₅₀ WT / IC₅₀ mutant.

ND: not detected

Table 2

Neutralization of Env glycosylation mutants by serum antibodies.

Env	region	Serum/ID ₅₀																			
		xj16	bj20	bj22	xj50	sc59r	gd64	gx66	gx75	gx76	yn148r	gx77	gx78	gx79	gx82	gx85	gx87	gx93	gx94	gx95	yn99r
FE(WT)	-	1494	75	239	800	302	205	156	90	246	1309	402	188	624	528	150	62	232	543	201	125
N87D	C1	1893	54	251	930	253	200	206	119	197	1152	559	354	555	319	122	62	152	514	202	137
N133Q	V1	2734	82	234	1130	531	216	191	90	217	1284	452	188	734	485	84	62	186	451	191	83
N142Q	V1	1961	109	445	1287	372	280	279	225	406	1989	730	222	1557	886	177	76	243	841	318	207
N197D	C2	1226	275	284	316	396	164	214	273	377	1498	540	249	1262	1214	223	85	145	1654	410	291
N289D	C2	1508	99	103	824	290	213	181	39	283	1244	492	183	832	561	122	62	140	527	222	131
N301Q	V3	3215	235	375	950	327	307	232	131	229	1510	1187	162	799	694	171	137	1182	1681	601	270
N339Q	C3	1611	98	250	976	263	241	207	152	333	1607	641	244	809	549	161	73	256	704	236	150
N355Q	C3	2253	59	208	1584	175	183	112	102	186	707	670	197	1005	986	115	58	248	1059	185	84
N392Q	V4	2058	67	200	1387	285	209	180	107	235	1344	461	174	628	414	126	70	294	636	216	111
N408Q	V4	1478	80	268	1129	261	225	183	157	309	1266	502	216	690	572	169	72	252	611	240	157
N411Q	V4	2051	116	317	1181	420	308	195	182	324	1096	415	217	613	487	124	63	220	600	203	109
N442Q	C4	2636	292	488	1228	563	366	278	481	303	1329	685	323	1240	1135	253	77	395	1017	414	174
N448Q	C4	1075	82	224	1092	151	204	180	69	248	1507	396	168	590	493	83	59	206	1164	221	127
N463Q	V5	1868	119	338	1492	347	249	208	130	325	1376	634	254	2250	1146	129	75	270	1836	192	196
N466Q	V5	1811	72	224	1222	382	197	154	116	337	1587	517	259	1121	741	187	77	211	790	261	144
N611Q	gp41	2322	68	223	1350	206	195	766	172	750	1108	368	200	1089	762	99	44	173	898	134	63
N625Q	gp41	2293	199	396	1506	413	242	242	181	260	1544	904	376	1034	2136	286	87	350	790	409	227
N637Q	gp41	1979	82	374	1402	267	180	160	117	249	6714	539	210	719	532	133	63	231	1090	219	137
M46	combination	2537	110	240	1008	386	215	128	190	320	1238	487	380	685	1580	165	75	225	1128	586	152
197M.2	combination	1904	563	311	544	403	320	394	237	375	1401	574	438	1404	2480	287	106	288	1850	594	292
197M.3	Combination	1860	483	365	525	413	332	251	865	423	2533	612	305	8210	8005	290	110	235	8350	581	525
197M.4	combination	2018	350	320	480	475	230	305	580	420	1580	690	428	1350	1860	235	80	230	1523	625	538
197M.5	combination	2033	375	335	456	660	256	330	883	415	1695	699	465	8335	6853	440	83	260	7895	674	550
197M.6	combination	2943	625	333	557	814	344	386	987	470	1671	1053	418	1437	2386	430	101	184	1592	613	425
197M.7	combination	2493	727	325	563	866	325	363	1996	654	2236	1904	553	2403	2987	447	93	212	3043	652	404
197M.8	combination	1223	567	291	476	345	341	440	446	471	3075	983	510	8737	7083	301	89	1329	12346	579	666

		Serum/ID ₅₀																			
Env	region	xj16	bj20	bj22	xj50	sc59r	gd64	gx66	gx75	gx76	yn148r	gx77	gx78	gx79	gx82	gx85	gx87	gx93	gx94	gx95	yn99r
197M.9	combination	2774	620	305	486	789	390	400	1143	438	2702	944	636	9166	7481	442	392	1252	9477	643	755
197M.10	combination	2062	617	241	413	1072	367	382	1071	695	4135	1493	434	16294	7786	353	109	3313	11378	582	658
197M.11	combination	2557	640	303	402	1038	358	3281	1085	3018	4742	1560	403	21096	7546	454	79	3452	11078	621	660

Boxes are color coded as follows: yellow, 2–10 fold increase of ID₅₀; orange, > 10-fold increase of ID₅₀; gray, > 2-fold decrease of ID₅₀.