Published in final edited form as:

ACS Chem Biol. 2017 June 16; 12(6): 1566–1575. doi:10.1021/acscchembio.7b00319.

Systematic Synthesis and Binding Study of HIV V3 Glycopeptides Reveal the Fine Epitopes of Several Broadly Neutralizing Antibodies

Jared Orwenyo[#], Hui Cai[#], John Giddens, Mohammed N Amin, Christian Toonstra, and Lai-Xi Wang^{*}

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland, USA

Abstract

A class of new glycan-reactive broadly neutralizing antibodies represented by PGT121, 10-1074 and PGT128 has recently been discovered that target specific N-glycans and peptide region around the V3 domain. However, the glycan specificity and fine epitopes of these bNAbs remain to be further defined. We report here a systematic chemoenzymatic synthesis of homogeneous V3 glycopeptides derived from HIV-1 JR-FL strain carrying defined N-glycans at N332, N301 and N295 sites. Antibody binding studies revealed that both the nature and site of glycosylation in the context of the V3 domain were critical for high-affinity binding. It was found that antibody PGT128 exhibited specificity for high-mannose N-glycan with glycosylation site promiscuity; PGT121 showed binding specificity for glycopeptide carrying a sialylated N-glycan at N301 site; and 10-1074 was specific for glycopeptide carrying a high-mannose N-glycan at N332 site. The synthesis and binding studies permit a detailed assessment of the glycan specificity and the requirement of peptide in the context of antibody-antigen recognition. The identified glycopeptides can be used as potential templates for HIV vaccine design.

Graphical abstract

Keywords

Glycopeptide; Glycosylation; N-Glycan; Chemoenzymatic synthesis; Neutralizing Epitope; Broadly Neutralizing Antibody; HIV Vaccine

^{*}To whom correspondence should be addressed: Wang518@umd.edu.

[#]These authors contributed equally to this work.

Associated Content: The Supporting Information is available free of charge on the ACS Publications website. Detailed experimental procedures; HPLC and MS profiles.

Introduction

The heavy glycosylation of the HIV-1 envelope glycoprotein gp120 constitutes a strong defense mechanism for viral evasion of host immune surveillance because of the generally weak immunogenicity of the viral N-glycans.¹⁻³ Nevertheless, the recent discovery of a new class of glycan-reactive broadly neutralizing antibodies (bNAbs) that recognize specific N-glycans and peptide regions around the variable (V1V2 and V3) domains suggests that the defensive glycan shield can be targets of bNAbs.⁴⁻¹⁵ Among these bNAbs, PGT128, 10-1074 and PGT121 are of outstanding viral neutralization breadth and potency. Antibody PGT128 neutralizes over 70% of globally circulating viruses.¹⁰ Antibody PGT121 are able to protect against high-dose vaginal SHIV challenge in passive immunization in macaques,¹⁶ and to suppress SHIV replication in chronically infected macaques.¹⁷ Similarly, 10-1074 has been shown to suppress viral load in combination with other bNAbs in mouse models and macaques.¹⁸⁻²⁰ Thus, characterization of the neutralizing epitopes of these bNAbs constitutes a critical step in HIV vaccine design aiming to elicit similar broadly neutralizing antibodies.

Structural and mutational studies have indicated that these HIV-neutralizing antibodies share common features in antigen recognition, i.e., targeting specific N-glycans around the V3 domain and a conserved peptide region at the base of the V3 loop of gp120.¹⁵ For example, X-ray crystallographic studies on antibodies PGT127 and PGT128 Fabs and their complexes with a recombinant gp120 outer domain have shown that PGT127 and PGT128 recognize two high-mannose N-glycans located at N332 and N301 sites and a peptide portion of the V3 domain.¹⁰ Structural studies and glycan microarray analysis have suggested that antibody 10-1074 is high-mannose dependent, while PGT121 recognizes complex-type glycans.⁹ Very recently, Danishefsky and co-workers have demonstrated that a synthetic V3 glycopeptide carrying a high-mannose glycan at N332 site are able to raise V3 glycan-targeted antibodies in rhesus macaques, suggesting that the glycosylated V3 domain can be a potential template for HIV-1 vaccine design.²¹ Despite these remarkable progresses, the precise neutralizing epitopes in terms of the fine structures of the glycans and the peptide context remain to be further characterized. The heterogeneous nature of gp120 glycosylation poses a significant challenge in epitope characterization as current recombinant technology is unable to selectively control or alter glycan structures at different sites for a multiply glycosylated protein during expression. To address the glycosylation heterogeneity of gp120 in epitope characterization, we have launched a project aiming at defining and reconstituting the minimal neutralizing epitopes through a systematic synthesis of homogeneous V3 glycopeptides carrying defined N-glycans at specific sites followed by antibody binding screening. Previously, several research groups including ours have applied synthetic chemistry and antibody binding analysis for characterizing the neutralizing epitopes of some glycan-reactive anti-HIV antibodies, including antibody 2G12 that recognizes a novel cluster of high-mannose N-glycans on gp120 and antibody PG9 that targets a glycopeptide epitope on the V1V2 domain.²²⁻²⁶ We describe in this paper the chemoenzymatic synthesis of a series of homogeneous V3 glycopeptides derived from HIV-1 JR-FL and the use of the glycopeptide library to approach the minimal neutralizing epitopes through binding studies with bNAbs PGT128, PGT121 and 10-1074. Our experimental data revealed that PGT128

was specific for a high-mannose glycan in the context of the V3 domain but was promiscuous for the site of glycosylation; PGT121 recognized a complex type N-glycan at the N301 site of V3 domain, but sialylation of the N-glycan was critical for a high-affinity binding; and the 10-1074 was highly specific for a high-mannose N-glycan located at the N332 glycosylation site. In addition, we found that the presence of a neighboring N-glycan at N301 had an adverse effect on the binding of 10-1074 to the high-mannose N-glycan at N332. Our studies suggest that the nature of the N-glycan, the site of glycosylation, and the V3 domain peptide context all are important for high-affinity binding by these broadly neutralizing antibodies.

Results and Discussion

Research Design

The gp120 V3 domain consists of 35-40 amino acids including three conserved N-glycosylation sites (N295, N301 and N332/N334), with a disulfide bond between C296 and C331 to form a loop structure. In this study, we selected mini-V3 glycopeptide domains as mimics of neutralizing epitopes, in which the highly variable and immune predominant tip was deleted to avoid elicitation of predominant strain-specific neutralizing antibodies and to focus the immune response to the conserved elements.²⁷ We selected an extended V3 domain sequence (aa 292-339, HIV-1 HXB2 numbering) from HIV-1 JR-FL strain as the synthetic targets, since the corresponding envelope glycoprotein gp120 of the strains was previously selected in structural studies.¹⁰ All the three conserved N-glycan sites (N295, N301 and N332) were considered to systematically address the binding specificity.

Synthesis of large, biologically relevant glycopeptides carrying complex natural N-glycans is still a challenging task.²⁸⁻³³ Here we chose to synthesize the target V3 glycopeptides by applying a chemoenzymatic method, which we have recently developed for N-glycoprotein synthesis.^{30,34} An N-acetylglucosamine (GlcNAc) moiety is installed at a pre-determined glycosylation site during automated solid-phase peptide synthesis (SPPS), and then a synthetic glycan, in the form of activated glycan oxazoline, is transferred to the GlcNAc moiety by a glycosynthase (an endoglycosidase mutant that is devoid of product hydrolysis activity) to form natural glycosidic linkages.³⁵⁻⁴⁶ Retrosynthetic analysis of the gp120 V3 glycopeptides leads to two kinds of building blocks: GlcNAc-peptides and glycan oxazolines (Figure 1). Finally, antibody binding screening of the glycopeptide library would assess the structural requirement in terms of the glycan structure, glycosylation site and peptide context in antigen recognition.

Chemoenzymatic Synthesis of the HIV-1 JR-FL mini-V3 glycopeptides carrying distinct N-glycans at the N332, N301, or N295 sites

We selected the sequence of the JR-FL mini-V3 domain corresponding to the residues 292-304 and 321-339 with a replacement of the 305-320 tip residues by a PG (Pro-Gly) dipeptide insert as the basic glycopeptide sequence. This mini-V3 peptide sequence with the same dipeptide insert at the tip was previously engineered in the JR-FL gp120 outer domain for the crystal structural study of antibody PGT128.¹⁰ Deletion of the highly variable and immune predominant tip sequence is expected to avoid elicitation of predominant strain-

specific neutralizing antibodies and to focus the immune response to the conserved epitope if such glycopeptide would be used as a component for vaccine in future immunization studies. We extended the sequence at the two ends (from V292 to N339) to include the natural disulfide bond (between C296 and C331) and additional potential peptide epitopes. To introduce a defined N-glycan at the N332 site, the precursor cyclic polypeptide **1** was synthesized by automated solid-phase peptide synthesis (SPPS), in which a GlcNAc moiety was installed at the pre-determined N332 site using the protected glycosylamino acid Fmoc-Asn(Ac₃GlcNAc)-OH as the building block. A biotin tag was introduced at the N-terminus to facilitate site-specific immobilization on streptavidin sensor chips for SPR binding analysis. A Man₉GlcNAc glycan was then transferred from the corresponding glycan oxazoline **2** by the glycosynthase EndoA-N171A³⁹ to give the glycopeptide **3** in 89% yield after HPLC purification. Similarly, the complex type glycopeptide **5** was obtained by transferring a complex type N-glycan from the sialoglycan oxazoline **4**, prepared from to the N332 site using EndoM-N175Q³⁸ as the catalyst (Scheme 1). The Man₉GlcNAc glycan (**2**) was prepared from Man₉GlcNAc₂Asn following the previously reported procedure.³⁷ The complex type N-glycan oxazoline (**4**) was synthesized from the sialoglycopeptide (SGP) isolated from egg yolk, following the previously reported procedure.⁴⁷

To introduce N-glycan at the N301 site, the precursor cyclic GlcNAc-peptide **6**, in which the GlcNAc moiety was installed at the N301 site, was first synthesized by SPPS, then a high-mannose type or a sialylated complex type glycan was transferred from the corresponding glycan oxazoline (**2** and **4**) by a glycosynthase to provide the corresponding glycopeptides **7** and **8** in excellent yields (Scheme 2a). An asialylated glycoform was also synthesized using the glycan oxazoline **9** as donor substrate for transglycosylation to obtain glycopeptide **10**. Using a similar synthetic strategy, glycopeptides **12** and **13** bearing a high-mannose or a complex type N-glycan at N295 site were constructed in excellent yields (Scheme 2b). The purity and identity of the synthetic V3 glycopeptides were confirmed by HPLC and ESI-MS analysis (Figures S1-S10, supporting information).

Synthesis of the JR-FL mini-V3 glycopeptides carrying N-glycans at both the N301 and N332 sites

For the synthesis of the JR-FL glycopeptides carrying two N-glycans, the precursor GlcNAc-polypeptide **14** carrying two GlcNAc moieties (at the N301 and N332 site, respectively) was synthesized by SPPS. Enzymatic transglycosylation of **14** with an excess amount of Man₉GlcNAc-oxazoline **2** under the catalysis of EndoA-N171A gave the doubly glycosylated peptide **15** in 75% yield. On the other hand, the synthesis of glycopeptide **16** carrying two complex type N-glycans was achieved by transglycosylation of **14** with the sialoglycan oxazoline **4** under the catalysis of EndoM-N175Q, giving glycopeptide **16** in 81% yield (Scheme 3a). The purity and identity of the synthetic glycopeptides were confirmed by HPLC and ESI-MS analysis (Figures S11-13).

To introduce two different N-glycans at N301 and N332, the precursor GlcNAc-peptide **17** carrying a free GlcNAc moiety at the N332 site and a protected Ac₃GlcNAc moiety at the N301 site was synthesized by SPPS, following our recently reported synthetic strategy that employs two orthogonally protected GlcNAc-Asn building blocks.⁴⁶ The high-mannose

glycan was first introduced to the N332 site using Man₉GlcNAc-oxazoline **2** under the catalysis of EndoA-N171A. After removal of the O-acetyl protecting groups using 5% hydrazine, glycopeptide **18** carrying high-mannose glycan at N332 and a GlcNAc moiety at the N301 site was obtained in 89% yield for two steps. Then a sialylated complex-type glycan or an asialylated complex type glycan was transferred from the corresponding glycan oxazoline (**4** and **9**) to provide the glycopeptides (**19** and **20**) in 84% and 80% yields, respectively (Scheme 3b). The purity and identity of the synthetic glycopeptides were confirmed by HPLC and ESI-MS analysis (Figure S14-S17).

Synthesis of biotinylated high-mannose and complex N-glycans

The corresponding biotinylated N-glycans were synthesized by reaction of the asparagine-linked N-glycans with an activated ester of biotin derivative to give the corresponding biotinylated N-glycans (Scheme 4). The high-mannose N-glycan Man₉GlcNAc₂-Asn (**21**) was prepared by pronase digestion of crude soybean agglutinin obtained from soybean flour, following the previously reported procedure.⁴⁸ The sialylated complex type N-glycan sialoglycan-Asn (**24**) was prepared from the sialoglycopeptide (SGP) isolated from egg yolk, following the previously reported procedure.⁴⁷ The resulting biotinylated Man₉GlcNAc₂-Asn-biotin (**23**) and sialoglycan-Asn-biotin (**25**) were purified by HPLC and their identities were confirmed by ESI-MS analysis (Figures S18-S19).

Binding analysis with PGT128

To evaluate the binding of PGT128, PGT121 and 10-1074 to the synthetic biotinylated V3 glycopeptides, neutravidin was immobilized on CM5 chips and then glycopeptides were captured on the chips. PGT128, PGT121- and 10-1074 IgGs were run as analytes individually on the chips for binding evaluation. While the potential involvement of avidity effect in the antibody-antigen interactions would make it difficult to obtain accurate kinetic parameters, the SPR analysis provided a quick assessment of the antibody-antigen recognition. For PGT128, no binding was observed to the GlcNAc-peptides or those glycopeptides carrying only complex type N-glycans at the N332, N301 and N295 sites (Figure 2A). These results confirm that PGT128 does not bind to complex type N-glycans or non-glycosylated V3 polypeptides. On the other hand, PGT128 showed apparent binding to all those synthetic glycopeptides carrying a Man₉GlcNAc₂ glycan attached at N332, N301 and/or N295 sites (glycopeptide **3**, **7**, **12**). In comparison, the V3 glycopeptides carrying a Man₉GlcNAc₂ glycan at the N332 or N301 site (**3** and **7**, respectively) showed about the same affinity for PGT128 ($K_D = 4 \mu\text{M}$), which were slightly better than that of the glycopeptide (**12**) having the high-mannose N-glycan at the N295 site ($K_D = 4 \mu\text{M}$). These results might partially explain the broad spectrum of neutralization of majority of HIV-1 strains by PGT128. Another interesting observation was that attachment of a second high-mannose N-glycan at the N301 in addition to N332 (glycopeptide **15**) slightly enhanced the apparent affinity of the glycopeptide antigen to PGT128. However, the presence of a secondary complex type N-glycan at the N301 site was found to adversely affect the antibody's recognition of the N332 glycan in the V3 glycopeptide, as reflected by the reduced affinity of PGT128 for glycopeptides **19** and **20**. Under the SPR assay conditions, the antibody could also recognize free high-mannose N-glycan **23** ($K_D = 96 \mu\text{M}$).

Nevertheless, the apparent affinity was estimated to be more than 20-fold lower than the glycopeptides carrying a high-mannose N-glycan at N332 or N301 site such as glycopeptides **3** and **7** ($K_D = 4 \mu\text{M}$). These results indicate that although V3 peptide alone does not bind to PGT128, the V3 polypeptide could synergize with the high-mannose glycan to achieve substantially enhanced affinity for PGT128. We also performed the binding analysis using the Fab fragments of PGT128, which precluded the potential avidity that would complicate the SPR analysis when whole IgG antibody was used. The binding of PGT128 Fab showed the same tendency of binding as the whole IgG of PGT128 (Figure S20). The binding of PGT128 IgGs to high-mannose glycopeptides was further confirmed by ELISA analysis. For the glycopeptides carrying a single N-glycan (Figure. 2B), PGT128 showed apparent binding to the high-mannose glycopeptides (**3**, **7** and **12**), while no binding was observed to the complex type glycopeptides (**5**, **8**, **10** and **13**). The ELISA binding of PGT128 to V3 glycopeptides carrying two N-glycans was consistent with the SPR binding (Figure 2C). Compared to the glycopeptide **3** carrying single high-mannose glycan at N332, the binding was slightly enhanced by attaching an additional high-mannose glycan at N301 (glycopeptide **15**) and significantly reduced by attaching a complex type glycan (glycopeptide **19** and **20**).

It was previously reported that in the case of the JR-CSF strain, individual alanine mutations at N332 and N301 site had little to no effect on neutralization by PGT128, but a combined double mutation at N301 and N332 resulted in complete abolishment of the neutralizing activity by PGT128.¹⁰ Our binding results are consistent with the mutational studies. Taken together, these results suggest that a presence of a critical high-mannose N-glycan (likely a $\text{Man}_{8-9}\text{GlcNAc}_2$) at either the N332 or the N301/N295 site is required and sufficient for recognition by PGT128. This promiscuous recognition mode partially explains the breadth of neutralization of HIV-1 strains by PGT128.

Binding analysis with PGT121

PGT121 is a bNAb that was previously shown to be specific for complex type N-glycans in the context of gp120 envelope glycoprotein.⁹ Our SPR binding analysis revealed that PGT121 showed a moderate affinity for those V3 glycopeptides carrying a complex type N-glycan at the N301 site (glycopeptides **8**, **16**, and **19**, K_D ca. $200 \mu\text{M}$), but it did not bind to the high-mannose type glycopeptides (**3**, **7** and **12**) or free complex type N-glycan **25** under the assay conditions (Figure 3A). The site specificity of glycosylation at N301 for PGT121 recognition was apparent. When the complex type N-glycan was shifted from N301 site to the N332, or N295 sites (glycopeptides **5**, **13**), the binding was completely abolished below the detection limit. Interestingly, PGT121 recognition of the N301 complex type N-glycan appeared to be sialylation dependent, as the corresponding glycopeptide **10** carrying an asialylated complex type N-glycan at the N301 site showed no detectable affinity under the SPR assay condition. Attaching an additional high-mannose or complex type glycan at N332 site has no influence on the binding affinity. The preference of PGT121 to sialylated N-glycans was previously implicated in a glycan array assessment.⁹ These results are consistent with the observed specificity of PGT121's recognition found in the ELISA binding analysis (Figure 3B and 3C). Although it was originally classified as an N332 dependent antibody,⁷ PGT121 could tolerate the loss of the N332 glycan. For isolate

92BR020, which has a glycan site at N332, PGT121 neutralization was greatly reduced by combined deletion of the N136 and N332 glycans or the double mutation at the N301 and N332 sites, whereas no large effects were observed for single substitution.¹² Taken together with the mutational studies, our results confirm that a complex type N-glycan at the N301 site or at a spaciouly proximal site (such as N136 at the V1V2 domain) would be critical for PGT121 recognition. Under our SPR binding conditions, no binding of PGT121 to peptide-free complex type glycan (**25**) was detected, probably due to the relatively low affinity and the detection limits. Moreover, our binding data clearly show that a sialylated complex type N-glycan around N301 has much higher affinity than the asialylated V3 glycopeptide.

Binding analysis with 10-1074

The binding of antibody 10-1074 with the synthetic V3 glycopeptides was assessed in a similar way. It was observed that antibody 10-1074 showed strong binding to glycopeptides **3** and **15** carrying a high-mannose glycan (Man₉GlcNAc₂) at the N332 site ($K_D = 0.4 \mu\text{M}$). However, shifting the high-mannose N-glycan from N332 to the N301 or N295 site completely abolished the affinity, as reflected by the non-detectable binding to glycopeptides **7** and **12** (Figure 4A). In addition, 10-1074 did not recognize complex type glycopeptides wherever the complex type N-glycan was placed, and it did not bind to peptide-free high-mannose N-glycan (**23**) under the SPR assay conditions. The binding specificity to the N332 high-mannose glycan was also confirmed by ELISA analysis (Figure 4B and 4C). These results are consistent with previous X-ray structural and glycan microarray analysis showing that 10-1074 exhibited remarkable binding to high-mannose glycan in the context of gp120, but no detectable binding to protein free N-glycans.⁹ Interestingly, the presence of a secondary complex type N-glycan at the N301 site abolished the antibody's recognition of the N332 glycan in the V3 glycopeptide, as no binding was observed for glycopeptides **19** and **20** in both SPR and ELISA binding, but an additional high-mannose N-glycan at the N301 only slightly reduced the antibody's recognition of the N332 glycan. These results indicate the high site-specificity of antibody 10-1074 for recognizing the glycan antigen.

Conclusion

Characterization of the fine epitopes of broadly neutralizing antibodies is an important step for HIV vaccine design. The chemoenzymatic method described here permits an efficient construction of a library of homogeneous gp120 V3 glycopeptides carrying well-defined N-glycans at specific conserved N-glycosylation sites. The binding studies with these V3 glycopeptides enable a detailed assessment of the glycan specificity and the requirement of the polypeptide context for antigen recognition by PGT128, PGT121 and 10-1074. This approach should be equally applicable for defining the glycan-dependent epitopes of other bNAbs. The minimal glycopeptide epitopes identified here, including the V3 glycopeptide carrying a sialylated complex type N-glycan at N301 as an epitope of PGT121 and the V3 glycopeptide carrying a high-mannose N-glycan at N332 as an epitope of 10-1074 and PGT128, should be valuable as important components for HIV vaccine design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Wang lab for technical assistance and discussions. We also thank P. Bjorkman and her lab members for kindly providing PGT128, 10-1074 and corresponding Fab proteins. This work was supported by the National Institutes of Health (NIH grants R01AI113896).

References

1. Stewart-Jones GB, Soto C, Lemmin T, Chuang GY, Druz A, Kong R, Thomas PV, Wagh K, Zhou T, Behrens AJ, Bylund T, Choi CW, Davison JR, Georgiev IS, Joyce MG, Kwon YD, Pancera M, Taft J, Yang Y, Zhang B, Shivatare SS, Shivatare VS, Lee CC, Wu CY, Bewley CA, Burton DR, Koff WC, Connors M, Crispin M, Baxa U, Korber BT, Wong CH, Mascola JR, Kwong PD. Trimeric HIV-1 Env structures define glycan shields from Clades A, B, and G. *Cell*. 2016; 165:813–826. [PubMed: 27114034]
2. Reitter JN, Means RE, Desrosiers RC. A role for carbohydrates in immune evasion in AIDS. *Nat Med*. 1998; 4:679–684. [PubMed: 9623976]
3. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. Antibody neutralization and escape by HIV-1. *Nature*. 2003; 422:307–312. [PubMed: 12646921]
4. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Kaminsky S, Zamb T, Moyle M, Koff WC, Poignard P, Burton DR. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science*. 2009; 326:285–289. [PubMed: 19729618]
5. Doores KJ, Burton DR. Variable loop glycan dependency of the broad and potent HIV-1-neutralizing antibodies PG9 and PG16. *J Virol*. 2010; 84:10510–10521. [PubMed: 20686044]
6. Bonsignori M, Hwang KK, Chen X, Tsao CY, Morris L, Gray E, Marshall DJ, Crump JA, Kapiga SH, Sam NE, Sinangil F, Pancera M, Yongping Y, Zhang B, Zhu J, Kwong PD, O'Dell S, Mascola JR, Wu L, Nabel GJ, Phogat S, Seaman MS, Whitesides JF, Moody MA, Kelsoe G, Yang X, Sodroski J, Shaw GM, Montefiori DC, Kepler TB, Tomaras GD, Alam SM, Liao HX, Haynes BF. Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors. *J Virol*. 2011; 85:9998–10009. [PubMed: 21795340]
7. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, Wang SK, Ramos A, Chan-Hui PY, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong CH, Phogat S, Wrin T, Simek MD, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature*. 2011; 477:466–470. [PubMed: 21849977]
8. Kong L, Lee JH, Doores KJ, Murin CD, Julien JP, McBride R, Liu Y, Marozsan A, Cupo A, Klasse PJ, Hoffenberg S, Caulfield M, King CR, Hua Y, Le KM, Khayat R, Deller MC, Clayton T, Tien H, Feizi T, Sanders RW, Paulson JC, Moore JP, Stanfield RL, Burton DR, Ward AB, Wilson IA. Supersite of immune vulnerability on the glycosylated face of HIV-1 envelope glycoprotein gp120. *Nat Struct Mol Biol*. 2013; 20:796–803. [PubMed: 23708606]
9. Mouquet H, Scharf L, Euler Z, Liu Y, Eden C, Scheid JF, Halper-Stromberg A, Gnanapragasam PN, Spencer DI, Seaman MS, Schuitemaker H, Feizi T, Nussenzweig MC, Bjorkman PJ. Complex-type N-glycan recognition by potent broadly neutralizing HIV antibodies. *Proc Natl Acad Sci USA*. 2012; 109:E3268–3277. [PubMed: 23115339]
10. Pejchal R, Doores KJ, Walker LM, Khayat R, Huang PS, Wang SK, Stanfield RL, Julien JP, Ramos A, Crispin M, Depetris R, Katpally U, Marozsan A, Cupo A, Malveste S, Liu Y, McBride R, Ito Y, Sanders RW, Ogohara C, Paulson JC, Feizi T, Scanlan CN, Wong CH, Moore JP, Olson WC, Ward AB, Poignard P, Schief WR, Burton DR, Wilson IA. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. *Science*. 2011; 334:1097–1103. [PubMed: 21998254]
11. Julien JP, Sok D, Khayat R, Lee JH, Doores KJ, Walker LM, Ramos A, Diwanji DC, Pejchal R, Cupo A, Katpally U, Depetris RS, Stanfield RL, McBride R, Marozsan AJ, Paulson JC, Sanders

- RW, Moore JP, Burton DR, Poignard P, Ward AB, Wilson IA. Broadly neutralizing antibody PGT121 allosterically modulates CD4 binding via recognition of the HIV-1 gp120 V3 base and multiple surrounding glycans. *PLoS Pathog.* 2013; 9:e1003342. [PubMed: 23658524]
12. Sok D, Doores KJ, Briney B, Le KM, Saye-Francisco KL, Ramos A, Kulp DW, Julien JP, Menis S, Wickramasinghe L, Seaman MS, Schief WR, Wilson IA, Poignard P, Burton DR. Broadly neutralizing antibody PGT121 allosterically modulates CD4 binding via recognition of the HIV-1 gp120 V3 base and multiple surrounding glycans. *Sci Transl Med.* 2014; 6:236ra63.
 13. McLellan JS, Pancera M, Carrico C, Gorman J, Julien JP, Khayat R, Louder R, Pejchal R, Sastry M, Dai K, O'Dell S, Patel N, Shahzad-ul-Hussan S, Yang Y, Zhang B, Zhou T, Zhu J, Boyington JC, Chuang GY, Diwanji D, Georgiev I, Kwon YD, Lee D, Louder MK, Moquin S, Schmidt SD, Yang ZY, Bonsignori M, Crump JA, Kapiga SH, Sam NE, Haynes BF, Burton DR, Koff WC, Walker LM, Phogat S, Wyatt R, Orwenyo J, Wang LX, Arthos J, Bewley CA, Mascola JR, Nabel GJ, Schief WR, Ward AB, Wilson IA, Kwong PD. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. *Nature.* 2011; 480:336–343. [PubMed: 22113616]
 14. Pancera M, Shahzad-Ul-Hussan S, Doria-Rose NA, McLellan JS, Bailer RT, Dai K, Loesgen S, Louder MK, Staupé RP, Yang Y, Zhang B, Parks R, Eudailey J, Lloyd KE, Blinn J, Alam SM, Haynes BF, Amin MN, Wang LX, Burton DR, Koff WC, Nabel GJ, Mascola JR, Bewley CA, Kwong PD. Structural basis for diverse N-glycan recognition by HIV-1-neutralizing V1-V2-directed antibody PG16. *Nat Struct Mol Biol.* 2013; 20:804–813. [PubMed: 23708607]
 15. Garces F, Sok D, Kong L, McBride R, Kim HJ, Saye-Francisco KF, Julien JP, Hua Y, Cupo A, Moore JP, Paulson JC, Ward AB, Burton DR, Wilson IA. Structural evolution of glycan recognition by a family of potent HIV antibodies. *Cell.* 2014; 159:69–79. [PubMed: 25259921]
 16. Moldt B, Rakasz EG, Schultz N, Chan-Hui PY, Swiderek K, Weisgrau KL, Piaskowski SM, Bergman Z, Watkins DI, Poignard P, Burton DR. Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proc Natl Acad Sci USA.* 2012; 109:18921–18925. [PubMed: 23100539]
 17. Barouch DH, Whitney JB, Moldt B, Klein F, Oliveira TY, Liu J, Stephenson KE, Chang HW, Shekhar K, Gupta S, Nkolola JP, Seaman MS, Smith KM, Borducchi EN, Cabral C, Smith JY, Blackmore S, Sanisetty S, Perry JR, Beck M, Lewis MG, Rinaldi W, Chakraborty AK, Poignard P, Nussenzweig MC, Burton DR. Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys. *Nature.* 2013; 503:224–228. [PubMed: 24172905]
 18. Klein F, Halper-Stromberg A, Horwitz JA, Gruell H, Scheid JF, Bournazos S, Mouquet H, Spatz LA, Diskin R, Abadir A, Zang T, Dorner M, Billerbeck E, Labitt RN, Gaebler C, Marcovecchio PM, Incesu RB, Eisenreich TR, Bieniasz PD, Seaman MS, Bjorkman PJ, Ravetch JV, Ploss A, Nussenzweig MC. HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. *Nature.* 2012; 492:118–122. [PubMed: 23103874]
 19. Shingai M, Nishimura Y, Klein F, Mouquet H, Donau OK, Plishka R, Buckler-White A, Seaman M, Piatak M Jr, Lifson JD, Dimitrov DS, Nussenzweig MC, Martin MA. Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. *Nature.* 2013; 503:277–280. [PubMed: 24172896]
 20. Gautam R, Nishimura Y, Pegu A, Nason MC, Klein F, Gazumyan A, Golijanin J, Buckler-White A, Sadjadpour R, Wang K, Mankoff Z, Schmidt SD, Lifson JD, Mascola JR, Nussenzweig MC, Martin MA. A single injection of anti-HIV-1 antibodies protects against repeated SHIV challenges. *Nature.* 2016; 533:105–109. [PubMed: 27120156]
 21. Alam SM, Aussedat B, Vohra Y, Ryan Meyerhoff R, Cale EM, Walkowicz WE, Radakovich NA, Anasti K, Armand L, Parks R, Sutherland L, Searce R, Joyce MG, Pancera M, Druz A, Georgiev IS, Von Holle T, Eaton A, Fox C, Reed SG, Louder M, Bailer RT, Morris L, Abdool-Karim SS, Cohen M, Liao HX, Montefiori DC, Park PK, Fernandez-Tejada A, Wiehe K, Santra S, Kepler TB, Saunders KO, Sodroski J, Kwong PD, Mascola JR, Bonsignori M, Moody MA, Danishefsky S, Haynes BF. Mimicry of an HIV broadly neutralizing antibody epitope with a synthetic glycopeptide. *Sci Transl Med.* 2017; 9:eaai7521. [PubMed: 28298421]
 22. Shivatare SS, Chang SH, Tsai TI, Tseng SY, Shivatare VS, Lin YS, Cheng YY, Ren CT, Lee CC, Pawar S, Tsai CS, Shih HW, Zeng YF, Liang CH, Kwong PD, Burton DR, Wu CY, Wong CH.

- Modular synthesis of N-glycans and arrays for the hetero-ligand binding analysis of HIV antibodies. *Nat Chem.* 2016; 8:338–346. 2016. [PubMed: 27001729]
23. Horiya S, MacPherson IS, Krauss IJ. Recent strategies targeting HIV glycans in vaccine design. *Nat Chem Biol.* 2014; 10:990–999. [PubMed: 25393493]
24. Wang LX. Synthetic carbohydrate antigens for HIV vaccine design. *Curr Opin Chem Biol.* 2013; 17:997–1005. [PubMed: 24466581]
25. Amin MN, McLellan JS, Huang W, Orwenyo J, Burton DR, Koff WC, Kwong PD, Wang LX. Synthetic glycopeptides reveal the glycan specificity of HIV-neutralizing antibodies. *Nat Chem Biol.* 2013; 9:521–526. [PubMed: 23831758]
26. Alam SM, Dennison SM, Aussedat B, Vohra Y, Park PK, Fernandez-Tejada A, Stewart S, Jaeger FH, Anasti K, Blinn JH, Kepler TB, Bonsignori M, Liao HX, Sodroski JG, Danishefsky SJ, Haynes BF. Recognition of synthetic glycopeptides by HIV-1 broadly neutralizing antibodies and their unmutated ancestors. *Proc Natl Acad of Sci USA.* 2013; 110:18214–18219. [PubMed: 24145434]
27. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao HX, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y, Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med.* 2012; 366:1275–1286. [PubMed: 22475592]
28. Gamblin DP, Scanlan EM, Davis BG. Glycoprotein synthesis: an update. *Chem Rev.* 2009; 109:131–163. [PubMed: 19093879]
29. Schmaltz RM, Hanson SR, Wong CH. Enzymes in the synthesis of glycoconjugates. *Chem Rev.* 2011; 111:4259–4307. [PubMed: 21749134]
30. Wang LX, Amin MN. Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem Biol.* 2014; 21:51–66. [PubMed: 24439206]
31. Unverzagt C, Kajihara Y. Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem Soc Rev.* 2013; 42:4408–4420. [PubMed: 23403448]
32. Wilson RM, Dong S, Wang P, Danishefsky SJ. The winding pathway to erythropoietin along the chemistry-biology frontier: a success at last. *Angew Chem Int Ed.* 2013; 52:7646–7465.
33. Danby PM, Withers SG. Advances in enzymatic glycoside synthesis. *ACS Chem Biol.* 2016; 11:1784–1794. [PubMed: 27176929]
34. Wang LX. Chemoenzymatic synthesis of glycopeptides and glycoproteins through endoglycosidase-catalyzed transglycosylation. *Carbohydr Res.* 2008; 343:1509–1522. [PubMed: 18405887]
35. Li B, Zeng Y, Hauser S, Song H, Wang LX. Highly efficient endoglycosidase-catalyzed synthesis of glycopeptides using oligosaccharide oxazolines as donor substrates. *J Am Chem Soc.* 2005; 127:9692–9693. [PubMed: 15998066]
36. Li H, Li B, Song H, Breydo L, Baskakov IV, Wang LX. Chemoenzymatic synthesis of HIV-1 V3 glycopeptides carrying two N-glycans and effects of glycosylation on the peptide domain. *J Org Chem.* 2005; 70:9990–9996. [PubMed: 16292832]
37. Umekawa M, Huang W, Li B, Fujita K, Ashida H, Wang LX, Yamamoto K. Mutants of *Mucor hiemalis* endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. *J Biol Chem.* 2008; 283:4469–4479. [PubMed: 18096701]
38. Umekawa M, Li C, Higashiyama T, Huang W, Ashida H, Yamamoto K, Wang LX. Efficient glycosynthase mutant derived from *Mucor hiemalis* endo-beta-N-acetylglucosaminidase capable of transferring oligosaccharide from both sugar oxazoline and natural N-glycan. *J Biol Chem.* 2010; 285:511–521. [PubMed: 19880511]
39. Huang W, Li C, Li B, Umekawa M, Yamamoto K, Zhang X, Wang LX. Glycosynthases enable a highly efficient chemoenzymatic synthesis of N-glycoproteins carrying intact natural N-glycans. *J Am Chem Soc.* 2009; 131:2214–2223. [PubMed: 19199609]

40. Schwarz F, Huang W, Li C, Schulz BL, Lizak C, Palumbo A, Numao S, Neri D, Aebi M, Wang LX. A combined method for producing homogeneous glycoproteins with eukaryotic N-glycosylation. *Nat Chem Biol.* 2010; 6:264–266. [PubMed: 20190762]
41. Huang W, Zhang X, Ju T, Cummings RD, Wang LX. Expedient chemoenzymatic synthesis of CD52 glycopeptide antigens. *Org Biomol Chem.* 2010; 8:5224–5233. [PubMed: 20848033]
42. Huang W, Giddens J, Fan SQ, Toonstra C, Wang LX. Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. *J Am Chem Soc.* 2012; 134:12308–12318. [PubMed: 22747414]
43. Li T, Tong X, Yang Q, Giddens JP, Wang LX. Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling. *J Biol Chem.* 2016; 291:16508–16518. [PubMed: 27288408]
44. Giddens JP, Lomino JV, Amin MN, Wang LX. Endo-F3 glycosynthase mutants enable chemoenzymatic synthesis of core-fucosylated triantennary complex type glycopeptides and glycoproteins. *J Biol Chem.* 2016; 291:9356–9370. [PubMed: 26966183]
45. Yamaguchi T, Amin MN, Toonstra C, Wang LX. Chemoenzymatic synthesis and receptor binding of mannose-6-phosphate (M6P)-containing glycoprotein ligands reveal unusual structural requirements for M6P receptor recognition. *J Am Chem Soc.* 2016; 138:12472–12485. [PubMed: 27500601]
46. Toonstra C, Amin MN, Wang LX. Site-selective chemoenzymatic glycosylation of an HIV-1 polypeptide antigen with two distinct N-glycans via an orthogonal protecting group strategy. *J Org Chem.* 2016; 81:6176–6185. [PubMed: 27380452]
47. Astronomo RD, Kaltgrad E, Udit AK, Wang SK, Doores KJ, Huang CY, Pantophlet R, Paulson JC, Wong CH, Finn MG, Burton DR. Defining criteria for oligomannose immunogens for HIV using icosahedral virus capsid scaffolds. *Chem Biol.* 2010; 17:357–370. [PubMed: 20416507]
48. Wang LX, Ni J, Singh S, Li H. Binding of high-mannose-type oligosaccharides and synthetic oligomannose clusters to human antibody 2G12: Implications for HIV-1 vaccine design. *Chem Biol.* 2004; 11:127–134. [PubMed: 15113002]

Figure 1.
Design and retrosynthetic analysis of HIV V3 glycopeptide antigens.

Figure 2. Binding analysis of PGT128 to the synthetic V3 glycopeptides

A) SPR sensorgrams of the binding between PGT128 and synthetic V3 glycopeptides. The synthetic glycopeptides was immobilized on neutravidin chips and PGT128 IgG was run as the analyte (3.9 nM to 500 nM in a 2-fold serial dilution). An attempt to fit the data with a 1:1 Langmuir binding model were made (fitting was shown in black line). The K_D data obtained serve as an estimate for comparison, as potential avidity might be involved in the interactions that could complicate the fitting results. B) ELISA binding of PGT128 to V3 glycopeptides carrying one N-glycan. C) ELISA binding of PGT128 to V3 glycopeptides carrying two N-glycans.

Figure 3. Binding analysis of PGT121 to the synthetic V3 glycopeptides

A) SPR sensorgrams of the binding between PGT121 and synthetic V3 glycopeptides. The synthetic glycopeptides were immobilized on neutravidin chips and PGT121 IgG was run as the analyte (3.9 nM to 500 nM in a 2-fold serial dilution). An attempt to fit the data with a 1:1 Langmuir binding model were made (fitting was shown in black line); B) ELISA analysis of the binding of PGT121 to V3 glycopeptides carrying one N-glycan; C) ELISA analysis of the binding of PGT121 to V3 glycopeptides carrying two N-glycans.

Figure 4. Binding analysis of 10-1074 to the synthetic V3 glycopeptides

A) SPR sensorgrams of the binding between antibody 10-1074 and synthetic glycopeptides. The biotinylated glycopeptides were immobilized on neutravidin chips and 10-1074 IgG was run as the analyte (3.9 nM to 500 nM in a 2-fold serial dilution); B) ELISA binding of 10-1074 to V3 glycopeptides carrying one N-glycan; C) ELISA binding of 10-1074 to V3 glycopeptides carrying two N-glycans.

Scheme 1. Chemoenzymatic synthesis of V3 glycopeptides carrying a defined N-glycan at the N332 glycosylation site

Scheme 2. Chemoenzymatic synthesis of V3 glycopeptides carrying a defined N-glycan at the N301 and/or N295 glycosylation site

Scheme 3. Chemoenzymatic synthesis of V3 glycopeptides carrying two N-glycans at the N301 and N332 sites

Scheme 4. Synthesis of biotinylated high-mannose and complex type N-glycans