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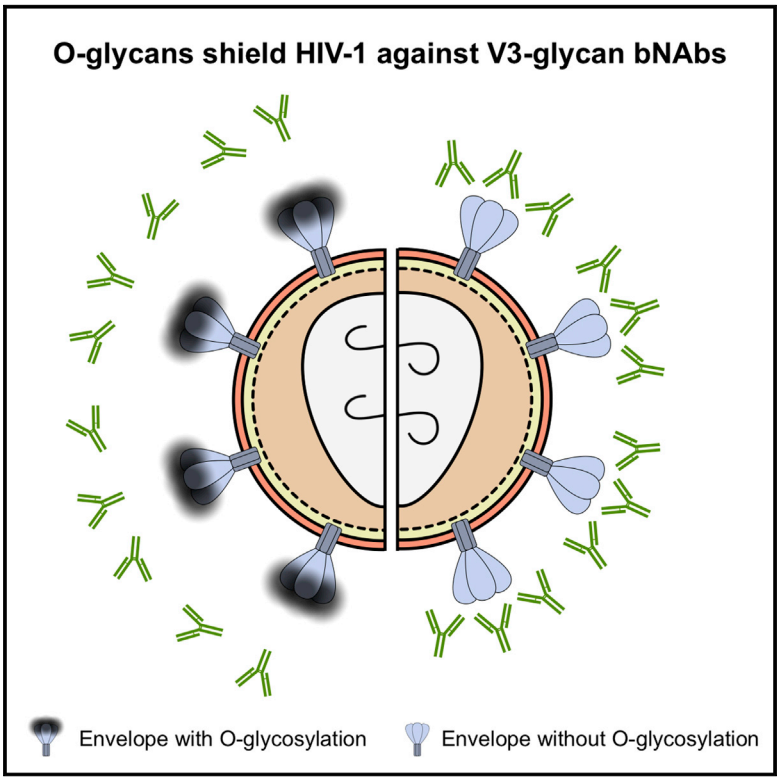
Discovery of O-Linked Carbohydrate on HIV-1 Envelope and Its Role in Shielding against One Category of Broadly Neutralizing Antibodies

Graphical Abstract



Highlights

- A subset of HIV-1 isolates possess O-linked carbohydrate on their Envelope glycoprotein
- This O-glycosylation is preferentially found on strains with unusually long V1 domains
- These O-glycans shield the virus from V3-glycan broadly neutralizing antibodies

Authors

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In Brief

Silver et al. demonstrate that certain HIV-1 isolates possess O-linked carbohydrate on their Envelope glycoprotein. These sugars allow the virus to evade V3-glycan broadly neutralizing antibodies. This work identifies a post-translational modification to the HIV-1 Envelope and sheds light on its role in shielding against the host antibody response.

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Discovery of O-Linked Carbohydrate on HIV-1 Envelope and Its Role in Shielding against One Category of Broadly Neutralizing Antibodies

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SUMMARY

Approximately 50% of the mass of the Envelope (Env) glycoprotein surface subunit (gp120) of human immunodeficiency virus type 1 (HIV-1) is composed of N-linked carbohydrate. Until now, the dogma has been that HIV-1 lacks O-linked carbohydrate on Env. Here we show that a subset of patient-derived HIV-1 isolates contain O-linked carbohydrate on the variable 1 (V1) domain of Env gp120. We demonstrate the presence of this O-glycosylation both on virions and on gp120 expressed as a secreted protein. Further, we establish that these O-linked glycans can confer a more than 1,000-fold decrease in neutralization sensitivity (IC_{50}) to V3-glycan broadly neutralizing antibodies. These findings uncover a structural modification to the HIV-1 Env and suggest a functional role in promoting viral escape from one category of broadly neutralizing antibodies.

INTRODUCTION

The addition of O-linked carbohydrate to proteins in the secretory pathway, or mucin-type O-glycosylation, is the most abundant type of O-linked carbohydrate attachment in humans (Tian and Ten Hagen, 2009). Mucin-type O-glycosylation always begins with the addition of N-acetylgalactosamine to the hydroxyl group of serine or threonine, thus forming the Tn antigen (GalNac-Ser/Thr) (Hart and Akimoto, 2009). The Tn antigen is then further modified to form one of eight core mucin-type O-linked carbohydrate structures. Although dense stretches of serines and threonines in a protein sequence can be preferential for O-glycosylation, little is known regarding the specific sequences or structural determinants that guide the attachment of O-linked carbohydrates (Gooley and Williams, 1994). However, informatic tools such as the NetOGlyc4.0 prediction server may be used to indicate the likelihood that a particular serine or threonine will be O-glycosylated (Steentoft et al., 2013).

The Envelope (Env) glycoprotein of HIV-1 consists of a heterotrimeric complex of gp120 and gp41 (Araújo and Almeida, 2013). Although HIV-2 and SIVmac239 are O-glycosylated on the V1 domain of gp120, the current dogma is that HIV-1 lacks such O-glycosylation (Stansell et al., 2011, 2015). Using the same lectin-binding procedures that readily demonstrated O-glycosylation on the gp120s of a panel of SIVmac, SIVsm, and HIV-2 strains, Stansell et al. (2011) failed to find evidence for O-glycosylation on 11 of 11 strains of HIV-1 and SIVcpz. When HIV-1 gp120 is expressed as a secreted product, the C-terminal threonine at position 499 is O-glycosylated (Go et al., 2013; Stansell et al., 2015). However, when HIV-1 gp120 is made naturally from its gp160 precursor and analyzed from purified virions, there is no O-linked carbohydrate (Stansell et al., 2015). Furthermore, when HIV-1 is produced in a knockout (KO) cell line that is incapable of adding O-linked carbohydrate, the HIV-1 retains full infectivity (Termini et al., 2017a, 2017b).

We recently described a primary HIV-1 isolate from an elite controller that displayed an unusually long V1 domain in Env gp120 (Silver et al., 2019). Careful analysis of this V1 domain revealed stretches of serines and threonines with positive O-glycosylation prediction scores by the NetOGlyc4.0 algorithm. We therefore hypothesized that, like its SIV and HIV-2 counterparts, some HIV-1 Envs with long V1 domains may be O-glycosylated on V1. In this study, we show that there indeed exists a subset of patient-derived HIV-1 strains whose long V1 domains are modified with O-linked carbohydrate. Furthermore, we show that these O-glycans can result in more than a 1,000-fold decrease in sensitivity to broadly neutralizing antibodies (bNAbs) of the V3-glycan category. Our results uncover a previously unknown structural modification to HIV-1 Env and suggest a role in viral escape from the host antibody response.

RESULTS

Long V1 Domains Have Increased O-Glycosylation Prediction Scores

To determine whether HIV-1 Envs with long V1 domains might be O-glycosylated, we downloaded a curated web alignment of

Table 1. gp120s with Varying V1 Domain Features

Sequence Name	Subtype	V1 Length	Number of S/T in V1	Number of Scored S/T in V1 (>0.90)[a]	V1 Domain Sequence[b]
AD8	B	23	4	0	CTDLRNVTNINNSSEGMRGEIKN
NL4-3	B	26	6	0	CTDLKNDTNTNSSSGRMIMEKGEIKN
VA40774	B	49	14	5	CTDWKYTNCTNSGNTTTTNTNC TDSGKATNTTGGGKKGKLKLEKGEIKN
HM215335	C	38	23	17	CTNVNSTAPNSTAATSTTTSSSTT SSSTTTATYNEMTN
KX693469	B	49	28	21	CTNLETNNTNSNTSSPPPTTTSSSN TSSTTTSTTSSYTRSTTEGGEMKN
GU329473	C	54	29	23	CENVNMTARAKGPASSPTSATSTT SPTSTTSTTSPTSATSTTSPTSTVEPTMKN

See also Figure S1.

[a]O-glycosylation prediction scores were determined by the NetOGlyc4.0 server (Steentoft et al., 2013).

[b]The V1 domain boundaries are those defined by the Los Alamos National Laboratory HIV Sequence Compendium and correspond to HXB2 amino acid positions 131–156.

6,112 Group M (A-K + recombinants) HIV-1 Env sequences from the Los Alamos National Laboratory (LANL) online database (https://www.hiv.lanl.gov). A total of 4,757 sequences remained in the amino acid alignment after removing sequences with characters (X, #, ?, and $) that interfere with the O-glycosylation prediction algorithm. These Env sequences were then systematically uploaded to the NetOGlyc4.0 prediction server to assign an O-glycosylation score between 0.00 and 1.00 to each serine or threonine in a particular Env sequence. A Python-based computer algorithm was devised to parse the following information from each of the 4,757 sequences: V1 domain length, number of serines and threonines within the V1 domain, number of serines and threonines within the V1 domain that have NetOGlyc4.0 prediction scores above 0.90, and mean O-glycosylation score of the V1 domain. The V1 domain boundaries are those defined by the LANL HIV Sequence Compendium and correspond to HXB2 amino acid positions 131–156.

The range of V1 domain lengths in our modified LANL HIV-1 Env sequence alignment falls between 8 and 63 amino acids, with a median length of 27 amino acids (Figure S1A). We divided these 4,757 sequences into V1 domain length groups with approximately equal numbers of sequences to determine whether groups with increasingly long V1 domains are increasingly likely to have predicted O-glycans on their V1 domains. For each sequence, we investigated predicted V1 O-glycosylation density, which we define as the number of serines and threonines in V1 with O-glycosylation prediction scores above 0.90 divided by the amino acid length of the V1 domain. The results, displayed in Figure S1B, show that predicted V1 O-glycosylation density increases with increasing V1 domain length.

A Subset of HIV-1 Strains with Long V1 Domains Are O-Glycosylated

To determine whether HIV-1 strains with long V1 domains may be O-glycosylated compared with those with short V1 domains, we selected a curated set of gp120s for initial testing (Table 1). The selected gp120s included two short V1 domain strains without meaningful O-glycosylation scores (NL4-3 and AD8), three long V1 domain strains with dense prediction scores (HM215335, KX693469, and GU329473), and our elite controller primary isolate with a long V1 domain and an intermediate prediction density (VA40774). Expression cassettes were constructed for the six HIV-1 gp120s (Table 1; Figure S2A), transfected into HEK293T cells, and the gp120s produced from them were analyzed.

The lectin jacalin is a valuable reagent for O-glycosylation assays because of its ability to bind Tn antigen, the basic building block of all O-linked glycans, as well as non- and mono-sialylated core 1 carbohydrates (Jeyaprakash et al., 2002; Sastry et al., 1986; Tachibana et al., 2006; Wu et al., 2003). SIVmac239 gp120, which has been shown unambiguously to be O-glycosylated on V1, was used as positive control (Stansell et al., 2011). The SIVmac239 gp120 reacted clearly and strongly with jacalin on western blots when produced from HEK293T cells but not when produced from a KO cell line incapable of forming O-linked carbohydrate (GaleGalk2 KO) (Figure 1A; Termini et al., 2017b). The HIV-1 gp120s from HM215335, KX693469, and GU329473 were clearly and strongly reactive with jacalin, while the HIV-1 gp120s from NL4-3, AD8, and VA40774 were not (Figure 1A). Mass spectrometry-based glycomic analysis of released glycans from HEK293T-produced GU329473 gp120 revealed an abundant core 1 structure that could also be mono- and disialylated (*m/z* 534, 895, and 1,256) (Figure 2A). Core 2 structures were also present, which again could be mono- and disialylated (*m/z* 779, 983, 1,140, 1,344, and 1,713). These sugars were absent when GU329473 gp120 was produced in the GaleGalk2 KO cells. Jacalin-binding epitopes are shown schematically in Figure 2B.

We next generated a set of V1 domain exchange recombinants to obtain evidence that the O-linked carbohydrate being detected was indeed present on the V1 domain (Figure S2B). Exchange of the V1 domain of strain VA40774 into the GU329473 gp120 resulted in loss of jacalin reactivity (Figure 1B, lane 6). Conversely, exchange of the V1 domain of strain GU329473 into the VA40774 gp120 resulted in a gain of jacalin reactivity (Figure 1B, lane 5). These results indicate that the jacalin

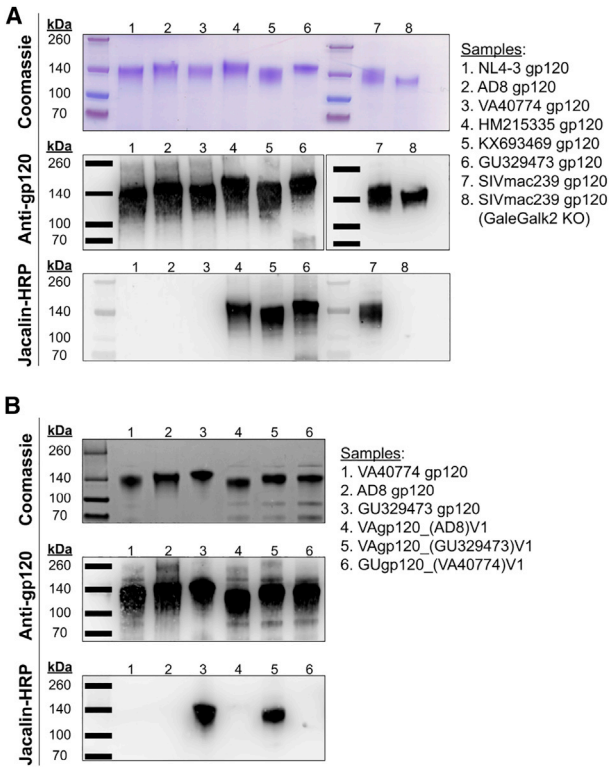

Figure 1. A Subset of HIV-1 Strains Are O-Glycosylated on the V1 Domain of Envelope

Equal amounts of monomeric gp120 were loaded into the wells of three 4%–12% SDS-PAGE gels. Recombinant proteins were quality checked by Coomassie (top rows) and gp120 antibody binding (middle rows). The presence of O-glycosylation was determined using jacalin-HRP (bottom rows).

(A) Identification of three O-glycosylated HIV-1 Envelopes.

(B) O-glycosylation of HIV-1 gp120 maps to the V1 domain.

See also Figure S2.

reactivity for GU329473 seen in Figure 1A is a result of O-linked carbohydrate on the V1 region. Jacalin binding of other V1 recombinants has shown that the O-linked carbohydrates on strains HM215335 and KX693469 are also associated with the V1 domain (see below).

Using mass spectrometry, Stansell et al. (2015) showed that Env gp120 from HIV-1 was O-glycosylated on its C-terminal threonine when produced as a secreted gp120 but not O-glycosylated when directly purified from virions. Therefore, we set out to demonstrate that the O-linked carbohydrate being detected on the V1 domains of HM215335, KX693469, and GU329473 is present not only on gp120 when made as the secreted product but also on virions when gp120 is produced naturally from the gp160 precursor. Several virus-encoding plasmids were constructed on the HIV-1 NL4-3 proviral backbone to contain gp160 regions derived from five different strains: AD8, VA40774, HM215335, KX693469, and GU329473 (Figure S2C). Again, SIVmac239 served as the positive control. Virus stocks were produced by transfection of HEK293T cells and the O-glycosylation-deficient GaleGalk2 KO cell line (Termini et al., 2017b). These virus stocks were used to infect the TZM-bl reporter cell line in the presence and absence of jacalin as an indicator for the presence or absence of O-linked carbohydrate on the gp120-containing virion spike. Jacalin inhibited the infectivity of the positive control SIVmac239 by approximately 1.5 logs when it was produced in the parental HEK293T cells but not when the SIVmac239 was produced in the KO cell line (Figure 3F). No jacalin inhibition was observed with the HIVs containing AD8 (Figure 3A) or VA40774 (Figure 3B) Env whether virus was produced in the parental or KO cell lines. In contrast, potent jacalin inhibition was again observed with HIVs containing the Envs of HM215335 (Figure 3C), KX693469 (Figure 3D), and GU329473 (Figure 3E), but only when virus was produced in the parental HEK293T cells. To further define this jacalin-mediated inhibition, we tested serial dilutions of jacalin for their ability to neutralize the infectivity of each HEK293T-produced virus (Figure S3). The viruses listed in order of decreasing sensitivity to jacalin inhibition are as follows: NL-GU329473, SIVmac239, NL-HM215335, and NL-KX693469. NL-AD8 and NL-VA40774 were not neutralized by jacalin. Taken together, these results confirm O-glycosylation of the HM215335, KX693469, and GU329473 gp120s as they exist on the surface of virions.

HIV-1 O-Glycosylation Shields against Broadly Neutralizing Antibodies

We next asked whether O-glycosylation on the V1 domain of HIV-1 virions contributes to evasion from bNAbs. The same five recombinant virus stocks detailed above, produced in either HEK293T or GaleGalk2 KO cells, were used in neutralization assays with 13 different monoclonal antibodies (mAbs) representing five different categories of potent broadly neutralizing activity (Figure 4; Table 2). The most remarkable results were observed with the V3-glycan antibodies PGT121, PGT128, and 10-1074. Production of the NL-HM215335, NL-KX693469, and NL-GU329473 viruses under conditions of no O-glycosylation from the KO cell line resulted in a profound increase in sensitivity to neutralization by antibodies in this V3-glycan bNAb category. The greatest effect was observed with the NL-HM215335 and NL-GU329473 viruses and the PGT121 and PGT128 mAbs, in which the increase in neutralization sensitivity was 830- to 1,300-fold (Table 2). No such effect was observed with these V3-glycan antibodies on the NL-AD8 and NL-VA40774 recombinant strains. Similarly, little or no difference was observed for the other 10 antibodies with all five recombinant viruses when virus produced from the parental HEK293T cells was compared with that from the KO line (Figure 4; Table 2).

We next asked to what extent the shielding effects of V1 O-linked carbohydrate against V3-glycan-directed antibodies could be manifest by monomeric gp120 in simple ELISAs. For the three O-glycosylated strains, we observed no difference between 10-1074 or PGT121 binding to O-glycosylated versus non-O-glycosylated gp120. On the other hand, PGT128 required more antibody to bind the O-glycosylated forms of these three gp120s (Figure S4). The magnitude of the effect was most substantial for PGT128 reactivity against the GU329473 gp120

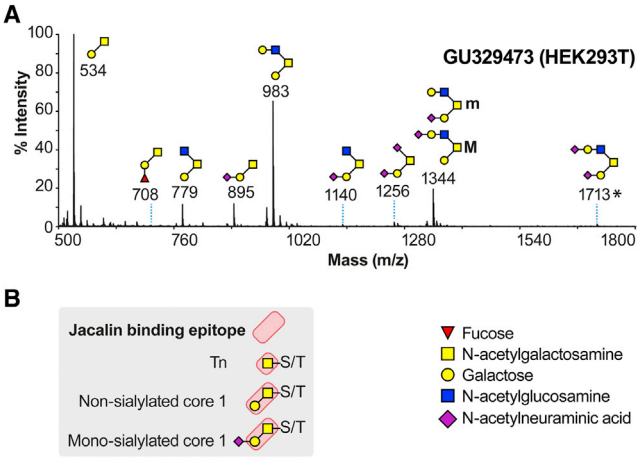

Figure 2. O-Linked Glycomic Analyses of Recombinant gp120

(A) Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectra of permethylated O-glycans isolated from HEK293T-produced HIV-1 GU329473 gp120. The sugar symbols in the represented glycan structures are those used by the Consortium for Functional Glycomics. Structural assignments are based on monosaccharide composition (obtained by MALDI-TOF mass spectrometry [MS]), fragmentation analyses (MALDI-TOF/TOF MS/MS), and knowledge of glycan biosynthetic pathways. All molecular ions are $[M+Na]^+$. "M" and "m" designations indicate major and minor abundances, respectively. The asterisk on the ion at *m/z* 1,713 indicates that the hydroxyl of the carboxylic group of a NeuAc residue of the corresponding glycan structure is sodiated shifting the *m/z* value 8 units higher than the permethylated one.

(B) Jacalin-binding epitopes.

See also Figure S2.

(Figure S4E); GU329473 is the strain with the greatest number of predicted O-linked sites and the highest level of jacalin reactivity among the strains we have analyzed (see below). These results suggest that the blocking effects of the GU329473 V1 O-linked carbohydrate on the V3-glycan region may be evident in crystal structures of monomeric gp120.

Identification of Additional O-Glycosylated HIV-1 Strains

Our analyses described thus far identify three naturally occurring gp120 sequences that are O-glycosylated on the HIV-1 virion. Furthermore, we have shown that these O-glycans can play a role in shielding virus from recognition by a particular category of bNAbs. We next set out to examine which V1 domain characteristics may be most predictive of whether an individual HIV-1 strain will be O-glycosylated. To do so, we synthesized the coding sequences of 32 V1 domains from our curated Group M Env alignment that displayed varying degrees of V1 domain length, NetOGlyc4.0 prediction scores, and numbers of serines and threonines in V1 (Table S1). These V1 domains were subcloned into the VAgp120 expression vector (Figure S2B), produced in HEK293T cells, and assessed for their ability to bind jacalin-HRP in an ELISA (Figure 5).

These experiments identified an additional 8 HIV-1 Envs with O-glycosylated V1 domains, thus bringing the total number of known O-glycosylated HIV-1 isolates to 11. These 11 O-glycosylated gp120s varied in V1 domain length from 31 to 54, with a median of 39 amino acids. Our analyses show statistically significant differences between the jacalin-reactive and non-jacalin-reactive V1 domains for four parameters: V1 length, number of serines and threonines in V1, number of serines and threonines in V1 with NetOGlyc4.0 scores above 0.50, and mean NetOGlyc4.0 score (Figures S5A–S5D). All 11 O-glycosylation-positive strains had mean NetOGlyc4.0 scores above 0.90. All 20 strains with mean NetOGlyc4.0 scores below 0.90 scored negative for the presence of O-linked carbohydrate on the basis of jacalin reactivity. However, 6 of 17 strains with mean NetOGlyc4.0 scores above 0.90 were negative for jacalin reactivity. Quantitative assessments revealed a large range of jacalin-binding capacities among positive samples (Figure S6). We determined the jacalin-binding coefficient for each of these samples, which we define as the amount of gp120 required to elicit an optical density (A450) of 0.50 by ELISA. Among the O-glycosylation-positive samples, three of the four parameters, excluding mean NetOGlyc4.0 score, showed statistically significant correlations with the jacalin-binding coefficient (Figure S7).

Nature of V1 Regions that Emerged in a Patient with V3-Glycan Broadly Neutralizing Activity

Bonsignori et al. (2017) described the emergence of broadly neutralizing activity of V3-glycan specificity in a Malawian man beginning about 3.5 years after the time of HIV-1 infection. A total of 1,215 Env sequences from peripheral blood mononuclear cell (PBMC) RNA over a span of 4.7 years have been deposited in the LANL sequence database from this individual (https://www.hiv.lanl.gov). Our analysis of those sequences shows the emergence of long V1 regions with high serine and threonine content late in the course of this sequence data collection. These V1 regions with high serine and threonine content and highest NetOGlyc4.0 prediction scores were recorded only after the emergence of this V3-glycan-specific broadly neutralizing activity, 1,400 days (3.84 years) or later from the time of infection (Table S2). V1 lengths as long as 42 amino acids were observed, with as many as 11 serines and threonines with NetOGlyc4.0 prediction scores greater than 0.50 and as many as 3 NetOGlyc4.0 prediction scores greater than 0.90.

DISCUSSION

Now 36 years after the discovery of HIV-1 as the cause of AIDS, there is an enormous amount of detailed knowledge regarding the structural elements and replication strategies of the virus. In fact, discovery of new fundamental features of HIV-1's structural elements, replicative strategies, and evasion strategies is now quite uncommon. Here we have documented that a subset of HIV-1 isolates possess O-linked carbohydrate on the V1 domain of their gp120 Env glycoprotein. This structural feature appears similar to what is more commonly observed among SIVmac/SIVsm isolates (three of three) and among HIV-2 isolates (two of three) (Stansell et al., 2011). Importantly, we have shown that this V1 O-glycosylation on HIV-1 can have a dramatic shielding effect against antibodies directed to the V3-glycan region of gp120. The expected location of these

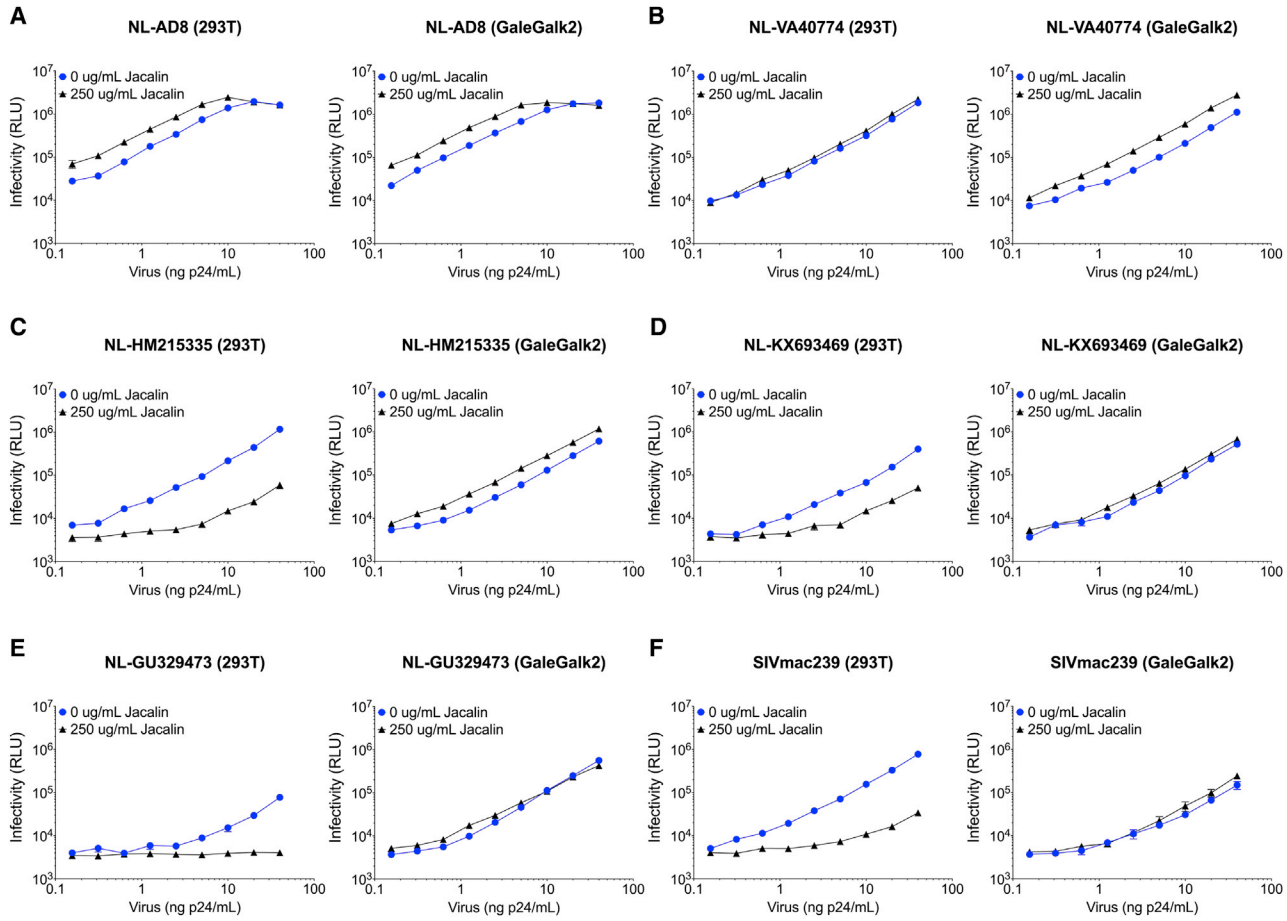

Figure 3. Jacalin Inhibition of Virus Infectivity

(A–F) Recombinant NL4-3 strains each containing a unique full-length gp160 were produced in either wild-type HEK293T or GaleGalk2 KO cells. TZM-bl cells were infected with each recombinant NL4-3 in the presence or absence of 250 μg/mL jacalin, a lectin that specifically binds O-linked glycans: (A) NL-AD8, (B) NL-VA40774, (C) NL-HM215335, (D) NL-KX693469, (E) NL-GU329473, and (F) SIVmac239. The black and blue curves represent the infectivity in the presence or absence of jacalin, respectively. Results are presented as mean ± SEM from three experiments.

See also Figures S2 and S3.

O-glycosylated V1 regions in the prefusion-closed HIV-1 Env trimer structure (Bartesaghi et al., 2013) appears to be analogously similar to the location of the mucin domain on the Ebola virus glycoprotein (Beniac and Booth, 2017), at the outer periphery of the trimer apex.

What selective forces might be responsible for driving the emergence and/or allowing the persistence of these unusual gp120s with long V1 regions and O-linked carbohydrate? It seems highly likely that these features are driven by selective pressures from the host antibody response. Emergence of anti-HIV-1 antibodies with potent neutralizing activity against a broad range of HIV-1 isolates occurs in only a fraction of HIV-1-infected individuals (Doria-Rose et al., 2009; Gray et al., 2011; Klein et al., 2013; Mikell et al., 2011; Mouquet, 2014; Sanchez-Merino et al., 2016). Nonetheless, the past decade has witnessed the characterization of a remarkable array of human mAbs with such potent broadly neutralizing activity (Stephenson and Barouch, 2016). One such category of bNAbs, the so-called V3-glycan category, recognizes sequences in the vicinity of the V3 region in a carbohydrate-dependent fashion. The specificity of this carbohydrate dependence varies with the individual mAb and can be quite complex (Anthony et al., 2017; Krumm et al., 2016; Sok et al., 2014). PGT128 appears to be quite dependent on the N-linked site at N301, but the presence of an additional N-linked site at N332 or N295 also appears largely necessary. PGT121 has co-dependency on N301, N332/334, and N136/156; absence of any one of these three still yields good neutralization; absence of any two of these three gives little or no neutralization. 10-1074 appears to have greater dependence on N332, but the 324–327 region is also important for 10-1074 recognition. We can speculate that antibody specificities may arise in some individuals, possibly including the combination of both V3-glycan specificities as well as other specificities, which make it difficult for virus to escape recognition simply by altering the locations of N-linked sites. Such a situation would be expected to put pressure on the virus to evolve some other means of escape.

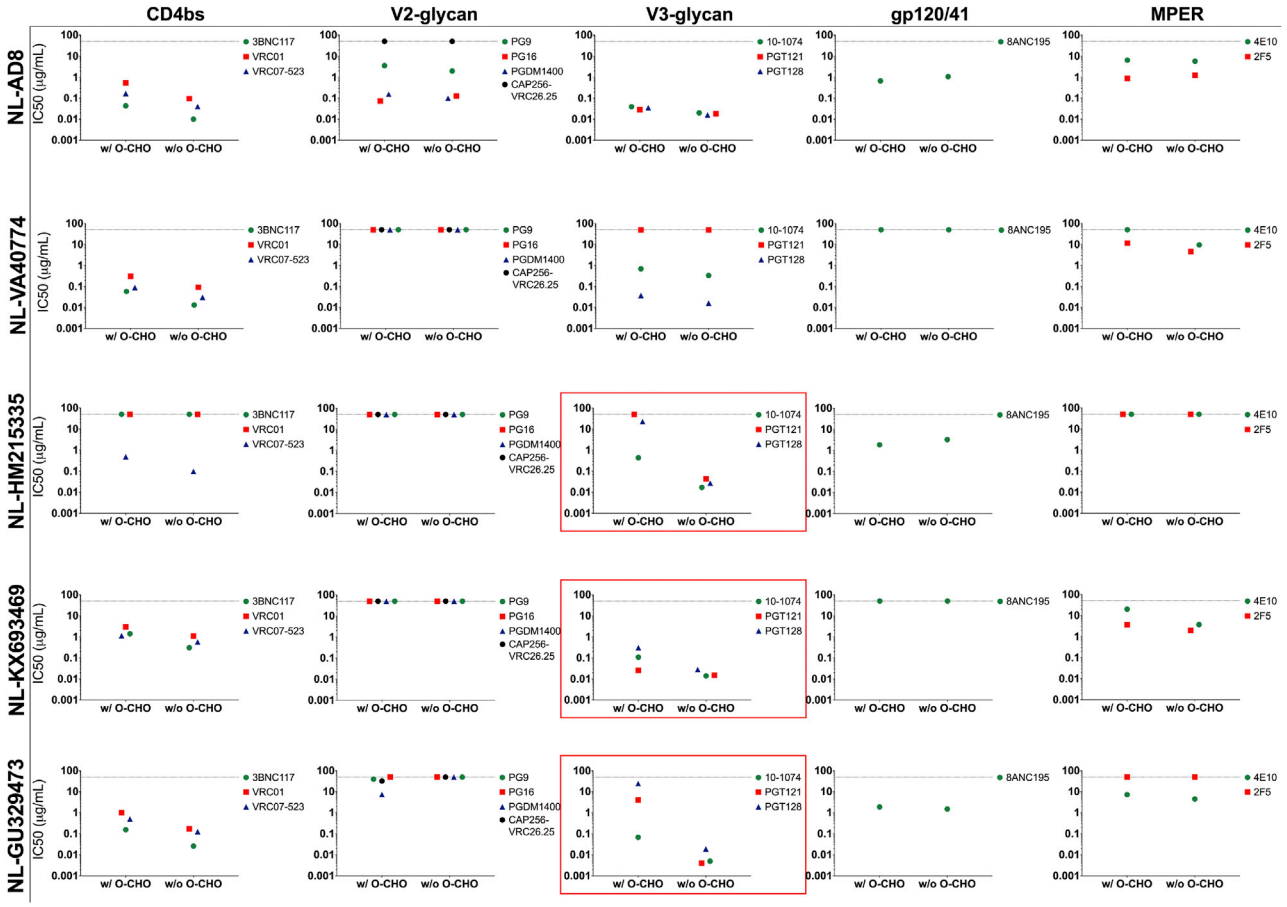

Figure 4. Effect of O-Glycosylation on Neutralization by Broadly Neutralizing Antibodies

Half maximal inhibitory concentration (IC_{50}) values for several categories of broadly neutralizing antibodies were determined as the average of two independent experiments for each recombinant Env virus produced with versus without O-glycosylation. Each column represents a single category of broadly neutralizing antibody, and each row represents a single strain of virus. The maximum concentration of antibody used for these assays was 50 μg/mL. Red boxes are placed around the viruses and antibody categories with the most dramatic changes in IC_{50}. The mAbs tested belong to the CD4bs (3BNC117, VRC01, and VRC07-523), V1/V2-glycan (PG9, PG16, PGDM1400, and CAP256-VRC26.25), V3-glycan (10-1074, PGT121, and PGT128), gp120/41 (8ANC195), and MPER (4E10 and 2F5) categories of bNAbs. See also Figures S2 and S4 and Table S2.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Cell lines
- METHOD DETAILS
  - Python program
  - Plasmids
  - Production of recombinant gp120s
  - Generation of recombinant virus
  - Jacalin ELISA
  - V3-glycan bNAb binding assay
  - Western blot
  - TZM-bl cell assays
  - Mass spectrometry
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.056.

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Table 2. Fold Change in bNAb IC_{50} When Virus Is Grown with or without O-Glycans

bNAb	Epitope	NL-AD8	NL-VA40774	NL-HM215335	NL-KX693469	NL-GU329473
3BNC117	CD4bs	4.3	4.5	-	4.6	6.0
VRC01	CD4bs	5.6	3.4	-	2.7	6.0
VRC07-523	CD4bs	4.3	3.0	5.0	2.0	4.0
PG9	V2-glycan	1.8	-	-	-	<0.8
PG16	V2-glycan	0.6	-	-	-	-
PGDM1400	V2-glycan	1.6	-	-	-	<0.1
CAP256-VRC26.25	V2-glycan	-	-	-	-	<0.7
10-1074	V3-glycan	2.0	2.1	25.5	7.8	13.6
PGT121	V3-glycan	1.6	-	>1162.8	1.7	994.3
PGT128	V3-glycan	2.2	2.3	833.9	11.0	1300.9
8ANC195	gp120/41	0.6	-	0.6	-	1.3
4E10	MPER	1.1	>3.2	-	5.4	1.6
2F5	MPER	0.7	2.5	-	1.9	-

Fold-change[a]
>1000
101-1000
11-100
1-10
<1
No Neutralization[b]

See also Figure S4.

[a]Fold change in IC_{50} is calculated as IC_{50} for HEK293T-produced virus divided by IC_{50} for GaleGalk2-produced virus. For some viruses and antibodies, the IC_{50} could not be determined when produced in HEK293T but could be determined when grown in GaleGalk2 KO cells. For these viruses, the fold change should be considered as at least the value given: NL-VA40774 (4E10), NL-GU329473 (PG9, PGDM1400, CAP256-VRC26.25), and NL-HM215335 (PGT121).

[b]If the IC_{50} of a particular bNAb is above the upper limit of detection (>50 μg/mL) for both HEK293T and GaleGalk2 KO virus, the value is defined as no neutralization (gray).

to A.D.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

AUTHOR CONTRIBUTIONS

Z.A.S. and R.C.D. conceived the idea. Z.A.S. wrote the Python computer algorithm, designed all plasmids, and was responsible for experimental design, execution, data analysis, and manuscript preparation. A.A., S.M.H., and A.D. performed and analyzed the mass spectrometry experiments. M.S.S. tested the recombinant viruses in neutralization assays. G.M.D. initiated the plans that led to this line of investigation. R.C.D. helped plan the experiments, supervised the research, interpreted the data, and contributed to the preparation of this manuscript.

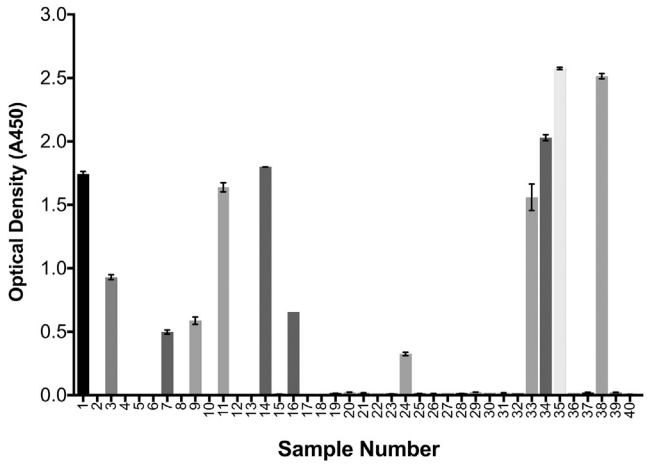

Figure 5. Screening HIV-1 Env V1 Domains for Jacalin Reactivity

The V1 domains of 32 HIV-1 Envelopes were screened using ELISA for their ability to elicit a jacalin-HRP signal in the context of the non-jacalin-reactive VAgp120 backbone. Samples 33–40 served as internal controls for the assay. The name and V1 domain characteristics of samples 1–37 are found in Table S1. Sample 38, GU329473 gp120; sample 39, GUgp120_(AD8)V1; sample 40, AD8 gp120. Results are presented as mean ± SEM from two experiments. See also Figures S2 and S5–S7 and Tables S1 and S2.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-gp120 (HIV-1, Pan)	Immune Technology	Cat# IT-001-Pan-M1
Anti-gp120 (SIVmac239, 3.11H)	Termini et al., 2017a	N/A
HRP-goat anti-mouse IgG (H+L)	Immune Technology	Cat# IT-200-010
HRP-goat anti-rhesus IgG (H+L)	Southern Biotech	Cat# 6200-05
10-1074	NIH AIDS Reagent Program	Cat# 12477
PGT121	NIH AIDS Reagent Program	Cat# 12343
PGT128	NIH AIDS Reagent Program	Cat# 13352
Bacterial and Virus Strains		
MAX Efficiency Stbl2 competent cells	Invitrogen	Cat# 10268019
Chemicals, Peptides, and Recombinant Proteins		
Artocarpus integrifolia (jacalin) HRP	United States Biological	Cat# A3590-11
Unconjugated jacalin	Vector Laboratories	Cat# L-1150-10
Critical Commercial Assays		
Britelite Plus reporter gene assay system	Perkin Elmer	Cat# 6066766
HIV p24 antigen capture assay	Advanced Bioscience Laboratories, Inc.	Cat# 5447
SIV p27 antigen capture assay	Advanced Bioscience Laboratories, Inc.	Cat# 5436
Experimental Models: Cell Lines		
HEK293T cells	ATCC	Cat# CRL-3216
HEK293T *gale* knockout cells	Termini et al., 2017b	N/A
HEK293T *gale* & *galk2* knockout cells	Termini et al., 2017b	N/A
TZM-bl cells	NIH AIDS Reagent Program	Cat# 8129
Recombinant DNA		
NL4-3	NIH AIDS Reagent Program	Cat# 114
NL-AD8	NIH AIDS Reagent Program	Cat# 11346
NL-VA40774	Silver et al., 2019	N/A
Recombinant NL4-3 virus (NL-HM215335, NL-KX693469, NL-GU329473)	This paper	N/A
gp120 expression cassettes (samples #1-37)	This paper	N/A
Software and Algorithms		
GraphPad Prism	https://www.graphpad.com	RRID: SCR_002798
MacVector	https://macvector.com	RRID: SCR_015700
SnapGene	https://www.snapgene.com	RRID: SCR_015052
GlycoWorkbench	https://code.google.com/archive/p/glycoworkbench/	RRID: SCR_000782
Python algorithm for analyzing V1 length and NetOGlyc4.0 score	This paper	N/A
Other		
jetPRIME	Polyplus-transfection S.A.	Cat# 114-15
BIO-MPM-1 serum-free medium	Biological Industries USA	Cat# 05-060-1A
HisPur Ni-NTA spin columns, 3 mL	ThermoFisher Scientific	Cat# 88226
Laemmli sample buffer	Sigma-Aldrich	Cat# S3401
iBlot 2 Gel Transfer Device	ThermoFisher Scientific	Cat# IB21001
iBind Western Device	ThermoFisher Scientific	Cat# SLF1000

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Ronald C. Desrosiers (r.desrosiers@med.miami.edu). All unique/stable reagents generated in this study are available from the Lead Contact with a completed Material Transfer Agreement.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

HEK293T and TZM-bl cells (American Type Culture Collection) were cultured in D10, a media preparation consisting of Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific) with 10% heat-inactivated fetal bovine serum (HI-FBS, ThermoFisher Scientific) and 100 μg/mL of Primocin (Invivogen). The Gale and GaleGalk2 KO cell lines, which are incapable of mucin-type O-glycosylation, were also cultured in D10.

METHOD DETAILS

Python program

A computer algorithm was devised to interrogate the 2016 Los Alamos National Laboratory curated alignment of 6,112 Group M (A-K + recombinants) HIV-1 Env sequences. The sequences were systematically uploaded to the NetOGlyc4.0 mucin-type O-glycosylation prediction server to generate a prediction score for each serine and threonine in a given sequence. The number of predicted O-glycosylation sites in each Env V1 domain was determined, along with the V1 domain length, number of serines and threonines in V1, and the mean V1 O-glycosylation score. All of these data were exported to an Excel spreadsheet.

Plasmids

To determine whether long V1 domain Envs are O-glycosylated, several expression cassettes encoding truncated secreted gp120s were designed (Figure S2A). HIV-1 gp120 lies between amino acids 1 and 511 of the HXB2 reference sequence (Foley et al., 2016). Codon-optimized nucleotide sequences for the HIV-1 gp120s (HXB2-aligned amino acids 1-505) of NL4-3, AD8, VA40774, HM215335, KX693469, and GU329473 were generated by Genscript gene synthesis service. Each gp120 open-reading frame was immediately preceded by a Kozak sequence (GCCGCCACC) and followed by six C-terminal histidine tags (CACCATCACCA TCACCAT). Each gp120 was cloned into the pcDNA6 vector (Invitrogen) using the BamHI and XhoI restriction sites and the integrity of each plasmid was verified by Sanger sequencing and restriction analysis. The SIVmac239 gp120 (HXB2-aligned amino acids 1-519) expression cassette served as the positive control and was generated in a similar fashion.

A V1 exchange recombinant on the VA40774 gp120 backbone (VAgp120) was utilized to screen an additional panel of V1 domains from the Los Alamos curated Env alignment for O-glycosylation (Figure S2B). Briefly, silent mutations were generated by PCR mutagenesis to create two new restriction sites on either side of the V1 domain (5′ = HindIII, 3′ = KpnI). The resulting plasmid, termed VAgp120, served as the backbone for our O-glycosylation screen. Genscript gene synthesis was used to generate codon-optimized oligonucleotides corresponding to the V1 domain of interest with VA40774 nucleotide sequences that flank the V1 domain through the restriction sites. Each gene synthesis product was then subcloned into VAgp120 and the integrity of the resulting plasmids was verified by restriction analysis and Sanger sequencing.

To determine whether O-linked glycans exist on the surface of HIV-1 virions, several recombinant Env virus strains were generated on the NL4-3 proviral backbone (Figure S2C). The full-length, native gp160 sequences of HM215335, KX693469, and GU329473 were gene synthesized (Genscript). These gp160 sequences were exchanged into NL4-3 using Gibson Assembly cloning to generate a set of NL4-3 Env chimerics termed NL-HM215335, NL-KX693469, and NL-GU329473. The remaining four recombinant Env virus-encoding plasmids – NL4-3, NL-AD8, NL-VA40774, and SIVmac239 – had previously been generated.

Production of recombinant gp120s

HEK293T, Gale KO, or GaleGalk2 KO cells were seeded in a T-75 flask such that their confluency would reach ~60% after 24 hours. The cells were transfected with 10 μg of each gp120-encoding plasmid using jetPRIME (Polyplus-transfection S.A.). At 18 hours post-transfection, the cell monolayer was washed once with Dulbecco's phosphate-buffered saline (D-PBS) and switched to BIO-MPM-1 serum free media (Biological Industries). The serum free media was supplemented with 2 mM L-glutamine, 100 μg/mL Primocin, and a 1:700 dilution of tissue-culture media protease inhibitor cocktail (Sigma-Aldrich). Cell culture supernatant was harvested at approximately 72 hours post-transfection and clarified by two rounds of centrifugation (500 g for 5 minutes, 2000 g for 10 minutes). Each protein-containing supernatant was stored overnight at 4°C and purified the next day with 3mL HisPur nickel-NTA columns (ThermoFisher Scientific) according to the manufacturer's instructions. The proteins were washed and concentrated in D-PBS using the Vivaspin 20 sample concentrators (GE Healthcare). A NanoDrop One instrument (ThermoFisher Scientific) was used to determine the concentration of each protein.

Generation of recombinant virus

HEK293T or GaleGalk2 KO cells were seeded in a T-75 flask such that their confluency would reach ~60% after 24 hours. The cells were transfected with five micrograms of each virus-encoding plasmid using jetPRIME (Polyplus-transfection S.A.) and complete media changes were performed at 24 and 48 hours post-transfection. At 72 hours, virus-containing supernatant was harvested and clarified by two rounds of centrifugation (500 g for 5 minutes, 2000 g for 10 minutes). The concentration of each virus stock was measured using a Gag-p24 antigen-capture kit (Advanced Bioscience Laboratories, Inc.). All viruses were stored at –80°C prior to experimentation.

Jacalin ELISA

Equal amounts of His-purified gp120 were loaded into duplicate wells of a high-protein-binding 96-well plate and left to incubate for 1 hour at 37°C. The wells were washed six times with 0.05% Tween 20 in PBS and blocked for 1 hour at 37°C in three hundred microliters of RIPA buffer (Life Technologies). The wells were then washed six times with 0.05% Tween 20 in PBS and one hundred microliters of horseradish peroxidase-conjugated jacalin (jacalin-HRP) (United States Biological, Swampscott, MA) was added at concentration of 2 μg/mL. After a one-hour incubation at 37°C, the wells were washed ten times with 0.05% Tween 20 in PBS. One hundred microliters of tetramethylbenzidine (TMB) solution (EMD Chemicals, Gibbstown, NJ) was added to each well. The wells were allowed to develop for 30 minutes before stopping the reaction with 100 μL of 1M phosphoric acid. The plates were read at 450 nm on a spectrophotometer.

V3-glycan bNAb binding assay

Three V3-glycan-dependent broadly neutralizing antibodies (PGT121, PGT128, and 10-1074) were tested for their ability to bind the HEK293T-, Gale- or GaleGalk2-produced HIV-1 gp120s. Test plates were coated with equal amounts of each gp120 overnight at 4°C. The plates were washed twice with 0.05% Tween 20 in PBS and blocked with 5% nonfat dry milk in PBS (Bio-Rad). Each bNAb was diluted to 2 μg/mL in blocking buffer and four-fold serial dilutions were made nine times. The serial dilutions of antibody were added to the corresponding wells, incubated for 1 hour at 37°C, and washed five times with 0.05% Tween 20 in PBS. An HRP-conjugated goat anti-rhesus IgG H^+L (SouthernBiotech) was then added to each well and allowed to incubate for 1 hour at 37°C. Next, the plates were washed 10 times, fifty microliters of TMB substrate was added to each well, and the reaction was stopped with TMB stop solution (SouthernBiotech) after 5 minutes. The plates were read at 450 nm on a spectrophotometer.

Western blot

Two micrograms of each purified gp120 was loaded into three 4%–12% SDS-PAGE gels, which were all run in parallel for 50 minutes at 165 V. The first gel was analyzed by Coomassie staining using the eStain 2.0 Protein Staining Device (Genscript). The proteins run on the second and third gels were transferred to two PVDF membranes using the iBolt Dry Blotting System (Life Technologies). The first membrane was probed for either SIVmac239 gp120 using 500 ng/mL rhesus anti-gp120 monoclonal antibody 3.11H (purified in-house) or HIV-1 gp120 using 1 μg/mL pan-HIV-1 mouse anti-gp120 (Immune Technology). The appropriate HRP-conjugated species-specific secondary antibodies were used in accordance with the manufacturer's instructions. The third PVDF membrane was blocked for 90 minutes in RIPA buffer, probed for O-glycosylation using 4 μg/mL of jacalin-HRP (United States Biological, Swampscott, MA) in RIPA buffer, and washed five times in RIPA buffer to remove excess jacalin-HRP. Specific signals were detected by an enhanced chemiluminescence system dependent upon the SuperSignal West Pico chemiluminescent substrate (Pierce). Each image was acquired using a Fujifilm LAS-4000 instrument.

TZM-bl cell assays

The TZM-bl cell infectivity assay was performed as previously described (Silver et al., 2019). To determine the particle infectivity of our recombinant viruses, each virus stock was diluted to a concentration of 80 ng Gag-p24/mL. Two-fold serial dilutions were performed eight times, in triplicate, in a 96-well plate. One hundred microliters of each dilution condition was transferred to a 96-well plate containing 10,000 TZM-bl cells per well. The 96-well plate containing virus and cells was stored in a 37°C incubator with 5% CO_2 for 48 hours, at which point viral infectivity per nanogram Gag-p24 was determined as a function of luciferase activity using the britelite plus kit (PerkinElmer).

The assay to assess jacalin inhibition of virus infectivity was performed similarly to the standard TZM-bl cell infectivity assay. However, to allow for the addition of jacalin while keeping the virus concentration constant, each virus stock was initially diluted to 107 ng/mL instead of 80 ng/mL. Seventy-five microliters of each virus dilution was transferred to a 96-well plate. Unconjugated jacalin (Vector Laboratories) was reconstituted in HEPES-buffered saline to a concentration of 2 mg/mL. Twenty-five microliters of jacalin was added to each virus condition and incubated for one hour prior to the addition of 10,000 TZM-bl cells to each well. After 48 hours, viral infectivity was measured using the britelite plus kit.

The neutralization assay was performed similarly to the infectivity assay except that a single concentration of virus was used that resulted in approximately 150,000 relative light units (RLU) after 48 hours. Each monoclonal antibody was tested at a primary concentration of 50 μg/mL and titrated five-fold, seven times. The antibody and virus were incubated together for one hour prior to the addition of 10,000 TZM-bl cells to each well. The panel of antibodies tested recognize the CD4 (3BNC117, VRC01, VRC07-523), V1/V2-glycans (PG9, PG16, PGDM1400, CAP256-VRC26.25), V3-glycans (10-1074, PGT121, PGT128), gp120/41

(8ANC195), and the membrane proximal external region (MPER) of gp41 (4E10, 2F5). The jacalin neutralization assay was performed similarly except that the lectin was tested at a primary concentration of 1 mg/mL and titrated four-fold, seven times.

Mass spectrometry

The gp120 glycoprotein variants, isolated as described above, were treated as previously described (Jang-Lee et al., 2006). Briefly, 200 μg of gp120 glycoprotein was subjected to reduction in 4 M guanidine-HCl (Pierce), carboxymethylation, and digestion with trypsin. The digested glycoprotein was then purified by Oasis Plus HLB Sep-Pak (186000132, Waters Corp.). O-glycans were released from the digested gp120 glycoprotein by reductive elimination (55 mg of KBH_4/mL in 0.1 M KOH), followed by 50W-X8 Dowex beads column (44509, Sigma). Released O-glycans were permethylated using the sodium hydroxide procedure and purified by a classic short C_{18}-Sep-Pak (WAT051910, Waters Corp.).

Permethylated O-glycans were analyzed using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF/ TOF MS/MS. MS and MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. Permethylated samples were dissolved in 10 μL of methanol, and 1 μL of dissolved sample was premixed with 1 μL of matrix (10 mg/mL 3,4- diaminobenzophenone in 75% (v/v) aqueous acetonitrile), spotted onto a target plate, and dried under vacuum. For the MS/MS studies, the collision energy was set to 1 kV, and argon was used as collision gas. The 4700 Calibration standard kit, calmix (Applied Biosystems), was used as the external calibrant for the MS mode, and [Glu1] fibrinopeptide B human (Sigma) was used as an external calibrant for the MS/MS mode.

For analysis of mass spectra, data were processed using Data Explorer 4.9 Software (Applied Biosystems). The processed spectra were subjected to manual assignment and annotation with the aid of a glycobioinformatics tool, GlycoWorkbench (Ceroni et al., 2008). The proposed assignments for the selected peaks were based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways. Proposed structures were then confirmed by data obtained from MS/MS experiments.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using the software GraphPad PRISM. Most of the data is represented as the mean ± standard error of the mean. t tests were used to discern differences between the means of two groups of samples. Linear regression analyses were used to assess the correlates of O-glycosylation signal intensity.

DATA AND CODE AVAILABILITY

There are currently restrictions to the availability of the Python computer algorithm due to licensing agreements with the University of Miami. Please contact the corresponding author with any requests.