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Characterization of a monoclonal antibody to a novel glycan-dependent epitope in the V1/V2 domain of the HIV-1 envelope protein, gp120

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Recent studies have described several broadly neutralizing monoclonal antibodies (bN-mAbs) that recognize glycan-dependent epitopes (GDEs) in the HIV-1 envelope protein, gp120. These were recovered from HIV-1 infected subjects, and several (e.g., PG9, PG16, CH01, CH03) target glycans in the first and second variable (V1/V2) domain of gp120. The V1/V2 domain is thought to play an important role in conformational masking, and antibodies to the V1/V2 domain were recently identified as the only immune response that correlated with protection in the RV144 HIV-1 vaccine trial. While the importance of antibodies to polymeric glycans is well established for vaccines targeting bacterial diseases, the importance of antibodies to glycans in vaccines targeting HIV has only recently been recognized. Antibodies to GDEs may be particularly significant in HIV vaccines based on gp120, where 50% of the molecular mass of the envelope protein is contributed by N-linked carbohydrate. However, few studies have reported antibodies to GDEs in humans or animals immunized with candidate HIV-1 vaccines. In this report, we describe the isolation of a mouse mAb, 4B6, after immunization with the extracellular domain of the HIV-1 envelope protein, gp140. Epitope mapping using glycopeptide fragments and in vitro mutagenesis showed that binding of this antibody depends on N-linked glycosylation at asparagine N130 (HXB2 numbering) in the gp120 V1/V2 domain. Our results demonstrate that, in addition to natural HIV-1 infection, immunization with recombinant proteins can elicit antibodies to the GDEs in the V1/V2 domain of gp120. Although little is known regarding conditions that favor antibody responses to GDEs, our studies demonstrate that these antibodies can arise from a short-term immunization regimen. Our results suggest that antibodies to GDEs are more common than previously suspected, and that further analysis of antibody responses to the HIV-1 envelope protein will lead to the discovery of additional antibodies to GDEs.

1. Introduction

Recombinant forms of the HIV-1 envelope (Env) protein have long been studied as HIV vaccine immunogens (Lasky et al., 1986; Berman et al., 1990). The Env protein is synthesized as a 160 kDa precursor, gp160, which then undergoes maturational cleavage to yield gp41, a membrane-bound protein that mediates virus fusion, and gp120, a peripheral membrane protein that is responsible for CD4 and chemokine receptor binding and virus tropism. In virus particles, the envelope proteins gp120 and gp41 are associated by non-covalent interactions and form a trimeric spike structure. Both gp120 and gp41 are highly glycosylated, with approximately 50% of their molecular mass attributed to N-linked glycosylation. Since both gp120 and gp41 possess epitopes recognized by neutralizing antibodies, multiple vaccine development efforts have investigated the immunogenicity of these proteins. However, after more than 30 years of effort, none of the candidate vaccines described to date have been effective in eliciting broadly neutralizing antibodies (bNAb). For many years, the inability to elicit bNAb was attributed to the inability to accurately replicate the trimeric structure of the Env protein found on the surface of viruses or virus-infected cells. However, the recent discovery of bNAb to glycan-dependent epitopes (GDEs) on monomeric HIV-1 (Walker et al., 2009, 2011; McLellan et al., 2011; Pejchal et al., 2011; Kong et al. 2013) has
raised the possibility that the inability to elicit bNAbS was due to:
(1) the inability to accurately replicate the specific glycan structure
of envelope proteins on the surface of viruses and virus-infected
cells and (2) our inability to direct antibody responses to GDEs.
Indeed, little is known about immunization regimens or adjuvant
formulations that favor the formation of antibodies to GDEs.

Of particular interest is the GDE landscape within the first and
second variable (V1/V2) domain of gp120. Although the V1/V2
domain is known as a “variable” region (Leonard et al., 1990),
numerous glycosylation sites within the V1/V2 domain exhibit
a high degree of conservation (Zolla-Pazner and Cardozo, 2010; Go
et al., 2011). Previously, it was thought that glycans on gp120 were
poorly immunogenic. This characteristic, in addition to the unusu-
ally large number of glycosylation sites on gp120, was thought to
be a major mechanism, glycan shielding, responsible for immune
escape (Wei et al., 2003; Wyatt et al., 1995; Bunnil et al., 2008; Rong
et al., 2007; van Gils et al., 2010, 2011). However, the recent dis-
covey of bN-mAbs to GDEs suggests that these epitopes are more
immunogenic than previously imagined and that a vaccine target-
ing GDEs might help to overcome the problem of virus variation.
Thus, we have begun to investigate the magnitude, specificity, and
frequency of antibodies to GDEs resulting from immunization with
recombinant Env proteins.

At this early stage of investigation, all antibodies to GDEs of
the HIV-1 envelope protein are informative; however, antibod-
ies to the V1/V2 domain of gp120 are of particular interest.
First, the V1/V2 domain contains the GDEs recognized by several bN-
mAbs (e.g., PG9, PG16, CH01-4, PG145) (Walker et al., 2009,
2011; Pejchal et al., 2011; Sanders et al., 2002; Trkola et al.,
1996; Mouquet et al., 2011; Bonsignori et al., 2011). Second, non-
neutralizing antibodies to the V1/V2 domain represent the only
antibody response found to correlate with protection in the RV144
HIV vaccine trial, which included immunization with the ALVAC-
HIV canarypox vector vaccine (vCP1521) and the AIDSVAX B/E
recombinant gp120 subunit vaccine (Berman, 1998; Berman et al.,
1999; Rerks-Ngarm et al., 2009; Haynes et al., 2012). The lack of
involvement between neutralizing antibodies and protection in the
RV144 trial caused investigators to consider numerous ways by
which non-neutralizing antibodies against the V1/V2 domain may
confer protection against infection (Liu et al., 2013). Such methods
may involve viral inactivation through antibody-dependent cell-
mediated virus inhibition, virion aggregation, or inhibition of virion
mobility and transport across mucosal surfaces (Liu et al., 2013).
Therefore, GDEs in the V1/V2 domain recognized by both neutral-
izing and non-neutralizing antibodies represent intriguing targets
for candidate vaccines to prevent HIV-1 infection.

2. Materials and methods

2.1. Production of HIV envelope proteins gp120, gp140, and
gp120 fragments

Recombinant gp140 was prepared as described previously
(Berman et al., 1989, 1990) from the 108060_Q655R clinical isola-
ted (O’Rourke et al., 2009). From this sequence, the extracellular
domain of gp160 (gp140), full length gp120, and nine overlapping
fragments of 108060_Q655R were created and expressed as pre-
viously described (Nakamura, 2012). All proteins contained a flag
epitope from herpes simplex virus glycoprotein D (gD) fused to
the amino terminus as described previously (Lasky et al., 1986;
Berman et al., 1988). All proteins were purified by immunoaffin-
ity chromatography using an immunosorbent prepared with 34.1,
a mAb to the gD flag epitope. Point mutations N130H or T132A were
introduced into a V1/V2 fragment of gp120 from the MN strain of
HIV-1 by site-directed mutagenesis, using a QuickChange Lightning
kit (Agilent, Santa Clara, CA). The resulting construct was verified
by confirmatory sequencing. Plasmids for protein expression were
transfected into FreeStyle™ 293-F cells (Invitrogen, Carlsbad, CA).

2.2. Deglycosylation studies

Digestion with the enzyme peptide-N-glycosidase F (PNGase F)
was used to remove N-linked glycans on HIV gp120 proteins or
fragments of gp120. The enzyme with its respective buffers was
obtained from New England Biolabs (Ipswich, Mass) and used per
manufacturer’s instructions. Briefly, 200 μg of recombinant gp120
or V1/V2 fragment was denatured in 10× denaturation buffer. Sam-
ple were boiled at 100 °C for 10 min, then mixed with 10× reaction
buffer and 5000 units of PNGase F. Digests were carried out at
37 °C for 12 h. To confirm digest completion, the digest product was
analyzed by polyacrylamide gel electrophoresis (PAGE) using pre-
cast polyacrylamide gels (4–12% Bis-Tris) in MOPS running buffer
(NUPAGE®, Invitrogen). Proteins were transferred to nitrocellulose
paper (Novex, Life Technologies, Carlsbad, CA). The 486 mAb was
used as the primary antibody, and the goat anti-mouse IgG/M con-
jugated to HRP (American Qualex Antibodies, San Clemente, CA)
was used as the secondary antibody. Glycan-independent control
mAbs included 34.1 (anti-gD) and 1088 (anti-V1/V2).

2.3. Immunizations

BALB/c mice from Charles River (Hollister, CA) were initially
immunized with 5 μg of 108060_Q655R gp140 incorporated in Fre-
und’s Complete Adjuvant, and then boosted multiple times over a
four-week period with 5 μg of immunogen incorporated in Freund’s
Incomplete Adjuvant.

2.4. Monoclonal antibody production

Splenocytes were harvested from immunized mice, and fused
to the mouse sp2/0 cell line to create immortalized hybridoma cell
lines using standard procedures (Kohler and Milstein, 1975). To
identify antibodies that bound the gp120 region, hybridomas were
selected using HAT media, and hybridoma subclones were screened
for secreted antibody reactivity against gD-tagged 108060_Q655R-
grpg120. Hybridoma supernatants that tested positive for reactivity
against 108060_Q655R-grpg120 in an enzyme-linked immunosor-
bent assay (ELISA) were subcloned, and the secreted mAbs were
further characterized by ELISA. These assays were carried out in flat
bottomed 96 well microtiter plates (Nunc Maxisorp®, Affymetrix,
Santa Clara, CA) and coated with 2 μg/mL of protein in PBS,
overnight at 4 °C. After 12 h, plates were blocked with PBS contain-
ing 1% BSA and 0.02% sodium azide for 1 h at room temperature.
After 1 h, PBS solution was removed, and plates were washed
four times. For initial screening, 60 μL of hybridoma supernatant
obtained directly from the 96 well culture plate was added to
the gp120-coated plates. Plates were incubated with gentle agi-
tation for 1 h at room temperature, washed four times in PBS, and
incubated for 1 h at room temperature with goat-anti-mouse, or
goat-anti-human, HRP-conjugated antibodies (Jackson ImmunoRe-
search Laboratories, West Grove, PA). Plates were then washed
four times in TBST. Plates were developed with OPD solution and
stopped with 50 μL of 3 M H2SO4. Absorbance was read at 492 nm.
The isotype of mAb 486 was determined using the Pierce Rapid
ELISA Mouse Antibody Isotyping kit (Thermo Fisher Scientific Inc.,
Rockford, IL).

2.5. Immunoassays

ELISAs were used to detect antibody binding to gp120s from
clade B, C, and CRF01_AE isolates and to overlapping fragments
of 108060_Q655R-rgp120. The gp120s and V1/V2 fragments used in these studies were similar to those described previously (Nakamura, 2012; Smith, 2010). Epitopes were further mapped using a library of overlapping peptides 15 amino acids in length derived from the MN sequence (NIH AIDS Reagent Program, Germantown, MD; catalog number 6541). Peptide ELISAs were performed with an initial coating of peptide at 5 μg/mL, and subsequent steps followed the ELISA described above. Antibody binding to peptide was tested in duplicate. The PG9 mAb used in competition assays was purchased from Polynum Scientific (Klosterneuburg, Austria).

2.6. Neutralization assays

A TZM-bl neutralization assay (Montefiori, 2005) was used to evaluate the neutralization potential of the mAbs described in this paper. The viruses used in this assay were from the clade C isolate MW965, and from clade B isolates MN, 108060_Q655R, QH0692, PV04, and JR-FL. Plasmids for the construction of all pseudoviruses with the exception of 108060_Q655R were kindly provided by Dr. David Montefiori (Duke University, Durham, NC). The positive control used for virus neutralization assays consisted of a mixture of the monoclonal antibodies b12, 2G12, and 2F5 (Tri-mAb).

2.7. Sequence alignments and amino acid numbering

The sequences of gp120s compared in this study were aligned using MAFFT (Katoh and Toh, 2008). The numbering of the amino acids described in this paper is provided using the HXB2 standard reference sequence (Los Alamos National Laboratories, HIV Sequence Compendium, Los Alamos, NM, hiv.lanl.gov/content/sequence/HIV/compendium.html).

3. Results

3.1. Immunization with 108060_Q655Rrgp140 and initial mAb screening

In previous studies, we characterized an envelope protein from the clade B clinical isolate 108060_Q655R (O’Rourke et al., 2009). This isolate contains a point mutation in gp41 that appeared to increase neutralization sensitivity to broadly neutralizing, monoclonal and polyclonal antibodies by destabilizing the pre-hairpin fusion intermediate. We postulated that immunization with this envelope protein might provide access to epitopes in gp120 and gp41 (e.g., the membrane proximal external region, or MPER) that are normally concealed until the formation of the 6-helix coiled-coil structure is triggered by the engagement of the CD4 and chemokine receptors (O’Rourke et al., 2009). Although immunization with gp140 prepared from the 108060_Q655R envelope protein did not result in antibodies with exceptional neutralizing activity, mouse hybridomas were isolated as reagents to investigate the antibody response against the unique structural features of this molecule.

To map the epitopes recognized by mAbs secreted by these hybridomas, a series of glycopeptide fragments was prepared. These were designed to contain overlapping sequences of the 108060_Q655R-gp140, similar to those previously described (Nakamura, 2012) (Fig. 1, Supplementary Fig. S1). Briefly, fragments of the 108060_Q655R-gp140 sequence were designed to contain consecutive constant and variable domains of HIV gp120. The smallest fragment contained only the V1/V2 domain (Supplementary Fig. S1F), while the largest fragment included sequences from the V1/V2 domain through the C4 domain (Supplementary Fig. S1B). Constructs were appended with an N-terminal gD flag epitope for purification purposes and were expressed by transient transfection in FreeStyle™ 293-F cells. In order to maintain as much tertiary structure as possible, the constructs were designed to preserve the disulfide bridges found in the full-length gp140. As described previously (Nakamura et al., 1993, 2012), fragments expressed by this approach are typically glycosylated, and maintain the disulfide structures required for recognition by a variety of conformation-dependent mAbs.

3.2. Isolation and characterization of mAb 4B6

Analysis of mAb binding to a panel of six different 108060_Q655R fragments (Fig. 1, Supplementary Fig. S1 panels A–F) led to the discovery of the hybridoma clone, 4B6. The mAb from this clone was found to bind to all 108060_Q655R fragments that contained the V1/V2 domain (UCSC522, UCSC545, UCSC323, and UCSC523), including the short fragment (UCSC523) that included only the V1/V2 domain (Fig. 1, Supplementary Fig. S1). In contrast, 4B6 did not bind to the two fragments (UCSC321 and UCSC521) that lacked the V1/V2 domain (Fig. 1). These data suggested that 4B6 recognizes an epitope located within the V1/V2

![Fig. 1](image-url)  
Fig. 1. Mapping of the 4B6 epitope using gD-tagged fragments of 108060-rgp120. Binding of 4B6 to various fragments of the 108060 envelope protein was assayed by ELISA. All fragments were bound by the positive control antibody to the gD tag (34.1). A mouse IgGx mAb served as a negative control. Values represent absorbance (492 nm) from single point ELISA using undiluted 4B6 cell culture supernatant. Shading indicates minimal length of epitope bound by the 4B6 antibody.
domain of 108060_Q655R_gR120. Isotype analysis of 4B6 revealed that it belonged to the IgG2a subclass (data not shown).

In order to further map the 4B6 epitope, we attempted to measure antibody binding to a series of overlapping synthetic peptides, 15 amino acids in length, from the V1/V2 domain of the MN strain of HIV-1 (NIH AIDS Reagent Program). These synthetic peptides contained neither the glycosylation nor the disulfide structures present in the secreted fragments used for the initial screening. In contrast to rabbit antibodies to MN-rgp120 that bound to multiple peptides, 4B6 was unable to bind to any of the synthetic peptides in this panel (Supplementary Table S1). This result suggested that 4B6 might recognize a conformation-dependent epitope. To further investigate this possibility, we measured binding of the native and the reduced and carboxymethylated (RCM) forms of the V1/V2 domain of MN-rgp120 (Supplementary Fig. S1, panel C). Surprisingly, we found that 4B6 bound to both the native and the RCM MN V1/V2 fragment (Fig. 2A). This result demonstrated that 4B6 recognized a conformation-independent epitope in the V1/V2 domain of both MN and 108060_Q655R gp120. The observation that 4B6 bound to the RCM form of MN V1/V2 but not to the synthetic peptides indicated that a factor other than conformation was required for 4B6 binding. We hypothesized that 4B6 binding may be dependent on the glycosylation present in the V1/V2 fragment expressed in mammalian cells, but not in the synthetic peptides.

3.3. Sequence comparison and in vitro mutagenesis to localize the 4B6 epitope

To further localize the epitope recognized by 4B6, we evaluated its ability to bind to a panel of seventeen purified gp120s with diverse sequences expressed in mammalian cells (Fig. 3). These included eleven clade B gp120s including MN, JR-FL, SC422, TR0.11, JRFL, WITO (Seaman et al., 2010), and five clinical isolates 108060_Q655R, UCSC101, UCSC109, UCSC127, and UCSC195. Additionally, we measured binding to five clade C gp120s described previously (CN97001, CN98005, IN98026, T297005, and ZA97010) (Smith, 2010) and to the CRF01 AE A244-rgp120 (Berman et al., 1999). Of the gp120s tested, only eight of the clade B sequences were bound by the 4B6 antibody (Fig. 3).

To localize the epitope recognized by 4B6, the sequences of the 4B6 binding and non-binding envelope proteins were aligned, and the amino acid sequences were compared to identify polymorphisms that segregated with 4B6 binding (Fig. 3). The sequence alignments implicated several predicted N-linked glycosylation sites (PNGSs) that were present in the gp120s that bound 4B6, and not present in gp120s unable to bind 4B6. The most promising site from this comparison was asparagine at position 130 (N130). Of note, while the clade B sequence JR-FL contains the N130 residue, it lacks the required the serine (S) or threonine (T) of the canonical N-X-S/T motif necessary for N-linked glycosylation. Therefore, mAbs dependent on glycosylation at N130 would not be expected to bind to JR-FL.

In vitro mutagenesis studies were then carried out to investigate the dependency of 4B6 binding on position 130. To prevent glycosylation at this position, we independently substituted for either the N or the T of the canonical N-X-S/T N-linked glycosylation motif. First, we replaced N at position 130 with histidine (H), because examination of multiple HIV sequence data sets revealed that histidine is the second most common amino acid at position 130 after N. The results of this study are shown in Fig. 4A. We found that replacement of N with H at position 130 completely abolished the binding of 4B6 to the V1/V2 fragment of MN-rgp120. To confirm this finding, we replaced threonine (T) at position 132 with alanine (A) in an independent construct. This substitution also disrupted the N130 PNGS and abolished the binding of 4B6 to the V1/V2 fragment of MN-rgp120 (Fig. 4B). In contrast, neither of these mutations had any effect on the binding of a positive control anti-V1/V2 mAb (1088), whose binding has been established to be independent of glycosylation (Nakamura, 2012). Together, these studies indicate that 4B6 binding is dependent on the PNGS at position 130.

3.4. PNGase treatment destroys the epitope recognized by the 4B6 mAb

To verify that 4B6 recognizes a glycan-dependent epitope, we treated the MN V1/V2 fragment with peptide-N-glycosidase F (PNGase F). This enzyme cleaves N-linked glycans at the first N-acetylglucosamine (GlcNac), effectively removing any carbohydrates on a glycoprotein. In ELISA assays (Fig. 2B), we observed that PNGase treatment destroyed the binding of the 4B6 mAb to the MN V1/V2 domain fragment. To confirm that the PNGase treatment ran to completion, and to verify the identity of the species bound by 4B6, immunoblot studies were carried out. For these experiments, the mock- and enzyme-treated proteins were probed with either 4B6 (Fig. 5A) or an anti-gD control mAb, 34.1 (Fig. 5B). We observed that the native and mock-treated V1/V2 fragment exhibited a molecular mass that was higher than expected, approximately 50 kDa. This anomalous migration was attributed to the high carbohydrate content of the V1/V2 domain which contains 9 PNGSs in 131 amino acids, including the gD tag. As expected, treatment with PNGase F resulted in a molecular mass consistent with the calculated (17 kDa) value. These results further suggested that 4B6 recognizes a GDE in the V1/V2 domain.
Fig. 3. Amino acid sequence alignment of V1/V2 domains from gp120s used for 4B6 binding studies. The binding of 4B6 to gp120s from 17 different isolates was measured by ELISA. The gp120s able to bind 4B6 are indicated by plus signs (+) and those unable to bind are indicated by minus signs (−). The sequences of all the gp120s were aligned using MAFFT (Katoh and Toh, 2008). The location of the N130 PNGs that matched the pattern of 4B6 binding is indicated by white on black lettering. The location of other predicted glycosylation sites that failed to match the pattern of 4B6 binding is indicated by black on blue lettering. Numbering is provided with reference to the standard HXB2 sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Location of N130 in the V1/V2 domain structure

Recent structural studies have shown that the V1/V2 domain forms a 4-stranded β-sheet structure with the different strands identified as A, B, C, and D (McLellan et al., 2011; Julien et al., 2013) (Fig. 6). We observed that N130 is located near the middle of the A strand and located between cysteines C126 and C131 that form disulfide bonds with cysteines in the D and B strands, respectively. Comparison of gp120 sequences showed that the N130 glycan is conserved among clade B viruses (Go et al., 2011). Examination of the 3-D structure of the V1/V2 domain showed that this N130 is located in close spatial proximity to the N160 and N156 glycosylation sites that are critical for the binding of the broadly neutralizing PG9 mAb (Fig. 6) (Walker et al., 2009; McLellan et al., 2011).
However, when we measured PG9 binding to envelope proteins lacking the N130 glycosylation site, the binding of PG9 appeared to be unaffected (data not shown).

3.6. Neutralization assays

The ability of 4B6 to neutralize various clade B or C viruses was determined in a TZM-bl neutralization assay (Montefiori, 2005). We found that 4B6 was not able to neutralize any of the clade B isolates MN, 108060, Q655R, QH0692, PV04, and JR-FL, nor the clade C isolate MW965 (Supplementary Table S2). Examination of the sequences of these viruses showed that all but JR-FL contained the glycosylation site at N130.

4. Discussion

This study documents the isolation of a mouse mAb (4B6) to a novel GDE in the V1/V2 domain of the HIV envelope protein, gp120. Four new findings derive from these studies. First, we show that antibodies to GDEs in the V1/V2 domain of gp120 can result from immunization with recombinant gp120, whereas previously such antibodies have been isolated only from HIV-infected humans or chimpanzees (Walker et al., 2009, 2011; Bonsignori et al., 2011; Trkola et al., 1995; Vlij-Warrier et al., 1996; Wu et al., 1995). Second, we show that antibodies to GDEs can result from a relatively short immunization schedule and that continuous exposure to gp120 over a long period of time, as occurs during chronic HIV infection, is not required to elicit antibodies to GDEs. Third, we show that the 4B6 epitope is common among clade B viruses. Finally, we show that while 4B6 binds to GDEs in the V1/V2 domain, this binding is not sufficient for virus neutralization, and that additional factors determine whether an antibody possesses virus neutralizing activity.

Historically, N-linked glycans have been considered poorly immunogenic, and it has been surprising to discover that a large percentage of the broadly neutralizing antibodies in sera from HIV-infected individuals are directed to GDEs on gp120 (Walker et al.,

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**Fig. 5.** Analysis of 4B6 binding to PNGase-treated MN V1/V2 fragments by immunoblot. A fragment corresponding to the V1/V2 domain of MN-gp120, expressed in 293 cells, was either treated with PNGase or treated with PNGase digest buffer alone (mock treated), and subjected to polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose paper (Novex, Life Technologies, Carlsbad, CA) and allowed to react with either the 4B6 mAb (panel A) or with 34.1, a mAb to the gD flag epitope (panel B). When probed with 4B6, untreated and mock-treated V1/V2 fragment migrates as a diffuse band of approximately 50 kDa (panel A, lanes 1 and 2); when treated with PNGase, the V1/V2 fragments runs as a sharp band of approximately 17 kDa (panel A, lane 3). When probed with the 34.1 mAb to HIV gD, untreated V1/V2 fragment migrates as a diffuse band of approximately 50 kDa (panel B, lane 1); when treated with PNGase, the V1/V2 fragment runs as a sharp band of approximately 17 kDa (panel B, lane 2). The mobility of molecular weight standards is shown in the left and right margins.

**Fig. 6.** Diagram of the V1/V2 domain. The 4-stranded β-sheet structure of the V1/V2 domain reported by McLellan et al. (2011) was modified to show the approximate location of glycosylation sites (red segments) recognized by the 4B6 mAb (N130) and the broadly neutralizing PG9 mAb (N156 and N160) (Walker et al., 2009). Letters (A–D) indicate each strand of the β-sheet structure. The two disulfide bonds at positions C126 and C131 flanking the N130 glycosylation site are also indicated (yellow). Other features including the B–C connecting peptide required for PG9 binding are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
While it has been well documented that N-linked glycans found on the HIV envelope protein function as a “glycan shield” to protect large regions of the gp120 structure from antibody binding by steric hindrance, we now know that this shield is imperfect, and that glycans can themselves become targets for neutralizing antibodies (Moore et al., 2012). Recent studies of mAbs from HIV-infected humans have identified at least two different clonal lineages of bNAb that recognize GDEs in the V1/V2 domain. These include the PG9 lineage that recognizes glycans at positions 156 and 160, and the PGT121/122 lineage that recognizes glycans at position 137 (Kwong and Mascola, 2012; Julien, 2013). Thus far, antibodies from humans recognizing the N130 GDE have not been described. However, recent pepscan analysis of clinical specimens from VAX003 and RV144 vaccine trials revealed that the portion of the V1/V2 domain containing N130 PNGS is highly immunogenic (Gottardo, 2013). The observation that the N130 site is contained within a region that is particularly visible to the humoral immune response supports the hypothesis that antibodies to the N130 GDE may additionally be found in human sera.

Studies showing that GDEs are major targets of bNAb have prompted considerable interest in developing vaccines able to elicit antibodies to this class of epitope. However, numerous uncertainties remain concerning the best approach to elicit antibodies to GDEs in gp120. We know little regarding the optimal formulation or immunization regimen to elicit antibodies to GDEs. In humans, bNAb to GDEs are seldom found until 2–3 years post-infection, following years of continuous exposure to viral antigens (Walker et al., 2009, 2011; Bonsignori et al., 2011). Based on these kinetics, it has been postulated that antibodies to GDEs might only occur as the result of continuous exposure to highly glycosylated viral antigens, or as a consequence of breakdown in immune tolerance of the glycan structures resulting from HIV infection. Indeed, several groups are pursuing the possibility of “guided” immunization strategy with viral proteins recovered from the sequential virus isolates thought to have guided the evolution of antibodies to GDEs with broadly neutralizing activity (Kwong and Mascola, 2012; Bonsignori et al., 2012; Klein et al., 2013). It is postulated that this approach might be effective in driving the evolution of antibodies with the atypical structures found in most bNAb, such as long CDR H3 domains (>20 amino acids) resulting from unusual VDJ splicing, or highly mutated antibody genes (>100 nucleotide changes) resulting from unusually high levels of somatic mutation (Kwong and Mascola, 2012). Moreover, bNAb to GDEs often appear to bind simultaneously to two different glycan moieties (e.g., N156 and N160 in the case of PG9, and N302 and N332 in the case of PGT128) (Kong et al., 2012). Our isolation of 486 demonstrates that antibodies to GDEs can arise in healthy, uninfected animals as a result of a short intensive immunization regimen with recombinant envelope protein. Further work to sequence and solve the structure of this antibody would provide insight as to whether its inability to neutralize HIV results from a lack of structural features (e.g., long CDR H3 domain, etc.) common to bNAb.

Finally, these studies highlight the possibility that antibodies to GDEs are more common than previously appreciated. This insight has implications for our understanding of the protective immune responses to the highly glycosylated proteins of other pathogens such as cytomegalovirus (Britt and Vugler, 1989), SARSCoV, influenza, and West Nile virus (Vigerust and Shepherd, 2007). Until recently, vaccines were developed to epitopes that were primarily amino acids or carbohydrates, but not a combination of both. It now appears that another important class of epitope exists, exemplified by 486 and by bNAb to HIV that depend on both amino acid and N-linked glycan contacts. Further studies evaluating the frequency and specificity of these antibodies, as well as studies aimed to elucidate the best method to elicit such antibodies, may present important medical insights and applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2014.06.025.

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