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V1/V2 domain scaffolds to improve the magnitude and quality of protective antibody responses to HIV-1

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V1/V2 DOMAIN SCAFFOLDS TO IMPROVE THE MAGNITUDE AND QUALITY OF THE PROTECTIVE ANTIBODY RESPONSE TO HIV-1

A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

CHEMISTRY

By

Javier F. Morales

December 2014

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Tyrus Miller

Vice Provost and Dean of Graduate Studies
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Abstract

V1/V2 DOMAIN SCAFFOLDS TO IMPROVE THE MAGNITUDE AND QUALITY OF THE PROTECTIVE ANTIBODY RESPONSE TO HIV-1

By Javier F. Morales

About 35 million people are living with HIV worldwide and in 2013 approximately 1.5 million people died from AIDS-related illnesses. There is an urgent need for a safe, effective, and affordable HIV vaccine. Ideally, a vaccine should stimulate a long-lasting protective antibody response to conserved regions of the viral surface protein Env, with a focus on gp120. Two lines of investigation have highlighted the importance of antibodies to the V1/V2 domain of gp120 in providing protection from HIV-1 infection. First, the recent RV144 HIV-1 vaccine trial documented a correlation between non-neutralizing antibodies to the V2 domain and protection. Second, multiple broadly neutralizing monoclonal antibodies to the V1/V2 domain (e.g., PG9) have been isolated from rare infected individuals, termed elite neutralizers. The binding of both types of antibodies appears to depend on the same cluster of amino acids in the B and C strands of the four-stranded V1/V2 domain β-sheet structure. However, the broadly neutralizing mAb, PG9, has an additional glycan requirement and depends on mannose-5 at position 160 for binding. Here, we describe monomeric gp120s and V1/V2 scaffolds from multiple strains of HIV-1 that can bind PG9. We further show that immunization with V1/V2 scaffolds in a prime/boost regimen with gp120, enhanced the antibody response to sequences in the V1/V2 domain associated with protection in the RV144 trial. The prime-boost approach we describe should be further investigated as a potential HIV-1 vaccine.
Acknowledgments

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Most importantly, I would like to thank my family for their love and support. I want to thank my parents for encouraging me to continue my studies. I want to thank my brothers and sisters, Chris, John, Olga, Gabe, Marisol, and brother-in-law Rogelio for always being there for me and being so supportive. Thanks Gabe for being a good listener and supporting me through school.
1.1 Introduction

The development of a vaccine able to provide protection from HIV-1 infection has long been a global public health priority (1–3). To achieve this goal, vaccine development efforts have focused on the discovery of immunogens able to elicit cellular immune responses (e.g. cytotoxic lymphocytes) or broadly neutralizing antibody (bNAb) responses (4). Cellular immune responses are detected soon after infection in most HIV-1-infected individuals (5), whereas bNAb responses are found in only 10 –20% of infected individuals (6 –12). Unfortunately, after more than 30 years of research, none of the candidate vaccines described to date have been effective in eliciting bNAbs (13–15). Thus, new approaches to elicit bNAbs must be considered. The recent isolation and characterization of multiple human bNAbs from HIV-1-infected subjects (16 –23) have now identified the epitopes responsible for much of the neutralizing activity in sera from HIV-1-infected humans (24). Over the past several years, the structures of several bNAbs in complexes with gp120 fragments have been elucidated (20, 25–31). Several of these, including PG9, PG16, CH01, CH03, and PGT145, appear to target glycan-dependent epitopes in the V1/V2 domain. PG9 and PG9-like antibodies are particularly interesting, because the epitope they recognize appears to over-
lap with an epitope associated with protection from HIV-1 infection in the RV144 HIV-1 vaccine trial (32). Structural studies showed that the binding of PG9 was highly dependent on mannose-5 glycans at positions 156 and 160, as well as basic amino acid side chains at positions 168–169 and 171 (25). These positions in the C strand are adjacent to the B-C junction of the four-stranded V1/V2 domain β-sheet structure (25).

In previous studies (33), we showed that this region contains contacts required for the binding of multiple neutralizing and non-neutralizing antibodies to the V1/V2 domain. Interestingly, although the RV144 correlates of protection analyses showed a correlation between protection and antibodies to this region, protection did not correlate with neutralizing antibodies (34, 35). Rather, protection correlated with antibody binding to the V1/V2 domain measured with a glycosylated fusion protein (V1/V2 sequences fused to murine leukemia virus gp70) and with nonglycosylated synthetic peptides from the V1/V2 domain (35–37). Based on these studies, antibody binding to positions 165–178 of the V1/V2 domain appeared to be the only immune responses, out of more than 40 examined, that correlated with protection. Additional support for the importance of this region was provided by sieve analysis (38), where lysine 169 (Lys-169) was highlighted as a residue subject to vaccine-induced immune selection. Sieve analysis is a method to detect immune selection in vaccine trials based on differences in the sequence of viruses from breakthrough infections in vaccinated
subjects with the sequences of viruses from infected placebo recipients (39 – 42).
Together, these results were surprising because they failed to support the prevailing hypothesis that has dominated HIV vaccine research for the last 2 decades, i.e. that neutralizing antibodies were required for protection from HIV-1 infection. Thus, antibodies to the V1/V2 domain might provide protection by mechanisms other than direct neutralization. These mechanisms might include antibody-dependent cellular cytotoxicity and antibody-dependent or cell-mediated virus inhibition, etc. (35, 43– 45).

As a consequence of these studies, strategies designed to enhance immune responses to the V1/V2 domain of gp120 have become the focus of intense interest for HIV-1 vaccine development. In previous studies, we showed that the two gp120 vaccine antigens (MN-rgp120 and A244-rgp120 produced in CHO cells) incorporated in the AIDSVAX B/E vaccine (42, 46) and used in the RV144 trial possess high levels of sialic acid-containing glycans and lacked the high mannose glycans required for the binding of some PG9-like bNAbs (PG9, CH01, and CH03) (47). Because PG9 binding requires mannose-5 at Asn-156 and Asn-160, we decided to investigate the immunogenicity of the gp120 and V1/V2 scaffolds produced in GnTI- cells. We reasoned that by restricting the glycosylation to mannose-5 structures, we might be able to induce broadly neutralizing PG9-like antibodies. Equally important was the possibility that this approach might enhance the production of the non-neutralizing protective antibodies observed
in the RV144 trial. In this report, we describe the construction, antigenicity, and immunogenicity of novel gp120s and V1/V2 scaffolds designed to focus the immune response to functionally significant epitopes in the V1/V2 domain. By improving the magnitude of the antibody response to the V1/V2 domain that correlated with protection, and by incorporating the glycans required for PG9 binding, we might improve the efficacy of the vaccine from the 31.2% level of protection observed in the RV144 trial to the level of 60% or higher that is widely regarded to be necessary for regulatory approval and clinical deployment (48, 49).
1.2 RESULTS

1.2.1. Binding of PG9 and PG9-like broadly neutralizing antibodies to gp120 and V1/V2 scaffolds expressed in 293F and GnT1- cells.

Previous studies suggested that PG9, the prototypic bN-mAb to the V1/V2 domain, as well as several PG9-like antibodies (including CH01 and CH03), recognized an epitope dependent on glycans at positions 156 and 160 (19, 25). In some cases, an N-linked glycosylation site at asparagine 173 can replace Asn at position 156 (25). These studies also reported enhanced binding of PG9 to trimeric forms of gp120 on the cell surface compared with monomeric gp120 and suggested that the epitope recognized by PG9 was dependent on quaternary interactions (19, 25, and 30). In previous studies, we analyzed PG9 binding to the two gp120 immunogens in the AIDSVAX B/E vaccine. Both proteins were expressed in CHO cells and found to incorporate high levels of neuraminidase-sensitive complex carbohydrate. We observed moderate binding by A244-gp120 and weak binding by MN-gp120. However, we found that PG9 binding to both antigens could be markedly improved by production of the immunogens in GnTI-293 cells that limit N-linked glycans to simple mannose-5-containing glycan structures (47). To further explore this observation, we measured the binding of PG9 to several other envelope proteins produced in either normal 293 cells or GnTI-293 cells (Fig. 1 and Table 1). As positive controls, we also measured the
binding of the 2158 mAb specific for a glycan-independent epitope in the V1/V2
domain (60) and the VRC01 mAb specific for the CD4-binding site (20). Isotype
matched IgG was used as a negative control. We observed modest binding of PG9
to gp120s from the MN and A244 isolates produced in 293 cells with EC50 values
for MN and A244 gp120 of ~0.5 and 0.4 ug/ml, respectively (Fig. 1, A and E, and
Table 1). However, when MN and A244 gp120 were expressed in GnTI- cells, we
observed increased PG9 binding with EC50 values of 0.06 and 0.007 ug/ml,
respectively (Table 1). Thus, expression in GnTI- cells resulted in an 8.3-fold
increase in relative binding affinity for MN-gp120 and a 57-fold increase in
relative binding affinity for A244-gp120. We next examined PG9 binding to gp120
from three additional clade B isolates (Fig. 1, B–D), including TRO.11, 108060, and
a variant of JRFL (JRFL_E168K) mutagenized at position 168 to enhance
neutralization by PG9 (19). In addition, we measured binding to gp120 from the
clade CRF01_AE isolate, TH023, that was incorporated in the poxvirus vector,
vCP1521, used for priming immunizations in the RV144 clinical trial (Fig. 1F) (32,
61). We found that PG9 bound to TRO.11, JRFL_E168K, and TH023 gp120s with
EC50 values of 0.4, 0.07, and 0.4 ug/ml, respectively (Table 1).
FIGURE 1.1. Effect of glycosylation on PG9 binding to monomeric gp120s from clade B and CRF01_AE isolates. The genes encoding gp120s from the clade B MN, TRO.11, 108060, and JRFL_E168K isolates and the CRF01_AE A244 and TH023 isolates were transfected into either normal 293F cells (open circles) or GnTI- 293 cells (closed circles) that restrict N-linked carbohydrate to mannose-5 glycans. Growth-conditioned cell culture supernatants from transient transfections were recovered, and the gp120 was purified by affinity chromatography. The proteins were then captured on microtiter plates, and the binding of PG9 was measured by ELISA. Isotype-matched IgG served as the negative control (open and closed triangles). Antibody binding by mAbs 2158 and VRC01 was included as positive control. The source of gp120 used in each assay is indicated in each panel (A–H).
Table 1.1
Relative affinity (EC$_{50}$) of PG9 binding to gp120 and scaffolds expressed in normal and GnTI- 293 cells

<table>
<thead>
<tr>
<th>Construct</th>
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<th>293</th>
<th>GnTI-</th>
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<tr>
<td>gp120</td>
<td>MN</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>TRO.11</td>
<td>0.4</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>108060</td>
<td>&gt;10</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>JRFL_E168K</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>A244</td>
<td>0.4</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>TH023</td>
<td>0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>V1/V2 scaffolds</td>
<td>MN</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>TRO.11</td>
<td>&gt;10</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>108060</td>
<td>&gt;10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>JRFL_E168K</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>A244</td>
<td>0.4</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>TH023</td>
<td>0.6</td>
<td>0.01</td>
</tr>
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aData represent the concentration of PG9 required for 50% binding (EC$_{50}$) to gp120s or V1/V2 scaffolds coated to wells of microtiter plates (see Methods).

The gp120 from the 108060 clinical isolate (62) was unable to bind PG9 at a concentration of 10 μg/ml. We next examined the binding of PG9 to these proteins produced in GnTI- cells. We found that the binding of PG9 to all six gp120s was greatly enhanced by restricting the glycans to mannose-5 structures. EC$_{50}$ values for TRO.11, 108060, JRFL_E168K, and TH023 were 0.08, 0.9, 0.01, and 0.03 μg/ml, respectively. Thus, there was a 5–13-fold increase in the relative binding affinity for PG9 when these proteins were produced in GnTI- cells. With the exception of 108060, all of these gp120 monomers appeared to possess structural features required for the binding of PG9 when expressed in 293 cells,
and this binding could be improved by incorporation of mannose-5 glycans (Table 1). From these studies, we also concluded that the A244 Env was unusual in its ability to bind PG9 with high affinity. This result agrees with previous studies demonstrating that gp120 from some viral strains of HIV-1 possess structural features that preserve the PG9 epitope better than others (25, 30, 63–65).

Because protection in the RV144 trial correlated with antibodies to the V2 domain (35–37, 66, 67), a new goal of HIV vaccine research has been to improve the magnitude and affinity of the immune response to this region. Although production of gp120 in GnTI- cells might improve the affinity of antibody binding to the PG9 epitope, it would not be expected to improve the immunogenicity of the V1/V2 domain nor the magnitude of the antibody response, which in the RV144 trial rarely exceeded serum dilutions of 1:300 (36, 37). To improve the magnitude of the immune response to sequences in the V1/V2 domain (residues 165–178) that correlated with protection (Fig. 2A), and at the same time elicit PG9-like antibodies, we investigated development of glycopeptide fragments (scaffolds) from the V1/V2 domain for use as potential vaccine immunogens. In previous studies (33, 52), we reported that fragments of gp120, expressed with the signal and N-terminal sequences from herpes simplex virus type 1 gD, could be secreted into growth-conditioned cell culture medium. These scaffolds were glycosylated and properly folded, as indicated by the binding of multiple conformation-dependent, functionally significant mAbs. In initial studies, we
examined PG9 binding to V1/V2 scaffolds (Fig. 2B) from six different strains of HIV-1 expressed in 293 and GnTI-293 cells. As positive controls, we also measured the binding of the 2158 and 697–30D mAb specific for glycan-independent epitopes in the V1/V2 domain (60, 68). Isotype-matched IgG was used as a negative control. The sequences of the V1/V2 domains for the six scaffolds are shown in Fig. 3. We found that three of the six scaffolds (e.g. MN, 108060, and JRFL_E168K) exhibited little or no binding to bind PG9 when expressed in either 293 or GnTI-293 cells (Table 1 and Fig. 4, A, C, and D). However, scaffolds from the A244, TRO.11, and TH023 isolates all bound PG9 with EC50 values of 0.004, 0.5, and 0.01 ug/ml, respectively, when expressed in GnTI- cells (Fig. 4, B, E, and F). The relative affinity of the A244 V1/V2 scaffold produced in GnTI- cells for PG9 was comparable with that observed for the full-length gp120 protein. The fact that gp120s and V1/V2 scaffolds from the two CRF01_AE isolates, A244 and TH023, both bind well to PG9 suggests that they possess structural features that enhanced the stability of the PG9 epitope compared with the four clade B isolates tested (Table 1). To further explore the antigenicity of the V1/V2 fragments, we measured the binding of five PG9-like antibodies, PG16, CH01, CH02, CH03, and CH04 (19, 22), to the A244 and TH023 fragments. We found that the A244 V1/V2 scaffold was able to bind PG9, CH01, and CH03 but none of the other PG9-like antibodies (data not shown). Interestingly, the TH023 scaffold bound PG9 with high affinity but not CH01 or
Thus, the A244 V1/V2 fragment better preserved the antigenic structure of the V1/V2 domain than the corresponding fragment from the TH023 Env. Based on these results, the A244 V1/V2 scaffold was selected for further studies.
FIGURE 1.2. Diagram of the V1/V2 domain and the A244 V1/V2 domain scaffold. A, ribbon diagram of the three-dimensional structure of the V1/V2 region based on the structure of McLellan et al. (25). The approximate location of N-linked glycosylation sites (asparagine residues 156 and 160) required for PG9 binding are shown. The side chain corresponding to Lys-169 identified by sieve analysis (38) is indicated in teal. Disulfide bonds are shown in yellow. The approximate location of the synthetic peptides used for antibody binding studies is indicated as follows: A–B (green), B–C (red), and C–D (blue). B, diagram of the A244 V1/V2 scaffold used for antibody binding studies and immunization based on the two-dimensional structure of Leonard et al. (97). The gD tag epitope used for immunoaffinity purification is shaded in black. C, sequence for the V1-V2 domain of A244 gp120 is shown along with the sequence of the synthetic peptides adjacent to the junctional regions used for antibody binding studies. The approximate location of the A–D strands is noted in gray arrows based on the structure of McLellan et al. (25). The location of predicted N-linked glycosylation sites in the V1/V2 sequence is indicated by red letters.
FIGURE 1.3. **Comparison of V1/V2 domain sequences from envelopes and scaffolds used for PG9 binding studies.** Sequence numbering was made with reference to the V1/V2 domain of HXB2. Sequences were aligned using MAFFT (98). Arrows above indicate the A, B, C, and D strands of the four stranded β-sheet structure described by McLellan et al. (25). Thin lines show the connecting peptides between the strands involved in the β-sheet.
FIGURE 1.4. Effect of glycosylation on PG9 binding to V1/V2 domain scaffolds from clade B and CRF01_AE isolates. The genes encoding gp120 V1/V2 scaffolds from clade B isolates (MN, TRO.11, 108060, and JRFL_E168K) and CRF01_AE isolates (A244 and TH023) were transfected into either normal 293F cells (open circles) or GnTI- 293 cells (closed circles) that restrict N-linked carbohydrate to mannose-5 glycans. Growth-conditioned cell culture supernatants from transient transfections were recovered, and the V1/V2 scaffolds were purified by immunoaffinity chromatography. The proteins were then captured on microtiter plates, and the binding of PG9 was measured by ELISA. Isotype-matched IgG served as the negative control (open and closed triangles). The binding of two mAbs to the V1/V2 domain that do not require glycans for binding (2158 and 697-30D) served as positive controls. The V1/V2 scaffold used in each experiment is indicated in each panel (A–L).
1.2.2. Physical Characterization of the A244 V1/V2 Scaffold

We next characterized the biophysical characteristics of the A244 V1/V2 scaffold. We first examined the size of the scaffolds produced in normal and GnTI- 293 cells by PAGE. The results of these studies are shown in Fig. 5. We observed that the V1/V2 scaffold produced in normal 293 cells ran as a diffuse smear of 38–55 kDa (Fig. 5, lanes 3 and 9). The apparent molecular mass was far greater than the 25-kDa mobility expected based on the amino acid sequence. Additionally, we were surprised to see that the A244 V1/V2 scaffold produced in GnTI- cells ran as three discrete bands of 38, 40, and 45 kDa (Fig. 5, lane 2). To determine whether this variation was a consequence of proteolysis or variation in carbohydrate structure, we treated the A244 V1/V2 scaffold produced in GnTI- cells with PNGase F to remove all of the N-linked carbohydrate or with endoglycosidase H specific for the high mannose carbohydrate (47, 69). For these experiments, the proteins were visualized by Coomassie staining and immunoblotting. Treatment of the A244 V1/V2 scaffold with PNGase F (Fig. 5, lanes 1, 4, and 8) resulted in a single band of 14 kDa, whereas treatment with endoglycosidase H (lane 5) resulted in a single band of ~17 kDa. These results demonstrated that the V1/V2 scaffold was highly glycosylated and that the differences among the three different bands seen in the scaffold produced in GnTI- cells were due to the amount of carbohydrate attached. A large difference in the size of the glycoprotein scaffold before and after treatment with PNGase F was
understandable in view of the fact that there are nine PNGSs within the V1/V2 fragment of A244. Our results suggested that the three bands observed with the scaffolds produced in GnTI- cells are likely to be attributable to differences in the usage of particular PNGSs.
FIGURE 1.5. Physical characterization of the A244 V1/V2 scaffold produced in GnTI- cells. A, purified A244-V1/V2 scaffolds were analyzed by mobility on 4–12% reducing SDS-polyacrylamide gels. Lanes 1–3 represent a Coomassie-stained gel of the purified V1/V2 fragments expressed in GnTI- cells (lanes 1 and 2) or 293 cells (lane 3) before (lanes 2 and 3) or after (lane 1) digestion with PNGase F. In lane 1, the band corresponding to PNGase F enzyme is indicated by an asterisk. Lanes 4 and 5 represent immunoblots of the A244-V1/V2 fragment expressed in GnTI- cells after digestion with PNGase F and endoglycosidase H. Lane 6 represents the untreated A244 V1/V2 scaffold produced in GnTI- cells. Lane 7 represents A244 V1/V2 scaffold purified by immunoprecipitation with the PG9 mAb. Lane 8 is the V1/V2 fragment expressed in 293F cells treated with PNGase F. Lane 9 is the V1/V2 fragment produced in normal 293F cells. Proteins in lanes 4–6, 8, and 9 were detected with a monoclonal antibody to the gD tag epitope. B, mass spectroscopy analysis of V1/V2 peptides resulting from the treatment of the V1/V2 scaffold with trypsin and PNGase F. Site occupancy (PNGS utilized) was determined by the change in mass of asparagine to aspartate. The calculation of glycan occupancy of each site is provided in D. C, table of the glycopeptides generated by trypsin digestion of the V1/V2 scaffold and the location of potential glycosylation sites (red N). D, predicted and observed mass of fragments from trypsic digest of V1/V2 fragments detected by mass spectroscopy. †, peptides potentially resulting from cleavage of the V1/V2 fragment by trypsin. Cleavage products of two or fewer amino acids are not shown. ‡, After PNGase F treatment, the N-linked asparagine in the canonical NX(S/T) sequon is converted to aspartic acid with +1 atomic mass unit shift. E, circular dichroism of purified A244-V1/V2 scaffold expressed in GnTI- cells before (solid blue line), and after (dashed red line) denaturation by reduction and carboxymethylation.
To explore this possibility, we carried out mass spectroscopy studies. For these studies, the A244 V1/V2 scaffold was digested with trypsin and then PNGase F. The resulting fragments were then analyzed by mass spectroscopy (Fig. 5, B–D). Six peaks (T4, T5, T6, T7, T9, and T10) containing PNGSs were generated. We found differences in the utilization of the PNGSs at positions 136 and 142 in the V1 domain (fragment T5), at positions 145 and 148 of the V1 domain (fragment T6), and position 186 in the V2 domain (fragment T9) (Fig. 5, C and D). However, we found that the glycosylation sites at positions 156 and 160, required for PG9 binding, were fully utilized in fragment T7 that contained both glycosylation sites. Thus, the differences between the sizes of the three bands seen in polyacrylamide gels were attributed to differences in glycosylation at positions in the V1/V2 domain that did not appear to affect PG9 binding. Additionally, immunoprecipitation experiments (Fig. 5A, lane 7) demonstrated that PG9 was able to bind all three bands. Although initial structural studies of PG9 (25) demonstrated that the V1/V2 domain is a four-stranded anti-parallel β-sheet, structural studies with other mAbs to the V1/V2 domain suggested that portions of the V2 domain can also assume an a-helical structure (64). To further investigate the structure of the A244-V1/V2 scaffolds produced in GnTI- cells, we carried out circular dichroic studies (Fig. 5E). We observed an absorbance band at 218 nm, clearly indicating the presence of the β-sheet structure. When we measured the absorbance of the same protein that had been reduced and
carboxymethylated to destroy the secondary and tertiary structure, the pattern changed to that characteristic of a random coil (70). Thus, circular dichroism provided evidence that the β-sheet structure was preserved in the A244 V1/V2 fragment produced in GnTI- cells.

1.2.3. Preliminary Immunogenicity Studies of A244-gp120 and V1/V2 Scaffolds Produced in GnTI- Cells

Although we had succeeded in identifying molecules (A244-gp120 and A244-V1/V2) with the antigenic structure required for the binding of some, but not all, PG9-like antibodies, we next wanted to characterize the immunogenic properties of these proteins. For this purpose, a pilot immunogenicity study was carried out in rabbits using a single adjuvant formulation (Freund’s adjuvant). The design of the experiment is provided in Table 2. Group 1 received A244-gp120 produced in 293 cells; group 2 received A244-gp120 produced in GnTI- 293 cells; group 3 received a priming immunization with A244-gp120 produced in GnTI- cells and booster immunizations with the A244 V1/V2 scaffold produced in GnTI- cells; and group 4 was immunized exclusively with the A244 V1/V2 scaffold produced in GnTI- cells. Because most bNAbs to HIV-1 are known to be highly evolved compared with their corresponding germ line genes, we carried out a prolonged (six dose) immunization schedule, using a minimal amount of antigen, intended
to drive clonal expansion and immune selection. Antibody binding studies were carried out with pooled sera from the last immunization.

We first looked at antibody titers to A244-gp120 produced in GnTI- cells (Fig. 6). The highest titers were observed in the group 1 animals, suggesting that A244-gp120 produced in normal 293 cells, and able to incorporate sialic acid, was somewhat more immunogenic than gp120 produced in GnTI- cells. Surprisingly, we observed titers in the group 3 animals (Fig. 6A) that were comparable with the animals in group 2, despite the fact that they had received only one immunization with full-length gp120. This result suggested that the duration of the immune response to gp120 is sustained far longer in rabbits than in humans or non-human primates immunized with gp120 (39, 41, 51, 71–74). We next examined the relative immunogenicity of the V1/V2 domain as a function of immunization regimen. We found that the animals in all four groups developed high titers to the V1/V2 scaffold (expressed with a His6 tag epitope), with 50% end point dilution titers between 1:10,000 and 1:30,000 (Fig. 6B). These studies suggested that the V1/V2 domain is highly immunogenic, either within the context of gp120 or as an isolated fragment. They also raised the possibility that far higher titers to the V1/V2 domain are detected using a properly folded fragment of the V1/V2 domain than previously reported using synthetic peptides from the V1/V2 domain (36, 37, 41).
**TABLE 1.2**

Immunization study to compare the immunogenicity of glycan-engineered envelope proteins and scaffolds from the A244 isolate of HIV-1

<table>
<thead>
<tr>
<th>Group(^a)</th>
<th>Primary</th>
<th>Booster</th>
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<tbody>
<tr>
<td>1</td>
<td>gp120 (293)</td>
<td>gp120 (293)</td>
</tr>
<tr>
<td>2</td>
<td>gp120 (GnTI-)</td>
<td>gp120 (GnTI-)</td>
</tr>
<tr>
<td>3</td>
<td>gp120 (GnTI-)</td>
<td>V1/V2 (GnTI-)</td>
</tr>
<tr>
<td>4</td>
<td>V1/V2 (GnTI-)</td>
<td>V1/V2 (GnTI-)</td>
</tr>
</tbody>
</table>

\(^a\) Each group consisted of three rabbits that were immunized as described under “Experimental Procedures.”
FIGURE 1.6. Immunogenicity of A244 gp120s and V1/V2 scaffolds expressed in GnTI- cells. Rabbits were immunized with A244-gp120 produced in 293 cells (group 1), A244-gp120 produced in GnTI- cells (group 2), or protocol was used in group 3, where rabbits received one injection of A244- gp120 and four injections of the V1/V2 scaffold, both produced in GnTI- cells. Data obtained with pooled sera from each group is show in A–E. F–I represent sera from the three individual rabbits in group 4. EC50 values were deter- mined by standard end point dilution ELISA for antibody binding to the A244 V1/V2 scaffold, the B-C peptide (NMTTELKDVKKVHA) from the V1/V2 domain, and the V3 crown peptide (SITIGPGQVYR) from A244 gp120. TZM-bl neutralization titers were carried out using pseudoviruses from the TH023 strain of HIV-1.
1.2.4. Antibody Binding to Synthetic Peptides Based on the V1/V2 Structure

We then examined the immune response to synthetic peptides from different regions of the V1/V2 scaffold. For this purpose, we measured antibody binding to a novel series of synthetic peptides designed on the basis of the three-dimensional structure (Fig. 2A) and previous epitope mapping studies (33). In those studies, we reported that the immunodominant epitopes recognized by most mAbs targeting the V1/V2 domain are located at sequences adjacent to the exposed turns and connecting peptides between adjacent strands in the four-stranded β-sheet structure of the V1/V2 domain (33). Antibody binding to these junctional regions was measured with synthetic peptides that included sequences from the A-B, B-C, and C-D junctions as shown in Figs. 2A and 3. In this regard, it is interesting to note that the B-C peptide contained the sequence that correlated with protection in the RV144 trial (36), Lys-169 identified by sieve analysis (38), as well as the basic amino acids at Lys-168, Lys-169, and Lys-171 required for PG9 binding (75). The C-D peptide was noteworthy because it possessed the canonical LD (I/V) sequence required for α4β7 binding to gp120 (33, 76). When we examined the titers to all three peptides (Table 3), we found that the highest levels of antibodies were to the A-B peptide, regardless of immunogen. Thus, the A-B peptide appears to be the immunodominant epitope in the V1/V2 domain. When antibody responses to the B-C peptide (Table 3 and Fig. 6C) were examined, we found that this region was poorly immunogenic in
animals immunized with full-length gp120 (titers in the range of 1:1500) regardless of glycan composition; however, the immunogenicity was enhanced 6–10-fold by immunization with the V1/V2 scaffold. Similarly, the immune response to the C-D peptide (Table 3) was enhanced by immunization with the V1/V2 scaffold. Thus, immunization with the V1/V2 scaffold increased the titers to all three regions of the V1/V2 domain, compared with what could be achieved by immunization with gp120 alone.

1.2.5. Peptide Microarray Analysis of Antibody Binding to the V1/V2 Domain

To further explore the immunogenicity of the V1/V2 domain, the sera from all four groups were analyzed by peptide microarray analysis with overlapping 15-mer synthetic peptides from A244 V1/V2 domain (Fig. 7). These studies suggested that the most immunogenic regions of the V1/V2 domain included the N terminus of the V1 domain (peptides 27–30) and the C-D connecting peptide containing the canonical LDI(L/L)DV α4β7-binding site (peptides 42–44). Interestingly, the lysine-rich peptides (peptides 39–40) containing the sequences that correlated with protection in the RV144 trial, and important for PG9 binding, were poorly immunogenic in the gp120-immunized animals. However, antibody binding to these peptides (39–40) was enhanced in animals immunized with the V1/V2 scaffolds (groups 3 and 4). We also noted that antibody binding to the N terminus of the A strand (peptides 28–30) was
reduced in animals that received the V1/V2 scaffold compared with those immunized with gp120. These results suggest that immunization with V1/V2 scaffolds enhanced the antibody response to the lysine-rich region of the V1/V2 domain that correlated with protection in the RV144 trial and is essential for the binding of PG9.

FIGURE 1.7. Peptide microarray analysis of rabbit sera to the V1/V2 domain of A244 gp120. Synthetic peptides, 15 amino acids in length with an overlap of 11 amino acids, were immobilized on microarray slides. The sequences of synthetic peptides spotted on the slides (27–46) are indicated. The slides were then incubated with pooled immune or preimmune rabbit sera from the experiment described in Table 2 at a 1:200 dilution. Antibody binding was detected with a secondary antibody and the fluorescence intensity measured. For each spot, the mean signal intensity was measured between 0 and 65,535 arbitrary units, with relative binding indicated by shading as noted on figure.
<table>
<thead>
<tr>
<th>Binding specificity to rabbit sera to gp120s and V1/V2 scaffolds</th>
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<tbody>
<tr>
<td>Antibody binding&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Junctional peptides</strong></td>
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<tr>
<td>A-B</td>
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<td>B-C</td>
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<td>C-D</td>
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<td><strong>mAb competition</strong></td>
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<tr>
<td>PG9</td>
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<tr>
<td>CH01</td>
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<td>CH03</td>
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<tr>
<td><strong>Cross-clade binding</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>UG37 (A)</td>
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<tr>
<td>Bal.01 (B)</td>
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<tr>
<td>CN54 (C)</td>
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<tr>
<td>96ZM651 (C)</td>
</tr>
<tr>
<td>UG21 (D)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pooled sera are from rabbit groups described in Table 2.

<sup>b</sup> Data represent 50% binding (EC50) determined by end point dilution ELISA for antibody binding to the A-B, B-C, and C-D peptides indicated in Fig. 2C.

<sup>c</sup> Data from competition ELISA indicate the dilution of rabbit serum required to inhibit a fixed amount of the PG9, CH01, and CH03 mAbs binding to the A244.

<sup>d</sup> Ability of rabbit antibodies to bind gp120s from different clades. Clades A–D are indicated in parentheses.

1.2.6. Neutralizing Antibodies

We next measured the neutralizing antibody responses to pseudoviruses prepared from the tier 1 TH023 strain of HIV-1 (Fig. 6D). The sequence of TH023 gp120 closely resembles that of A244 gp120 in both the V1/V2 and V3 domains.

We observed high titers of neutralizing antibodies (1:6000 to 1:8000) to TH023.
in groups 1 and 2 that received the full-length gp120 immunogens, modest titers (1:1600) in sera from group 3 resulting from the prime/boost regimen, and low titers (1:96) in group 4 that received the V/V2 scaffold alone. The higher titers in animals that received gp120 were not unexpected, because full-length gp120 possesses multiple epitopes known to be recognized by neutralizing antibodies, including the V3 domain and the CD4-binding site (51, 77–79). However, these epitopes were not present in the V1/V2 scaffold. Because tier 1 viruses such as TH023 are particularly sensitive to antibodies directed to the V3 domain, we next examined the antibody titers to a synthetic peptide corresponding to the principal neutralizing determinant at the crown of the V3 domain (Fig. 6E). The sequence of this peptide is identical in both the A244 and TH023 strains of HIV-1. We found that the antibody titers to the V3 peptide closely paralleled the neutralizing antibody response in the gp120-immunized animals, suggesting that most of the neutralizing activity was directed to the V3 domain. The presence of V3 antibodies in group 3 was somewhat surprising, because these animals received full-length gp120 containing the V3 domain only on day 0 in the primary immunization and received the V1/V2 scaffold alone for the five subsequent immunizations. Subsequent time course studies (data not shown) demonstrated that the V3 response in rabbits is far more sustained than in humans or non-human primates (41, 51, and 71). As expected, there was no significant antibody response to the V3 domain in group 4, because these animals only received the
V1/V2 immunogen. Although the majority of the neutralizing response in groups 1–3 was likely to be due to V3 antibodies, the low (e.g. 1:96) but significant level of neutralizing antibodies in group 4 could only be due to antibodies to the V1/V2 domain.

To better understand the neutralizing antibody response in group 4, we measured the antibody response in each animal individually. When we examined the antibody responses to gp120 and to the V1/V2 scaffold (Fig. 6, F and G), we found that only rabbits 2 and 3 developed robust titers. Thus, rabbit 1 appeared to be a nonresponder. When we examined antibody titers to the B-C peptide, we found that rabbit 2 developed titers greater than 1:5000, whereas rabbit 3 possessed a titer of 1:2000 (Fig. 6G). When we examined the titers of neutralizing antibodies in these three rabbits (Fig. 6H), we found that rabbit 2 had the highest titer (1:304); rabbit 3 had an intermediate titer (1:60), and rabbit 1 had no detectable neutralizing response. Thus, there was an excellent correlation (R² = 0.9922) between neutralizing antibodies and antibodies to the B-C peptide.

1.2.7. Peptide Competition Studies

To further explore the specificity of the neutralizing antibodies, we carried out peptide competition studies with the sera from groups 2–4 in a manner similar to those described by Beddows et al. (79). In these studies, antisera were first incubated with a large molar excess of V3 peptide, and this mixture was then
added to viruses used for infectivity studies. Thus, the majority of antibodies
directed to the V3 domain should bind to the synthetic peptide and should not
be available for binding to the virus particles. The results of this assay are shown
in Fig. 8A. Preincubation of sera with the V3 peptide reduced neutralization titers
by 100-fold (e.g. from 1:10,000 to 1:100) in the group 2 animals immunized with
gp120 produced in GnTI- cells. The neutralizing activity in group 3 that received
the gp120 prime and scaffold boost was reduced from 1:2200 to ~1:300. As
expected, the neutralizing activity in group 4 was undiminished by pre-
adsorption with the V3 peptide because this group lacked V3 antibodies. To
verify that the residual neutralizing activity in sera pre-adsorbed with V3 peptide
was due to antibodies to the V1/V2 domain, additional adsorption studies were
carried out using V1/V2 domain peptides for groups 3 and 4 (Fig. 8, B and C). In
group 3, we found that pre-adsorption with the A-B peptide eliminated
approximately half the remaining neutralizing activity. With the B-C and C-D
peptides, most of the remaining neutralizing activity was eliminated; the A-D
peptide had no effect. For group 4 (Fig. 8C), there was a slight reduction in
neutralizing activity for the A-B peptide. However, a significant change in
neutralizing activity was seen with the addition of the B-C and C-D peptides.
These results demonstrate that immunization with the V1/V2 scaffolds is able to
elicit neutralizing antibodies to the B-C and C-D strands of the V1/V2 domain of
CRF01_AE viruses. However, preliminary studies (data not shown) suggest that
these V1/V2 antibodies did not exhibit the broad PG9-like neutralizing activity that we had sought to elicit.

![Graph showing neutralization of TH023 before and after adsorption with synthetic peptides from the V1/V2 and V3 domains.](image)

**FIGURE 1.8. Neutralization of TH023 before and after adsorption with synthetic peptides from the V1/V2 and V3 domains.** Pooled sera from the rabbit immunization study described in Table 2 were examined for neutralization activity with and without preincubation with synthetic peptides from the V1/V2 domain and V3 domain (see under “Experimental Procedures”). A shows the neutralization titers data for groups 2–4 with and without preincubation of serum-saturating amounts of V3 peptide. B shows neutralization data for group 3 (prime-boost immunization) after preincubation with V3 peptide alone or with V1/V2 peptides. C shows neutralization data for group 4 (V1/V2 scaffold alone immunization) before and after preincubation with excess peptides from the V1/V2 domain (corresponding to the A-B, B-C, C-D, and A-D region) alone or in combination.

### 1.2.8. Competitive Binding with PG9-like mAbs

The studies described above demonstrate that the V1/V2 scaffolds were able to elicit neutralizing antibodies to the V1/V2 domain. However, these antibodies did not exhibit neutralizing activity similar to PG9. To investigate this further, competitive binding studies were carried out with PG9 and two PG9-like mAbs...
(CH01 and CH03) reported to possess broad neutralizing activity (22).

Surprisingly, we found that the sera from both the gp120-immunized groups and the scaffold-immunized groups were able to inhibit the binding of all three of these bN-mAbs (Table 3). However, the group that received the V1/V2 scaffold alone possessed the highest inhibitory activity (1:8100 dilution), and the rabbits that were immunized by the prime/boost regimen had higher titers (e.g. 1:2700) compared with the rabbits that received gp120 alone (1:100 –1:300).

Interestingly, all four groups of rabbits possessed antibodies able to inhibit the binding of the CH01 and CH03 mAbs at high dilution. In these animals, the groups that received the scaffold (groups 3 and 4) had the highest titers of inhibitory antibodies, but the magnitude of the response in all of the groups was 10 –200-fold higher than that seen for inhibition of PG9 binding. This result suggests that CH01 and CH03 bind to distinct epitopes that are more immunogenic than the PG9 epitope. Thus, all of the immunogens and immunization regimens described here were effective in eliciting antibodies able to inhibit the binding of PG9, but immunization with the V1/V2 scaffold alone was the most effective way to elicit these antibodies. This result was consistent with the peptide microarray data.

1.2.9. Cross-reactivity Studies

Finally, we examined the cross-re-activity of the antibodies to the V1/V2 domain. The sequence of the V1/V2 domain was highly variable, due to numerous and
large insertions and deletions, and many of the mAbs reactive with the V1/V2 domain were strain-specific. However, the PG9 epitope appears to be in a highly conserved portion of this variable region, which accounts for its breadth of neutralizing activity. To determine whether the antibodies that we elicited to the V1/V2 domain recognize conserved epitopes, we measured the binding of antibodies from the four groups to recombinant envelope proteins from clades A to D. These proteins were obtained from commercial sources and did not include the highly immunogenic gD tag epitope present in the gp120 and V1/V2 constructs used for immunization. We observed (Table 3) broad cross-reactivity to gp120s from different clades in pooled sera from all four groups. As expected, the magnitude of the cross-reactive response was higher in the groups that were immunized with full-length gp120, because it possesses many more epitopes than the V1/V2 scaffold. The ability of the antibodies in group 4 to bind recombinant envelope proteins from five different clades of HIV-1 (A–D and CRF01_AE) demonstrated that this scaffold possessed epitopes that were conserved across the major clades of HIV-1.

1.3 DISCUSSION

In this paper, we describe efforts to improve the vaccine that provided protection in the RV144 trial (32). To accomplish this, we developed immunogens and immunization regimens designed to enhance antibody responses to functionally
significant epitopes in the V1/V2 domain of gp120. These include the epitopes in
the V1/V2 domain that correlated with protection in the RV144 clinical trial (32,
35) and epitopes in the V1/V2 domain targeted by bNAbss such as PG9, CH01, and
CH03 (18, 19, 22, 80). A number of significant conclusions can be drawn from
these studies. First, we found that gp120 from the A244 strain of HIV-1, included
in the AIDSVAX B/E vaccine, and used in the RV144 trial (42, 46, 81), could be
modified to bind multiple PG9-like antibodies with high affinity provided that the
proper carbohydrate (mannose-5) was incorporated. Second, we found that
fragments of A244 gp120 (e.g. scaffolds) could be isolated that also bound
multiple PG9-like antibodies with high affinity provided that mannose-5 glycans
were present. Third, we found that immunization with A244 gp120 and V1/V2
scaffolds, administered according to a prime/boost regimen, could improve
antibody responses to epitopes in the V1/V2 domain that correlated with
protection in the RV144 trial. Finally, we found that immunization with
immunogens possessing epitopes recognized by PG9-like antibodies was not
sufficient to elicit antibodies with PG9-like neutralizing activity.

Our studies demonstrate that PG9 and some PG9-like antibodies (e.g. CH01 and
CH03) are able to bind monomeric gp120s and V1/V2 scaffolds with high affinity.
This binding can be observed with proteins from some strains of HIV-1 (e.g. A244)
but not others (e.g. 108060) and is glycan-dependent, requiring trimmed
mannose structures (e.g. mannose-5). In contrast, other PG9-like antibodies (e.g.
PG16 and PGT141–145) appear much more dependent on quaternary interactions in the envelope trimer for binding and may, in the case of PG16, also depend on sialylated glycans at Asn-156 and sequences outside of the V1/V2 domain for binding (26). Several studies have also reported the binding of PG9 and PG9-like antibodies to monomeric gp120s and/or V1/V2 scaffolds from selected strains of HIV-1 (22, 25, 30, 54, 63–65, 82). In this regard, the ability to bind one PG9-like antibody does not predict the ability to bind other PG9-like antibodies. Thus, A244 V1/V2 scaffold was able to bind PG9, CH01, and CH03, whereas the V1/V2 scaffold from the closely related TH023 V1/V2 scaffold bound PG9 but not the CH01 and CH03 mAbs. The recent three-dimensional structure of the trimeric gp140 (29–31) has improved our understanding of PG9 binding. These studies showed the PG9 epitope in the BG505 isolate includes intermolecular contacts with the Asn-160 glycans from an adjacent V1/V2 protomer as well as amino acid and glycan contacts within monomeric gp120 (30, 31). However, quaternary inter-actions do not appear to be an absolute requirement for high affinity binding, because the Kd value for PG9 Fab binding to monomeric gp120 produced in GnTI- cells (31 nM) was roughly comparable with trimeric gp140 produced in GnTI- cells (36 nM). Moreover, a 10-fold difference in PG9 binding affinity (110 nM versus 11 nM) was observed between monomeric gp120 and trimeric gp140 expressed in 293 cells (30), suggesting that it is differences in glycosylation between monomeric and trimeric gp120, rather
than oligomerization, that account for the difference in PG9 binding activity. This conclusion is also supported by studies with chemically synthesized V1/V2 glycopeptides where the affinity of PG9 binding was shown to be 29.4 nM with mannose-5 at Asn-156 and Asn-160 (83).

Thus, the enhanced binding of PG9-like antibodies to trimeric envelope proteins (19, 25, and 63) is attributable to two factors. The first factor is differences in glycosylation between the HIV-1 envelope protein expressed as monomers or as trimers. Expression of gp120 monomers in normal cell lines typically leads to the incorporation of complex sialic acid-containing glycans at positions 156 and 160 in the V1/V2 domain that prevents binding by most PG9-like antibodies (47). This is also supported by a study of synthetic glycopeptides that showed that PG9 is unable to bind when sialylated glycans are present at Asn-160 (84). In contrast, gp120 expressed in the context of gp160 trimers appears to be enriched for the mannose structures required for the binding of PG9-like antibodies (13, 29, 30,65, 85–87). The second factor is that some, PG9-like antibodies are dependent on both glycans and quaternary interactions for binding. This appears to be the case for PG16 and PGT141–145 (18, 19). Thus, it may not be possible to produce gp120s or V1/V2 scaffolds able to bind all of the known PG9 family members. Recently, cleaved, soluble trimeric gp140s have been described that appear to bind most PG9-like antibodies (30, 65). However, unlike A244-gp120,
these molecules have not been produced in large quantities, tested in humans, nor associated with protection in large scale HIV vaccine trials.

A major finding of these studies was the fact that scaffolds from the V1/V2 domain of A244-gp120 expressed in GnTI- cells preserved the β-sheet structure required for PG9 binding and improved immune responses that correlated with protection in the RV144 trial. The V1/V2 domain of A244 is a complex glycan-dense structure that includes nine PNGS in the span of only 97 residues. Several previous studies have described the construction of V1/V2 scaffolds; however, they differed from those described in this report in several respects. Pinter et al. (88) described a V1/V2 scaffold from the CASE-A2 strain of HIV-1 fused to gp70 of murine leukemia virus (MLV). Antibody binding to this scaffold correlated with protection in the RV144 trial (35, 36). Immunogenicity studies found that sera to the fusion protein was cross-reactive with other V1/V2 scaffolds and neutralized two primary isolates (88). However, the scaffolds were produced in CHO cells and lacked the glycans required for the binding of PG9-like mAbs. Another series of chimeric V1/V2 scaffolds was developed for x-ray crystallography studies (25). The V1/V2 domains from various clade B and C viral strains were fused onto the protein G B1 domain (1FD6) or stabilized Saccharomyces cerevisiae Abp1 SH3 domain (JO8) (25). The scaffolds were produced with the correct glycans for binding PG9; however, the immunogenicity of these scaffolds was not reported. Recently, several groups have produced synthetic V1/V2 glycopeptides with
mannose-5 at Asn-156 and Asn-160 (83, 84, and 89). Alam et al. (83) reported the affinity of PG9 and CH01 to dimerized A244 V1/V2 glycopeptide to be 29.4 and 45.5 nM, respectively. The glycopeptides were disulfide-stabilized to preserve the β-sheet structure, and mannose-5 was chemically added to both Asn-156 and Asn-160. Additionally, Liao et al. (64) reported PG9 and CH01 binding to V1V2 fragments with EC50 values of 1.1 and 8.9 nM respectively. The relatively high affinities for these glycopeptides provide further evidence that some PG9-like antibodies can bind V1/V2 scaffolds with high affinity.

A novel aspect of this study is the use of V1/V2 scaffolds expressed in GnTI- cells for immunization studies. This is the first study that has taken glycosylation and PG9 binding into consideration in evaluating the immunogenicity of V1/V2 scaffolds. In terms of inducing broadly neutralizing antibodies, the initial results were disappointing. The rabbit sera neutralized only one neutralization-sensitive Tier 1 isolate (TH023) and failed to neutralize other tier 2 or tier 3 viruses (data not shown). However, neutralizing antibodies appear to be only one aspect of the protective immune responses that target the V1/V2 domain. The RV144 correlates of protection analysis suggested that non-neutralizing antibodies to the V1/V2 domain might be equally important in providing protection from HIV-1 infection (35, 90).
A potentially important result from our pilot immunization studies was the observation that immunization with V1/V2 scaffolds alone, or in combination with gp120 in a prime/boost strategy, appeared to improve the magnitude of the antibody response to residues (e.g. 165–178) that correlated with protection in the RV144 trial. The response obtained in the two groups immunized with V1/V2 scaffolds appeared higher than that achieved in the two groups immunized with gp120 alone. The prime/boost immunization regimen also appeared to enhance the response to the C-D junction containing the α4β7- binding site. However, the small size of the treatment groups in this experiment precluded the calculation of statistical significance, and these conclusions will require confirmation in subsequent studies with larger treatment groups.

Natural history studies of HIV suggest that bNAbs such as PG9 are only detected 1–3 years post-infection (91, 92). In this regard, it is interesting to note that the modest neutralizing activity we have observed to the V1/V2 scaffolds only occurred after a 5-month immunization regime, and that the neutralizing activity had not plateaued by the time the study terminated. Therefore, the true immunogenic potential of the gp120s and V1/V2 scaffolds that we have described will require a longer immunization schedule, larger treatment groups, and adjuvants less likely to denature the immunogens compared with Freund’s adjuvant used in this study. Additionally, prime boost studies with other vaccine
concepts (e.g. virus vectors, DNA vectors, or trimeric envelope proteins) may select for antibodies with broader neutralizing activity.

Our results highlight the differences between antigenicity and immunogenicity that must be overcome before we can elicit bNAbs. Thus, although the molecules described in this paper and the recently described cleaved and soluble gp140 trimers (29–31, 65) appear to have solved the problem of creating immunogens that replicate the PG9 epitope, we have not solved the problem of enhancing its immunogenicity. B-cell immunodominance and protein immunogenicity are poorly understood and are likely the result of a multiple factors, including the evolution of B-cell receptors as well as antigen processing and presentation. Gp120 likely contains scores of epitopes, many of which are more highly immunogenic than those recognized by PG9-like antibodies. Immunization or boosting with V1/V2 scaffolds may be the most effective way to improve the relative magnitude of antibody responses of the V1/V2 domain. In previous studies, we demonstrated that the V1/V2 domain possesses a highly conserved cathepsin D cleavage site that may diminish the immunogenicity of key epitopes in the V1/V2 domain by proteolytic degradation in vivo (93). Deletion of this site might also enhance the immunogenicity of this region. The development of vaccine immunogens able to bind at least some of the PG9 family members represents a significant improvement over the vaccines developed to date.
Finally, a major challenge to all efforts to elicit bNAbs relates to the fact that many of the bN-mAbs described to date are highly mutated and possess immunoglobulin heavy chains with long complementarity determining region H3 domains. Long complementarity determining region H3 domains are uncommon in most species available for preclinical testing (94), and a non-human primate model may not predict the immune response seen in humans. Thus, it is possible that the immuno- genic potential of vaccines designed to elicit bNAbs or induce non-neutralizing antibodies to the V1/V2 domain can only be assessed in small human immunogenicity studies (95). The fact that A244-rgp120 has an established record of safety in more than 9000 subjects (32, 96) and is one of the few HIV-1 Env proteins known to bind to the inferred germ line precursor of PG9-like antibodies (22) provides additional rationale for the continued development of immunogens based on A244-gp120 and V1/V2 scaffolds.
1.4 Methods

1.4.1 Ethics Statement

Animal experiments were performed according to the guidelines of the Animal Welfare Act. The immunization protocol (PRF2A) was reviewed and approved by the Animal Care and Use Committee of the Pocono Rabbit Farm and Laboratory, a facility that is fully accredited by AAALAC International with a current Animal Welfare Assurance on file (OLAW A3886-01).

1.4.2 Construction of gp120 and V1/V2 Fragments

Recombinant forms of gp120 from the A244, MN, TRO.11, 108060, JRFL_E168K, and TH023 isolates, as well as glycopeptide fragments of the V1/V2 domain, were expressed as described previously (33, 42, 50–52). To enhance expression, the genes were codon-optimized (53), and the Env sequences beginning 12 amino acids from the mature N terminus were fused to a fragment of herpes simplex virus glycoprotein D (gD) containing the signal sequence and a 27-amino acid N-terminal tag epitope. The tag epitope provided a convenient sequence for immunoaffinity purification using a monoclonal antibody to gD (54). To preclude the possibility of measuring antibodies to the gD tag epitope, fragments of gp120 lacking the gD tag epitope were expressed for use in antibody binding assays. For these studies, a hexahistidine tag epitope (His6) replaced the gD tag epitope.
1.4.3 Production and Purification of gp120 and V1/V2 Fragments

Plasmids for the expression of proteins were transfected into FreeStyleTM 293-F cells (Invitrogen) or into GnTI-293 cells (293 cells deficient in N-acetylglucosaminyltransferase I; ATCC No. CRL-3022) that limit N-linked glycans to simple, mannose-5-containing glycan structures (American Type Culture Collection, Manassas, VA). Transfections were carried out with polyethyleneimine, and the supernatant was collected on day 3 or day 4 (55). For gD-tagged constructs, immunoaffinity chromatography was used to purify the proteins as described previously (47). To purify His6-tagged constructs, HisTrap HP columns (GE Healthcare) were used. All proteins were buffer-exchanged into PBS post-purification. The resulting proteins were analyzed by PAGE using 4–12% precast gradient gels (Invitrogen). The purity of the preparations was estimated to be ~95%. Recombinant gp120s and gp140s used for cross-clade binding studies were obtained from the AIDS Reagent Program (CN54 product number 7749, 96ZM651 product number 10080, and Bal.01 product number 4961) (56, 57) or Polymun (UG37 number ENV001 and UG21 number ENV003).

1.4.4 Synthetic Peptides

Synthetic peptides corresponding to sequences adjacent to the junctions of the A-B, B-C, and C-D strands of the V1/V2 domain from A244 gp120 were synthesized by Genscript, Inc. (Piscataway, NJ). The sequence of the A-B peptide
(residues 132–153) was TNANLTANKNVTNVSNI-IGNITDE, the sequence of the B-C peptide (residues 160–174) was NMTTELKRDQKVHA, and the sequence of the C-D peptide (residues 173–192) was HALFYKLDVIEDNNDSEYR. A cyclized synthetic peptide (CVKLTPPSVTIARAAALINSNT-SVIKQASPKISFDPC) with spatially adjacent discontinuous sequences from the beginning of the A strand and the end of the D strand was synthesized and used in some assays.

1.4.5 Peptide Microarrays

Peptide scanning of antibody responses to the V1/V2 domain was carried out using peptide microarrays (Repliotope) synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). Briefly, 15-mer peptides from A244 gp120 were synthesized and conjugated to epoxy glass slides at the C terminus using a short hydrophilic linker in triplicates. The peptides overlapped by 11 amino acids (4-amino acid jump). Microarrays were first incubated with a 1:200 dilution of pooled immune or preimmune rabbit sera for 60 min at 30 °C. The microarrays were then washed before the addition of the secondary antibody (anti-rabbit DyLight 649 IgG, Pierce) and incubated again for 60 min at 30 °C. Following a final wash, the microarrays were then dried. Fluorescent intensity was measured and analyzed with an Axon GenePix 4200AL scanner and software. Microarrays were scanned and the resulting images were analyzed and quantified using spot recognition software (GenePix, Molecular Devices, and Sunnyvale, CA). For each
spot, the mean signal intensity was extracted (between 0 and 65535 arbitrary units), and the mean intensity value for the triplicates for each peptide was calculated. In control experiments, microarrays were incubated with secondary antibodies alone. No cross-reactivity of the secondary antibody with the peptide microarray was detected.

1.4.6 Antibodies

The PG9 and PG16 monoclonal antibodies (19) were purchased from Polymun Scientific GmbH (Vienna, Austria) and also kindly provided by the International AIDS Vaccine Initiative (New York). The CH01-CH04 antibodies were kindly provided by Dr. Barton Haynes at Duke University (Durham, NC). MAbs 2158 and 697-30D were kindly provided by Dr. Susan Zolla-Pazner. The 34.1 mAb is a mouse monoclonal antibody specific for the N-terminal gD tag epitope of HSV-1 and is used as a capture antibody in indirect ELISAs.

1.4.7 Indirect ELISAs

Assays to detect monoclonal antibody binding to gp120 were carried out with Maxisorp microtiter plates (Nunc, Rochester, NY) coated with 1 ug/ml of the 34.1 anti-gD antibody in PBS overnight at 4 °C. The plates were then washed four times with PBS containing 0.05% Tween 20 and blocked for 2 h with PBS containing 1% BSA and Tween 20 (blocking buffer). Saturating amounts of recombinant gD- gp120 were added at 5 ug/ml. Serial dilutions of PG9, CH01, or
CH03 were added beginning at 10 μg/ml. Peroxidase-conjugated AffiniPure goat anti-human IgG (Fcγ-specific) (Jackson ImmunoResearch, West Grove, PA) was used at a 1:5000 dilution. O-Phenylenediamine dihydrochloride substrate (Fisher) was developed for 10 min following the manufacturer’s suggested development time and stopped with 3 M H2SO4. The absorbance was measured at 490 nm. All steps, except coating, were done at room temperature on a plate shaker; incubation steps were for 1 h (except blocking), and all dilutions were done in blocking buffer. To detect monoclonal antibodies to the V1/V2 scaffold expressed with the gD tag epitope, the same conditions used in the gp120 ELISA were used, with the exception that the V1/V2 scaffolds were captured onto microtiter plates coated with anti-gD tag antibody 34.1 at 2 μg/ml.

1.4.8 Immunization Studies

Four groups of three New Zealand White rabbits per group were immunized using an IACUC-approved protocol designed to elicit high affinity antibodies (Pocono Laboratories, Canadensis, PA). All of the immunogens were incorporated in Complete Freund’s adjuvant for the primary immunizations, and in incomplete Freund’s adjuvant for the booster immunizations. The animals were immunized according to a 6-dose protocol designed to elicit high affinity antibodies. The protocol involved immunizations at weeks 0, 2, 4, 8, 12, and 18 with decreasing amounts of antigen and test bleeds ~2 weeks after each immunization. In the
primary immunization, group 1 received 200 ug of gp120 produced in normal 293 cells, and groups 2 and 3 received 100 ug of gp120 produced in GnTI- cells. Group 4 received 33 ug of V1/V2 scaffold produced in GnTI- cells. Booster immunizations were given at weeks 2 and 4 with 50% of the initial dose. Booster immunizations were given at weeks 8 and 12 with 25% of the initial dose. At week 18, groups 1 and 2 were boosted with the original dose of gp120, and groups 3 and 4 were boosted with 83 ug of V1/V2 scaffold. The first two immunizations were given intradermally in a volume of 400ul or less; all subsequent immunizations were given subcutaneously in a volume not exceeding 200ul.

1.4.9 Assays to Detect the Binding of Rabbit Antibodies

All rabbit antibody binding assays were done with His6-tagged versions of gp120 or V1/V2 scaffold. Binding assays were carried out by directly coating protein or peptide in PBS (0.2 and 0.5 ug, respectively) onto 96-well plates. After washing four times in PBS/Tween and blocking with PBS supplemented with 1% BSA, serum was sequentially diluted to 1:102 to 1:106 and added to the plates. The assay plates were washed four times, and HRP- labeled goat anti-rabbit IgG (Fc-specific) antibody (Jackson ImmunoResearch) diluted 1:5000 in PBS, 0.1% BSA was used for detection. O-Phenylenediamine dihydrochloride substrate was used as described above.
1.4.10 Deglycosylation Studies, SDS-PAGE, and Western Blot Analysis

PNGase F and endoglycosidase H were purchased from New England Biolabs (Ipswich, MA). Proteins were digested as described previously (47). Samples were run on NuPAGE® (Invitrogen) precast gels (4–12% BisTris) with MES running buffer. Gels were stained with SimplyBlue stain (Invitrogen) following the manufacturer’s protocol. For Western blot analysis, the 34.1 mAb was used as the primary antibody and goat anti-mouse IgG/M conjugated to HRP as the secondary antibody (American Qualex Antibodies, San Clemente, CA). Chemiluminescent substrate was used for detection.

1.4.11 Immunoprecipitation Experiments

50 ug of PG9 and 50 ug of the A244 V1/V2 scaffold were mixed in PBS buffer at room temperature for 30 min. Immune complexes were then purified using protein a resin contained in an LTS pipette tip (E4 XLS electronic pipette system, Rainin-Mettler, Toledo, OH). After washing in PBS, proteins were eluted with acid (0.1 M glycine) and fractionated by SDS-PAGE (4–12% BisTris, NuPAGE, Invitrogen). The fractionated proteins were transferred to PVDF membranes and further analyzed by immunoblot using the mouse 34.1 mAb to the gD tag epitope on the V1/V2 scaffold. Proteins were visualized by treatment with a secondary goat anti-mouse IgG/M coupled to horseradish peroxidase (American Qualex Antibodies) and visualized using chemiluminescent substrate detection.
1.4.12 Mass Spectrometry

Purified A244 V1/V2 scaffold produced from GnTI- cells (100ug in PBS) was first digested with immobilized trypsin (Thermo Fisher Scientific) resin overnight at 37 °C. The slurry was spun down, and the supernatant was collected. Digested fragments were further processed with PNGase F according to the manufacturer’s protocol (New England Biolabs, Ipswich, MA) overnight at 37 °C and reduced with 5 mM DTT. The fragments were run on a BetaBasic C18 column for separation, and the spectra were collected on a Thermo Finnigan LTQ LC-MS/MS located in the Mass Spectrometry Facility, Department of Chemistry and Biochemistry, University of California at Santa Cruz.

1.4.13 Circular Dichroism (CD) Measurements

Purified A244 V1/V2 scaffold produced from GnTI- cells was analyzed before and after being reduced and carboxymethylated (RCM). For RCM V1/V2 scaffold, 1 mg of protein was reduced with 5 mM DTT under 6 M guanidine HCl and carboxymethylated with iodoacetic acid. The resulting protein was dialyzed in PBS buffer. CD spectra for both native and RCM A244 V1/V2 scaffold produced from GnTI- cells were collected on an AVIV model 62DS spectrometer. The spectra were measured from 200 to 280 nm using a 1-nm step with an integration time of 2 s; 10 repeat scans were collected with a 1-mm path length cell. The background from the buffers was subtracted, and the data were
converted into ellipticity per residue according to the protein concentrations and path length. Data analysis was performed using GraphPad Prism software.

1.4.14 Antibody Competition Experiments

Competition experiments were performed by ELISA in a manner similar to the antibody binding experiments described above. Briefly, 0.01 µg of PG9, CH01, or CH03 was added to wells of microtiter plates coated with 0.2 µg of gp120 produced in GnTI- cells after the incubation with specific rabbit sera. In control experiments, PG9-like antibodies were added to wells incubated with preimmune rabbit sera. The binding of the human mAbs was detected using HRP-labeled secondary antibody at a 1:5000 dilution (American Qualex).

1.4.15 Virus Neutralization Studies

Virus neutralization studies were carried out using the TZM-bl assay described by Montefiori (58). A plasmid for the expression of pseudovirions from the TH023 isolate of HIV-1 was kindly provided by Drs. Victoria Polonis and Jerome Kim of the Military HIV Research Program (Walter Reed Army Institute of Research, Silver Spring, MD). Peptide adsorption of neutralizing antibodies was carried out according to the method of Beddows et al. (59), with the additional step of adding 5 µg of peptide to the rabbit sera, incubating for 1 h to adsorb specific antibodies, and then adding the mixture to the HIV-1 pseudovirions.
1.5 REFERENCES


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Chapter 2: Engineering a V1/V2 scaffold of gp120 to bind the glycan-dependent broadly neutralizing antibody PG9 when produced in normal cell lines

2.1 Introduction

The development of an effective HIV vaccine is a global health priority [1, 2]. A major goal of HIV vaccine research is to develop a vaccine able to induce broadly neutralizing antibodies (BNabs). Several efforts to stimulate BNabs have focused on structure-based approaches using scaffolds. Scaffolds provide an approach to selectively stimulate an immune response to conserved regions of the virus while at the same time minimizing the response to immunodominant variable epitopes [3-5]. We describe the development of engineered fragments of gp120 (scaffolds) designed to target antibody responses to the V1/V2 domain of gp120.

The V1/V2 is an attractive target for vaccine development [6]. Several BNabs have been found to target this region, and passive transfer of BNabs protects against HIV-1 infection in animal models at serum concentrations attainable by vaccination [7]. Further support for the V1/V2 as a target for vaccine development comes from the RV144 clinical trial. The correlates of protection analysis from this trial showed non-neutralizing antibodies to the V1/V2 correlated with a reduced rate of HIV-1 infection [8, 9].

PG9 is a BNab that targets the V1/V2 domain of gp120 [10, 11], and is one of the few quaternary-dependent BNabs that can bind some monomeric gp120’s or V1/V2 scaffolds provided the proper glycosylation (mannose-5) is present [10,
However, monomeric gp120’s or V1/V2 scaffolds from only a few strains of HIV have the capacity to bind PG9. Expression of scaffolds in normal cell lines, such as 293F and CHO cells, is problematic because these cells typically incorporate complex glycans that preclude PG9 binding [17-20]. The binding activity can be improved for some strains by expression in a mutant cell line (293-GnT1−/− cells) that lacks N-acetylglycosaminyl trasferase I (GnT1) [21], and limits glycosylation to mannose-5 glycans [10, 22]. In previous studies, we have shown several BNabs: PG9, CH01, and CH03 could bind to a V1/V2 scaffold with high affinity when produced in this cell line [22]. However, a better alternative would be to produce V1/V2 scaffolds in normal cell lines suitable for vaccine production.

In this study, we have combined HIV-1 sequence analysis with protein engineering to develop scaffolds able to bind PG9 when expressed in normal cell lines suitable for vaccine manufacturing. We show strains unable to bind PG9 could be engineered to bind this antibody through a combination of point mutations in the β-hairpin formed by the B and C strands of the four stranded V1/V2 β-sheet structure. We show hydrophobic amino acids in the B and C strands along with changes in the B-C turn effect PG9 binding. More importantly, we describe a novel V1/V2 scaffold from a clade C virus that can be engineered to bind PG9 with high affinity when produced in cell lines amenable for large scale cGMP vaccine production.
2.2 Results

2.2.1 PG9 binding to V1/V2 scaffolds from multiple clades

Scaffolds consisting of the V1/V2 domains of viral clades B, C, and E were constructed and screened for binding of PG9, a prototypic BNab targeting a glycan-dependent epitope in the V1/V2 domain. Since previous studies have shown PG9 requires mannose-5 at N156 and N160, scaffolds were expressed in normal 293F cells and 293-GnT1- cells [10, 23]. Cells were transfected with genes encoding the V1/V2 scaffolds and the resulting cell culture supernatants were tested for PG9 binding by indirect ELISA. We found that PG9 bound V1/V2 scaffolds from several strains of HIV-1 (Figure 1b; Supplemental Figure 2). The most interesting was the clade C, ZM233 isolate, which showed moderate binding to PG9 even when produced in 293F cells. This isolate is particularly interesting because it lacks a glycosylation site at N156 where isoleucine (I) replaces asparagine (N).

2.2.2 Amino acid alignment of V1/V2 scaffolds

Sequence alignment of scaffolds able to bind PG9 with those unable to bind PG9 allowed us to identify elements within the V1/V2 region that influenced binding (Figure 1c). The purpose of the alignment was to identify amino acid changes, variations in loop length, and number of N-linked glycosylation sites within the V1/V2 that affect the folding and exposure of the PG9 epitope. The crystal
structure of the V1/V2 domain (Figure 2a) shows it adopts a four stranded antiparallel β-sheet consisting of strands A, B, C, and D and the connecting loops [10]. Strands B and C form a β-hairpin with a short turn region (Figure 2b and 2c). The 3-D structure of PG9 co-crystallized with the V1/V2 domain shows the antibody interacts with the hydrophilic side of the β-hairpin. Critical contacts are made with glycans at N156 and N160 as well as basic amino acids at 168, 169, and 171. Recognizing that PG9 interacts with the hairpin we focused on amino acid changes that may stabilize this structure and enhance the binding of this antibody. Previous studies with synthetic glycopeptides have shown stabilizing the hairpin with disulfide bonds improves PG9 binding [27], and stability of the B-C hairpin may correlate with a change in neutralization sensitivity [28]. It is well-known that cross-strand interactions between side-chains and the turn region are important factors for hairpin formation and stability [29-35]. Therefore, we sought to identify amino acids from the alignment that would improve formation of the hairpin structure. Modeling the identified amino acids onto the crystal structure seemed to show favorable cross-strand interactions and turn propensity. Interestingly, many of these interactions do not appear to be contact sites required for antibody binding and may effect binding indirectly.
Figure 2.1. **Sequence alignment of V1/V2 scaffolds to identify amino acids that influence PG9 binding.** A. Diagram of the V1/V2 scaffolds used in this study. B. Summary of ELISA data. V1/V2 scaffolds from multiple clades were expressed in 293F and 293-GnT1- cells and tested for PG9 binding (Supplemental Figure 2). Several scaffolds showed good binding (e.g. A244, ZM109, and ZM233), while others did not (MN and 108060). C. A sequence alignment was constructed for the PG9 positive and PG9 negative scaffolds in order to identify elements within the V1/V2 that affect binding. As shown in the alignment many of the previously identified amino acids important for neutralization are present in these scaffolds. Amino acids in the B-C hairpin appeared to be associated with improved binding (e.g. A244 and ZM233). These included changes in the B and C strands (highlighted with green rectangles) along with changes in the turn region (highlighted with red rectangles). Strand changes identified from the alignment include the hydrophobic amino acids I156, M161, and V172, and the turn changes G164 and G167. β-strands A-D are indicated by grey arrows. The B-C turn (positions 164-167), the V1 loop, and V2 loop are shown with connecting lines.
Figure 2.2. **Mapping the mutations onto the CAP.45 V1/V2 crystal structure.** Amino acids identified from the alignment were mapped onto the CAP.45 crystal structure (ref). (A) The crystal structure of the V1/V2 shows it adopts a four-stranded β-sheet composed of strands A-D with the V1 loop connecting strands A and B and the V2 loop connecting strands C and D. (B) Strands B and C form a β-hairpin connected by a short turn region (a.a. 164-167). Positions 159, 161, and 172 are illustrated because these sites may form favorable cross-strand interactions that could improve hairpin stability. From the alignment, M161 and V172 were identified. A cluster of hydrophobic amino acids: F159, M161, and V172 may improve hairpin formation and enhance PG9 binding. (C) Amino acids at positions 164 and 167 may alter the turn propensity, improve hairpin formation, and enhance PG9 binding. From the sequence alignment glycine at 164 and 167 were identified.
2.2.3 Effect of amino acid mutations in the B and C strands on PG9 binding

We next carried out experiments where mutations identified from the alignment were sequentially introduced into a clade B V1/V2 scaffold (108060) that did not bind PG9 (Figure 3a). This isolate contains many of the amino acids required for PG9 binding with the exception that it is missing a critical contact at position 169 [10] where isoleucine (I) replaces a critical a lysine (K). We found that replacement of I for K at position 169 (I169K mutant) improved PG9 binding when expressed in GnT1· cells, but had no effect in 293F cells (Figure 3b). Since K169 is known to be a required contact, the I169K mutant became our template for further analysis.

The next mutation made was E172V. Valine was selected for several reasons. First, it is an amino acid frequently found in β-sheet structures [30]. Valine, isoleucine, and threonine have a β-branched carbon that limits the conformations the main-chain backbone can adopt [30]. Second, valine is a hydrophobic amino acid and can form cross-strand interactions with hydrophobic amino acids on opposing strands. In the B-C hairpin, V172 is in the middle of strand C and could interact with F159 on strand B. Finally, valine was found to be an important amino acid for antibodies that targeted the C-strand in RV144 vaccinated individuals [36, 37]. As shown in Figure 3c, the combination of I169K/E172V improved PG9 binding. Interestingly, position 172 is not a contact
site for PG9; the amino acid is oriented on the opposite side of the PG9 contact surface. Therefore, valine on strand C could indirectly influence antibody binding by interacting with F159 on strand B, and this change may improve hairpin stability.

The I169K and E172V mutant of the 108060 scaffold became the new template. When a mutation, or a combination of mutations, showed an improvement in binding it was added and became the starting template for the next round of mutagenesis. We next selected T161M from the alignment for the next round of mutagenesis. We found this mutant did not improve binding when expressed in GnT1− cells, but did show a slight improvement in 293F cells (data not shown). Similar to the E172V polymorphism, T161M is oriented on the opposite side of the PG9 contact surface and does not interact directly with the antibody; the improvement in binding is indirect and cross-strand hydrophobic interactions between F159, 161M and V172 may change hairpin structure and lead to better binding.

It is known that PG9 and PG9-like antibodies do not always require glycosylation at N156 for binding and neutralization [38, 39] [40-43]. However, this has not been explored in the context of a V1/V2 scaffold. Based on the alignment and the observation that ZM233 has an isoleucine at position 156 we decided to remove the glycosylation site on our mutant 108060 V1/V2 scaffold and substituted
asparagine for isoleucine. As shown in Figure 3d, the scaffold bound PG9. We found the loss of the glycan at N156 and substitution with isoleucine did not reduce binding. The isoleucine substitution actually showed a slight improvement in binding for both cell lines compared to the previous mutant. This was surprising because the crystal structure of the V1/V2 domain from the CAP.45 isolate shows PG9 makes considerable contacts with the glycan at N156 [10].

Figure 2.3. Effect of mutations identified from the sequence alignment on a PG9 negative scaffold. Amino acids taken from the alignment were sequentially introduced into a clade B V1/V2 scaffold (108060) that did not bind PG9. The mutations were added and then tested for PG9 binding by ELISA. Figure 3a shows PG9 binding to the wildtype strain. It showed no binding when it was produced in 293F (blue) and little binding in 293-GnT1- cells (red). The first mutation made was I169K (Figure 3b). Lysine (K) is a critical contact essential for PG9 binding. No binding was observed when the mutant was produced in 293F cells and weak binding was observed for GnT1- cells. The next mutant was I169K/E172V (Figure 3c). Valine was selected because it could potentially form a cross-strand hydrophobic interaction with F159. The combination improved binding in GnT1- cells. The next mutant was I169K/E172V/T161M (data not shown). This combination slightly improved binding in 293F cells compared to the previous mutant. We next removed the glycosylation site at N156, and mutated
asparagine to isoleucine (N156I). Surprisingly, the I169K/E172V/T161M/N156I mutant (Figure 3d) showed a slight improvement in PG9 binding. The combination of mutations shown in Figure 3d are referred to as the β-strand changes. Next, we tested amino acids in the turn region. Figure 3e shows the β-strand changes combined with S164G/D167G. Figure 3f shows the β-strand changes combined with S164P/L165G. The results show the combination of mutations, rather than single mutations, had the most dramatic effect on PG9 binding. Isotype controls are shown for 293F (black) and GnT1- (grey).

2.2.4 Effect of mutations in the B-C turn on PG9 binding

The B-C turn (residues 164 to 167) is the short segment connecting the B and C strands. The turn region has been shown to influence formation of β-hairpins [32, 34, 35, 44, 45]. Statistical analysis of amino acids found in turns show a preference for small amino acids, such as glycine, and also a preference for proline, asparagine, and aspartate [30, 46, 47]. Based on the alignment, several amino acids in the B-C turn were identified as possibly being important for stabilizing this structure. Most viral sequences contain a glutamate (E) at 164 and aspartate (D) at 167, where E occupies the i position and D the i+3 position in the turn sequence. A hydrogen bond between the main chain carbonyl group from i and the amino group from i+3 helps stabilize the turn. However, the presence of two negatively charged amino acids at the i and i+3 positions may affect hairpin formation in the context of the V1/V2 scaffold. To investigate this 164 and 167 were mutated to glycine. Mutating these sites served several functions. First, glycine can adopt a wider range of Φ and Ψ angles necessary for β-turns. Second,
removing the negative amino acids occupying the i or i+3 positions may increase
the turn propensity. Third, position 167 is a key site for the development of some
PG9-like and strain-specific quaternary antibodies to the V2 domain [40, 41, 48,
49]. Glycines at 164 and 167 were added onto the 108060 mutant. As shown in
Figure 3e, these changes had a dramatic effect on PG9 binding with EC50’s of
0.05ug/ml for 293F cells and 0.005ug/ml for GnT1- cells.

To further investigate the role the turn has on PG9 binding, we tested another
turn sequence. This sequence was not identified from the alignment, but rather
identified from the literature as a strong promoter of hairpin formation. The
optimized turn was a proline-glycine combination [34, 50]. Proline is statistically
preferred at the i position for many common turn types [46, 47], and adding a
proline introduces a kink into strand B that restricts the φ to -60. Proline was
added at position 164 and glycine at position 165 on the 108060 mutant. We
found the proline-glycine mutations had a similar effect on PG9 binding as the
glycine-glycine mutations. As shown in Figure 3f, an EC50 of 0.1ug/ml was
observed for 293F cells and 0.008ug/ml for GnT1- cells.

2.2.5 Incorporating the mutations into other viral strains

Next, we wanted to know if the mutations introduced in the 108060 V1/V2
scaffold would similarly be effective in V1/V2 scaffolds from other viral strains.

V1/V2 scaffolds for MN and JRFL E168K were constructed and tested for PG9
binding. As shown in Figure 4a-b, no binding was observed with the wild-type sequences. ZM233 V1/V2 scaffold was also included (Figure 4c). Next, we incorporated the amino acid changes to the B and C strands and turn that were effective in improving binding to the 108060 scaffold. MN and JRFL E168K contained additional changes in the V1 domain. MN had glycosylation sites at N130 and N143 removed, and a deletion of 8 amino acids in the V1 loop. JRFL E168K had glycosylation sites at N138 and N143 removed. Previous work has suggested a shorter V1 domain with fewer glycosylation sites may change the exposure of the epitope for PG9 and other quaternary structure specific V1/V2 antibodies [10, 41, 48, 51, 52]. Interestingly, ZM233 has a short V1 domain [53] and showed good binding when tested by ELISA. As shown in Figures 4d-f, the mutations added to MN, JRFL E168K, and ZM233 had a dramatic effect on PG9 binding. The greatest change was seen for ZM233 (Figure 4f). The ZM233 mutant produced in both GnT1- and 293F cells had EC50 values of 0.01ug/ml and 0.002ug/ml, respectively. ZM233 already contains many of the mutations identified from the alignment and only required changes to the turn region (E164G and D167G).
Figure 2.4. Effect of mutations identified from the sequence alignment on additional viral strains. To determine if the mutations applied to 108060 would apply to other viral strains we took additional PG9 negative scaffolds and added the mutations. The PG9 negative scaffolds were MN and JRFL E168K; ZM233 was also included. As shown in Figures 4a-c, PG9 showed no binding to MN or JRFL E168K when they were produced in 293F (blue line) and GnT1- (red line) cells, and tested by indirect ELISA. ZM233 showed moderate binding when the scaffold was produced in 293F cells and high binding when the scaffold was produced in GnT1- cells. Figures 4d-f shows PG9 binding to the V1/V2 scaffolds when the mutations are added. All mutant scaffolds showed binding to PG9. The most striking change was seen for the ZM233 mutant; the mutant showed EC50 values of 0.002ug/ml and 0.01ug/ml for 293F and GnT1- cells, respectively. Interestingly, ZM233 required mutations only in the turn region (E164G/D167G). Isotype controls are shown for 293F (black) and GnT1- (grey).
2.2.6 Endo H treatment of V1/V2 scaffolds

The binding of PG9 normally depends on mannose-5 at N160 for binding [10, 23]. To determine if the V1/V2 mutants expressed in 293F cells still required high mannose glycosylation at N160 for binding we treated the scaffolds with the glycosidase Endo H and observed their ability to bind PG9. Endo H cleaves high mannose and some hybrid N-linked oligosaccharides; it will not cleave complex oligosaccharides. If the V1/V2 mutants produced in 293F cells contain mannose-5 or other high mannose glycans at N160, Endo H will remove these and should inhibit PG9 binding. If complex glycans are present at N160 they will be resistant to Endo H and be unable to bind PG9. We found (Figure 5a-b), that mock digested V1/V2 scaffolds bound PG9 whereas Endo H digested V1/V2 scaffolds (Figure 5c-d) eliminated PG9 binding activity. Therefore, it appeared that PG9 binding still depended on high mannose or hybrid oligosaccharide at N160 for binding. Future work will quantitate the amount of mannose-5 present at N160 in the mutant scaffolds when they are expressed in normal 293F cells.
Figure 2.5. **Endo H digestion of V1/V2 mutants produced in normal 293F cells.**
The V1/V2 mutants showed moderate to high affinity binding to PG9 when they were produced in normal 293F cells. To determine the glycan dependency, V1/V2 scaffolds were treated with Endo H and tested by direct ELISA. Endo H cleaves high mannose oligosaccharides and some hybrid types. If mannose-5 is present at N160, Endo H will cleave the glycan and no PG9 binding will be observed. Figures 5a-b shows PG9 binding to mock treated V1/V2 scaffold. Figures 5c-d show PG9 binding to Endo H treated V1/V2 scaffold. A loss of PG9 binding occurred after treatment with Endo H. PG9 binding to V1/V2 mutant is shown in blue, negative isotype IgG1 control is shown in black, and an anti-His6x positive control is shown in light gray.
2.2.7 Affinity of PG9 binding to V1/V2 scaffolds

We used surface plasmon resonance (SPR) to measure the binding affinity of PG9 to the mutant scaffolds expressed in 293F cells. For these measurements purified goat anti-human Fc antibodies were coated onto SPR detection chips and used to capture PG9. The V1/V2 scaffolds were then flowed over the chip at concentrations of 40μg/ml, 30μg/ml, 20μg/ml, and 10μg/ml. As shown in Figure 6, the wildtype scaffold for MN showed no binding to PG9, the MN mutant scaffold showed moderate binding with a $K_D$ value of 74nM, and the ZM233 mutant showed relatively high binding with a $K_D$ of 33nM. Interestingly, the ZM233 mutant has a much faster on-rate compared to the other V1/V2 scaffolds. This faster on rate may be due to the lack of the N156 glycosylation site, a more stable hairpin structure, or better exposure of the PG9 epitope due to the short V1 domain.
Figure 2.6. **Surface Plasmon resonance of PG9 binding to V1/V2 mutant scaffolds.** A. The affinity of PG9 to V1/V2 mutant scaffolds produced in normal 293F cells was measured by surface plasmon resonance on a Biacore 3000. CM5 chips were coupled to anti-human Fc using standard amine chemistry. PG9 or isotype was added to 50-100 RU. V1/V2 scaffold was added at 50ug/ml, 30ug/ml, and 10ug/ml. A negative isotype control was added to subtract background. Each sensogram shows PG9 binding with increasing concentration of V1/V2 scaffold. Global curve fitting is to a 1:1 Langmuir model. Each experiment was repeated 3 times. B. Summary of ELISA data and SPR data.
Table 2.1. Summary of ELISA and SPR data

<table>
<thead>
<tr>
<th>V1/V2 scaffolds</th>
<th>293F</th>
<th>GnT1-</th>
<th>293F</th>
</tr>
</thead>
<tbody>
<tr>
<td>108060 wild-type</td>
<td>ND</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>108060 mutant</td>
<td>0.05</td>
<td>0.005</td>
<td>118nM</td>
</tr>
<tr>
<td>MN wild-type</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MN mutant</td>
<td>0.02</td>
<td>0.008</td>
<td>74nM</td>
</tr>
<tr>
<td>ZM233 wild-type</td>
<td>0.08</td>
<td>0.02</td>
<td>nM</td>
</tr>
<tr>
<td>ZM233 mutant</td>
<td>0.002</td>
<td>0.01</td>
<td>31.9nM</td>
</tr>
</tbody>
</table>

ND, indicates not detectable.

K_D = Disassociation constant (nM)
2.3 Discussion

In this paper we describe efforts to engineer fragments of the V1/V2 domain that enable binding of the prototypic broadly neutralizing mAb PG9 when expressed in normal cell lines. Comparison of sequences that differed in the ability to bind PG9 revealed naturally occurring polymorphisms from viral sequences that affected binding. These mutations occurred in the β-hairpin formed by the B and C strands. We found mutations associated with improving hairpin formation and stability, were also associated with enhanced PG9 binding. In particular, hydrophobic amino acid changes in the B and C strands along with changes in the turn region had a dramatic effect on binding.

PG9 critically depends on mannose-5 at N160, but the amino acid and glycan occupying position 156 can vary. Previous work has shown PG9 can recognize complex glycans at N156 [23, 54]. Here we show the glycan at N156 is not necessary for PG9 binding in the context of a V1/V2 scaffold. In fact, the V1/V2 mutant for 108060 showed a slight improvement in binding when N was mutated to I. This was surprising because the crystal structure of the CAP.45 V1/V2 domain showed 27% of the contact surface for PG9 is made with the glycan at N156. This observation follows a pattern seen with other HIV BNab’s. Particularly, the ability of some broadly neutralizing antibodies to recognize both high mannose and complex glycans [55, 56], and the ability to recognize nearby
glycans reveals how some BNabs have evolved to recognize multiple variations of highly related epitopes [57]. PG9 binding may follow a similar strategy. This may explain why it can bind to V1/V2 scaffolds that lack N156 and can also bind when high mannose or complex glycans are present at this site. It appears mannose-5 at N160 is essential, but position 156 can vary in both site occupancy and glycan type. A vaccine designed to induce PG9-like antibodies should therefore take this into consideration to broaden the scope of viral strains recognized [57].

The ZM233 mutant scaffold produced in 293F cells showed high affinity binding when tested by surface plasmon resonance. The K_D for PG9 binding to the scaffold was 33nM. This is comparable to values obtained for disulfide stabilized synthetic glycopeptides containing mannose-5 at N156 and N160 [27]. While the affinity of PG9 to the V1/V2 scaffolds is not as high as trimeric gp140 from the BG505 strain of HIV-1 (11nM), our data nevertheless shows high affinity binding is possible for a V1/V2 scaffold produced in 293F cells. Additionally, development of a vaccine based on the ZM233 V1/V2 mutant may be significant. ZM233 is a clade C virus and this subtype represents the clade of virus predicted to be responsible for the majority of new infections over the next decade [24-26]. The predicted germline gene for PG9 has been shown to neutralize ZM233 [38]. Several studies have suggested that immunogens capable of direct binding to the germline immunoglobulin genes of BNabs may represent a new class of superior vaccine immunogens [58-61]. However, future studies will need to address
whether recombinantly produced V1/V2 scaffolds described in this study can
bind to the predicted PG9 germline antibody [62].

Several conclusions can be drawn from these studies. First, mutations in the B-C
hairpin significantly improved PG9 binding for the viral strains tested even
though many of the changes do not occur at antibody contact sites and may
influence binding indirectly by altering hairpin formation. Future work will
measure hairpin stability for both the wildtype and mutant V1/V2 scaffolds.
Second, antigenicity can be engineered for some V1/V2 scaffolds and many of
the factors responsible for PG9 binding have been identified. However, variables
such as V1 loop length, V2 loop length, or location of glycosylation sites may be
equally important for exposing the PG9 epitope. Third, the ZM233 mutant
scaffold showed high affinity binding when it was produced in normal 293F cells.
This is the first report of a V1/V2 scaffold showing high affinity binding when
expressed in a normal cell line. Finally, the development of scaffolds able to bind
PG9 with high affinity appears to require combinations of mutations rather than
single point mutations. The scaffolds created provide an approach to selectively
stimulate and focus the antibody responses to an important glycan-dependent
epitope in the V1/V2 domain without stimulating antibodies to other
immunodominant regions of Env. Preliminary immunogenicity studies with V1/V2
scaffolds produced in GnT1- cells suggest they can improve protective antibody
responses compared to immunization with gp120 alone [22]. However, BNabs
such as PG9 have exceptionally long CDRH3 domains, and it is unlikely the true immunogenic potential of V1/V2 scaffolds can be determined in animal immunogenicity studies alone. The development of scaffolds that can be produced in normal 293 and CHO cell lines, such as the such as the ZM233 scaffold described above, will allow for the cGMP production of V1/V2 scaffolds that can be tested in small clinical trials.

2.4 Methods

2.4.1 Construction of V1/V2 scaffolds

Bal.01; CAP45.2.00.G3, SVPC16; SC422661, Clone B SVPB8; ZM109F.PB4, SVPC13; ZM197M.PB7, SVPC6; ZM233M.PB6, SVPC9 were obtained from the NIH AIDS Reagent Program (Germantown, MD). A244, 108060, MN, and JRFL E168K V1/V2 constructs have been previously described [22]. gD tagged V1/V2 constructs contain the herpes simplex virus (HSV) signal sequence, an N-terminal HSV gD tag epitope for affinity purification, and V1/V2 domain (amino acids 116-207). His6x-StrepTag constructs contain the ICAM signal sequence, a Hexa-histidine (His6x) tag and StrepTag for tandem affinity purification, and V1/V2 domain (a.a. 116-207). The amino acid sequences for all V1/V2 scaffolds used in this study are provided in Supplemental Table 1.
2.4.2 Production and purification of V1/V2 scaffolds

Plasmids were transfected into FreeStyle™ 293F cells (Invitrogen, Carlsbad, CA) or into GnTI- -293 cells (293 cells deficient in N-acetylglucosaminyltransferase I) that limit N-linked glycans to simple, mannose-5 containing glycan structures (American Type Culture Collection Manassas, VA). Transfections were carried out with polyethyleneimine (PEI) and the supernatant was collected on day 3 or day 4. For gD-tagged constructs, immunoaffinity chromatography was used to purify the proteins as described previously [22]. To purify His6x-Streptag constructs, HisTrap and StrepTactin sepharose high performance columns (GE Healthcare Biosciences) were used for purification. Purified proteins were run on a HiLoad Superdex 200 (26/60) size exclusion column (GE Healthcare). All proteins were buffer exchanged into TBS and analyzed by SDS-PAGE using 4-12% precast gradient gels (Invitrogen, Carlsbad, CA).

2.4.3 Antibodies

PG9 and PG16 monoclonal antibodies were purchased from Polymun Scientific GmbH. (Vienna, Austria) 34.1 is an in-house mouse monoclonal antibody specific for the N-terminal gD flag epitope of HSV-1 and was used as a capture antibody in indirect ELISA assays (Morales, JF et al., unpublished data). Mouse anti-His6x Mab was purchased from R&D systems - Clone# AD1.1.10. (Minneapolis, MN)
Human IgG1 Isotype control Mab was purchased from Sigma-Aldrich. (St. Louis, MO)

2.4.4 Indirect ELISAs of wildtype V1/V2 scaffolds with cell culture supernatant

The following protocol was used to measure PG9 binding to V1/V2 scaffold from cell supernatant. Nunc Maxisorp ELISA plates (Nunc, Rochester, NY) were coated with 2 μg/ml of 34.1 anti-gD antibody in PBS overnight at 4°C. The following day the plates were washed 4 times with PBS containing 0.05% Tween-20, and blocked for 1-2 hrs with PBS containing 1% BSA (blocking buffer). Cell supernatant containing V1/V2 scaffold from 3-4 days post-transfection was added at 100μl/well. (Western blot was used to confirm scaffold expression.) Dilutions of PG9 or Isotype control was added from 10μg/ml to 0.001μg/ml. Peroxidase-conjugated AffiniPure Goat Anti-Human IgG, (Fcy specific) (Jackson ImmunoResearch, West Grove, PA) was used at a 1:5000 dilution. OPD substrate (Fisher Scientific, Pittsburg, PA) was developed for 10 min and stopped with 3 M H₂SO₄. The absorbance was measured at 490 nm. All steps, except coating, were done at room temperature on a shaking platform; incubation steps were for 1 hr. All dilutions (except coating) were done in blocking buffer. Washes with PBS + 0.05% Tween-20 (4X) were included after incubation steps.
2.4.5 Indirect ELISAs of V1/V2 mutants with purified protein

PG9 binding to V1/V2 mutant scaffolds was done by capture ELISA. Maxisorp microtiter plates (Nunc, Rochester, NY) were coated with 2 μg/ml of the 34.1 anti-gD antibody in PBS overnight at 4°C. The plates were then washed 4 times with PBS containing 0.05% Tween-20, and blocked for 2 hrs with PBS containing 1% BSA (blocking buffer). Saturating amounts of recombinant gD-V1/V2 scaffold were added at 10 μg/ml. Serial dilutions of PG9 were added from 10 μg/ml to 0.001μg/ml. Peroxidase-conjugated AffiniPure Goat Anti-Human IgG, (Fcy specific) (Jackson ImmunoResearch, West Grove, PA) was used at a 1:5000 dilution. OPD substrate (Fisher Scientific, Pittsburg, PA) was developed for 10 min and stopped with 3 M H₂SO₄. The absorbance was measured at 490 nm. All steps, except coating, were done at room temperature on a plate shaker; incubation steps were for 1 hr (except blocking), and all dilutions were done in blocking buffer.

2.4.6 Endo H digests and ELISA

Endo H cloned from Streptomyces picatus and expressed in E.coli was purchased from New England Biolabs, (Boston, MA). Endo H cleaves high mannose and some hybrid N-linked oligosaccharides between the two N-acetylglucosamine residues in the diacetylchitobiose core. V1/V2 scaffolds used in the Endo H assays were expressed in FreeStyle™ 293-F cells and were digested under native
conditions. Briefly, 30ug of V1/V2 scaffold in 50 mM sodium citrate buffer pH 5.5 (G5 buffer) was digested with 500U of Endo H overnight at 37°C. Mock digestions were run under the same conditions except Endo H was not included. Maxisorp ELISA plates were coated with 5ug/ml of Endo H-digested V1/V2 scaffold overnight at 4°C. The following day, the plates were washed 4X with PBS + 0.05% Tween-20 and blocked for 2hrs using 1% BSA in PBS. PG9, IgG1 Isotype control, or anti-His6x was added at 10ug/ml to 0.001ug/ml. HRP-labeled anti-human IgG Fc or anti-mouse IgG Fc was added at 1/5000 dilution. OPD substrate was developed for 10min. and stopped with 3M H2SO4. The absorbance was measured at 490 nm. All incubation steps (except blocking) were for 1hr at room temperature on a plate shaker. All dilutions (except coating) were done with 1% BSA and PBS. Washes were included after incubation steps.

2.4.7 Surface plasmon resonance

Kinetic analysis of V1/V2 scaffold binding to PG9 was run on a Biacore 3000 instrument. Human antibody capture kit (GE Healthcare/Biacore) was used to immobilize anti-human IgG Fc to a CMS sensor chip using primary amine chemistry. Briefly, the carboxymethylated dextran surface was activated using EDC/NHS, anti-human IgG Fc was conjugated to the chip, and the reaction blocked using 1M ethanolamine-HCl. HBS-EP was the running buffer and was also used for antibody dilutions. Isotype control and PG9 was captured to 50-100 RU.
Five concentrations of V1/V2 scaffold were tested: Analysis was done with Biaevaluation software and fit to a 1:1 Langmuir model. Background was subtracted from the Isotype control.
2.5 References


Supplemental Figures and Tables

Supplemental Figure 2.7. SDS-PAGE of V1/V2 scaffolds

SDS-PAGE of V1/V2 scaffolds
ZM233 mutant  MN mutant

Red.  Red.
Non-Red.  Non-Red.
Supplemental Figure 2.8. **PG9 binding to V1/V2 scaffolds from multiple HIV strains by indirect ELISA.** V1/V2 scaffolds from Clade B (Bal.01, MN, & 108060), Clade C (CAP45, ZM109, ZM197, & ZM233) and Clade E (A244) viral strains were made and tested for PG9 binding by indirect ELISA. V1/V2 scaffolds were expressed in either normal 293F (blue) or GnT1- cells (red). GnT1- cells do not express N-acetylglucosaminyltransferase I and glycosylation is limited to Man9-GlcNAc2. Cell supernatant was collected 3 days post-transfection. ELISA plates were coated with anti-gD tag antibody to capture V1/V2 scaffolds from cell culture supernatant. PG9 was added from 10μg/ml → 0.001μg/ml. Detection was with HRP-labeled anti-human Fc and OPD substrate. As shown in Figures 1a-h, PG9 can bind some viral strains as V1/V2 scaffold. In particular, the best binding was observed for scaffolds from the A244, ZM109, and ZM233 isolates produced in GnT1- cells. Weak binding was observed for most of the scaffolds produced in normal 293F cells, however we noted that scaffolds from the clade B viruses (MN and 108060) showed no binding to PG9. Figure 1a = Bal.01; 1b = CAP.45; 1c = ZM109; 1d = MN; 1e = A244; 1f = ZM197; 1g = ZM233; 1h = patient sample 108060. Isotype controls are shown for 293F (black) and GnT1- (grey).
Supplemental Figure 2.9. **Adding N156 into ZM233 V1/V2 mutant scaffold reduces binding to PG9.** The ZM233 mutant contains an isoleucine at 156. To determine the effect of glycosylation at N156, isoleucine was mutated to asparagine. The ZM233 V1/V2 mutant with and without N156 was produced in normal 293F cells and tested for PG9 binding by direct ELISA. As shown in Figure 7, adding N156 into the ZM233 V1/V2 mutant scaffold reduced binding to PG9.

Supplemental Figure 2.10. **PG9 binding to ZM233 V1/V2 mutant scaffold produced in 293F and CHO cells.** PG9 binding to ZM233 V1/V2 mutant scaffold produced in 293F and CHO cells was tested by indirect ELISA. PG9 can bind with relatively high affinity to 293F produced material (blue line) and CHO produced material (yellow line). Binding to CHO is slightly weaker due to the higher sialic acid content of proteins expressed in this cell line.
Chapter 3: Identification of protease cleavage sites in conserved regions of gp41

3.1 Introduction

An HIV-1 vaccine remains a global health priority. After 30 years of HIV vaccine research and numerous clinical trials, a safe and effective vaccine is still urgently needed [1]. The diversity of viruses circulating worldwide, viral evasion mechanisms, an error-prone reverse transcriptase, and integration of the virus into host cells have made the development of an effective HIV vaccine challenging. However, the recent success of the RV144 clinical trial and the identification of broadly neutralizing antibodies has reinvigorated the field [2, 3]. Over the past several years, several broadly neutralizing antibodies (BNabs) have been identified in HIV-1 infected individuals [4]. Sites of vulnerability have been exposed and are now being targeted for vaccine development. These include the V1/V2 region targeted by the PG9-like class of antibodies [5-9], the CD4 binding site targeted by the VRC01-like class of antibodies [10], the base of the V3 targeted by the PGT128-like class of antibodies [7, 11], and the gp120/gp41 interface targeted by the PGT151-like class of antibodies [12]. One of the more interesting classes of antibodies is the 2F5-like class that targets the MPER region of gp41 [13-17]. This class includes 2F5, 4E10, m66.6, Z13, and 10e8. These antibodies have proven to be exceptionally broad and potent. However, they
rarely develop in HIV infected individuals, and inducing them by vaccination has proven to be challenging despite our knowledge about MPER structure [18, 19].

Several reasons may explain why 2F5-like antibodies rarely develop [18, 20]. First, several of these antibodies have been shown to be polyreactive to self-antigens [21-23]. Self-reactivity and immune tolerance may explain why these antibodies rarely develop in HIV infected individuals [18, 24]. Second, the MPER is a temporal epitope [25]. In the native state, the MPER is partially buried in the viral membrane. In the pre-hairpin conformation, the MPER becomes highly exposed; it has been shown the broadly neutralizing antibodies, 2F5 and 4E10, preferentially bind this conformation [25]. However, the pre-hairpin conformational state is temporary, and formation of the 6-helical bundle again shields the MPER. Third, it has become clear that the viral membrane plays an important role in the development of this class of antibodies [26-31]. Vaccine efforts are now focused on incorporating a membrane component for MPER-based vaccines [19, 32, 33]. Fourth, similar to other BNabs, a long CDR3 and variable regions with high mutation rates may be needed [2, 26].

Here we offer an additional explanation. Previous work in our lab has identified protease cleavage sites in highly conserved regions of the virus that are important for receptor binding or the binding of neutralizing antibodies [34]. It is possible protease cleavage sites have evolved in the virus in order to escape the
humoral immune response. Destruction of conserved epitopes by serum or cellular proteases may prevent the formation of neutralizing antibodies. Conserved cleavage sites may also alter antigen processing, affect T-cell help, and indirectly influence B-cell development. Our lab has previously identified conserved protease cleavage sites in the C4 region, the V3 crown, and the V1/V2 loop of gp120. In this study we have identified protease cleavage sites in gp41.

3.2 Results

3.2.1 Digestion of HIV-1 gp140 with Cathepsins L, S, and D.

To identify protease cleavage sites in gp41, we used gp140, the ectodomain of Env (e.g. gp120 + gp41). Gp41 is a hydrophobic protein that is difficult to purify on its own. A time-course digestion with cathepsins L, S, and D was done with a clade B clinical isolate 108060_Q655R. This isolate contains a point mutation in gp41 that may alter the exposure of the membrane proximal external region (MPER) to broadly neutralizing antibodies and may also make it more susceptible to proteolytic cleavage [35]. Western blot with monoclonal antibodies, 2F5 and 4E10, was used to analyze the cleavage products. These two antibodies are broadly neutralizing antibodies that are specific for the MPER of gp41. 2F5 recognizes the epitope ELDKWAS and 4E10 recognizes NWFDIT.

As shown in Figure 1, several cathepsin cleavage products were detected by Western blot. 2F5 recognized considerably more protease cleavage products
compared to 4E10, which appeared to recognize only gp41. 2F5 is a high affinity antibody that works well by Western blot, and is much more sensitive than 4E10 under these conditions. As shown in Figure 1a, digestion with cathepsin S produced several cleavage products in gp41. Cathepsin L (Figure 1c) did not digest as well as cathepsin S and produced very few cleavage products. Cathepsin D also did not digest as well and showed a very similar digestion profile as cathepsin L (data not shown).

Interestingly, the 4E10 blot showed a loss of signal for gp41 after 1hr incubation with cathepsin S, which indicates the presence of a protease cleavage site within the epitope (Figure 1b). For the 2F5 blot, small cleavage products were detected around 5kD, also indicating a cleavage site near the MPER. Protease cleavage sites in the MPER could be significant because this region is conserved and targeted by several broadly neutralizing antibodies. Cathepsin S is an antigen processing enzyme, but has been shown to be active at physiological pH and has been found in serum under inflammatory conditions [36-39]. Inflammation associated with HIV-1 infection, in particular, inflammation associated with the gut could lead to the release of proteases and targeted destruction of this epitope.
Figure 3.1. Cathepsin S and cathepsin L time-course digestion with gp140. Monoclonal antibodies 2F5 and 4E10 were used to detect cleavage products in the MPER region of gp41. Cathepsin D showed a similar cleavage pattern as cathepsin L. (data not shown) Figure 1b shows the loss of signal for gp41 at 1hr post-digestion. This shows a protease cleavage site is present within or near the 4E10 epitope.
3.2.2 N-terminal sequencing to identify protease cleavage sites in gp41

N-terminal sequencing (Edman degradation) was used to identify cathepsin cleavage sites in gp140. N-terminal sequencing was preferred for several reasons. First, it identifies several amino acids at the N-terminus of the cleavage site. Second, N-terminal sequencing is quantitative. The strongest bands on the SDS-PAGE gel correspond to major cleavage products and can be selected for sequencing. However, the downside of N-terminal sequencing is that masking could occur between major and minor cleavage products.

Gp140 was digested with cathepsin L, S, or D for 2-4hrs. The cleavage products were separated by SDS-PAGE, transferred to PVDF, and the resulting bands analyzed by Edman degradation. As shown in Figure 2, several cleavage sites were identified for cathepsin L, S, and D. In gp120, five cathepsin S cleavage sites were identified. These include T\textsuperscript{209}-F\textsuperscript{210}, A\textsuperscript{265}-E\textsuperscript{266}, and N\textsuperscript{287}-E\textsuperscript{288} in the C2 domain, Y\textsuperscript{435}-A\textsuperscript{436} in the C4 domain, and G\textsuperscript{495}-V\textsuperscript{496} in the C5 domain. In gp41, four cathepsin S cleavage sites were identified. These include G\textsuperscript{513}-T\textsuperscript{514} in the fusion peptide, G\textsuperscript{531}-A\textsuperscript{532} and T\textsuperscript{536}-L\textsuperscript{537} in the polar segment, and T\textsuperscript{620}-D\textsuperscript{621} before the carboxy-terminal heptad repeat. Two cathepsin D cleavage sites were identified and they occurred only in gp120. The first site identified occurred at V\textsuperscript{89}-T\textsuperscript{90} in the C1 domain. The second site, L\textsuperscript{287}-N\textsuperscript{288}, is in the C2 domain. Two cleavage sites were identified for cathepsin L and both were in gp120. The first site
occurred at \( F^{313-314} \) in the V3 domain, and the second site is \( L^{483-484} \) in the C5 domain.

![Diagram showing cleavage sites](image)

**Figure 3.2.** Summary of cathepsin S, L, and D cleavage sites in gp140 identified by Edman degradation

### 3.2.3 Identification of cathepsin L, S, and D cleavage sites in the MPER with soluble peptide

The results from the cathepsin S time-course digestion with gp140 and Western blot analysis with 2F5 and 4E10 suggested a cleavage site occurred in the MPER region of gp41. However, we were unable to identify the cleavage site using Edman degradation and an alternative strategy was needed. The approach we took was to use a soluble MPER peptide as a substrate for the protease...
digestions. The MPER is very hydrophobic and a peptide of this region would be insoluble at physiological pH. In order to increase the solubility, polylysines were added at both the N- and C- termini. Cathepsin S was the only protease that showed cleavage in the MPER of gp140 as determined by Western blot analysis (See Figure 1). Therefore, we only digested the peptide with cathepsin S. The peptide was digested for 30min., 4hrs., and 24hrs., and the resulting digestion products analyzed by LC MS/MS. The results are shown in Figure 3.

Two major cleavage sites were identified after 30 minutes. The first cleavage site occurred between S668 – L669 and the second cleavage site occurred between E662 – L663. Both cleavage sites occur at the epitope of 2F5, which is ELDKWAS (See Figures 3 & 4). A third cleavage site was identified between W680-Y681, but this was only after 4hrs of incubation with cathepsin S. The third cleavage site is located near the 4E10 and 10E8 epitopes (See Figures 3 & 4). The identification of cleavage sites in a conserved region of the virus that are important epitopes of broadly neutralizing antibodies 2F5, 4E10, and 10e8 may provide a further explanation of why these antibodies rarely develop.

Additionally, cleavage sites within the MPER may explain why neutralizing antibodies are difficult to induce by vaccination. Adjuvants are often included as part of immunization protocols to strengthen the immune response. Inflammation associated with injection of the immunogen and the adjuvant may
cause proteases to be secreted into the surrounding tissue. Proteases may alter the immunogenicity of the antigen and affect the quality of the antibody response. The identification of cathepsin S cleavage sites in the MPER peptide may explain why previous vaccination studies (e.g. MPER-KLH conjugates) showed limited neutralization breadth.

**Figure 3.3.** Time-course digestion with cathepsin S and soluble MPER peptide. LC MS/MS was used to identify the cleavage sites in the peptide.
3.2.4 Identification of cathepsin S cleavage sites using a pre-hairpin intermediate.

Gp41 is a trimer on the surface of the virus and exists in three conformational states: the native state, the pre-hairpin conformation, and the 6-helical bundle (See Figure 5). In the first state, gp41 is in the native conformation and the MPER is partially buried in the viral membrane [40-42]. In this conformation, the MPER would be partly exposed to serum proteases. In the second state, the pre-hairpin conformation, the fusion peptide of gp41 inserts into the target membrane, gp41 is extended, and the MPER becomes highly exposed (See Figure 5). Published work has shown broadly neutralizing antibodies 2F5 and 4E10 preferentially bind to the MPER in the pre-hairpin conformation [25]. It is in the

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**Figure 3.4.** Summary of cleavage sites identified in the MPER peptide by LC MS/MS
pre-hairpin conformation that the MPER would be most susceptible to proteolytic cleavage. In the third state, gp41 folds back on itself, brings the opposing membranes together, and forms a 6-helical bundle. The 6-helical bundle is stable and would be resistant to proteolysis. Therefore, out of the three conformational states of gp41, the pre-hairpin conformation would be the best model to study protease sensitivity.

A previously described recombinant protein, GCN-gp41-inter, representing the trimeric pre-hairpin intermediate [25] was used in our studies to examine the susceptibility of the MPER to protease digestion. As shown in Figure 5, a time-course digestion with cathepsin S and Western blot analysis with 2F5 shows several cleavage products. However, it is clearly apparent from the Western blot that the trimeric pre-hairpin intermediate is stable and mostly resistant to protease degradation. The signal for the uncleaved GCN-gp41-inter remained relatively unchanged for up to 6 hours after the addition of the protease. Bands corresponding to digestion products were present, but they appear to be minor. The smaller band above 3.5kD could represent a protease cleavage site in the MPER, but this digestion product appears only after several hours of incubation with cathepsin S. 4E10 was unable to detect any of the cleavage products seen with 2F5. However, the 4E10 blot shows the signal for the uncleaved GCN-gp41 remained relatively unchanged. This is in contrast to the time-course digestion of gp140 with cathepsin S; the results from this experiment showed a loss of
signal for gp41 after 1hr incubation and detection with 4E10 (Figure 1b). Based on these results, the MPER in GCN-gp41-inter is structurally more stable and resistant to proteolysis. This is not surprising because the N- and C-terminus are both constrained by domains that would limit the flexibility of the MPER and its susceptibility to proteolysis. This is not the case for gp140 or the MPER peptide, which have unrestrained C-termini.
A. Conformational states of gp41

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B. GCN-gp41-inter

C. GCN-gp41-inter

D. 2F5

E. 4E10

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Figure 3.5. A. The conformational states of gp41. B and C. Description of GCN-gp41-inter. D. Time-course digestion with cathepsin S and GCN-gp41-inter. 2F5 was used for detection. E. Time-course digestion with cathepsin S and GCN-gp41-inter. 4E10 was used for detection
3.2.5 Identification of cleavage sites in GCN-gp41-inter by mass spectrometry (Bin Yu did this work)

A protease digestion with cathepsin S and cathepsin D with GCN-gp41-inter identified cleavage sites in the MPER. The cathepsin S cleavage site is E$^{662}$ – L$^{663}$. The cathepsin D cleavage site is W$^{680}$-Y$^{681}$.

![Figure 3.6. Identification of cleavage sites in GCN-gp41-inter by Edman degradation](image)

3.3 Discussion

The identification of conserved cleavage sites in regions of the virus that are important for receptor binding and neutralizing antibodies may be one more reason why broadly neutralizing antibodies rarely develop. In this study we identified several cathepsin cleavage sites in the MPER of gp41. In particular, we identified cathepsin S cleavage sites that affect the binding of the broadly neutralizing antibodies 2F5 and 4E10. Cathepsin S is a protease that is active at physiological pH and has been found in serum under inflammatory conditions. It is possible, upon vaccination with inflammatory adjuvants, proteases are
secreted into the surrounding tissue and consequently alter the immunogenicity of the antigen.
3.4 Materials and Methods

3.4.1 Antibodies

Monoclonal antibodies 2F5 and 4E10 were purchased from Polymun Scientific GmbH (Vienna, Austria). 2F5 recognizes the epitope ELDKWA, and 4E10 recognizes the epitope NWFDIT. Rabbit polyclonal anti-human IgG Fc conjugated to HRP was used for Western blot analysis.

3.4.2 Synthetic peptides

A synthetic peptide corresponding to the MPER region of gp41 was synthesized by Genscript, Inc. (Piscataway, NJ). The sequence of the MPER peptide was KKKNEQELLELDKWASLWNWFDITNWLWYIRKKK-NH2. Polylsines were added at the N- and C- terminus to increase the solubility of the peptide. The peptide was resuspended in DI H2O to a concentration of 1mg/ml, and aliquots were frozen at -80°C until further use.

3.4.3 Construction and expression of gp140

Recombinant gp140 from patient sample 108060 (clade B) was constructed as previously described [43]. The plasmid for protein expression was transfected into HEK-293T cells (American Type Culture Collection, Manassas, VA). Transfection was carried out with polyethyleneimine, and the supernatant was collected on day 4. Affinity chromatography was used to purify the protein as described previously [44]. All proteins were buffer exchanged into PBS post-
purification. The resulting proteins were analyzed by PAGE using 4–12% precast gradient gels (Invitrogen), and the purity of the preparation was estimated to be ~95%.

3.4.4 Protease digestions with gp140

Time-course digestions with cathepsin S, L, or D were done with gp140. For cathepsin S digestions, fifty micrograms of 108060 gp140 was mixed with 0.5μg of cathepsin S (1:50 enzyme: protein ratio) in 50ul of 50mM sodium phosphate and 50mM sodium chloride, pH 6.5 buffer. The reaction was incubated at 37°C for 15’, 30’, 1hr, 2hrs, 4hrs, 6hrs, and 24hrs. Aliquots were taken at each of the time points; an aliquot was also taken prior to the addition of cathepsin S. The reaction was stopped by the addition of the protease inhibitor X. Aliquots of the cathepsin S digestions were mixed with sample buffer and reducing agent, boiled for 2min., and run on a 4-12% gradient gel. For Western blot analysis, the gel was transferred to a PVDF membrane, blocked, and probed with 2F5 or 4E10. For N-terminal sequence analysis, the gel was transferred to a PVDF membrane, stained with SimplyBlue staining reagent, and destained. Bands corresponding to protease cleavage products were cut and sent out for Edman degradation. Edman degradation was performed at the proteomics core facility (UC Davis).

For cathepsin L digestions, the same conditions were used, except, the digestion buffer was 100mM sodium acetate, pH 5.5, and the reaction was stopped with
the protease inhibitor ALLM. For cathepsin D digestions, similar conditions were used, except, the digestion buffer was 100mM sodium acetate, pH 3.3, and the reaction was stopped by the addition of the protease inhibitor Pepstatin A.

3.4.5 Cathepsin S digestion with soluble MPER peptide

Cathepsin S time-course digestions with soluble MPER peptide were done under the following conditions: 5ugs of MPER peptide was mixed with 0.1ug of cathepsin S (1:50 ratio) in 50ul of 50mM sodium phosphate and 50mM sodium chloride, pH 6.5 buffer. The reaction was incubated at 37°C for 30’, 4hrs, and 24hrs. Reactions were stopped by addition of the irreversible protease inhibitor E-64. Digested peptide was run on a C18 column for separation, and analyzed on an LTQ LC-MS/MS (Thermo Finnigan) located in the Mass Spectrometry Facility at the University of California, Santa Cruz.

3.4.6 Cathepsin S digestion of GCN-gp41-inter

GCN-gp41-inter was constructed as previously described (ref). Time-course digestions with cathepsin S were done with the following conditions: 5ugs of GCN-gp41-inter was mixed with 0.1ug of cathepsin S (1:50 ratio) in 50ul of 50mM sodium phosphate and 50mM sodium chloride, pH 6.5 buffer. The reaction was incubated at 37°C for 15’, 30’, 1hr, 2hrs, 4hrs, 6hrs, and 24hrs. Aliquots were taken at each of the time points; an aliquot was also taken prior to the addition of cathepsin S. The reaction was stopped by addition of the irreversible protease
inhibitor E-64. Aliquots of the cathepsin S digestions were mixed with sample buffer and reducing agent, boiled for 2min., and run on a 4-12% gradient gel. For Western blot analysis, the gel was transferred to a PVDF membrane, blocked, and probed with 2F5 or 4E10. For N-terminal sequence analysis, the gel was transferred to a PVDF membrane, stained with SimplyBlue staining reagent, and destained. Bands corresponding to protease cleavage products were cut and sent out for Edman degradation. Edman degradation was performed at the proteomics core facility (UC Davis).
3.5 References


