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HIV-1 Envelope Proteins and V1/V2 Domain Scaffolds with Mannose-5 to Improve the Magnitude and Quality of Protective Antibody Responses to HIV-1*

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Background: Antibodies to glycan-dependent and -independent epitopes in the V1/V2 domain of HIV-1 gp120 have been associated with protective immunity.
Results: Novel gp120s and scaffolds able to target antibodies to both types of epitopes in the V1/V2 domain were developed.
Conclusion: Improved antibody responses to the V1/V2 domain were observed.
Significance: The immunogens described may provide improved HIV-1 vaccines.

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. Interestingly, the binding of both types of antibodies appears to depend on the same cluster of amino acids (positions 167–171) adjacent to the junction of the B and C strands of the four-stranded V1/V2 domain β-sheet structure. However, the broadly neutralizing mAb, PG9, additionally depends on mannose-5 glycans at positions 156 and 160 for binding. Because the gp120 vaccine immunogens used in previous HIV-1 vaccine trials were enriched for complex sialic acid-containing glycans, and lacked the high mannose structures required for the binding of PG9-like mAbs, we wondered if these immunogens could be improved by limiting glycosylation to mannose-5 glycans. Here, we describe the PG9 binding activity of monomeric gp120s from multiple strains of HIV-1 produced with mannose-5 glycans. We also describe the properties of glycopeptide scaffolds from the V1/V2 domain also expressed with mannose-5 glycans. The V1/V2 scaffold from the A244 isolate was able to bind the PG9, CH01, and CH03 mAbs with high affinity provided that the proper glycans were present. We further show that immunization with A244 V1/V2 fragments alone, or 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 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)[5] 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 overlap 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).

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[5] The abbreviations used are: bN, broadly neutralizing; gp120, glycoprotein 120 from HIV-1; rgp120, recombinant gp120; V1/V2, the first and second variable regions of gp120; gD, fragment of herpes simplex virus glycoprotein D containing the signal sequence and a 27-amino acid N-terminal tag epitope; GnTI$^-$, 293 cells deficient in *N*-acetylglucosaminyltransferase I; RCM, reduced and carboxymethylated; PNGS, predicted N-linked glycosylation sites; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PNGase F, peptide:*N*-glycosidase F.

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).

EXPERIMENTAL PROCEDURES

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).

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 (His_6) replaced the gD tag epitope.

Production and Purification of gp120 and V1/V2 Fragments—Plasmids for the expression of proteins were transfected into FreeStyle™ 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 His_6-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).

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 TNANLTKANLTNVNNRTNVSNI-IGNITDE, the sequence of the B-C peptide (residues 160–174) was NMTTELRDKKQKVHA, and the sequence of the C-D peptide (residues 173–192) was HALFYKLDIVPIEDNNDSSEYR. A cyclized synthetic peptide (CVKLTPPSVTLAAAAAALINSNT-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.

Peptide Microarrays—Peptide scanning of antibody responses to the V1/V2 domain was carried out using peptide microarrays (Replitope) 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, 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.

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.[6]

Indirect ELISAs—Assays to detect monoclonal antibody binding to gp120 were carried out with Maxisorp microtiter plates (Nunc, Rochester, NY) coated with 1 μg/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 μg/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 H_2SO_4. 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.

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 μg of gp120 produced in normal 293 cells, and groups 2 and 3 received 100 μg of gp120 produced in GnTI$^-$ cells. Group 4 received 33 μg 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 μg of V1/V2 scaffold. The first two immunizations were given intradermally in a volume of 400 μl or less; all subsequent immunizations were given subcutaneously in a volume not exceeding 200 μl.

Assays to Detect the Binding of Rabbit Antibodies—All rabbit antibody binding assays were done with His_6-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 μg, 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 $1:10^2$ to $1:10^6$ and added to the plates. The assay plates were washed four times, and HRP-labeled goat anti-rabbit IgG (Fcγ-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.

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.

Immunoprecipitation Experiments—50 μg of PG9 and 50 μg 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.

[6] J. F. Morales, unpublished data.

Mass Spectrometry—Purified A244 V1/V2 scaffold produced from GnTI$^-$ cells (100 μg 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.

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.

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).

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.

RESULTS

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, 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 EC_{50} values for MN and A244 gp120 of ~0.5 and 0.4 μg/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 EC_{50} values of 0.06 and 0.007 μg/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. 1*F*) (32, 61). We found that PG9 bound to TRO.11, JRFL_E168K, and TH023 gp120s with EC_{50} values of 0.4, 0.07, and 0.4 μg/ml, respectively (Table 1). 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 TR0.11, 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

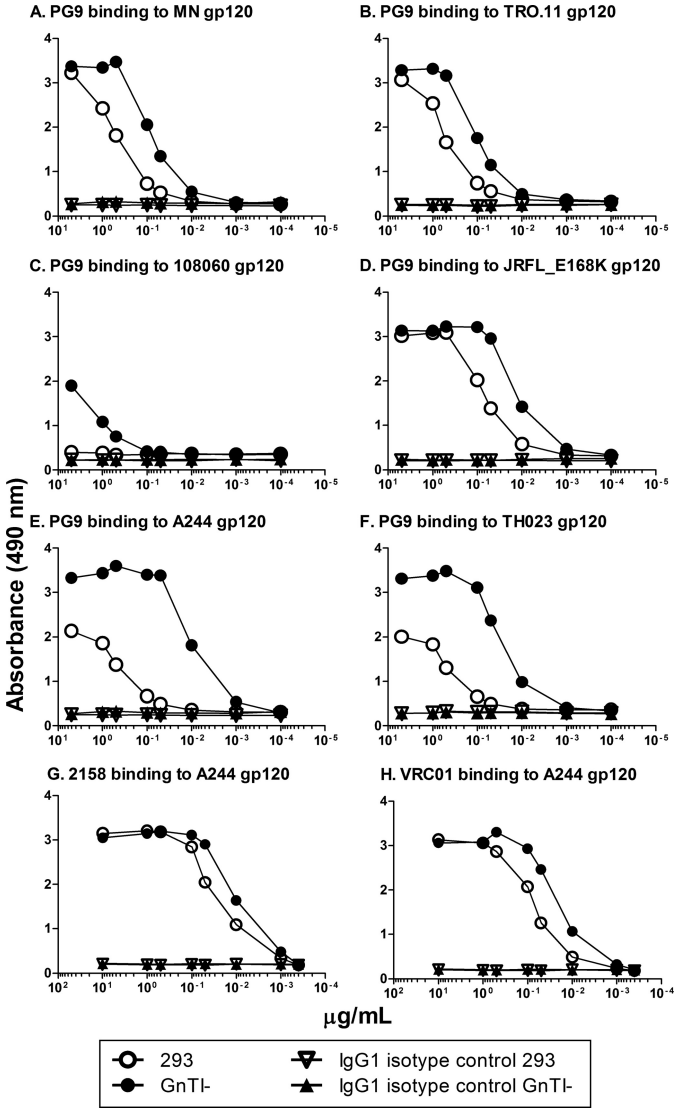


FIGURE 1. **Effect of glycosylation on PG9 binding to monomeric gp120s from clade B and CRF01_AE isolates.** The genes encoding gp120s from the clade B MN, TRO.11, 108060, and JRFL_E168K isolates and the CRF01_AE A244 and TH023 isolates were transfected into either normal 293F cells (*open circles*) 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
Relative affinity (EC_{50}) of PG9 binding to gp120 and scaffold constructs expressed in normal and GnTI$^-$ 293 cells

Construct	Strain	EC_{50} (μg/ml)[a]	
		293	GnTI$^-$
gp120			
	MN	0.5	0.06
	TRO.11	0.4	0.08
	108060	>10	0.9
	JRFL_E168K	0.07	0.01
	A244	0.4	0.007
	TH023	0.4	0.03
V1/V2 scaffolds			
	MN	>10	>10
	TRO.11	>10	0.5
	108060	>10	1
	JRFL_E168K	>10	>10
	A244	0.4	0.004
	TH023	0.6	0.01

[a] Data represent the concentration of PG9 required for 50% binding (EC_{50}) to gp120s or V1/V2 scaffolds coated to wells of microtiter plates (see "Experimental Procedures").

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. 2*A*), 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. 2*B*) 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 EC_{50} values of 0.004, 0.5, and 0.01 μg/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 CH03. 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.

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

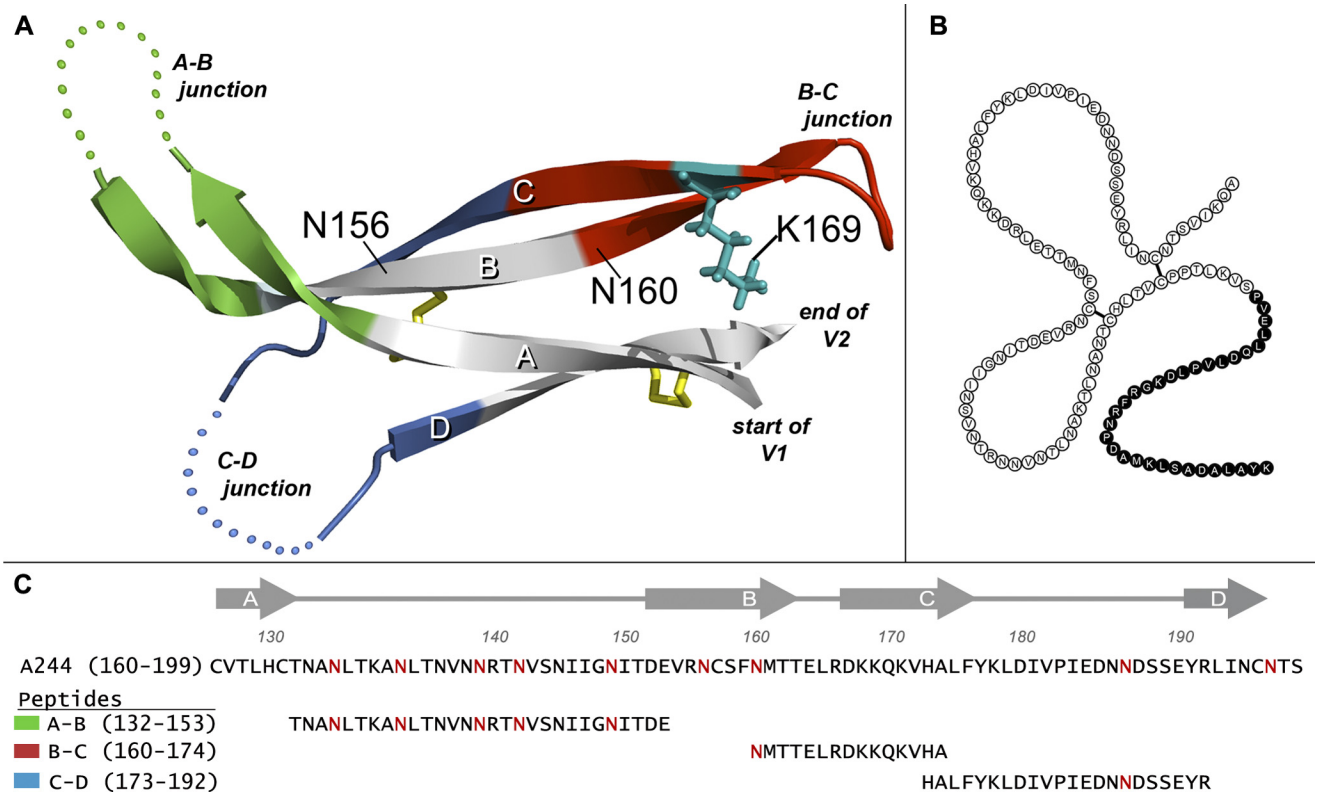


FIGURE 2. **Diagram of the V1/V2 domain and the A244 V1/V2 domain scaffold.** *A,* ribbon diagram of the three-dimensional structure of the V1/V2 region based on the structure of McLellan *et al.* (25). The approximate location of *N*-linked glycosylation sites (asparagine residues 156 and 160) required 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*.

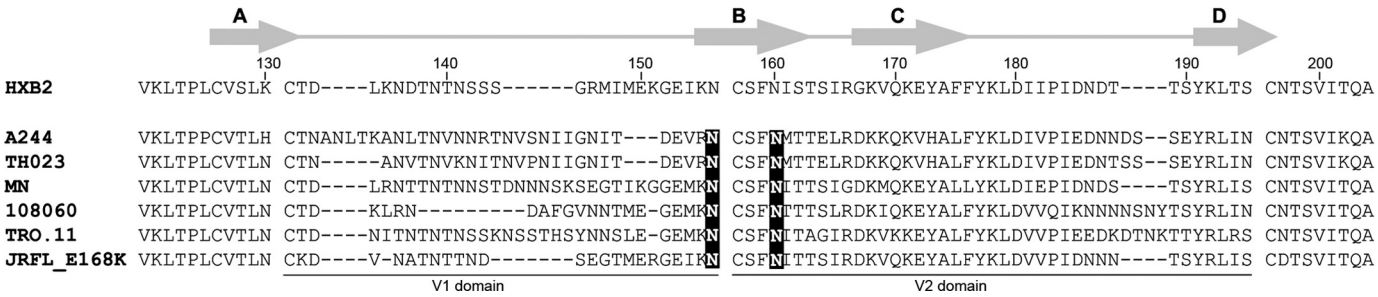


FIGURE 3. **Comparison of V1/V2 domain sequences from envelopes and scaffolds used for PG9 binding studies.** Sequence numbering was made with reference to the V1/V2 domain of HXB2. Sequences were aligned using MAFFT (98). *Arrows* above indicate the *A, B, C,* and *D* strands of the four stranded β-sheet structure described by McLellan *et al.* (25). *Thin lines* show the connecting peptides between the strands involved in the β-sheet.

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.

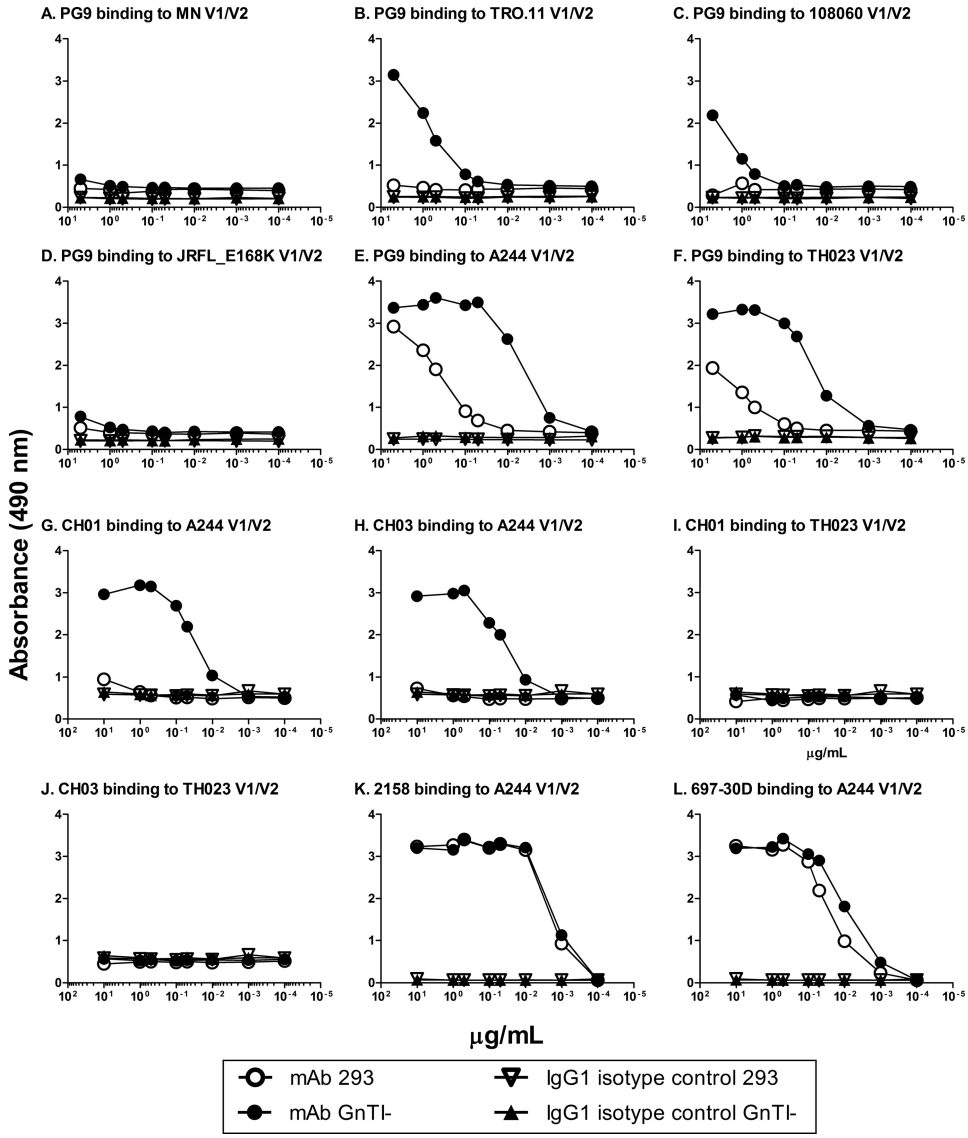
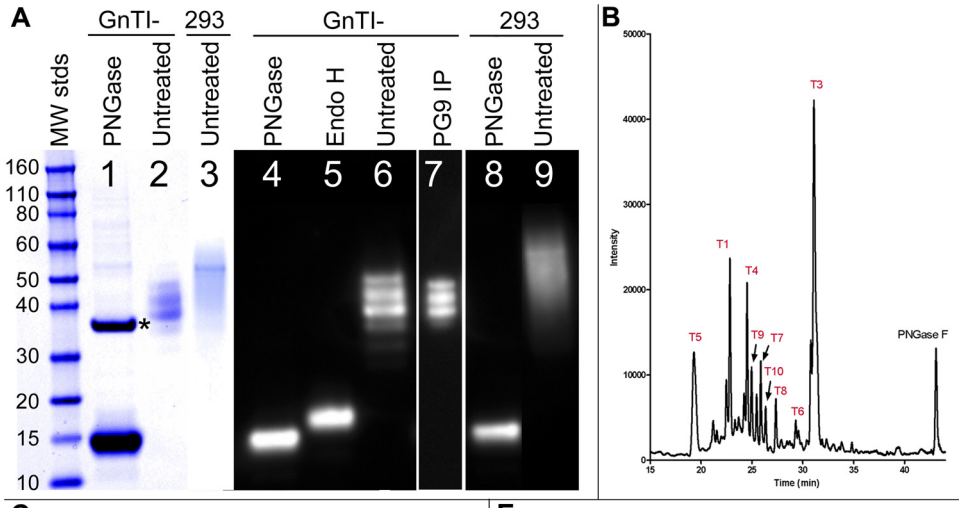


FIGURE 4. **Effect of glycosylation on PG9 binding to V1/V2 domain scaffolds from clade B and CRF01_AE isolates.** The genes encoding gp120 V1/V2 scaffolds from clade B isolates (MN, TRO.11, 108060, and JRFL_E168K) and CRF01_AE isolates (A244 and TH023) were transfected into either normal 293F cells (*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*).

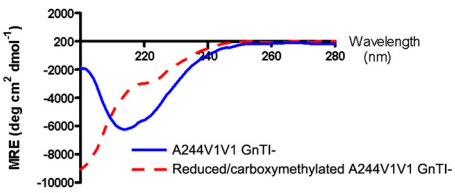
To explore this possibility, we carried out mass spectroscopy studies. For these studies, the A244 V1/V2 scaffold was digested with trypsin and then PNGase F. The resulting fragments were then analyzed by mass spectroscopy (Fig. 5, *B–D*). Six peaks (T4, T5, T6, T7, T9, and T10) containing PNGSs were 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 was attributed to differences in glycosylation at positions in the V1/V2 domain that did not appear to affect PG9 binding. Additionally, immunoprecipitation experiments (Fig. 5*A*, *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 α-helical structure (64). To further investigate the structure of the A244-V1/V2 scaffolds produced in GnTI$^-$ cells, we carried out circular dichroism studies (Fig. 5*E*). 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



C

V1/V2 peptide	Glycopeptides	PNGS	PNGS utilized
T4	LTPPCVTLHCTNANLTK	1	1
T5	ANLTNVNNR	2	0, 1, and 2
T6	TNVSNIIGNITDEVR	2	1 and 2
T7	NCSFNMTTELR	2	2
T9	LDIVPIEDNNDSSEYR	1	0 and 1
T10	LLINCNTSVIK	1	1



D

†Number	Peptides	Theoretical mass	Observed mass
T1	YALADASLK	951.5	951.5
T2	MADPNR	703.3	N/A
T3	DLPVLDQLLEVPSVK	1664.9	1665.4
T4	‡LTPPCVTLHCTNANLTK	1825.9	1827.2
T5	‡ANLTNVNNR	1015.5	1015.7, 1016.8, 1017.8
T6	‡TNVSNIIGNITDEVR	1644.9	1646.2,1647.2
T7	‡NCSFNMTTELR	1315.6	1317.6
T8	VHALFYK	877.5	877.7
T9	‡LDIVPIEDNNDSSEYR	1878.9	1879.2, 1880.2
T10	‡LINCNTSVIK	1104.6	1105.7

FIGURE 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 tryptic 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.

was preserved in the A244 V1/V2 fragment produced in GnTI$^-$ cells.

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 min-

TABLE 2
Immunization study to compare the immunogenicity of glycan-engineered envelope proteins and scaffolds from the A244 isolate of HIV-1

Group[a]	Immunization	
	Primary	Booster
1	gp120 (293)	gp120 (293)
2	gp120 (GnTI$^-$)	gp120 (GnTI$^-$)
3	gp120 (GnTI$^-$)	V1/V2 (GnTI$^-$)
4	V1/V2 (GnTI$^-$)	V1/V2 (GnTI$^-$)

[a] Each group consisted of three rabbits that were immunized as described under "Experimental Procedures."

imal 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. 6*A*) 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 His_6 tag epitope), with 50% end point dilution titers between 1:10,000 and 1:30,000 (Fig. 6*B*). 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).

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. 2*A*) 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. 2*A* 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 $\alpha4\beta7$ binding to gp120 (33, 76). When we examined the titers to all three peptides (Table 3), we found that the highest levels of antibodies

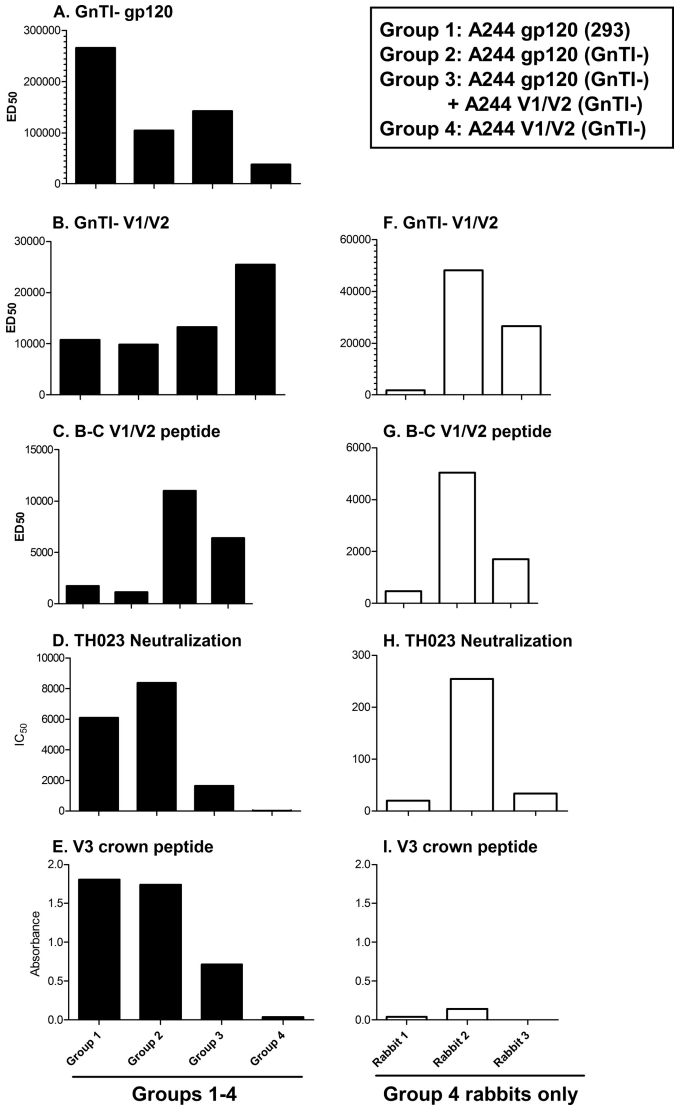


FIGURE 6. **Immunogenicity of A244 gp120s and V1/V2 scaffolds expressed in GnTI$^-$ cells.** Rabbits were immunized with A244-gp120 produced in 293 cells (group 1), A244-gp120 produced in GnTI$^-$ cells (group 2), or the A244-V1/V2 scaffold produced in GnTI$^-$ cells (group 4). A prime/boost protocol 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. EC_{50} values were determined by standard end point dilution ELISA for antibody binding to the A244 V1/V2 scaffold, the B-C peptide (NMTTELRDKKQKVHA) from the V1/V2 domain, and the V3 crown peptide (SITIGPGQVFYR) from A244 gp120. TZM-bl neutralization titers were carried out using pseudoviruses from the TH023 strain of HIV-1.

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. 6*C*) 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

TABLE 3
Binding specificity to rabbit sera to gp120s and V1/V2 scaffolds

	Antibody binding[a]			
	Group 1	Group 2	Group 3	Group 4
Junctional peptides[b]				
A-B	6747	10192	26194	24926
B-C	1712	1131	10996	6393
C-D	1520	3897	11144	5994
mAb competition[c]				
PG9	100	300	2700	8100
CH01	21817	46468	68653	114671
CH03	21210	48829	67645	96725
Cross-clade binding[d]				
UG37 (A)	13228	11288	2824	813
Bal.01 (B)	20304	20078	7931	878
CN54 (C)	28321	24387	9630	2202
96ZM651 (C)	19356	20800	5633	1829
UG21 (D)	19455	15538	5252	1042

[a] Pooled sera are from rabbit groups described in Table 2.
[b] Data represent 50% binding (EC_{50}) determined by end point dilution ELISA for antibody binding to the A-B, B-C, and C-D peptides indicated in Fig. 2*C*.
[c] 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 GnTI$^-$ gp120 protein.
[d] Ability of rabbit antibodies to bind gp120s from different clades. Clades A–D are indicated in parentheses.

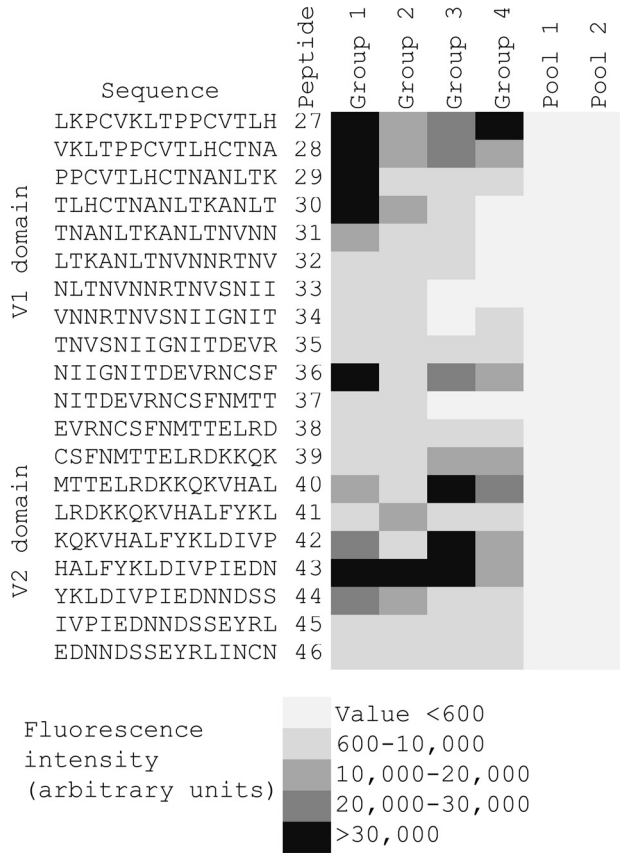


FIGURE 7. **Peptide microarray analysis of rabbit sera to the V1/V2 domain of A244 gp120.** Synthetic peptides, 15 amino acids in length with an overlap of 11 amino acids, were immobilized on microarray slides. The sequences of synthetic peptides spotted on the slides (27–46) are indicated. The slides 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.

three regions of the V1/V2 domain, compared with what could be achieved by immunization with gp120 alone.

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 LD(I/L)DV $\alpha4\beta7$-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.

Neutralizing Antibodies—We next measured the neutralizing antibody responses to pseudoviruses prepared from the tier 1 TH023 strain of HIV-1 (Fig. 6*D*). 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. 6*E*). 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, 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

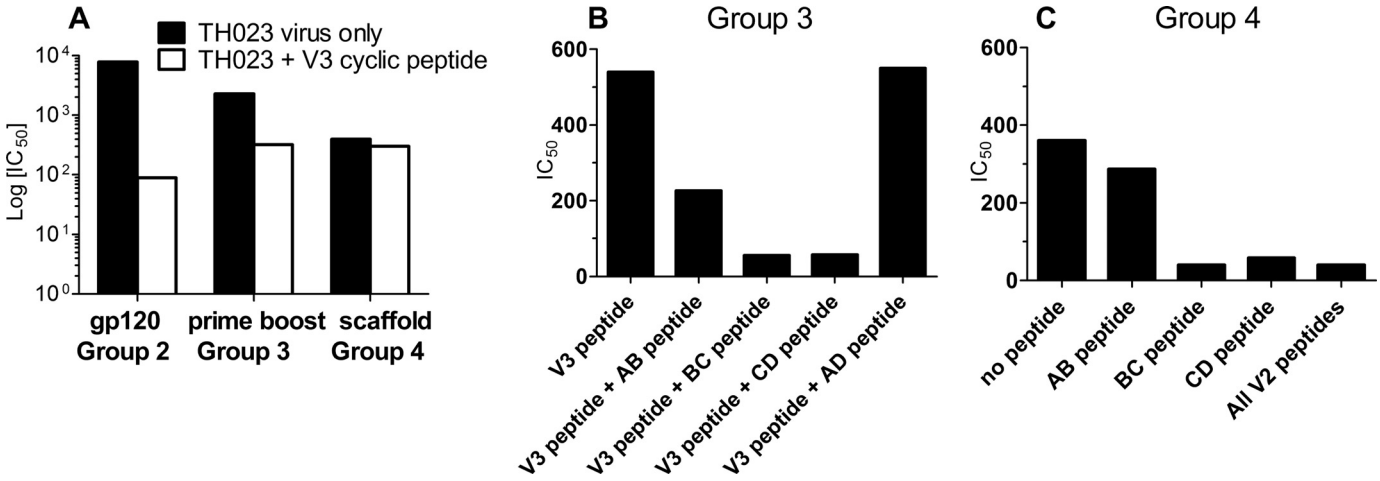


FIGURE 8. **Neutralization of TH023 before and after adsorption with synthetic peptides from the V1/V2 and V3 domains.** Pooled sera from the rabbit immunization study described in Table 2 were examined for neutralization activity with and without preincubation with synthetic peptides from the V1/V2 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.

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. 6*G*). When we examined the titers of neutralizing antibodies in these three rabbits (Fig. 6*H*), 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 ($R^2 = 0.9922$) between neutralizing antibodies and antibodies to the B-C peptide.

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. 8*A*. 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. 8*C*), 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.

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.

Cross-reactivity Studies—Finally, we examined the cross-reactivity 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.

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 bNAbs 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 theV1/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 interactions do not appear to be an absolute requirement for high affinity binding, because the K_d 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, 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, 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 EC_{50} 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 $\alpha4\beta7$-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 bNAb responses. 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 immunogenic 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.

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HIV-1 Envelope Proteins and V1/V2 Domain Scaffolds with Mannose-5 to Improve the Magnitude and Quality of Protective Antibody Responses to HIV-1

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