Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9


*Department of Integrative Structural and Computational Biology, International AIDS Vaccine Initiative Neutralizing Antibody Center and Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery, and Staggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037; and Well Medical College of Cornell University, New York, NY 10021; †Department of Medical Microbiology, Academic Medical Center, 1105 AZ, Amsterdam, The Netherlands; and ‡International AIDS Vaccine Initiative, Design and Development Laboratory, New York, NY 10038

Edited by Michel C. Nussenzweig, The Rockefeller University, New York, NY, and approved January 29, 2013 (received for review October 8, 2012)

PG9 is the founder member of an expanding family of glycan-dependent human antibodies that preferentially bind the HIV (HIV-1) envelope (Env) glycoprotein (gp) trimer and broadly neutralize the virus. Here, we show that a soluble SOSIP.664 gp140 trimer constructed from the Clade A BG505 sequence binds PG9 with high affinity (~11 nM), enabling structural and biophysical characterizations of the PG9:Env trimer complex. The BG505 SOSIP.664 gp140 trimer is remarkably stable as assessed by electron microscopy (EM) and differential scanning calorimetry. EM, small angle X-ray scattering, size exclusion chromatography with inline multilateral light scattering and isothermal titration calorimetry indicate that only a single PG9 fragment antigen-binding (Fab) binds to the Env trimer. An ~18 Å EM reconstruction demonstrates that PG9 recognizes the trimer asymmetrically at its apex via contact with two of the three gp120 protomers, possibly contributing to its reported preference for a quaternary epitope. Molecular modeling and isothermal titration calorimetry binding experiments with an engineered PG9 mutant suggest that, in addition to the N156 and N160 glycan interactions observed in crystal structures of PG9 with a scaffolded V1/V2 domain, PG9 makes secondary interactions with an N160 glycan from an adjacent gp120 protomer in the antibody–trimer complex. Together, these structural and biophysical findings should facilitate the design of HIV-1 immunogens that possess all elements of the quaternary PG9 epitope required to induce broadly neutralizing antibodies against this region.

Rational immunogen design is an increasingly promising approach for development of an effective human immunodeficiency virus-1 (HIV-1) vaccine. The recent discovery of many new and potent broadly neutralizing antibodies (bnAbs) has helped define conserved sites of vulnerability on the HIV-1 envelope (Env) glycoprotein (gp) complex that mediates viral entry into cells (refs. 1–6 and reviewed in refs. 7–11). Passive immunization studies show that sterilizing immunity can be achieved if sufficient amounts of bnAbs are present before virus challenge in macaques (12–16). Hence, intensive efforts are ongoing to design immunogens capable of re-eliciting these types of bnAbs by vaccination.

The major difficulty in mounting an effective antibody response against HIV-1 resides in the multiple evasion strategies that have evolved in Env. An error-prone reverse transcriptase drives a high degree of Env sequence diversity (17–19). The few conserved regions of Env are shielded by an extensive array of glycans (20–24) and are often occluded by more variable structures, such as the V1–V5 loops. However, because some HIV-1–infected individuals can develop bnAbs over the course of infection, these various evasion strategies are not insurmountable (1, 25–27). Although bnAbs do not seem to confer significant protection against disease progression in infected individuals (28, 29), their induction through vaccination might prevent the acquisition of infection. Thus, the epitopes recognized by bnAbs are now being carefully scrutinized to serve as templates for rational vaccine design.

Conserved elements in the V1/V2 variable loops on gp120 contain epitopes for a family of glycan-dependent bnAbs, including PG9 and PG16. These quaternary-prefering bnAbs were isolated from an African donor and neutralize 70–80% of circulating HIV-1 isolates with high potency (2, 6). Both antibodies possess an elongated (28 residues), hammerhead-shaped, complementarity-determining region 3 of the heavy chain (HCDR3) that contains tyrosine sulfation sites (30, 31). Other bnAbs that target the same epitopes in this region, such as the PGT140 and CH01 series, share both of these unusual structural features (6, 32). Whether other V1/V2 bnAbs have similar characteristics is as yet unclear (33). Early functional studies showed that the interaction between PG9 or PG16 and the V1/V2 loop highly depends on a glycan at position N160 and the overall cationic character of protein segments in this region (2). Recently, cryostal structures of protein scaffolds bearing V1/V2 loops from two different isolates showed that PG9 interacts with two glycans and a β-sheet (32). More specifically, the HCDR3 hammerhead penetrates the glycan shield to mediate mostly charged interactions with strands C of a disulfide-linked, antiparallel β-sheet in the V1/V2 region, whereas glycans at positions N160 and either N156 or N173 are accommodated in the surrounding antibody paratope (32). Although these structures clearly revealed some of the key interactions between PG9 and the V1/V2 loops at an atomic level, they did not clarify why bnAbs in this family are generally trimer-specific (i.e., why they do not bind to most monomeric gp120 proteins, despite neutralizing the corresponding virus).

Here, we elucidate how PG9 recognizes soluble Env trimers. These trimers are based on the BG505 Env sequence, cleaved at the gp10/gp41 junction, stabilized by SOSIP mutations, and truncated at residue 664 of the gp41 ectodomain (34–37). Several biochemical and biophysical techniques, alone and in combination, clearly show that only a single PG9 fragment antigen-binding (Fab) binds each BG505 SOSIP.664 glycoprotein 140 (gp140) trimer. Overall, these findings may have significant implications for guiding immunogen design efforts intended to induce PG9-like bnAbs by vaccination; it now seems important to display all of the components of these quaternary epitopes in an appropriate, asymmetric manner.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Frequently available online through the PNAS open access option.

Data deposition: The reconstruction data reported in this paper has been deposited in the Electron Microscopy Data Bank, www.emdatabank.org (EMDB ID code EMDB-2241).

1To whom correspondence should be addressed. E-mail: abward@scripps.edu.

*To whom correspondence should be addressed. E-mail: abward@scripps.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217537110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1217537110

PNAS | March 12, 2013 | vol. 110 | no. 11 | 4351–4356
Experimental error, to one PG9 Fab bound per trimer (Fig. 1B). The SEC-purified PG9 Fab:trimer complex was monodisperse and able to bind additional PG9 IgG in immunoprecipitation assays and negatively interfering with one another (Fig. S2). The excluded volume determined by analysis of the Porod invariant was 786 nm$^3$ (Fig. S2). This volume is directly related to molecular mass by a factor of 0.5 for large globular proteins (38), so the implied mass of the Fab:trimer complex is $\sim 393$ kDa. Hence, the SAXS-determined molecular mass of the monodisperse sample, which has a theoretical calculated mass of 392 kDa (Table S1), corroborated the SEC-MALS data and provided further evidence that only one PG9 Fab binds to each Env trimer. An ab initio density map was generated from scattering data with $q_{\text{max}} = 0.2 \text{ Å}^{-1}$ that corresponds to a $\sim 30$ Å nominal resolution for the scattering curve (38). The resulting low-resolution density map allowed the previously determined crystal structure of monomeric gp120 to be fitted into a trimeric arrangement and the crystal structure of a complex containing one PG9 Fab and gp120 V1/V2 scaffold (Fig. 2A).

**Molecular Structure Revealed by Negative Stain Single Particle EM.**

The interaction between the PG9 Fab and the SOSIP.664 gp140 trimer was also analyzed by negative stain electron microscopy (EM). The reference free 2D class averages of a purified complex, and an unpurified sample containing a 10-fold molar excess of the Fab, unequivocally showed that the Fab recognizes the trimer in a unique and asymmetric manner (Figs. S3 and S4 and Table S2); the interaction is dissimilar to those for all other structurally characterized bnAbs, where one Fab is bound to each gp120 protomer in a threefold symmetric arrangement (39–41). In addition to corroborating the low-resolution SAXS solution structure (Fig. S5), the EM reconstruction enabled high-fidelity docking of the PG9:V1/V2 scaffold crystal structure (Fig. 2B and Fig. S6). In the fitted model, the PG9 HCDR3 hammerhead sits directly atop the trimer and the N160 glycan lies immediately adjacent to the trimer axis, whereas the glycan at positions N156 or N173 resides at the outer edge of the density ascribed to V1/V2 (Fig. 2B). One copy of core gp120 can be fitted into each of the three lobes of density that correspond to each protomer of the Env trimer. Two of these gp120 protomers were positioned in close proximity to PG9, whereas the third appears to have moved away from the trimer center, explaining the loss of C3 symmetry (Fig. 2C). This observation led to the hypothesis that the preference of PG9 for a quaternary epitope might be attributable to additional interactions involving one or more non-structural factors.
glycan, comes into close proximity to elements at the trimer interface, consistent with the EM-derived trimer model.

Stability of the BG505 SOSIP.664 Trimer and the Effect of PG9 Binding. The EM reconstruction of the unliganded BG505 SOSIP.664 trimer showed a slightly more compact structure than the corresponding unliganded trimer based on the KNH1144 sequence (Fig. 3A and Fig. S7). In accord with these structural observations, the melting temperature of these two constructs (Tm), as probed by differential scanning calorimetry (DSC), differed significantly. Whereas the KNH1144 gp140 trimer showed thermal transitions at three different temperatures (51.3 °C, 61.4 °C, and 68.1 °C), its BG505 counterpart remained intact up to a temperature of 67.9 °C (Fig. 4B). These data imply that the BG505 SOSIP.664 gp140 trimer is unusually stable; overall, we saw no signs of subunit dissociation until complete protein unfolding occurs. Of additional note is that PG9 binding appears to have both

both of the other two V1/V2 loop structures that are also present at the trimer apex. A secondary interaction site of this nature could not have been observed in the PG9:V1/V2 scaffold crystal structure, which involves only monomeric components.

High-Affinity PG9 Binding and a Putative Secondary Site of Interaction on the Env Trimer. Fitting the PG9:V1/V2 crystal structure into the EM reconstruction suggested that the PG9 Fab made additional trimer contacts via its HCDR1, HCDR3, and complementary determining region 2 of the light chain (LCDR2) regions. To probe whether this element of the PG9 binding site interacts with a different V1/V2 moiety at the trimer interface, a PG9 mutant was designed. A bulky Man$_5$GlcNAc$_2$ glycan was introduced into PG9 to interact with the pocket around the predicted secondary V1/V2 interaction by substituting residues S$_{58}$GV$^{59}$ at the edge of LCDR2 with a canonical NGT glycosylation motif (Fig. 3A). This strategy enabled contacts or steric clashes lying within the ∼16 Å × 14 Å × 10 Å footprint of the Man$_5$GlcNAc$_2$ glycan to be probed by assessing changes to antibody function. Accordingly, when tested in HIV-1 neutralization assays, the glycan mutant of PG9 was ~10-fold less potent than the WT antibody (Fig. 3B). Of note is that the WT and glycan-mutant forms of PG9 bound to monomeric BG505 gp120 with almost identical affinities (∼20–30 nM), as assessed by isothermal titration calorimetry (ITC), but the glycan mutation caused a >10-fold reduction in the Fab affinity for the BG505 SOSIP.664 gp140 trimer (Fig. 3C and Table S3). Thus, the introduced glycan moiety has quantitatively similar adverse effects on both trimer binding and virus neutralization. Together, these results indicate that PG9 LCDR2, the site of the introduced glycan, comes into close proximity to elements at the trimer interface, consistent with the EM-derived trimer model.

Stability of the BG505 SOSIP.664 Trimer and the Effect of PG9 Binding. The EM reconstruction of the unliganded BG505 SOSIP.664 trimer showed a slightly more compact structure than the corresponding unliganded trimer based on the KNH1144 sequence (Fig. 3A and Fig. S7). In accord with these structural observations, the melting temperature of these two constructs (Tm), as probed by differential scanning calorimetry (DSC), differed significantly. Whereas the KNH1144 gp140 trimer showed thermal transitions at three different temperatures (51.3 °C, 61.4 °C, and 68.1 °C), its BG505 counterpart remained intact up to a temperature of 67.9 °C (Fig. 4B). These data imply that the BG505 SOSIP.664 gp140 trimer is unusually stable; overall, we saw no signs of subunit dissociation until complete protein unfolding occurs. Of additional note is that PG9 binding appears to have both

both of the other two V1/V2 loop structures that are also present at the trimer apex. A secondary interaction site of this nature could not have been observed in the PG9:V1/V2 scaffold crystal structure, which involves only monomeric components.

High-Affinity PG9 Binding and a Putative Secondary Site of Interaction on the Env Trimer. Fitting the PG9:V1/V2 crystal structure into the EM reconstruction suggested that the PG9 Fab made additional trimer contacts via its HCDR1, HCDR3, and complementary determining region 2 of the light chain (LCDR2) regions. To probe whether this element of the PG9 binding site interacts with a different V1/V2 moiety at the trimer interface, a PG9 mutant was designed. A bulky Man$_5$GlcNAc$_2$ glycan was introduced into PG9 to interact with the pocket around the predicted secondary V1/V2 interaction by substituting residues S$_{58}$GV$^{59}$ at the edge of LCDR2 with a canonical NGT glycosylation motif (Fig. 3A). This strategy enabled contacts or steric clashes lying within the ∼16 Å × 14 Å × 10 Å footprint of the Man$_5$GlcNAc$_2$ glycan to be probed by assessing changes to antibody function. Accordingly, when tested in HIV-1 neutralization assays, the glycan mutant of PG9 was ~10-fold less potent than the WT antibody (Fig. 3B). Of note is that the WT and glycan-mutant forms of PG9 bound to monomeric BG505 gp120 with almost identical affinities (∼20–30 nM), as assessed by isothermal titration calorimetry (ITC), but the glycan mutation caused a >10-fold reduction in the Fab affinity for the BG505 SOSIP.664 gp140 trimer (Fig. 3C and Table S3). Thus, the introduced glycan moiety has quantitatively similar adverse effects on both trimer binding and virus neutralization. Together, these results indicate that PG9 LCDR2, the site of the introduced glycan, comes into close proximity to elements at the trimer interface, consistent with the EM-derived trimer model.

Stability of the BG505 SOSIP.664 Trimer and the Effect of PG9 Binding. The EM reconstruction of the unliganded BG505 SOSIP.664 trimer showed a slightly more compact structure than the corresponding unliganded trimer based on the KNH1144 sequence (Fig. 3A and Fig. S7). In accord with these structural observations, the melting temperature of these two constructs (Tm), as probed by differential scanning calorimetry (DSC), differed significantly. Whereas the KNH1144 gp140 trimer showed thermal transitions at three different temperatures (51.3 °C, 61.4 °C, and 68.1 °C), its BG505 counterpart remained intact up to a temperature of 67.9 °C (Fig. 4B). These data imply that the BG505 SOSIP.664 gp140 trimer is unusually stable; overall, we saw no signs of subunit dissociation until complete protein unfolding occurs. Of additional note is that PG9 binding appears to have both

both of the other two V1/V2 loop structures that are also present at the trimer apex. A secondary interaction site of this nature could not have been observed in the PG9:V1/V2 scaffold crystal structure, which involves only monomeric components.

High-Affinity PG9 Binding and a Putative Secondary Site of Interaction on the Env Trimer. Fitting the PG9:V1/V2 crystal structure into the EM reconstruction suggested that the PG9 Fab made additional trimer contacts via its HCDR1, HCDR3, and complementary determining region 2 of the light chain (LCDR2) regions. To probe whether this element of the PG9 binding site interacts with a different V1/V2 moiety at the trimer interface, a PG9 mutant was designed. A bulky Man$_5$GlcNAc$_2$ glycan was introduced into PG9 to interact with the pocket around the predicted secondary V1/V2 interaction by substituting residues S$_{58}$GV$^{59}$ at the edge of LCDR2 with a canonical NGT glycosylation motif (Fig. 3A). This strategy enabled contacts or steric clashes lying within the ∼16 Å × 14 Å × 10 Å footprint of the Man$_5$GlcNAc$_2$ glycan to be probed by assessing changes to antibody function. Accordingly, when tested in HIV-1 neutralization assays, the glycan mutant of PG9 was ~10-fold less potent than the WT antibody (Fig. 3B). Of note is that the WT and glycan-mutant forms of PG9 bound to monomeric BG505 gp120 with almost identical affinities (∼20–30 nM), as assessed by isothermal titration calorimetry (ITC), but the glycan mutation caused a >10-fold reduction in the Fab affinity for the BG505 SOSIP.664 gp140 trimer (Fig. 3C and Table S3). Thus, the introduced glycan moiety has quantitatively similar adverse effects on both trimer binding and virus neutralization. Together, these results indicate that PG9 LCDR2, the site of the introduced glycan, comes into close proximity to elements at the trimer interface, consistent with the EM-derived trimer model.

Stability of the BG505 SOSIP.664 Trimer and the Effect of PG9 Binding. The EM reconstruction of the unliganded BG505 SOSIP.664 trimer showed a slightly more compact structure than the corresponding unliganded trimer based on the KNH1144 sequence (Fig. 3A and Fig. S7). In accord with these structural observations, the melting temperature of these two constructs (Tm), as probed by differential scanning calorimetry (DSC), differed significantly. Whereas the KNH1144 gp140 trimer showed thermal transitions at three different temperatures (51.3 °C, 61.4 °C, and 68.1 °C), its BG505 counterpart remained intact up to a temperature of 67.9 °C (Fig. 4B). These data imply that the BG505 SOSIP.664 gp140 trimer is unusually stable; overall, we saw no signs of subunit dissociation until complete protein unfolding occurs. Of additional note is that PG9 binding appears to have both
protomers in the trimer (those to which it binds), while a mechanism by which PG9 binding stabilizes two of the gp120 open conformation (42). Our DSC data therefore support changes in trimers are reported to drive conversion to a more
with other studies where soluble CD4-induced conformational
bilizing effect of soluble CD4 binding (Fig. 4
of the BG505 SOSIP.664 gp140 trimer contrasts with the destabi-
ness of stability than its KNH1144 counterpart, where thermal transitions
particularly at the spike apex. Images were generated with UCSF Chimera
25-Å resolution. The latter appears to adopt a more compact conformation,
rapidly add new elements to those previously identi-
ated N160 Man5GlcNAc2 glycan. The model on the left was generated from the EM reconstruction of the unliganded
PG9 WT had similar (∼30 nM) binding affinities for the SOSIP.664 gp140 trimer and gp120 monomer from 293S cells, but the anti-
body bound with much higher affinity to the trimer (11 nM) than to the corresponding monomer (110 nM), when both Env proteins
were produced in 293T cells (Fig. S9). The proximity of additional
PG9 components, such as HCDR1, HCDR3, and LCDR2, to elements located at the trimer apex appears to create a higher
affinity interaction when the SOSIP.664 gp140 trimers are pro-
duced in 293T cells. These data imply that specific glycoforms on
the Env trimer, which may include complex and high mannose
sugars, are important components of the complete PG9 epitope.

Discussion
It was shown that the PG9 bnAb is directed against an epitope in
the V1/V2 region of gp120 (2, 32). Here, we took advantage of the identification and production of a more stable, cleaved
Env trimer (BG505 SOSIP.664) to conduct multiple structural (EM and SAXS) and biophysical studies (SEC-MALS, ITC, and
DSC) of the PG9:trimer complex. All of these data indicate that
a destabilizing and a stabilizing effect on the BG505 SOSIP.664 gp140 trimer. Upon PG9 binding to the trimer, two thermal
transitions at 52.2 °C and 73.8 °C (Fig. 4C) occur in addition to those seen with the individual unbound PG9 Fab and trimer
components (Fig. 4B and Fig. S8). The thermal stability profile
of the BG505 SOSIP.664 gp140 trimer contrasts with the destabi-
lying effect of soluble CD4 binding (Fig. 4C) and is consistent with other studies where soluble CD4-induced conformational
changes in trimers are reported to drive conversion to a more
open conformation (42). Our DSC data therefore support a mechanism by which PG9 binding stabilizes two of the gp120 protomers in the trimer (those to which it binds), while destabilizing the third protomer that, as a consequence, disrupts the overall integrity of the trimer.

Role of Glycosylation in PG9 Recognition of Env. Expression of glyco-
coproteins in 293S cells, in which glycan maturation pathways are
blocked, leads to an accumulation of unprocessed, high-mannose
sugars (43, 44). However, 293T cells can process immature gly-
cans to complex forms. EM data confirmed that one PG9 binds
to the trimer in a similar asymmetric manner whether the trimer is
produced in 293S or 293T cells (Fig. S4). However, we saw marked
differences in the affinities of PG9 for monomeric gp120 and trimeric gp140, depending on the expression system used.
PG9 WT had similar (∼30 nM) binding affinities for the SOSIP.664 gp140 trimer and gp120 monomer from 293S cells, but the anti-
body bound with much higher affinity to the trimer (11 nM) than to the corresponding monomer (110 nM), when both Env proteins
were produced in 293T cells (Fig. S9). The proximity of additional
PG9 components, such as HCDR1, HCDR3, and LCDR2, to elements located at the trimer apex appears to create a higher
affinity interaction when the SOSIP.664 gp140 trimers are pro-
duced in 293T cells. These data imply that specific glycoforms on
the Env trimer, which may include complex and high mannose
sugars, are important components of the complete PG9 epitope.

Fig. 4. EM and DSC studies of the unliganded SOSIP.664 trimer structure and stability. (A) Comparison of the EM reconstructions of the KNH1144 (blue mesh) and BG505 (orange surface) SOSIP.664 gp140 trimers, both at 25Å resolution. The latter appears to adopt a more compact conformation, particularly at the spike apex. Images were generated with UCSF Chimera (S8). B) The melting profile of the BG505 trimer suggests it has a higher degree of stability than its KNH1144 counterpart, where thermal transitions are initiated at a 16.6 °C lower temperature. Raw data are shown in black and the fitted curves from which Tm values were obtained for the different peaks observed are colored in red. C) Whereas soluble CD4 (scD4) binding destabilizes the BG505 SOSIP.664 gp140 trimer (initiation of thermal de-
naturation 16.8 °C lower than for the unliganded sample), PG9 binding appears to both destabilize and stabilize elements of the trimer (note the appearance of events with Tms at 52.2 °C and 73.8 °C). The unliganded PG9 Fab and soluble CD4 have Tms of 67.7 °C and 61.7 °C, respectively (Fig. S8).

A

B

C

Fig. 5. Model of the asymmetric recognition of the HIV-1 Env trimer by bnAb PG9. (A) One PG9 Fab interacts closely with two gp120 protomers atop the HIV-1 spike. For clarity, the third gp120 protomer has been omitted. PG9 (shades of gray) sits directly above the trimer axis and potentially accom-
modates additional elements to those previously identi-
fied in Figs. 2 and 3. A second V1/V2 model (purple) with −120° rotation relative to the primary V1/V2 model (magenta) was generated to fit the density images were generated with UCSF Chimera (S8). (B) Cartoon representation of the PG9 epitope on the Env trimer, showing the N156 glycan (yellow), the N160 glycan (magenta), cationic elements of strand C (cyan) from the first V1/V2 gp120 protomer, and the suggested involvement of a second N160 glycan (purpel) from a neighboring protomer. (C) Cartoon representation of how the interaction of PG9 with two V1/V2 elements at the Env trimer apex results in the third gp120 protomer slightly peeling away from the trimer axis. Glycans are shown as spheres, gp41 as orange rectangles, whereas other elements are colored as in
previous figures.
a single PG9 Fab interacts asymmetrically with the Env trimer with nanomolar binding affinity and appears to engage two V1/V2 elements at the trimer apex. Overall, our EM, neutralization, and binding data indicate that PG9 has a more extended site of interaction with the trimer than was revealed by the crystal structure of the monomeric PG9:V1/V2 scaffold complex (32). Elements of the extended paratopes, including HCDR1, HCDR3, and LCDR2, come into close proximity to a neighboring V1/V2 moiety at the trimer apex; these secondary interactions appear to involve the glycan moiety located at position N160. In the context of the Env trimer, PG9 could, therefore, contact a total of three glycans and a β-strand (Fig. 5). The additional protein and glycan elements emanating from a neighboring protomer may, wholly or in part, explain the previously reported quaternary nature of the PG9 epitope (2). Thus, the PG9:BG505 SOSIP.664 gp140 trimer structure reported here constitutes a paradigm shift in antibody recognition of HIV-1 Env, where only a single Fab binds to the Env trimer by interacting asymmetrically with two of the three gp120 protomers. Previous mixed-trimer neutralization experiments led to the hypothesis that PG9 recognized a single protomer of a trimer (2, 43), but that test system could not detect the weaker secondary interactions that we deduce from structural studies.

Our EM-derived model suggests that the N160 glycans from each protomer are arranged at the Env apex in close proximity to one another around the threefold axis (Fig. S10). We postulate that the elongated anionic, tyrosine-sulfated HCDR3 hammerhead of PG9 penetrates through a dense trimeric cluster of N160 and N156/175 glycans to reach a sequestered cationic groove in the center of the trimer atop the spike (Fig. 5). PG9 recognition of its quaternary epitope at the Env trimer apex represents a unique asymmetric binding mode when compared to other HIV-1 bnAbs or any other viral glycoprotein–antibody complex. Although additional work is needed to confirm the exact carbohydrate glycoform(s) present at the interacting N-glycosylation sites, steric constraints imposed by gp120 trimerization would restrict processing of the glycans that are located in this region near the trimer apex (45–47). Our comparative studies of BG505 gp120 and SOSIP.664 gp140 trimers expressed in 293S vs. 293T cells suggest that the precise glycoforms in and around the PG9 epitope influence binding affinity, but not the binding mode. Further studies demonstrating the exact glycan composition of these interacting sites in monomers and trimers in different cell types, and how differential glycosylation correlates with antigenicity, is likely to be very informative.

To date, only a few gp120 monomers, from clones such as BG505, have been shown to bind PG9, for reasons that have been poorly understood (31, 32, 36, 43, 48, 49). It is likely that the PG9 epitope on these select monomers is broadly similar to, but also critically different from, the bona fide epitope present on the native Env trimer. Glycoform profiles may be particularly important in this regard; although the BG505 gp120 monomer binds PG9, the gp120 has to be produced in a cell line (293S) in order to reach the nanomolar range. When the same gp120 is made in 293T cells, it has a lower affinity for PG9 and only a small fraction is competent for binding. We suggest that, for most other gp120 monomers, these glycan-dependent restrictions on the formation of the PG9 epitope are more profound, leading to significantly reduced or no affinity. Additionally, in the context of the trimer, the V1/V2 loops may be constrained in a particular orientation that is not generally exhibited in the monomer.

We hypothesize that the bona fide (i.e., high affinity) form of the PG9 epitope is present only on properly folded trimers and absent from nonfunctional, but highly immunogenic, forms of Env also present on virions. Accordingly, such a quaternary epitope might elicit a particularly productive response from the host’s humoral immune system. Here, we show that the BG505 SOSIP.664 gp140 trimer not only binds PG9, but is also more thermally stable than gp120 monomers (50, 51) and previously described SOSIP gp140 trimers (34). Studies comparing how neutralizing and nonneutralizing Abs recognize the CD4bs on Env highlight the importance of epitope presentation in the context of the functional trimer (50). Thus, this Env trimer may be useful in a vaccine context for presentation of quaternary and other broadly neutralizing epitopes that are now being defined from analyses of bnAbs generated during the course of natural HIV-1 infection.

Materials and Methods

The PG9 Fab, BG505 gp120 monomer, and BG505 SOSIP.664 gp140 trimer were expressed as recombinant proteins and purified as described (31, 34, 41). Melting temperatures of trimers and Fab/trimer complexes were measured by DSC, whereas PG9 Fab binding to Env proteins was assessed by SEC-MALS, ITC, SAXS, and EM. The SAXS data were analyzed by using PRIMUS (52), GASBOR (53), and DAMAVER (54). EM reconstructions were carried out by using Xmipp (55), IMAGIC (53), SPARX (56), and EMAN (57). Fitting of X-ray models into the EM reconstruction was carried out by using UCSF Chimera (58). For more details, see SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Y. Hua, D. C. Diwanji, P. S. Lee, L. Kong, R. S. Stanfield, and C. Dreyfus for technical assistance; W. C. Koff and D. R. Burton for valuable discussions; and the staff at the Stanford Synchrotron Radiation Lightsource (SSRL) BIO-SAXS beamline 4-2, particularly Thomas Weiss, for assistance with SAXS data collection and analysis. Portions of this research were carried out at SSRL, a Directorate of the Stanford Linear Accelerator Center (SLAC) National Accelerator Laboratory, and an Office of Science User Facility operated for the Department of Energy (DOE) Office of Science by Stanford University. This work was supported by National Institutes of Health (NIH) Grants HIVRAD P01 AI082362 and R01 AI136082; the International AIDS Vaccine Initiative Neutralizing Antibody Center; Scripps Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery Grant UM1 AI010663; the University of California, San Diego Center for AIDS Research; NIH-funded program Grant P30 AI036214 (which is supported by the following NIH Institutes and Centers: National Institute of Allergy and Infectious Diseases; National Cancer Institute; National Institute of Mental Health; National Institute on Drug Abuse; National Institute of Child Health and Human Development; National Heart, Lung, and Blood Institute; National Institute on Aging); a Vidi grant from the Netherlands Organization for Scientific Research (to R.W.S.); a Starting Investigator Grant from the European Research Council (to R.W.S.); AIDS Fonds Grant 2011032 (to R.D.); and a Canadian Institutes of Health Research fellow to R.W.S.; a European Research Council (to R.W.S.); AIDS Fonds Grant 2011032 (to R.D.); and a Canadian Institutes of Health Research fellowship (to J.P.J.). The three-dimensional reconstructions were conducted at the National Resource for Automated Molecular Microscopy, which is supported by NIH through the National Center for Research Resources P41 Program RR017573. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research; NIH National Center for Research Resources, Biomedical Technology Program P41RR001209; and the National Institute of General Medical Sciences. This work is manuscript 21950 from The Scripps Research Institute.


Supporting Information
Supporting Information Corrected January 15, 2014
Julien et al. 10.1073/pnas.1217537110

SI Materials and Methods

Protein Expression and Purification. The PG9 Fab was expressed as a recombinant protein by following a protocol similar to that described (1). To ensure a maximum level of tyrosine sulfation, the heavy and light chain genes were cotransfected in 293T cells with a gene encoding tyrosylprotein sulfotransferase 1. The secreted PG9 Fab was harvested 6–7 d after transfection, and the supernatant was directly loaded on an antihuman λ light chain affinity matrix (CaptureSelect Fab λ; BAC): After elution with 100 mM glycine at pH 2.7, the sample was exchanged into buffer containing 20 mM sodium acetate at pH 5.6 and loaded onto a MonoS cation exchange column (GE Healthcare). After a gradient elution with potassium chloride, the PG9 Fab was further purified by size-exclusion chromatography in a buffer containing 20 mM Tris at pH 8.0 and 150 mM sodium chloride. The PG9 Fab glycan mutant was generated by mutation-Polymerase Incomplete Primer Extension (PIPE) PCR and the resulting DNA was sequenced to ensure the correct introduction of the desired mutations. A high level of purity for both the WT and mutant PG9 Fabs was confirmed by ESI-TOF high accuracy mass spectrometry.

The BG505 gp120 monomer and BG505 SOSIP.664 gp140 trimer were expressed and purified using methods similar to those previously described (2). The BG505 (BG505.W6M.ENV.C2) env gene (GenBank accession nos. ABA61516 and DQ208458) is derived from a clade A virus from an infected infant (3). It has 73% identity to the proposed PG9-sensitive progenitor virus from the PG9 donor subject, based on computational analysis of the most recent common ancestor sequence (4). The BG505 gp120 monomer has the rare characteristic of binding PG9 (4). Brieﬂy, after expression in either 293T or 293S (GNT T-deﬁcient) cells, the secreted BG505-derived proteins were harvested from supernatants. The gp120 monomer was puriﬁed by using a Galanthus Nivalis Lectin (GNL) afﬁnity matrix, the SOSIP.664 gp140 trimer using a 2G12-coupled afﬁnity column (2, 5). After elution from the respective columns, the monomers and trimers were puriﬁed to size homogeneity by using a Superdex 200 or Superose 6 SEC matrix, respectively (GE Healthcare).

Size-Exclusion Chromatography Coupled with Multilight Light Scattering. To obtain a pure PG9 Fab:BG505 SOSIP.664 gp140 trimer sample, PG9 Fab was added in a sufﬁx molar excess over the trimer. After incubation for 15 min on ice, the sample was loaded on a Superose 6 10/30 SEC column (GE Healthcare), which was coupled in-line on an AKTA Avant FPLC system (GE Healthcare) with the following calibrated detection systems: (i) HP 1050 Hewlett-Packard UV detector; (ii) MiniDawn Treos multilight light scattering (MALS) detector (Wyatt); (iii) quasielastic light scattering (QELS) detector (Wyatt); (iv) Optilab T-reX refractive index (RI) detector (Wyatt). Analysis of the light scattering data coupled to UV and refractive index protein concentration measurements allowed determination of the molar mass of the eluting protein by using the protein conjugate template in Astra V, as reported for KNH1144 SOSIP.664 gp140 (5).

Immunoprecipitation Assays. Immunoprecipitation assays were performed essentially as described (6). Brieﬂy, 2.0 μg of BG505 SOSIP.664 or 2.0 μg of SOSIP.664:PG9 Fab complex was incubated overnight at 4°C, with rotation, with PG9 IgG at equimolar amounts, 10-fold lower amounts, or 10-fold excess, in 500 μL of radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris·HCl at pH 7.0, 150 mM sodium chloride, 1% Nonidet P-40, 0.25% sodium deoxycholate, and protease inhibitors (Complete protease inhibitor tablets; Roche)]. Next, protein G-coated agarose beads (Pierce/Thermo Fisher) were added and incubated for 2 h at 4°C with rotation. The beads were then washed three times with RIPA buffer (supplemented with 0.01% Tween 20), after which the bound proteins were eluted by heating at 95°C for 10 min in 50 μL of 2x SDS-loading buffer containing 100 mM DTT. The immunoprecipitates were fractionated by SDS/PAGE (8% polyacrylamide) at 125 V for 1.5 h. Env detection was performed by using MAb ARP3119 and standard Western blot techniques.

Small-Angle X-Ray Scattering. Samples of the purified PG9:BG505 SOSIP.664 gp140 complex isolated from the SEC-UV/MALS/RI experiment were concentrated to 0.16, 0.34, or 0.65 mg/mL for later study by small-angle X-ray scattering (SAXS) at the Stanford Synchrotron Radiation Lightsource BIO-SAXS beamline 4-2. For each concentration, 15 exposures of 2 s each were collected and the resulting scattering curves were corrected with ones derived from buffer-control samples. Inspection of the SAXS data using the PRIMUS program (7) showed no signs of aggregation, long-range interactions or radiation damage. An ideal scattering curve was generated by merging the low resolution data collected from a more dilute sample (0.34 mg/mL) with the larger angle data of the most concentrated sample (0.65 mg/mL). Scattering data up to q = 0.2 Å⁻¹ were included. Agreement between Rg and I(0) values determined from the Guinier plot [log[I(q)] vs. q²] and the pair-distribution function, P(r) further confirmed the good quality of the data. As such, determination of the Pmax overall shape, excluded volume, and molar mass were performed, with high confidence, via analysis of the P(r) function, Kratky plots [I(q)q² vs. q] and Porod invariant. The ab initio shape of the monodisperse sample was determined by using the program GASBOR without using any symmetry (8). Ten independent reconstructions were calculated, and then aligned and averaged, by using the program DAMAVER to yield a final probability low-resolution map (9).

Electron Microscopy and Sample Preparation. Purified PG9 Fab: BG505 SOSIP.664 gp140 complexes were recovered from the SEC-UV/MALS/RI experiment and analyzed by EM. To study the structure/stoichiometry of PG9 binding to trimers produced in 293S or 293T cells, trimers were also incubated with a 10-fold excess of PG9 Fab for 30 min. The samples were then prepared for EM analysis. A 3-μL aliquot of the complex (0.03 mg/mL) was applied for 5 s onto a carbon-coated 400 Cu mesh grid that had been glow discharged at 20 mA for 30 s, then negatively stained with Nano-W (Nanoprobes) for 30 s. Data were collected by using a FEI Tecnai F20 electron microscope operating at 120 keV using an electron dose of 30 e⁻/Å² and a magnification of 100,000× that resulted in a pixel size of 1.09 Å at the specimen plane. Images were acquired with a Gatan 4K×4K CCD camera by using a nominal defocus range of 500–900 nm in 5° tilt increments from 0 to 55°. A PG9 Fab:BG505 SOSIP.664 gp140 sample with bound Protein G was prepared by adding 1:10 equivalents of Protein G to the Fab–trimer complex (0.2 mg/mL) and incubating on ice for 4 h. The sample was then diluted fivefold by adding 1X TBS pH 7.4 buffer. The grid was prepared as outlined above and collected automatically at 0° tilt by using Legoion software operating on the same microscope and imaging settings as stated (10).

Image Processing of the SEC Purified Complex. Particles were picked automatically by using DoG Picker and put into a particle stack.
using the Appion software package (11, 12). Initial reference-free 2D class averages were calculated by using particles binned by 5 via the Xmipp Clustering 2D Alignment (13) and sorted into 440 classes. Particles were selected into a substack and binned by four before another round of reference-free alignment was carried out by using the Xmipp Clustering and 2D alignment and IMAGIC softwares (14). A template stack of 154 images of 2D class averages was used to generate an ab initio 3D model, using the unliganded HIV-1 BaL trimer tomogram (Electron Microscopy Data Bank (EMDB) ID 5019; (15) or the unliganded BG505 SOSIP.664 gp140 trimer as the initial model for 89 iterations without imposing symmetry. The Fab density was visible after 10 iterations, and the final volumes were nearly identical whether the EMDB 5019 trimer or the unliganded BG505 SOSIP.664 gp140 trimer reconstruction was used as a starting model. Notably, enforcing C3 symmetry resulted in three lobes of density that floated in a disconnected manner around the trimer axis. Thus, symmetry was not enforced during any part of the reconstruction. Using the map generated with the EMDB 5019 reconstruction as a starting model, further refinement was carried out against raw particles binned by 2, without imposing symmetry, for 80 cycles. EMAN (16) was used for the 3D reconstruction procedures described. Refinement information of all presented reconstructions is noted in Table S2.

Image Processing of Non-SEC Purified Samples. Three separate datasets were analyzed—(i) a SEC-purified PG9 Fab:BG505 SOSIP.664 gp140 complex, and non-SEC purified complexes to which 10-fold excess PG9 Fab were added to (ii) BG505 SOSIP.664 gp140 and (iii) BG505 SOSIP.664 gp140. Particles were picked and sorted as stated. For each dataset, a common lines model was generated with the EMAN2 software package (17) by using bin 4 reference-free class averages. The resulting three independent initial models were refined against their respective bin 2 raw particles for 10 iterations by using the SPARX package (18) without imposing symmetry. To obtain a direct comparison, the final models were low-pass filtered to the resolution of the lowest resolution reconstruction of the three.

Image Processing of the Protein G-Bound Sample and Statistical Analysis. The dataset for Protein G bound to PG9 Fab:BG505 SOSIP.664 gp140 was sorted by the aforementioned 2D classification methods. Reference-free class averages were used to generate common lines models by using the EMAN2 package (17). The final datasets for the complex with or without out Protein G were randomly split into thirds, and three independent reconstructions were calculated using SPARX (18). Each of the six models was refined for 20 iterations without symmetry imposed, each starting with a different initial model generated by common lines. Final real space averages for each experiment (with or without Protein G) were generated by using the three independently refined models. Next, a difference map between the averages was calculated. The variance between the three unlabeled complex reconstructions was compared with the Protein G labeled reconstructions by using a Student’s t test (19). The largest peak in the difference map at \( \sigma = 17 \) (99.7% confidence level) corresponded to the predicted position of Protein G (Fig. S6).

Fitting of gp120 and PG9 Crystal Structures into the EM Density. The crystal structures of PG9 Fab in complex with a V1/V2 scaffold based on the CAP45 Env sequence (PDB ID 3U4E) and with the gp120 core of HXB2 sequence (PDB ID 3DNN) were fitted into the 18-A PG9 Fab:SOSIP.664 gp140 trimer reconstruction manually and refined by using the Fit in Map function in UCSF Chimera (20) based on correlation optimization. Nearly all of the gp120 core trimERIC model and PG9:V1/V2 structures could be positioned within the 3D reconstruction without significant parts protruding. The boat-shaped density of the Fab, with a dipole between the constant and variable domains, allowed unambiguous docking of the Fab glycan onto the trimer. The correct orientation of the PG9 Fab relative to the trimer was validated by adding Protein G to the complex (Fig. S6). Two additional V1/V2 elements (from PDB ID 3U4E) were fitted in the density map above the other gp120 protomers in the same relative position as the protomer that interacts directly with PG9. This docking process produced no significant clashes and placed glycan N160 from an adjacent protomer in close proximity to the PG9 Fab.

Neutralization Assays. Single-cycle infection and inhibition experiments with serially diluted Fab were performed as described by using TZM-bl cells (21). The 50% neutralization concentrations (IC50 values) were determined by using GraphPad.

Isotermal Titration Calorimetry. A MicroCal iTC200 or an Auto- iTC200 instrument (GE Healthcare) was used to perform isothermal titration calorimetry (ITC) measurements. All proteins were extensively dialyzed against 20 mM Tris and 150 mM sodium chloride at pH 8.0 buffer before the titration experiment. PG9 Fab was placed in the syringe at concentrations ranging between 30 and 200 μM, depending on the binding partner, whereas the BG505 gp120 monomer or SOSIP.664 gp140 trimer was in the cell at concentrations from 3 to 25 μM. The protein and glycoprotein concentrations were determined by UV absorbance at 280 nm using calculated extinction coefficients (22). Experiments were carried out at least in duplicate at 25 °C and consisted of 12 injections of 3.5 μL each, with injection duration of 5 s, injection interval of 180 s, and reference power of 5 μCal. Fitting of the integrated titration peaks with Origin 7.0 software by using a single-site binding model allowed direct determination of the reaction affinity constant (Kd) and molar reaction enthalpy (ΔH). In addition, the change in Gibbs free energy, ΔG, and the entropic change, ΔS, were derived from the basic thermodynamic relationships ΔG = RTInK and ΔG = ΔH − TΔS, respectively.

Differential Scanning Calorimetry. Thermal denaturation was probed with a VP-differential scanning calorimetry (DSC) calorimeter (GE Healthcare). Before the experiments, all test samples were extensively dialyzed against Dulbecco’s phosphate buffer saline. The protein concentration was subsequently adjusted to 0.1–0.3 mg/mL, as assessed by UV absorbance at 280 nm by using theoretical extinction coefficients (22). For complexes analyzed by DSC, the ligand (either PG9 or soluble CD4) was present in at least twofold molar excess over the glycoprotein. After loading the protein sample into the cell, thermal denaturation was probed at a scan rate of 90 °C/h. After buffer correction, normalization and baseline subtraction, the data were analyzed by using the Origin 7.0 software. Data were fit by using a non–two-state model, which ensues from the asymmetry of some of the peaks.


Julien et al. pnas.org/cgi/content/short/1215753110


Fig. S1. Immunoprecipitation assays. (A) Immunoprecipitation assays confirm that the size-exclusion chromatography coupled with multiangle light scattering (SEC-MALS)–purified SOSIP.664:PG9 Fab complex with only one Fab bound is saturated and not competent for binding additional PG9 IgG. As a control, SEC-MALS–purified SOSIP.664 is competent for binding PG9 IgG. (B) Control lanes show that similar amounts of SOSIP.664 were used in both experiments. Western blots are from SDS/PAGE experiments run under reducing conditions. Env detection was performed by using MAb ARP3119.
Fig. S2. SAXS data analysis. (A) SAXS data of samples of the PG9 Fab:BG505 SOSIP.664 gp140 complex at different concentrations. (B) Superposition of the two datasets chosen as low and high concentrations after normalization. (C) An ideal dataset for further analysis was obtained by merging the lower q data from the lower concentration dataset with the higher q data from the higher concentration dataset. Inset shows the result of the Guinier plot analysis. (D) The pair-distribution function, \( P(r) \), is an autocorrelation function that provides information about the distances between electrons in the scattering particles. \( D_{\text{max}} \), \( R_g \) and \( I_0 \) are parameters obtained from this analysis. (E) Kratky plot showing parabola-shaped peaks that indicate the presence of folded domains. (F) Porod invariant analysis provides information about the volume of scattering macromolecules in the sample.
Fig. S3. Negative stain EM data of the PG9 Fab:BG505 SOSIP.664 gp140 sample. (A) Representative negative stain image. (B) Two-dimensional (2D) class averages. (C) Projection matching showing the 3D model (Left) and the matching 2D class average (Right). (D) Fourier shell correlation (FSC) curve used to determine the ∼18-Å resolution of the final reconstruction.

Fig. S4. Negative stain EM comparison of PG9 complexes with BG505 SOSIP.664G gp140 trimers produced in 293S and 293T cells. (A) Representative class averages of SEC-purified complexes formed between PG9 Fab and BG505 SOSIP.664G gp140 trimers from 293S cells at a 1:1 molar ratio (Left); the same trimers but with PG9 present in 10-fold molar excess (Center); BG505 SOSIP.664G gp140 trimers from 293T cells, also with PG9 in 10-fold molar excess (Right). All three experiments show that a single PG9 Fab is bound to the top of the Env trimer. (B) Three-dimensional (3D) reconstructions of each of the three BG505 SOSIP.664G gp140 trimer preparations demonstrate that highly similar models are obtained from each of these different experimental conditions.
Fig. S5. Comparison of the SAXS and EM reconstructions. The SAXS density map and the EM reconstruction are colored blue and red, respectively. Both are contoured at a threshold that gives the same volume of $\sim 780 \text{ nm}^3$ and show similar reconstructions.

Fig. S6. Verification that the PG9 Fab:V1/V2 scaffold crystal structures are correctly fitted into the EM reconstruction by addition of Protein G. (A) Side views (Upper) and top views (Lower) of the real space averages ($n = 3$) of PG9 Fab:BG505 SOSIP.664 gp140 trimer EM reconstructions with (gray mesh) and without (red surface) Protein G. The crystal structure of the PG9 Fab in complex with gp120 V1/V2 scaffolds based on the CAP45 (gray, PDB ID 3U4E) sequence was fit into the PG9 Fab:BG505 SOSIP.664 gp140 trimer EM density. The crystal structure of a mouse Fab constant domain (yellow) in complex with Protein G Domain III (cyan) (PDB ID 1IGC) was superimposed onto the PG9 Fab constant region. The extra density next to the PG9 Fab constant domain indicates the site of interaction of Protein G and, hence, the location of the PG9 heavy chain. (B) The difference in density between Protein G bound and unbound maps from a Student’s $t$ test with a 99.7% confidence level shown as a blue mesh corresponds to the location of Protein G in the fitted Fab structure. (C) Same as in B, but showing only the difference map with Protein G added to the complex as a cyan surface for clarity.

Julien et al. www.pnas.org/cgi/content/short/1217537110
Fig. S7. Negative stain EM data of the unliganded BG505 SOSIP.664 gp140 sample. (A) Projection matching showing the projection of the 3D model (Left) and the matching 2D class average (Right). (B) A FSC curve was used to determine the ~25-Å resolution of the final reconstruction.

Fig. S8. Differential scanning calorimetry (DSC) thermal scanning of ligands. Raw data are shown in black and fitted curves from which Tm values were obtained are colored in red. (A) PG9 Fab. (B) Soluble CD4 (sCD4).
Fig. S9. ITC comparison of PG9 binding to Env proteins produced in either 293T or 293S (GnT I-deficient) cells. PG9 interacts better with BG505 gp120 monomers produced in 293S cells vs. 293T cells, as illustrated by the higher stoichiometry and higher affinity. In contrast, PG9 binds to BG505 SOSIP.664 gp140 trimers produced in 293T cells with a higher affinity than those from 293S cells. Of note is that PG9 binds the Env trimers from 293T cells with a 10-fold higher affinity than the corresponding gp120 monomers, but this difference does not arise when the trimers and monomers are both produced in 293S cells. Representative raw data, isotherms, and reported values are representative of at least two ITC experiments.
Fig. S10. The PG9 epitope potentially includes two N160 glycans and is located immediately adjacent to the trimer axis at the apex of the Env trimer. Model of the Env trimer was generated from the EM reconstruction of the unliganded, membrane-bound, virus-derived HIV-1 trimer (gray mesh, EMDB ID 5019; ref. 1), the soluble gp120 core structure (PDB ID code 3DNN; refs. 1 and 2), the gp120 mini-V3 loop (PDB ID code 3TYG; ref. 3), and the gp120 V1/V2 loops (PDB ID 3U4E; ref. 4). Potential N-linked glycosylation representing the glycan shield from the Asp-X-Thr/Ser consensus sequences is shown as medium blue spheres, and protein components are shown as dark blue spheres. The putative positions of missing regions of the gp120 V1/V2 and V3 loops that are not represented in any of the crystal structure models used are labeled. The position of the N160 glycans on all three protomers, and the gp120 V1/V2 strand C residues that form the cationic groove on one protomer, are colored in magenta and green, respectively. The model indicates that N160 is the closest glycan to the trimer axis and, as such, its specific glycoform may be dictated by how much access glycosidases have to this region of the trimer during posttranslational modification in the Golgi apparatus. The appropriate modification of this glycan might be a hallmark of truly native HIV-1 spikes. Consequently, bnAbs such as PG9 may preferentially interact with functional HIV-1 spikes via these N160-dependent epitopes.


Table S1. Molar mass

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected total MM_{glycoprotein}* g/mol</th>
<th>MM_{glycoprotein} from SEC-UV/MALS/RI, g/mol</th>
<th>MM_{glycoprotein} from SAXS, g/mol</th>
<th>Hydrodynamic radius r_H, nm</th>
<th>Radius of gyration r_g, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unliganded BG505. SOSIP.664</td>
<td>343,233</td>
<td>357,200 ± 25,000</td>
<td>ND</td>
<td>8.1</td>
<td>ND</td>
</tr>
<tr>
<td>PG9 Fab + BG505 SOSIP.664</td>
<td>392,270†</td>
<td>387,000 ± 31,000</td>
<td>393,000</td>
<td>8.6</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Molar mass analysis of unliganded and PG9-bound BG505 SOSIP.664 gp140 trimers produced in GnT-1-deficient cells, as assessed by SEC-UV/MALS/RI and SAXS. ND, not determined.

*Expected MM_{protein} of BG505 SOSIP.664 trimer (g/mol) = 225,233, estimated MM_{glycan} of BG505 SOSIP.664 trimer (g/mol) = 118,000, expected MM_{protein} of PG9 Fab (g/mol) = 49,037.
†Associated error as determined by Astra V.
†One trimer plus one Fab.
Table S2. EM structure statistics

<table>
<thead>
<tr>
<th>Model</th>
<th>Particle count</th>
<th>Resolution at FSC = 0.5 cutoff, Å</th>
<th>Low-pass filter resolution cutoff, Å</th>
<th>Refinement program</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC purified PG9:SOSIP.664&lt;sub&gt;293S&lt;/sub&gt;</td>
<td>33,431</td>
<td>18.1</td>
<td>18</td>
<td>EMAN</td>
<td>Fig. 2 B and C</td>
</tr>
<tr>
<td>SEC purified PG9:SOSIP.664&lt;sub&gt;293S&lt;/sub&gt;</td>
<td>26,222</td>
<td>19.2</td>
<td>20</td>
<td>SPARX</td>
<td>Fig. S4B</td>
</tr>
<tr>
<td>10X PG9:SOSIP.664&lt;sub&gt;293S&lt;/sub&gt;</td>
<td>19,114</td>
<td>20.0</td>
<td>20</td>
<td>SPARX</td>
<td>Fig. S4B</td>
</tr>
<tr>
<td>10X PG9:SOSIP664&lt;sub&gt;293T&lt;/sub&gt;</td>
<td>15,748</td>
<td>19.3</td>
<td>20</td>
<td>SPARX</td>
<td>Fig. S4B</td>
</tr>
<tr>
<td>PG9:SOSIP664&lt;sub&gt;239S&lt;/sub&gt; t test split 1</td>
<td>8,741</td>
<td>23.8</td>
<td>27</td>
<td>SPARX</td>
<td>Fig. S6</td>
</tr>
<tr>
<td>PG9:SOSIP664&lt;sub&gt;239S&lt;/sub&gt; t test split 2</td>
<td>8,741</td>
<td>24.0</td>
<td>27</td>
<td>SPARX</td>
<td>Fig. S6</td>
</tr>
<tr>
<td>PG9:SOSIP664&lt;sub&gt;239S&lt;/sub&gt; t test split 3</td>
<td>8,740</td>
<td>23.6</td>
<td>27</td>
<td>SPARX</td>
<td>Fig. S6</td>
</tr>
<tr>
<td>Protein G+PG9: SOSIP664&lt;sub&gt;239S&lt;/sub&gt; t test split 1</td>
<td>4,794</td>
<td>25.7</td>
<td>27</td>
<td>SPARX</td>
<td>Fig. S6</td>
</tr>
<tr>
<td>Protein G+PG9: SOSIP664&lt;sub&gt;239S&lt;/sub&gt; t test split 2</td>
<td>4,794</td>
<td>27.4</td>
<td>27</td>
<td>SPARX</td>
<td>Fig. S6</td>
</tr>
<tr>
<td>Protein G+PG9: SOSIP664&lt;sub&gt;239S&lt;/sub&gt; t test split 3</td>
<td>4,793</td>
<td>27.0</td>
<td>27</td>
<td>SPARX</td>
<td>Fig. S6</td>
</tr>
</tbody>
</table>

Table shows the number of particles and the program used for each refinement. The resolution of each map is determined at a Fourier Shell Correlation (FSC) cutoff of 0.5. Each model was low-pass filtered to the indicated resolution for analysis and comparison.

Table S3. Thermodynamic data derived from ITC

<table>
<thead>
<tr>
<th>Binding experiment</th>
<th>ΔG,* kcal·mol⁻¹</th>
<th>ΔH, kcal·mol⁻¹</th>
<th>−TΔS, kcal·mol⁻¹</th>
<th>K&lt;sub&gt;d&lt;/sub&gt;, nM</th>
<th>N&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG9 WT into BG505 monomer&lt;sup&gt;§&lt;/sup&gt;</td>
<td>−10.2</td>
<td>−27.4</td>
<td>17.2</td>
<td>31 ± 2</td>
<td>0.4</td>
</tr>
<tr>
<td>PG9 mutant into BG505 monomer&lt;sup&gt;§&lt;/sup&gt;</td>
<td>−10.5</td>
<td>−23.5</td>
<td>13.0</td>
<td>19 ± 3</td>
<td>0.4</td>
</tr>
<tr>
<td>PG9 WT into BG505 SOSIP trimer&lt;sup&gt;§&lt;/sup&gt;</td>
<td>−10.1</td>
<td>−20.6</td>
<td>10.5</td>
<td>36 ± 10</td>
<td>0.6</td>
</tr>
<tr>
<td>PG9 mutant into BG505 SOSIP trimer&lt;sup&gt;§&lt;/sup&gt;</td>
<td>−8.8</td>
<td>−23.7</td>
<td>14.9</td>
<td>339 ± 76</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*The change in Gibbs free energy (ΔG) was determined by using the relationship: ΔG<sub>binding</sub> = RTlnK<sub>d</sub> (1).
†Associated errors are the SD calculated from three independent measurements.
§The stoichiometry of binding (N) is directly affected by protein concentration measurements, and perhaps by glycan heterogeneity on gp120 and, hence, sample heterogeneity, that is incompatible with PG9 binding. For the heavily glycosylated gp120 monomer/trimer, discrepancies in glycoprotein concentrations determined from different techniques (UV<sub>280</sub>, BCA, Bradford) result in variations in N of ± 0.15.
‡The Env proteins were produced in 293S GnT I-deficient cells. Differences in PG9 binding to proteins produced in 293T and 293S cells are reported in Fig. S9.