Redesigned HIV antibodies exhibit enhanced neutralizing potency and breadth

Jordan R. Willis, … , Jens Meiler, James E. Crowe Jr.


Several HIV envelope-targeting (Env-targeting) antibodies with broad and potent neutralizing activity have been identified and shown to have unusual features. Of these, the PG9 antibody has a long heavy chain complementarity determining region 3 (HCDR3) and possesses unique structural elements that interact with protein and glycan features of the HIV Env glycoprotein. Here, we used the Rosetta software suite to design variants of the PG9 antibody HCDR3 loop with the goal of identifying variants with increased potency and breadth of neutralization for diverse HIV strains. One variant, designated PG9_N100F_Y, possessed increased potency and was able to neutralize a diverse set of PG9-resistant HIV strains, including those lacking the Env N160 glycan, which is critical for PG9 binding. An atomic resolution structure of the PG9_N100F_Y fragment antigen binding (Fab) confirmed that the mutated residue retains the paratope surface when compared with WT PG9. Differential scanning calorimetry experiments revealed that the mutation caused a modest increase in thermodynamic stability of the Fab, a feature predicted by the computational model. Our findings suggest that thermodynamic stabilization of the long HCDR3 in its active conformation is responsible for the increased potency of PG9_N100F_Y, and strategies aimed at stabilizing this region in other HIV antibodies could become an important approach to in silico optimization of antibodies.

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Introduction
Recent studies have described the isolation of a number of human mAbs directed to the HIV envelope (Env) that exhibit broad and potent neutralizing activity, many of which exhibit unusual features (1–5). One of the most interesting mAbs described to date is PG9, which possesses an antibody heavy chain complementarity determining region 3 (HCDR3) loop with a “hammerhead” structure formed by stabilizing interactions within the loop (2). PG9 and the related antibody PG16 interact with HIV Env using similar binding modes but diverge in amino acid sequences with 24% amino acid differences in their antibody heavy chain variable regions, and 33% amino acid differences in their HCDR3 sequences (6). Therefore, we hypothesized that the HCDR3 loop of PG9 could be redesigned to achieve improved affinity of binding, increased potency, and increased breadth of neutralization for diverse HIV strains. The large number of potential variants of the loop made systematic synthesis and testing of all possible variants resource intensive, if not prohibitive. Therefore, we used a hybrid method for optimization, in which we coupled structure-based computational design and experimental validation to identify candidate variant antibodies with minimal alteration that exhibited increased potency and breadth. Remarkably, we found PG9 variant antibodies with single amino acid changes in noncontact regions of the HCDR3 loop that exhibited increased affinity and neutralizing activity for HIV Env, and they achieved these enhanced functional activities without altering the paratope surface of the HCDR3. Instead, the variant amino acid caused an increase in thermodynamic stability of the Fab. The crystal structure of the variant Fab and direct measurements of antibody thermodynamic stability are in agreement with the model. The results suggest an exciting approach to improving the functional activity of HIV-neutralizing mAbs with long HCDR3s through thermodynamic stabilization of the HCDR3 in its active conformation. The work also illustrates the power of integrating Rosetta computational design with experimental verification for rapid design of antibodies with enhanced properties.

Results
Design process. We retrieved the atomic resolution structure of the mAb PG9 in complex with the HIV CAP45.2.00.G3 variant V1/V2 scaffold from the Protein Data Bank (PDB ID: 3U4E) (2). The structure reveals interesting interaction features of the complex (Supple-
to that of the glycan-specific mAb 2G12, the antibody that was used to affinity purify the trimer (Supplemental Figure 2 and ref. 18).

**Neutralization assays.** We next tested the panel of redesigned PG9 variants and WT PG9 for neutralizing activity against a panel of PG9-susceptible or -resistant viruses using a TZM-bl neutralization assay (19). PG9_N100_Y exhibited increased neutralization potency for all viruses tested, including viral variants for which WT PG9 did not have activity (i.e., had neutralization concentration >3.3 μg/ml; Figure 2). We also tested HIV strains that lacked the glycan at Env position N160, using naturally occurring or engineered knockouts for Env sequences (20). Remarkably, PG9_N100_Y neutralized 7 out of the 9 viruses tested that lacked N160 glycan, with inhibitory activity at concentrations as low as 2.7 μg/ml (Figure 2). PG9_N100_L also exhibited an increase in potency against HIV strains compared with WT PG9, although not at the same level as PG9_N100_Y (P value < 0.001). Although the magnitude of the improvement was modest in some cases, the improvement was consistent over a wide variety of HIV strains (P value < 0.001, geometric mean of 0.64 PG9_N100_Y vs. WT PG9 2.31). We also found a decrease in potency to be statistically significant for the virus panel tested for PG9_D100_N (P value < 0.001).

**Crystal structure of PG9_N100_Y.** To validate the Rosetta models for the structure of the PG9_N100_Y antibody, we determined the crystal structure for its Fab at 2.3 Å resolution (Supplemental Table 1). All residues of the HCDR3 could be resolved in the electron density, including the N100_Y mutation (Figure 3A). Overall, the HCDR3 of PG9_N100_Y Fab adopts the same hammerhead conformation as in the WT PG9-gp120 V1/V2 complex structure, with a Cu root mean square deviation (RMSD) of 0.43 Å for this loop compared with the complexed structure (PDB ID: 3U4E), and a Cu RMSD of 0.68 Å against the Rosetta complexed model (Figure 3B), i.e., the Fab crystal structure and its modeled structure adopt the same conformation. We also note that the side of the HCDR3 hammerhead that harbors the N100_Y mutation has increased B values but is significantly more ordered than in the previous crystal structure of WT PG9, in which the HCDR3 hammerhead could not be resolved (PDB ID: 3U36). We interpret in this observation that the N100_Y mutation provides additional stability to the HCDR3 conformation for recognition of the glycoprotein by forming π-π stacking interactions with the WT residues P99 and Y100. The PG9_N100_Y Fab electron density, albeit weak, indicates that the substituted tyrosine at this position does not possess an associated sulfation. Two sulfated tyrosines (indicated in this manuscript as Y — specifically Y100_Q and Y100_P) in WT PG9 remain sulfated in the variant Fab PG9_N100_Y.

**Mechanism of improved binding.** We sought to understand the molecular basis for the increased potency and breadth of these PG9 variants using Rosetta analysis of variants with single mutations. N100_Q was mutated in WT PG9 to N100_Y, and the interaction with Env was compared with that of WT PG9 using the Rosetta scoring function (Figure 3C). We calculated stabilization and binding energy for the HCDR3, stabilization of the full HCDR3 loop, and binding energy for the antibody-Env interaction (further explained in Supplemental Table 2). For each metric calculated, we observed statistically significant improvements in HCDR3 only for N100_Y (P < 0.001). Consistent with the WT PG9 structure, antibody position 100 is located on an antiparallel β-sheet at the apical...
tip of the HCDR3, forming a hydrophobic pocket near the interface of the antigen (Figure 3D). The pocket is formed by Y100A, YS100G, and P99 of the antibody heavy chain. In addition, the asparagine at position 167 on the antigen face of gp120 contacts this pocket. The structural models reveal that a large, hydrophobic amino acid at position 100F is predicted to fill the hydrophobic pocket there, thereby likely stabilizing the HCDR3 conformation for interaction with the HIV V1/V2 epitope (Figure 3D). In addition, the polar group on the designed N100Y mutant points into accessible solvent space. Both the attractive forces of the hydrophobic bulk and the improved solvation potential were reflected in the Rosetta scoring function (Supplemental Figure 3). Further, the PG9_N100Y adds a π-π stacking interaction with YS100G that is not present with the asparagine side chain in WT PG9 (Supplemental Figure 3).
Figure 2. Rosetta redesigned mutants exhibit increased breadth and potency against HIV. Binding and neutralization profiles for the characterized PG9 mutants. Antibody mutants are shown using Kabat numbering. The virus name is shown in the left column, and amino acid sequence for position 160 (HIV strain HXBc2 numbering) is shown in the second column. Strains lacking a potential N-linked glycosylation site are indicated with bold rows. A yellow-to-green color scale indicates ranges of values, from less than 0.01 μg/ml to greater than 100 μg/ml, respectively, for concentrations needed for half maximum signal in ELISA (EC 50) in the left panel. A yellow-to-green scale from 0.4 ng/ml to greater than 33 μg/ml, respectively, is shown for half-maximal concentration needed to inhibit virus infection (IC 50), in the right panel. Each ELISA experiment was done in triplicate, and neutralization was done in duplicate. A Wilcoxon test was used to determine if the distributions for binding and neutralization were statistically significant from WT PG9. The P value for comparison of each variant against WT PG9 (comparing the pattern of results in a single variant antibody column against the results in the left column for WT PG9) is shown at the bottom of the panels and reflects a 2-sided significance at a level of 0.05.
**Differential scanning calorimetry.** The structure of the PG9_N100,Y Fab suggested that the observed increase in breadth and neutralization potency resulted not from an alteration in the contact surface of the HCDR3, but rather from a stabilization of the loop through increased thermodynamic stability. To test this parameter directly, we compared the WT PG9 and PG9_N100,Y variant Fabs and full-length IgG mAbs using differential scanning calorimetry (DSC). The DSC curves of the Fabs showed a biphasic transition, while the full-length IgG mAbs showed a quadriphasic transition (Table 1 and Supplemental Figure 4). The 1st and 2nd peaks observed from Fab denaturation appeared to correspond to the 1st and 3rd peaks observed from mAb denaturation. The thermo-denaturation is irreversible under the conditions tested, which has been observed in many other antibody unfolding experiments (21–24); hence, the melting temperature and enthalpy deconvolution reported are apparent values. The apparent melting temperature ($T_m$) derived from the 1st peak from WT PG9 mAb/Fab was about 1°C lower than that from PG9_N100,Y mAb/Fab. Deconvolution of the DSC thermogram showed higher apparent calorimetric enthalpy changes for PG9_N100,Y during thermal denaturation, which indicates that more heat was absorbed during denaturation (24). The difference in apparent calorimetric enthalpy indicates that the PG9_N100,Y mutation causes an altered transition state during thermo-denaturation. Taking the shift in apparent $T_m$ by 1°C and higher denaturation heat together, the DSC results suggested that PG9_N100,Y is thermodynamically more stable than WT PG9.

**Absence of autoreactivity for PG9_N100,Y.** Antibodies with long HCDR3s are thought to exhibit a high frequency of autoreactivity (25). Indeed, we have shown previously that the length of antibody genes in B cells encoding long HCDR3s usually are generated at the time of original recombination, not by insertional mutagenesis during somatic hypermutation; however, their frequency is reduced as B cells move from the naive B cell compartment to memory B cell compartments (26). Additionally, some broadly neutralizing antibodies against HIV have been shown to be autoreactive (27). Thus, we considered whether the mutation that we made synthetically in PG9_N100F,Y might inadvertently increase autoreactivity, and therefore the increase in breadth of binding could be attributed to nonspecific binding. We compared the binding to human HEp-2 cell antigens of PG9wt, PG9_N100FY, 4E10 (an HIV mAb with known autoreactive properties),
or a heterologous control antibody, motavizumab, directed to the respiratory syncytial virus fusion protein (Supplemental Figure 5 and ref. 28). PG9_N100FW exhibited a comparable level of reactivity as PG9 and a lower level of reactivity than the other control antibodies, suggesting the increased thermodynamic stability of the HCDR3 did not cause undesired acquisition of autoreactive or nonspecific binding.

**Discussion**

These results have important implications for antibody design because they reveal the potential of computational modeling to design antibodies with improved function. The crystal structure shows that the mutations recovered from RosettaDesign are pointed away from the antigen V1/V2 strand-C face. Based on the DSC results, we conclude that the increased potency and breadth of the PG9 variant might result from a thermal stabilization of the HCDR3, which allows the loop to adopt a more lock-and-key–like binding mode, limiting the entropic cost when engaging the antigen. Our ability to resolve the HCDR3 of an unliganded PG9 variant — in contrast to WT PG9 — also is consistent with increased thermal stabilization of the HCDR3, although we cannot rule out contributions from a different crystal packing in the PG9 variant. This finding of increased loop thermostability was obtained in the context of a holistic model and facilitated identification of a variant antibody that bound in a glycan-independent manner and exhibited increased potency. This finding is consistent with recent mutagenesis experiments showing that noncontact residues are essential for antigen recognition by many broadly neutralizing antibodies, suggesting the increased thermodynamic stability of the HCDR3 did not cause undesired acquisition of autoreactive or nonspecific binding.

**Table 1. DSC for mAbs or Fabs of WT PG9 or PG9_N100FW**

<table>
<thead>
<tr>
<th>Transition</th>
<th>WT PG9 mAb</th>
<th>N100FW mAb</th>
<th>WT PG9 Fab</th>
<th>N100FW Fab</th>
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<tr>
<td></td>
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<td>ΔHm (kJ/mol)</td>
<td>Tm (°C)</td>
<td>ΔHm (kJ/mol)</td>
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<td>4</td>
<td>80.1</td>
<td>423</td>
<td>81.1</td>
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</tr>
</tbody>
</table>

Transition temperature (Tm) and changes of enthalpy (ΔHm) after deconvolution.

**Methods**

**Redesign of PG9 HCDR3.** Using the RosettaDesign algorithm (9), iterative rounds of design, docking, and minimization were applied to each position in the HCDR3, with a small energetic bonus applied to recovery of the native sequence. The small energetic bonus is commonly applied to redesigned models to reduce complexity of sequence space for large designs (9). 100 models were generated using this protocol (http://www.crowelab.com/protocol-captures).
For each mutated position, mutations seen in greater than 10% of the models and conveying an energetic bonus of greater than 1.0 Rosetta energy units (REU) were inspected manually using PyMOL (Version 1.5.0.4; Schrödinger LLC) and compared with the native sequence along with the native PG9 structure (PDB ID: 3U4E) (2). The change in fitness for any mutation was determined to be the sum of the contribution to binding energy, as compared with WT for each mutation (ΔΔΔG) (39), and increased thermodynamic stability energy compared with WT PG9 (ΔΔG). Both energies were approximated by the Rosetta scoring function.

**Antibody and gp120 expression.** We used recombinant expression in mammalian cells to recover natural posttranslational modifications, as previously described (40). Briefly, the mAb PG9 heavy- and light-chain genes were cloned into the pEE6.4 and pEE12.4 vectors, respectively (Lonza). BsiWI and Xhol cloning sites were generated at amino acid position 93 and 103, respectively. Variant HCDR3 sequences were synthesized and cloned into the PG9 backbone (GeneArt) using the unique cloning sites. The DNA was cotransfected at a 1:1 heavy-light ratio into FreeStyle 293-F cells (Human Embryonic Kidney; Invitrogen) using 25 kDa linear polyethyleneimine (PEI; Polysciences Inc.) transfection reagent at a ratio 2:1 of PEI to DNA. IL of culture was used for each variant, and the supernatant was collected on day 5. Antibody was purified and concentrated from supernatant on a protein G column (GE Healthcare). The expression levels for all PG9 variants were comparable to that of WT PG9.

A cDNA encoding each gp120 was cloned into pcDNA3.1 (Invitrogen) using HindIII and EcoRI restriction sites. A CD5 signal peptide was added to the N-terminus of each molecule (Clontech), according to the manufacturer’s specifications. The protein was concentrated using Amicon Ultra centrifugal filters with a 100 kD cutoff (Millipore) and further purified on a Superdex column (GE Healthcare) using size exclusion.

BG505 SOSIP.664 trimmer protein was received as a gift from John P. Moore, the Weill Medical College of Cornell University, New York, New York, USA.

**PG9 variant characterization.** ELISA plates were coated with 3 μg/ml of gp120 and incubated overnight at 4°C. The wells were washed with phosphate-buffered saline with 0.05% Tween (PBS-T) in the following steps. The uncoated sites on the wells were blocked with 2% milk and 1% goat serum in PBS-T for 2 hours at room temperature. All antibodies were diluted serially in 2-fold steps starting from 25 μg/ml, for 24 dilutions. Horseradish peroxidase–conjugated goat anti-human IgG (catalog 2040-05; Southern Biotechnology Associates) was added to each well, allowed to incubate for 1 hour at 37°C, and color developed with 3,3',5,5'-tetramethylbenzidine (Thermo). The reaction was stopped with 1N HCl and read at 450 nm. The concentration and absorbance were fit with a nonlinear robust fit using Prism default parameters to generate an EC_{50}. The EC_{50} of each PG9 variant was compared with that of the WT PG9 positive control.

For BG505 SOSIP.664 trimer, ELISAs were performed according to the protocol as previously described (18). Maxisorp 96-well plates (Nunc) were coated overnight with Ab D7324 (product code D7324; Aalto Bio Reagents) at 5 μg/ml in 0.1 M NaHCO_3, pH 8.6 (100 μl/well). After the washing and blocking steps, purified D7324-tagged BG505 Env proteins were added at 800 ng/ml in PBS and 2% milk for 2 hours at ambient temperature, and the unbound Env proteins were washed away. WT PG9 and PG9 variants were diluted to 25 μg/ml in PBS with 10% sheep serum/2% milk, diluted serially 2-fold, and then allowed to incubate for 2 hours at room temperature followed by 3 washes with PBS-T. Horseradish peroxidase–conjugated goat anti-human IgG was added for 1 hour at a 1:3,000 dilution (final concentration 0.33 μg/ml) in 10% sheep serum/2% milk, followed by 5 washes with PBS-T. Color development, optical density measurement, and EC_{50} calculations were done as above.

**Neutralization assays.** Neutralization was measured as a function of reduction in luciferase (Luc) reporter gene expression after a single round of infection in TZM-bl cells, as described (19, 41). This assay has been optimized and validated (42) and was performed in compliance with Good Clinical Laboratory Practices. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. Briefly, virus at a dose of 50,000–150,000 relative luminescence unit (RLU) equivalents was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 μl for 1 hour at 37°C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 μl of growth medium containing 75 μg/ml DEAE dextran) were added to each well.

One set of control wells received a combination of the cells and the virus (virus control) and another set received cells only (background control). After a 48-hour incubation, 100 μl of cells was transferred to a 96-well black solid plate (Corning Inc.) for measurements of luminescence using the Biotite Luminescence Reporter Gene Assay System (PerkinElmer). Neutralization titers are the dilution at which RLUs were reduced by 50%, compared with virus control wells, after subtraction of background RLUs. Assay stocks of molecularly cloned Env-pseudotyped viruses were prepared by transfection in 293T cells and were titrated in TZM-bl cells as described (19). For all neutralization assays, the starting concentration for PG9 and PG9 mutants was 53 μg/ml. However, for viruses BG505ΔCT/T322N, YU2, HxB2CP3.2, CAP45.2.00.G33, and BaL.26, the starting concentration for WT PG9 was 3.3 μg/ml. For RHPA.LucR.T2A.ecto, the PG9 starting concentration was 10 μg/ml. Additional details of the assay and all supporting protocols may be found at (http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.html).

**Crystallization, data collection, structure determination, and refinement.** Crystallization trials with the PG9_N100 Y Fab fragment were set up with the IAVI/Center for Structural Genomics (ICGS)/TSRI CrystalMation robotic system (Rigaku), at a concentration of 10 mg/ml. A crystal was obtained in a condition containing 5%PEG 3000, 40% PEG 400, and 0.1M MES. Data were collected at SSRL beamline 11-1. Data were processed using XDS (43). The structure was solved using PHASER (44) with WT PG9 (PDB ID: 3U4E; ref. 2) as the initial search model. COOT (15) and PHENIX (45) were used for refinement, and the statistics are reported in Supplemental Table 1. Pymol (Version 1.5.0.4; Schrödinger LLC) and UCSF Chimera (46) were used for the rendering.

**DSC.** The samples were loaded in a MicroCal VP-DSC (GE Healthcare) sample cell against the reference cell containing PBS. The samples were heated at a constant heating rate of 1°C/min. Experiments using mAbs were performed with protein concentration at 0.5 μM, while experiments using Fabs were performed with protein concentration at 1 μM. Data analysis was done using Origin.
for DSC package. Deconvolution of the excess heat capacity function was done using 4 “two-state” transitions for mAbs, and 2 “two-state” transitions for Fabs.

**Cell staining for autoreactivity.** HEp-2 cells were plated on glass coverslips within 48 well plates 24 hours prior to staining. Cells stained for intracellular autoreactivity were fixed with freshly prepared 4% paraformaldehyde and permeabilized with 3% BSA/0.3% Triton-X-100, while cells stained for cell-surface autoreactivity were fixed with 4% paraformaldehyde only. Fixed cell monolayers were incubated with 25 μl/g of HIV mAb 4E10, which is known to exhibit autoantigen properties; WT PG9; the PG9_N100,Y variant; or motavizumab, a control mAb directed to the respiratory syncytial virus fusion protein. Secondary goat anti-human IgG antibodies containing Alexa Fluor 488 (Invitrogen) were used to detect mAb staining of human antigens. TO-PRO3-iodine dye were used for detection of DNA in the cell nuclei, to identify cells. Images were captured at ×40 magnification on a Zeiss LSM 710 META Inverted laser scanning confocal microscope with a ×40/1.10 LD C-APOCHROMAT water objective and analyzed using ZEN Blue software platform (Zeiss).

**Statistics and graph generation.** All statistics were calculated in the R-programming language (http://www.r-project.org) or Prism package (GraphPad Software) through the Rinterface (www.ipyn-thon.org). All graphs were generated in Prism package or the ggplot2 library (http://ggplot2.org) in the R-programming language.

To facilitate comparison of both WT PG9 and variants for all viruses tested, separate Wilcoxon signed-rank tests for paired data were used to test for differences in IC₅₀ or EC₅₀ between WT PG9 and each of the 4 antibody variants. We compared the EC₅₀ or IC₅₀ of all viruses using a 2-sided significance level of 0.05. A P value of less than 0.05 was considered significant.

**Acknowledgments**

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Address correspondence to: James E. Crowe Jr., Vanderbilt University, Vanderbilt Vaccine Center, and the Departments of Pediatrics, Pathology, Microbiology and Immunology, 11475 Medical Research Building IV, 2213 Garland Avenue, Nashville, Tennessee 37232-0417, USA. Phone: 615.343.8064; E-mail: james.crowe@vanderbilt.edu. Or to: Jens Meiler, Vanderbilt University, Department of Chemistry, 7330 Stevenson Center, Station B 351822, Nashville, Tennessee 37235, USA. Phone: 615.936.5662; E-mail: jens@meilerlab.org.

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