The human monoclonal antibody 2G12 is a member of a small group of broadly neutralizing antibodies against human immunodeficiency virus type 1. 2G12 adopts a unique variable heavy domain-exchanged dimeric configuration that results in an extensive multivalent binding surface and the ability to bind with high affinity to densely clustered high mannose oligosaccharides on the “silent” face of the gp120 envelope glycoprotein. Here, we further define the amino acids responsible for this extraordinary domain-swapping event in 2G12.

Several mechanisms have been proposed to be involved in three-dimensional domain swapping in proteins. These include 1) destabilization of the monomer, 2) accumulation of mutations in the dimer interface, and 3) modifications (e.g. deletions or mutations) in the hinge loop (1–3). All such modifications have been suggested to play a role in the domain-swapping capability of the broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 (4–7). The crystal structure of Fab 2G12 revealed a tightly packed Fab dimer created by three-dimensional swapping of the variable heavy (VH)3 domains. This uncommon assembly results in an additional antigen-binding site at the novel VH/VH interface and “elbow” region of 2G12. We have generated three IgG1 2G12 heavy chain mutants based on the Fab 2G12 crystal structure and tested their potential for domain swapping. We discovered that the domain exchange-enabling properties of Pro1113 at the elbow region of 2G12 can be emulated by other residues. Our results furthermore support a combination of up to four somatic mutations at the VH/VH interface and elbow region as being responsible for promoting domain exchange in 2G12.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of IgG1 2G12 Mutants**—The modified genes of the IgG1 2G12 mutants were codon-optimized and synthesized by GeneArt (Regensburg, Germany) and stably transfected into protein-free cultivated Chinese hamster ovary dehydrofolate reductase-deficient suspension cells (ATCC CRL-9096) as described earlier (10). 24 h after transfection with polyethyleneimine/DNA, selection was started with hypoxanthine/thymidine-deficient medium. Stably transfected clones were propagated and purified over a protein A column. Quality control of purified IgG fractions was assessed by SDS-PAGE analysis. Final IgG concentrations were determined by A280 measurement.

**Circular Dichroism (CD) Spectrometry**—CD analysis was performed on a PiStar-180 spectrometer equipped with a thermostatic cell holder from Applied Photophysics (Leatherhead, U.K.). For recording near-UV spectra (240–340 nm) the quartz cuvette had a path length of 10 mm. Spectral bandwidth was 2 nm; step size, 1 nm; scan time, 21 min; protein concentration, 0.1 mg/ml. All CD measurements were performed in 5 mM PBS, pH 7.3, at 25 °C (11). Each spectrum was automatically corrected with the base line to remove birefringence of the cell. The instrument was flushed with nitrogen with a flow rate of 5 liters min⁻¹.

**Electron Microscopy**—For the preparation of the 2G12 IgG samples, freeze drying and heavy metal shadowing were performed according to Kellenberger and Kistler (12). Electron micrographs of the antibody preparations were taken on a CM 12 electron microscope (Philips, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV.

**Papain Digestion**—Buffer-exchanged (1 mM EDTA, 50 mM sodium phosphate buffer, pH 6.3) and concentrated (1 mg/ml) 2G12 IgG samples were mixed with activated papain solution (1 mM EDTA, 10 mM cysteine, 50 mM sodium phosphate buffer, pH 7.0) to give a final papain/antibody ratio of 4% (w/w). The
resulting mixtures were incubated for 2 h at 37 °C and stopped by adding 30 mM iodoacetamide. Subsequently, the cleaved antibody solution mix was buffer-exchanged (PBS) and incubated twice with PBS-plequilibrited protein A-Sepharose beads for 1 h at room temperature under gentle agitation on a shaker. Finally, the Fab-containing supernatants were collected and further characterized by SDS-PAGE.

**Gel Electrophoresis**—Each purified antibody sample (IgG and Fab) was mixed with Laemmli sample buffer (Bio-Rad), heated for 5 min at 100 °C, and examined by denaturing gel electrophoresis in a Bio-Rad Mini-Protean 3 cell system. Subsequently, the 2G12 samples were visualized with Simply-Blue SafeStain (Invitrogen) by following the manufacturer's instructions.

**ELISA**—96-well microplates (eBioscience) were sensitized with recombinant gp160 MN (100 ng/well) overnight at 4 °C. All of the following steps were carried out at room temperature. After washing the plates with TPBS (PBS containing 0.01% Tween 20), the wells were blocked for 1 h with 4% nonfat dry milk in TPBS. The 2G12 Fab samples (2 μg/ml) were serially diluted in 1% nonfat dry milk/TPBS and added to the antigen-coated wells. After 1 h, bound 2G12 Fab was detected with a peroxidase-conjugated goat anti-human IgG Fab conjugate (Sigma) diluted 1:1,000 in 1% nonfat dry milk/TPBS. Finally, bound conjugate was visualized with tetramethylbenzidine substrate (Pierce) and measured at 450 nm.

**Gel Filtration**—The papain-digested 2G12 Fab variants (100 μg/ml) were loaded in PBS (50 μl) onto a PBS-equilibrated Superdex 75 10/30 column (GE Healthcare). The protein was eluted in PBS at a flow rate of 0.5 ml/min and detected by UV absorbance.

**Virus Neutralization Assay**—JR-FL pseudovirus was generated as described previously (13) by co-transfection of a gp160 JR-FL envelope with the pSG3ΔEnv backbone. Pseudotyped virus was added at a 1:1 ratio to serially diluted (1:3) 2G12 variants (starting at 200 μg/ml) and incubated at 37 °C, for 1 h. TZM-bl cells were then seeded (1:1 by volume) at 1 × 10^4 cells/well in a final concentration of 10 μg/ml DEAE-dextran. After a 48-h incubation at 37 °C, the cells were washed, lysed, and finally developed with luciferase assay reagent according to the manufacturer’s instructions (Promega). Luminescence in relative light units was measured using an Orion microplate luminometer (Berthold Detection Systems).

**In Solution Virus Capture Assay**—ELISA microplates were coated overnight with 2 μg/ml rabbit anti-human Fab fragment in PBS at 4 °C. The capture antibodies (2G12 Fab variants) were mixed with 100-fold concentrated JR-FL pseudovirus at a final antibody concentration of 5 μg/ml. The resulting antibody/virus mix was incubated at 37 °C for 2 h and subsequently pelleted for 50 min at 16,000 × g at 4 °C. In the meantime, the coated plates were washed with PBS and blocked (3% nonfat dry milk) for 1 h at room temperature. The resulting antibody/virus pellets were resuspended in PBS, transferred onto the ELISA plates, and incubated for 1 h at 37 °C. Finally, plates were washed and overlaid with 1 × 10^4 TZM-bl cells in 100 μl/well. After 48 h, the luciferase activity was measured as described above. All experiments were performed in duplicate.

**Antibody Modeling**—The antibody models were generated with COOT (14) and PyMOL (15).

**RESULTS AND DISCUSSION**

To investigate the mechanism of domain swapping in 2G12 further, particularly the roles of previously highlighted residues at the V_H/V_H’ dimer interface and elbow region, three IgG1 2G12 heavy chain mutants with multiple “germ line” amino acid substitutions in the VDJ region were created (Fig. 1A). An amino acid alignment of the V_H domains of these 2G12 mutants with that of wild-type 2G12 (2G12-wt) is shown in Fig. 1B. In the first mutant, 2G12-JH3, the J region of 2G12-wt was replaced by a corresponding germ line region IGHJ3*01, resulting in five amino acid substitutions and one deletion (Fig. 1B). Two of these substitutions and the amino acid deletion occur in the complementarity-determining region (CDR) 3, whereas the remaining three substitutions are located in the J region. For the second mutant, 2G12-GL, four additional germ line amino acid substitutions are found in the V_H region, including one substitution in the framework region (FR) 1, one in the CDR2, and two in the FR3. In a third construct, 2G12-3H6, the 2G12 V_H region was completely exchanged by a different V_H region derived from a previously described antibody (16, 17) to verify the responsibility of the 2G12 D region for domain swapping.

All modified 2G12 heavy chain genes and the corresponding 2G12-wt light chain genes (codon-optimized and synthesized by GeneArt) were stably transfected into protein-free cultivated Chinese hamster ovary dehydrofolate reductase-deficient suspension cells (ATCC CRL-9096). The protein A-purified IgG1 2G12 mutants exhibited electrophoretic characteristics similar to those of IgG 2G12-wt as revealed by SDS-PAGE (supplemental Fig. S1).

The IgG1 2G12-wt and IgG1 2G12 mutants were further analyzed by CD spectrometry. Measurements in the near-UV region (240–340 nm) are shown in Fig. 2A. IgG1 2G12-JH3 exhibited a spectrum identical to that of IgG1 2G12-wt, indicating that the exchange of the J region of IgG1 2G12-wt had no influence on the overall structure. Substitution of four additional amino acids in the IgG1 2G12-GL mutant was reflected by an apparent structural change. However, this difference may have derived from subtle changes in the local environment of particular aromatic residues, which are not necessarily associated with any major structural change (i.e. domain exchange).

The spectrum of the third mutant, IgG1 2G12-3H6, revealed the largest difference from IgG1 2G12-wt. In this mutant, the whole V_H region was exchanged, which appeared to be accompanied by major structural changes. To investigate potential structural differences between the IgG1 2G12-wt and the IgG1 2G12 mutants further, electron microscopy was carried out. We used freeze drying and heavy metal shadowing to visualize the frozen IgG samples in the electron microscope. Although the resolution of this preparation method was slightly lower compared with a previously described staining method (18), we were able to discriminate between I-shaped and Y-shaped conformations of the 2G12 samples (6, 18). The IgG1 2G12-wt, IgG1 2G12-JH3, and IgG1 2G12-GL displayed a successively decreasing predominance of the I-shaped conformation (~90%, ~75%, and ~40%, respectively). In contrast, mutant
2G12-3H6 IgG1 existed predominantly in a Y-shaped conformation (~90%). Taken together, these electron microscopy results indicate qualitative structural differences between the different IgG1 2G12 samples (supplemental Fig. S2).

We next examined the binding capacities of Fab 2G12 mutants (produced by papain digestion) to recombinant gp160 MN (Fig. 2B). Relative affinities (i.e. apparent affinities determined from the antibody concentration at half-maximal binding) of Fab 2G12-JH3, Fab 2G12-GL, and Fab 2G12-3H6 compared with Fab 2G12-wt were less than 1%. These findings are in good correlation with those of Calarese et al. (6), who discovered that most of their mutations to the primary combining site, secondary binding site, and VH/VH interface resulted in a dramatic loss of binding to gp120. Considering that the gp160 specificity ELISA might not represent an optimal environment for the 2G12 mutants, the same samples were analyzed further.
for binding activity to native gp120 (i.e. in solution virus capture assay) as well as neutralization potency on JR-FL pseudovirus. In contrast to 2G12-wt, the mutants were neither able to cap-ture free virus nor exhibited any neutralization capacity (data not shown).

Finally, the Fab 2G12 variants were examined for their ability to form three-dimensional domain-swapped dimers. For this experiment, Fab 2G12 fractions were concentrated, visualized by SDS-PAGE (Fig. 3A), and separated by gel filtration. Fig. 3B shows the normalized spectrograms. Fab 2G12-wt eluted as two dominant peaks at 26.5 and 30 min, corresponding to dimeric and monomeric forms, respectively. Fab 2G12-JH3 also eluted as two peaks at 26.5 and 30 min. However, the proportion of dimer was significantly less than that for Fab 2G12-wt. In contrast, Fab 2G12-GL, Fab 2G12-3H6, and the Fab 3H6 control exhibited dominant peaks at 30 min, indicating monomer only.

The fact that 2G12-JH3 still forms a dimer (Fig. 3B) indicates that the ProH113 to Ser substitution in the J region does not prevent V_{H} domain exchange. The high resolution crystal structure of Fab 2G12 (Protein Data Bank code 1op3) (6) revealed that Pro N, Cα, Cβ at the elbow, or hinge region of the domain-exchanged dimer are within van der Waals (VDW) contact of ValH84 Cα2 (Fig. 4A). These hydrophobic interactions were proposed to stabilize the conformation of the hinge region in which the V_{H} domain pivots around ProH113 to facilitate dimerization. Modeling of the ProH113 to Ser substitution, as found in 2G12-JH3, by simply mutating the ProH133 side chain to that of the most probable rotamer of the Ser side chain using the program COOT (14) suggests that these interactions could be conserved in the mutant (Fig. 4A). Moreover, the ϕ and ψ angles of the modeled Ser lie within the preferred regions of the Ramachandran plot. Consequently, the conformation of the hinge as observed in the Fab 2G12 structure may be maintained in 2G12-JH3 via hydrophobic interactions between ValH84 and SerH113 that are analogous to those between ValH84 and ProH113.

On the other hand, the fact that 2G12-GL does not form a dimer (Fig. 3B) could be due to one or a combination of the four additional substitutions found in this mutant over 2G12-JH3. As mentioned above, in the Fab 2G12 structure, ValH84 Cγ2 was observed to form several VDW interactions with ProH113 at the elbow region as well as with ValH111 O (6) (Fig. 4A).
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![Diagram showing structural analysis of sequence differences among 2G12-wt, 2G12-JH3, and 2G12-GL.]

Modeling of the Val\(^{H84}\) to Ala substitution suggests that these interactions would be lost in 2G12-GL, and this may be enough to destabilize the hinge region and, hence, inhibit dimerization.

In regard to the Ile\(^{H19}\) to Arg substitution, the region of the V\(^{H}/V\(^{H}\) interface around Ile\(^{H19}\) was revealed to be very tightly packed in the Fab 2G12 structure (6) (Fig. 4B), with the hydrophobic side chain of Ile\(^{H19}\) being within VDW contact of Ser\(^{H72}\), Leu\(^{H20}\), Ser\(^{H21}\), and Tyr\(^{H79}\) in the V\(^{H}/V\(^{H}\) interface of the domain-swapped dimer. C, Arg\(^{H57}\) forms a salt bridge with Asp\(^{H72}\) at the V\(^{H}/V\(^{H}\) interface of the domain-swapped dimer. This interaction not only likely plays an important role in stabilization of the V\(^{H}/V\(^{H}\) interface, but also likely plays an indirect role in 2G12 binding to clustered high mannose oligosaccharides on gp120.

any rotamers of the Arg side chain at this position using COOT without substantially affecting the location and rotamers of the surrounding side chains. The Ile\(^{H19}\) to Arg substitution may, therefore, not even be tolerated within the dimer interface.

In relation to the Phe\(^{H77}\) to Ser substitution, the aromatic side chain of Phe\(^{H77}\) was observed to be within VDW contact of Thr\(^{H68}\) and Gln\(^{H81}\) in the Fab 2G12 structure (6). Modeling a Ser at this position suggests that these interactions would be lost in the 2G12-GL mutant.

The Arg\(^{H57}\) to Ile substitution would break a salt bridge with Asp\(^{H72}\) observed at the V\(^{H}/V\(^{H}\) dimer interface of Fab 2G12 (Fig. 4C) (6). In addition to the putative role of this salt bridge in stabilizing the V\(^{H}/V\(^{H}\) interface, it is also likely to be important for positioning of the neighboring Tyr\(^{H56}\) which, in the complex with Man\(_{α,α,α}\)Man (6), forms pi-stacking interactions with Tyr\(^{L94}\) at the V\(^{H}/V\(^{H}\) interface. These interactions in turn may be important for the positioning of Gly\(^{H93}\) which H-bonds with the terminal mannose O6 in the Man\(_{α,α,α}\)Man containing arms of high mannose sugars. Notably, a Tyr\(^{H56}\) to Ala substitution was reported to cause a dramatic loss in affinity of 2G12 for gp120 (6). Moreover, Ser\(^{H54}\), Thr\(^{H55}\), and Arg\(^{H57}\) to Ala substitutions were similarly observed to lead to significantly decreased gp120 binding (6). These effects could similarly arise from changes in the conformation of the aromatic side chain of Tyr\(^{H56}\) which prevent it from forming pi-stacking interactions with Tyr\(^{L94}\).

Consequently, the unusual domain-exchanged configuration of 2G12 could be fostered by the single or combined effects of four amino acid side chains that either help to stabilize the elbow region (H113) or the V\(^{H}/V\(^{H}\) dimer interface. Moreover, a proline at H113 has been shown here to not be required for the domain swapping capability of 2G12. Rather, the hydrophobic contacts between the residue at this position and the side chain of residue His\(^{H84}\) appear to be predominantly responsible for stabilization of the elbow region.

The lack of domain swapping capability observed for the mutant 2G12–3H6 clearly indicates that the 2G12-wt CDR3 and J region alone are not sufficient to promote V\(^{H}\) domain exchange when the complete V\(^{H}\) region of 2G12 is replaced by another antibody V\(^{H}\) region. Taken together, the present study shows that the I-shaped conformation of 2G12 cannot be traced back to a single amino acid mutation but rather involves a complex interplay of amino acid replacements. It remains to be further investigated at which stage these mutations occurred in the evolution of this very unique antibody.

Acknowledgments—We thank Zara Fulton (Dept. of Molecular Biology, The Scripps Research Institute, La Jolla, CA) for providing the 2G12 models and for critically reading the manuscript and M. Löscher, R. Marvan, and K. Bauer for excellent technical support.

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