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A peptide inhibitor of HIV-1 neutralizing antibody 2G12 is not a structural mimic of the natural carbohydrate epitope on gp120

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Abstract

MAb 2G12 neutralizes HIV-1 by binding with high affinity to a cluster of high-mannose oligosaccharides on the envelope glycoprotein, gp120. Screening of phage-displayed peptide libraries with 2G12 identified peptides that bind specifically, with K_d s ranging from 0.4 to 200 μ M. The crystal structure of a 21-mer peptide ligand in complex with 2G12 Fab was determined at 2.8 Å resolution. Comparison of this structure with previous structures of 2G12-carbohydrate complexes revealed striking differences in the mechanism of 2G12 binding to peptide vs. carbohydrate. The peptide occupies a site different from, but adjacent to, the primary carbohydrate-binding site on 2G12, and makes only slightly fewer contacts to the Fab than Man₉GlcNAc₂ (51 vs. 56, respectively). However, only two antibody contacts with the peptide are hydrogen bonds in contrast to six with Man₉GlcNAc₂, and only three of the antibody residues that interact with Man₉GlcNAc₂ also contact the peptide. Thus, this mechanism of peptide binding to 2G12 does not support structural mimicry of the native carbohydrate epitope on gp120, since it neither replicates the oligosaccharide footprint on the antibody nor most of the contact residues. Moreover, 2G12.1 peptide is not an immunogenic mimic of the 2G12 epitope, since antisera produced against it did not bind gp120.—Menendez, A., Calarese, D. A., Stanfield, R. L., Chow, K. C., Scanlan, C. N., Kunert, R., Katinger, H., Burton, D. R., Wilson, I. A., Scott, J. K. A peptide inhibitor of HIV-1 neutralizing antibody 2G12 is not a structural mimic of the natural carbohydrate epitope on gp120.

Keywords

peptide mimics; HIV-1 envelope proteins; crystal structure; phage-displayed peptide libraries

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Human monoclonal antibody (MAb) 2G12 efficiently neutralizes a broad range of HIV-1 primary isolates *in vitro* (1–3) and protects from *in vivo* viral challenge in macaques in combination with other antibodies (4–6). MAb 2G12 binds with high affinity to a unique, conserved epitope on the HIV-1 envelope that is formed by a cluster of *N*-linked, high-mannose glycan groups on the “silent” face of gp120 (7–10). Crystal structures of 2G12 Fab alone, and in complex with oligosaccharides Man₉GlcNAc₂ and Man α 1-2Man, revealed that two Fab monomers assemble into an interlocked, domain-swapped dimer, that forms an extensive, multivalent binding surface (11). Four Man₉GlcNAc₂ moieties were observed bound to the Fab dimer in the crystal structure, occupying the two canonical antigen-binding sites, and two novel ones located at the assembled interface of the two interlocked V_H domains. Since the identification of its epitope, MAb 2G12 has received significant attention as a template for HIV-1 vaccine development. Considerable effort has been directed at characterizing its oligosaccharide-binding profile (12–15), and the generation of novel antigens in the form of synthetic oligomannoses (13,16,17) or short, synthetic glycopeptides (18–20). In an attempt to generate immunogens that elicit 2G12-like antibodies, we chose to isolate peptides that bind to 2G12 and inhibit antibody binding to the native oligomannose epitope on gp120, by screening a set of phage-displayed peptide libraries with MAb 2G12.

Short peptide ligands that bind to anti-carbohydrate antibodies have previously been isolated and tested as templates for vaccine development (21–26), under the working principle that a peptide recognized by the antibody is likely to “mimic” its corresponding carbohydrate epitope. It is implicit in this concept that such a peptide would be a “structural mimic,” in that it would produce a good molecular fit in the same binding site as the carbohydrate, and would replicate the nature and location of most carbohydrate-antibody contacts, especially those of high affinity. In addition, this concept infers that such a peptide mimic might elicit its cognate antibody on immunization (*i.e.*, act as an immunogenic mimic of the antibody's native epitope). However, very little is actually known about the molecular mechanisms of carbohydrate-peptide cross-reactivity. Only two comparative structural analyses of carbohydrate and peptide ligands in complex with carbohydrate-binding proteins are available: the lectin concanavalin A specific for Man(α 1–6) Man(α 1–3) Man trimannose cores in mannose-containing carbohydrates, and the MAb SYA/J6, directed against the O-antigen polysaccharide of *Shigella flexneri* Y (27,28). These two studies have suggested that structural mimicry is not a major mechanism by which carbohydrate-binding proteins interact with peptides.

Here, we present the isolation, optimization, and first structural characterization of peptide ligands specific for anticarbohydrate antibody 2G12. The crystal structure of MAb 2G12 in complex with a synthetic peptide (2G12.1) was compared with previously published structures of 2G12 in complex with Man₉GlcNAc₂ and Man α 1-2Man (11,15). The 2G12-bound peptide exhibited minimal spatial overlap with the bound oligosaccharides, and common contacts with the antibody were limited to a few residues, which reveals that the mechanism of antibody-peptide recognition differs from that for the oligomannose epitope on gp120. Our results demonstrate that the peptide ligands that we have generated for MAb 2G12 are not structural mimics of the cognate oligomannose epitope on HIV-1 and support the notion that structural mimicry of polysaccharides is not the major mechanism by which peptides are recognized by carbohydrate-binding proteins. Sera from rabbits immunized with recombinant phage displaying the 2G12.1 peptide produced strong titers against the peptide, but no cross-reactivity with gp120. The implications for the use of peptides as immunogenic mimics of carbohydrate epitopes are discussed.

Materials and Methods

Materials

The phage-displayed peptide libraries are as described previously (29). Human MAb 2G12 Fab was produced as before (11). The 2G12.1 sequence was synthesized as a peptide (sequence: NH₃-ACPPSHVLDMRS GTCLAAEGK(biotin)-NH₂) by Multiple Peptide Synthesis (San Diego, CA, USA). Recombinant gp120_{Ba-L} was a kind gift from T. Fouts (Institute of Human Virology, Baltimore, MD, USA). Protein A-coated paramagnetic beads were purchased from Dynal (Lake Success, NY, USA). Purified *Escherichia coli* maltose binding protein (MBP) and a MAb against MBP were from New England Biolabs (Beverly, MA, USA). Man α 1-2 Man (α 1-2 mannosidase) was obtained from Dextra Laboratories (Reading, UK).

Bacterial strains and DNA constructs

Phage were produced in *E. coli* K91 cells, following Bonnycastle *et al.* (29). Electrocompetent, *F*[−] *E. coli* MC1061 cells were used for library construction, and strain CJ236 was used to amplify the phage used as a source of single-stranded viral DNA for site-directed mutagenesis. *E. coli* ER2507 (a gift from New England Biolabs) was used for production of MBP fusion proteins.

The 2G12.1 peptide sublibrary was constructed using the f88-4 phage vector (30); single-stranded, covalently closed circular DNA was used as template, following the procedure described in (29), and a degenerate oligonucleotide was synthesized using the two-column, “divide-couple-recombine method,” as described by Haaparanta and Huse (31). In the resulting library, the amino acids at each position were either from the 2G12.1 peptide or a random residue encoded by a degenerate NNK codon (in which *n*=A, G, C, or T and K=G or T). Also in preparing the sublibrary, the original 2G12.1 DNA sequence was modified to optimize codon usage for *E. coli*. Site-directed mutagenesis was performed using covalently closed, circular single-stranded phage DNA as a template, as described by Kunkel *et al.* (32). The transfer of peptide coding sequences to pMALX and the conditions for culture and protein purification are as described (33). The DNA from partially purified phage was sequenced using the Thermo Sequenase II Dye Terminator Cycle Kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions.

Screening of the phage-displayed peptide libraries

Several primary phage-displayed peptide libraries were mixed in Tris-buffered saline (TBS) containing 1% (w/w) BSA and 0.5% (v/v) Tween 20, and a total of 10¹² phage particles were used in the first round of screening. Theoretically, ~60–80 copies of every clone from each library were represented in this mixture. To minimize the selection of protein-A-binding phage, 12 μ l of protein-A-coated magnetic beads (Dynal, Burlington, ON, Canada) were added to the library mixture and incubated for 4 h at 4°C, with gentle shaking. The beads were removed from the phage with a magnet (Dynal) and discarded. 2G12 IgG was added to the remaining phage to a final concentration of 200 nM, and the mixture was incubated on a rotator at 4°C overnight. Phage-antibody complexes were captured out of solution with 12 μ l of protein-A beads for 1 h at 4°C. The beads were separated from the unbound phage with the magnet and washed five times with 1 ml of TBS-containing 0.5% Tween 20. The recovered beads were used to directly infect 75 μ l of starved K91 cells (34), and the infected cells were grown for 20 h at 37°C in 3 ml NZY medium (34) supplemented with 10 μ g/ml tetracycline (Tet) and 1 mM isopropyl- β -D-thiogalactoside (IPTG) (NZY/Tet/IPTG). Amplified phage were partially purified and concentrated by precipitation with PEG/NaCl (34), then resuspended in TBS containing 0.02% (w/v) sodium azide, and stored at 4°C. A second round of selection was carried out similar to the first round, using as input 10¹⁰ phage particles from the first-round amplification. Beads bearing bound phage were used to infect starved K91 cells, and individual

clones were isolated by plating the infected cells on NZY agar supplemented with 40 µg/ml tetracycline. Independent colonies were transferred to liquid NZY/Tet/IPTG media, grown overnight, and the phage were purified as described above. PEG-purified phage clones were used for ELISAs and DNA sequencing.

The 2G12.1 sublibrary was subjected to three successive rounds of screening with 200 and 100 nM of 2G12 IgG (for rounds one and two, respectively), and 100 nM 2G12 Fab for round three. Phage-IgG complexes were captured out of solution with protein-A beads, as described above, whereas Fab-bound phage were captured by protein-A magnetic beads coated with goat anti-human IgG (Fab-specific). The isolation of clones was performed as described above.

Enzyme-linked immunosorbent assays

Phage enzyme-linked immunosorbent assays (ELISAs) were performed as described previously (35,36). MAb 2G12 was used at 50 nM and incubated for three hours at 4°C. The plates were washed six times with cold (4°C) TBS containing 0.1% Tween 20, and 35 µl of a 1:500 dilution of horseradish peroxidase (HRP) conjugated to protein-A or goat anti-human, Fab-specific antibody (Pierce, Rockford, IL, USA) was added for 2 h at 4°C. The plates were washed six times with cold buffer, and the reactions were developed at room temperature with 0.03% (v/v) H₂O₂ and 400 µg/ml 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in citrate/phosphate buffer (34). Optical density was measured using a Biotek EL 312e plate reader at 405 and 490 nm for up to 45 min. The optical density at 405 nm minus that at 490 nm (OD₄₉₀₋₄₀₅) was recorded. Replicate, phage-coated wells were reacted with a rabbit, polyclonal antiphage antibody to verify that similar amounts of phage particles had been adsorbed to the plate. Bound antiphage antibody was detected with a protein-A-HRP conjugate and H₂O₂/ABTS. For phage competition ELISAs, 50 nM 2G12 MAb was incubated overnight at 4°C with 300 nM of soluble Ba-L gp120, and then reacted with the phage as described above.

Capture ELISAs, using synthetic biotinylated 2G12.1 peptide in solution and plate-adsorbed 2G12 IgG, were performed essentially as described by Menendez *et al.* (36), except that captured peptide was detected with Neutravidin-HRP (Pierce) and H₂O₂/ABTS. For titration ELISAs, 200 ng of 2G12.1 peptide were captured in microtiter wells coated with 1 µg streptavidin, followed by BSA blocking and incubation with 2G12 Fab or IgG, as described above. Bound antibodies were detected with a goat anti-human (Fab specific) antibody conjugated to alkaline phosphatase (Pierce) and a substrate solution containing 5 mg/ml p-nitrophenyl phosphate in the manufacturer's recommended buffer. Optical density was read at 405 nm (OD₄₀₅). For peptide competition ELISAs, 200 ng 2G12.1 peptide was captured in streptavidin-coated microtiter wells and reacted with 100 nM 2G12 IgG, which was preincubated for 16 h at 4°C with varying concentrations of glucose, fructose, mannose, Man α 1-2Man, or 2G12.1 peptide. Bound antibody was detected with an alkaline phosphatase conjugate. Competitions with gp120 were performed in a similar fashion, except that 100 ng gp120 (Ba-L isolate) was adsorbed directly to each microtiter well overnight at 4°C, and 1 nM 2G12 IgG was used. All ELISAs were performed at least twice.

Crystal structure determination of the Fab 2G12–2G12.1 peptide complex

Fab 2G12 at 56 mg/ml was mixed with biotinylated 21-mer peptide 2G12.1 at a 7:1 (peptide:Fab) molar ratio. Fab 2G12 + peptide 2G12.1 crystals were grown by the sitting drop vapor diffusion method with a well solution of 1.33 M Na/potassium phosphate, pH 7.8, and 0.2 M isopropanol. For crystallization, 0.6 µl of protein was mixed with an equal volume of reservoir solution. Data were collected at the Advanced Light Source (ALS; Berkeley, CA, USA) beamline 5.0.1 at 100 K. Crystals were cryoprotected by a quick plunge into a solution containing the reservoir conditions augmented with 25% glycerol. Data to 2.8 Å resolution for the Fab-peptide complex were reduced in monoclinic space group P2₁ with unit cell dimensions

$a = 66.3 \text{ \AA}$, $b = 171.3 \text{ \AA}$, $c = 119.6 \text{ \AA}$, and $\beta = 105.6^\circ$. All data were indexed, integrated, and scaled with HKL2000, using all observations $>-3.0\sigma$.

The Matthews' coefficient (V_m) for the Fab-peptide crystals was estimated to be $3.24 \text{ \AA}^3/\text{Da}$ (corresponding to a solvent content of 62%), with four Fab-peptide complexes per asymmetric unit. Rotation and translation solutions were found with AMoRe using the previously determined coordinates from the $1.75\text{-\AA}$ crystal structure of Fab 2G12 as a molecular replacement model. Positional refinement of the four individual Fab molecules (2 dimers) in the asymmetric unit gave an overall correlation coefficient of 60.6% and an R_{cryst} of 43.4%. The Fab 2G12 and peptide 2G12.1 model building was then performed using TOM/FRODO and refined with CNS version 1.1 and REFMAC using TLS refinement. Refinement and model building were carried out using all measured data ($F > 0.0\sigma$). Tight, noncrystallographic symmetry restraints were maintained throughout model building and refinement that included $F_{\text{obs}} - F_{\text{calc}}$ and $2F_{\text{obs}} - F_{\text{calc}}$ electron density maps. An R_{free} test set (5%) was maintained throughout refinement. Coordinates and structure factors have been deposited in the PDB (2oqj).

Affinity determinations

The binding of 2G12 to various antigens was investigated using KinExA (Boise, ID, USA) and Biacore 3000 (Biacore AB, Uppsala, Sweden) instruments. The 2G12.1 synthetic peptide was analyzed with the KinExA following the procedure described by Craig *et al.* (37). Biacore analyses were done at The Centre for Biothermodynamics, University of British Columbia (Vancouver, BC, Canada). The 2G12.1 synthetic peptide was immobilized at high density on streptavidin-coated sensor chips (Biacore) by two sequential injections of $1 \mu\text{g}$ of peptide, with a total of ~ 800 resonance units (RUs) captured. An unrelated 2F5-binding peptide E4.6 (36), with sequence $\text{NH}_3\text{-LHEESMDKWSNLMQCCTAAEGK(biotin)-NH}_2$, was immobilized in the reference cell, as a control. The binding-inhibition method was used to determine the in-solution affinity at equilibrium (38,39); separate reactions of 2G12 IgG mixed with 2G12.1 peptide in buffer HBS-EP (Biacore) were allowed to reach equilibrium by incubation at 4°C for 20 h. The final 2G12 IgG concentration was 40 nM , which consistently produced $\sim 100 \text{ RU}$ on injection over the 2G12.1-coated surface. Concentrations of competitor, ranging from 1 mM to $3 \mu\text{M}$ peptide, were explored in every experiment. After reaching equilibrium, the reactions were injected in duplicate for 100 s over the 2G12.1-coated chip at a flow rate of $3 \mu\text{l/min}$ at 25°C . Bound antibody was recorded as RU early in the association phase. The chip was regenerated with a one-minute pulse of 50 mM NaOH . The concentration of free antibody for each reaction was estimated by comparison of the experimental data with an antibody calibration curve generated with 2G12 IgG concentrations from 2.5 to 80 nM , in the absence of antigen. The free antibody concentration in each reaction was plotted against its respective competitor concentration, and the curves were fitted to the "in-solution" affinity model in the BiaEvaluation 4.1 software (Biacore).

For MBP fusion proteins bearing the sequences of peptides 2G12.1 and 2G12.1-D10, affinity was determined using a kinetic assay. MAb 2G12 was immobilized on a CM5 sensor chip using the amine-coupling kit (Biacore) following the manufacturer's instructions. Serial two-fold dilutions of the MBP fusions, from 2 mM to 31 nM , were passed over the chip for 2 min at a flow rate of $50 \mu\text{l/min}$ at 25°C . RUs were recorded during the association phase and the first 20 min of the dissociation phase. After each cycle, the surface was regenerated with a $50\text{-}\mu\text{l}$ injection of 3 M MgCl_2 .

Rabbit immunization study

Two rabbits were immunized with CsCl-purified 2G12.1 phage, and immune sera were titrated and tested in ELISA for binding to phage, synthetic 2G12.1 peptide and recombinant gp120, as described in Supplemental Data (see also refs. 40, 59).

Results

Identification of the 2G12-binding peptide 2G12.1

Two consecutive rounds of screening of the primary phage-displayed peptide libraries with 2G12 IgG yielded pools enriched in 2G12-reactive phage. Isolation, sequencing and 2G12 binding assays of 30 independent clones identified a phage bearing the sequence ACPPSHVLDMRSGTCL (clone 2G12.1), which bound specifically to MAb 2G12 (see Supplemental Tables S1 and S2). A cyclic, biotinylated peptide (2G12.1, NH₂-ACPPSHVLDMRSGTCLAAEGK(bio)-NH₂) was synthesized and tested for binding to 2G12 IgG and Fab; 2G12.1 reacted with both 2G12 IgG and Fab (Fig. 1A). In addition, plate-adsorbed 2G12 IgG captured 2G12.1 peptide out of solution (Fig. 1B). The K_d of 2G12 IgG for 2G12.1 peptide, at equilibrium, was 180 μ M by KinExA and 200 μ M by Biacore analysis.

MAb 2G12 binds to terminal Man α 1-2Man groups present in a cluster of high-mannose oligosaccharides on the silent face of HIV-1 gp120 (8,9). 2G12 also reacts with monosaccharides, such as mannose and fructose, with much lower affinity (8,11). To assess whether 2G12.1 peptide interacts with the carbohydrate-binding sites of MAb 2G12, competition assays were performed between 2G12.1 peptide and a variety of carbohydrates. Binding inhibition experiments, using a fixed concentration of Man α 1-2Man (64 μ M), showed a 75% reduction of 2G12 IgG binding to 2G12.1 and a 50% reduction in IgG binding to gp120 (data not shown). Preincubation of 2G12 with mannose and fructose inhibited the reactivity of 2G12 with both 2G12.1 peptide and gp120 in a dose-dependent manner in ELISA (Fig. 2), whereas preincubation of 2G12 IgG with 2G12.1 peptide inhibited 2G12 binding to the peptide (Fig. 2A), but caused only a modest reduction (30%) in gp120 binding, even at very high peptide concentration (Fig. 2B). Thus, 2G12.1 peptide reacts specifically with MAb 2G12, with low affinity. This reactivity can be blocked by monosaccharides and disaccharides that also bind to the paratope of 2G12, suggesting that peptide and carbohydrates bind to the same or overlapping sites on 2G12.

Critical binding residues on the 2G12.1 peptide

Single substitutions of every residue of the 2G12.1 sequence were generated by site-directed mutagenesis in the context of the phage. Most residues were changed to Ala with the exception of Ala^{P1}, which was changed to Gly, and Cys^{P2} and Cys^{P15}, which were changed to Ser. PEG/NaCl-purified phage were assayed for binding to two concentrations of 2G12 IgG in ELISA (Fig. 3). Several classes of substitution were revealed, based on their effect on binding to 5 nM 2G12 IgG (Fig. 3B): 1) substitutions that do not affect reactivity or have only a modest effect (Ala^{P1}, Ser^{P5}, Val^{P7}, and Arg^{P11}), 2) substitutions that have a significant effect (Pro^{P4}, His^{P6}, Leu^{P8}, Asp^{P9}, Met^{P10}, Ser^{P12} and Thr^{P14} and Leu^{P16}), and 3) those that ablate binding and thus define critical binding residues (Cys^{P2}, Pro^{P3}, Gly^{P13}, Cys^{P15}). These results correspond well with those from the 2G12.1 sublibrary screening, since most sequence deviations from the 2G12.1 parental clone are concentrated at sites that are not strongly affected by substitution. Nonetheless, we have previously observed that moderate differences in binding to recombinant phage clones may go undetected in IgG-based assays due to bivalent binding of IgG to multicopy peptide on the phage (36,40); thus, several substitutions that showed a weak effect on binding may mask significant affinity.

Crystal structure of the 2G12.1 peptide in complex with Fab 2G12

As previously demonstrated, 2G12 adopts an unusual configuration in which the heavy-chain variable (V_H) regions are exchanged (domain-swapped) between the antibody's two equivalent Fab domains, thus creating a tight interface between them. The close packing of Fv domains leads to multivalent carbohydrate binding at up to four sites: two within each Fv domain and two in the interface between the exchanged V_H regions (11,41). Because of V_H exchange, 2G12 Fabs are dimeric (*i.e.*, comprising 2 V_H - C_H1 chains and 2 light chains).

The crystal structure of Fab 2G12 bound to the 2G12.1 peptide was determined at 2.8 Å resolution (Table 1). Each crystallographic asymmetric unit contains four Fab-peptide complexes (*i.e.*, two dimers), which are almost identical. Thus, only one of the four complexes is described, and its light- and heavy-chain and peptide residues are designated by chain identifiers L, H and P, respectively.

Each Fv domain binds a single peptide, whereas peptide is not bound at the V_H - V_H' interface (Fig. 4A). A total of 426 Å² of Fab and 360 Å² of peptide molecular surface are buried in the complex, indicating that a significant portion of the peptide contacts Fab. Surprisingly, the 2G12.1 peptide does not bind within the primary carbohydrate-binding site of 2G12, but rather adjacent to it, with the peptide centered over CDR L3 and wedged between CDRs H2 and L1 (Fig. 4B, C). Four independent copies of the 21-residue, 2G12.1 peptide complexed with Fab (Mol1–4) are present in the crystallographic asymmetric unit; peptide residues 1–18, 1–20, 1–17, and 1–19 could be reliably built into each electron density for the Mol1–4 complexes, respectively. The electron density, which corresponds to the longest of these (Mol2) after refinement, is shown in Fig. 5. This peptide is stabilized at its C-terminus (residues P18-P20) by crystal packing contacts that are not present for the other three peptides. All four peptide structures reveal that 2G12.1 forms a two-stranded (β -sheet that is stabilized by a disulfide bridge between Cys^{P2} and Cys^{P15}, along with two turns and nine intrapeptide hydrogen bonds (Fig. 6A).

The intrapeptide and Fab-peptide interactions in the peptide-Fab complex are illustrated in Fig. 6B, in which peptide residues are highlighted that are designated as critical to (*) or important for (+) Fab binding, as determined by amino acid substitutions. Two hydrogen bonds and 49 van der Waals' interactions comprise the contacts between Fab 2G12 and the 2G12.1 peptide, with a third hydrogen bond observed in two of the four complexes in the asymmetric unit (Mol2 and Mol4; Table 2). Interestingly, the peptide residues that make the two hydrogen bonds with Fab are critical to (Gly^{P13}) or important for binding (Met^{P10}); these residues contact CDRs L3 and L1, respectively. In contrast, His^{P6} and Asp^{P9} are examples of residues that do not directly contact Fab yet are also important for binding, probably because of intrapeptide hydrogen bonds made by their sidechains. Thus, the complex is stabilized both by peptide residues involved in intrapeptide disulfide and hydrogen bonds and those making direct contacts with the paratope. The bulk of the buried peptide molecular surface and van der Waals' interactions with the antibody are contributed through contact of Ala^{P1}-Pro^{P3}, Leu^{P8}, and Met^{P10}-Cys^{P15} with Fab CDRs L1, L3, H2, and H3. Most of these peptide residues are either important to or critical for binding to Fab, indicating that, although the affinity of 2G12.1 is relatively low (~ 200 μ M) as compared to many peptide-antibody interactions (~ 1 μ M to 10 nM), the functional interaction involves a number of residues in the 2G12.1 peptide. Moreover, all significant contacts with the peptide are with the Fab CDRs, rather than framework regions, reflecting interactions that are characteristic for most antigens.

Optimization of 2G12.1 peptide sequence

With the objective of optimizing the affinity of clone 2G12.1, we constructed a phage-displayed peptide sublibrary of 10^9 independent clones. Screening of this sublibrary yielded phage clones

with a range of reactivities for 2G12, with the majority of selected sequences differing by one or two residues from the parental 2G12.1 (average 1.68 substitutions per peptide) (Table 3).

The coding sequences of several peptides (2G12.1, 2G12.1-4, 2G12.1-D3, and 2G12.1-D10) were cloned into the 5' end of the MBP coding sequence, and the resulting fusion proteins were expressed and purified. The purity and the monomeric status of the MBP fusion proteins were verified by nonreducing SDS-PAGE (not shown). Binding of the MBP fusions to immobilized 2G12 IgG was tested by a capture ELISA and confirmed that the peptides expressed by clones 2G12.1-4, 2G12.1-D3, and 2G12.1-D10 bind MAb 2G12 tighter than the parental 2G12.1 clone (data not shown). The affinity of 2G12 IgG for recombinant fusions 2G12.1-MBP and 2G12.1-D10-MBP was determined by SPR, in kinetic experiments. As observed for other phage-display derived peptides (36) (and our unpublished results), the 2G12.1 fusion protein displayed higher affinity than the free synthetic peptide, in this case by almost 1 log ($K_{dMBP\text{ fusion}}=28\text{ }\mu\text{M}$ vs. $K_{dsynt.pept}=200\text{ }\mu\text{M}$). In addition, 2G12.1-D10-MBP, which differs from 2G12.1-MBP by only three amino acids, displayed a K_d of 400 nM, almost two orders of magnitude lower than that of 2G12.1-MBP.

Sublibrary clones 2G12.1-D10 and 2G12.1-D3 displayed similar binding to 2G12 IgG, even though 2G12.1-D3 has a single substitution with respect to the parental clone 2G12.1 (vs. three substitutions on 2G12.1-D10; Table 3). Further optimization of the 2G12.1-D10 sequence was explored by modeling the single amino acid change observed in clone 2G12.1-D3 (Ser^{P1} compared to the parental Ala^{P1}), into the crystal structure of the 2G12.1 peptide-Fab. Analysis of the modeled peptide showed potential for additional hydrogen bonds of Ser^{P1} with CDR H2 Tyr^{H56} and Asp^{H58}, (Fig. 7A, B). Substitution of Ala^{P1} on clone 2G12.1-D10 with Ser^{P1} produced a new phage clone 2G12.1-KH3, with sequence SCPPSHYLDKSGTCR. 2G12 IgG bound this clone more tightly than clones 2G12.1, 2G12.1-D3 or 2G12.1-D10 (Fig. 7C).

Immunogenicity of 2G12.1 peptide displayed on phage

Two rabbits were immunized four times, at 2-week intervals, with 6×10^{12} CsCl-purified 2G12.1 phage particles. Immune and prebleed sera were tested with an ELISA for binding to wild-type phage (f88), 2G12.1 peptide, and gp120. As shown in Supplemental Table 3 and Supplemental Fig. 1, strong reactivity was observed for f88 phage and 2G12.1 peptide, with half-maximal titers ranging from $1\text{--}2.5 \times 10^5$ and 800-2000, respectively. Yet, even at a 1:50 dilution, neither serum cross-reacted with gp120. Thus, 2G12-like reactivity was not elicited in face of significant titers against the 2G12.1 peptide.

Discussion

Peptide 2G12.1 described here is specific for the HIV-1 neutralizing MAb 2G12, but it is not a structural mimic of the 2G12 oligomannose epitope on gp120. 2G12.1 binds at a site that only partially overlaps the primary carbohydrate-binding site (Fig. 8) utilized by the oligosaccharides described by Calarese *et al.* (11,15). The 2G12 Fab dimer has up to four possible antigen-binding sites for the Man₉GlcNAc₂ moieties that form its epitope on gp120 (two conventional and two at the V_H-V_{H'} interface) (11). The 2G12.1 peptide contacts the conventional antigen-binding site of the antibody on the outside of CDR L3 rather than in the carbohydrate-binding site between H3 and L3 (Fig. 4). 2G12 binding to carbohydrate and peptide occurs through different antibody interactions. 2G12 interacts with Man α 1-2Man (PDB 1op3) *via* 4 hydrogen bonds, plus 8 additional OH-O or NH-O contacts (*n.b.*, these contacts are within hydrogen bonding distance but do not have optimal geometry), and 32 van der Waals' interactions. The antibody contacts Man₉GlcNAc₂ (PDB 1op5) *via* 6 hydrogen bonds, 6 additional OH-O or NH-O contacts, and 44 van der Waals' interactions. The MAb-carbohydrate interactions involve 11 (for Man α 1-2Man) or 13 (for Man₉GlcNAc₂) Fab residues, most of which are located in V_H (10 for Man α 1-2Man and 11 for Man₉GlcNAc₂).

(11). In contrast, 13 antibody residues, 9 from the V_L domain, are involved in contacting the 2G12.1 peptide through three hydrogen bonds, two OH-O or NH-O contacts, and 45 van der Waals' interactions. All of the peptide-contacting residues are located on the 2G12 CDRs, and the peptide does not bind cyanovirin, a mannose binding lectin, in ELISA or Biacore (data not shown), which indicates that the interaction is, indeed, specific for 2G12.

Only two residues from the 2G12 paratope are unequivocally implicated in binding to both carbohydrate and 2G12.1 peptide: Gly^{L93} (CDR L3) and Asn^{H100c} (CDR H3). A third common contact residue may be inferred based on the recent observation of a water-mediated hydrogen bond between Tyr^{H56} (CDR H2) and oligomannoses Man₄ and Man₇ (15). A similar interaction is likely present in the Man₉GlcNAc₂-2G12 complex, but the lower resolution of that crystal structure (3.0 Å) may mask its detection. 2G12 Gly^{L93} makes backbone contact with the carbohydrates, and substitution of Asn^{H100c} and Tyr^{H56} results in 1000-fold and 50-fold reduction in reactivity with gp120, respectively, indicating that they are key residues for this interaction (see Supplemental Data in ref. 11). Gly^{L93}, Tyr^{H56}, and Asn^{H100c} contact peptide residues Ala^{P1}, Cys^{P2}, Pro^{P3}, Leu^{P8}, Gly^{P13}, and Cys^{P15}, most of which are critical or important for peptide binding to 2G12. The mutagenesis and sublibrary screening results are in good agreement with the structural data; most residues defined as critical or important by mutagenesis are engaged in interactions with the antibody or in intrapeptide hydrogen bonds (Fig. 6). Moreover, only 5 of 27 amino acid changes (18.5%) observed on clones selected in the 2G12.1 sublibrary screening occurred at locations that we have determined to be important residues, and no substitutions were selected at positions defined as critical binding residues. No changes were observed for Cys^{P2}, Pro^{P3}, Leu^{P8}, Asp^{P9}, Met^{P10}, Gly^{P13}, Thr^{P14}, and Cys^{P15} (Table 3), which are all critical or important residues.

The peptide-Fab structure was superimposed on those of bound Man₉GlcNAc₂ and Man_α1-2Man (11), as a means of comparing the peptide and oligosaccharide structural shapes, spatial overlap, and occupancy of the antibody paratope. The peptide- and carbohydrate-bound 2G12 Fabs adopt similar conformations, with only small differences in their carbohydrate-binding sites, primarily in CDRs L3 and H3. When the 2G12.1-bound 2G12 is superimposed onto Man_α1-2Man-(1op3) and Man₉GlcNAc₂-(1op5) bound structures, using framework residues, the resulting RMS deviations (in Å) for CDR C α atoms are 0.19, 0.18, 0.84, 0.27, 0.41, and 1.22 (for L1, L2, L3, H1, H2, and H3 in 1op3) and 0.24, 0.30, 1.10, 0.39, 0.47, and 1.55 (for L1, L2, L3, H1, H2, and H3 in 1op5). The H3 movement maintains the same overall loop conformation but reflects movement of this loop as a rigid body. The L3 conformational change is localized to Gly^{L93}, where the C α atom moves by 2.1 Å. The D2 and D3 arms of the bound Man₉GlcNAc₂ clash with the complexed 2G12.1 peptide (Fig. 8), indicating the possibility of competition between these two ligands; indeed, reactivity with 2G12.1 phage was blocked by gp120 (Supplemental Table 2). Only partial inhibition of 2G12 binding to gp120 was observed on preincubation of 2G12 with a high concentration of 2G12.1 peptide, most likely as a result of the comparatively low affinity of 2G12.1, or residual interaction of gp120 Man₉GlcNAc₂ with the secondary binding sites on 2G12 (at the V_H-V_{H'} interface), which may favor binding to gp120 and displace competing peptide binding at the two conventional antigen-binding sites. Man_α1-2Man and the 2G12.1 peptide do not occupy the same space in the bound structures; in fact, their footprints barely overlap (Fig. 8). However, competition ELISA showed that Man_α1-2Man interfered with 2G12 binding to 2G12.1, similar to its inhibition of 2G12 binding to gp120. This suggests that inhibition of peptide binding by Man_α1-2Man must be mediated by the competition of these two ligands for their common contacts with Gly^{L93} and Asn^{H100c}. Gly^{L93}, in particular, moves by ~2 Å between the two complexes, supporting the notion that competing interactions with this residue may be part of the mechanism that prevents 2G12 from binding both peptide and Man_α1-2Man simultaneously.

Significant efforts are in progress to develop synthetic oligosaccharide immunogens that elicit 2G12 (14,16,18–20,42,43). Two other groups have reported isolation of phage clones that bind 2G12, from phage-displayed random peptide libraries (44,45), but their peptides display very low affinity for 2G12. For example, one sequence identified by Pashov *et al.* (45) binds 2G12 only as phage-borne peptide, but not as a free, synthetic peptide. The 2G12.1 sequence is clearly different from the peptides found by Tumanova *et al.* (44) and Pashov *et al.* (45); in fact, under close examination, we noticed that the clone most frequently isolated by Pashov *et al.* (45), SVSVGMKPSRP, has been previously isolated in screenings with unrelated antibodies (46, 47), suggesting that this peptide is not specific for MAb 2G12 (see also ref. 48). In contrast, our results show that, although the K_d of 2G12.1 peptide is also low (200 μ M), its binding to 2G12 MAb can be detected in a standard ELISA. In addition, K_d s of around 400 nM were achieved for an optimized peptide sequence fused to MBP; we also showed that third-generation peptides with better affinity for 2G12 (such as the one displayed by clone 2G12.1-KH3) are possible to obtain by rational design. Other substitutions observed in tight-binding clones selected from the 2G12.1 sublibrary screening (*e.g.*, Thr^{P5} instead of Ser^{P5}, as in clones 2G12.1-C11 and 2G12.1-D11, Table 3), may also be incorporated and tested in further rounds of optimization.

Peptide ligands for antibodies against carbohydrate epitopes have been mostly sought as immunogens to overcome the poor immunogenicity of carbohydrate antigens, and the difficulties associated with synthesis of complex polysaccharides. For a peptide to succeed as an immunogen that can generate a specific anticarbohydrate antibody response, carbohydrate-peptide cross-reactivity must translate into immunogenic mimicry (*i.e.*, the ability of a cross-reactive peptide to elicit that same antibody, or functionally similar ones). Immunogenic mimicry of carbohydrate epitopes by peptides has been previously reported (22–26,49–51), which suggests that cross-reacting peptides may elicit antibodies functionally equivalent to those elicited by carbohydrate epitopes. In contrast, our preliminary immunization of rabbits with recombinant 2G12.1 phage produced a significant anti-peptide response but no cross-reactivity with gp120, indicating that 2G12.1 elicits an antibody response that is qualitatively different from 2G12 (see Supplemental Table 3 and Supplemental Fig. 1).

It has been widely assumed that immunogenic mimicry by peptides is most likely based on replication of high-energy contacts, shape, and occupancy of the antibody's paratope by such peptide ligands, (*i.e.*, structural mimicry). Structural mimicry has been suggested (but not demonstrated) for peptide and carbohydrate ligands of anti-Lewis Y MAb BR55-2, using molecular modeling (52), and proposed for a peptide mimic of the group B streptococcal type III capsular polysaccharide by Pincus and colleagues (53,54). However, structures of both peptide and carbohydrate with the cognate antibody are not available. Comparison of crystal structures of an anticarbohydrate antibody against the O-antigen polysaccharide of *S. flexneri* Y (SYA/J6) with an octapeptide ligand and a synthetic pentasaccharide antigen revealed that peptide and pentasaccharide both bind to an overlapping site on SYA/J6, but the antibody-peptide contacts and the thermodynamics of binding are very different from those of the pentasaccharide (28). Similar to our results, peptide ligands for concanavalin A (ConA) bind at sites different from those used by carbohydrate ligands (27). Furthermore, molecular modeling of nonasaccharide and peptide ligands of an antibody against the 5a O-specific polysaccharide of *S. flexneri*, suggests different contacts between antibody and the two ligands and, hence, different mechanisms of binding (55). Only traces of structural mimicry can be identified in these examples, mostly in the form of a few shared contacts, or from partial occupancy of the same region of the binding site by both carbohydrate and peptide ligands.

As suggested by our immunization results, poor structural mimicry of carbohydrate epitopes by peptide ligands makes it unlikely that a peptide will elicit the corresponding anticarbohydrate antibodies in conventional immunization protocols, even in the case of strong

cross-reactivity of antibodies with the two ligands. This notion is further supported by the combined results of Young *et al.* (56) and Valadon *et al.* (57) using a peptide mimic of the glucuronoxylomannan capsular polysaccharide of *Cryptococcus neoformans*. They showed a relationship between the ability to elicit antibodies with the same light-chain V gene as the original antibody (2H1) and a tight peptide fit in the antigen-binding site mediated by interactions with the V_L domain. Immunization with peptide ligand elicited antibodies with the same or related V_L genes but different V_H genes from 2H1, suggesting that proper peptide contacts with a V region are necessary to recall the same or related V genes (57). In addition, none of the antibodies produced by immunization with their peptide mimic cross-reacted with glucuronoxylomannan. Importantly, a peptide similar to 2H1 successfully elicited strong cross-reactivity as a boosting immunogen following priming with the capsular polysaccharide (58).

Our data, as well as those reviewed above, suggest that antibody cross-reactivity with carbohydrate and peptide occurs in the absence of significant structural mimicry. The data are also difficult to reconcile with the notion that peptides can act directly as immunogenic mimics of carbohydrates and elicit the same antibody response as the native polysaccharide antigens. Interestingly, similar conclusions emerge from studies involving a peptide ligand of MAb b12, another HIV-1 neutralizing antibody that recognizes a conformational protein epitope on gp120 (40,59). Clearly, more structural studies and in-depth molecular characterization of the antibody responses against peptide mimics of carbohydrate epitopes are required to determine whether the lack of structural mimicry is the general rule for peptide-carbohydrate cross-reactivity and to establish what relationships, if any, exist between such potential mimicry and the type of antibody response elicited by those mimics in experimental immunizations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Binding of 2G12 MAb to 2G12.1 peptide. A) Titration ELISA of 2G12 IgG and Fab on 2G12.1 peptide captured with streptavidin. Bound antibodies were detected with a goat anti-human antibody conjugated to alkaline phosphatase and pNPP. Values are expressed as optical density at 405 nm. B) Titration of 2G12.1 peptide by capture from solution with plate-adsorbed MAb 2G12. Bound peptide was detected with neutravidin-horseradish peroxidase and ABTS. Values are expressed as optical density at 405 – 490 nm. One representative experiment of two is shown.

Figure 2. Inhibition of 2G12 binding to 2G12.1 peptide (A) and gp120 (B) in the presence of monosaccharides and 2G12.1 peptide. Binding of 2G12 was assayed after preincubation of 1 or 100 nM IgG (for gp120 and peptide binding assay, respectively) with serial dilutions of glucose, mannose, fructose, and 2G12.1 peptide. Antibody binding in the absence of a competitor was considered 100%; values are expressed as percent binding.

Figure 3.

Alanine-substitution scanning of the 2G12.1 peptide in the context of the phage. The relative binding of 20 nM (A) and 5 nM (B) 2G12 IgG to the alanine-substituted phage is expressed as percentage binding of each mutant phage with respect to wild-type (wt) 2G12.1 phage. The f88 is a negative control for wild-type phage expressing no recombinant peptide. One representative experiment of two is shown.

Figure 4.

A) Overall crystal structure of the Fab 2G12 dimer bound to the biotinylated 21-mer 2G12.1 peptide. Each Fab of the domain-swapped dimer interacts with 2G12.1 peptide (shown in a yellow ball-and-stick representation), only on one side of the primary carbohydrate-binding site. The light chains are shown in cyan; the heavy chains from the individual Fabs in the dimer are shown in red and purple for clarity. The CDR L1, L2, L3, H1, H2, and H3 loops are labeled and colored orange, pink, green, blue, purple, and yellow, respectively. This color scheme is followed throughout all the subsequent figures. B) Top view of the 2G12.1 peptide-Fab complex, looking down onto the combining site, asterisk indicates the binding site for oligosaccharide antigens. C) Side view of the Fab-bound 2G12.1 peptide.

Figure 5.
Stereoview of 2G12.1 peptide electron density. The final 2Fo-Fc electron density for the peptide after refinement, with the final peptide coordinates, is contoured at 1.8σ .

Figure 6.

Hydrogen bonding in the 2G12.1-2G12 structure. A) Stereoview of the 2G12.1 peptide with intrapeptide hydrogen bonds represented as black dotted lines. The peptide forms a 2-stranded β -sheet. B) Schematic representation of direct contacts between 2G12.1 peptide and Fab. Black lines represent 1–4 van der Waals' interactions between residues, green lines represent 5–10 van der Waals' interactions, and red lines represent hydrogen bonds. Peptide residues shown to be critical for binding to 2G12 by alanine mutagenesis are indicated by an asterisk, and other important residues are indicated by a plus sign. Fab residue Gly^{L93} (L3), which contacts both Man₉GlcNAc₂ and 2G12.1 peptide, is underlined. Tyr^{H56} (H2) may also contact Man₉GlcNAc₂ (see text for details). Note that the hydrogen bond Ala^{P1}-2G12 D^{H58} is only observed in two of the four MAb-peptide complexes, and therefore is not shown here.

Figure 7.

A) Hydrogen bond between 2G12.1 Ala^{P1} with 2G12 D^{H58} (H2). B) Modeled hydrogen bonds between 2G12.1-KH3 peptide Ser^{P1} with 2G12 D^{H58} (H2) and Tyr^{H56} (H2). Dotted black lines represent hydrogen bonds. C) Binding of 2G12 IgG to 2G12.1 phage clone and to optimized derivatives. Values are expressed as optical density at 405 – 490 nm.

Figure 8.

A) Overlap of the $\text{Man}_9\text{GlcNAc}_2$ -Fab 2G12 and peptide 2G12.1-Fab 2G12 structures. The $\text{Man}_9\text{GlcNAc}_2$ glycan is shown in red, while the 2G12.1 peptide is shown in blue. The 2G12.1 peptide sterically clashes with the D2 and D3 arms of the $\text{Man}_9\text{GlcNAc}_2$ moiety, illustrating why this peptide is capable of competing with this glycan for 2G12 binding. B) Molecular surface representation of a 2G12 monomer showing antibody areas in contact with $\text{Man}_9\text{GlcNAc}_2$, (cyan shading) and with 2G12.1 peptide (black outline). The $\text{Man}_9\text{GlcNAc}_2$ is shown in a yellow ball-and-stick representation, and the 2G12.1 peptide is shown as a red $\text{C}\alpha$ tube. C) Same as in B, but the surface in contact with $\text{Man}\alpha 1\text{-}2\text{Man}$ is shown in cyan, with

its sugar coordinates. PCBS refers to the primary carbohydrate-binding site, as defined in Calarese *et al.* (11,15).

Table 1
Summary of crystallographic data and refinement

Characteristic	Fab 2G12 + 2G12.1
Data collection	
Space group	P2 ₁
Unit cell dimensions (Å)	$a = 66.3, b = 171.3, c = 119.6, \beta = 105.6^\circ$
Resolution (Å) ^a	48.7–2.8 (2.85–2.80)
No. observations	194,291 (9427)
No. unique reflections	62,018 (3095)
Completeness (%)	98.2 (97.9)
R_{sym} (%) ^b	11.5 (47.6)
Average I/σ	9.8 (2.5)
Refinement statistics	
Total no. of reflections	58,837
No. in test set	3138
R_{cryst} (%) ^c	23.4 (44.3)
R_{free} (%) ^d	27.5 (52.5)
Fab atoms	13,172
Ligand atoms	513
Wilson B^e	67.9(Å ²)
Average B values (Å ²)	
Fabs	68.8
Peptides	78.2
Ramachandran plot (%) ^f	
Most favored	89.6
Allowed	9.7
Generously allowed	0.3
Disallowed	0.5 ^g
Rms deviations	
Bond lengths (Å)	0.021
Angles (°)	1.9

^aNumbers in parentheses are for highest resolution shell.

^b $R_{\text{sym}} = \sum_{\text{hkl}} |I - \langle I \rangle| / \sum_{\text{hkl}} I$.

^c $R_{\text{cryst}} = \sum_{\text{hkl}} |F_{\text{obs}} - F_{\text{calc}}| / \sum_{\text{hkl}} F_{\text{obs}}$.

^d R_{free} is the same as R_{cryst} but calculated for 5% of the reflections excluded from refinement.

^eAll Wilson B values are calculated using data from 4.0–2.8 Å.

^fCalculated using Procheck.

^gResidues Ala^{L51} and Glu^{L30} are in the disallowed regions. L51 is in a conserved γ -turn in almost all other antibodies, while L30 is in clear strong density.

Hydrogen bond distances and number of van der Waals' contacts between Fab 2G12 and peptide 2G12.1

Table 2

Fab atom	Peptide atom	Distance (Å)			
		mol1	mol2	mol3	mol4
Trp ^{L32} NE1	Met ^{P10} O	2.8	3.0	2.8	3.0
Gly ^{L93} NH	Gly ^{P13} O	2.7	3.0	3.0	3.0
Asp ^{H58} OD2	Ala ^{P1} NH	3.8 ^a	3.1	3.6 ^a	3.2
Number of van der Waals' contacts		47	48	51	44

Interactions are listed for each of the four Fab-peptide complexes (1–4) in the crystallographic asymmetric unit. Hydrogen bonds evaluated with HBPLUS (60) and contacts with Contaesym (61).

^aNot a hydrogen bond in mol1 and mol3.

Table 3
Deduced amino acid sequence of peptides displayed by phage clones isolated in the screening of the 2G12.1 sublibrary

Clone	Deduced amino acid sequence ^a	2G12 binding ^b
2G12.1-1	SCPSSSVLDMRSGTCL	-/+
2G12.1-2	ACPPHHVLDMPSGTCL	-/+
2G12.1-5	ACPPSTALDMRSGTCK	-/+
2G12.1	ACPPSHVLDMRSGTCL	+
2G12.1-3 ^c	ACPPSHVLDMRSGTCL	+
2G12.1-6	TCPPSHVLDMRSGTCL	+
2G12.1-8	ACPPSHVLDMRSGTCV	+
2G12.1-9	GCPPTHVLDMRSGTCL	+
2G12.1-10 ^c	ACPPSHVLDMRSGTCL	+
2G12.1-C11	QCPPTHVLDMRSGTCL	++
2G12.1-D11	GCPPTLVLDMRSGTCL	++
2G12.1-4	ACPPSHVLDMRSGTCI	++
2G12.1-C2	GCPPSHVLDMRSGTCL	+++
2G12.1-C12	GCPPSHRLDMRSGTCI	+++
2G12.1-D3	SCPPSHVLDMRSGTCL	++++
2G12.1-D10	ACPPSHYLDMKSGTCR	++++

^a Amino acids different from the parental 2G12.1 clone are shown in bold.

^b Relative binding to 2G12 IgG with respect to the parental clone 2G12.1, as determined by phage ELISAs. -/+, <0.5-fold binding; +, 0.5–1.5; ++, 1.5–2.5; +++, 2.5–3.5; +++, >3.5.

^c Sequence identical to the 2G12.1 parental clone.