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THE CARBOHYDRATE EPITOPE OF THE NEUTRALIZING ANTI-HIV-1 ANTIBODY 2G12

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2G12 is a broadly neutralizing human monoclonal antibody against human immunodeficiency virus type-1 (HIV-1) that has previously been shown to bind to a carbohydrate-dependent epitope on gp120. Here, site-directed mutagenesis and carbohydrate analysis were used to define further the 2G12 epitope. Alanine scanning mutagenesis showed that elimination of the N-linked carbohydrate attachment sequences associated with residues N295, N332, N339, N386, and N392 by N → A substitution produced significant decreases in 2G12 binding affinity to gp120_{JR-CSF}. The mutagenesis studies provided no convincing evidence for the involvement of gp120 amino acid side chains in 2G12 binding. Antibody binding was inhibited when gp120 was treated with *Aspergillus saitoi* mannosidase, Jack Bean mannosidase, or endoglycosidase H, indicating that Man α 1 → 2Man-linked sugars of oligomannose glycans on gp120 are required for 2G12 binding. Consistent with this finding, the binding of 2G12 to gp120 could be inhibited by monomeric mannose but not by other hexoses. The data presented here suggests that the most likely epitope for 2G12 is formed from a specific cluster of mannose residues on the outer face of gp120, with the other glycans playing an indirect role in maintaining epitope conformation. [Journal of Virology, July 2002, pp. 7306–7321, Vol. 76, No. 14.]

1. INTRODUCTION

According to the World Health Organisation there are over 40 million individuals worldwide living with HIV, all of whom are expected to develop AIDS. The development of an effective vaccine, providing sterilizing immunity, remains the primary goal in efforts to control the epidemic.

1.1. Neutralizing Antibodies against HIV-1

Most vaccine initiatives, aimed at eliciting a humoral response, have been targeted to the external HIV glycoproteins: gp120 and gp41 (Burton, 1997). Although the immune response to HIV and its structural proteins is generally characterized by low levels of neutralizing antibodies (Burton and Montefiori, 1997; Connor *et al.*, 1998; Kostrikis *et al.*, 1996; Moog *et al.*, 1997; Moore *et al.*, 1996; Parren *et al.*, 1999), a few such antibodies, cross reactive to many different isolates of the virus have been characterized (Burton *et al.*, 1994; Conley *et al.*, 1994; D'Souza *et al.*, 1994; Thomas *et al.*, 2002; Trkola *et al.*, 1996). The epitopes of these neutralizing antibodies may act as a template for improved vaccine design. Three of these MAbs bind to the surface glycoprotein, gp120, which is the viral receptor for CD4 and chemokine receptors CCR5 and CXCR4. The most potent of these MAbs are b12, which recognizes an epitope overlapping the CD4 receptor site (Burton *et al.*, 1994; Roben *et al.*, 1994), and 2G12 (Kunert *et al.*, 1998; Trkola *et al.*, 1996), which recognizes an epitope based around the C4/V4 region of gp120 and is highly sensitive to the presence of N-linked glycans in this region. Another Fab with broad neutralizing ability, X5, recognizes a region close to the coreceptor binding site on gp120 and overlapping the epitope recognized by CD4-induced MAbs, such as 17b (Moulard *et al.*, 2002). GP41 is also a target for broadly neutralising antibodies: one MAb, 2F5, binds to an epitope involving a linear motif (ELDKWA) on the membrane proximal region of the transmembrane envelope protein gp41 (Conley *et al.*, 1994; Kunert *et al.*, 1998; Parker *et al.*, 2001; Zwick *et al.*, 2001). Recently, two MAbs, Z13 and 4E10, have been described which recognize a region close to the C terminus of the 2F5 epitope (Stiegler *et al.*, 2001; Zwick *et al.*, 2001).

1.2. 2G12 Binds to the "Silent" Face of gp120

The MAb 2G12 has been shown, *in vitro*, to neutralize a wide spectrum of different HIV-1 isolates, including those from different clades, with the notable exception of clade E (Trkola *et al.*, 1995, 1996). *In vivo*, the MAb protects macaques against vaginal challenge with the chimeric virus SHIV 89.6P (Mascola *et al.*, 2000). The antibody recognizes a unique epitope that does not compete with any of the large panel of MAbs to gp120 that have been produced (Moore and Sodroski, 1996). The binding of 2G12 to gp120 had previously been shown to be inhibited by a number of mutations that disrupted sequences encoding attachment of N-linked carbohydrates. These sequences were located in the C2 and C3 regions around the base of the V3 loop, the C4 region, and the V4 loop (Trkola *et al.*, 1996) and probably clade C (Sanders *et al.*, 2002). The crystal structure of the core of gp120 suggests that these carbohydrate attachment sites are clustered together on a region of the gp120 known as the "silent face" (Kwong *et al.*, 1998, 2000). This solvent-accessible face is largely covered by carbohydrate and expected to be relatively weakly immunogenic and, hence, is described as immunologically silent (Wyatt *et al.*, 1993, 1998).

1.3. Glycosylation and Antibody Recognition

Glycosylation can limit the immunogenicity of glycoproteins such as gp120. This may be for a number of reasons. Firstly, glycoproteins exhibit micro-heterogeneity; a single protein sequence might display multiple antigenic glycoforms leading to the dilution of any single response (Rudd and Dwek, 1997). Secondly, the carbohydrates added to potential antigens are the same as those added to "self"-proteins and therefore immunological tolerance may come into play. Thirdly, the affinities of protein-carbohydrate interactions are generally weaker than those between proteins so that high affinity antibodies to carbohydrates do not generally develop. Furthermore large, dynamic glycans can cover an otherwise immunogenic protein epitope (Wilson and Stanfield, 1995; Woods *et al.*, 1994).

Despite large variations in gp120 sequence the 25 consensus *N*-linked glycosylation sites are well-conserved between strains. Mass spectrometric analysis of gp120 has indicated that oligomannose-type glycans are clustered around the C3/C4 base, whereas complex glycans are found on the V1/V2, V3, V4, and V5 domains (Zhu *et al.*, 2000).

1.4. Neutralizing Antibody 2G12

The antibody 2G12 is thought to bind to the silent outer domain of gp120. A previous study showed that loss of *N*-linked glycans around the C3/V4 stem prevented 2G12 binding (Trkola *et al.*, 1996). Additionally, mutations deleting the *N*-link coding sequences have been observed in escape mutants (Poignard *et al.*, 1999). These data were, however, ambiguous. 2G12 might either bind directly to gp120 carbohydrates, or it may bind to a protein epitope with a glycosylation dependent conformation. In this study, the potential contributions of both carbohydrate and protein to the 2G12 epitope were investigated. Site directed alanine-scanning mutagenesis showed that protein side chains contributed little to the specificity of the 2G12 epitope. Glycosidase digestion of gp120 glycans, mutation of *N*-linked coding sequences and alteration of the *N*-linked glycosylation pathway all reduced or abrogated the affinity of 2G12 for gp120. The binding of 2G12 to its epitope can be specifically inhibited by monosaccharides. From the data reported here, it appears that the 2G12 epitope requires $\alpha 1 \rightarrow 2$ linked D-mannose structures present within a cluster of *N*-linked oligomannose glycans on the silent outer face of gp120 (Scanlan *et al.*, 2002).

2. RESULTS

2.1. Alanine Scanning Mutagenesis of gp120_{JR-CSF} to Identify Residues Important for 2G12 Binding

Several mutations which disrupt attachment sites for *N*-linked carbohydrates on gp120 have previously been shown to reduce 2G12 binding to monomeric gp120 (Trkola *et al.*, 1996) significantly. However, no systematic mutagenesis analysis has been carried out, and the involvement of extensive regions of gp120 protein surface in the interaction with 2G12 cannot be excluded. Therefore, we decided to carry out extensive alanine scanning mutagenesis. We targeted amino acids predicted to be accessible to antibody from the

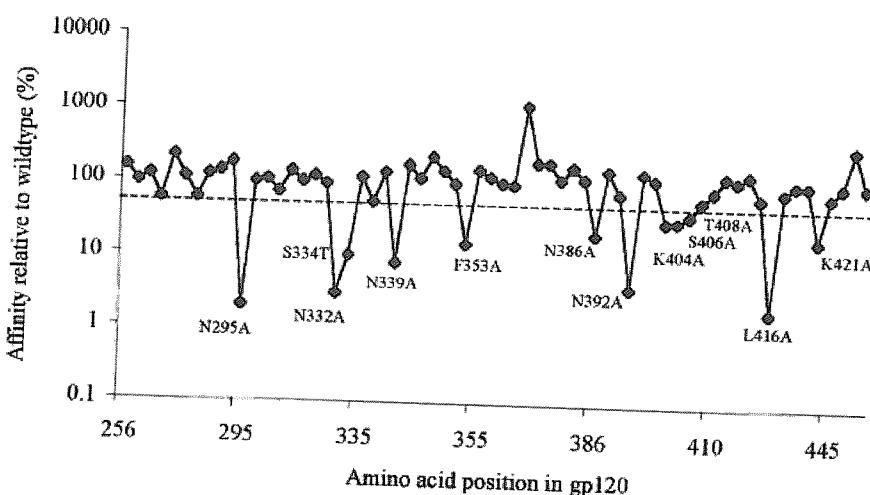


Figure 1. Apparent affinity of 2G12 for alanine mutants of gp 120_{JR-CSF} relative to that of parent gp 120_{JR-CSF}. HXB2 sequence numbering is used (Korber *et al.*, HIV Sequence Database, 2001 [<http://hiv-web.lanl.gov>]). The substitutions that caused a more than 50% (dashed line) reduction in apparent affinity are labeled.

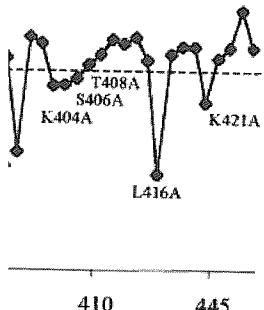
only available structures of gp120: those of core gp120 complexed to CD4 and the Fab fragment 17b (Kwong *et al.*, 1998, 2000).

Mutagenesis was carried out using gp120 from the isolate JR-CSF as the parent. Sixty-three single-amino-acid variants were generated; all of these substituted alanine for the amino acid in the parent gp120 with a small number of exceptions. Where alanine occurs in the parent gp120, it was substituted by lysine. In two cases where threonine and serine form part of an N-linked carbohydrate signal sequence (specifically, T297 and S334), they were substituted by serine and threonine, respectively, to maintain the signal (NXS/T) while altering the residue at these positions. Mutant monomeric gp120s from recombinant pseudovirions were captured onto ELISA wells and probed with various concentrations of 2G12 to generate a binding curve for each mutant. Apparent binding affinities were determined from the concentration of 2G12 at half-maximal binding. The apparent affinity of 2G12 for each mutant gp120 was then related to that for wild-type gp120.

The overwhelming majority of alanine substitutions in gp120 had a limited effect on 2G12 binding. Twelve amino acid substitutions resulted in significant decreases in 2G12 affinity. Five of these substitutions altered triplet sequence motifs (NXS/T) coding for potential N-linked glycosylation sites: N295A, N332A, N339A, N386A, and N392A (Figure 1). Other substitutions producing lowered affinity for 2G12 were on the silent face of gp120 (S334T, L416A), in the V4 loop (K404A, S406A, T408A), in the coreceptor binding site (K421A), and at the junction between the inner and outer domains of gp120 (F353A).

2.2. Conformation of gp120 Measured by IgG b12

For those mutants showing decreased affinities for 2G12, we also investigated binding of the human neutralizing antibody b12, which recognizes an epitope overlapping



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itive to that of parent gp 120_{JR-CSF-001} [http://hiv-web.lanl.gov]). The t affinity are labeled.

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the CD4 binding site (Burton *et al.*, 1994; Roben *et al.*, 1994). N295A and N332A mutants showed essentially unchanged b12 binding affinities (see also Table 2 in Scanlan *et al.*, 2002). However, N339A, N386A, and N392A mutants all displayed significantly lowered b12 affinities, implying that the substitutions may induce extensive misfolding or conformational perturbation. Therefore, the corresponding carbohydrate chains may not be involved in 2G12 binding. The retention of 2G12 binding by the mutant T388A, in which the carbohydrate signal sequence at position 386–388 is eliminated, suggests that the carbohydrate chain at N386 is indeed not involved in 2G12 binding.

To further investigate the importance of the carbohydrates at N339 and N392, N → Q substitutions were generated. The N339Q mutant bound 2G12 with an affinity similar to that of the parent gp120 and bound b12 with an enhanced affinity. This implies that the carbohydrate chain at position 339 may not be crucial for 2G12 binding but that substitution of Asn with Ala, although not with Gln, disrupts the conformation of the 2G12 (and b12) epitope. In contrast, an N392Q mutant, like the N392A mutant, bound 2G12 with considerably lower affinity than the parent gp120, but bound b12 with unchanged affinity. This is consistent with there being a supporting role for the carbohydrate chain at N392 in 2G12 binding.

Of the remaining mutants that showed decreased affinities for 2G12 relative to the parent gp120, four (S334T, L416A, E353A, and K421A) displayed a similar reduction in affinity for b12. The substitutions involved may, therefore, result in some disruption of global conformation or misfolding. Three substitutions in the V4 loop—K404A, S406A, and T408A—produced very modest decreases in the affinity of 2G12 for gp120 while maintaining b12 affinity.

For two amino acid substitutions, G458A and S365A, significant increases in both 2G12 and b12 binding affinities were observed. Both of these residues are located in the CD4 binding site of gp120. Mutations of these residues thus appears to lead to global conformational changes of gp120.

2.3. Mapping of Mutagenesis Results onto Model of gp120

Data from alanine scanning experiments were mapped onto the crystal structure of the complexed gp120 core of HIV-1_{HXB2} (Figure 2). This approach was thought to be valid as, although the mutagenesis studies used gp120_{JR-CSF}, the structure of the core seems to be highly conserved between isolates. One caveat is that the structure of gp120 is that of the core molecule complexed to CD4 and Fab 17b, and some differences between liganded and unliganded core gp120 have been proposed (Myszka *et al.*, 2000). The carbohydrates attached to N295, N332, N339, N386, and N392 lie on either side of the V3 and V4 loops of the outer face of gp120 (Figure 3). Previous site-specific analysis of N-linked glycosylation of gp120 revealed that oligomannose sugars are attached to these five asparagine residues (Zhu *et al.*, 2000).

The proximity of the carbohydrate chains to the V3 and V4 loops raises the question as to whether these loops could be involved in 2G12 binding. The very modest effect of a V3 deletion on 2G12 binding (67% of wild type affinity) argues against involvement of the V3 loop. Similarly, deletion of the V1 or V1/V2 regions has modest effects, suggesting that the V1/V2 loop does not significantly impact 2G12 binding. On the other hand, the modest decreases in affinity for 2G12 associated with substitutions in the V4 loop described above suggest that the V4 loop may have some role in 2G12 binding. A V4 loop-deleted

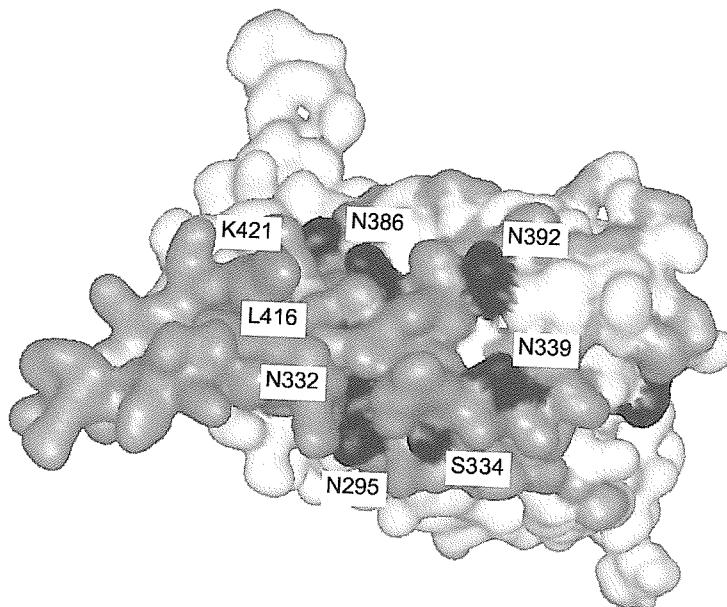


Figure 2. gp120 protein structure with amino acids colored to denote the effects of alanine substitutions on 2G12 affinity. The view shows the surface of the C4-V4 face of gp120. Coordinates were taken from the structure of the CD4-ligated core of gp120_{HXB2} (Kwong *et al.*, 2000). Mutations that did not cause a significant decrease in affinity are shown in gray, and those that caused a decrease in relative affinity are indicated in black. For clarity, the V4 loop has been omitted.

mutant was not generated, as previous studies revealed that the envelope of such a mutant is not processed and only gp160 can be immunoprecipitated from transfected cells. However, alignments of primary sequences of isolates that are effectively neutralized by 2G12 indicated little conservation of amino acid type or loop length between strains. Therefore, it seems unlikely that V4 loop residues are specifically involved in 2G12 binding.

2.4. Glycosidase Digestion of gp120 N-linked Glycans

Exoglycosidase and endoglycosidase digestion of the oligomannose glycans of monomeric gp120 gave further insight into the carbohydrate structures that might be required for the 2G12 epitope. Removal of mannose residues from gp120 by endoH, leaving only the core asparagine-linked GlcNAc, dramatically reduced the affinity of 2G12 for gp120. In contrast, the affinity of IgG1 b12 for gp120 was unaffected, indicating that endoH treatment does not have global conformational effects on gp120. Removal of either the Man α 1 → 2Man-linked residues or Man α 1 → 2,3,6Man-linked residues by linkage-specific mannosidases greatly reduced the affinities of both 2G12 and CVN for gp120, but not that of b12.

From these experiments, it appears that the epitope of 2G12 is either formed mainly from the outer Man α 1 → 2Man residues of oligomannose chains or also involves Man α 1 → 2,3,6Man residues in the context of Man α 1 → 2Man residues (for more details see Figures 5 and 6 in Scanlan *et al.*, 2002).

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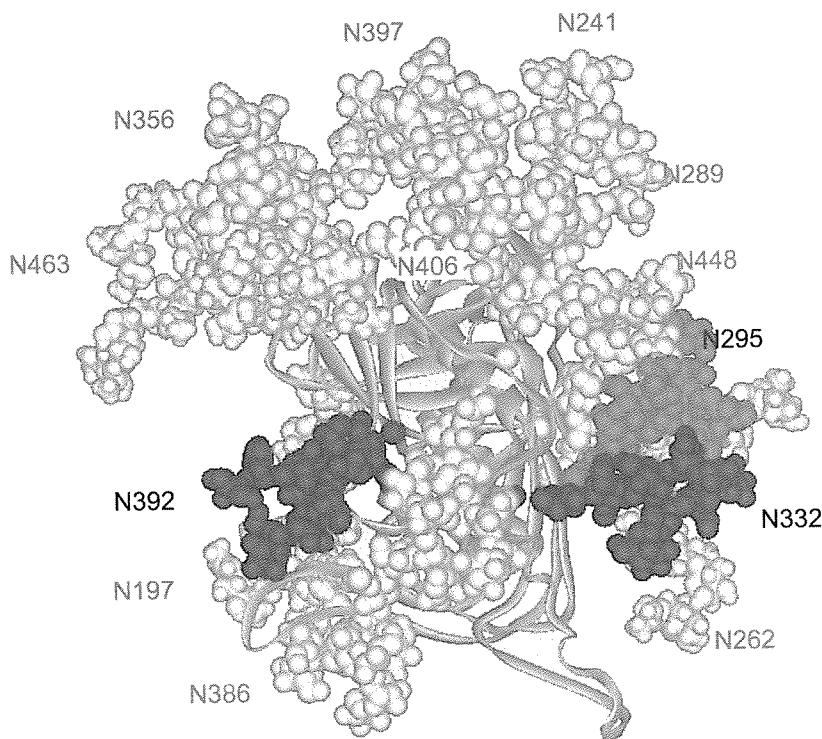


Figure 3. Model of gp120 showing the location of N-linked carbohydrates relevant to this study. The oligomannose-type carbohydrates attached to N332 and to N332 are strongly conserved amongst 2G12-sensitive strains (black). The carbohydrate attached to N295 (dark gray) is strongly implicated by mutagenesis as being essential for the 2G12 epitope. The remaining glycans are of both the complex (N463, N197, N262) and oligomannose (N356, N397, N242, N289, N448, N406, N386) types.

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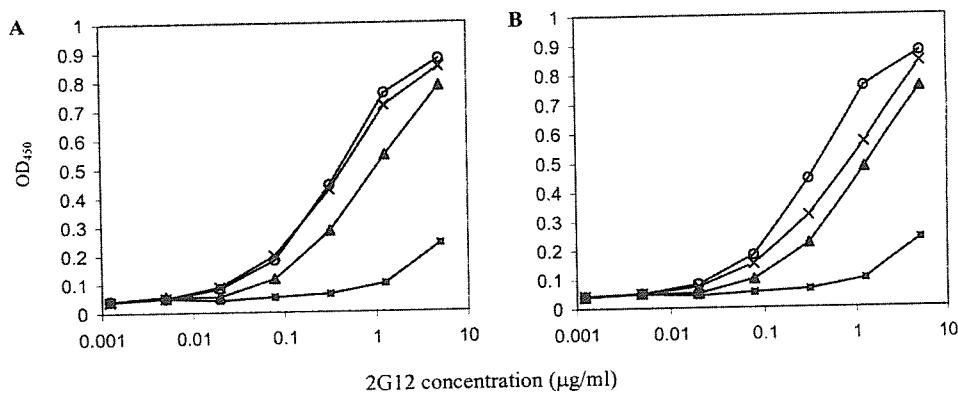


Figure 4. Monosaccharide inhibition of 2G12 binding to gp120. (A) Binding of 2G12 to gp120_{JR-FL} in the presence of 0 (○), 5 (×), 50 (▲), or 500 mM mannose (■). (B) Binding of 2G12 to gp120_{JR-FL} in the presence of 500 mM mannose (■), galactose (▲), N-acetylglucosamine (×), or buffer (○).

2.5. Carbohydrate Inhibition of the Interaction of 2G12 and gp120

The results frommannosidase digestion strongly suggest that mannose residues are involved in the 2G12 epitope. Consistent with this, high concentrations of D-mannose were able to inhibit the interaction of 2G12 and gp120. The specificity for the inhibition of 2G12 for gp120 appears to be restricted to mannose structures. A comparison of several monosaccharides shows that only mannose (Figure 4) and fructose (data not shown) significantly inhibits 2G12 binding (Figure 4).

3. DISCUSSION

The human MAb 2G12 is one of the few known broadly neutralizing anti-HIV-1 antibodies. Definition of its epitope at the molecular level may contribute to the design of an immunogen able to elicit 2G12-like antibodies. The antibody has previously been shown to bind an epitope on gp120 that is sensitive to changes in N-linked glycosylation and that does not overlap that of any other known antibody to gp120 (Moore and Sodroski, 1996). Here, site-directed mutagenesis and glycan modification of gp120 have been used to characterize the protein and carbohydrate contributions to the unique 2G12 binding site.

3.1. Mutagenesis of gp120

Alanine scanning mutagenesis showed that elimination of the N-linked carbohydrate attachment sequences associated with residues N295, N332, N339, N386, and N392 by N→A substitution produced significant decreases in 2G12 binding affinity to gp120_{JR-CSF}. The N295A and N332A substitutions had specific effects on 2G12 binding to gp120 in that binding of the anti-CD4 binding site antibody b12 was unaffected. In contrast, the N339A, N386A, and N392A substitutions also affected b12 binding, suggesting that they may produce conformational perturbation or protein misfolding, thus bringing into question the involvement of the carbohydrates at these positions in 2G12 binding. Indeed, the retention of 2G12 binding by a T388A mutant in which the carbohydrate attachment sequence at positions 386 to 388 was eliminated confirmed that the carbohydrate chain at N386 is not involved in 2G12 binding. The retention of 2G12 binding by an N339Q mutant similarly argued against the importance of the carbohydrate at N339 in 2G12 binding. Since an N392Q substitution significantly reduced 2G12 binding with little effect on b12 binding, the carbohydrate chain at N392 is likely to be important for 2G12 binding. We also noted that, whereas previous studies had suggested that the carbohydrate at N448 might be important in 2G12 binding, we found this conclusion unlikely for gp120_{JR-CSF} since a mutant (T450A) in which the carbohydrate signal sequence was eliminated at this position still bound 2G12. Therefore, the mutagenesis studies implicated carbohydrate chains at N295, N332, and N392 as most likely to be important in 2G12 binding. Previous site-specific analysis of gp120_{SF2} has shown that oligomannose chains are attached at these positions (Zhu *et al.*, 2000).

3.2. Sequence Alignments of 2G12 Sensitive Strains

Primary sequence comparisons of gp120s known to interact with 2G12 could help to illuminate the relative importance of the residues highlighted by the mutagenesis studies. A comparison of the primary sequences (HIV Sequence Database, 2001

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[<http://hiv-web.lanl.gov>] of gp120 from a panel of isolates efficiently neutralized by 2G12 showed that the N-linked carbohydrate signal sequences associated with N295, N332, and N392 are particularly highly conserved. In one of the isolates in which a carbohydrate signal sequence is lost at position 332, it is replaced by another one immediately adjacent. The conservation of the carbohydrate signal sequence associated with N339 is less pronounced than that at the other positions which is, again, consistent with the notion that the carbohydrate at this position is not as crucial for 2G12 binding.

3.3. Hypervariable Loops of gp120

The carbohydrate chains implicated in 2G12 recognition are close to both the V3 and V4 loops. Involvement of the V3 loop was excluded for gp120_{JR-CSF}, since deletion of the loop had minimal effect on affinity for 2G12. On the other hand, alanine substitutions in the V4 loop produced very modest decreases in 2G12 affinity. The variability of primary sequences in this region, among isolates neutralized by 2G12, argues against a direct role for the V4 loop in 2G12 binding. However, some role for the V4 loop in modulating or maintaining the 2G12 epitope is consistent with all the data. This is further supported by the observation that clade E isolates, most of which have an additional disulfide bond internal to the V4 loop, are not recognized by 2G12.

3.4. Investigation of the Carbohydrate Moiety of the 2G12 Epitope

Cleavage of specific mannose linkages was found to be sufficient to dramatically reduce 2G12 binding to gp120. EndoH treatment leaving only the core asparagine-linked GlcNAc, Jack Bean mannosidase treatment leaving Man₁GlcNAc₂, and *A. saitoi* mannosidase treatment leaving Man₅GlcNAc₂ structures were all effective in essentially eliminating 2G12 binding. *A. saitoi* mannosidase removes a single mannose from each of the D2 and D3 arms and two mannoses from the D1 arm of Man₉GlcNAc₂, suggesting that one or more of these residues is critical for 2G12 recognition.

Mannose inhibited 2G12 binding to gp120. In contrast, other hexoses did not significantly affect the binding of 2G12 to gp120 (Figure 4). This further indicates a direct interaction of the carbohydrate with the antibody combining site. The high concentrations of D-mannose required for the inhibition suggests that multiple mannose sub-units may be accommodated in the 2G12 paratope.

The requirement for specific mannose structures is consistent with the inhibition of 2G12 binding by CVN. CVN binds specifically to the Man₁ → 2Man termini of Man₉GlcNAc₂(D1D3) and Man₉GlcNAc₂.

Overall, therefore, our studies support a cluster of mannose residues contributed by upto three different oligomannose chains on the outer face of gp120 as being critical for 2G12 binding, while providing no indication of any direct involvement of protein side chains. The positioning of the epitope, a model of trimeric gp120 suggests that the attachment of 2G12 would not be hindered either by CD4, co-receptors or by other 2G12 molecules attached to adjacent monomeric units (Figure 5). The dense clustering of oligomannose residues, surrounding N295, N332, and N392 is evident. As previously noted (Kwong *et al.*, 2000), the only solvent exposed regions of gp120 not occluded by carbohydrate are the receptor binding sites, some portions of the hypervariable loops and the C and N terminus (which would be expected to covered by gp41).

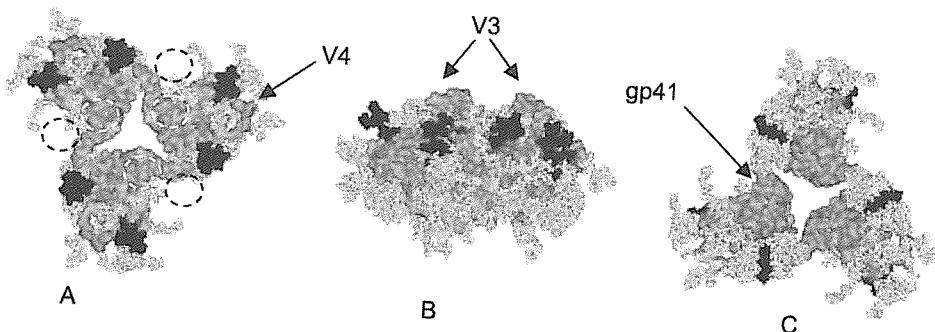
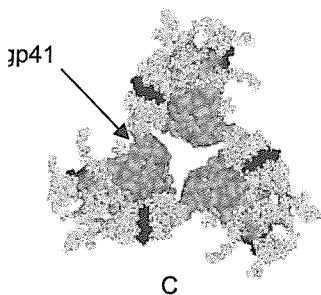


Figure 5. Positioning of the 2G12 epitope on a trimeric model of gp120. The glycans attached to N392, N332, and N295, and which are proposed to form the basis of the 2G12 interaction with gp120, are marked (black). The perspectives shown are from (A) the target cell membrane—looking towards the virus, from (B) the side, and from (C) the viral membrane. The approximate sites of CD4 binding (black broken circle), and the cellular co-receptor (white broken circle) are indicated.

3.5. 2G12 and Carbohydrate Recognition

Exclusive recognition of the carbohydrate of a glycoprotein by an antibody is unusual and raises a number of issues. First, such recognition might be thought to be excluded by tolerance mechanisms, as discussed earlier. However, the cluster of tightly packed oligomannose sugars in the region of the 2G12 epitope is unrepresentative of mammalian glycosylation and may, therefore, invoke an antibody response. There are few, if any, mammalian glycoproteins that are extensively mannosylated. Usually when mannose structures are present, they are mainly associated with one particular site, for example, CD2 (Wyss *et al.*, 1995), Thy-1 (Parekh *et al.*, 1987), and tissue plasminogen activator (Parekh *et al.*, 1998). Even when proteins contain oligomannose sugars at more than one site, a survey of N-glycosylated mammalian proteins in the protein database gave no examples of tightly packed oligomannose clusters such as those on gp120 (A. Petrescu and M. R. Wormald, unpublished data). One factor militating against heavy mannosylation of mammalian proteins is effective clearance by mannose receptors (Taylor and Drickamer, 1993; Weis *et al.*, 1998). Certain pathogens that are heavily mannosylated, such as yeast, are cleared rapidly by this mechanism (Neth *et al.*, 2000). The second unusual aspect of the 2G12–gp120 interaction is that 2G12 has a high affinity for gp120; typically, the K_d for the interaction is in the nanomolar range. However, the affinities of protein–carbohydrate interactions are generally much weaker (Thomas *et al.*, 2000; Wilson and Stanfield, 1995). Surprisingly, the high affinity is also observed for the Fab fragment of 2G12 (R. Pantophlet and D. R. Burton, unpublished data). One way in which higher affinity may be achieved is if multivalency is exercised within a single antibody combining region, that is, if the antibody, for instance, bridges between two or more “subsites” corresponding to mannose residues from different carbohydrate chains. The increase in affinity of proteins for carbohydrates achieved through multiple interactions between the sugar and the protein binding site has been discussed (Cygler *et al.*, 1991; Lee and Lee, 2000; Perkins *et al.*, 1981).

The oligomannose structures located at N295, N332, N339, N386, and N392 are clustered together with the asparagine residues within a 40 by 25 Å² area on the protein surface (Figure 3). N295 and N332 are located in close proximity to one another, as are N339,



The glycans attached to N392, N332, and N339, with gp120, are marked (black). The virus, from (B) the side, and lack broken circle), and the cellular

coprotein by an antibody is tion might be thought to be however, the cluster of tightly e is unrepresentative of mam- v response. There are few, if ated. Usually when mannose icular site, for example, CD2 asminogen activator (Parekh gars at more than one site, a atabase gave no examples of 20 (A. Petrescu and M. R. avy mannosylation of mam- Taylor and Drickamer, 1993; osylated, such as yeast, are second unusual aspect of the 120; typically, the K_d for the es of protein-carbohydrate Wilson and Stanfield, 1995). ent of 2G12 (R. Pantophlet affinity may be achieved is g region, that is, if the anti- corresponding to mannose finity of proteins for carbo- gar and the protein binding 9; Perkins *et al.*, 1981). N339, N386, and N392 are 25 Å² area on the protein to one another, as are N339,

N386, and N392. It appears that the glycan at N332 is likely to be very constrained, and the asparagine residue and the GlcNAc₂ (chitobiose) core have extensive interactions with the protein, while the glycan side chain is packed against the V3 loop and the glycan at N295. The mobility of the glycan at N295 is itself expected to be restricted by glycans at N448 and N262. The glycan at N339 is likely to be restricted in its mobility by interactions of the asparagine residue and the chitobiose core with the protein and of the glycan with the V3 and V4 loops. The presence of glycan at N392 may further constrain the glycan at N339. The glycans at N386 and N392 probably have extensive conformational freedom. The reduced dynamic freedom of the glycans on N295, N332, and N339 is not unprecedented (Garcia *et al.*, 1996; Wilson *et al.*, 1981), but it is in contrast to that suggested for a number of other glycoproteins studied (Wormald and Dwek, 1999). The positioning of these carbohydrates and their unusual packing is predicted to form the basis for the 2G12 epitope.

In general, sugar recognition by antibody combining sites involves three to four distinct subsites. The shape and topology of these subsites determine the specificity for a particular 3D arrangement of sugars. In the case of gp120, it is possible to construct a novel sugar epitope (i.e., nonself) from the tight clustering of the different oligomannose structures on the silent face of gp120.

3.6. Immunogenicity of the 2G12 Epitope

2G12 is a neutralizing anti-HIV antibody with a unique epitope requiring specific glycan structures. This requirement for epitope-glycosylation has implications for vaccine design in the light of the generally poor immunogenicity of carbohydrate antigens. The suitability of the silent face of gp120 as a template for vaccine design depends on both the ability of the human immune repertoire to form paratopes against glycosylated antigens and, more importantly, the probability of these arising through somatic mutation and clonal selection.

As discussed above, multivalency is required for high affinity antibody-carbohydrate binding. This is because the free energy gained from the accommodation of a hydroxyl group within a receptor is low. Therefore a B-cell, that acquires a mutation allowing the recognition of an additional hydroxyl by its BCR, may not gain a replicative advantage during clonal selection. The evolution of high avidity antibodies whose paratopes contain multiple low affinity sites is an improbable event and might be expected to arise infrequently during B-cell maturation, perhaps explaining why no other known antibodies bind to the "silent" face of HIV.

4. ACKNOWLEDGMENTS

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