Improved design of an antigen with enhanced specificity for the broadly HIV-neutralizing antibody b12

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In an attempt to design immunogens that elicit broadly HIV-neutralizing antibodies, we recently engineered monomeric HIV-1 gp120 to bind preferentially b12, a broadly neutralizing antibody to the CD4-binding site (CD4bs) on gp120, by mutating four central residues in the CD4bs to alanine and introducing extra N-glycosylation sites potentially to mask unwanted B-cell epitopes. Despite the favorable antigenicity of this mutant, it harbors two potential caveats that may limit its effectiveness to elicit b12-like antibodies: (i) b12-binding affinity is reduced relative to wild-type gp120 and (ii) binding of some non-neutralizing antibodies to the N-terminal C1 region of gp120 is still observed. Here, we sought to correct these potential limitations. By reverting one of the added N-glycosylation sites on the gp120 core, b12 binding was improved without affecting the epitope-masking properties of the original mutant. Furthermore, truncation of the gp120 N-terminus eliminated binding of the anti-C1 antibodies. Finally, based on the binding profiles of additional non-neutralizing antibodies tested here, further N-glycosylation sites were incorporated to mask their corresponding epitopes. The resulting hyperglycosylated gp120 variants bind b12 and another broadly neutralizing antibody, 2G12, with apparent affinities approaching that of wild-type gp120, but do not bind 21 non- or weakly neutralizing antibodies to seven different epitopes on gp120. These hyperglycosylated variants expand our panel of glycoengineered gp120s that are currently being evaluated for their ability to elicit broadly neutralizing antibodies.

Keywords: AIDS vaccine/antigen engineering/gp120/HIV-1 neutralizing antibodies

Introduction

Ever since human immunodeficiency virus type 1 (HIV-1) was identified as the cause of AIDS, investigators worldwide have been attempting to design an effective vaccine to stop the spread of the virus. The emerging consensus is that such a vaccine will probably need to confer both humoral and cellular immunity (Burton, 2002; Letvin et al., 2002; McMichael and Hanke, 2003). A few antigens have been described that are capable of inducing potent cellular immune responses that provide short-term reduction in viral loads during experimental HIV infection [see Barouch and Letvin (2002) and references therein]. In contrast, no antigens are currently available that can induce broadly protective antibody responses in simian models of HIV infection, although many do generate strong humoral responses upon immunization (Graham, 2002).

Design of an effective antigen that induces a neutralizing antibody response has been hampered by the fact that the virus has devised a number of ways to minimize antibody recognition of its surface envelope spikes, the major targets for any neutralizing antibody response. For example, antibodies are apparently largely incapable of accessing the gp41 transmembrane domain prior to binding of virus to target cells (Sattentau et al., 1995, 1999), presumably owing to masking by the bulky gp120 surface domain, which partially caps gp41. Adjacent gp41 protomers and the close proximity of gp41 to the viral membrane most likely also limit access for antibody–antigen recognition. Gp120 is itself extensively covered by a dense array of host-derived carbohydrates, which render the underlying protein surfaces largely non-immunogenic. Furthermore, a number of highly variable loops serve to direct immune responses away from conserved epitopes within the CD4-binding site (CD4bs) (Kwong et al., 1998, 2000; Wyatt and Sodroski, 1998; Wyatt et al., 1998). Another potential target for antibodies, the conserved coreceptor-binding site (Rizzuto et al., 1998; Rizzuto and Sodroski, 2000), is partially masked by variable loops prior to CD4 binding (Wu et al., 1996). The coreceptor binding site does become transiently exposed after conformational changes initiated by binding of gp120 to CD4 (Wu et al., 1996; Xiang et al., 2002), but it appears that spatial constraints resulting from binding of the virus to the target cell sterically hinder access of intact immunoglobulin molecules to epitopes within this region (Laabrijn et al., 2003).

Despite the plethora of viral defense mechanisms, a few broadly neutralizing monoclonal antibodies (mAbs) have been isolated that recognize conserved epitopes on gp41 and gp120. The two most potent, broadly neutralizing antibodies to gp120 are 2G12 and b12 (Burton et al., 2004). MAb 2G12 recognizes a conserved cluster of terminal mannose residues on the carbohydrate-covered face of gp120 (Trkola et al., 1996; Sanders et al., 2002; Scanlan et al., 2002). Interestingly, this antibody has a highly unusual domain-exchanged structure (Calarese et al., 2003), which enables it to recognize its multivalent carbohydrate epitope with high specificity and affinity (Calarese et al., 2003). This dense array of oligomannoses, which has not been found as such on any other host cell or host glycoprotein, may, therefore, be an exploitable target for vaccine design (Calarese et al., 2003; Lee et al., 2004; Li and Wang, 2004). The second mAb, b12, recognizes a conserved epitope that overlaps the CD4bs on gp120 (Burton et al., 1991; Roben et al., 1994). Because of its potency and broad neutralizing properties (Burton et al., 1994; D’Souza et al., 1997; Parren et al., 2001; Veazey et al., 2003), this antibody is considered a promising template for the design of an AIDS vaccine component aimed at the induction of highly cross-reactive antibodies with equivalent neutralizing properties (Poignard et al., 2001; Saphire et al., 2001; Burton, 2002).
In this study, we focused primarily on mAb b12. To gain insight into the molecular requirements for b12 binding and, by inference, on how to design an antigen that specifically induces broadly neutralizing anti-CD4bs antibodies, gp120 was previously subjected to extensive alanine-scanning mutagenesis to identify residues that are critical for antibody binding (Pantophlet et al., 2003a). Four residues (G473, D474, M475 and R476) on the outer edge of the so-called Phe43 cavity (Kwong et al., 1998; Wyatt et al., 1998) were identified that, when replaced with alanine, abrogated or reduced binding of several non-neutralizing CD4bs mAbs (Pantophlet et al., 2003a), whereas b12 binding was not affected. However, these alanine substitutions apparently did not affect binding of non- or weakly neutralizing antibodies to other gp120 epitopes. To inhibit binding of these antibodies, we adopted a strategy that could potentially dampen antibody responses in a selective manner (Garrity et al., 1997). We have dubbed this approach immunofocusing (Pantophlet and Burton, 2003). Consensus sequences for an N-glycosylation site with high probability of glycan incorporation (Gavel and von Heijne, 1990; Kasturi et al., 1997), Asn-Xaa-Thr (in which Xaa is any amino acid except Pro), were inserted into the gp120 sequence so as to incorporate glycan moieties at select positions on the envelope glycoprotein in order to mask non-neutralizing epitopes (Pantophlet et al., 2003b) (Figure 1). Incorporation of these additional glycans did indeed abolish binding of a broad panel of non- and weakly neutralizing anti-gp120 antibodies (Pantophlet et al., 2003b). Although b12 binding was preserved, the binding affinity relative to wild-type gp120 was reduced. Furthermore, binding was still observed with antibodies to epitopes in the N-terminus of gp120 (Pantophlet et al., 2003b).

It is unknown at present whether the reduced affinity of mAb b12 for the hyperglycosylated gp120 mutant translates into difficulties in eliciting b12-like neutralizing antibodies upon immunization. However, given that some non-neutralizing antibodies still bind the prototypic hyperglycosylated mutant, we reasoned that a variant with better antigenic properties, and one to which b12 is able to bind with an affinity equivalent to wild-type gp120, would probably improve the chances of eliciting such antibodies. We report here that we have, indeed, succeeded in engineering such a variant that, together with the mutants described previously, should permit a comprehensive assessment of this strategy as a
means selectively to divert and direct antibody responses and, hence, assess its potential as an immunogen to elicit broadly neutralizing antibodies.

**Materials and methods**

**Antibodies**

A total of 23 mAbs and one polyclonal immunoglobulin preparation (HIVIG) were tested. In addition to antibodies (n = 19) from our previous study (Pantophlet et al., 2003b), mAbs 39F and CO11 (kindly provided by J. Robinson) and mAb F425 B4e8 (kindly provided by L. Cavacini) were also used here. The latter three antibodies are V3-loop specific (Grundner et al., 2002; Cavacini et al., 2003). We also included mAb 8.22.2 (kindly provided by A. Pinter), which binds to the V2 loop of gp120 (He et al., 2002). These four mAbs neutralize primary HIV-1 isolates weakly (Grundner et al., 2002; He et al., 2002; Cavacini et al., 2003). Affinity-purified polyclonal antibody against the C5 region of gp120, which binds the same epitope (APTKAKRRVQREKR) at the extreme C-terminal region of gp120 as recognized by mAb D7324 (Wyatt et al., 1995), was purchased from Cliniqa (Fallbrook, CA).

**MAB biotinylation**

MAB 2G12 was biotinylated using N-hydroxysuccinimido-biotin (Sigma) according to the manufacturer’s instructions.

**Plasmids and mutagenesis**

The generation of plasmid pCMV-Tag4A-tpa was described in an earlier study (Pantophlet et al., 2003a). The plasmid was derived from plasmid pCMV-Tag4A (Stratagene) and contains a tissue plasminogen activator (tpa) leader sequence for extracellular expression of recombinant proteins. The generation of plasmid pCMV-Tag4A-tpaJR_fgp120-mCHO-GDMR, which encodes the hyperglycosylated gp120 mutant that was previously termed mCHO*-GDMR and is now designated mCHO-GDMR, has been described recently (Pantophlet et al., 2002, 2003b). Briefly, glycoproteins were captured on ELISA plate wells using the anti-C5 polyclonal antibody preparation, unless indicated otherwise. Antibodies against CD4-induced epitopes were tested in the absence of soluble CD4. MAB 2G12, or in some cases cyano‐vinir (CVN) (Boyd et al., 1997), was used to ensure that similar amounts of envelope proteins were captured in each experiment. In general, plates were developed with p-nitrophenyl phosphate (Sigma) and absorbance was measured at 405 nm. When peroxidase-conjugated secondary antibody (Pierce) was used, plates were developed with 3,3’,5,5’ tetramethyl benzidine/hydrogen peroxide substrate (TMB/H2O2; Pierce) and absorbances measured at 450 nm. In this case, the color reaction was stopped with sulfuric acid (2 M) prior to spectrophotometric measurement. For detection of biotinylated 2G12, peroxidase-conjugated avidin (Pierce) was used in combination with the TMB system. All assays were performed in duplicate. Apparent binding affinities were calculated as the antibody concentration at half-maximal binding.

**Results**

**Improving b12-binding affinity to mutant mCHO-GDMR by reverting newly added N-glycosylation sites on the gp120 core to wild-type sequence**

In a recent study (Pantophlet et al., 2003b), we reported on a gp120 mutant, termed mCHO-GDMR, containing four alanine-substituted residues (G473A, D474A, M475A and R476A) at the center of the CD4-binding site and seven extra N-glycosylation sites, which were incorporated to mask non-neutralizing epitopes with carbohydrates. Mutant mCHO-GDMR and variants thereof are of particular interest for immunogenicity studies because most non-neutralizing mAbs do not bind this antigen, whereas the broadly neutralizing antibody b12 can still bind (Pantophlet et al., 2003b). However, despite the unique antigenic properties of mutant mCHO-GDMR, the efficacy of this antigen to induce b12-like antibodies may be restricted because b12 binds this mutant with an apparent affinity that is lower than the corresponding affinity for wild-type gp120 and also because binding of a few non-neutralizing antibodies to mutant mCHO-GDMR was still observed (Pantophlet et al., 2003b). In the present study, we sought to correct these potential limitations to improve the antigenic quality of this mutant as a b12-tailored antigen.

First, added N-glycans that might potentially be involved in reducing the binding of b12 to mutant mCHO-GDMR were identified by reverting the newly introduced glycosylation sites on the gp120 core to wild-type sequence (see Figure 1); glycosylation sites on the core were reverted first because the inserted glycans are relatively close to the putative b12-binding site on gp120 (Saphire et al., 2001). Reverting
the glycosylation signal sequence inserted at positions 92–94 to wild-type sequence (designated variant H92Nx; in this study, the suffix ‘x’ denotes the reversion of an added glycosylation site to wild-type sequence), which lies closest to the putative b12 epitope, resulted in a slight increase in b12-binding affinity relative to mutant mCHO-GDMR but did not improve b12 binding affinity to wild-type levels (Figure 2). To determine whether reverting additional N-glycosylation signal sequences might further rescue b12-binding without loss of the epitope-masking properties of mutant mCHO-GDMR, we tested a small panel of non- or weakly neutralizing CD4bs antibodies and mAb b12 for binding to glycosylation site variants of mutant mCHO-GDMR. We noted that reversion of additional glycosylation sites on the gp120 core did not improve b12 affinity further (Figure 3). Reassuringly, however, reversion of the added N-glycan attachment site at position 92 did not lead to enhanced binding of non-neutralizing anti-CD4bs antibodies tested here (Figure 3A). Significantly increased binding was also not observed for these non-neutralizing CD4bs antibodies with a variant in which the added glycosylation sites at positions 92 and 114 were simultaneously reverted to wild-type sequence (Figure 3B). However, when all three glycosylation sites surrounding the CD4bs were reverted to wild-type sequence (variant H92Nx/Q114Nx/I423Nx), binding of mAbs 15e and F91 was no longer completely abolished (Figure 3C). Given that reversion of the N-glycosylation site at position 92 allowed better b12 binding without affecting the epitope masking properties of the parental mutant, this variant, designated H92Nx, was selected as a template for further mutagenesis.

Definition of epitopes masked by added glycans in mutant mCHO-GDMR

To identify regions on gp120 that might be masked by the incorporation of additional glycans on to gp120, we tested mAbs to the V2 loop, to CD4-induced (CD4i) epitopes and to the V3 loop with variants of mutant mCHO-GDMR in which added glycosylation signal sequences were reverted to wild-type sequence. Of the two anti-V2 loop antibodies tested here,
mAbs 8.22.2 and G3-4, only the former bound the variant (K171Nx) in which the glycosylation site in the V2 loop was reverted to wild-type sequence (Figure 4A). This result suggested that one or more glycans that had been introduced elsewhere contributed to steric hindrance of mAb G3-4 to the V2 loop. To investigate further, we generated two additional variants, E150Nx/K171Nx and P313Nx/K171Nx, in which the glycosylation sites that were inserted in the V1 loop (E150N) and the V3 loop (P313N), respectively, were reverted to wild-type sequence in the background of the K171Nx variant. We observed that by reverting the added glycosylation site at position 150, the apparent binding affinity of mAb G3-4 was improved, whereas reverting the glycosylation site in the V3 loop did not enhance G3-4 binding (Figure 4B). However, binding of G3-4 to variant E150Nx/K171Nx still did not achieve wild-type levels, and an explanation for this effect is not readily apparent.

For mAbs 17b, 48d and X5, which recognize CD4i epitopes overlapping the coreceptor-binding site on gp120 and bind weakly to wild-type gp120 in the absence of soluble CD4 (Figure 5A), unexpectedly we did not observe any binding to a variant (I423Nx) in which the added N-glycosylation site at position 423 in the coreceptor-binding site was reverted to wild-type sequence in the background of mutant mCHO-GDMR (Figure 5B). Considering the close proximity of CD4i antibodies to the V3 loop (Kwong et al., 1998; Wyatt et al., 1998) and the observation that some V3 loop antibodies inhibit binding of CD4i antibodies to gp120 (Moore and Sodroski, 1996), we next tested binding of mAbs 17b, 48d and X5 to another variant, P313Nx, in which the extra glycosylation site inserted into the apex of the V3 loop was reverted to wild-type sequence. The CD4i antibodies were also unable to bind this
variant (data not shown). Given that a Met→Ser substitution at position 475 and an Asp→Val substitution at position 477 negatively affect 17b binding (Thali et al., 1993), we next tested the CD4i antibodies with mutant GDMR, in which residues at positions 473–476 at the center of the CD4bs are substituted by alanine (Pantophlet et al., 2003a). Surprisingly, all three antibodies were unable to bind mutant GDMR (Figure 5C), thus indicating the reason for their inability to bind mutant mCHO-GDMR. Notably, the mAbs also failed to bind mutant DR, in which only Asp474 and Arg476 were mutated to alanine (Figure 5D). These observations suggest not only that Asp474 and Arg476 play critical roles in the binding of several non-neutralizing CD4bs antibodies to gp120 (Pantophlet et al., 2003a), but also that these two residues influence binding of CD4i antibodies to gp120. In the absence of sCD4, these mAbs are able to bind wild-type gp120 weakly (Moulard et al., 2002; Xiang et al., 2002), indicating that gp120 is able to assume a ‘CD4-induced’-like conformation in the absence of CD4; the remote location of the binding sites of mAbs 17b, 48d and X5 relative to residues Asp474 and Arg476 (Kwong et al., 1998, 2000) suggests that the propensity of gp120 to adopt a CD4i-like conformational state may be allosterically inhibited as a result of the alanine substitutions.

For the anti-V3 loop mAbs, reversion of the glycosylation site at position 313 at the apex of the V3 loop (variant P313Nx) restored binding of the anti-V3 loop mAb 447–52D, in addition to mAb CO11 (Figure 6A). The latter mAb was not tested in our previous study (Pantophlet et al., 2003b), but in these subsequent analyses did not bind mutant mCHO-GDMR (not shown). For both antibodies, replacement of either the Pro residue at position 313 by Asn in the background of wild-type gp120 (mutant P313N-2) or the Arg residue at position 315 by Thr in the background of wild-type gp120 (mutant R315T) was sufficient to abolish binding (Figure 6B and C). Hence incorporation of a glycan at the apex of the V3 loop was not necessarily required to block reactivity of these particular anti-V3 loop mAbs, as point mutations of conserved residues in the apex of the V3 loop were sufficient to block binding.

Abolition of binding of anti-C1 antibodies to hyperglycosylated gp120

Although removal of the glycosylation site at position 92 improved b12-binding affinity relative to the parental hyperglycosylated mutant mCHO-GDMR, antibodies to the C1 region of gp120 were still reactive with this variant (not shown). To abolish binding of these antibodies, the N-terminus of variant H92Nx was truncated up to residue E83, which is analogous to the truncated gp120 construct used for crystallization of the gp120 core (Kwong et al., 1998, 2000). We reasoned that truncation of the N-terminus would allow for more efficient elimination of several epitopes at once, rather than introducing multiple glycosylation sites in an attempt to eliminate antibody binding to a likely highly flexible N-terminus (Kwong et al., 1999). ELISA experiments showed that the anti-C1 antibodies tested were indeed not able to bind the variant with the truncated N-terminus, designated ΔN-mCHO-GDMR (Figure 7A). More importantly, b12 binding was not affected by truncation of the N-terminus. However, as seen previously with mutant mCHO-GDMR (Pantophlet et al., 2003b), truncation of both N- and C-termini severely diminished b12 binding (Figure 7B).

Improving the antigenic properties of hyperglycosylated gp120 by incorporating additional N-glycans

To evaluate the antigenicity of hyperglycosylated variant ΔN-mCHO-GDMR, we tested a panel of 23 mAbs and a polyclonal immunoglobulin preparation by ELISA (Figure 8). Included in this panel were two mAbs, 39F and F425 B4e8, which had not been tested previously with the parental mutant mCHO-GDMR or with any of the variants described so far. Both of these antibodies have been reported to recognize epitopes at the base of the V3 loop (Grundner et al., 2002; Cavacini et al., 2003; J.Robinson, personal communication). Only four antibodies were able to bind variant ΔN-mCHO-GDMR: mAbs 2G12, b12, 39F and F425 B4e8. The fact that the last two V3-loop antibodies were able to bind this variant suggested that the glycan incorporated at position 313 in the apex of the V3 loop was not sufficient to obscure the base of the loop. To eliminate binding of these antibodies, a series of additional hyperglycosylated variants were generated, in which glycosylation signal sequences were added sequentially to the base of the loop. Glycosylation sites were first introduced simultaneously at positions 320 and 325, which are both located at the C-terminus of the V3 loop and then in the V3 loop N-terminal region at residue 306. Binding of
mAb F425 B4e8 was diminished by the first two substitutions in the C-terminal end of the base (Figure 9A). Surprisingly, 39F binding was not significantly affected, even when a glycosylation site at position 306 was incorporated (Figure 9B). However, substituting residues at positions 304 (Arg) and 305 (Lys) with Ala completely abolished 39F binding (Figure 9C). These two substitutions also further reduced binding of F425 B4e8 compared with the hyperglycosylated variant containing only the three added glycosylation sites in the V3 loop, suggesting that this antibody may also be interacting with or affected by N-terminal residues at the base of the V3 loop. Taking into consideration that the single glycan at the apex of V3 was insufficient to prevent binding of mAbs directed to the base of the loop and given that the V2 loop in our hyperglycosylated variants contained only one extra glycan (at position 171), a second N-glycosylation site was inserted in the V2 loop at position 180 in variant ΔN-mCHO-GDMR containing the additional modifications in V3. The resulting variant was designated ΔN2-mCHO-GDMR. Introduction of the additional glycosylation site in V2 had no effect on b12 binding (data not shown).

Comparison of the apparent binding affinity of mAb b12 for wild-type gp120 and hyperglycosylated gp120 variants

Given the superior antigenic properties of variant ΔN2-mCHO-GDMR relative to the parental hyperglycosylated mutant mCHO-GDMR, we next compared b12 affinity for our hyperglycosylated variants with that of the parental mutant and wild-type gp120, to determine whether we had also succeeded in improving b12 binding relative to the original mutant (Figure 10). As reported previously, the apparent b12 binding affinity was 5-fold lower for mutant mCHO-GDMR than for

Fig. 7. Binding of biotinylated mAb 2G12 to hyperglycosylated variant ΔN-mCHO-GDMR (A), in which the gp120 N-terminus has been truncated up to residue E83 and variant ΔN/C-mCHO-GDMR (B), in which the C-terminus of variant ΔN-mCHO-GDMR has additionally been truncated after residue 492. Glycoproteins were captured on microtiter plate wells with the anti-C1 mAbs 133/237 and 522-149 and with mAb b12 at concentrations indicated on the x-axis.

Fig. 8. Binding of anti-gp120 mAbs to variant ΔN-mCHO-GDMR. Antibody binding curves are color coded according to the gp120 epitope recognized by the respective mAb. Black, mAbs to the C1, C5, C1/C4 and C1/C5 domains; cyan, mAbs to CD4-induced epitopes; red, mAbs to epitopes overlapping the CD4bs; green, anti-V2 and anti-V3 loop mAbs; blue, mAb 2G12 to the carbohydrate-covered silent face of gp120 (Wyatt et al., 1998); pink, polyclonal antibody preparation (HIVIG). Owing to the inability of several mAbs to bind variant ΔN-mCHO-GDMR, not all antibody-binding curves are visible in the graph.
wild-type gp120 (Pantophlet et al., 2003b). In comparison, the apparent antibody affinity for variant ΔN2-mCHO-GDMR was equivalent to that for wild-type gp120. For variant ΔN2-mCHO-GDMR, a slight decrease in b12 affinity was observed relative to wild-type gp120. However, b12 affinity for variant ΔN2-mCHO-GDMR was higher than the antibody affinity for the parental mutant, indicating that, in addition to enhancing the antigenic qualities of the original mutant, we had also succeeded in improving b12 binding.

**Discussion**

A major problem in AIDS vaccine development has been the inability to design immunogens that elicit high titers of antibodies to neutralizing epitopes on the HIV envelope glycoproteins, gp120 and gp41. For gp120, it has been postulated that this problem may be associated, at least in part, with the overwhelming exposure of non-neutralizing epitopes on monomeric gp120 when used as an immunogen and, consequently, skewing of the antibody response to these ‘decoy’ epitopes (Wyatt and Sodroski, 1998; Poignard et al., 2001). In an attempt to address this problem from a perspective that is fundamentally different from other current approaches, we sought to engineer monomeric gp120 in such a way as to reduce its capacity to induce non-neutralizing antibodies, by insertion of extra N-glycans over a range of sites in order to reduce the immunogenicity of unwanted, non-neutralizing epitopes on the glycoprotein. We monitored this desired antigenicity by comparing the ability of the engineered mutants to bind to a large set of non-neutralizing mAbs and the broadly neutralizing antibody b12 (Pantophlet et al., 2003b). Although our prototypic hyperglycosylated mutant, mCHO-GDMR, abolished binding of many non-neutralizing antibodies (Pantophlet et al., 2003b), the reduced binding of b12 for this mutant relative to wild-type gp120, combined with the failure to abolish binding of all non-neutralizing antibodies, could potentially restrict the efficacy of this mutant to elicit b12-like antibodies. The aim of the present study was, therefore, to (i) improve the binding affinity of b12 for the hyperglycosylated mutant and (ii) enhance the desired antigenic properties of the mutant.

To address the first goal, the added glycosylation sites on the gp120 core in mutant mCHO-GDMR were reverted to wild-type sequence. Reverting a single glycosylation site, at position 92, was sufficient to enhance b12 affinity relative to its affinity for the parental hyperglycosylated mutant (Figure 2). Notably, removal of the other two added N-glycan attachment sites on the gp120 core at positions 114 and 423, combined with the removal of the added glycosylation site at position 92, did not improve b12 binding further (Figure 3). Hence the decreased b12 affinity for mutant mCHO-GDMR appears to be due either to removal of an antibody contact residue as a result of the introduced glycosylation site, to localized conformational changes surrounding the CD4bs associated with the mutations needed to introduce the glycosylation site or through incorporation of the glycan itself, which may hinder b12 binding. Although it is not obvious at present which of these three possibilities is most likely, results from a previous study suggest that alterations in this region may negatively affect b12 binding (Pantophlet et al., 2003a). For two other anti-CD4bs antibodies, 15e and F91, binding was nearly fully restored only when all three added glycosylation sites on the gp120 core were reverted simultaneously to wild-type sequence, but not when the sites were reverted individually (Figure 3 and data not shown). As it seems unlikely that all three glycosylation sites harbor antibody contact residues, the most likely explanation in this case for the observed increase in...
Antigen Binding of non-neutralizing virion spike (Kwong observations substantiate the generally accepted notion that the position 150 in the V1 loop was reverted to wild-type sequence, (Figure 4A). However, when the extra glycosylation site at position 150 in the V1 loop was reverted to wild-type sequence, G3-4 binding affinity for gp120 improved (Figure 4B). These observations substantiate the generally accepted notion that the V1 and V2 loops are in close proximity to each other on monomeric gp120 and probably even more so on the functional virion spike (Kwong et al., 1998; Wyatt et al., 1998). The second anti-V2 loop antibody, mAb 8.22.2, was able to bind the variant with the reverted V2-glycosylation site, indicating that the masking of neighboring epitopes by the added glycans may in some cases only be partial.

In contrast, the observed inhibition of binding of mAbs CO11 and 447-52D to mutant mCHO-GDMR appears not to be due solely to the presence of the added glycans at position 313, but to substitution of critical antibody contact residues in the apex of the loop, in particular for mAb 447-52D (Gorny et al., 1997; Stanfield et al., 2004). Whether this is also true for mAb CO11 is currently not assessable because the epitope recognized by this antibody has not been mapped. In addition to substituting crucial antibody contact residues, mutation of residues P313 and/or R315 may also cause an alteration in the type I and II β-turns that are characteristic of the apex of the V3 loop and are required for proper presentation of the antibody epitopes (Stanfield et al., 2003). We make particular note here of the specific elimination of the 447-52D epitope. This antibody neutralizes ~50% of clade B isolates (Binley et al., 2004) and is therefore considered a somewhat broader neutralizing antibody than other ‘typical’ V3 loop antibodies with only limited neutralizing capacities (Kwong, 2004). However, we can accept the loss of 447-52D binding in the context of our hyperglycosylation strategy, given that our goal is to obtain b12-like antibodies which, ideally, would have broader cross-clade neutralizing properties than those exhibited by mAb 447-52D.

Having improved binding of b12 relative to mutant mCHO-GDMR, we next sought to improve the antigenicity of the hyperglycosylated variant H92Nx. First, we truncated the N-terminus of gp120 up to residue E83 to abolish binding of non-neutralizing anti-C1 antibodies (Figure 7). Binding of b12 to the resulting variant, designated ΔN-mCHO-GDMR, was not negatively affected. However, b12 binding was severely reduced when both N- and C-termini were truncated. Although we have not explored whether truncation of the C-terminus alone affects b12 binding, the above results indicate that the C-terminal region of gp120 may play a role in the conformational integrity of the b12 epitope. ELISA experiments with a panel of more than 20 antibodies showed that variant ΔN-mCHO-GDMR had not lost the epitope-masking properties of the parental mutant (Figure 8).

However, two anti-V3 loop antibodies, F425 B4e8 and 39F, which had not been tested previously, were able to bind variant ΔN-mCHO-GDMR. Hence the antigenic properties of this variant needed to be improved further, given that the single glycan incorporated in the apex of the V3 loop was insufficient to mask the entire loop. Similar observations have been made in a previous study (Garrity et al., 1997) in which immunogenicity studies showed that incorporation of a glycan on the C-terminal side of V3 caused a shift in antibody response to the N-terminal side of the loop, whereas the opposite effect was observed when a glycan was incorporated at the N-terminal side. Such findings illustrate the remarkable inherent antigenicity and immunogenicity of the V3 loop. To address this problem here, we inserted further N-glycosylation sites at the base of the V3 loop to mask the putative epitopes of antibodies F425 B4e8 and 39F. Although this strategy proved successful for F425 B4e8, two alanine substitutions, R304A and K305A, were required in addition to the incorporated glycans to eliminate binding of mAb 39F (Figure 9).

We also inserted an additional glycosylation site in the V2 loop, at position 180, to mask epitopes that might not be covered by the single glycan at position 171 that was incorporated into the parental mutant. We termed our newly generated hyperglycosylated variant ΔN2-mCHO-GDMR. This variant only binds the broadly neutralizing mAbs b12 and 2G12 and a monospecific antibody preparation against the extreme C-terminal region of gp120 (Table I). Although one could argue that the C-terminus may also elicit unwanted non-neutralizing antibodies, essentially all antibodies to the C5 region that have been characterized to date appear to preferentially bind denatured gp120 (http://www.hiv.lanl.gov/content/immunology/index.html), indicating that anti-C5 antibodies may not be elicited readily when immunizations are performed with native gp120 formulated in a non-denaturing adjuvant preparation.

Hence we have engineered a hyperglycosylated variant of gp120, designated ΔN2-mCHO-GDMR, to which the broadly

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<tr>
<td>ΔN2-mCHO-GDMR</td>
<td>No</td>
<td>–</td>
<td>75</td>
</tr>
</tbody>
</table>

<sup>a</sup>Apparent affinities were calculated as the antibody concentration at half-maximal binding. Apparent affinities relative to those for wild-type gp120 were calculated with the formula (apparent affinity for the wild type/apparent affinity for the mutant) × 100.

<sup>b</sup>Only certain anti-C1 mAbs are able to bind mutant mCHO-GDMR.

<sup>c</sup>Binding only observed for mAbs to epitopes at the base of the V3 loop.
HIV-1-neutralizing mAb b12 binds with nearly 3-fold better apparent affinity than the previously reported parental mutant mCHO-GDMR (Figure 10). Immunogenicity studies are currently ongoing for mutant mCHO-GDMR and previously described variants thereof and we plan to report on those shortly (S.Selvarajah, R.Pantophlet, B.Puffer, R.W.Doms and D.R.Burton, unpublished data). The superior antigenic properties of variant AN2-mCHO-GDMR make this antigen a potentially more interesting candidate for similar immunogenicity studies. Because of the uncertainty as to the effect of the four alanine-substituted residues at the center of the CD4bs—the so-called GDMR mutation—on antibody responses, we also plan to immunize with variants in which these residues are unaltered. These studies will, then, reveal whether our attempt to focus the antibody response on key cross-neutralizing epitopes by hyperglycosylating gp120 is applicable to the rational design of an AIDS vaccine.

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References


