Polysaccharide mimicry of the epitope of the broadly neutralizing anti-HIV antibody, 2G12, induces enhanced antibody responses to self oligomannose glycans

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Immunologically, “self” carbohydrates protect the HIV-1 surface glycoprotein, gp120, from antibody recognition. However, one broadly neutralizing antibody, 2G12, neutralizes primary viral isolates by direct recognition of Manα1→2Man motifs formed by the host-derived oligomannose glycans of the viral envelope. Immunogens, capable of eliciting antibodies of similar specificity to 2G12, are therefore candidates for HIV/AIDS vaccine development. In this context, it is known that the yeast mannann polysaccharides exhibit significant antigenic mimicry with the glycans of HIV-1. Here, we report that modulation of yeast polysaccharide biosynthesis directly controls the molecular specificity of cross-reactive antibodies to self oligomannose glycans. Saccharomyces cerevisiae mannans are typically terminated by α1→3-linked mannoses that cap a Manα1→2Man motif that otherwise closely resembles the part of the oligomannose epitope recognized by 2G12. Immunization with S. cerevisiae deficient for the α1→3 mannosyltransferase gene (ΔMnn1), but not with wild-type S. cerevisiae, reproducibly elicited antibodies to the self oligomannose glycans. Carbohydrate microarray analysis of ΔMnn1 immune sera revealed fine carbohydrate specificity to Manα1→2Man units, closely matching that of 2G12. These specificities were further corroborated by enzyme-linked immunosorbent assay with chemically defined glycoforms of gp120. These antibodies exhibited remarkable similarity in the carbohydrate specificity to 2G12 and displayed statistically significant, albeit extremely weak, neutralization of HIV-1 compared to control immune sera. These data confirm the Manα1→2Man motif as the primary carbohydrate neutralization determinant of HIV-1 and show that the genetic modulation of microbial polysaccharides is a route towards immunogens capable of eliciting antibody responses to the glycans of HIV-1.

Keywords: 2G12/glycan array/GnT I-deficient HEK 293S/human immunodeficiency virus/kifunensine/oligomannose/vaccine/yeast

Introduction

Neutralizing antibodies are a dominant protective component of most, if not all, prophylactic vaccines. In the context of HIV-1, it has been established that naturally occurring, neutralizing antibodies exert a significant selection pressure on established infection (Frost et al. 2005) and that passive transfer of neutralizing antibodies provides sterilizing immunity to viral challenge in animal models of infection (Mascola et al. 2000; Hessell, Poignard, et al. 2009; Hessell, Rakasz, et al. 2009). However, neutralizing antibodies elicited during infection or by vaccination are generally directed to subtype-specific epitopes (Parren et al. 1997; Parren et al. 1999; Wei et al. 2003; Burton et al. 2005; Frost et al. 2005; Deeks et al. 2006). These narrowly neutralizing antibodies are unable to neutralize closely related escape variants and hence fail to contain infection. For the same reason, most antibodies generated by vaccination fail to provide protection against heterologous primary viral challenge (Pantophlet and Burton 2006). The antigenic diversity of HIV remains a fundamental barrier to the design of a prophylactic vaccine for HIV/AIDS.

In contrast to the subtype specificity of most neutralizing antibodies, a small group of broadly neutralizing antibodies (BNAbs) have been described that are each capable of neutralizing a wide range of circulating HIV isolates (Burton et al. 2005; Walker et al. 2009). The conserved elements defined by BNAbs are therefore under active evaluation as components of novel HIV immunogens, an epitope-based vaccine design process described as “reverse vaccinology” (Burton 2002; Burton et al. 2004). One such broadly neutralizing antibody is IgG 2G12, which recognizes a cluster of Manα1→2Manα1→2Man residues formed by the oligomannose glycans on the outer domain of the envelope glycoprotein, gp120 (Sanders et al. 2002; Scanlan et al. 2002; Calarese et al. 2003). In addi-
tion to this canonical motif, further modes of binding have been proposed (Sanders et al. 2002; Calarese et al. 2005) and have informed vaccine development (Dudkin et al. 2004; Lee et al. 2004).

Epitope-based vaccine design first requires the characterization of the antigenic structure of a broadly neutralizing epitope, then the synthesis/discovery of an immunogen that presents this epitope as part of a vaccine. Central to this concept of reverse vaccinology is the relationship between the epitope recognized by a broadly neutralizing antibody and that epitope’s ability to elicit similar antibodies. However, any such relationship (formally between “antigenicity” and “immunogenicity”) is unlikely to be simple, as the native antigen for a broadly neutralizing antibody is, almost by definition, unable to normally elicit such antibodies. This troubled relationship between broadly neutralizing antibodies and their epitopes may also be reflected in their unusual structures and/or modes of binding: If immunological solutions to these epitopes were routinely generated, the epitopes would be selected against during natural infection and would not be broadly conserved. In the case of 2G12, the F(ab’)2 has evolved to adopt a highly mutated, domain-exchanged configuration so as to engage its constrained carbohydrate epitope (Calarese et al. 2003).

Given the poor immunogenicity of the native carbohydrates of gp120, we have previously argued that the search for an immunogen capable of eliciting antibodies to the 2G12 epitope should include structures which also contain the Manα1→2Man motif but in a more immunogenic format (Scanlan, Offer, et al. 2007; Scanlan, Ritchie, et al. 2007). Support for this approach comes from the consideration of immune responses to self glycans generated in response to self-mimicking microbial carbohydrates (Willison 2005; Scanlan, Offer, et al. 2007; Scanlan, Ritchie, et al. 2007). In this context, we have reported that, in addition to its known epitope on gp120, 2G12 also binds selectively to yeast mannans which display extended arrays of the (Manα1→2Man)n motif, branching from a repeating Manα1→6Man backbone (Dunlop et al. 2008). Moreover, it has previously been reported that antibodies, generated to such yeast mannans, can cross react with the mannose residues found on the HIV-1 envelope in a serotype-dependent manner (Muller et al. 1991; Tomiyama et al. 1991). Together, these results provide some support for the existence of an overlap between the motif recognized by a broadly neutralizing antibody and that motif’s ability to reliably elicit specific antibodies to HIV carbohydrates. Further support for this strategy comes from the identification of self ligands capable of binding 2G12 which elicit Manα1→2Man-specific antibodies (Scanlan, Offer, et al. 2007; Scanlan, Ritchie, et al. 2007; Luallen et al. 2008). However, the molecular basis for this link between antigenicity and immunogenicity remains largely unexplored. In this study, we begin to determine the rules governing this relationship: We find that the antibodies elicited to yeast mannans display remarkable reproducibility and selectivity in their cross-reactivity to oligomannose glycans of gp120. The deletion of the Mmn1 gene, responsible for the variable and polydisperse Manα1→3Man “cap” found on the nonreducing termini of the Manα1→2Man branches of Saccharomyces cerevisiae mannan, focused the specificity of these antibodies towards Manα9GlcNAc2 glycans (Supplementary Figure S1). Although the resultant serum closely mimicked 2G12 in its fine carbohydrate reactivity, the neutralization of primary HIV isolates was barely detectable above background and showed considerable inter-isolate variation. This molecular basis for this differential serum reactivity between Manα1→2Man and Manα1→3Man immune serum and between HIV isolates was confirmed by biosynthetic and enzymatic manipulation of gp120 N-linked glycosylation. We show that the identity of glycoforms present on gp120 has a dramatic impact on its antigenicity for yeast immune sera. The implications of our findings for HIV vaccine design are discussed.

Results and discussion

Carbohydrate microarray analysis of immune sera

Groups of New Zealand white rabbits were immunized with whole-cell preparations of either wild-type S. cerevisiae (WT) or S. cerevisiae deficient in the α1→3 mannosyltransferase gene (∆Mmn1). Four animals for each group were given secondary immunizations with hypermannosylated gp120 (Kif-gp120WT, as described in Materials and methods). We sought to characterize the binding of immune sera to carbohydrate antigens using the carbohydrate microarray of the Consortium of Functional Glycomics (CFG; http://www.functionalglycomics.org), which consists of 406 unique carbohydrate structures and conjugation densities. Before conducting our analysis, we first established the scale of the natural variation among pre-immune animals for the glycans present on the array (Figure 1A). Each individual serum revealed a wide range of carbohydrate reactivities, consistent with our previous studies of mammalian immune sera (Blixt et al. 2004). Moreover, our analysis revealed considerable variation in the reactivities between individuals. This has important implications for the use of glycan microarrays in the analysis of responses to carbohydrate-based immunogens and highlights the possibility of potentially misleading false positives which may have occurred independently of any immunization. We note that natural variation also extends towards the oligomannose glycans pertinent to HIV vaccine design (Figure 1B).

Despite the wide heterogeneities in specificities between individual immune systems, the responses were nonetheless consistent in their general reactivity to blood group antigens, Lewis structures and, significantly, from the perspectives of this study, yeast glucan-like motifs and α-linked mannose conjugates; the complete set of array data is presented in Supplementary Information.

Our primary aim was to determine if there were significant differences in specificities which correlated with exposure to the particular immunogens used in this study. Although the Kif-gp120WT boost did not alter the anticarbohydrate serum specificity, differential antibody responses were observed when the sera from animals immunized with the different yeast strains were compared. Analysis of the entire dataset of over 400 glycans revealed that the only significant (p < 0.05) difference between the WT and ∆Mmn1 groups was in the serum binding to two Manα1→2Man terminating glycan probes (Figure 2). This sensitivity is exquisite: Whole cells present a wide range of possible antigens and inevitably elicit a stochastic, heterogeneous antibody response. Despite this immunological diversity and despite the antigenic space represented by the numerous and
diverse glycan structures, the only difference to emerge between groups exactly recapitulated the genetic basis of the differential immunization. Specifically, Man\(\alpha_1 \rightarrow 2\)Man\(\alpha_1 \rightarrow 2\)Man\(\alpha_1 \rightarrow 3\)Man\(\alpha_1 \rightarrow 2\)Man\(\alpha_1 \rightarrow 6\)Man\(\alpha_1 \rightarrow R\) (CFG glycan number 311) and Man\(\alpha_1 \rightarrow 2\)Man\(\alpha_1 \rightarrow 2\)Man\(\alpha_1 \rightarrow 3\)Man\(\alpha_1 \rightarrow 6\)Man\(\alpha_1 \rightarrow R\) (CFG glycan number 195) revealed elevated reactivity to \(\Delta Mnn1\) immune sera over WT (Figure 2). The only shared motif between these two antigenic structures closely corresponds to the (Man\(\alpha_1 \rightarrow 2\))n epitope presented in the \(\Delta Mnn1\) polysaccharide and to the Man\(\alpha_1 \rightarrow 2\)Man\(\alpha_1 \rightarrow 2\)Man motif recognized by 2G12. Indeed, the glycan 311 is chemically identical to the mannosyl moiety of “self” Man\(_9\)GlcNAc\(_2\), abundant on the HIV envelope. Given the reactivity of \(\Delta Mnn1\) sera to the structure 311, we next investigated the ability of this serum to neutralize HIV-gp120.

Neutralization assays
A panel of HIV-1s, including primary circulating isolates, was exposed to both sets of yeast immune sera (animals that had received the gp120 boost were excluded from this analysis). No potent neutralization was observed in either group (Figure 3). Nonetheless, there was a small but statistically significant elevation in apparent antiviral IC\(_{50}\) titer for the \(\Delta Mnn1\) sera against three viral isolates (92BR020, IAVI C22, NL43). As the immunized animals had never been exposed to viral antigens, we hypothesized that this slight reactivity may be directed against the carbohydrates. Interestingly, a control virus used in the panel, murine leukemia virus (MLV), was also...
weakly neutralized by the ΔMnn1 sera compared to WT sera. Although antigenically unrelated to HIV-1, MLV does contain an envelope glycoprotein bearing oligomannose glycans (Geyer et al. 1990). These data indicate that viral isolates may differ in their susceptibility to anti-Manα1→2Man-specific antibodies present in the ΔMnn1 sera. Interestingly, this reactivity does not strongly correlate with 2G12 neutralization. For example, 94UG103 is 2G12-resistant yet is the most sensitive of the strains tested here to ΔMnn1 sera; in contrast, JRCSF is 2G12-sensitive but displays complete resistance to ΔMnn1 sera (Simek et al. 2009). Although the neutralization titers observed for the ΔMnn1 sera are far below anything that would constitute realistic protection against HIV-1, a molecular understanding of even this modest differential might guide vaccine design towards a more potent immunogen capable of eliciting higher titers against this carbohydrate target.

Construction of an HIV glycoform array
To validate our hypothesis that differential neutralization of isolates by ΔMnn1 sera was attributable to differences in viral carbohydrates, we constructed an HIV envelope glycoform array via the targeted manipulation of mammalian glycan biosynthesis. We chose as our starting point gp120αΔMnn1, which showed no sensitivity to ΔMnn1 sera in our neutralization assay and sought to manipulate its glycosylation to alter this sensitivity. We have previously shown that such inhibition of the biosynthesis of gp120 and other heavily glycosylated mammalian glycoproteins provides defined ligands for 2G12 (Scanlan, O'Flaherty, et al. 2007; Scanlan, Ritchie, et al. 2007). In that study, we used the endoplasmic reticulum (ER) α1→2 mannosidase I inhibitor, kifunensine, to trap gp120 glycans as ManαGlcNAc2. Subsequently, it has been shown that genetic manipulation of yeast glycan biosynthesis can generate similar glycoproteins with ManαGlcNAc2 glycans displaying 2G12 reactivity (Luallen et al. 2008). Here, we sought to extend our defined glycoform library to help delineate the specificity of immune sera. We characterized the glycoforms present on our glycoform array by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Figure 4) and validated the isomeric assignments using negative ion fragmentation [tandem mass spectrometry (MS/MS)] (Figure 5).

HIV gp120αΔMnn1 was expressed in human embryonic kidney (HEK) 293T cells, and the N-linked glycans were characterized by MALDI-TOF-MS (Figure 4A). The spectrum of the released glycans revealed a pattern of complex glycosylation characteristic of the cell line (Bowden et al. 2008; Bowden et al. 2009; Crispin et al. 2009), dominated by galactosylated bi- and triantennary structures with variable core fucosylation (glycan masses are presented in Table S1, Supplementary Information). However, despite the expected differences between this profile and previously published analyses of gp120 glycans from Chinese hamster ovary cells (Zhu et al. 2000), the persistence of the oligomannose patch is evident. This highlights the fact that the biosynthesis of the mannose patch, and by extension the 2G12 epitope, is a protein-directed phenomenon, whereas the complex-type glycosylation is largely determined by cell type (Zhu et al. 2000; Scanlan et al. 2002; Cutalo et al. 2004; Scanlan, O’Flaherty, et al. 2007; Scanlan, Ritchie, et al. 2007).
We have previously reported that stable expression of gp120 in Chinese hamster ovary cells in the presence of kifunensine (an inhibitor of ER and Golgi apparatus type 1 α-mannosidases) yielded a glycoform with abundant Man \(_9\)GlcNAc \(_2\) (Scanlan, Offer, et al. 2007; Scanlan, Ritchie, et al. 2007). MALDI-TOF-MS of glycans from gp120 transiently expressed in HEK 293T cells under similar conditions revealed an equivalent glycoform (Figure 4B). ESI-MS/MS of the peak at \(m/z\) 1979.7 (phosphate adduct, equivalent to the \([M+Na]^+\) ion at \(m/z\) 1905.7 in the MALDI-TOF spectrum) confirmed this species as Man\(_9\)GlcNAc\(_2\) (Figure 5A).

This defined gp120 glycoform was used as the starting material for the generation of a specific isomer (D1,D3-Man\(_8\)GlcNAc\(_2\)) of Man\(_8\)GlcNAc\(_2\). We cloned and expressed human ER \(\alpha\_1\rightarrow 2\) mannosidase I which specifically hydrolyses the terminal mannose residue from the D2 arm of Man\(_9\)GlcNAc\(_2\) to generate D1,D3-Man\(_8\)GlcNAc\(_2\). Incubation of the purified ER \(\alpha\_1\rightarrow 2\) mannosidase I with Kif-gp120 BaL yielded a glycoform dominated by a species with a mass corresponding to Man\(_8\)GlcNAc\(_2\) (Figure 4C). ESI-MS/MS of the peak at \(m/z\) 1756 (chloride adduct, equivalent to the \([M+Na]^+\) ion of Man\(_8\)GlcNAc\(_2\) in the MALDI-TOF spectrum at \(m/z\) 1743) confirmed this species as the D1,D3-Man\(_8\)GlcNAc\(_2\) isomer of Man\(_8\)GlcNAc\(_2\) (Figure 5B and C). Spectral interpretation of the negative ion MS/MS spectrum was performed as previously described (Harvey 2005b; Harvey et al. 2008) and follows the nomenclature of Domon and Costello (1988). The chitobiose core region of both compounds is defined by the two cross-ring \(2^4\)A\(_6\) and \(2^4\)A\(_5\) and B\(_5\) glycosidic fragments. The ions towards the center of the spectrum define the composition of the 6-antenna; thus, the ion labeled D contains the branching mannose residue and the mannose residues of the 6-antenna, and the \(0^3\)A\(_4\) ion is a cross-ring fragment of the branching mannose containing the same antenna. The mannose residues at the nonreducing terminus give rise to the C\(_1\) fragment at \(m/z\) 179 and the B\(_2\) and C\(_2\) fragments at \(m/z\) 323 and 341, respectively. The D' fragment contains the mannose residues of the 6-branch of the 6-antenna and appears at the same mass (\(m/z\) 485) in the spectra of both Man\(_8\)GlcNAc\(_2\) and Man\(_9\)GlcNAc\(_2\) showing that Man\(_8\)GlcNAc\(_2\) has lost the mannose residue from the 3-branch of the 6-antenna.

Given the importance of Man\(_\alpha\_1\rightarrow 2\)Man residues in both 2G12 and \(\Delta M_{\text{nn1}}\) serum reactivity to the CFG array, we expressed gp120 in a cell line expressing glycoproteins dominated by terminal Man\(_\alpha\_1\rightarrow 5\)Man and Man\(_\alpha\_1\rightarrow 6\)Man structures. By utilizing a HEK 293S cell line lacking GnT I activity (Reeves et al. 2002) to express gp120, complex glycan processing was trapped at the Man\(_9\)GlcNAc\(_2\) biosynthetic intermediate (Figure 4D; GnT I-deficiency is also known as Lec1). An additional Man\(_9\)GlcNAc\(_2\)Fuc\(_1\) species is present as
a result of a minor pathway that leads to the α1→6 fucosylation of the reducing terminal GlcNAc of Man$_5$GlcNAc$_2$ (Figure 4) (Crispin et al. 2006). Importantly, in addition to the Man$_5$GlcNAc$_2$-based glycans, we observed an oligomannose series corresponding to the mannose patch of normally glycosylated gp120. The maintenance of the mannose patch in the GnT I-deficient HEK 293S cells is consistent with the known biosynthetic pathway of N-linked glycans; Man$_5$GlcNAc$_2$ glycans are an obligate intermediate in the formation of complex- and hybrid-type glycans. The Man$_5$GlcNAc$_2$ glycans form 54% of the total glycan population, as assessed by the relative abundances of the five highest peaks on the MALDI-TOF-MS spectrum (Figure 6). The glycans that contribute to the Man$_5$GlcNAc$_2$ signal correspond to the complex, hybrid and Man$_5$GlcNAc$_2$ glycans of the gp120 BaL spectra from HEK 293T cells, whereas the Man$_9$GlcNAc$_2$ peaks (46% relative abundance) correspond to the remaining sterically protected structures of the mannose patch. The gp120$_{bal}$ expressed in the GnT I-deficient HEK 293S cell line binds to 2G12 with a broadly equivalent affinity to that expressed in the HEK 293T cells (data not shown).

Reactivity of mannan immune sera to HIV-1 gp120

The binding of the immune sera from all animals to recombinant gp120$_{bal}$ was determined by enzyme-linked immunosorbent assay (ELISA) (Figure 7). Sera from rabbits that had received gp120$_{bal}$ boosts showed predictable reactivity against the cognate antigen (data not shown). However, animals vaccinated with either WT or ΔMnn1 showed no detectable serum titers against gp120$_{bal}$ compared to pre-immune control sera (Figure 7A), consistent with the result from the neutralization study. However, this picture was dramatically altered when the Man$_9$GlcNAc$_2$ (Figure 7B) and Man$_8$GlcNAc$_2$ (Figure 7C) gp120$_{bal}$ glycoforms were assessed. Both hypermannosylated glycoforms exhibited clear antigenicity for the ΔMnn1 but not for WT sera. This differential reactivity can be attributed to the non-reducing terminal Man$_9$→2Man residues as no binding was observed to gp120$_{bal}$ expressed from the GnT I-deficient HEK 293S (Figure 7D), which displays predominantly Man$_1$→3Man and Man$_1$→6Man over Man$_1$→2Man terminating glycans (Figure 7D), again recapitulating the differential results yielded by glycan microarray analysis (Figure 2). We note that WT sera, which might be expected to have reactivity to these Man$_1$→3Man terminating structures, do not bind to gp120 expressed in GnT I-deficient HEK 293S cells.
One possible explanation for this observation is that Man$_{\alpha 1\rightarrow 3}$Man terminating glycan structures are somewhat abundant on the surface of self cells, for example on hybrid-type glycans, and therefore may be more tolerated than the rarer unprocessed Man$_{\alpha 1\rightarrow 2}$Man terminating branches. We also note that the residual Man$_{\alpha 1\rightarrow 2}$Man-containing oligomannose patch recognized by 2G12 is not productively recognized by $\Delta$Mnn1 serum in contrast to the oligomannose glycans presented by the pure Man$_9$GlcNAc$_2$ glycoform of gp120. This may suggest that not all antibodies generated to Man$_9$GlcNAc$_2$ recognize this glycan in the context of the dense oligomannose network presented on the outer domain of gp120.

Reactivity of 2G12 and mannan immune sera to the neoglycolipid array

The results from the CFG glycan array, the neutralization assay and the viral envelope array indicate that Man$_{\alpha 1\rightarrow 2}$Man structures are the key mediators of serum reactivity. While 2G12 is known to bind to this motif (Scanlan et al. 2002; Calarese et al. 2003), the specificity of the serum seems narrower than some alternative modes of binding postulated for 2G12 (Sanders et al. 2002; Calarese et al. 2005). In addition to the 3-branch of the trimannosyl core which supports the D1 motif, the 6-branch of the trimannosyl core has been proposed to provide either the D3 arm (Calarese et al. 2005) or hybrid-type glycans (Sanders et al. 2002) as alternative motifs for 2G12 binding. Immunogens based on both of these alternative non-D1 arm binding modes have been synthesized for evaluation as vaccine candidates (Dudkin et al. 2004; Lee et al. 2004). In light of the central role of the D1-motif highlighted by this study, we sought to reconcile these data using a neoglycolipid (NGL) array presenting a wide range of homogeneously presented oligomannose-type glycans. We also selected representative immune sera to determine whether the enhanced Man$_{\alpha 1\rightarrow 2}$Man reactivity shown by the $\Delta$Mnn1 serum paralleled that of the only confirmed neutralizing monoclonal antibody to HIV-1 carbohydrates, 2G12 (Figure 8).

We have previously argued that 2G12 binds through the D1-arm of oligomannose-type glycans. This mode of binding was supported by the observation that 2G12 binding was inhibited by Aspergillus saitoi $\alpha_1\rightarrow 2$-mannosidase and by the observation that gp120 expressed in the presence of the $\alpha$-glucosidase inhibitor N-butyldeoxynojirimycin, which induces the presence of glucosyl moieties capping the D1-arm of oligomannose glycans, blocks 2G12 binding but not the binding of the conformationally specific monoclonal antibody, b12 (Scanlan et al. 2002). Finally, the crystal structure of the 2G12Man$_9$ GlcNAc$_2$ complex revealed that the protein–carbohydrate interface was dominated by the D1-arm (Calarese et al. 2003). However, a subsequent crystal structure of 2G12 in complex with the D3,D2-terminating Man$_3$ fragment, Man(D3)$\alpha 1\rightarrow 2$Man$_{\alpha 1\rightarrow 6}$(Man(D2)$\alpha 1\rightarrow 2$Man$_{\alpha 1\rightarrow 3}$Man, revealed an...
alternative mode of recognition through the D3 arm (Calarese et al. 2005). However, while 2G12 binds to Man9GlcNAc2, D1-Man7GlcNAc2 and D1,D3-Man8GlcNAc2, on the neoglycolipid array (Figure 8A), no detectable binding was revealed for the related D3-terminating structure, Man(D3)α1→2Manα1→6[Manα1→2Manα1→3]Man (green) (Calarese et al. 2005) with the remaining saccharide residues generated by docking the solution-state NMR structure of ManαGlcNAc2 onto the reducing terminal mannose (cyan). (C) The crystal structure of 2G12 in complex with Manα1→2Man (yellow) (Calarese et al. 2003). (D) The Manα1→3Man terminal residue of a hybrid-type glycan (cyan) docked onto the crystal structure of 2G12 in complex with Manα1→2Man using the most abundant torsion angles (Petrescu et al. 1999; Wormald et al. 2002).

Fig. 9. Molecular basis of 2G12-carbohydrate recognition. Glycans are represented in sticks and the protein surface in gray. (A) The crystal structure of 2G12 in complex with Man9GlcNAc2 (Calarese et al. 2003). (B) The crystal structure of 2G12 in complex with the D3-terminating glycan, Man(D3)α1→2Manα1→6[Manα1→2Manα1→3]Man (green) (Calarese et al. 2005) with the remaining saccharide residues generated by docking the solution-state NMR structure of Man6GlcNAc2 onto the reducing terminal mannose (cyan). (C) The crystal structure of 2G12 in complex with Manα1→2Man (yellow) (Calarese et al. 2003). (D) The Manα1→3Man terminal residue of a hybrid-type glycan (cyan) docked onto the crystal structure of 2G12 in complex with Manα1→2Man using the most abundant torsion angles (Petrescu et al. 1999; Wormald et al. 2002).
what wider degree of monosaccharide or linkage specificity compared to 2G12. We have previously argued that 2G12 may in fact represent an antimicrobial response, which through domain-exchange acquired avidity to HIV-1 (Scanlan, Offer, et al. 2007; Scanlan, Ritchie, et al. 2007; Dunlop et al. 2008). The remarkable similarity in molecular specificities between yeast immune sera and 2G12 would be entirely consistent with such hypothesis. There is, regardless, a convergence, revealed by this study, in the antigenic specificity of the serum reactivity generated in this study and the carbohydrate selectivity of the broadly neutralizing antibody, 2G12. This confirms the principle that genetic modulation of microbial polysaccharides can yield immunogenic mimics capable of eliciting antibody responses to the self glycans of HIV-1.

### Materials and methods

#### Protein expression and purification

The full-length soluble ectodomain of HIV-1 gp120<sub>BaL</sub> (corresponding to amino acid residues 1 to 507, numbering based on alignment with the HxB2 reference strain) and the C-terminal catalytic domain of human ER class I α1→2-mannosidase (corresponding to amino acid residues 237 to 699) were cloned and expressed as previously described (Aricescu et al. 2006). cDNA was cloned into the pHLsec vector encoding an in-frame C-terminal hexahistidine tag, and the target proteins were transiently expressed in HEK 293T (ATCC CRL-1573). The HEK 293T and GnT I-decient HEK 293S (Reeves et al. 2002) cell lines were maintained in Dulbecco’s modified Eagle medium supplemented with penicillin, streptomycin and 10% fetal bovine serum. Transient transfections were performed using a mixture of polyethylenimine and 2 mg DNA per liter cell culture. When used, the α-mannosidase inhibitor kifunensine (Cayman Europe, Estonia) was added at the stage of transfection at a concentration of 4 mg/mL (Chang et al. 2007). Culture supernatants were collected and replaced with new serum-free media 2 days post transfection, and the replacement media was collected an additional 3 days later. Protease inhibitor was added to the pooled supernatants. Following concentration by ultrafiltration (Vivaspin 20, Sartorius), gp120 was initially isolated by immobilized metal affinity chromatography then further purified by size exclusion chromatography using a Superdex 200 Prep Grade column (GE Health Care) with a 150-mM NaCl, 10-mM Tris (pH 8.0) buffer. Typical gp120 yields per liter of cell culture were approximately 5 mg/mL from the HEK 293S and HEK 293T cells and approximately 6 mg/mL from HEK 293T with kifunensine. Yields for the ER α-mannosidase I per liter cell culture were typically 1–2 mg/mL.

#### Mass spectrometry of oligosaccharides

Glycans were released enzymatically following the method of Küster et al. (1997). Coomassie blue-stained SDS-PAGE bands containing approximately 10 μg of target glycoproteins were excised, washed with 20 mM NaHCO<sub>3</sub> pH 7.0 and dried in a vacuum centrifuge before rehydration with 30 μL of 30 mM NaHCO<sub>3</sub> pH 7.0, containing 100 U/mL of protein N-glycanase F (PNGase F; Prozyme, San Leandro, CA, United States). After incubation for 12 h at 37°C, the enzymatically released N-linked glycans were eluted with water. Aqueous solutions of glycans were cleaned with a Nafion 117 membrane (Börnsten et al. 1995) prior to mass spectrometry.

Positive ion MALDI-TOF mass spectra were recorded with a Shimazu AXIMA TOF<sup>2</sup> MALDI-TOF/TOF mass spectrometer fitted with delayed extraction and a nitrogen laser (337 nm). Samples were prepared by adding 0.5 μL of an aqueous solution of the glycans to the matrix solution (0.3 μL of a saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile) on the stainless steel target plate and allowing it to dry at room temperature. The sample/matrix mixture was then recrystallized from ethanol. Further samples were examined after removal of sialic acids by heating at 80°C for 1 h with 1% acetic acid.

Electrospray mass spectrometry was performed with either a Waters quadrupole-time-of-flight (Q-ToF) Ultima Global instrument or a Q-ToF model 1 spectrometer (Waters MS Technologies, Manchester, United Kingdom) in negative ion mode. Samples in 1:1 (v:v) methanol:water were infused through Proxeon nanospray capillaries (Proxeon Biosystems, Odense, Denmark). The ion source conditions were as follows: temperature, 120°C; nitrogen flow, 50 L/h; infusion needle potential, 1.1 kV; cone voltage, 100 V; RF-1 voltage 150 V (Ultima Global instrument). Spectra (2-s scans) were acquired until a satisfactory signal:noise ratio had been obtained. For MS/MS data acquisition, the parent ion was selected at low resolution (about 5 m/z mass window) to allow transmission of isotope peaks and fragmented with argon as a fast atom (recorded on the instrument’s pressure gauge) of 0.5 mBar. The voltage on the collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale. Typical values were 80–120 V. Other voltages were as recommended by the manufacturer. Instrument control, data acquisition and processing were performed with MassLynx (Waters) software version 4.0 (Q-ToF 1) or 4.1 (Ultima Global). Analysis of fragmentation spectra was performed as previously described (Harvey 2005a, 2005b, 2005c, 2005d; Harvey et al. 2008).

#### Immunization

Wild-type (WT) <i>S. cerevisiae</i> (MATα, BY4742 strain) and an identical strain containing a homozygous deletion of the <i>Mnn1</i> gene (Δ<i>Mnn1</i>) were obtained from Open Biosystems. The strains were grown at 30°C in YPD medium (1% [w/v] yeast extract; 2% [w/v] bactopeptone; 2% [w/v] dextrose) with 120 μg/mL kanamycin and shaken at 170 rpm. Cultures were harvested after 26 h, pelleted and then resuspended in phosphate-buffered saline (PBS) pH 7. Preparations of each strain were heat-killed (100°C for 1 h) then aliquoted and frozen at set doses of 10<sup>5</sup> cells per animal per inoculation.

A high-frequency vaccination protocol similar to that used by Ballou was performed (Ballou 1990). Sixteen rabbits (New Zealand White) were divided into four groups. Groups 1 and 2 received three weekly intravenous marginal ear vein injections of WT and Δ<i>Mnn1</i>, respectively, for 9 weeks. Groups 3 and 4 followed an identical protocol for the first 4 weeks with Group 3 receiving WT and Group 4 receiving Δ<i>Mnn1</i>. After 4 weeks, these animals were switched to a protocol of three successive boosts of 50 μg of Kif-gp120<sub>BaL</sub> at fortnightly intervals. The Kif-gp120<sub>BaL</sub> was administered intramuscularly by an injection to each quadriceps. Injections of Kif-gp120<sub>BaL</sub> were co-administered with a lecithin and acrylic...
polymer emulsion adjuvant, Adjuplex, that has been reported to increase vaccine-induced serum IgG antibody titers (Gupta et al. 1995). Neutralization assays were performed as previously described (Simek et al. 2009).

Carbohydrate microarray analysis
Serum was analyzed for carbohydrate reactivity using the glycan array developed by the Consortium for Functional Glycomics (http://www.functionalglycomics.org) following their standard protocol (Blixt et al. 2004; Astronomo et al. 2008). In addition, carbohydrate reactivities were also probed using the Neoglycolipid Array (Imperial College, United Kingdom) (Palma et al. 2006).

ER α1→2 mannosidase I digestion of Kif-gp120Bat.
Kif-gp120Bat (20 μg) was incubated with purified ER α1→2 mannosidase I (1 μg) in a reaction buffer consisting of 80 mM 1,4-piperazinediethanesulfonic acid, 1 μg/μL bovine serum albumin (BSA), 4 mM CaCl₂ and 0.016% NaN₃ (pH 6.5). Incubation volumes were typically 100–1000 μL, and the reactions were carried out at 20–25°C overnight. Following the digestion, the modified Kif-gp120Bat was purified by size exclusion chromatography using a Superdex 200 Prep Grade column (GE Health Care) with a 150 mM NaCl, 10 mM Tris (pH 8.0) buffer. The enzyme was removed using Amicon Micropure-EZ™ spin columns.

Glycoform array analysis
Serum ELISA experiments were performed with half area microtiter plate wells (Costar type 3690, Corning) coated overnight at 4°C with 1 μg/mL glycoform-defined gp120Bat. Plates were subsequently blocked with 3% (w/v) BSA for 1 h. Serum was diluted during the blocking step using low-protein-binding 96-well Sero-Wel plates (Bibby Sterilin). After each incubation, the plates were washed five times with PBS. Binding was detected using alkaline-phosphatase conjugated secondary antibodies (1 μg/mL in PBS, with 1% BSA) from Pierce (Rockford, IL, United States).

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of statement
None declared.

Abbreviations
BNAbs, broadly neutralizing antibodies; BSA, bovine serum albumin; CFG, Consortium of Functional Glycomics; ELISA, enzyme-linked immunoabsorbant assay; ER, endoplasmic reticulum; ESI, electrospray ionization; HEK, human embryonic kidney; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MLV, murine leukemia virus; MS/MS, tandem mass spectroscopy; NGL, neoglycolipid; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; Q-ToF, quadrupole-time-of-flight.

References


