Expression-System-Dependent Modulation of HIV-1 Envelope Glycoprotein Antigenicity and Immunogenicity

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glycan type

Recombinant expression systems differ in the type of glycosylation they impart on expressed antigens such as the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins, potentially affecting their biological properties. We performed head-to-head antigenic, immunogenic and molecular profiling of two distantly related Env surface (gp120) antigens produced in different systems: (a) mammalian (293 FreeStyle™ cells; 293F) cells in the presence of kifunensine, which impart only high-mannose glycans; (b) insect cells (Spodoptera frugiperda, Sf9), which confer mainly paucimannosidic glycans; (c) Sf9 cells recombinant for mammalian glycosylation enzymes (Sf9 Mimic™), which impart high-mannose, hybrid and complex glycans without sialic acid; and (d) 293F cells, which impart high-mannose, hybrid and complex glycans with sialic acid. Molecular models revealed a significant difference in gp120 glycan coverage between the Sf9-derived and wild-type mammalian-cell-derived material that is predicted to affect ligand binding sites proximal to glycans. Modeling of solvent-exposed surface electrostatic potentials showed that sialic acid imparts a significant negative surface charge that may influence gp120 antigenicity and immunogenicity. Gp120 expressed in systems that do not incorporate sialic acid displayed increased ligand binding to the CD4 binding and CD4-induced sites compared to those expressed in the system.
Introduction

The only viral targets for neutralizing antibodies (NAb) against human immunodeficiency virus type 1 (HIV-1) are the viral envelope glycoproteins (Env), composed of a heterotrimer of three surface subunits (gp120) noncovalently linked to three transmembrane subunits (gp41). An ideal Env-based vaccine against HIV-1 would induce long-lived NAb responses recognizing a broad range of viral strains. To move toward this goal requires considerable optimization of the antigen, adjuvant and delivery strategy. This will include an understanding of the impact of Env glycosylation with regard to its effects on antigenicity and immunogenicity. The choice of expression system might be especially relevant in the context of HIV-1 Env, since glycans contribute approximately 50% of the molecular mass of gp120, and enzymatic glycosylation machinery differs substantially between, for example, insect and mammalian cell expression systems. When expressed in mammalian systems, between 13 and 16 of the N-linked glycans of gp120 are of the complex-type, while the remainder (of a total of 20–26) are of the high-mannose or hybrid types. High-mannose glycans occur more frequently in the conserved regions, whereas those attached to variable loops, being more exposed to the glycosylation machinery of the Golgi, are more frequently processed into complex glycans. Conversely, in wild-type (wt) insect cell systems, glycosylation is restricted to paucimannosidic structures and the degree of sequon occupancy may be lower.

Insect cell expression systems have the advantage of producing large quantities of proteins cheaply and without many of the potential biohazards associated with mammalian systems. In addition, insect cells carry out many posttranslational modifications, including high-mannose-type N- and O-linked glycosylation, resulting in glycoproteins generally considered to be of similar antigenicity and functionality to those prepared in mammalian cells. When expressed in insect cell systems, Env gp160 undergoes appropriate cleavage to generate the gp41 and gp120 subunits, and the gp120 subunit retains CD4 binding. For these reasons, insect cells have been used since the early years of HIV-1 vaccine research to produce Env antigens for immunogenicity and challenge studies in animals and immunogenicity in humans. However, given the differences in glycan enzyme machinery between these different expression systems, the criteria for selection of an optimal system might go beyond yield and ease of glycoprotein production, potentially affecting parameters such as antigenicity and immunogenicity.

Few studies have compared the effect of expression system on antigenicity in a head-to-head fashion, although one recent analysis compared gp120 expressed in different human cell lines for glycan content and relative binding to polyclonal sera. By contrast, many have examined the effects of enzymatic removal of glycans or mutations deleting selected glycan sequons. While the outcomes of individual studies vary, together they suggest that partial or complete deglycosylation generally increases the binding of antisera and monoclonal antibodies (mAbs). However, the effects can be epitope specific, with some regions being unaffected or even showing decreases in mAb binding. The site-specific deletion of glycan sequons from Env increased recognition by important neutralizing mAbs (NmAbs) such as b12, 447-52D and 2F5, especially when the mutants were expressed in insect cells. Moreover, deletion of three to five Env glycan sequons from wt SIVmac239 virus resulted in dramatic attenuation in rhesus macaques and the induction of substantial protection against the wt virus with higher titers of NAb than those obtained after wt virus challenge. Disappointingly, however, subunit antigens based on such Env glycan mutants proved to be less good than, or at best equivalent to, wt Env antigens at inducing NAb responses or controlling viremia in animal models. Moreover, since deletion of glycan sequons may alter glycoprotein processing and folding, this may have unpredictable effects on the presentation of conformational and discontinuous NAb epitopes such as those present on gp120. However, one study showed that only a small number of protein-proximal glycan residues are

that do, and imparted other more subtle differences in antigenicity in a gp120 subtype-specific manner. Non-sialic-acid-containing gp120 was significantly more immunogenic than the sialylated version when administered in two different adjuvants, and induced higher titers of antibodies competing for CD4 binding site ligand–gp120 interaction. These findings suggest that non-sialic-acid-imparting systems yield gp120 immunogens with modified antigenic and immunogenic properties, considerations that should be considered when selecting expression systems for glycosylated antigens to be used for structure–function studies and for vaccine use.
Expression System-Dependent HIV-1 Env Properties

important for folding, while the rest may function to block antibody (Ab) binding, suggesting that for immunogen design, glycans may need to be reengineered or otherwise modified rather than deleted.27,28 Such an approach has shown promise in a recent study in which complex glycans on gp120 were replaced with trimmed oligomannose structures by expression in a cell line lacking N-acetylgalcosamine transferase I, resulting in increased binding of ligands to the CD4 binding site (CD4bs) and the V3 loop.27

The choice of expression system might also affect the immunogenicity of glycoproteins such as gp120 in diverse ways. Immunogenicity might be reduced in insect-cell-expressed gp120, since sialic acid residues on complex glycans, not present on insect-cell-expressed material, are important for extending glycoprotein half-life in vivo by shielding mannose from interaction with mannose receptors.29 Conversely, it has recently been demonstrated that mannose receptors on professional antigen-presenting cells mediate uptake into compartments involved in cross-presentation: this function might be enhanced in the presence of insect-cell-produced glycoproteins expressing predominantly terminal mannose glycans.30 The latter supposition is in agreement with the finding that gp120 expressed in insect cells was better able to induce cytotoxic T lymphocyte responses in Balb/c mice than that produced in mammalian Chinese hamster ovary (CHO) cells.31

The reduced response to the CHO cell material was overcome by enzymatic deglycosylation, suggesting that mammalian glycans were responsible for the poor immunogenicity.31 An alternative view was recently proposed—that terminal mannose groups might downmodulate Ab responses to gp120 via lectin interactions on antigen-presenting cells leading to production of the immunosuppressive cytokine IL-10.32,33 In addition to regulating interaction with cell surface lectins including mannose receptors, the sialic acid residues of complex glycans also negatively regulate the interaction of gp120 with mannose-binding lectin in the serum,34 which upon binding to an antigen can trigger the complement cascade resulting in complement opsonization and improved antigen uptake. Finally, sialic acid has been demonstrated to suppress B-cell responses via interaction with CD22, a potential mechanism to avoid self-recognition.35,36

To investigate the effect of expression system on glycoprotein antigenicity and immunogenicity, we compared two insect systems [wt Sf9 (Sf9wt; from Spodoptera frugiperda) and Sf9 Mimic™] with a mammalian system [293 Freestyle™ (293F)] in the presence and absence of kifunensine, in a head-to-head fashion, using gp120 from two distantly related HIV-1 strains in order to describe both general and virus-strain-specific effects. Sf9 Mimic™ cells are a recombinant Sf9 cell line that express five mammalian glycosylation enzymes and produce the majority of complex mammalian glycan modifications10 with the exception that they lack a donor for sialic acid; thus, the complex glycans they produce have terminal galactose residues.37 The inclusion of this additional cell line allows the contribution to antigenicity and immunogenicity of complex glycans lacking sialic acid to be assessed without the need for enzymatic desialylation.

Results

Comparison of the sequence identity and glycosylation of gp120 from strains 97CN54 and Ba-L

HIV-1 is a highly diverse virus with strains differing by up to 20% within clades and 35% between clades in terms of the amino acid sequence, with Env being the most variable gene.38 To study general effects on recombinant gp120 antigenicity and immunogenicity of the expression system used, we selected CCR5-tropic strains from two different clades: 97CN54, a CRF07_BC primary isolate in which the gp120 region, with the exception of part of the leader sequence, is entirely of clade C origin39,40 (accession number: AF286226), and the clade B isolate Ba-L41 (accession number: AB221005). Alignment of the amino acid sequences of gp120 from these two strains with ClustalW (v1.83)42 showed that the strains are 26.3% divergent, with gp12097CN54 having 14 additional amino acids: 10 extra residues in the V1 loop and 6 in the V2 loop, but 2 fewer in the V4 loop (data not shown). Analysis of potential sites of N- and O-linked glycosylation using N-GlycoSite43 and NetOGlyc (v3.1)44 revealed that gp12097CN54 has 23 sequons for N-linked glycosylation and no predicted sites for mucin-type O-linked glycosylation, whereas gp120Ba-L has 22 sequons for N-linked glycosylation and 1 potential site for mucin-type O-linked glycosylation. Fifteen of the N-linked glycosylation sites were conserved between the two strains, with 12 occurring in conserved regions of the glycoprotein.

gp12097CN54 has one additional N-linked glycosylation sequon in each of the V1 and V2 loops and the C4 region, but one fewer in the V4 loop (data not shown). Analysis of potential sites of N- and O-linked glycosylation using N-GlycoSite43 and NetOGlyc (v3.1)44 revealed that gp12097CN54 has 23 sequons for N-linked glycosylation and no predicted sites for mucin-type O-linked glycosylation, whereas gp120Ba-L has 22 sequons for N-linked glycosylation and 1 potential site for mucin-type O-linked glycosylation. Fifteen of the N-linked glycosylation sites were conserved between the two strains, with 12 occurring in conserved regions of the glycoprotein. gp12097CN54 has an additional N-linked glycosylation sequon in each of the V1 and V2 loops and the C4 region, but one fewer in the V4 loop and C3 region when compared to gp120Ba-L (data not shown).

Characterization of the glycan content of gp120 expressed in different systems

To inform our modeling analysis of glycan coverage, we carried out mass spectrometric analysis of the glycan types present on gp120Ba-L produced in Sf9 cells, untreated 293F cells and 293F cells treated with 5 and 20 μM kifunensine, an
inhibitor of an inhibitor of class I α-mannosidases. We confirm results from a previous study\textsuperscript{10} that Sf9-expressed gp120 contains mostly Man\textsubscript{3}GlcNAc\textsubscript{2} but retains a minor population of untrimmed oligomannose structures that includes the 2G12 NAb epitope (Fig. 1). Mammalian-cell-expressed gp120 contained the expected proportions of complex and high-mannose glycans, implying that the purification process did not impose any dramatic bias in the selection of glycan types on the different glycoprotein forms. It has been reported that insect-cell-expressed material may have reduced numbers of glycans due to sequon skipping.\textsuperscript{11,12} Reduced numbers of glycans would be expected to modify the antigenicity of a highly glycosylated protein and might also influence immunogenicity. To limit differences between S9wt- and 293F-expressed gp120 to glycosylation type rather than number, we expressed gp120\textsubscript{Ba-L} in 293F cells in the presence or absence of 5 or 20 μM kifunensine. To confirm that kifunensine treatment inhibited the processing of Man\textsubscript{9}GlcNAc\textsubscript{2}, we performed mass spectrometry on gp120 produced in the presence and absence of the inhibitor (Fig. 1). Cultivation of gp120\textsubscript{Ba-L}-expressing 293F cells in the presence of 5 μM kifunensine completely prevented complex glycosylation and resulted in the unprocessed Man\textsubscript{9}GlcNAc\textsubscript{2} being the most prevalent glycan type (29.1%). However, the inhibition of class I α-mannosidases was incomplete, as the remaining species in order of abundance were Man\textsubscript{8}GlcNAc\textsubscript{2}, Man\textsubscript{7}GlcNAc\textsubscript{2}, Man\textsubscript{5}GlcNAc\textsubscript{2} and Man\textsubscript{6}GlcNAc\textsubscript{2}. Increasing the kifunensine concentration to 20 μM better inhibited the mannosidases, as only three glycan types could be detected: Man\textsubscript{9}GlcNAc\textsubscript{2}, Man\textsubscript{8}GlcNAc\textsubscript{2} and Man\textsubscript{7}GlcNAc\textsubscript{2}.

Models of gp120 glycan coverage and surface charge predict significant differences between antigens expressed in different systems

Potential differences in the antigenicity and immunogenicity of gp120 may result from differential glycan type, conformation, sequon usage, or all of these. To investigate the first possibility, we constructed molecular models of gp120 based on predicted N-linked glycosylation of the 293F, kifunensine-treated 293F and S9wt cell systems as described in Materials and Methods. A caveat for these models is that we were unable to include glycan mobility as a major factor in protein surface masking, since little is known of the flexibility of clusters of glycans in dense populations as found on gp120. In the absence of precise assignments for each glycan position, we modeled glycan heterogeneity by representing the proportion of each glycan species determined by mass spectrometry and distributed them, using a random assignment algorithm as detailed in Materials and Methods. In this way, we generated three models for each kind of expression system. Molecular dynamics was used to optimize the stereochemistry for each of these models, allowing for a limited account of flexibility. The exact assignments of glycans to the models are shown in Tables S1–S3. Glycans conferred by S9 Mimic™ cells are essentially the same as those conferred by mammalian cells except for sialic acid termini. Therefore, models of glycan coverage from 293F cells should be largely representative of glycans from S9 Mimic™ cells in terms of protein coverage, but not in terms of surface charge. Glycans conferred by kifunensine-treated 293F cells are retained as oligomannose structures, whereas those conferred by S9 cells are principally processed to Man\textsubscript{3}F\textsubscript{1}-containing structures with a minor population of oligomannose structures. A representative of the refined models for gp120\textsubscript{HxBc2} expressed in 293F, kifunensine-treated 293F and S9 cells are shown in Fig. 2a, b and c, respectively. The extent of gp120 glycosylation is
evident from the models, in which the protein core is labeled red, glycans containing terminal mannose are cyan, and complex glycans green. Using all these models, we quantified glycan coverage by calculating the amount of protein surface exposed to hypothetical spherical probes of different radii, approximating to the penetration of an amino acid side chain (1.4 Å), beta turns (2.5 and 5 Å) and an Ab combining region (10 Å) (Fig. 3). Although models for the same expression system vary greatly in glycan composition (Tables S1–S3), they are quite similar to each other in terms of glycan coverage. The occlusion of the solvent-exposed protein surface by the glycan canopy was extensive in all expression systems, and the difference in surface coverage between gp120 from kifunensine-treated cells and gp120 from 293 cells was not significant for small ligands of radii equivalent to amino acid side chains or polypeptide beta turns, components that can make up portions of an antigen–Ab combining region interface. Only when we modeled the interaction with a probe of radius 10 Å, equivalent to an entire Ab combining region, did a significant difference in total percentage occlusion of the solvent-exposed protein surface emerge between kifunensine-treated and untreated 293F-expressed material (P<0.01). However, this difference amounted only to an extra 8% total coverage (a 10.5% increase in coverage) compared to gp120 produced in the kifunensine system. A more profound difference in coverage was observed for gp120 produced in the Sf9 system, which revealed a 10% reduction in total coverage (a 13.6% reduction in coverage) compared to the 293F-produced gp120. This decrease in protein surface coverage imparted by the Sf9 cell production system would probably exert substantial effects on the exposure of ligand binding sites proximal to glycans, such as the IgG1b12 Ab epitope and the CD4bs.45

Another property that sialic acid imparts onto a glycoprotein that might influence Ab binding and/or immunogenicity, by altering interaction with antigen-presenting cells, is increased negative charge, which would be lacking on insect-cell-expressed material. We therefore modeled how the typical Sf9wt- and 293F-imported glycan structures affect the protein surface electrostatic potential (Fig. 4 a and b). As predicted, gp120 expressed in untreated mammalian cells is modeled to have a net negative average electrostatic potential of $-2.1 \, kT/e$. However, when expressed in other

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**Fig. 2.** Refined 3D gp120Ba-L glycan models. The gp120 protein is shown as a red ribbon model; high-mannose glycans are shown in cyan and complex glycans are shown in green. The orientation shown faces the glycan-free CD4bs with the V3 loop and associated glycan projecting left. Models of gp120 expressed in (a) 293F cells, (b) kifunensine-treated 293F cells, and (c) Sf9 cells.
systems not imparting sialic acid, the solvent-exposed protein surface had an average weak positive charge, a reversal of the electrostatic potential and a difference of $2.3 \, kT/e$ ($P<0.001$).

### The effect of expression system on gp120 antigenicity

All env constructs were subcloned into the vector pTri-Ex1.1, allowing expression from the same plasmids in both insect and mammalian systems. Equal amounts of gp120$_{\text{CN54}}$ expressed in 293F, Sf9wt and Sf9 Mimic™ and gp120$_{\text{Ba-L}}$ expressed in 293F and Sf9wt, as determined by BCA assay, were probed by Western blot for purity and assayed by ELISA with a panel of mAbs, the polyclonal antibody (pAb) D7324, soluble CD4 (sCD4), and polyclonal antisera, for expression-system-dependent antigenic differences. The median 50% binding titers obtained from three independent ELISAs for each condition were established and are represented in Tables 1 and 2 as fold changes in ligand binding to Sf9wt- or Sf9 Mimic™-expressed material compared to 293F-

<table>
<thead>
<tr>
<th>Probes (Å) and Example</th>
<th>Antibody Combining Region</th>
<th>β-Turn</th>
<th>Amino Acid Sidechain</th>
<th>Kifunensine</th>
<th>293</th>
<th>Sf9</th>
</tr>
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<tr>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td>32%</td>
<td>30.5%</td>
<td>30.5%</td>
</tr>
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<td></td>
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<td>p = 0.055</td>
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<tr>
<td>2.5</td>
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<td>64.5%</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.197</td>
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<td>10.0</td>
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<td>83.3%</td>
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<td></td>
<td></td>
<td></td>
<td>p = 0.002</td>
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</tbody>
</table>

Fig. 3. Relative protein surface occlusion by glycan in gp120$_{\text{Ba-L}}$ expressed in 293F cells, kifunensine-treated 293F cells or Sf9wt cells. The solvent-accessible protein surface is shown in red, high-mannose glycans in cyan and complex glycans in green. Calculations of the percentage coverage of the protein surface were determined for the three expression systems based on probes of a variety of radii ranging from amino acid side chains to the full footprint of an Ab Fab fragment (1.4–10 Å). This was done by subtracting the total solvent-accessible surface area from the same surface area after having the area covered by the glycans removed from it. The resulting value was then divided by the total solvent-accessible surface area. The calculation was performed for all three models of each of the three cell expression systems. Unpaired $t$ test was used for comparisons.
expressed material. Antigenic differences were detected in most regions in both strains of gp120, although some differences in antigenicity were specific for the individual gp120s. For gp120_{Ba-L}, the binding of the NmA b IgG1b12, which binds an epitope overlapping the CD4bs and 2G12 that binds a conserved glycan epitope, was significantly increased by expression in Sf9wt cells (Table 1). For gp120_{CN54} expression in the Sf9 systems significantly decreased binding of a C1 region mAb and the NmA b 447-52D, which binds a V3 loop epitope. As is common in a number of C-clade isolates, 2G12 failed to bind to gp120_{CN54} expressed in any system.46,47 Both gp120s showed substantial and significantly increased binding of sCD4 (8.5- and 15.7-fold for Ba-L and CN54, respectively), suggesting a dramatic increase in exposure of this surface on the Sf9-expressed material, which is in accord with the enhanced binding of IgG1b12 to gp120_{Ba-L} and computational modeling of glycan coverage (Fig. 2). By contrast, recognition of both strains of Sf9-produced gp120 by polyclonal antisera raised against mammalian-cell-expressed gp120 (ARP421 and ARP422) was significantly reduced. mAb binding to the CD4-induced (CD4i) surface was also increased for insect-cell-derived compared to mammalian-cell-derived antigen, and increases in CD4i site mAb binding were generally greater when sCD4 was present (Table 2), probably reflecting the increased sCD4 binding. Similarly, binding of mAb A32 to its complex conformational epitope was also increased for both strains when expressed in the Sf9wt system, and this binding was further enhanced for gp120_{CN54} in the presence of sCD4. The Sf9 Mimic™ and Sf9wt

**Table 1.** Comparative antigenicity of gp120_{CN54} expressed in 293F, Sf9wt and Sf9 Mimic™ cells and gp120_{Ba-L} expressed in 293F and Sf9wt cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Epitope</th>
<th>Strain</th>
<th>Sf9wt</th>
<th>Sf9 Mimic™</th>
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<tbody>
<tr>
<td>CA13</td>
<td>1</td>
<td>Ba-L</td>
<td>0.73</td>
<td>(0.12)</td>
</tr>
<tr>
<td></td>
<td>19b</td>
<td>CN54</td>
<td>(0.08)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>447-52D</td>
<td>2</td>
<td>CN54</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>LA21</td>
<td>2</td>
<td>Ba-L</td>
<td>0.08</td>
<td>0.08</td>
</tr>
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<td>2G12</td>
<td>3</td>
<td>Ba-L</td>
<td>5.11</td>
<td>ND</td>
</tr>
<tr>
<td>b12</td>
<td>4</td>
<td>Ba-L</td>
<td>3.68</td>
<td>3.68</td>
</tr>
<tr>
<td>4</td>
<td>CN54</td>
<td>0.63</td>
<td>1.29</td>
<td></td>
</tr>
<tr>
<td>sCD4</td>
<td>4</td>
<td>Ba-L</td>
<td>8.50</td>
<td>5.70</td>
</tr>
<tr>
<td>D7324</td>
<td>5</td>
<td>CN54</td>
<td>(2.49)</td>
<td>(27.00)</td>
</tr>
<tr>
<td>ARP421</td>
<td>6</td>
<td>Ba-L</td>
<td>0.22</td>
<td>ND</td>
</tr>
<tr>
<td>ARP422</td>
<td>6</td>
<td>Ba-L</td>
<td>0.55</td>
<td>0.68</td>
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</table>

Values indicate the fold increase or decrease in the 50% binding titer for the Sf9wt- or Sf9 Mimic™-expressed gp120 compared to that produced in 293F cells. Bold type indicates a statistically significant difference (P < 0.05). Where the 50% binding titer did not fall within the range of ligand utilized, the fold difference in absorbance at the maximal concentration of ligand is reported in parentheses. Epitope 1, C1 region; 2, V3 loop; 3, glycans; 4, CD4bs; 5, C5 region; 6, all regions (polyclonal antisera to gp120). ND, assay not done.
systems imparted broadly similar antigenic profiles to gp120\textsubscript{CN54} differences in the binding of the CD4i site mAb E51 in the absence of sCD4 and the C1–C4-region-specific mAb A32 in the absence or presence of sCD4 were not significant (Table 2). These data suggest that the presence of more complex glycans is not sufficient, in the absence of sialic acid, to recapitulate the mammalian cell glycosylation phenotype.

The effect of expression system on the immunogenicity of gp120

Having established that there were gp120-strain-dependent and -independent antigenic differences between gp120s expressed in the various systems, we tested their immunogenicity in Balb/c mice (Fig. 5). Since HIV-1 gp120 is a weakly immunogenic glycoprotein with potential immunosuppressive properties,\textsuperscript{32} we carried out immunization of mice with antigen in extrinsic adjuvant and compared two different adjuvants to control for the outcome of adjuvant-specific effects on gp120 immunogenicity observed by others.\textsuperscript{33,48} Groups of mice were primed subcutaneously with 10 μg of gp20\textsubscript{CN54} produced in Sf9, Sf9 Mimic™ or 293F cells, and gp120\textsubscript{Ba-L} produced in Sf9 or 293F cells formulated in a CpG-containing commercial adjuvant that triggers innate immune activation via TLR-9,\textsuperscript{49} and boosted 3 weeks later with the same amount of antigen alone. Serum samples were assayed by ELISA against a CHO mammalian-cell-expressed trimeric CN54 gp140 glycoprotein. We assayed against glycoprotein produced in a distinct system for two reasons: (i) to avoid assay bias that might occur when using glycoproteins produced in the original expression systems used to generate the test antigens; (ii) to reduce any potential cross-reactivity present in the sera against contaminants of the expression system unrelated to the recombinant

Table 2. Comparative antigenicity of the CD4i site and the A32 epitope on gp120\textsubscript{CN54} expressed in 293F, Sf9wt and Sf9 Mimic™ cells and gp120\textsubscript{Ba-L} expressed in 293F and Sf9wt cells

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Epitope</th>
<th>Strain</th>
<th>sCD4</th>
<th>Sf9wt</th>
<th>Sf9 Mimic™</th>
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<tbody>
<tr>
<td>E51</td>
<td>1</td>
<td>CN54</td>
<td></td>
<td>(2.76)</td>
<td>(1.80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>(2.67)</td>
<td>(2.82)</td>
</tr>
<tr>
<td>X5</td>
<td>1</td>
<td>Ba-L</td>
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<td>(8.09)</td>
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<td></td>
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<td></td>
<td>+</td>
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<td></td>
<td>+</td>
<td>10.10</td>
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<tr>
<td>17b</td>
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<td>Ba-L</td>
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<td>16.90</td>
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</table>

Table format is similar to Table 1 except that the extra column denotes the presence or absence of recombinant sCD4 added to the ELISA prior to and during the incubation with the ligand shown at a final concentration of 1 μg/mL; underlined type indicates that the addition of sCD4 significantly increased the binding of the ligand to the gp120 antigen from the indicated expression system. Epitope 1, CD4i site; 2, complex conformational epitope involving residues of regions C1–C4. NR, no ratio calculated due to the net ELISA absorbance equaling zero for the assay with 293F-cell-expressed gp120\textsubscript{CN54} and sCD4 plus mAb A32. Both the Sf9wt and Sf9 Mimic™ produced positive ELISA signals.

Fig. 5. The influence of expression system on gp120 immunogenicity. (a) Groups of five Balb/c mice were primed with Sf9wt, Sf9 Mimic™ or 293F-cell-expressed gp120\textsubscript{CN54}, or Sf9wt or 293F-expressed gp120\textsubscript{CN54} formulated in CpG-based adjuvant at week 0 and boosted with antigen alone at week 3. Results show reciprocal antigen-specific IgG endpoint titers at 6 weeks after the boost. (b) Groups of six Balb/c mice were primed with Sf9wt or 293F-cell-expressed gp120\textsubscript{CN54} or gp120\textsubscript{Ba-L} emulsified in FCA. Results show antigen-specific reciprocal IgG endpoint titers at 3 weeks after the prime. Box-and-whisker plots denote the median, 25th and 75th percentile and the outlier total IgG titer determined from three independent repetitions of the ELISAs. *P<0.05.
antigen under analysis. We found that antigens expressed in Sf9 and Sf9 Mimic™ cells induced significantly greater antigen-specific serum IgG responses compared to 293F-expressed gp120 (Fig. 5a). We subsequently immunized mice with a subset of the mammalian- and insect-cell-derived antigens in Freund’s complete adjuvant (FCA), a potent adjuvant that acts largely independently of TLR-receptor-mediated mechanisms, and hence has a mode of action different from that of the CpG adjuvant used previously. As before, the titers of antigen-specific IgG produced against CN54 and Bal-L gp120 produced in insect cells were significantly higher than those against the gp120s produced in mammalian cells (Fig. 5b). We therefore conclude that insect-cell-derived gp120 is more immunogenic than its mammalian-cell-expressed counterpart irrespective of the type of adjuvant used or its mode of action.

Since the antigenicity analysis demonstrated that certain epitopes were differentially recognized on the insect- compared to the mammalian-cell-expressed gp120, we considered that this might translate into differential recognition of these epitopes by B cells in vivo. We therefore analyzed sera derived from mice immunized with gp120Bal-L or gp120CN54 produced in the two systems for their ability to compete with mAbs or soluble receptor probes of defined specificity. For the competition assay, we used CHO-cell-derived gp140CN54 detection as before. Initial titrations of the sera into this ELISA determined the optimal dilutions for use in the competition assay (data not shown). Because the volumes of mouse serum were limiting we were only able to analyze competition with a small number of probes, and so we chose ligands that bound gp140CN54 with high avidity that were specific for the CD4bs (mAb HJ16 and CD4-IgG2) and mAbs specific for the gp120 V2 and V3 loops (HG68 and HR10, respectively). Figure 6 shows that sera from the mice immunized with Sf9-derived material competed significantly more with both

![Figure 6. Analysis of serum Ab specificity by competition ELISA.](image)
CD4bs-specific reagents than sera from mice immunized with 293F-derived material. These results are in line with the antigenicity data presented in Table 1 that demonstrate increased binding of CD4bs-specific probes to insect-cell-derived compared to mammalian-cell-derived gp120. A similar relative increase in competition was seen with gp120CN54-derived serum for the V3 mAb but not for the V2 mAb, demonstrating that the increased competition observed with sera derived from insect cell-produced gp120 was not global in nature, but restricted to specific epitopes. Only modest competition was observed for the V2 and V3 loop mAbs with sera from gp120Ba-L-immunized mice: this result was to be expected, since the gp140CN54 detection antigen would be unlikely to contain many appropriately cross-reactive epitopes within these hypervariable regions.

The influence of kifunensine on gp120 antigenicity and immunogenicity

The antigenicity of the 5 and 20 μM kifunensine-treated gp120Ba-L was probed with mAbs, sCD4 and a recombinant immunoglobulin–CD4 chimeric protein expressing four gp120 binding sites, CD4-IgG2: very similar results were obtained for both, and so only the 5 μM data are shown here. Few significant differences were observed for the wt compared to kifunensine-treated gp120 (Table 3). Exceptions were the CD4bs ligands IgG1b12 and CD4-IgG2 that showed significantly increased binding to the kifunensine-treated material. This is consistent with the increased binding of ligands to the CD4bs observed with insect-cell-derived compared to mammalian-cell-derived material. In addition, the binding of the V3 loop mAb 19b and the glycan-specific broadly neutralizing mAb 2G12 were significantly increased by the treatment.

The immunogenicity of gp120 from untreated wt and kifunensine-treated 293F cells was analyzed by immunization of Balb/c mice in the CpG-based adjuvant, and sera were titrated on both untreated and kifunensine-treated immunogens to take into account assay bias introduced by the modification. Sera were tested for antigen-specific IgG 3 weeks after the antigen-alone boost at week 6. Antigen-specific IgG responses were detected against gp120 from both wt and kifunensine-treated 293F cells (Fig. 7). The responses against gp120 from the kifunensine-treated cells were greater than those against wt gp120, but the differences did not reach significance when titrated on wt gp120. By contrast, mice immunized with gp120 from the kifunensine-treated cells achieved a 3.8-fold higher IgG titer against the kifunensine-treated gp120 compared to wt gp120, and this difference was significant (P=0.0496). This result implies that eliminating sialic acid by kifunensine treatment increased Ab responses to gp120, but this was only detectable using kifunensine-treated material in the ELISA, presumably reflecting recognition of epitopes better exposed on the modified compared to unmodified gp120.

<p>| Table 3. Comparative antigenicity of gp120Ba-L expressed in 293T cells in the presence or absence of 5 μM kifunensine |</p>
<table>
<thead>
<tr>
<th>Ligand</th>
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<th>Kifunensine</th>
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<td>447-52D</td>
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<tr>
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<td>CD4-IgG2</td>
<td>3</td>
<td>6.08</td>
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<tr>
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</tr>
<tr>
<td>21h</td>
<td>3</td>
<td>(0.96)</td>
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<tr>
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<tr>
<td>ARP440</td>
<td>4</td>
<td>1.38</td>
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</table>

Numbers indicate the fold increase or decrease in the 50% binding titer for the gp120Ba-L, expressed in the presence of kifunensine compared to that produced in untreated cells. Bold type indicates a statistically significant difference (P<0.05). Where the 50% binding titer did not fall within the range of ligand utilized, the fold difference in absorbance at the maximal concentration of ligand is reported in parentheses. Epitope 1, V3 loop; 2, glycans; 3, CD4bs; 4, all regions (polyclonal immunoglobulin from HIV-1-infected patients or rabbit antisera to gp120).
Discussion

Here we have examined the influence of expression system on the antigenic and immunogenic characteristics of HIV-1 gp120 and constructed three-dimensional (3D) models to facilitate our understanding of mechanisms underlying these differences. Production of gp120 in insect cells resulted in substantially different antigenicity from the equivalent material produced in mammalian cells and immunogenicity that was increased compared to mammalian-cell-expressed gp120 (Fig. 8). These results have obvious implications for the design of vaccine antigens based on expression of recombinant HIV-1 Env. Firstly, alterations in antigenicity will influence the exposure and/or conformation of particular epitopes and therefore their presentation to B cells, which will influence the specificity of the Ab response. This might be beneficial or detrimental for induction of NAb, since alterations in antigenicity will modify both neutralization-relevant and -irrelevant epitopes. gp120 expression in insect cell systems preferentially increased mAb and soluble receptor binding to the CD4bs and CD4i surfaces, probably as a result of decreased glycan bulk increasing exposure of the underlying protein surface. Since IgG1b12 is a broadly neutralizing mAb recognizing a conserved surface on gp120, the increase in the binding of this NmAb to gp120Ba-L expressed in insect cells might favor the induction of similar specificities by immunization. In this respect, our competition ELISA data support the idea that the CD4bs is not only more exposed on insect-cell-produced gp120, but is also more immunogenic, a finding of potential importance for Ab-based vaccine design. Although thus far it has not proved possible to recapitulate IgG1b12-like responses in vivo by immunization, such responses have been detected in HIV-1-infected individuals, suggesting that this region is immunogenic. Since increased IgG1b12 binding to insect-cell-expressed gp120 was viral isolate specific, occurring in gp120Ba-L but not in gp120CN54, this may be epitope-context-dependent and not a broadly applicable observation. Indeed, virus strain selectivity of IgG1b12 binding to glycan variants may explain why a previous study found that gp120 derived from the IIIB isolate produced under

![Fig. 8. Outline of experiment and summary of results. Arrows denoting gp120 produced in untreated 293F cells (continuous black lines), kifunensine-treated 293F cells (dotted black), Sf9 Mimic™ cells (dotted gray) and Sf9 cells (gray) indicate conclusions drawn about them from computational modeling, in vitro Ab binding assays and measurements of anti-gp120 serum titers.](image)
kifunensine treatment showed increased binding of the 2G12 Ab compared to wt gp120, but failed to show substantially increased IgG1b12 binding. However, the improved recognition of the CD4bs by sCD4 on both Ba-L and CN54 insect-derived gp120 may stimulate increased presentation of other neutralizing epitopes on this relatively conserved surface to B cells in a more generalized manner, such as that recognized by the novel CD4bs-specific mAbs HJ1654 and VRC01, VRC02 and VRC03. Kifunensine treatment of gp120Ba-L increased binding of IgG1b12 and CD4-IgG2, consistent with the reduction in glycan bulk rendering the CD4bs more accessible to ligands. The increased binding of NmAb 2G12 to gp120Ba-L is also of interest for vaccine development and may reflect a higher avidity of 2G12 for its epitope when the gp120 expresses a higher proportion of terminal mannose glycans. Consistent with this interpretation, kifunensine treatment also increased 2G12 binding to gp120Ba-L in a manner analogous to that previously described for enhanced 2G12 recognition of kifunensine-treated mammalian cells and mammalian-cell-produced gp120. The increase in CD4i epitope exposure was, with two exceptions (X5 on gp120CN54 and 17b on gp120Ba-L), generally applicable to both gp120s. It may be that reducing the bulk of the glycans allows greater exposure of CD4i surfaces that are otherwise masked in mammalian-cell-expressed material. A second explanation may be that increased CD4i mAb binding reflects increased numbers of gp120 with bound sCD4. Alternatively, reducing glycan complexity and charge may influence folding of gp120 molecules. Regardless of the mechanism, it seems unlikely that the CD4i surface is a useful target for induction of neutralizing antibodies, as it is largely inaccessible to IgG on the native viral spike either prior to or during viral entry. Our analysis shows that glycan structure will impact upon analyses of glycoprotein structure and function and should be taken into account. One obvious example from our work is that the affinity of sCD4 for insect-cell-derived material is substantially higher than for mammalian-cell-derived material and may bias interpretations of HIV-1 Env–receptor interaction energetics. Caution should therefore be exercised when extrapolating from data acquired with expression systems that differ from those in which virus would normally be produced in vivo.

We enriched the insect-cell-derived and mammalian-cell-derived gp120s by using a Galanthus nivalis lectin column that has high affinity for mannose groups. We cannot exclude that this enrichment selected for specific glycoforms of gp120 that could influence the antigenicity of the material. However, since both insect- and mammalian-cell-derived gp120 naturally presents clusters of terminal mannose groups, it seems unlikely that this would substantially bias selection of particular variants. This conclusion is consistent with the mass spectral analysis, which suggests that the glycan composition of the differentially produced gp120s reflects that described by others using different purification strategies. It is possible that a proportion of the gp120 populations that we have probed antigenically may consist of mixtures of conformers or potentially even some misfolded material, leading at least in part to the differences in antigenicity observed. However, one of the principal tools for probing the conformational integrity of an antigen is by Ab binding to conformational and discontinuous epitopes. Since all forms of gp120 tested here reacted with all conformation-dependent mAbs and with sCD4, we assume that a major fraction of the glycoproteins is most likely to be properly folded. Some differences observed in antigenicity were Env isolate dependent rather than expression system dependent, adding weight to the idea that the differences observed were due to intrinsic structural features of the glycoproteins rather than protein misfolding.

Our finding that the insect-cell-expressed gp120 antigens are more immunogenic in the context of adjuvant than the same antigens expressed in mammalian cells is of interest for vaccine development. To our knowledge, only one other study has compared immunogenicity of insect cell (Drosophila S2) and mammalian-cell-derived (293F) gp120. Grundner and colleagues administered mammalian-cell-expressed gp120 in Ribi adjuvant with insect-cell-expressed wt or gp120 containing additional T-helper epitopes administered in Ribi and FCA/Freund’s incomplete adjuvant (FIA). In this study, immunogenicity appeared to be dependent on the adjuvant used, since no immunogenicity for the S2-derived gp120 was observed in Ribi, whereas robust immunogenicity was observed for the same glycoprotein in FCA/FIA and also in the presence of the T-helper epitope. However, further comparison between the studies is difficult, since Grundner et al. assayed sera pooled from each group and hence were unable to carry out quantitative statistical analysis. Another study compared demannosylated mammalian-cell-derived gp120 with its untreated counterpart, which differs from our analysis of insect- compared to mammalian-cell-produced gp120, and concluded that demannosylation increased immunogenicity in an adjuvant-dependent context. Thus, when administered in alum, there was a significant increase in immunogenicity of the demannosylated material compared to the untreated gp120, but this difference disappeared when administered in QuilA adjuvant. Therefore, we conclude that gp120 immunogenicity is dependent on both the presence and the proportion of terminal mannose-containing glycans and on the adjuvant used to elicit the immune response. Our findings of
increased humoral immunogenicity with insect-cell-expressed gp120 concur with prior findings of increased cellular immunogenicity and support the idea that the insect-cell-expressed gp120 may be processed and presented more efficiently by antigen-presenting cells in vivo.

Full consideration of the effects of expression system on the antigenicity and immunogenicity of a candidate vaccine antigen should be taken into account when assessing which system should be employed. This is particularly true for antigens based on HIV-1 Env, which is a highly conformational, highly glycosylated antigen in development as a recombinant subunit immunogen for eliciting neutralizing antibodies. In this respect, the choice of adjuvant will be of central importance, since this will influence the folding and presentation of the antigen to the immune system. Based on our data, an insect cell system or kifunensine treatment of mammalian-cell-derived material, which increases immunogenicity while simultaneously increasing the presentation of epitopes, including those of IgG1b12 and 2G12, might be beneficial. However, the final choice must be further informed by the relative ability to induce durable, high-titer and broadly active NAb responses and cellular immune responses, a question best assessed empirically in vivo.

Materials and Methods

Reagents

Abs, mAbs and other reagents were obtained from the same sources as previously described, with the exception of CD4-IgG21 (manufactured by Progenics Inc. and obtained from The International AIDS Vaccine Initiative Neutralizing Antibody Consortium Repository); CD4bs-specific mAbs 15e and 21h [obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program]; the rabbit antiserum to gp120, ARP421 [obtained from the Centralised Facility for AIDS Reagents CFAR, National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK; donated by S. S. Ranjan]; D7324, the goat pAb to the gp120 C-terminal region (from Aalto Bio Reagents Ltd, Dublin, Ireland); human mAbs H16 (CD4bs), HR10 (V3) and HG68 (V2) from D. Corti and A. Lanzavecchia. Anti-species immunoglobulin–HRP conjugates were obtained from Jackson ImmunoResearch Europe Ltd (Soham, UK). HIV-1 Env coding for soluble gp140CN54, gp120CN54 and gp120bs-L were subcloned into the vector pTri-Ex1.1 (Invitrogen) with a C-terminal 6×His tag. Vectors were expressed in 293F cells following mass transfection and gp120CN54 and gp120bs-L in S9 and S9 Mimic™ cells (S9wt and S9 Mimic™, respectively) following infection with recombinant baculoviruses. gp120bs-L was expressed in 293F cells in the presence or absence of 5 or 20 μM kifunensine (TRC, Canada). Glycoproteins were purified by a combination of lectin followed by nickel (reactive with 6×His tag)-affinity chromatography. Briefly, supernatants were initially enriched on the G. nivalis Sepharose column (Vector Laboratories), followed by buffer exchange with ProfBond native binding buffer (Invitrogen) supplemented with 10 mM imidazole and purification using the Invitrogen ProfBond purification system. Eluted glycoproteins were buffer-exchanged with 20 mM Tris (pH 7.4), aliquoted and frozen at −70 °C until use. Glycoprotein purity was confirmed by SDS-PAGE and Coomassie blue staining, and all preparations were estimated to be ≥90% pure.

Mice, immunization and sampling

Balb/c mice (8–12 weeks old) were obtained from the specified pathogen-free animal breeding facility of the Sir William Dunn School of Pathology (University of Oxford, UK). All experiments were performed under appropriate license in accordance with the UK Animals (Scientific Procedures) Act 1986. Antigens were screened for endotoxin content and contained ≤ 1 EU/mL. Antigens at doses of 2, 5 or 10 μg as described in figure legends were formulated in phosphate-buffered saline (PBS) alone, emulsified with FCA at a 1:1 volume ratio, or with the CpG-based adjuvants ImmunEasy (Qiagen) (10 μL per vaccine dose) or CpG 1018 (MWG Biotech) (50 μg per vaccine dose) formulated in 25-kDa branched polyethyleneimine (PEI, Sigma-Aldrich) at an N/P ratio of 15. The immunogen preparations were made up to 100 μL using sterile, endotoxin-free 5% (w/v) D-glucose (Sigma-Aldrich) and injected subcutaneously into the right flank. Typically, immunizations were given at weeks 0, 3 and 6, with test tail bleeds (50 μL) collected immediately prior to each immunization.

ELISAs

ELISAs were performed as described previously except the plates were washed six times following the addition of antisera or mAbs and after the addition of the anti-species IgG–HRP conjugates. The absorbance of preimmune serum was subtracted from that of the postvaccine bleeds before calculation of the endpoint titer by interpolation of the point of intersect between the assay cutoff and a sigmoid dose–response curve was fitted to the dilution series in GraphPad Prism v5.0.

mAb, sCD4 and CD4-IgG2 binding assays

Antigens produced in the various expression systems were coated onto ELISA plates and probed with anti-Env Abs or CD4-IgG2 as described. For the sCD4 binding study, sCD4 was coated onto the ELISA plate at concentrations ranging from 0.31 to 20 nM. The gp120 antigens were then added at a concentration of 1 μg/mL and coincubated for 1 h at room temperature (RT) before the addition of sample buffer (10 μL per well) containing sixfold the median binding titer of ARP422, such that the final concentration of ARP422 equated its 50% binding titer. After a further 1 h at RT, the plates were washed and the ARP422 was detected using anti-rabbit IgG–HRP followed by the standard ELISA development protocol. For the assays in which sCD4 was added before the ligand, gp120-coated wells were incubated with 50 μL of
a 2 μg/mL solution of scD4 for 30 min at RT before the addition of 50 μL of a twofold concentrated dilution series of the ligand without washing out the scD4. Tittrations were performed in three to five replicates per plate and on at least two independent occasions, and comparisons were made between the median relative binding avidity (in nM⁻¹) or the 50% binding titer in the case of antiseras. Ligands that did not reach saturating levels were compared by analysis of the absorbance obtained at the highest concentration of ligand used. The mAb competition ELISA to assay for specificity in immune mouse serum was carried out as follows. CHO-derived CN54 gp140 was coated onto the ELISA plate and the plate was blocked and washed as described previously. Test or control (immune mouse serum from mice immunized with hen egg lysozyme formulated in FCA) serum diluted to 1:50 in 50 μL of PBS-1% bovine serum albumin was added to the plate for 1 h at RT, followed by one of the competition mAbs to yield a final concentration previously determined to give 50% binding in this assay format. Plates were washed three times and bound mAb was detected with anti-human IgG–HRP at 1:1000 in PBS-1% bovine serum albumin for 1 h at RT followed by washing, addition of substrate, stopping and reading as previously described. The percent inhibition was calculated using the equation 100 − [(test value − control value)/(maximum value − control value)] × 100.

Modeling N-linked glycans on gp120 expressed in Sf9 and kifunensine-treated and untreated 293F cells

We produced models of gp120 as it would be produced by expression in Sf9, untreated 293F or kifunensine-treated 293F cells. To mimic gp120 produced in 293F cells under kifunensine treatment, we modeled high-mannose glycans, which have been characterized on gp120 of the HIV-1BH8 isolate expressed in insect cells, on the structure glycans, which have been characterized on gp120 of the under kifunensine treatment, we modeled high-mannose treated 293F cells. To mimic gp120 produced in 293F cells by expression in Sf9, untreated 293F or kifunensine- and kifunensine-treated and untreated 293F cells

Modeling N-linked glycans on gp120 expressed in Sf9 infected lymphoblastoid (H9) cells, on the structure of N-linked glycan sites at amino acids 289, 397, 406, 448 and − was calculated using the equation 100 − [(test value − control value)/(maximum value − control value)] × 100. A particular glycan was modeled on a site if the numeric range equal to the molar percentage of the chromatography fraction, in which the glycan was characterized. The numeric ranges for complex glycans were separated from those for high-mannose glycans, forming two groups. The ranges in each group were normalized to 100. Because several chromatography fractions from the attempt to characterize the glycan types remained unknown, their molar percentages were evenly distributed among the other known fractions. Again, each N-linked glycan site was given a random number between 0 and 100. Each site was also labeled high-mannose or complex depending on the type of glycan determined by Leonard et al. A particular glycan was modeled on a site if its random number fell within that glycan’s numeric range and if the site’s label corresponded to the glycan’s type. Three variants of gp120 from 293F cells were generated with this method (Table S2).

To mimic Sf9 glycosylation, we stripped all complex sugars from the models for 293F glycosylation and replaced them with Man3F1. Furthermore, all high-mannose sugars containing nine mannose residues were trimmed down to six mannose residues. This was done so that the model would better match the mass spectrometry data on gp120 expressed from Sf9 cells (Table S3). Glycans were modeled on the structure of gp120HxBc2 with Glyprot. These models were refined in two steps with CNS. In the first step, they underwent simulated annealing, in which they were heated to 300–3000 K, freeing them from the local energy minimum. Then, they were slowly cooled in steps of 25–30 K so that they approached a global energy minimum. Three trials were done for each condition, and the condition that yielded models that differed most from the starting model was selected. In the second step, a model from the selected condition was subject to further molecular dynamics at 310 K for up to 10⁶ steps at 10-fs steps until its rmsd from the initial model approached a plateau. This effectively placed the model in an energy-minimized region at physiological temperature.

To quantify the extent of glycan coverage of protein surface, we analyzed the refined models with spherical probes of different radii as previously described. A 1.4 Å probe approximates the penetration of a side chain and a 2.5 or 5.0 Å probe approximates that of a beta hairpin turn. A 10 Å probe approximates to the reach of an entire Ab combining region. These probes were used to calculate the accessible surface of the models. Using GRASP, we determined the accessible surface area excluded by the glycans by finding the surface area of the polypeptide within carbon’s van der Waal’s radius, or 1.7 Å, of all glycan surfaces. These values were normalized over the accessible surface area of the entire polypeptide to obtain the percent glycan coverage.

Statistics

Analyses were performed with GraphPad Prism v 5.0. Raw or log₁₀-transformed data were only treated as

‡ http://www.glycosciences.de/modeling/glyprot/php/main.php
normally distributed if this was first demonstrated by the Kolmogorov–Smirnov normality test with a Dallal–Wilkinson–Lilliefors P value. For normally distributed data, comparisons of more than two groups were carried out using the one-way ANOVA test. Comparison of two data sets used unpaired t tests. For data that were not normally distributed, Kruskal–Wallis analysis was performed to compare more than two data sets. The Mann–Whitney test was used to compare two data sets. Bonferroni’s correction was applied when multiple two-sample tests were performed to compare more than two data sets. For data that were not normally distributed, the Wilcoxon–Mann–Whitney test was used to compare two data sets.

Accession numbers

The nucleotide sequences for HIV-1 97CN001 (CN54), Ba-L and HxBc2 have been deposited in the GenBank database under the GenBank accession numbers AF286226, AB221005 and K03455, respectively. The atomic coordinates for the crystal structure of gp120_{Ba-L} and gp120_{HxBc2} are available in the Molecular Modeling Database (MMDB) under MMDB number 8099.

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2010.08.033

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