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EXPRESSION SYSTEM-DEPENDENT MODULATION OF HIV-1 ENVELOPE GLYCOPROTEIN ANTIGENICITY AND IMMUNOGENICITY

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Abstract

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 (293F) cells in the presence of kifunensine which impart only high mannose glycans; b) insect (*Spodoptera frugiperda*, Sf9) cells, which confer mainly paucimannosidic glycans; c) Sf9 cells recombinant for mammalian glycosylation enzymes (Sf9 MimicTM), which impart high mannose, hybrid and complex glycans without sialic acid; d)

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The nucleotide sequences for HIV-1 97CN001 (CN54), Ba-L and HxBc2 have been deposited in the GenBank database under the GenBankAccession Numbers AF286226, AB221005 and K03455 respectively. The atomic coordinates for the crystal structure of gp120HxBc2 are available in the Molecular Modeling Database (<http://www.ncbi.nlm.nih.gov/structure/MMDB/mmdb.shtml>) under MMDB # 8099.

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- and wild-type mammalian cell-derived material that is predicted to impact upon ligand binding sites proximal to glycans. Modelling 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 that does, 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 titres 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 which should be considered when selecting expression systems for glycosylated antigens to be used for structure/function studies and for vaccine use.

The only viral target for neutralizing antibodies (NAb) against HIV-1 are the viral envelope glycoproteins (Env), comprised of a heterotrimer of three surface subunits (gp120) non-covalently 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 towards this goal, considerable optimization of the antigen, adjuvant and delivery strategy will be required. This will include an understanding of the impact of Env glycosylation with regard to its effects upon 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^{1; 2; 3; 4; 5} 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^{2; 6; 7; 8}. 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^{6; 9}. Conversely, in wild-type insect cell systems glycosylation is restricted to paucimannosidic structures¹⁰ and the degree of sequon occupancy may be lower^{11; 12}.

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 post-translational 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 cells¹³. 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^{14; 15; 16}. 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 animals¹⁷ and immunogenicity in humans¹⁸. 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 impact of expression system on antigenicity in a head-to-head fashion, although one recent analysis compared gp120 expressed in different human cells lines for glycan content and relative binding to polyclonal sera⁸. By contrast, many have examined the effects of enzymatic removal of glycans or mutations deleting selected glycan sequons^{19; 20; 21; 22; 23; 24}. 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^{25; 26}. The site-specific deletion of glycan sequons from Env increased recognition by important neutralizing (N)mAbs such as b12, 447-52D and 2F5, especially when the mutants were expressed in insect cells²⁰. Moreover, deletion of three-to-five Env glycan sequons from wild-type SIVmac239 virus resulted in dramatic attenuation in rhesus macaques and the induction of substantial protection against the wild-type virus with higher titers of NABs than those obtained after wild-type virus challenge^{21; 22}. Disappointingly however, subunit antigens based on such Env glycan mutants proved to be less good, or at best equivalent to, wild-type Env antigens at inducing NAb responses or controlling viremia in animal models^{19; 21; 23; 24}. 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 important for folding while the rest may function to block antibody binding, suggesting that for immunogen design glycans may need to be re-engineered 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-acetylglucosamine transferase I, resulting in increased binding of ligands to the CD4bs and the V3 loop²⁷.

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 receptors²⁹. 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 glycans³⁰. The latter supposition is in agreement with the finding that gp120 expressed in insect cells was better able to induce CTL responses in Balb/c mice than that produced in mammalian Chinese hamster ovary (CHO) cells³¹. The reduced response to the CHO-cell material was overcome by enzymatic deglycosylation, suggesting that mammalian glycans were responsible for the poor immunogenicity³¹. An alternative view was recently proposed that terminal mannose groups might downmodulate antibody 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 serum³⁴, 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: wild-type Sf9 (Sf9wt) and Sf9 MimicTM, with a mammalian system: 293 FreeStyleTM (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 MimicTM 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 and thus the complex glycans they produce have terminal galactose residues³⁷. The inclusion of this additional cell line allows the contribution of

complex glycans lacking sialic acid to antigenicity and immunogenicity 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 gene³⁸. 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 origin^{39; 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 using ClustalW (v1.83)⁴², showed that the strains are 26.3% divergent, with gp120_{CN54} 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 Net-O-Glyc (v3.1)⁴⁴ revealed that gp120_{CN54} has 23 sequons for N-linked glycosylation and no predicted sites for mucin-type O-linked glycosylation, whereas gp120_{Ba-L} has 22 sequons for N-linked glycosylation and 1 potential site for mucin-type O-linked glycosylation. 15 of the N-linked glycosylation sites were conserved between the two strains with 12 occurring in conserved regions of the glycoprotein. Gp120_{CN54} 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 gp120_{Ba-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 gp120_{Ba-L} produced in Sf9 cells, untreated 293F cells and 293F cells treated with 5 and 20 μ M kifunensine, an inhibitor of an inhibitor of class I α -mannosidases. We confirm results from a previous study¹⁰, that Sf9-expressed gp120 contains mostly Man₃GlcNAc₂ but retains a minor population of untrimmed oligomannose structures that includes the 2G12 NmAb 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^{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 Sf9wt- and 293F-expressed gp120 to glycosylation type rather than number, we expressed gp120_{Ba-L} in 293F cells in the presence or absence of 5 or 20 μ M kifunensine. To confirm that kifunensine treatment inhibited the processing of Man₉GlcNAc₂, we performed mass spectrometry on gp120 produced in the presence and absence of the inhibitor (Fig. 1). Cultivation of gp120_{Ba-L}-expressing 293F cells in the presence of 5 μ M kifunensine completely prevented complex glycosylation and resulted in the unprocessed Man₉GlcNAc₂ 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: Man₈GlcNAc₂, Man₇GlcNAc₂, Man₅GlcNAc₂ and Man₆GlcNAc₂. Increasing the kifunensine concentration to 20 μ M better inhibited the mannosidases as only three glycan types could be detected: Man₉GlcNAc₂, Man₈GlcNAc₂ and Man₇GlcNAc₂.

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 upon predicted N-linked glycosylation of the 293F, kifunensine-treated 293F and Sf9wt 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 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 S1–3. Glycans conferred by Sf9 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 for glycans from Sf9 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 Sf9 cells are principally processed to Man₃F₁-containing structures with a minor population of oligomannose structures.

A representative of the refined models for gp120_{HxBc2} expressed in 293F, kifunensine-treated 293F and Sf9 cells are shown in Figure 2a, b and c respectively. The extent of gp120 glycosylation is evident from the models, in which the protein core is labelled red, glycans containing terminal mannose are cyan, and complex glycans green. Using all these models, glycan coverage was quantified 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 (1.4Å), beta turns (2.5Å and 5Å) and an antibody combining region (10Å) (Fig. 3). Although models for the same expression system vary greatly in glycan composition (Tables S1–3), 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 293F 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 modelled the interaction with a probe of radius 10Å, equivalent to an entire antibody-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 cell-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 antibody epitope and the CD4bs⁴⁵.

Another property that sialic acid imparts onto a glycoprotein that might influence antibody 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 modelled how the typical Sf9wt- and 293F-imparted glycan structures affect the protein surface electrostatic potential (Fig. 4 a and b). As predicted, gp120 expressed in

untreated mammalian cells is modelled to have a net negative average electrostatic potential of -2.1 k/Te. However, when expressed in other 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_{CN54} expressed in 293F, Sf9wt and Sf9 MimicTM and gp120_{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 pAb D7324, sCD4, and polyclonal antisera, for expression system-dependent antigenic differences. The median 50% binding titers obtained from 3 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 MimicTM-expressed material compared to 293F-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 NmAbs IgG1b12, which binds an epitope overlapping the CD4bs⁴⁵ and 2G12 which binds a conserved glycan epitope, were 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 NmAb 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 modelling 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 MimicTM and Sf9wt systems imparted broadly similar antigenic profiles to gp120_{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 properties³², 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^{33; 48}. Groups of mice were primed subcutaneously with 10 μ g of gp120_{CN54} produced in Sf9, Sf9 MimicTM or 293F cells, and gp120_{Ba-L} produced in Sf9 or 293F cells formulated in a CpG-containing commercial adjuvant that triggers innate immune activation via TLR-9⁴⁹, and boosted three 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 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), an potent adjuvant that acts largely independently of TLR receptor mediated mechanisms⁵⁰, and hence has a different mode of action to the CpG adjuvant used previously. As before, the titres of antigen-specific IgG produced against CN54 and Ba-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 gp120_{Ba-L} or gp120_{CN54} 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 gp140_{CN54} 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 gp140_{CN54} with high avidity that were specific for the CD4bs (mAb HJ16 and CD4IgG2) 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 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 gp120_{CN54}-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 gp120_{Ba-L}-immunized mice: this result was to be expected since the gp140_{CN54} 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 μ M kifunensin-treated gp120_{Ba-L} was probed with mAbs, sCD4 and a recombinant immunoglobulin-CD4 chimeric protein expressing 4 gp120 binding sites, CD4-IgG2⁵¹: very similar results were obtained for both and so only the 5 μ M data are shown here. Few significant differences were observed for the wt compared to kifunensin-treated gp120 (Table 3). An exception was 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 neutralising mAb 2G12 were significantly increased by the treatment.

The immunogenicity of gp120 from untreated wt and kifunensine-treated 293F cells was analysed 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 titre 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 antibody 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.

DISCUSSION

In the present study we have examined the influence of expression system on the antigenic and immunogenic characteristics of HIV-1 gp120, and constructed 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 upon 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 antibody response. This might be beneficial or detrimental for induction of NAb, since alterations in antigenicity will modify both neutralization-relevant and neutralization-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 recognising a conserved surface on gp120⁴⁵, the increase in the binding of this NmAb to gp120_{Ba-L} expressed in insect cells might favour 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 antibody-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 immunogenic⁵². Since increased IgG1b12 binding to insect cell-expressed gp120 was viral isolate-specific, occurring in gp120_{Ba-L} but not gp120_{CN54}, 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 kifunensine treatment showed increased binding of the 2G12 antibody compared to wt gp120, but failed to show substantially increased IgG1b12 binding⁵³. 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 VRC03⁵⁵. Kifunensine treatment of gp120_{Ba-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 gp120_{Ba-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 gp120_{Ba-L}, in a manner analogous to that previously described for enhanced 2G12 recognition of kifunensine-treated mammalian cells and mammalian cell-produced gp120⁵³. The increase in CD4i epitope exposure was, with two exceptions (X5 on gp120_{CN54} and 17b on gp120_{Ba-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 entry⁵⁶. 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 using expression systems that differ from those in which virus would normally be produced *in vivo*.

We enriched the insect cell and mammalian cell-derived gp120s 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 present clusters of terminal mannose groups, it seems unlikely that this would substantially bias selection of particular variants. This conclusion is consistent with the MS analysis which suggests that the glycan composition of the differentially-produced gp120s reflects that described by others using different purification strategies^{10; 57; 58; 59}. 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 antibody 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) gp120⁴⁸. Grunder 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/FIA adjuvants. In this study, immunogenicity appeared to be dependent both upon 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 on the presence of the T helper epitope⁴⁸. 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 de-mannosylated mammalian cell-derived gp120 with its untreated counterpart, which differs from our analysis of insect compared to mammalian cell-produced gp120, and concluded that de-mannosylation increased immunogenicity in an adjuvant-dependent

context³³. 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 adjuvant³³. Our conclusion is therefore that gp120 immunogenicity is dependent upon both the presence and 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 immunogenicity³¹ 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 upon 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 upon 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

Antibodies (Abs), mAbs and other reagents were obtained from the same sources as previously described⁶⁰, with the exception of: CD4-IgG2⁵¹ manufactured by Progenics Inc. and obtained from The International AIDS Vaccine Initiative Neutralizing Antibody Consortium Repository; CD4-binding site (CD4bs)-specific mAbs 15e and 21h obtained from the National Institute of Health (NIH) AIDS Research and Reference Reagent Program; the rabbit antiserum to gp120, ARP421 obtained from the Centralised Facility for AIDS Reagents (CFAR, NIBSC, Potters Bar, UK. Donated by S. S. Ranjbar); D7324, the goat polyclonal Ab (pAb) to the gp120-C-terminal region, from Aalto Bio Reagents Ltd (Dublin, Ireland); human mAbs HJ16 (CD4bs), HR10 (V3) and HG68 (V2) from D. Corti and A. Lanzavecchia⁵⁴. Anti-species immunoglobulin-HRP conjugates were obtained from Jackson ImmunoResearch Europe Ltd (Soham, UK). HIV-1 *env* coding for soluble gp140_{CN54}, gp120_{CN54} and gp120_{Ba-L} were subcloned into the vector pTri-Ex1.1 (Invitrogen) with a C-terminal 6 × His tag. Vectors were expressed in 293 FreeStyle™ (293F) cells following mass transfection and gp120_{CN54} and gp120_{Ba-L} in Sf9 and Sf9 Mimic™ cells (Sf9wt and Sf9 Mimic™ respectively) following infection with recombinant baculoviruses. Gp120_{Ba-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 Galanthus nivalis sepharose column (Vector Laboratories), followed by buffer exchange with ProBond native binding buffer (Invitrogen) supplemented with 10 mM imidazole and purification using the Invitrogen ProBond purification system. Eluted glycoproteins were buffer exchanged with 20 mM Tris pH 7.4, aliquotted 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

8–12 week old Balb/c mice 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 licence in accordance with the UK Animals (Scientific Procedures) Act 1986. Antigens were screened for endotoxin content and contained $\leq 1 \text{ EU.mL}^{-1}$. Antigens at doses of 2, 5 or 10 μg as described in figure legends were formulated in PBS alone, emulsified with Freund's Complete Adjuvant (FCA) at a 1:1 volume ratio, or with the CpG-based adjuvants ImmunEasy (Qiagen) (10 μL /vaccine dose) or CpG 1018 (MWG Biotech) (50 μg /vaccine dose) formulated in 25 kDa, branched 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 previously⁶⁰ 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 pre-immune serum was subtracted from that of the post vaccine bleeds before calculation of the endpoint titre by interpolation of the point of intersect between the assay cut-off and a sigmoid dose-response curve fitted to the dilution series in GraphPad Prism v5.0⁶¹.

MAB, soluble (s)CD4 and CD4-IgG2 binding assays

Antigens produced in the various expression systems were coated on to ELISA plates and probed with the 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 $\mu\text{g/mL}^{-1}$ and coincubated for 1 h at room temperature (RT) before the addition of 10 μL /well of sample buffer containing six-fold 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 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 $\mu\text{g/mL}$ solution of sCD4 for 30 min at RT before the addition of 50 μL of a 2-fold concentrated dilution series of the ligand without washing out the sCD4. Titrations were performed in 3–5 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 antisera. 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 blocked and washed as described previously⁶⁰. Test or control (immune mouse serum from mice immunized with hen egg lysozyme formulated in FCA) serum diluted to 1/50 in 50 μL PBS/1% BSA 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 3 times and bound mAb detected with anti-human IgG-HRP at 1/1000 in PBS/1%BSA for 1 h at RT followed by washing, addition of substrate, stopping and reading as previously described⁶⁰. The % inhibition was calculated using $100 - [(\text{test value} - \text{control value}) / (\text{maximum value} - \text{control value}) \times 100]$.

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, high mannose glycans, which have been characterized on gp120 of the HIV-1_{BH8} isolate expressed in insect cells⁹, were modeled on the structure of gp120_{HxBc2} (PDB ID = 1GC1⁶¹). N-linked glycan sites at amino acids 289, 397, 406, 448 and 463 of gp120_{HxBc2} do not have conserved counterparts in gp120_{BH8}, so glycans on gp120_{BH8} closest to those amino acids were incorporated. Yeh *et al.* found that each site contains a distribution of glycans ranging in size from five to nine mannose residues⁹ that closely matched the distribution of glycans on gp120 produced from kifunensine-treated cells as determined by mass spectrometry (Figure 1). A method was incorporated to account for this microheterogeneity. Each N-linked glycan was assigned a numeric range equal to the percent that this glycan is found at a site. This was done for all glycans at each site. Next, each site was given a random number between 0 and 100. A particular glycan was modeled on a site if the random number fell within that glycan's numeric range. This method was used to generate three variants of gp120 from kifunensine-treated cells (Table S1). To mimic wild-type 293F glycosylation, complex and high mannose glycans, which were characterized on gp120_{HxBc2} expressed in CHO cells⁶ and chronically-infected lymphoblastoid (H9) cells⁷, were modeled on the structure of gp120_{HxBc2}. Leonard *et al.* determined whether each N-linked glycan site contains a high mannose or a complex glycan⁶. To assign glycans on gp120 and to account for microheterogeneity, a method similar to the one described above for Sf9 glycan modeling was developed. Each N-linked glycan was assigned a 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, all complex sugars were stripped from the models for 293F glycosylation and replaced by 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 gp120_{HxBc2} using Glyprot (<http://www.glycosciences.de/modeling/glyprot/php/main.php>). These models were refined in two steps using CNS^{62; 63}. In the first step, they underwent simulated annealing, in which they were heated up to 2500–3000 K, freeing them from the local energy minimum. Then, they were slow 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, the refined models were analyzed with spherical probes of different radii as previously described⁶⁴. 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 antibody combining

region. These probes were used to calculate the accessible surface of the models. Using GRASP, the accessible surface area excluded by the glycans was determined 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 using GraphPad Prism v 5.0. Raw or log₁₀-transformed data were only treated as 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-group comparisons were made. Error bars represent the range unless stated otherwise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

293F	293 FreeStyle™ cells
Ab	antibody
CD4bs	CD4 binding site
CD4i	CD4-induced site
CHO	Chinese hamster ovary cells
Env	envelope glycoprotein
mAb	monoclonal antibody
pAb	polyclonal antibody
sCD4	soluble CD4
Sf9	<i>Spodoptera frugiperda</i> cells
wt	wild-type

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Fig. 1. Mass spectrometric analysis of glycan diversity in differentially-expressed gp120s. Glycan diversity on gp120_{Ba-L} expressed in sf9, untreated or kifunensine-treated (5 or 20 μ M) 293F cells by mass spectrometry. F – fucose, H – hexose, M – mannose and N – Glc₂NAc₂. Bars represent % prevalence of total glycan content of each glycan type.

Fig. 2. Refined 3D gp120_{Ba-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. a) Model of gp120 expressed in 293F cells. b) Model of gp120 expressed in kifunensine-treated 293F cells. c) Model of gp120 expressed in Sf9 cells.

Fig. 3.

Relative protein surface occlusion by glycan in gp120_{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 by 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.

Fig. 4. Relative protein surface electrostatics in gp120_{Ba-L} expressed in 293F or kifunensine-treated 293F and Sf9 cells (labelled “mannose” in the figure). a) 3D models of gp120 with the same glycan colour scheme and orientations as in Fig 2, but with the solvent accessible protein surface colour coded to reflect electrostatic charge as shown on the scale bar. b) The electrostatic charge at each vertex of three kifunensine-treated models ($n = 68485$ vertices) and of the three untreated 293F models ($n = 72493$). *** $P < 0.0001$ from unpaired t-test.

Fig. 5.

The influence of expression system on gp120 immunogenicity. (a) Groups of 5 Balb/c mice were primed with Sf9wt, Sf9 Mimic™ or 293F cell-expressed gp120_{CN54} or Sf9wt or 293F-expressed gp120_{CN54} formulated in CpG-based adjuvant at week 0 and boosted with antigen alone at week 3. Results show reciprocal antigen-specific IgG endpoint titres at 6 weeks after the boost. (b) Groups of 6 Balb/c mice were primed with Sf9wt or 293F cell-expressed gp120_{CN54} or gp120_{Ba-L} emulsified in FCA. Results show antigen-specific reciprocal IgG endpoint titres 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. * $P < 0.05$.

Fig. 6.

Analysis of serum antibody specificity by competition ELISA. Individual sera from groups of 5 mice immunized with insect or mammalian cell-derived gp120_{Ba-L} or gp120_{CN54} in FCA were assayed for competition with (a) CD4IgG2; (b) HJ16; (c) HR10 and (d) HG68 in a binding assay to gp140_{CN54}. Results show % inhibition normalized to control sera. Bars represent means of individual sera + 1 SD and the Mann-Whitney test was used to determine statistical significance, * $P < 0.05$, ** $P < 0.01$.

Fig. 7.

The immunogenicity of gp120_{Ba-L} expressed in 293F cells in the presence or absence of kifunensine. Female Balb/c mice (5/group) were primed and boosted 3 weeks later with 10 μ g of untreated or 5 μ M kifunensine-treated gp120_{Ba-L} formulated in CpG-based adjuvant at week 0 and with 5 μ g antigen alone at weeks 3 and 6 after the prime, and antigen-specific reciprocal serum IgG titers assays 3 weeks after the final boost. Sera were titrated on gp120_{Ba-L} from untreated and kifunensine-treated 293F cells. 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. * $P < 0.05$.

Fig. 8.

Outline of experiment and summary of results. Arrows denoting gp120 produced in untreated 293F cells (solid black), kifunensine treated 293F cells (dotted black), Sf9 Mimic™ cells (dotted grey) and Sf9 cells (grey) indicate conclusions drawn about them from computational modelling, *in vitro* antibody binding assays and measurements of anti-gp120 serum titres.

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

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 utilised, 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.

Ligand	Epitope	Strain	Sf9wt	Sf9 Mimic
CA13	1	Ba-L	0.73	
		CN54	(0.08)	(0.12)
19b	2	Ba-L	0.59	
		CN54	1.06	2.05
447-52D	2	Ba-L	1.34	
		CN54	0.02	0.04
LA21	2	Ba-L	0.08	
2G12	3	Ba-L	5.11	
		CN54	(0.12)	ND
b12	4	Ba-L	3.68	
		CN54	0.63	1.29
sCD4	4	Ba-L	8.50	
		CN54	(15.70)	(27.00)
D7324	5	Ba-L	(2.49)	
		CN54	(1.25)	ND
ARP421	6	Ba-L	0.22	
ARP422	6	Ba-L	0.55	0.68

Table 2

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

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⁻¹; and 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 equalling zero for the assay with 293F cell-expressed gp120^{CN54} and sCD4 plus mAb A32. Both the Sf9wt and Sf9 Mimic™ produced positive ELISA signals.

Ligand	Epitope	Strain	sCD4	Sf9wt	Sf9 Mimic
E51	1	CN54	–	(2.76)	(1.80)
			+	(2.67)	(2.82)
X5	1	Ba-L	–	(8.09)	
			+	18.50	
		CN54	–	(1.28)	ND
			+	10.10	ND
17b	1	Ba-L	–	(0.59)	
			+	27.20	
412d	1	Ba-L	–	7.68	
			+	6.42	
		CN54	–	4.57	ND
			+	4.32	ND
A32	2	Ba-L	–	(19.10)	
			+	1.22	
		CN54	–	(16.90)	(0.55)
			+	(NR)	(NR)

Table 3
Comparative antigenicity of gp120_{Ba-L} expressed in 293T cells in the presence or absence of 5 μ M kifunensine

Numbers shown indicated the fold increase or decrease in the 50% binding titer for the gp120_{Ba-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).

Ligand	Epitope	Kifunensine
19b	1	3.43
447-52D	1	0.80
2G12	2	3.08
b12	3	82.06
F105	3	4.73
CD4-IgG2	3	6.08
15e	3	(9.14)
21h	3	(0.96)
HIVIg	4	1.46
ARP440	4	1.38