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Global N-glycan Site Occupancy of HIV-1 gp120 by Metabolic Engineering and High-Resolution Intact Mass Spectrometry

Weston B. Struwe[*,§], **Alexandra Stuckmann**[†], **Anna-Janina Behrens**[§], **Kevin Pagel**[†], and **Max Crispin**[*,§,¶]

[§]Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, OX1 3QU, United Kingdom

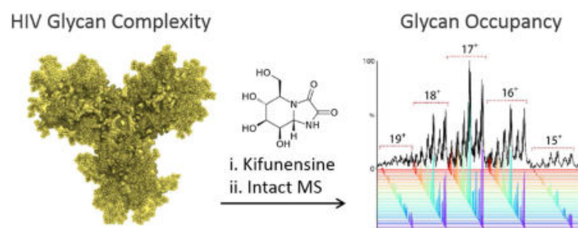
[†]Free University Berlin, Department of Chemistry and Biochemistry, Takustrasse 3, 14195 Berlin, Germany

[¶]Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA

Abstract

A vital step in HIV vaccine development strategies has been the observation that some infected individuals generate broadly neutralizing antibodies that target the glycans on the surface of HIV-1 gp120. These antibodies target glycan epitopes on viral envelope spikes and yet the positions and degree of occupancy of glycosylation sites is diverse. Therefore, there is a need to understand glycosylation occupancy on recombinant immunogens. The sheer number of potential glycosylation sites and degree of chemical heterogeneity impedes assessing the global sequon occupancy of gp120 glycoforms. Here, we trap the glycan processing of recombinant gp120 to generate homogenous glycoforms, facilitating occupancy assessment by intact mass spectrometry. We show that gp120 monomers of the BG505 strain contain either fully occupied sequons or missing one and sometimes two glycans across the molecule. This biosynthetic engineering approach enables the analysis of therapeutically important glycoproteins otherwise recalcitrant to analysis by native mass spectrometry.

Abstract



*Corresponding Authors weston.struwe@bioch.ox.ac.uk & max.crispin@bioch.ox.ac.uk.

ASSOCIATED CONTENT

Supporting Information

Materials and methods, Figures S1–S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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The human immunodeficiency virus (HIV-1) viral spikes have an extensive and dense coat of N-linked glycans that act to shield the underlying protein from antibody recognition[1–7]. The attachment glycoprotein (gp120) within these spikes is a key target for antibody-mediated neutralization[8, 9]. Over time, many infected individuals produce broadly neutralizing antibodies (bnAbs) against HIV viral spike epitopes. These antibodies offer broad protection to infection in passive transfer experiments[10] and eliciting bnAbs by vaccination with viral spike mimics is a key goal in the control of the pandemic[11]. The epitopes targeted by the majority of bnAbs contain one or more glycans[12, 13]. Although gp120 N-glycans are largely restricted to high-mannose type, the number and location of N-glycans may change during the viral life span[1, 14]. Finally, glycan occupancy of key sites modulates the development of a broad antibody response against heterologous viruses[15, 16]. To this end, it is important that recombinant candidate immunogens are fully characterized[17], invoke a suitable T-cell response[18] and efficiently display target bnAb epitopes to B-cells[11, 19, 20].

The extensive role of glycans in forming the epitopes of bnAbs and the emerging importance of viral site occupancy has necessitated detailed glycosylation analysis of recombinant mimics of the viral spike. This is important in guiding immunogen design and also in evaluating biotherapeutic glycoproteins for use in the clinic. Glycoproteins are known to consist of an ensemble of 'glycoforms'. These arise during cellular biosynthesis and the heterogeneity is driven by variable occupancy of the glycan sites and the chemical heterogeneity that arises from the action of an array of glycosidases and glycosyltransferases in the Golgi apparatus[21]. Partial occupancy of N-glycan sequons can have substantial impact on biological activity and is an important parameter in the characterization of biologics.

Significant progress has been made in site-specific analysis of gp120 glycosylation[22–24] but little is known about the overall occupancy of glycosylation sites. While glycopeptide analysis can reveal the occupancy of any particular site[25, 26], measuring the overall distribution of partially occupied sites across the spectrum of glycoproteins has not been tractable by current methods. As such, glycan heterogeneity obscures global occupancy information that could be derived by intact mass spectrometry (MS)[27].

Here, we circumvent this barrier by using metabolic engineering with a potent α-mannosidase inhibitor, kifunensine[28], to homogenize the processing of N-linked glycans on recombinant gp120 (BG505 strain) transiently expressed in human embryonic kidney (HEK) 293F cells (Fig. 1A and Fig. S1).

Homogeneous gp120 glycoforms could be resolved using a modified high-resolution Orbitrap mass spectrometer designed to evaluate high molecular weight proteins and their complexes[29]. High-resolution MS has been applied to glycoproteins with only one[30] or two glycan sites[31, 32], but not to highly glycosylated proteins due to overlapping glycoforms.

Kifunensine has previously been used to augment the crystallization of glycoproteins and is sufficiently potent to almost entirely eliminate chemical heterogeneity of N-linked glycosylation[33–35]. MS of released N-linked glycans from BG505 gp120 expressed in the presence of kifunensine shows a spectrum dominated by $Man_9GlcNAc_2$ (Man9) with only a

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trace of $Man_8GlcNAc_2$ (Man8) (Fig. 1B). Tandem MS reveals the known isomers of the mammalian glycosylation pathway (Fig. 1C)[36]. This is consistent with efficient blockade of both endoplasmic reticulum and type-I Golgi-resident α-mannosidase activity.

Native and deconvoluted mass spectra of the resulting glycan-engineered gp120 revealed a charge state distribution spanning 15–19+ (Fig. 2A). Within each charge state, six species were observed, with an evident mass shift between the three major peaks matching the mass of a single Man9 (1864 Da) demonstrating variability in occupied glycan sites in the intact gp120 (Fig 2A, inset). The gp120 structure is dominated by Man9 glycans, the cumulative effect of the low levels of Man8 structures gives rise to an evident hexose series within each major peak demonstrating Man8 and Man9 microheterogeneity.

Further examination of the spectra reveals mass shifts corresponding to variable modification with O-glycans (Fig 2A, pink circles). These observations are consistent with previous glycopeptide analysis where recombinant gp120 contained O-linked glycans[24, 26]. Notably, the measured mass of gp120 (101 kDa) was 1.6 kDa greater than that corresponding to the gp120 peptide backbone (~54.6 kDa) with 23 or 24 Man9 N-glycans (97.5 and 99.4 kDa, respectively) (Fig. S2). Analysis of released O-glycans confirmed mono- and di-sialylated core-1 structures (Fig. 2B) and accounts for the observed intact gp120 masses. Therefore, recombinant gp120 contains a single sialyl core-1 (656 Da) ± di-sialyl core-1 (947 Da) O-glycans. From the deconvoluted spectra (Fig. 2C) we can conclude that the dominant species matched to both fully occupied and *n*-1 (i.e. lacking a single N-glycan) gp120 protein moieties with variable O-glycosylation. The approximate relative abundance of gp120 glycoforms were 40.6 % fully occupied, 41.6% (*n*-1), and 17.8% (*n*-2).

To confirm the assigned peaks to N-glycans, we measured a BG505 gp120 variant mutated at a single highly conserved glycosylation site (N332) that is a critical component of many bnAb epitopes. Expectedly, the N332A mass spectrum lacked the $n = 24$ peaks, most evident from the 16^+ and 17^+ charge states (Fig. 3). The depletion of two clusters of peaks per charge state also substantiates the presence both O-glycans per gp120 monomer. Interestingly, the relative occupancy was maintained with *n*-1 and *n*-2 as the major glycoforms suggesting that N332 is largely completely occupied and that the global loss of glycan equivalents arises from the cumulative partial absence of glycans across other sites (Fig. S3).

Native high-resolution mass spectrometry holds enormous promise for glycoprotein characterization, but has only been demonstrated on relatively simple systems and has failed for more complex targets[30–32]. We have chosen one of the most heavily glycosylated glycoproteins in nature which is currently under investigation for clinical use[37]. Our results demonstrate that the assessment of global occupancy of glycosylation sites is tractable by intact mass spectrometry with biosynthetic engineering to eliminate heterogeneity arising from glycan processing. The occupancy information is ostensibly preserved as the engineered glycan processing occurs downstream of the initial oligosaccharyl-transferase that initiates N-glycosylation. Intact MS can rapidly assess the presence and structure of variable O-glycosylation which may otherwise be obscured by masses arising from heterogeneous N-link glycosylation. Detection of O-linked glycosylation remains an active

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area of investigation in the assessment of HIV immunogens[24, 38]. Furthermore, this approach is widely applicable for any N-glycosylated protein and will prove valuable in biotherapeutic characterization. The clinical use of complex glycoproteins requires a detailed understanding of structure-function relationships and the monitoring of key critical quality attributes, such as glycan site occupancy[39]. In particular, we expect assessment of global site occupancy to emerge as an important parameter in HIV immunogen production systems as these are assessed for the manufacture of clinical grade material[17].

Glycan engineering has already accelerated developments in structural biology by preventing glycan heterogeneity impeding glycoprotein crystallization[33]. We suggest that glycan engineering could similarly solve the 'glycosylation problem' in native mass spectrometry by simplifying the spectra for any studies dealing with heavily glycosylated glycoproteins and their interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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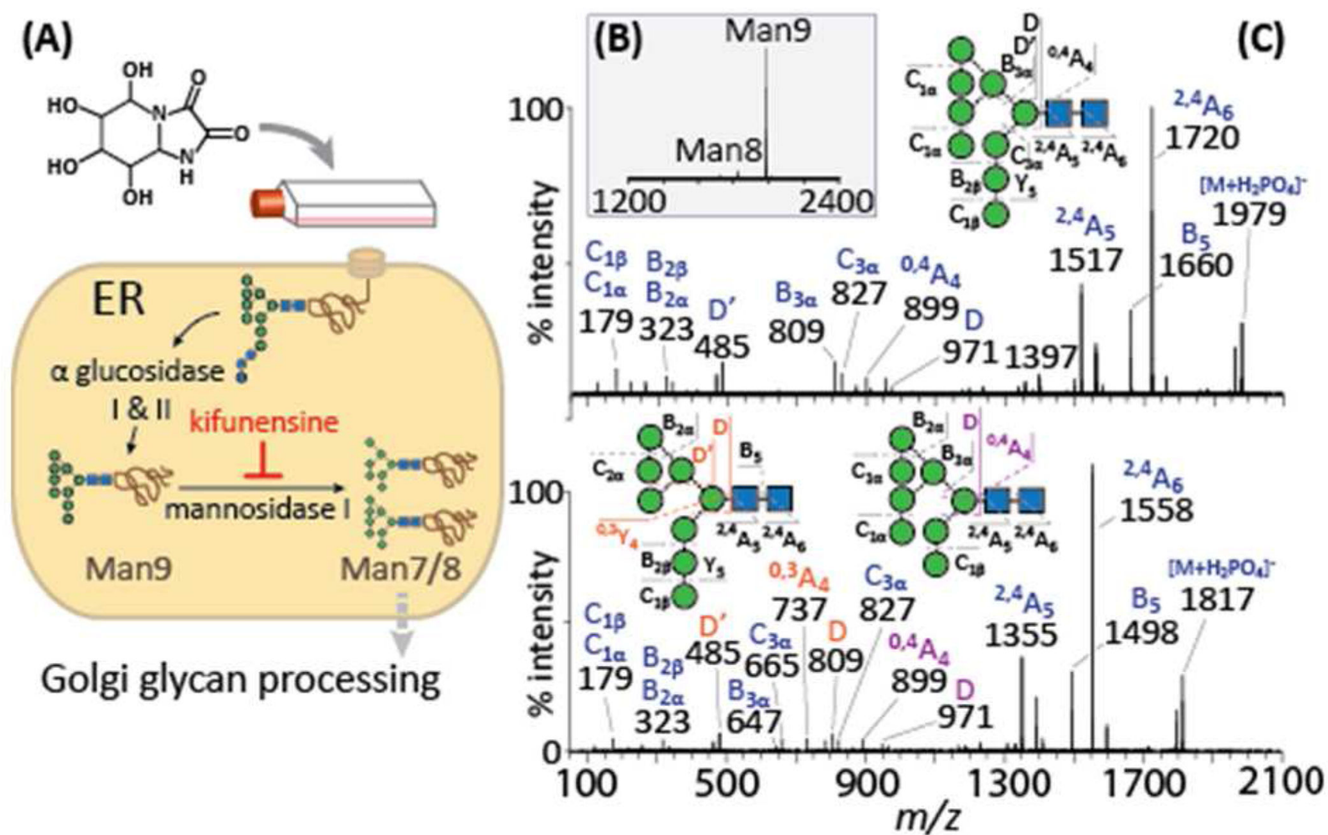

Figure 1.

(**A**) Expression strategy to produce an oligomannose-type glycoform of gp120. Kifunensine inhibits endoplasmic reticulum (ER) and Golgi mannosidase I during recombinant HIV gp120 expression resulting in predominantly $Man_9GlcNAc_2$ (Man9) N-glycans. (**B**) ESI-MS of Nlinked glycans released by protein N-glycanase F. (**C**) Tandem mass spectrometry of negative N-glycan ions (diagnostic ions for each isomer are in orange or purple). Green circles, mannose; Blue squares, GlcNAc.

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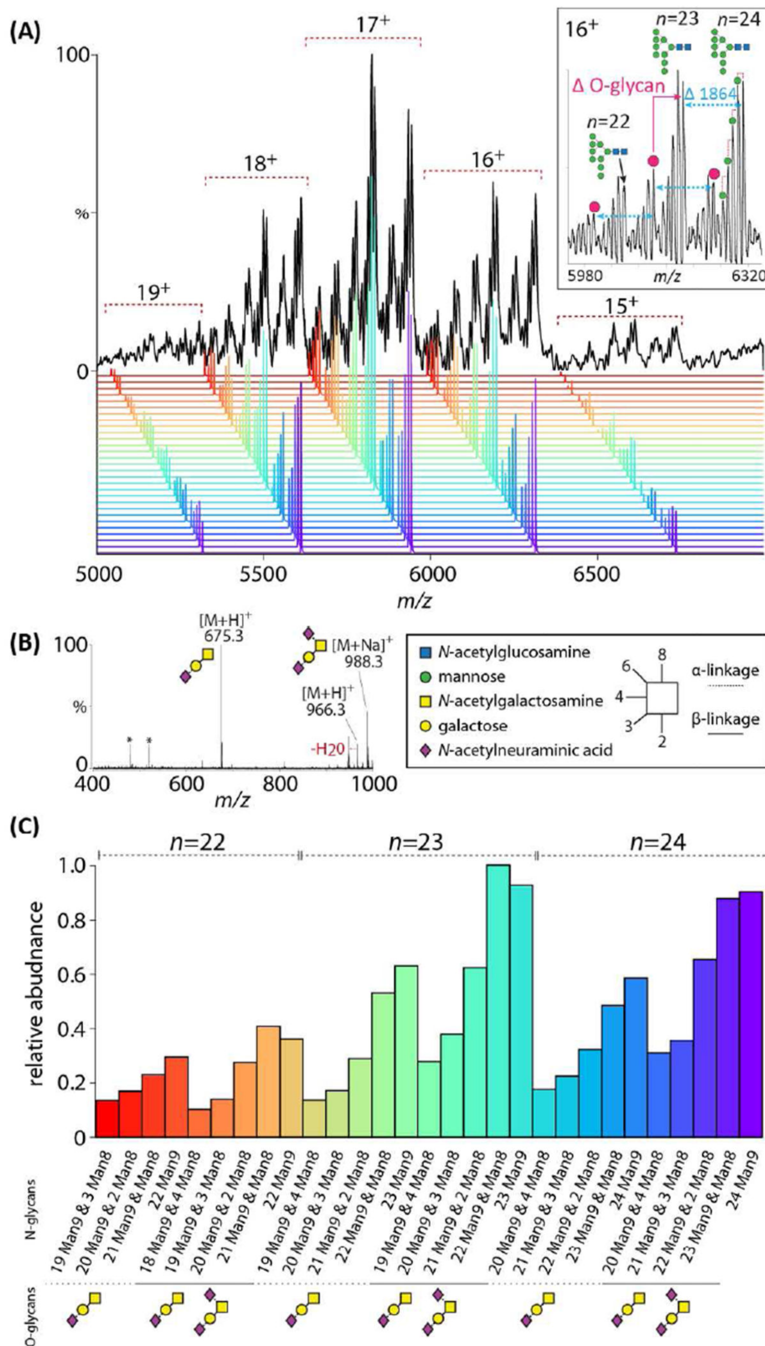

Figure 2.

Intact mass spectrum of BG505 gp120 transiently expressed in the presence of kifunensine. (**A**) Spectrum revealing six distinct ion species within each charge state (inset). A 162 Da mass difference corresponding to Man9/Man8 microheterogeneity is present within each species (inset, green circles). The major ions are separated by an 1864 Da mass shifts (blue, inset) equal to a single Man9. (**B**) Positive ion MS spectrum of released O-glycans identify mono- and di-sialyl core-1 structures that correspond to the peak shifts observed in the intact spectrum (pink circles, top). Peaks marked with asterisks are non-carbohydrate

contaminants. (**C**) Deconvoluted spectrum to quantify the major peaks in **A** identify fully occupied $n = 24$ or $n = 23$ glycoforms with sialyl core-1 ± disialyl core-1 O-glycans.

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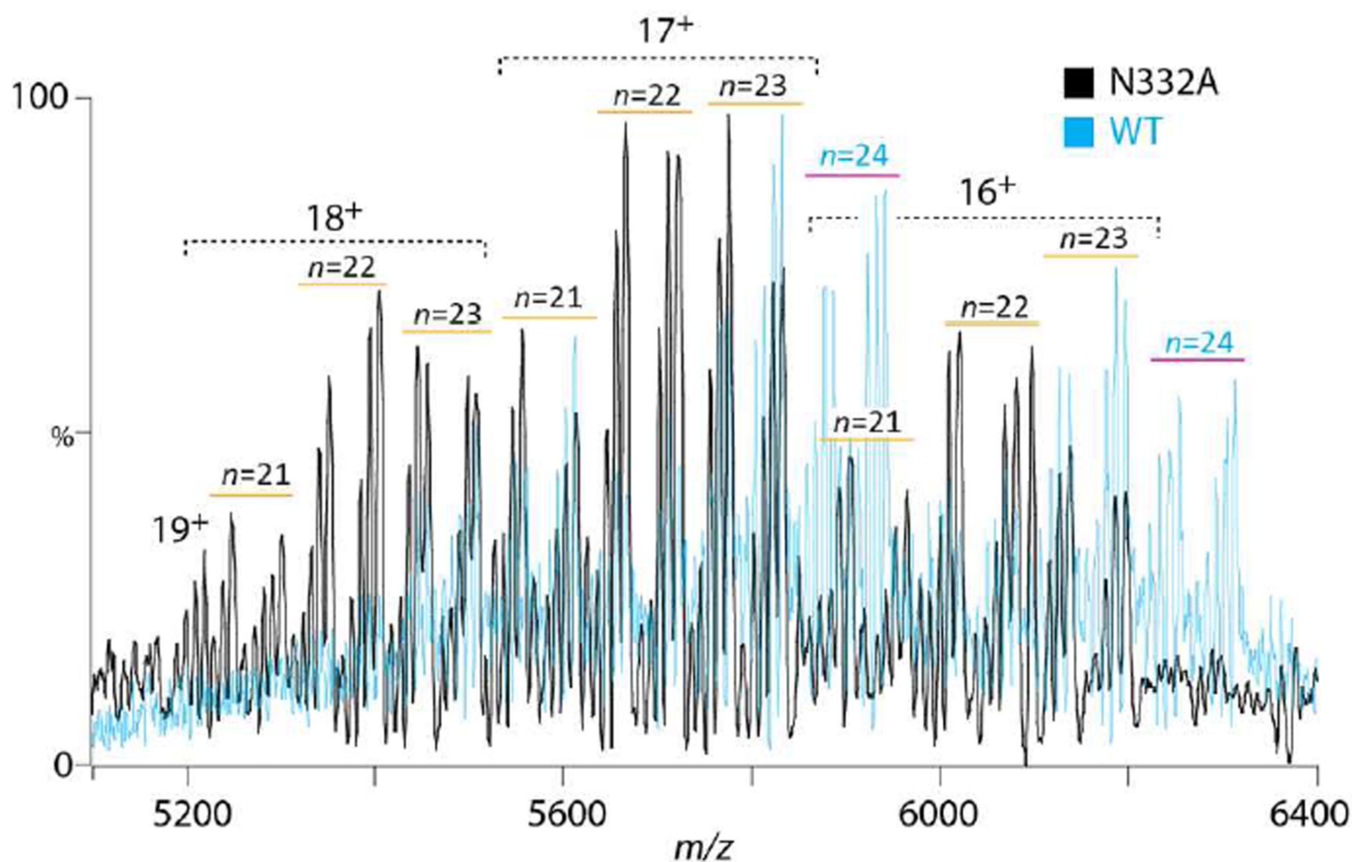

Figure 3.

Intact mass spectra of N332A gp120 (black) and wild-type (WT) gp120 (blue) expressed in the presence of kifunensine. The number of N-glycosylation sites at each cluster of peaks is indicated (n). WT and the N332A mutant of BG505 gp120 have a maximum N-glycosylation site occupancy potential of $n = 24$ and 23, respectively.