

# Chapter 1

## HIV Glycomics and Glycoproteomics

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**Abstract** The HIV-1 surface glycoprotein, gp120, is made of a rapidly mutating protein core, encoded by the viral genome, and an extensive carbohydrate shield which is synthesized by the host cell. HIV gp120 is a highly glycosylated protein, with an average of 25 potential N-linked glycosylation sites (PNGS). Determination of the site occupancy, microheterogeneity, and chemical structure of glycans attached to the potential glycosylation sites on gp120 have been performed on recombinant gp120 and gp140 by site analysis of glycosylation involving a combination of chromatography and mass spectrometry techniques. These studies were complemented by lectin-binding studies, and finally by mass spectrometric glycosylation analysis of gp120 isolated directly from infectious virions produced in peripheral blood mononuclear cells (PBMCs). In contrast to host cell glycoproteins, gp120 was shown to contain a population of incompletely processed oligomannose-type glycans that interact with host lectins, promote HIV infection, and alter cell signaling. These glycans also form the basis of the epitopes of several highly potent HIV broadly neutralizing antibodies isolated from HIV-infected individuals, making them a key feature for immunogen design. Furthermore, an elevated level of oligomannose-type glycans was evidenced on gp120 isolated from HIV-1 virions produced in PBMCs, compared to recombinant material, along with a subset of highly processed and sialylated, bi-, tri-, and tetra-antennary complex-type glycans.

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The effect of variation in viral production systems has also been reported, with envelope glycoprotein derived from pseudoviral particles produced in human embryonic kidney (HEK) 293T cells exhibiting predominantly an oligomannose population, compared to gp120 isolated from a single-plasmid infectious molecular clone. The gp120 glycan profile is remarkably similar across primary viral isolates from Africa, Asia, and Europe and consequently represents an attractive target for vaccine development. Finally, glycan remodeling and mutagenesis can also be employed for pseudoviral particle production and recombinant protein expression, to probe broadly neutralizing antibody specificity, structural analysis, and immunogen design.

**Keywords** Mass spectrometry • Oligomannose • Glycosylation • gp120 • Envelope glycoprotein • Glycan remodeling

## 1.1 Introduction

The HIV envelope glycoprotein, critical for HIV infectivity, consists of a metastable, non-covalently associated trimer of gp120/gp41 heterodimers. The attachment glycoprotein gp120 is extensively modified with covalently attached, host-derived O-linked (Bernstein et al. 1994; Corbeau et al. 1995) and N-linked oligosaccharides with up to 50 % of its mass comprising carbohydrate (Allan et al. 1985; Montagnier et al. 1985; Ratner et al. 1985; Lasky et al. 1986; Leonard et al. 1990; Zhu et al. 2000). The host-cell-derived N-linked glycans play important roles in assisting correct protein folding (Li et al. 1993; Trombetta and Helenius 1998), in shielding conserved regions of gp120 from recognition by the immune system, and in disease transmission through interaction with host receptors.

The glycans on gp120 have often been referred to as “the glycan shield” or “silent face” due to their poor immunogenicity and “self” nature (Calarese et al. 2003; Scanlan et al. 2007; Wei et al. 2003). However, a number of broadly neutralizing anti-HIV antibodies (bnAbs) have recently been isolated from HIV-infected individuals that interact directly with these N-linked glycans (Walker et al. 2009, 2011). These bnAbs target two distinct regions on gp120, the glycans around N332 (e.g., 2G12, PGTs 121–123, PGTs 125–131, and PGTs 135–137) (Pejchal et al. 2011; Walker et al. 2011; Calarese et al. 2003; Scanlan et al. 2002) and the glycans around N160 (e.g., PG9, PG16, PGTs 141–145, and CH01-04) (Walker et al. 2009, 2011; McLellan et al. 2011; Bonsignori et al. 2011). Therefore in these HIV-infected individuals, the virus is under constant pressure from both strain-specific antibodies targeting exposed protein epitopes and bnAbs targeting the N-linked glycans and thus the HIV glycan shield is constantly evolving and shifting in response to the host immune system (Wei et al. 2003; Moore et al. 2012).

As a gp120 antigen is likely to be a component of a successful B cell-based AIDS vaccine and N-linked glycans play important biological roles, a thorough understanding of the glycan composition of both virus-derived and recombinantly expressed gp120 may be critical for the design of successful vaccine candidates and

understanding pathogenesis. In particular, knowledge of the glycans bound by the carbohydrate-specific HIV bnAbs may be key for immunogen design strategies that aim to elicit similarly broad and potent neutralizing antibodies through vaccination. This chapter will discuss what is known currently about the glycosylation on both virus-derived and recombinantly expressed HIV envelope glycoproteins.

## 1.2 Biogenesis of HIV-1 gp120 N-Linked Glycans

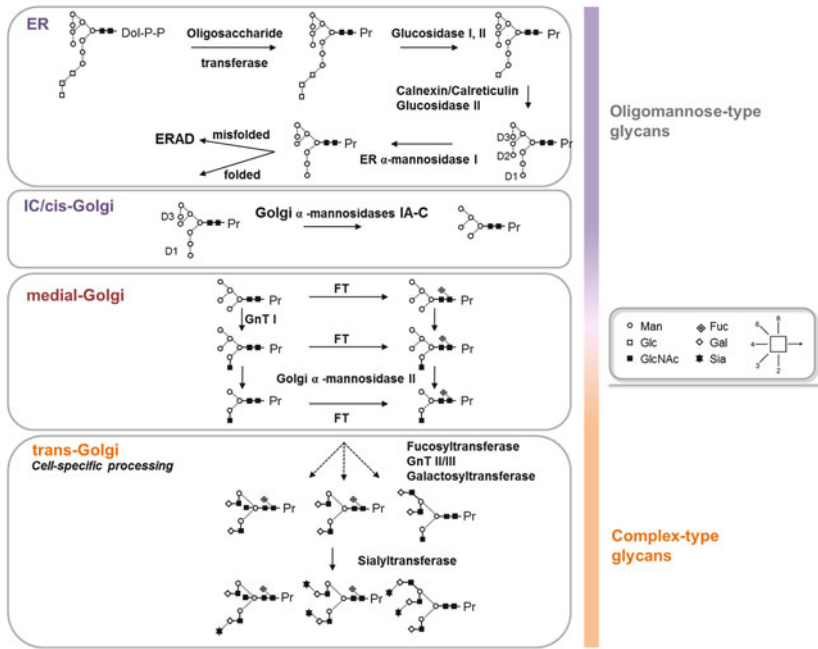
Protein N-glycosylation is one of the main post-translational modifications. Whilst O-linked glycans are attached to surface-exposed serine or threonine residues, predicted PNGS are asparagine residues within an N-X-S/T sequon, where X is any amino acid except proline (Kornfeld and Kornfeld 1985). Although the sequence of a protein is under direct genetic control, N-linked glycosylation is determined by the host-cell glycosylation machinery and therefore glycosylation can be very heterogeneous and a single glycoprotein can exist as a number of different glycoforms.

The mammalian N-linked glycosylation machinery follows a strictly ordered pathway in the endoplasmic reticulum (ER) and the Golgi apparatus (Fig. 1.1a). Following co-translational translocation of the envelope glycoprotein precursor gp160 in the ER, and cleavage by furin in the *trans*-Golgi into gp120 and gp41, a dolichol-pyrophosphate-linked glucosylated oligomannose precursor, Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>, is added to the free amide of asparagine residues by the oligosaccharyltransferase (Kornfeld and Kornfeld 1985). Trimming of this glycan species by ER  $\alpha$ -glucosidase I and II yields a monoglucosylated intermediate that interacts with the molecular chaperones calnexin and calreticulin. Competing with the hydrolysis reaction of ER  $\alpha$ -glucosidase II is the transferase reaction of the UDP-Glc:glycoprotein glucosyltransferase that use misfolded glycoprotein substrates. Once gp160 has reached a proper folded state, the ER  $\alpha$ -glucosidase II further trims the GlcMan<sub>9</sub>GlcNAc<sub>2</sub> intermediate into a Man<sub>9</sub>GlcNAc<sub>2</sub> species. The structure of this oligomannose-type glycan is highlighted in Fig. 1.1b. The oligomannose moiety can be cleaved during biogenesis in the ER by ER  $\alpha$ -mannosidase I and subsequently  $\alpha$ -mannosidases IA–C in the intermediate compartment (IC)/*cis*-Golgi to form Man<sub>5</sub>GlcNAc<sub>2</sub>, the last species of the oligomannose series, which comprises Man<sub>5-9</sub>GlcNAc<sub>2</sub> glycans.

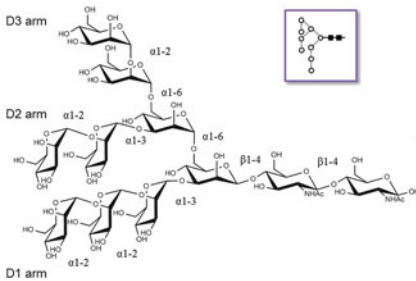
The diversification into complex-type glycans occurs after the essential step performed by the medial-Golgi-resident enzyme *N*-acetylglucosaminyltransferase I (GnT I). Addition of a single  $\beta$ 1→2-linked *N*-acetylglucosamine residue (GlcNAc) to the Man<sub>5</sub>GlcNAc<sub>2</sub> intermediate performed by GnT I, is followed by further trimming and processing by Golgi-resident glycosidases and glycosyltransferases, leading to the assembly of a wide array of hybrid and complex-type glycans. The diversity observed between complex-glycan structures between cell types is typically due to the tissue-specific expression of Golgi glycosyltransferases.

A particular example is the tissue-specific expression of sialyltransferases, which catalyze the addition of a terminal sialic acid (neuraminic acid; NeuNAc) residue in

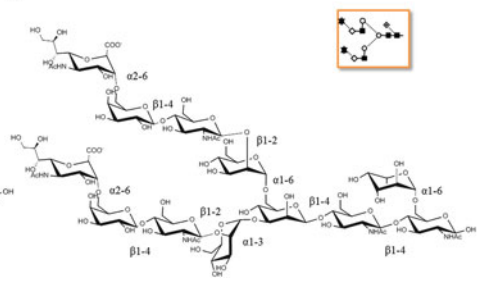
a



b



c



**Fig. 1.1** N-linked glycosylation pathway. **(a)** N-linked glycosylation of a glycoprotein (Pr) results in co-translational attachment of glycans to the amide group of asparagine residues within an N-X-T/S sequon (where X is any amino acid except proline) in the ER lumen. Folding of the nascent chain starts in the ER, accompanied by the formation of disulfide bonds in this oxidizing milieu (10 in total for gp160, including 9 for gp120), and helped by the protein chaperones calnexin and calreticulin. Unprocessed, immature oligomannose-type glycans arise through processing by ER and Golgi  $\alpha$ -mannosidases. Upon GnT I transfer of an *N*-acetylglucosamine residue in the medial Golgi, diversification of glycans occurs, leading to the presence of branched, galactosylated, and sialylated complex-type glycans in the *trans*-Golgi network. Glycan representation is shown according to Harvey et al. (2008) guidelines. Chemical structures of **(b)** the Man<sub>5</sub>GlcNAc<sub>2</sub> glycan highlighting the D1, D2, and D3 arm, and **(c)** an  $\alpha$ 2→6 sialylated, bi-antennary, and fucosylated complex-type glycan. *N*-acetyl-5-neuraminic acid (Neu5Ac) is the dominant form of sialic acids found in humans

a  $\alpha 2 \rightarrow 3$ ,  $\alpha 2 \rightarrow 6$ , or  $\alpha 2 \rightarrow 8$  linkage (Harduin-Lepers et al. 2001). Sialic acids are negatively charged moieties that are involved in recognition mechanisms as “self” elements, through receptors highly specific of particular linkages (Varki and Gagneux 2012). The NeuAc $\alpha 2 \rightarrow 6$ Gal linkage, catalyzed by the  $\beta$ -galactoside  $\alpha 2,6$ -sialyltransferase (ST6Gal I), a glycosylation enzyme that has been found to be up-regulated in several cancer cells (Dall’Olio and Chiricolo 2001), is illustrated in Fig. 1.1c. A differential sialic acid expression pattern has for instance been observed between  $T_{H1}$ ,  $T_{H2}$  and  $T_{H17}$  polarized cells, with ST6Gal I expressed at higher levels during  $T_{H2}$  differentiation, leading to the formation of  $\alpha 2-6$  linked sialylated glycans that prevent galectin-1 binding to T cells surface glycoprotein and subsequent cell death (Toscano et al. 2007).

Finally, in contrast to this cell-directed glycosylation model, whereby the structure of complex-type glycans is directly related to the cell-specific expression of glycosyltransferases, several proteins have been reported to exhibit a restricted set of glycans, dependant of glycan accessibility, protein structure and/or trafficking, referred to as protein-directed glycosylation. Human IgG bears a conserved glycosylation site at residue Asn297 in the Fc region, which has been shown to be a complex-type bi-antennary glycan. However, alternative glycosylation is observed for this glycan (Wang et al. 2011), and has high implication for IgG Fc-mediated effector functions. It was indeed shown that core fucosylation of the heavy chain glycan reduces binding to the activatory Fc $\gamma$ RIIIa receptor (Iida et al. 2006), whilst sialylation confers anti-inflammatory properties (Kaneko et al. 2006). Lack of terminal galactose residues on IgG Fc N-linked glycan is observed in autoimmune disease such as rheumatoid arthritis (Parekh et al. 1985) and is associated with low enzyme activity of the  $\beta 4$ -galactosyltransferase in B cells of rheumatoid arthritis patients (Keusch et al. 1998). Agalactosyl IgG has been shown to bind to mannose-binding lectin and activate the complement cascade. Likewise, the three-dimensional shape of the protein and limited glycan accessibility can also lead to protein-directed glycosylation, as reported for HIV envelope glycoproteins.

### 1.3 Methods for Analyzing gp120 N-Linked Glycosylation

A variety of techniques have been employed in the analysis of the glycans of HIV. Antibody or lectin binding can be used to detect specific mono- or oligosaccharides present on a glycoprotein, as well as probe for linkage specificity, with the glycan chemical structure being elucidated by a combination of mass spectrometry (MS) and chromatography on protein-released glycans. The analysis of the glycome is somewhat complicated by the complex-branched structure of carbohydrates compared to DNA and protein molecules, the microheterogeneity observed at a PNGS, i.e., different glycan structures can be present at the same site on different protein molecules, and the presence of isomeric structures that result from different stereochemistry of individual monosaccharides and linkages between them.

### 1.3.1 *Chromatographic and Mass Spectrometric Analyses of Protein-Released Glycans*

Glycan profiling aims to characterize the glycan pool present on a glycoprotein. The main challenges faced in HIV glycomics is the isolation of sufficient quantity of high quality native virions—a problem not encountered when analyzing recombinant envelope glycoprotein. The further challenges are to obtain high resolution and separation of glycan structures that can lead to quantitative measurements, high detection sensitivity, to overcome the low amount of glycans typically obtained from isolated glycoproteins, and finally not to discriminate against a certain type of glycans during isolation, preparation, and analysis.

Chromatography can be performed on fluorescently labeled glycans, obtained by conjugating a fluorescent molecule (such as 2-aminobenzoic acid 2-AA or 2-aminobenzamide 2-AB) to the reducing end of the protein-released glycans by reductive amination (Bigge et al. 1995). The labeled glycans can then be analyzed using normal-phase high-performance liquid chromatography (NP-HPLC) (Guile et al. 1996). The reproducibility of this technique allows for the retention time of each peak to be converted into glucose units (GU) values by comparison with a dextran standard, and further compared against databases of glycan standards to lead to preliminary assignment of the glycan structure. Similarly, reverse-phase (RP) HPLC can be performed and retention times can be calibrated with an arabinose ladder yielding arabinose units (AU). The use of various exoglycosidases, that cleave terminal monosaccharide residues on the nonreducing end of the glycan, can then be used to confirm/infirm the assignment. Finally, peak identification can also be achieved with coupling the NP-HPLC analysis with mass spectrometry (MS).

Different mass spectrometry techniques can be used depending on the ionization source. The two main techniques used for glycan analysis are electrospray ionization (ESI), which forms charged sample droplets, and matrix-assisted laser desorption ionization (MALDI), where the sample is co-crystallized with a matrix (2,5-dihydroxybenzoic acid being the one commonly used for glycans analysis) which transfers the energy from the laser to desorb and ionize glycans. More recently, the ion-mobility separation technique has been coupled with ESI-MS (Harvey et al. 2011) and MALDI-MS (Harvey et al. 2012), resulting in improved glycan sensitivity, even in the presence of contaminants. However, negatively charged glycans, such as sialylated glycans, can behave differently than neutral glycans in positive-ion mode, and it is thus necessary to remove the sialic acid residues, or perform permethylation to analyze such samples. Time-of-flight (TOF) MS relates the velocity of the ion to its mass and charge (usually singly charged ions for MALDI and multiply charged for ESI). The  $m/z$  ratio obtained for each peak thus gives an indication of the glycan structure; however, where there is a possibility of isobaric structures, MS/MS fragmentation is necessary to determine the monosaccharide sequence, branching, and linkage specificity.

These techniques have been used to analyze the nature of the glycans on virion-associated HIV envelope glycoproteins as well as recombinantly expressed trimer

mimics and monomeric gp120 (Sects. 1.5 and 1.6). Furthermore, several glycosylation site analyses have been conducted on either recombinant monomeric gp120 or trimeric gp140 to assign glycan structures at specific gp120 glycosylation sites and assess the occupancy and glycan heterogeneity at PNGS.

### 1.3.2 *Glycosylation Mapping*

These studies involve a combination of chromatography fractionation, ESI and MALDI-based MS and MS/MS analyses on protease-digested glycopeptides containing one or more glycosylation site(s) and are highlighted in Fig. 1.2. Challenges in this field arise from the low ionization efficiency of heterogeneous glycopeptides compared to peptides, glycan heterogeneity, the possible presence of several PNGS on one glycopeptide, and complex data analysis.

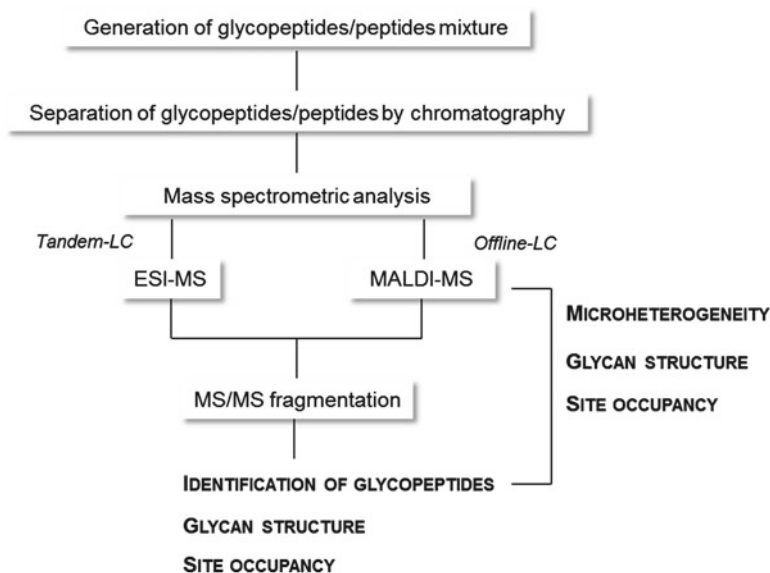
Improvements in separation and enrichment techniques have led to overcome some of these limitations (Zhang et al. 2008). RP-HPLC is now routinely used to separate glycopeptides and can furthermore be coupled to ESI-MS. Enrichment methods, such as lectin-binding chromatography, have also been developed to improve coverage (Zhang et al. 2008). Hydrophilic affinity methods can also be used to separate glycopeptides depending on their global charge. Charged residues, such as sialic acid, which have a low efficiency in positive-ion mode that is used for fragmentation, have to be permethylated in order to be further characterized.

## 1.4 **Recombinant gp120/gp140 N-Linked Glycosylation Site Analysis**

The number of sequons within the gp120 sequence varies from different viral isolates, with a median at 25 PNGS (Korber et al. 2001), whilst gp41 ectodomain contains a cluster of three to four PNGS. Host glycoproteins have on average two thirds of the PNGS occupied, with the occupancy rate of potential glycosylation sites increased by the presence of a threonine instead of a serine in the glycosylation sequon, and by the presence of neighboring hydrophobic residues positioned two amino acids before Asn (Petrescu et al. 2004). The experimentally determined mass of gp120 closely matches that theoretically predicted for a complete occupancy of all the N-linked glycosylation sites present in the envelope gene. This has been confirmed also by several site-analysis glycosylation studies that are detailed below.

Glycosylation site analysis has been reported for recombinant gp120 and gp140 proteins expressed in several mammalian and insect cells. Both gp120 and gp140 lack gp160's transmembrane domain and are expressed in the supernatant. The various constructs analyzed are described in Fig. 1.3.

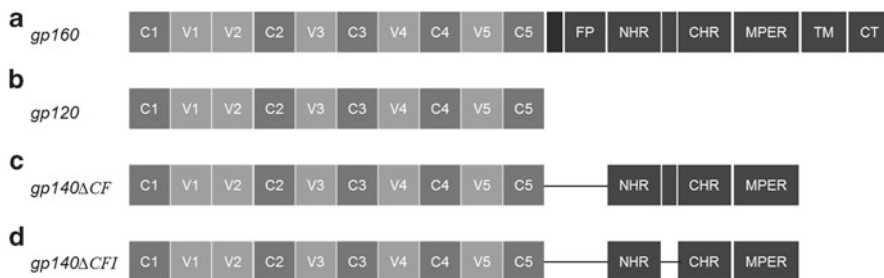




**Fig. 1.2** General glycosylation site analysis strategy. A mixture of peptides and glycopeptides is generated by digestion with proteases such as trypsin. Separation is then achieved by chromatography. Whilst ESI-MS can be used directly in tandem with RP-HPLC (LC-ESI-MS), peptide/glycopeptides are firstly separated off-line via RP-HPLC prior to analysis of the collected fractions on MALDI-MS. Identification of the peptide portion of glycopeptides is based on comparing the cross-ring cleavage fragment ion ( $^{0,2}X$  in MALDI-MS/MS and  $Y_1$  in LC-ESI-MS/MS) obtained upon collision-induced dissociation (CID) in MS/MS with databases or prediction. Once the glycopeptide has been identified, glycoforms and thus microheterogeneity can be identified in the corresponding MS profile, with for instance different oligomannose glycans separated by a mannose unit (162 Da), and more generally by comparing  $m/z$  with database and predicted values for different glycan attached. Additionally, a specific glycan structure can be isolated from the MALDI-MS profile and subjected to MALDI-MS/MS fragmentation. This is particularly relevant for potential isobaric and isomeric structures. Fragment ions resulting from glycosidic cleavage leads to the determination of the glycan sequence and branching, and analysis of cross-ring fragment ions gives information on monosaccharides linkage. Glycosidase digests can also be performed at different stages of this process to help assess site occupancy, glycan sequence and linkage (Go et al. 2011). Endo H is commonly used to probe for oligomannose and hybrid-type glycans, and various exoglycosidases can be used for terminal monosaccharide units. Digestion with PNGase F converts the Asn into an Asp residue, resulting in a shift of 1 Da on the MS profile for glycosylated sites. Analysis of deglycosylated glycopeptides profiles can thus assist to determine site occupancy, especially for glycopeptides bearing more than one PNGS

The first study was performed by Leonard *et al.* on recombinant monomeric HIV<sub>IIIb</sub> gp120 expressed in Chinese hamster ovary (CHO) cells (Leonard et al. 1990). Oligomannose and complex-type glycans were released from tryptic peptides by using the peptide-N-glycosidase F (PNGase F), a routinely used endoglycosidase that cleaves the bond between the GlcNAc moiety and the Asn residue, converting it to Asp. Oligomannose-type glycans were cleaved using the endoglycosidase H (Endo H) that cleaves oligomannose- and some hybrid-type glycans





**Fig. 1.3** Recombinant gp120 and gp140 constructs. **(a)** Schematic sequences of the envelope glycoproteins gp120 and gp41, highlighting gp120 constant (C1–C5) and variable (V1–V5) regions in *grey* and *light grey*, respectively, and gp41 domains in *dark grey*, where FP is the fusion peptide, NHR the N-terminal heptad repeat helix, CHR the C-terminal heptad repeat helix, MPER the membrane proximal external region, TM the transmembrane domain, and CT the cytoplasmic tail. The cleavage site between gp120 and gp41 is highlighted in *black*. **(b)** The recombinant gp120 construct analyzed by Cutalo et al. (2004), Leonard et al. (1990), and Zhu et al. (2000). **(c)** The recombinant gp140 $\Delta$ CF analyzed by Go et al. (2008, 2009), that lack the cleavage site, gp41’s fusion peptide, transmembrane domain and cytoplasmic tail. **(d)** The gp140 $\Delta$ CFI also lacks the region between the NHR and CHR and was developed for the consensus sequence HIV<sub>CON-S</sub> and for the HIV<sub>97ZA012</sub> strain (Go et al. 2008, 2009)

between the two core GlcNAc residues, but not complex-type glycans. Tryptic peptides were analyzed by RP-HPLC and identified by amino acid analysis. Fourteen glycopeptides were identified and their sensitivity to Endo H treatment was then used to determine the nature of the glycan structure, i.e., oligomannose or complex-type glycans (Table 1.1). Overall, the 24 PNGS on gp120<sub>IIB</sub> were found to be fully occupied, with 13 bearing complex-type glycans. The 11 oligomannose-type glycans occupied PNGS were amongst the 13 most highly conserved glycosylation sites found on gp120<sub>IIB</sub>. As this analysis was based on relative retention time shifting upon PNGase F and Endo H digests only, no defined chemical structure of the glycan at each PNGS was determined.

Progress in mass spectrometry allowed for greater precision in the characterization of gp120 glycans, including microheterogeneity at individual PNGS. The analysis of monomeric HIV<sub>SF2</sub> gp120 expressed in CHO cells was conducted with MALDI and nanoelectrospray MS and MS/MS techniques on RP-HPLC-fractionated glycopeptides (Zhu et al. 2000). Of HIV<sub>SF2</sub> gp120’s 26 potential glycosylation sites, the glycans’ structures at 25 of these sites were characterized further (Table 1.1). The relative abundance between different glycan species was also determined at the majority of PNGS. As observed on the previously characterized gp120<sub>IIB</sub>, gp120<sub>SF2</sub> exhibits a population of unprocessed oligomannose-type glycans, together with bi- and tri-antennary complex-type glycans, that can be fucosylated and sialylated. A follow-up study using nanocapillary HPLC coupled with ESI and MALDI-MS aimed to further characterize the sialylation, and eight sialylated glycans on monomeric gp120<sub>SF2</sub> were observed (Cutalo et al. 2004).

Mass spectrometric analyses were also conducted on recombinant gp140s from consensus Env sequences for the group M (CON-S) and the clade B HIV<sub>JRFL</sub> (Go et al.

**Table 1.1** Glycosylation site analysis of recombinant monomeric gp120

Region	Glycan position	Predominant glycan type	Minor glycan type
C1	N88	Complex	
	N136	Complex	Oligomannose
V1/V2	N141	Complex	Oligomannose
	N156	Complex	Oligomannose
	N160	Complex	Oligomannose
	N186	Complex	Oligomannose
	N197	Complex, oligomannose	
C2	N230	Complex, oligomannose	
	N234	Oligomannose	
	N241	Oligomannose	Complex
	N262	Oligomannose	Complex
	N276	Complex, oligomannose	
	N289	Oligomannose	Complex
	N295	Complex, oligomannose	
V3	N301	Complex	Oligomannose
C3	N332	Oligomannose	Complex
	N339	Oligomannose	Complex
	N356	Complex	Oligomannose
V4	N386	Oligomannose	Complex
	N392	Complex, oligomannose	
	N397	Complex, oligomannose	
	N406	Complex, oligomannose	
C4	N448	Complex	Oligomannose
V5	N463	Complex	oligomannose

In different studies where specific PNGS have been shown to bear different glycan types, the predominant and minor glycan structures are indicated. The precise chemical composition of each glycans is described in further detail in the relevant studies. The numbering is based on the HIV<sub>HXB2</sub> isolate Env sequence. The positions of the constant and variable regions are indicated. This table was derived from the results of Leonard et al. (1990), Zhu et al. (2000), Cutalo et al. (2004), and Go et al. (2008, 2009) describing the analyses of recombinant monomeric gp120 derived from the HIV<sub>IIIB</sub> and HIV<sub>SF2</sub> strains, and gp140 from HIV<sub>JRFL</sub> and HIV<sub>97ZA012</sub>

2008), as well as for the clade C consensus sequence (C.CON), and a clade C primary isolate HIV<sub>97ZA012</sub> (Go et al. 2009). Glycopeptides were analyzed by MALDI-MS, LC-ESI-MS, and MS/MS fragmentation. Based on the number of different glycan structures found at a given PNGS, a ratio between oligomannose- and complex-type glycans was determined that might not reflect entirely the relative abundance between these structures. It was found that the gp140 derived from the clade C consensus sequence exhibited a higher proportion of oligomannose-type glycans than gp140 derived from primary isolates; both variants had oligomannose-type glycans predominantly distributed around the immunodominant V3 loop, within the C2 and C3 regions. For these analyses, gp140 was expressed in HEK 293T cells and purified using *Galanthus nivalis*, a lectin that binds  $\alpha$ 1–3mannose residues preferentially.

In conclusion, nearly full site occupancy of gp120 PNGS was observed in all studies. The presence of the oligomannose patch on gp120 outer domain, around the

immunodominant V3 loop is also a characteristic feature of HIV Env glycosylation. On the folded protein, these oligomannose glycans arrange on what has been named the “immunologically silent face,” as the underlying peptide epitopes are covered by carbohydrates which prevent antibody recognition (Wyatt et al. 1998).

Whilst the microheterogeneity at each site was determined in the most recent studies, it is however important to note that the structure of complex-type glycans is likely to depend on the specific glycosylation machinery and tissue-specific expression of glycosyltransferases of the cell in which the glycoprotein has been expressed (Raska et al. 2010). The described analyses have been performed on recombinant material expressed in CHO or HEK 293T cells that are routinely used for protein expression. However, the glycan structures at each specific site might differ on viral Env, as gp120 trimerization would affect glycan processing, and the glycosylation machinery of human HIV-infected cells is different to the one of CHO and HEK 293T cells (Lee et al. 1989). Analysis of gp120 directly isolated from virions produced in PBMCs is described in the next section.

## 1.5 Virus Analysis

Although there have been a number of studies examining the glycosylation on recombinantly expressed gp120, the closest analysis of the glycosylation on HIV virions *in vivo* has come from studies of gp120 isolated from virus derived from HIV-infected cells.

### 1.5.1 Chromatography Analysis of Viral Glycans

The first step towards the determination of the chemical structure of viral envelope glycans was achieved in 1988, when Geyer *et al.* analyzed by HPLC the N-linked glycosylation of envelope glycoprotein isolated from cultures of radiolabeled HIV-infected cells (Geyer et al. 1988). An intracellular fraction, isolated from lysates of infected lymphoblastoid H9 cells, was shown to be comprised mainly of oligomannose glycans, whereas another fraction isolated from culture supernatant (shed gp120) displayed an array of oligomannose-type glycans as well as a set of fucosylated and sialylated bi-/tri-/tetra-antennary complex-type glycans. Two years later, a study conducted on cell-associated gp120 showed that this gp120 displayed a wide range of oligomannose, hybrid, and complex glycans, with 16 % of bi-/tri-/tetra-antennary with bisecting GlcNAc residues and sialylated complex glycans (Mizuochi et al. 1990). A wide range of lectin-binding studies have confirmed these results on both monomeric and viral trimeric gp120 (Zou et al. 2011; Alexandre et al. 2010). These initial studies highlighted differences between recombinantly expressed gp120 and virion-associated gp120.

### ***1.5.2 Env Glycosylation of PBMC-Derived Virus***

More recently, mass spectrometry was used to determine the precise chemical structure of glycans on the envelope spike derived directly from infectious viral particles. PBMCs were infected with HIV-1<sub>JRCSF</sub>, gp120 was detergent solubilized from the virus membrane and immunoprecipitated with a cocktail of bnAbs (Doores et al. 2010). The PNGase F-released N-linked glycans were then analyzed using mass spectrometry. The MALDI-TOF MS spectrum for viral Env glycosylation showed a higher abundance of oligomannose-type glycans compared to recombinant, monomeric gp120<sub>JRCSF</sub>, together with a population of highly processed, sialylated, bi-, tri-, and tetra-antennary complex-type glycans. Overall, the HIV<sub>JRCSF</sub> envelope derived from PBMCs showed an extended array of oligomannose-type glycans showing that  $\alpha$ -mannosidase processing during the early steps of viral glycoprotein assembly and secretion is greatly limited on the native virus in comparison to recombinant monomeric gp120. Moreover, in contrast to monomeric gp120, complex-type glycans isolated from viral envelope spikes form a restricted set of sialylated bi-/tri-/tetra-antennary glycans. MALDI-TOF MS analyses of gp120<sub>JRCSF</sub> envelope isolated from PBMC-derived virus, both before and after desialylation, showed that a majority of complex-type glycans present on gp120 are sialylated (Bonomelli et al. 2011; Doores et al. 2010).

### ***1.5.3 Conservation of Env Glycosylation Pattern for Viral Isolates from Different Clades***

Analyses of gp120 derived from virus prepared by infection of PBMCs with viruses from clade A (92RW009), clade B (JRCSF), and clade C (93IN905) showed a predominantly oligomannose glycan composition (62–79 % Man<sub>5–9</sub>GlcNAc<sub>2</sub>), with a distribution similar to that of PBMC-derived gp120<sub>JRCSF</sub> (Bonomelli et al. 2011). The series of branched, fucosylated complex-type corresponding to the neutral derivatives of sialylated bi-, tri-, and tetra-antennary glycans, were also conserved in all three spectra. This demonstrates that in marked contrast to the underlying protein epitopes, there is a high degree of conservation of the surface-exposed glycan structures, with a set of unprocessed oligomannose-type glycans, and an additional conserved set of highly processed, fucosylated, sialylated bi-, tri-, and tetra-antennary complex-type glycans.

However, the relative abundance of glycans within the oligomannose series differs slightly between isolates from different clades. The ratio of oligomannose-type glycans that terminate with Man $\alpha$ 1 $\rightarrow$ 2Man-linked mannose residues, compared with those that do not, is higher for gp120 derived from HIV-1<sub>92RW009</sub> (clade A, 5.4) and HIV-1<sub>JRCSF</sub> (clade B, 5.6) compared to that of HIV-1<sub>93IN905</sub> (clade C, 2.3). A likely explanation for this difference in glycan processing, in clade C envelope, is the absence of key glycosylation site(s) which reduce the density of the intrinsic

mannose patch and consequently increase the processing of adjacent Man $\alpha$ 1 $\rightarrow$ 2Man termini. Notably, the oligomannose glycan attached to Asn295 is absent in most clade C isolates, including HIV-1<sub>93IN905</sub>, and is critical for efficient neutralization by a number of mannose-specific antibodies, including 2G12.

## 1.6 Analysis of Mimics of the Viral Spike

Mimics of “real” HIV virions are commonly used for neutralization assays with both bnAbs and immune sera. Although HEK 293T cells are not naturally infected by HIV-1, they are routinely employed to produce pseudoviral particles. It is therefore important to know how the glycosylation of envelopes from pseudoviral particles resembles that of viral envelope glycosylation.

### 1.6.1 Infectious Molecular Clone Envelope Glycosylation

Envelope glycoprotein isolated from a replication-competent virus prepared in HEK 293T cells using an infectious pLAI-JRCSF Env molecular clone showed a more even distribution between oligomannose and complex-type glycans compared to the glycosylation profile obtained for gp120 isolated from virus produced in PBMCs (Bonomelli et al. 2011). The complex-type glycans were predominantly of the bi- or tri-antennary type with variable galactosylation and fucosylation typical for HEK 293T cell-derived glycoproteins.

### 1.6.2 Pseudoviral Particles Envelope Glycosylation

Pseudoviral particles generated by co-transfection of HEK 293T cells with plasmids carrying the JRCSF envelope gene and the HIV-1 backbone are also routinely used. MALDI-TOF MS analysis of isolated gp120 envelope glycans revealed an almost exclusive oligomannose population (Doores et al. 2010). Most notably, the Man<sub>5</sub>GlcNAc<sub>2</sub> glycan, found only as a minor species on monomeric gp120, or at a comparable level as the other oligomannose structures on viral envelope, is observed as the single most abundant species on gp120 derived from pseudoviral particles. Similar to the infectious molecular clone system, an uncleaved, nonfunctional gp160 band also showed the presence of higher levels of oligomannose glycans and Man $\alpha$ 1 $\rightarrow$ 2Man terminating glycans. Further, a distinct gp120 glycoform, shed in the supernatant, and proposed to be derived solely from cleaved functional trimers (Crooks et al. 2011), shows a decrease in  $\alpha$ 1 $\rightarrow$ 2-mannosidase trimming, together with a population of residual, complex-type glycans. This resembles the glycosylation of gp120 isolated from functional virus. Moreover, the complex-type glycans

seen in this shed gp120 were accompanied by a corresponding reduction in the Man<sub>5</sub>GlcNAc<sub>2</sub> peak compared to virion-associated gp120. This indicates that this species does not evade processing by GnT-I and subsequent Golgi-resident glycosidases and glycosyltransferases.

### 1.6.3 Analysis of Recombinant Trimers

Further mimics of the Env trimer on “real” HIV virus are recombinant trimers. The HIV Env trimer is relatively unstable, therefore there are very few examples of recombinantly expressed soluble trimers and little is known of their glycosylation. A common HIV trimer stabilization strategy has been to introduce mutations that stabilize the gp120/gp41 interface. One example has been the introduction of additional cysteine residues at the gp120/gp41 interface that stabilize the trimer (referred to as SOSIP trimer) through formation of disulfide bonds (Binley et al. 2000; Sanders et al. 2002). Sanders and co-workers expressed a KNIH144 SOSIP trimer in the *N*-acetylglucosaminyltransferase I-deficient HEK 293S cell line and analyzed the glycans using mass spectrometry and HPLC (Eggink et al. 2010). Man<sub>9</sub> and Man<sub>8</sub> glycans accounted for 53 % of the N-linked glycans in comparison to the typical 14–22 % found on recombinant monomers (Bonomelli et al. 2011; Doores et al. 2010) suggesting that  $\alpha$ 1→2-mannosidase trimming is reduced by the steric constraints imposed by gp120 trimerization leading to a “trimer-associated” oligomannose population in addition to the “intrinsic” mannose patch in a manner similar to that observed on virion-associated gp120.

The full assignment of the 27 PNGS present on soluble recombinant trimeric gp140 protein from the clade C HIV-1<sub>CNS4</sub> strain expressed in CHO cells was performed by LC-ESI-MS (Pabst et al. 2012). The *m/z* values then obtained by ESI-MS for each glycopeptides were analyzed to assess site occupancy, glycan structure, and microheterogeneity at each PNGS. In agreement with previous studies, the cluster of oligomannose-type glycans on gp120 outer domain was also observed.

### 1.6.4 Comparison of Trimer Mimics to PBMC-Derived Viruses

The analysis of N-linked glycans from gp120 isolated from virions produced in PBMCs showed that oligomannose-type glycans are a key feature of viral envelope glycoproteins, together with a conserved set of highly processed, sialylated bi-/tri-/tetra-antennary complex-type glycans. Overall, the distribution of oligomannose-type glycans for the PBMCs-derived virus is similar to the one observed for the single-plasmid infectious pLAI-JRCSF molecular clone, and the shed material from the pseudoviral system, with the presence of complex-type glycans, but without an elevated Man<sub>5</sub>GlcNAc<sub>2</sub> peak. A common feature of glycosylation of envelope

isolated from pseudoviral particles is the high abundance of the Man<sub>5</sub>GlcNAc<sub>2</sub> glycan. Furthermore, the decrease in complex-type glycans abundance demonstrates that further along the glycosylation pathway, the envelope spikes show a partial resistance to processing by GnT I or by subsequent Golgi-resident enzymes (Fig. 1.1). This data is consistent with recent reports that pseudoviral particles contain a large proportion of Endo H sensitive, nonfunctional envelope glycoprotein (Crooks et al. 2011). It was suggested that the cellular stress induced by the extended stay of Env in the ER lead to incorporation of uncleaved gp160 from the ER on nascent virions (Tong et al. 2012).

Viral envelope glycoproteins exhibit a further level of divergence in their glycosylation compared to host-cell glycosylation and monomeric glycosylation. Processing by  $\alpha$ -mannosidase during the early steps of viral glycosylation assembly and secretion is limited to a greater extent on the native virus than on monomeric gp120. This would be compatible with a model where envelope oligomerization occurs in cellular compartments with active  $\alpha 1 \rightarrow 2$  mannosidases, and where structural constraints, such as glycan–glycan and glycan–protein interactions at the trimer interface, alter HIV glycan processing.

## 1.7 Conservation of gp120 Oligomannose Patch

MALDI-TOF MS (Bonomelli et al. 2011; Doores et al. 2010) and lectin-binding studies (cyanovirin-N, griffithsin, scytovirin) (Alexandre et al. 2010) have shown that the population of oligomannose-type glycans is a conserved feature of all viral isolates from both virion-associated and recombinantly expressed gp120/gp140.

The presence of a dense cluster of glycans on gp120 outer domain is thought to decrease glycosidase accessibility to glycan structures and result in the presence of unprocessed oligomannose-type glycans on the surface of gp120. Consequently, the number of PNGS, as well as mutations involving PNGS, may alter the overall levels of oligomannose-type glycans, and the relative abundance of glycans within the oligomannose series. These unprocessed oligomannose-type glycans form the mannose-patch which is the target of a number of HIV bnAbs.

Site-analysis data suggest that the mannose-patch includes the N-linked glycans at positions N295, N332, N339, N386, and N392 which are 59 %, 73 %, 66 %, 87 %, and 79 % conserved, respectively. The 73 % conservation of the N332 glycan explains the breadth of neutralization of PGT128. However, for antibodies such as 2G12 and PGT131–135 that recognize additional and less well-conserved glycans, a more limited breadth of neutralization is observed (Walker et al. 2011; Binley et al. 2004), and the deletion of one or several glycosylation site(s) has a more significant impact on their neutralization sensitivity (Scanlan et al. 2002). Likewise, resistance to the cyanovirin-N lectin, whose binding site overlaps 2G12 epitope, is associated with mutations at the N230, N289, N332, N339, N386, N392, and N448 sites (Balzarini et al. 2006; Hu et al. 2007).



The conservation of the population of oligomannose-type glycans upon mutation of one or two key glycosylation sites, as well as between isolated from clade A, B, and C indicates that oligomannose-type glycans are a conserved feature of viral glycosylation, and therefore a potential template for immunogen design.

## 1.8 Conservation of a Restricted Set of Complex-Type Glycans on Viral Env

Whilst the oligomannose-type glycans form a cluster on gp120 outer domain distal to the CD4-binding site, it has been proposed that gp120 complex-type glycans arrange as a tight patch next to the receptor-binding site (Sato et al. 2012). The role of complex-type glycans in HIV infection and pathogenesis, however, remains poorly understood.

It was shown by MALDI-TOF MS analysis that the population of complex-type glycans was composed of a restricted set of highly processed, sialylated, bi-, tri-, and tetra-antennary glycans (Bonomelli et al. 2011). In addition to glycan profiling, lectin-binding studies have shown that gp120 exhibit glycans bearing terminal lactosamine (LacNAc or GalGlcNAc) residues, as evidenced by binding to Galectin-1 (St-Pierre et al. 2011), which has been shown to promote HIV binding to CD4<sup>+</sup> T-cells and facilitate infection.

The crystal structure of the bnAb PGT121 shows that this antibody can bind to bi-antennary sialylated complex-type glycans (Mouquet et al. 2012; Julien et al. 2013). PGT121 was also shown to neutralize virions produced in GnT I-deficient cells displaying oligomannose-type glycans only and virus produced in the presence of kifunensine displaying only Man<sub>9</sub>GlcNAc<sub>2</sub> glycans suggesting that PGT121 recognition of this complex glycan is not absolutely required for neutralization.

A similar pattern of glycosylation and glycan structures were observed on virion-associated gp120 from different viral isolates, suggesting that similarly to the oligomannose patch, complex-type glycans are also a conserved, typical feature of viral Env glycosylation.

## 1.9 Remodeling of HIV-1 gp120 Glycosylation

Although the glycosylation on glycoproteins is determined by the host-cell glycosylation machinery and the protein-directed effects discussed above (e.g., trimerization), it is possible to control the type of glycosylation displayed to some extent using glycosidase inhibitors that inhibit the enzymes within the N-linked glycosylation pathway. These inhibitors have been used in production of both pseudoviral particles and recombinantly expressed gp120s.

### 1.9.1 Remodeling of Viral Glycoforms

Glycosidase inhibitors have been used to manipulate the glycosylation on HIV pseudovirions.  $\alpha$ -Glucosidase inhibitors (e.g., *N*-butyldeoxynojirimycin (NB-DNJ) and castanosperimine) are potent inhibitors of HIV replication (Fleet et al. 1988; Gruters et al. 1987; Karpas et al. 1988; Walker et al. 1987) whereas inhibitors of the later stages of glycan processing (e.g., deoxymannojirimycin (DMJ), kifunensine, and swainsonine) have no effect (Elbein et al. 1990; Gruters et al. 1987; Montefiori et al. 1988). Glycosidase inhibitors have been used to measure the effect changes in gp120 glycosylation has on the sensitivity of viruses to neutralization by bnAbs and subsequently to evaluate the role glycans play in the epitope of the bnAb.

NB-DNJ inhibits  $\alpha$ -glucosidase I and II, resulting in glycoproteins displaying Glc<sub>1-3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> glycans by preventing removal of glucose residues (Fischer et al. 1995, 1996; Karlsson et al. 1993). However, the presence of an endomannosidase in HEK 293T cells, which cleaves glucosylated glycans between the terminal mannose residues of the D1 arm into (D2,D3)-Man<sub>8</sub>GlcNAc<sub>2</sub> (Roth et al. 2003) represents an alternative pathway to more processed glycans. Therefore virus made in HEK 293T cells in the presence of NB-DNJ will not display oligomannose glycans bearing Man $\alpha$ 1 $\rightarrow$ 2Man residues at the terminus of the D1 arm. NB-DNJ treated pseudovirus has therefore been used to identify bnAbs that neutralize HIV through interaction with terminal Man $\alpha$ 1 $\rightarrow$ 2Man residues of the D1 arm in a manner similar to the mannose-specific bnAb 2G12 (Calarese et al. 2003, 2005; Pejchal et al. 2011).

Kifunensine, a potent ER  $\alpha$ -mannosidase I as well as type I Golgi  $\alpha$ -mannosidase inhibitor, has now been used extensively to generate pseudovirions displaying homogeneous Man<sub>9</sub>GlcNAc<sub>2</sub> glycans (Agrawal-Gamse et al. 2011; Doores and Burton 2010). Kifunensine-treated pseudovirus is resistant to neutralization by N160-sensitive, trimer preferring bnAbs such as PG9, PG16, and PGT145 (Doores and Burton 2010) and this assay has now become a diagnostic tool for measuring the presence of this type of bnAb in HIV-infected individuals (Walker et al. 2010).

Swainsonine, a potent inhibitor of the enzyme Golgi  $\alpha$ -mannosidase II, prevents the formation of complex sugars by inhibiting the trimming of mannose residues from GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>. Therefore swainsonine-treated virus will display GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> instead of more processed complex glycans. Further, a GnT I-deficient HEK 293S cell line does not add a GlcNAc residue to the Man<sub>5</sub>GlcNAc<sub>2</sub> structures in the Golgi apparatus. Therefore, glycoproteins or pseudovirus prepared in this cell line will display Man<sub>5</sub>GlcNAc<sub>2</sub> instead of more processed complex glycans (Reeves et al. 2002). Any Man<sub>6-9</sub>GlcNAc<sub>2</sub> glycans naturally occurring on the virion-associated gp120 will be unchanged when using these two expression systems. These systems are useful for determining the role of complex glycans in pathogenesis and the epitopes of bnAbs (Doores and Burton 2010; Eggink et al. 2010; Pejchal et al. 2011; Binley et al. 2010).

### 1.9.2 *Remodeling of Recombinant Protein Glycoforms*

In a manner similar to that described for pseudovirus, recombinant glycoproteins can be expressed in the presence of glycosidase inhibitors and in the GnT I-deficient HEK 293S cell line (Reeves et al. 2002). These expression systems are useful tools for reducing the glycan heterogeneity of glycoprotein antigens used in structural studies of HIV envelope glycoproteins in complex with bnAbs and in immunogen development. By disrupting specific steps of the glycosylation pathway, gp120 glycoforms closely representing biosynthetic intermediates of the early ER and the IC/*cis*-Golgi based on the known localization of the processing enzymes can be generated. When gp120 is expressed in HEK 293T cells in the presence of kifunensine, Man<sub>9</sub>GlcNAc<sub>2</sub> is the predominant glycan displayed and this glycoform thus mimics an early ER gp120 glycoform. Expressing gp120 in GnT I-deficient HEK 293S cells creates a glycoform representing an IC/*cis*-Golgi gp120. Glycan processing in this cell line is stalled at the Man<sub>5</sub>GlcNAc<sub>2</sub> biosynthetic intermediate.

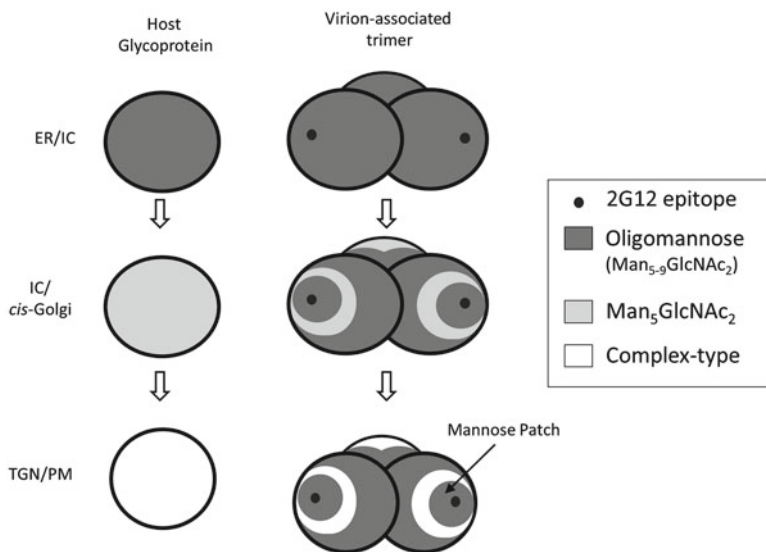
These two different glycoforms were used by Scanlan and co-workers to study the kinetics of hydrolysis of Man<sub>9</sub>GlcNAc<sub>2</sub> to D1,D3-Man<sub>8</sub>GlcNAc<sub>2</sub> (Doores et al. 2010) to understand the persistence of a core population of Man<sub>6-9</sub>GlcNAc<sub>2</sub> glycans on HIV envelope, a mannose patch that is intrinsic to the monomer and is independent of the effects of trimerization discussed above. It has been proposed that the dense clustering of glycans on gp120 outer domain might form a protected patch of unprocessed oligomannose glycans that are maintained as a result of their inaccessibility to ER and Golgi  $\alpha$ 1 $\rightarrow$ 2mannosidases. A biphasic hydrolysis was observed with an initial rapid trimming of Man<sub>9</sub>GlcNAc<sub>2</sub> to D1,D3-Man<sub>8</sub>GlcNAc<sub>2</sub> on the early gp120-ER glycoform described above (displaying homogeneous Man<sub>9</sub>GlcNAc<sub>2</sub> glycans) whereas the remaining Man<sub>9</sub>GlcNAc<sub>2</sub> were trimmed at an approximately 100 times slower rate. A significant population of Man<sub>9</sub>GlcNAc<sub>2</sub> (30 %) was left unprocessed after exhaustive digestion. This provides direct support for a model in which the high-mannose patch is conserved on gp120 due to the inaccessibility of the highly clustered N-linked glycans to the ER  $\alpha$ -mannosidase I enzyme. This patch includes the N332 glycan that forms the central contact for several of the HIV bnAbs (e.g., 2G12, PGT128, and PGT121).

### 1.9.3 *Remodeling of N-Linked Glycan Positioning*

In addition to manipulation of the composition of the N-linked glycans on gp120, individual glycosylation sites can be removed through site-directed mutagenesis, through mutation of the Asn or Thr/Ser residues of the glycosylation sequon removal of specific glycosylation sites using this method has allowed identification of the N-linked glycans that are important in the epitopes of some HIV bnAbs (Scanlan et al. 2002; Walker et al. 2011; Wang et al. 2013).

## 1.10 Discussion/Conclusion

In summary, the HIV envelope glycoprotein is heavily glycosylated with host-derived N-linked glycans. The above-described studies have shown that the glycosylation of this protein diverges from typical host-cell glycosylation as illustrated in the model in Fig. 1.4. The presence of a dense cluster of glycans on gp120 outer domain has been shown to decrease mannosidase accessibility to glycan structures and result in the presence of unprocessed oligomannose-type glycans on gp120 surface. The clustering of N-linked glycans and the steric constraints of trimerization gives rise to a population of oligomannose glycans on viral Env. A higher level of these  $\text{Man}\alpha 1 \rightarrow 2\text{Man}$  terminating glycans has been reported for viral trimeric spike, indicating that these additional structures derive from a mechanism separate from the one leading to the formation of the monomer-intrinsic mannose patch. This suggests that given the extensive opportunities for glycan–glycan and glycan–protein



**Fig. 1.4** Model for HIV Env glycosylation processing. As folded glycoproteins transit through the ER, IC, and *cis*-Golgi apparatus, terminal mannose residues are removed from the  $\text{Man}_9\text{GlcNAc}_2$  glycan by ER  $\alpha$ -mannosidase I and Golgi mannosidase A-C. The resulting  $\text{Man}_5\text{GlcNAc}_2$  intermediate is then processed by GnT I and subsequent glycosyltransferases to yield a variety of complex-type glycans, which composition depends on the cell from which they derive. However, whilst gp120/gp41 glycans are processed by the host-cell glycosylation machinery, the chemical composition of monomeric gp120 and native trimeric HIV Env glycans was shown to exhibit markedly constrained, protein-directed glycan processing when compared to that of the host cell. Firstly, dense clustering of glycans on gp120 outer domain leads to lack of mannosidase processing and persistence of a patch of unprocessed oligomannose type glycans. Secondly, further glycan–glycan and protein–glycan interactions due to trimerization lead to an additional “trimer associated” oligomannose population

interactions at the trimer interface, these structural constraints of trimerization do alter HIV glycan processing.

The population of oligomannose-type glycans on virion-associated gp120 is conserved regardless of the virus production system, the envelope expression level, bnAb sensitivity, or the envelope sequence. This divergence from host-cell glycosylation gives rise to the high-mannose epitopes that are targeted by a number of the HIV bnAbs including 2G12, PGT128, and PG9. Finally, from the perspective of viral transmission and pathogenesis, it is proposed that the oligomannose glycans are required for the interaction with the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on peripheral dendritic cells and promote the capture and dissemination of HIV in the early stage of infection. The broad conservation of the oligomannose array indicates that such interactions are highly efficient for any isolate or clade of HIV.

## 1.11 Future Perspectives

As there are noticeable differences between virion-associated and recombinantly expressed gp120 and an increasing number of HIV bnAbs isolated bind to the N-linked glycans on the HIV envelope glycoprotein, knowledge of the type of glycan present at each site on virion-associated gp120 may be critical for design of immunogens that correctly mimic the glycan shield on HIV. However, due to the amount of material required for site-specific analysis using the current methodologies, studies using virion-associated gp120 have not been carried out. More sensitive analytical methodologies and improved protocols for making large quantities of HIV may be necessary.

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