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Structural principles controlling HIV envelope glycosylation

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Abstract

The heavily glycosylated, trimeric HIV-1 envelope (Env) protein is the sole viral protein exposed on the HIV-1 virion surface and is thus a main focus of antibody-mediated vaccine development. Dense glycosylation at the outer domain of Env constrains normal enzymatic processing, stalling the glycans at immature oligomannose-type structures. Furthermore, native trimerization imposes additional steric constraints, which generate an extensive ‘trimer-induced mannose patch’. Importantly, the immature glycans present a highly conserved feature of the virus that is targeted by broadly neutralizing antibodies. Quantitative mass spectrometry of glycopeptides together with structures of the trimeric viral-spike define the steric principles controlling processing and provide a detailed map of the glycan shield.

Graphical abstract

Introduction

The extensively glycosylated envelope spike (Env) of the type-1 human immunodeficiency virus (HIV-1) mediates host cell infection while at the same time constituting a target for broadly neutralizing antibodies (bNAbs). Env is a trimer of gp120-gp41 heterodimers, which is generated by protease cleavage, usually furin, of the pro-protein gp160. The trimer is covered in a dense array of glycans constituting about half of its mass [1]. Although the glycan shield protects the highly mutative protein surface from the immune system and

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constantly evolves to escape antibody recognition [2–4], very potent bNAbs have been shown to overcome this barrier by evolving to tolerate the presence of glycans, or more commonly, incorporating glycans directly into their epitopes or [5–12]. The majority of characterized bNAbs have matured in ways that allow access to the shielded protein surface whilst exhibiting affinity maturation in response to the surrounding glycans. In fact, the majority of characterized bNAbs recognizes glycan-dependent epitopes such as at the V1/V2 loops including a glycan at N160 (e.g. PG9), a region on the outer domain of gp120 centered around glycan N332 (e.g. PGT128), the gp120-gp41 interface (e.g. PGT151) and at the CD4 binding site (e.g. 179NC75) [6,8,13,14]. Elicitation of bNAbs by rational immunogen design is a main focus of structural-guided HIV-1 vaccine development [15]. Thus, understanding the fine structure of the HIV-1 glycan shield on the virus and recombinant mimics of the trimeric viral spike is essential in guiding vaccination strategies. Here, we review how the formation of a natively glycosylated Env spike is shaped by its intrinsic structural features (Figure 1).

Strategies to analyse envelope glycosylation

Global and site-specific analysis of Envelope glycosylation has been performed using a variety of chromatographic, enzymatic and mass spectrometric methods. Assessment of glycosylation by hydrophilic interaction chromatography of released and fluorescently labelled glycans is the leading method for the quantitation of the glycan composition of Env [16]. Mass spectrometric glycomic analyses of released Env glycans has typically been performed by either matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis of permethylated glycans [17,18] or by ion-mobility mass spectrometry (IM MS) [19–21]. The latter provides a valuable tool for the separation of glycan isomers as well as for cleaning up MS profiles from possible contaminants, thus being suitable for low abundance viral samples [19,20]. Both strategies, chromatographic and mass spectrometric – possibly in combination with exoglycosidase digests – are complementary approaches to gain information on overall Env glycosylation fingerprints.

Mapping of the HIV-1 glycan shield requires glycopeptide analysis by mass spectrometry. This has been performed on recombinant monomeric Env [22–26], soluble native-like trimers [27–29], membrane-associated trimers [30] and virion-derived gp120 [17]. These approaches usually include protease digestion of the target glycoprotein followed by tandem liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS) using varying mass analysers and fragmentation modes. While clades, constructs, cell lines and purification methods varied between these different studies they reveal interesting insights into the glycan shield that will be further elaborated in the next sections. Relative quantitation of the individual glycan structures at any one site has been a challenge and most studies have not attempted to quantify glycan abundances [23,31]. However, a quantitative method has now been established and validated involving LC-ESI MS analysis of glycopeptides in which the data analysis was guided by target-specific library created by IM MS of released glycans [27,29].

Glycan clustering in the intrinsic mannose patch

Virion-derived HIV-1 Env has been shown to carry a large abundance of underprocessed, oligomannose-type glycans ($\text{Man}_5\text{-}_9\text{GlcNAc}_2$) usually not found in great abundance on human glycoproteins [17,21,32,33]. This triggered speculation as to whether this phenomenon can be attributed to the bypassing of the conventional N-glycosylation pathway or to an intrinsic property of the glycoprotein, which limits glycan processing [32,34]. Env released from replication-incompetent pseudoviruses shows similarly large populations of oligomannose-type glycans. In fact, a model of steric hindrance on glycan processing enzymes imposed by the high glycan density is strongly supported by the resistance of a subpopulation of glycans on recombinant gp120 to *in vitro* digestion with recombinant α -mannosidases [34]. Additionally, mutations eliminating glycosylation sites have measurable effect on the fine processing state of neighboring glycans [35]. However, steric constraints do not apply uniformly across the spike and location determines processing fate. One feature that is conserved in both native-like trimers and recombinant gp120 monomers is a region of underprocessed glycans on the outer domain termed the ‘intrinsic mannose patch’ (IMP) [34]. The IMP is a highly conserved feature among all HIV-1 clades that persists throughout the course of HIV-1 infection [32,36]. A quantitative map of the fine structure of the IMP has recently been determined (Figure 2) [29]. It is formed by an interlocked network of two main oligomannose microclusters that center around the N295, N332, N392 glycans. Many of these oligomannose-dominated glycan sites are among the most conserved glycans on Env (Figure 2B) [11] and are targets for multiple families of bNAbs [11,37–41].

Trimer-associated mannose patch of native-like Envelope

Glycan analyses of virion-associated Env and recombinant trimers show an elevated oligomannose content compared to gp120 monomers [16,29,32,34]. In the context of a natively-folded trimer, interprotomer glycan-protein and glycan-glycan interactions are thought to lead to the emergence of an additional, “trimer-associated mannose patch” (TAMP) [16,34,42]. In practice, the TAMP does not always arise by a complete switch in processing state from entirely complex to entirely oligomannose but represents an increase in the abundance of underprocessed glycans [27,29]. The formation of the TAMP is dependent on cleavage-induced, native-like envelope folding and is not a feature of uncleaved and incorrectly assembled trimers [16,29]. Site-specific N-glycosylation analysis of soluble, fully cleaved, native-like trimers compared to both gp120 monomers and soluble, uncleaved pseudotrimers by LC-ESI MS revealed a detailed molecular map of trimer-associated glycan remodelling (Figure 3) [29]. Uncleaved pseudotrimers, similar to gp120 monomers, lack a native TAMP and show a non-native elevation in glycan processing (Figure 1B). The impact of a trimeric rather than monomeric context can have a dramatic impact on glycan processing. For example, when in the trimeric rather than monomeric context, there were 60 and 70 % point increase in levels of oligomannose at positions N160 and N273, respectively. These observations highlight that both trimerization and cleavage are required for the integrity of the HIV-1 glycan shield [16,29].

Protease cleavage of Env, usually by furin, is an essential step in the formation of natively folded trimers [43,44]. The influence of furin cleavage on Env glycosylation processing

points towards two possible models of Env maturation. Furin cleavage of the proprotein, gp160, could generate compactly folded trimers, which are then protected against subsequent post-furin α -mannosidase activity [16,45]. Alternatively, it could be hypothesized that the protease selectively cleaves particular glycoforms of Env. Given the observation that furin overexpression leads to complete gp160 cleavage and that uncleaved gp160 can be partially processed by *in vitro* incubation with recombinant furin [46,47], we suggest that post-furin α -mannosidase activity is important in Env maturation. One aspect to be resolved will be how to reconcile these observations with the proposed distribution of the enzymes and the substrate along the secretory pathway.

Impact of membrane-association on glycan processing

Most site-specific studies on Env glycosylation have been performed on truncated, soluble monomers or trimers, which lack the membrane proximal external region (MPER) and the C-terminal domain. In the context of virions, however, the envelope spike is membrane-bound which triggers the question of a possible additional constraint on processing by the proximity of the membrane. This is not a simple question as membrane localisation brings the glycoprotein in closer proximity to the enzymes and membrane association is known to enhance the processing of complex-type glycans [48]. However, a study on the comparison of recombinant, membrane-bound and uncleaved, soluble Env indicates a higher abundance of oligomannose, in particular in the gp41 region [30]. In addition, analysis of virion-derived gp120 suggests the membrane proximal glycan N88 to be occupied by oligomannose-type glycans [17], whereas only a very minor population of such glycans were observed on recombinant Env constructs [49]. The site-specific effect of membrane proximity on virion-derived gp41 is unknown, although bNAbs PGT151 and PGT152 are known to interact with gp41 glycans and preferentially binds complex-type glycans [5,6]. This is consistent with glycan profiling of the virally-derived gp41, which revealed highly processed complex-type glycans with a sub-population of oligomannose- and hybrid-type glycans [21].

Cellular shaping of complex-type glycosylation

The array of oligomannose-type glycans on the viral spike is punctured by regions of enzyme accessibility and associated complex-type glycosylation. This is not a binary process and many sites show an intermediate processing state. The regions exhibiting predominantly complex-type glycans are the apex, some key sites next to the CD4-receptor binding site, and the trimer base. While the oligomannose-type glycans are mainly determined by their steric environment, the portfolio of complex-type glycans are substantially influenced by the glycosyltransferase activities of the producer cell [21]. Importantly, the glycan processing state is known to influence binding of many bNAbs targeting these regions. For example, in addition to the complex glycans of the PGT151 epitope discussed above [5,6], complex-type glycans are known to influence neutralization by apex-targeting PG9, PG16 bNAbs [13]. It is also becoming apparent that many bNAbs can recognise glycans in a variety of processing states including both oligomannose-type and complex-type [13,50]. This underscores that bNAbs can be promiscuous in their glycan recognition. While it is clear that the conserved mannose patches are one of the key properties of Env that should be preserved in immunogen design, as they include the

epitopes for many bNAbs, a possible fine impact of the producer cell's glycosylation machinery on immunogen efficacy needs to be considered.

Resolving the mannose paradox

There is an apparent contradiction in that glycans are directed to be in an oligomannose-type state by steric restriction and yet those same glycans are accessible for antibody and lectin recognition [51]. With the advent of recent structural data, this apparent conflict can now be resolved [35,52]. Overlaying the crystal structure of the enzyme-substrate complex of the ER α 1,2-mannosidase I [52] with the model of the fully glycosylated Env trimer derived from cryo-EM [53] and mass spectrometry analysis [29] shows how the mannosidase enzyme wraps around target glycans. In contrast, bNAbs are able to recognise glycans while also penetrating the glycan shield [8,53]. We illustrate this principle by the comparison of the attempted digestion of the N332 glycan by ER α 1,2-mannosidase I with the recognition mode of bNAb PGT128 (Figure 4). Enzymatic processing is limited by multiple surrounding glycans that clash with the enzyme and thus prevent mannose trimming (Figure 4A), highlighting the persistence of the mannose patch despite an evolving glycan shield [35,36]. Broadly neutralizing antibodies, such as PGT128, however, manage to adjust to the high density of glycosylation by reaching in between glycans and incorporating them into their epitopes [8,53] (Figure 4B).

Strategies to elicit anti-mannose immune responses

In order to elicit potent and broad neutralizing antibodies against glycan epitopes, immunological tolerance to 'self' glycan structures must be overcome. In addition, bNAbs must tolerate microheterogeneity at target glycosylation sites and exhibit avidity effects to overcome the low affinity often exhibited by carbohydrate-protein interactions [42,54]. In terms of HIV-1 vaccine design, the exploitation of non-self features of the envelope glycan shield is nicely exemplified by bNAb 2G12, a uniquely domain-exchanged antibody, which exclusively recognizes an oligomannose glycan epitope on the outer domain of gp120 [39]. Mimicry of the 2G12 epitope has been exploited in various different immunogen design approaches, including glycan clustering on rationally designed peptide, dendrimer, carbohydrate, DNA, steroid, gold nanoparticle and biomacromolecular scaffolds (reviewed in [49]). To date, none of these synthetic approaches have yielded neutralizing antibodies. Encouragingly, however, yeast-based immunogens engineered to mimic the terminal motifs of human oligomannose-type glycans did elicit a weak antibody response mirroring 2G12 specificity [55]. Mimicry of the natural immune response to glycans in infection and in the development of anti-glycolipid autoimmune conditions has been proposed to be a fertile ground for developments in HIV-1 immunogen design [54,56]. One avenue could be to produce envelope immunogens in ways to carry potentially more immunogenic yeast-type oligomannose glycans to exhibit an additional 'non-self' stimulus to trigger antibody production and maturation.

Exploiting gaps in the glycan shield

It rather recently was discovered that autologous neutralizing antibodies developed during rabbit immunization experiments with native-like trimers often target holes in the HIV-1 glycan shield [57]. In fact, it was shown that most HIV strains indeed lack one or more conserved glycan sites that could potentially create an area of vulnerability for the virus [57]. The discovery of these breaches in the glycan shield has encouraging implications for immunogen design. Mainly, by following a prime-boost strategy in which immunization of glycan-depleted trimers designed to target key B-cell lineages followed by immunization of fully glycosylated trimers. In this way, the immunization regimen is designed to mimic the host-pathogen dynamic within patients that manage to develop potent bNAbs [58].

Conclusion

There are multiple structural and cellular factors that shape Env glycosylation. Firstly, the high density of glycans on the outer domain of gp120 gives rise to the presence of the intrinsic mannose patch through steric restriction of the processing enzymes that require extensive glycan recognition interfaces. Secondly, cleavage-induced trimerization imposes additional steric constraints at the protomer interface leading to the emergence of a trimer-associated mannose patch. Thirdly, there may be an elevation in oligomannose-type glycans in membrane proximal regions. Finally, the producer cell can shape the portfolio of complex-type glycans. Broadly neutralizing antibodies targeting the HIV-1 glycan shield need to demonstrate a substantial amount of plasticity in their recognition and tolerate viral diversity and glycan escape mutants. While this seems to be a very challenging goal, successes in cancer vaccine research, observations from autoimmunity and natural occurrence of bNAbs during HIV-1 infection support the oligomannose clusters on the viral surface as an attractive target for rational immunogen design.

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Highlights

- The HIV-1 glycan shield is a conserved target for broadly neutralizing antibodies
- Mass spectrometry analyses have revealed fine structural details of HIV-1 envelope glycosylation
- Dense glycan packing, trimerization and potentially membrane-association lead to the emergence of oligomannose-microclusters
- The large array of underprocessed glycans is an important target for antibody-based vaccine design

Figure 1. Envelope cleavage induces a trimer-associated mannose patch

(A) The conformational and oligomeric state of recombinant or natural Env glycoprotein has significant impact on glycan processing. The Env proprotein precursor is synthesized in the endoplasmic reticulum and glycosylated with oligomannose-type glycans at Asn-X-Ser/Thr (X is any amino acid except proline) sequons. Trafficking through the secretory pathway exposes the glycoprotein to α -mannosidases and other glycan processing enzymes, which generally lead to the conversion of oligomannose-type glycans (green) to complex-type glycans (pink). However, intrinsic structural features of Env determine how well individual glycans can be accessed and processed by these enzymes. The outer domain of monomeric gp120 is characterised by a dense cluster of glycans that limits accessibility of ER and Golgi α -mannosidases and thus leads to the emergence of an intrinsic mannose patch. Cleaved Env engages a compact quaternary structure that further constrains glycan processing and induces the formation of an additional trimer-associated mannose population. Uncleaved, non-native Env pseudotrimers, however, have an open and irregular structure (inset EM image in B) that allows easier access to processing glycans and hence leads to an elevation in complex-type glycans. (B) Glycan profiles of released and fluorescently labelled N-glycans from three soluble, recombinant clade A BG505 constructs (monomeric gp120, pseudotrimeric uncleaved BG505.SEKS and trimeric cleaved BG505 SOSIP.664) generated by hydrophilic interaction chromatography-ultra performance liquid chromatography (HILIC-UPLC) [29].

Figure 2. Fine structure of the intrinsic mannose patch of recombinant gp120

(A) Glycan crowding in the outer domain of recombinant monomeric gp120 (BG505 strain) leads to an intrinsic mannose patch (IMP) consisting of two main oligomannose-microclusters, centered around N295 and N392 (see magnifications). Model of the glycosylated gp120 monomer is derived from the one previously described elsewhere [27]. (B) Bar graphs of the percentage of conservation of the 24 N-glycosylation sites on BG505 gp120 [11]. (C) Site-specific glycosylation profiles of IMP gp120 glycans, categorized as follows: oligomannose series (M4 to M9; $\text{Man}_4\text{GlcNAc}_2$ to $\text{Man}_9\text{GlcNAc}_2$) in green; hybrid- and complex-type glycans in pink (grouped by the number of their branching antennae and by the presence or absence of a core fucose) [27,29].

Figure 3. The trimer-associated mannose patch

Models of the glycosylated BG505 SOSIP.664 trimer are derived from the one previously described elsewhere [27]. (A) The glycans are colored according to their oligomannose content [29]. (B) Heat map of the effect of native-like trimerization on the percentage point of oligomannose-type glycans of recombinant gp120 [29]. (C) Schematic of the localization of the intrinsic and the trimer-associated mannose patches on Env.

Figure 4. Enzyme resistance and antibody recognition of the HIV-1 glycan shield
 (A) ER α 1,2-mannosidase I (PDB ID 5KIJ) [52] (cyan) was modelled to bind the $\text{Man}_9\text{GlcNAc}_2$ N332 glycan (green) on a fully glycosylated model of a previously described model of BG505 SOSIP.664 [27]. The surrounding glycans that sterically clash with enzyme recognition of N332 are highlighted in red. (B) The Fab variable region of broadly neutralizing antibody PGT128 (PDB ID 5ACO) (orange) targets the N301 glycan and also interacts and depends on additional nearby glycans [53]. Green: oligomannose-type glycans; pink: complex-type glycans. Removal of the N137 glycan has been shown