ANNUAL REVIEWS

Annual Review of Virology

Structure and Role of O-Linked Glycans in Viral Envelope Proteins

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Annu. Rev. Virol. 2023. 10:283–304

First published as a Review in Advance on June 7, 2023

The *Annual Review of Virology* is online at virology.annualreviews.org

https://doi.org/10.1146/annurev-virology-111821-121007

Keywords

mucin-like domain, heparan sulfate, chondroitin sulfate, vaccine, attachment, virion, egress

Abstract

N- and O-glycans are both important constituents of viral envelope glycoproteins. O-linked glycosylation can be initiated by any of 20 different human polypeptide O-acetylgalactosaminyl transferases, resulting in an important functional O-glycan heterogeneity. O-glycans are organized as solitary glycans or in clusters of multiple glycans forming mucin-like domains. They are functional both in the viral life cycle and in viral colonization of their host. Negatively charged O-glycans are crucial for the interactions between glycosaminoglycan-binding viruses and their host. A novel mechanism, based on controlled electrostatic repulsion, explains how such viruses solve the conflict between optimized viral attachment to target cells and efficient egress of progeny virus. Conserved solitary O-glycans appear important for viral uptake in target cells by contributing to viral envelope fusion. Dual roles of viral O-glycans in the host B cell immune response, either epitope blocking or epitope promoting, may be exploitable for vaccine development. Finally, specific virus-induced O-glycans may be involved in viremic spread.

INTRODUCTION

Glycan: a covalent assembly of monosaccharides; may be free or covalently linked to proteins or lipids

Golgi region: a vesicular organelle, stratified as *cis*-, medial-, and *trans*-Golgi, situated between the endoplasmic reticulum and the plasma membrane

N-acetylneuraminic acid (NeuAc): dominant sialic acid in human tissue; negatively charged monosaccharide; mostly terminal in O-glycans via its carbon atom 2

Glycosylation is the most important post-translational modification of viral envelope proteins both in quantitative terms and regarding biological implications. Glycans may account for up to 50% of the molecular mass of an envelope glycoprotein (1), with a wide range of functions involving the viral replicative cycle, colonization of the host, and manipulation of the immune response. Human viruses seem not to encode any glycan-forming enzyme; they all rely on the host cell glycosylation machinery. Essentially all glycans of viral glycoproteins belong to one of two types (2): N-glycans, which are bound to an asparagine nitrogen of the viral glycoprotein, and O-glycans, which are linked to a threonine (Thr) [(or serine (Ser)] oxygen of the viral glycoprotein (O-glycan structures in **Figure 1*a***). (For a detailed compilation of viral glycoproteins containing N- and O-glycans, see Reference 3.) The formation and biology of N-glycans of viral glycoproteins are well investigated (4, 5), but an extreme complexity in initiation of O-linked glycosylation has hampered studies on structure and function of viral O-glycans. However, novel mass spectrometry (MS) procedures for combined assignment of the glycoprotein O-glycan sites and determination of the actual glycan structures (6–9) have paved the way for modern biophysical tools for exploring the interactions with enveloped viruses and their target cells (10, 11). Here, we review the emerging knowledge on O-linked glycosylation in virus-infected cells and new insights into the versatile glycobiology of O-linked glycans of viral glycoproteins.

O-LINKED GLYCOSYLATION IN THE VIRUS-INFECTED CELL: A PLAY WITH MANY ACTORS

Initiation of O-Glycosylation

O-linked glycosylation of viral and host glycoproteins is initiated by addition of N-acetylgalactosamine (GalNAc) to a Ser or Thr of the target polypeptide via special enzymes, polypeptide GalNAc transferases (GalNAc-Ts). The human genome encodes 20 different GalNAc-Ts (isoforms enumerated GalNAc-T1 to GalNAc-T20) with different target peptide motif preferences (**Figure 1*b***). As the combination of expressed GalNAc-Ts is different from tissue to tissue, the O-glycan landscape is also variable with respect to utilized/vacant O-glycan sites (13, 14). This is in sharp contrast to the straightforward initiation of N-glycosylation that is carried out by one single enzyme activity, common to all Metazoa (15).

Structural Variants of O-Glycans and Their Dependence on Expressed Glycosyltransferases

The size of O-glycans found on viral glycoproteins ranges from 1 to 10 or more monosaccharides (13) (see **Figure 1*a***). All glycans are assembled by combinations of altogether more than 100 human glycosyltranferases (16), and each one adds one specific monosaccharide to a growing glycan in a nontemplate manner (**Figure 1*c***). Thus, it theoretically takes 8 different glycosyltransferases to form the sLex O-glycan shown in **Figure 1*a***. Formation of mature O-glycans is entirely a Golgi region–associated process, divided into four parts (reviewed in 17, 18) (**Figure 1*a***). Viral interference with Golgi functions may partially disturb core extension (9), resulting in truncated glycans (**Figure 1*a***). Therefore, O-glycans of viral glycoproteins may range from truncated structures, normally absent in human cells, to large mature O-glycans (19, 20).

Two types of mature O-glycans are of special interest in the viral context: (*a*) O-glycans terminated by N-acetylneuraminic acid (NeuAc) (**Figure 1*a***), whose negatively charged carboxyl group can be relevant for early viral interactions (21), and (*b*) large O-glycans terminated with rarely expressed carbohydrate epitopes [e.g., sialyl Lewis X (sLex)] (**Figure 1*a***) that are

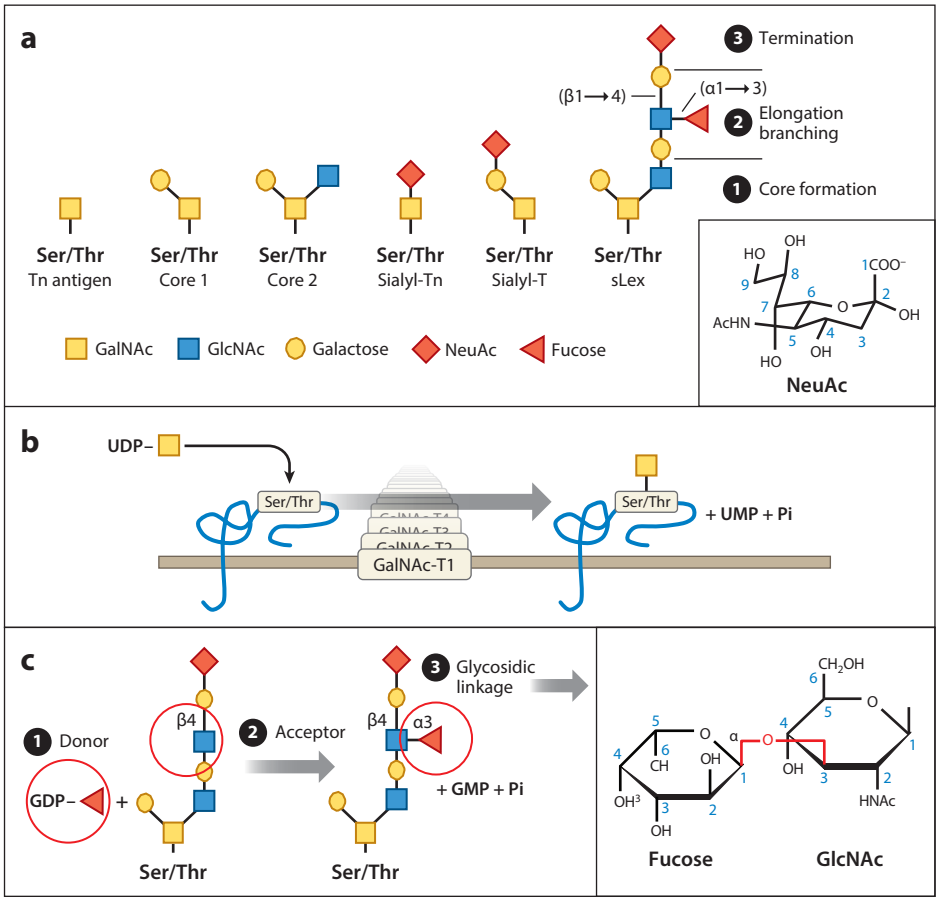


Figure 1

(*a*) Structures of O-glycans occurring in viral glycoproteins. Five small structures and one large, mature O-glycan are depicted. Cores 1 and 2 are precursors to larger O-glycans in viral glycoproteins. sLex is induced by some viruses. Critical sLex glycosidic bonds are indicated. Three consecutive phases in maturation of larger O-glycans (12) are indicated. The insert box presents the structure of NeuAc, an important representative of the sialic acid family, with an N-acetyl group at carbon 5 and a negatively charged carboxyl group at carbon 1. For a presentation of virologically important sialic acids, see Reference 10. (*b*) Heterogeneity of the initiation step of O-linked glycosylation, initiated in the *cis*-Golgi by addition of GalNAc to a Ser or Thr. This is carried out by any of 20 different human polypeptide GalNAc-Ts (GalNAc-T1 through GalNAc-T20). GalNAc-Ts are distributed differently in various tissues, addressing different (sometimes overlapping) target peptide sequences. For a review, see Reference 13. (*c*) Glycosyltransferases form an ether (here identified as glycosidic) linkage between carbon atom 1 of an activated glycan donor monosaccharide and a growing acceptor glycan. The fucosyl transferase depicted completes sLex (see *panel a*) formation by addition of Fuc in an α-1–3 position; structural representation is shown in the insert box. The linkage may be α or β, dependent on the carbon 1 hydroxyl orientation of the donated monosaccharide in the final glycan. Carbon atoms 3, 4, and 6 of the target GlcNAc may participate in a glycosidic linkage, but only carbon atom 3 is addressed by the specific fucosyl transferase discussed here. The high glycosyltransferase specificity is the sum of three subspecificities as indicated. Abbreviations: GalNAc, N-acetylgalactosamine; GalNAc-T, GalNAc-transferase; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; P_i, inorganic phospate; Ser, serine; sLex, sialyl Lewis X; T, Thomsen–Friedenreich antigen; Thr, threonine; Tn, Thomsen-nouveau.

induced by retroviruses (22) or herpesviruses (23, 24), due to viral manipulation of the target cell glycosylation system.

Clustered and Single, Scattered O-Glycans of Viral Glycoproteins

O-glycans of viral glycoproteins appear in two types of patterns (**Figure 2*a***): (*a*) single O-glycans scattered along the amino acid sequence of certain viral glycoproteins (9, 25) and (*b*) multiple

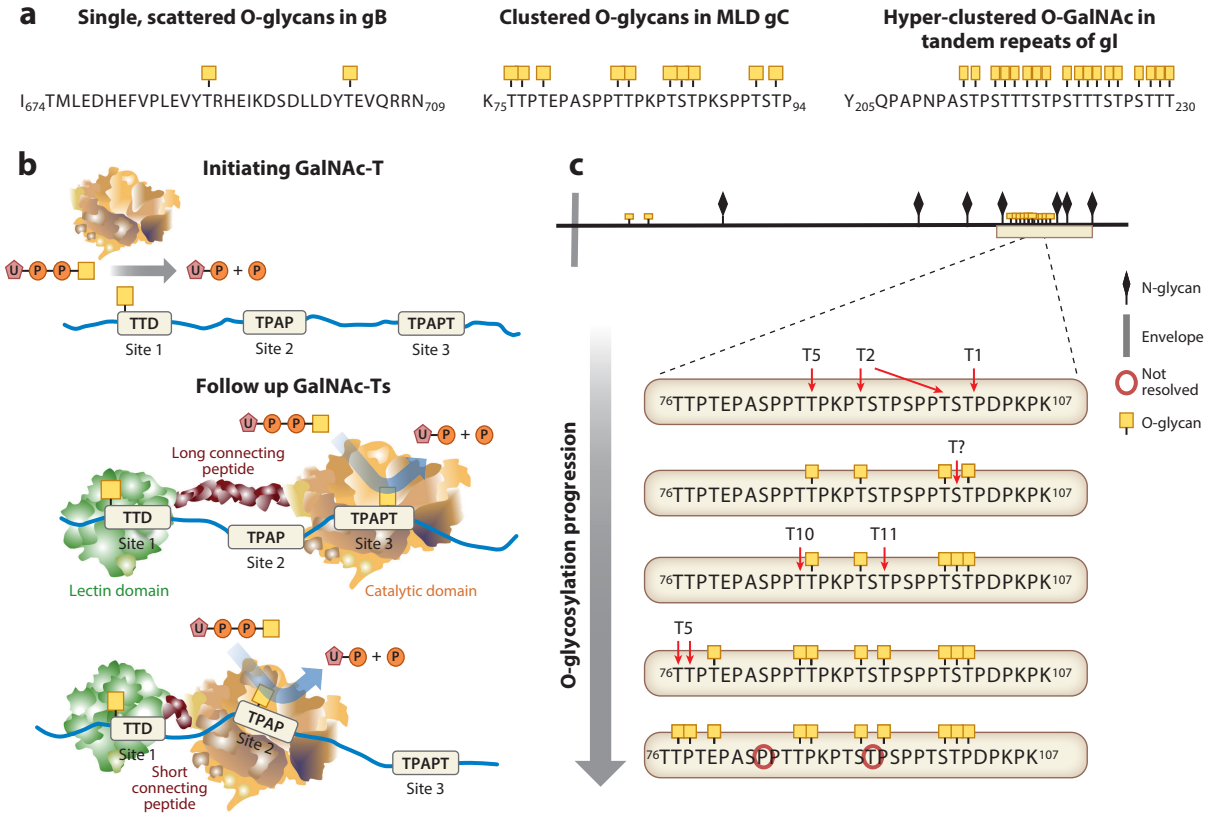


Figure 2

O-glycosylation targets in viral glycoproteins and the temporal regulation of O-glycosylation. (*a*) Examples of scattered and clustered O-glycans from the HSV-1 glycoproteins gB, gC, and gI: solitary, scattered O-glycans in gB, clustered O-glycans exemplified by a section of the HSV-1 gC MLD (a heterogeneous, nonrepetitive amino acid sequence), and hyperclustered O-glycans of the HSV-1 gI MLD consisting of multiple tandem repeats (three repeats depicted). Owing to its extreme concentration of Ser/Thr units, the O-glycan cluster of the gI MLD is so crowded that extension beyond the GalNAc monosaccharide is spatially forbidden. Data are from References 8, 9, and 27. (*b*) Cooperation between initiating and follow-up, glycopeptide-specific GalNAc-Ts with different operation ranges. Pre-glycosylation at site 1 by a peptide-specific, lectin-independent GalNAc-T is a prerequisite for action by subsequent cooperating/competing glycopeptide-specific GalNAc-Ts with different operation ranges. Data are from References 28 and 29. (*c*) GalNAc-T cooperation in practice: the temporal O-glycosylation initiation of the MLD of HSV-1 gC. The sequential actions of different GalNAc-Ts are indicated (each GalNAc-T is denoted by "T" and a numeral): Initiating transferases form a seed for subsequent follow-up transferases by concomitant additions of four GalNAc units to Thr87, Thr91, Thr99, and Thr101, respectively, followed by waves of follow-up GalNAc-T actions. Data are from Reference 8. A predominant final glycoform of the gC MLD peptide stretch is shown, representing only one of the multiple different O-glycan occupancy patterns (within a given tissue signature) in MLDs of the thousands of gC copies produced (reviewed in 26). Abbreviations: GalNAc, N-acetylgalactosamine; GalNAc-T, GalNAc-transferase; HSV-1, herpes simplex virus 1; MLD, mucin-like domain; P, inorganic phosphate; Ser, serine; Thr, threonine; U, uridine.

Table 1 Examples of mucin domain sequences of glycoproteins from different enveloped viruses

Virus/glycoprotein	Accession number[a]	Mucin domain sequence[b]
Human respiratory syncytial virus/ glycoprotein G	AAR14265.1	$_{227}$TTTNPTKKPTLKTTEGDTSTSQSTVLDTTTSKHTIQQQSLHSITSENTPNSTQIPTAT EASTSNST$_{292}$
Marburg virus, Musoke strain, 1980/ glycoprotein GP	CAA78117.1	$_{268}$TSGSGSGEREPHTTSDAVTKQGLSSTMPPTPSPQPST$_{301}$
Varicella zoster virus/ glycoprotein E	P09259.1	$_{309}$TSTYATFLVTWKGDEKTRNPTPAVTPQ$_{335}$

[a]Accession number from the Protein database of the National Center for Biotechnology Information.
[b]Threonine and serine units are indicated in dark and light blue, respectively, and proline units are indicated in red.

O-glycans densely packed in clusters, called mucin-like domains (MLDs) (reviewed in 26). Typically, the viral glycoprotein MLD peptide backbone is a heterogeneous 20- to 80-amino acid sequence, containing 20 or more potential O-glycosylation sites. However, there are single examples of biologically enigmatic viral MLDs that are based on several tandem repeats of a short Ser/Thr-rich sequence and sometimes equipped with a great number of hyperclustered, extremely short O-glycans (27) (**Figure 2*a***). Despite its high concentration of Ser, Thr, and proline (Pro) residues (**Table 1**), essentially each viral glycoprotein MLD appears unique with respect to sequence diversity and absence of serological cross-reactivity (reviewed in 26).

Two Unique Types of O-Glycan Variability: A Viral Key for Regulation of Glycoprotein Activities

One biologically important feature of the viral MLD is that not all of its multiple O-glycosylation sites are necessarily utilized concomitantly. The 20 human GalNAc-Ts have individual (although sometimes partly overlapping) preferences for different potential O-glycan sites (**Figure 2*b***). Hence, a particular Ser/Thr unit of a viral MLD can acquire an O-glycan only when located in a peptide motif, targeted by any of the subset of human GalNAc-Ts expressed in the particular virus-infected cell. Some sites are universal, targeted by several different GalNAc-Ts and therefore carrying O-glycans independent of tissue origin (13, 14), whereas other sites are addressed only in the particular tissue where the appropriate GalNAc-Ts are expressed (14). The tissue-dependent combinations of different Ser/Thr sites being either forbidden or permitted for O-glycosylation are provisionally identified here as *tissue signatures*.

Despite the tissue signature constraint, the MLD occupation patterns of permitted O-glycan sites are highly variable, even for a glycoprotein population formed in one single virus-infected cell. This is because of a special mode of interactive behavior of the GalNAc-Ts expressed in a given cell. Thus, in addition to their catalytic domain, some human GalNAc-Ts are also equipped with a critical GalNAc-Ser/Thr-specific lectin domain (**Figure 2*b***). Such GalNAc-Ts do not add GalNAc unless the lectin domain binds to a previously added GalNAc adjacent to the intended Ser/Thr target (28). Only GalNAc-Ts that are independent of a lectin domain are able to add GalNAc to Ser/Thr units of a naïve, naked MLD peptide target (initiating transferases), whereas GalNAc-Ts, relying on binding to a preexisting GalNAc unit, constitute follow-up transferases (13). Hence, GalNAc-Ts interact in sequence, sometimes cooperatively, sometimes competitively, resulting in an expansion in the number of MLD GalNAc occupancy patterns in a given target cell (reviewed for viruses in 26). Dissection of this interplay for the MLD of the herpes simplex virus 1 (HSV-1) attachment glycoprotein C (gC) (8) revealed several rounds of sequential

Herpesviruses: large, enveloped DNA viruses; latent state after primary infection and occasional reactivation events; divided into α, β, and γ-herpesviruses

Mucin-like domain (MLD): a section of a glycoprotein containing 20 or more clustered O-linked glycans; first discovered in mammalian mucins

Lectin domain: protein substructure that may bind to a glycan motif; here O-linked GalNAc to Ser/Thr of a glycoprotein

operation involving at least 6 GalNAc-Ts that expands the spectrum of the gC MLD O-glycan site occupancy patterns in HSV-1-infected human cells (**Figure 2*c***). This heterogeneity, limited by the tissue signature restriction (forbidden/permitted O-glycan sites), is here provisionally defined as *natural heterogeneity*. The phenomenon of different superordinate tissue-specific O-glycan signatures, each with a subordinate natural heterogeneity, of viral glycoproteins is of relevance for viral-host interactions.

Glycosaminoglycans (GAGs): long, linear, mostly negatively charged glycans consisting of repeating disaccharide units; constituents of cell surface proteoglycans

Glycocalyx: a thick, gel-like, often negatively charged layer surrounding most cells; proteoglycans are major constituents of the glycocalyx

Single-particle tracking: a biophysical real-time technique involving fluorescent virus for tracing migration of individual virions along the cell surface

Biological Relevance of O-Glycans in Viral Glycoproteins

Viral glycoprotein O-glycans are biologically relevant primarily in three respects: (*a*) early and egress phases of the infectious cycle, (*b*) viral interactions with the immune response, and (*c*) strategies for viral colonization of the host. These roles of O-glycans were disclosed mostly for human herpesviruses, in particular HSV-1 and HSV-2, but the knowledge also appears applicable for other enveloped viruses. Other functions of O-glycans, such as in intracellular sorting of human glycoproteins or as antiproteolytic agents (17, 18), are possible also for viral glycoproteins (9).

SIGNIFICANCE OF O-LINKED GLYCANS IN THE VIRAL REPLICATIVE CYCLE

Functions of Clustered Viral Mucin-Like Domain O-Glycans in Viral Attachment and Egress

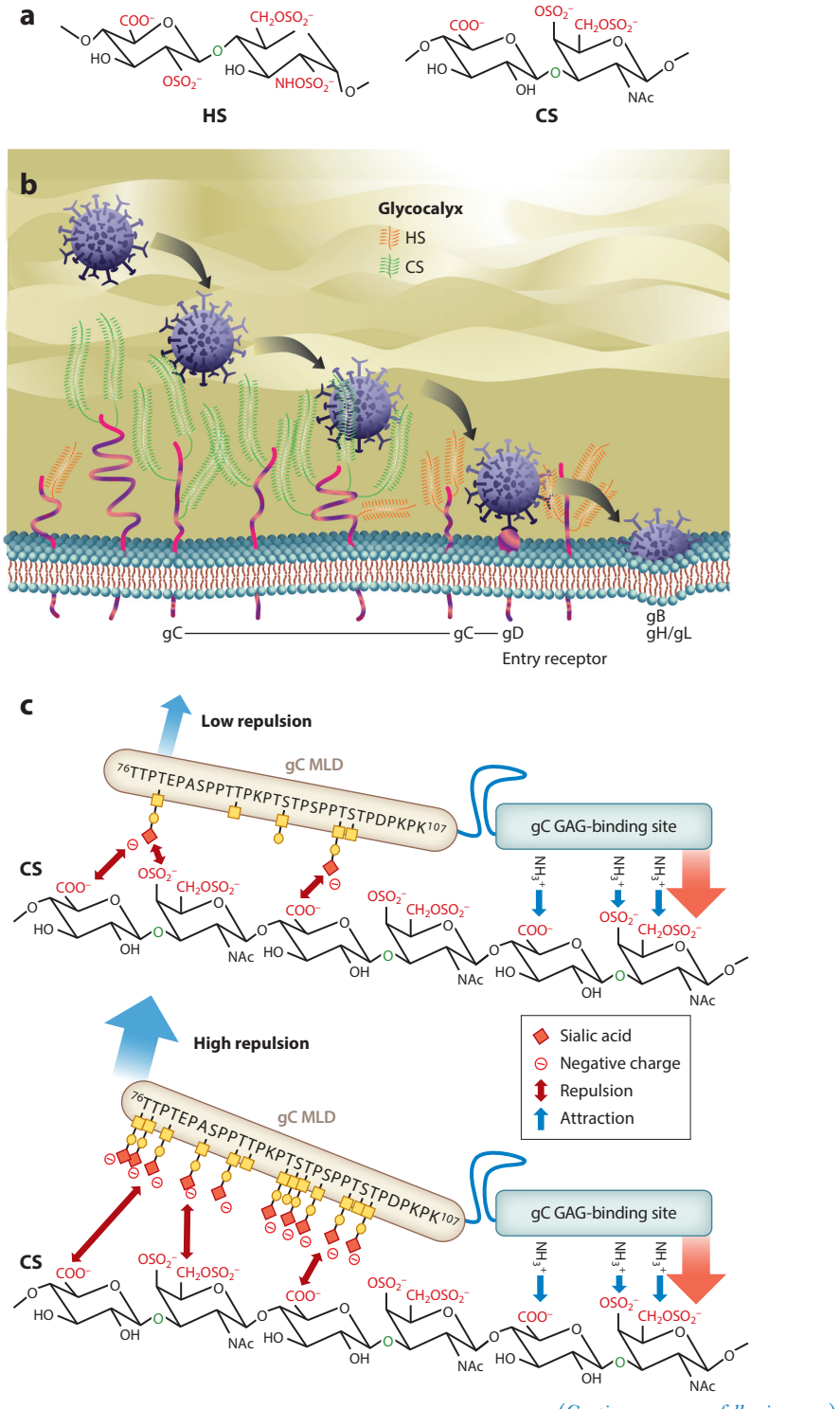
A multitude of human viruses contain envelope glycoproteins with O-linked glycan-rich MLDs, the biological functions of which have largely remained unknown. However, a picture is now emerging that viral MLDs may be involved in a combined function necessary for early viral receptor interactions and also, late in the infectious cycle, for promoting efficient viral egress.

A Class of Target Cell Receptors Interacting with O-Glycans of Viral Envelope Glycoproteins

Several viruses representing a wide range of families—e.g., Flaviviridae, Herpesviridae, Paramyxoviridae, and Retroviridae—use glycosaminoglycans (GAGs) of the target cell as primary attachment receptors (reviewed in 30, 31). Several GAG-binding viruses express at least one MLD-equipped envelope glycoprotein, involved in modulating viral behavior at the cell surface (21, 32–34). Two GAG types, heparan sulfate (HS) and chondroitin sulfate (CS) (structure in **Figure 3*a***), appear especially relevant for viral attachment (30). The GAG-containing proteoglycans are ubiquitous constituents of the cell surface glycocalyx (**Figure 3*b***), which can approach 1,000 nm in thickness on certain cells (35). The glycocalyx is heterogeneous and can form a structure gradient with asymmetric distribution of, for example, CS and HS (36, 37). In real life, GAGs function as ligand organizers in phenomena from organogenesis to inflammation (38, 39). Viral use of GAGs for attachment is favored by the GAG accessibility at the target cell periphery (**Figure 3*b***), but diffusion of GAG-binding viruses toward the cell surface through the thick and sticky GAG layers requires supporting regulatory mechanisms offered by O-glycans of the viral MLDs.

Chondroitin Sulfate and Heparan Sulfate May Have Opposite Functions in Viral Navigation at the Cell Surface

The early interactions between GAG-binding viruses and their target cells were dissected for HSV-1 by single-particle tracking that follows migration patterns of individual virus particles at



(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

The interactions between envelope glycoproteins and cell surface GAGs, constituting viral attachment factors. (*a*) Structural representations of the disaccharide units that are building blocks of HS and CS, two major GAG types. Each CS or HS strand is a linear polymer ($n \geq 15$) of the disaccharides depicted; CS chains are usually longer than HS chains. Positions of charged groups are indicated in red, but differences in the number of sulfates exist. Typically, each CS disaccharide contains 0.9 sulfates, and each HS disaccharide contains 1,4 sulfates (40). For biochemical details, biosynthesis, and the viral receptor spectrum, see Reference 30. The interactions between envelope glycoproteins and cell surface GAGs constitute viral attachment factors. (*b*) Proposed gC-dependent HSV-1 navigation through the target cell glycocalyx. The model is based on biophysical data (41) and refers to particular target cells with an outer glycocalyx layer enriched in CS and an inner layer enriched in HS. The virus hops (diffusion) from CS strand to CS strand until HS, closer to the cell surface, is reached. The interaction conditions between HSV-1 and the CS strands appear optimized to prevent the virus from bouncing away yet prevent arrest of the virus in the outer CS layer. These early contacts and the first contact with HS are carried out exclusively by the envelope glycoprotein gC (42), but subsequent transfer (primed by the gC/HS interaction) to the entry receptor is catalyzed by sequential actions of other HSV-1 envelope glycoproteins (gD, gH/gL, and gB) (see 43). (*c*) Hypothesis explaining the high regulatory potential of a viral glycoprotein MLD. The model is based on the interactions of three key actors: the GAG-binding site and the MLD of gC on one hand and the surface GAGs (here CS) of the target cell on the other. Thus, charged O-glycans in the gC MLD determine the MLD ability to electrostatically counteract gC binding to CS (21, 41). The regulatory capacity of a given gC MLD reflects the variable electrostatic prying force (*blue gradient arrow*) that in turn depends on the heterogeneity in number of sialylated O-glycans and their positions in the gC MLD. The three positive charges indicated for the gC GAG-binding domain belong to arginines, critical for GAG binding (44). The situations of a scarcely sialylated and an abundantly sialylated MLD are depicted. For brevity, other factors possibly affecting the number of MLD negative charges, such as possible suboptimal expression of sialyl transferases in some target tissues, are not discussed. Abbreviations: CS, chondroitin sulfate; GAG, glycosaminoglycan; HS, heparan sulfate; HSV-1, herpes simplex virus 1; MLD, mucin-like domain.

the cell surface (11, 41). Herpesviruses are particularly complex enveloped viruses as they carry at least 10 different glycoproteins engaged in viral attachment and uptake (43). Of these, one glycoprotein, gC, which is equipped with a large MLD with multiple clustered O-glycans (8, 9), is responsible for the initial viral contacts with CS and HS, whereas the other envelope glycoproteins participate in subsequent events leading to cell entry (outlined in **Figure 3*b***). In the early attachment phase HSV-1 switches frequently between immobility, confined diffusion, and free Brownian diffusion (41). In some tissues CS promotes HSV-1 diffusion, whereas HS restricts mobility, indicating competing roles of CS and HS in the interactions with virus-associated gC (41). This suggests a mode of viral hopping between the distal parts of the diffusion-promoting CS chains that facilitates viral navigation in the distal CS layer (proposed model in **Figure 3*b***), a notion supported by the fact CS chains can be considerably longer and more distally exposed than the HS chains (31). This mechanism precludes arrest of virus in the outer CS layer yet ensures migration until more firm HS binding is possible. After this, other HSV-1 envelope glycoproteins take over and promote viral transfer to the entry receptor. The model is based on studies on HSV-1 attachment to only two target cell types, and other target tissues may behave differently. Yet, the viral ability to capitalize structural differences between different GAG types, including HS and CS, into different migration modes appears as an appropriate means for virus navigation in the complex GAG landscape at the target cell surface.

Confined diffusion: diffusion during which motion is restricted to a certain area

Brownian diffusion: diffusion during which a particle moves randomly

Charged Clustered O-Glycans of Viral Glycoproteins Are Major Regulators of Viral Navigation at the Cell Surface

As detailed below, the intriguing HSV-1 navigation in the glycocalyx as well as the wide target tissue spectrum of HSV-1 may be explained by a model (**Figure 3*c***) that involves three key actors: (*a*) the various virus-binding GAGs, especially their negatively charged sulfate/carboxyl

groups with differences in number, orientation, and position on the saccharide chain (30, 38); (*b*) the positively charged GAG-binding domain [receptor-binding domain (RBD)] of gC, of which especially three arginines appear positioned for optimal binding to cognate sulfates/carboxyls of the target GAG (44); and (*c*) the regulatory gC MLD (adjacent to the RBD), especially its content of multiple clustered, often NeuAc-terminated O-glycans (8, 45) (structures in **Figure 1*a***), whose negative charges are instrumental for regulating the gC RBD binding to CS (21).

Receptor-binding domain (RBD): a viral (glyco)-protein substructure, specifically binding to target cell receptors or attachment factors

The basic MLD effector mechanism for regulating gC-GAG interactions is the repulsion caused by the accumulation of negative charges originating from juxtaposition of the NeuAc units of the gC MLD glycans and the adjacent GAG sulfate/carboxyl groups at the gC-GAG interface (21, 41) (**Figure 3*c***). We suggest that the broad regulatory capacity of the MLD to handle a wide range of different GAG targets depends on the many different possible MLD O-glycan occupancies, which is also characteristic for envelope gC of single virions. As discussed above (**Figure 2*c***), such patterns vary with respect both to the number and positions of the charged O-glycans, thereby generating the flexibility that facilitates interactions with different viral GAG targets. The regulatory outcome of two MLD occupancy situations is presented in **Figure 3*c***.

The model is supported by studies at the molecular gC level addressing a 20-kDa CS chain with 15 potential gC-binding sites (**Figure 4*a***). Here, only one single copy of gC with an intact MLD is bound per CS strand, whereas mutagenized gC, with excised MLD ($gC_{\Delta MLD}$), will bind quantitatively to all 15 sites, indicating a regulatory range of more than one order of magnitude (21). This is also mirrored at the virus level (**Figure 4*a***): While wild-type HSV-1 readily navigates toward the entry receptors at the cell surface, the diffusion capacity of mutagenized HSV-1, expressing $gC_{\Delta MLD}$ (entirely devoid of negatively charged MLD glycans), is severely impaired (41) (modeled in **Figure 4*a***). Evolutionarily, the positions of the possible gC MLD O-glycan sites are conserved over 100,000 years of HSV-1 evolution (46). Moreover, small mutational changes of the gC MLD, not affecting the actual O-glycan site positions, may selectively influence viral CS binding (47). Thus, the adapted cooperation between the two extremes, a highly conserved MLD peptide backbone and a heterogeneous O-glycosylation, may be one key to the wide range of possible HSV-1 target tissues. Evidence is accumulating that viral MLDs may also be relevant in a similar way for other GAG-binding viruses (33, 34, 48).

The Mucin-Like Domain–Dependent Regulation of Viral GAG Binding Is Also Relevant During Viral Egress

The MLD control of viral GAG binding is also important late in the infectious cycle for preventing a dead-end infection, caused either by a lack of detachment of the egressing virions from the parent cell or by their reattachment. This was demonstrated for mutant viruses (lacking the relevant MLD) of equine herpesvirus 1 (48), murine herpesvirus 4 (34), and HSV-1 (21, 49) and confirmed also for HSV-2 (32). Obviously, the viral MLD regulation overcomes the seemingly irreconcilable combination of a strict need for receptor binding during viral attachment with an equally necessary absence of such binding to the parent cell during viral egress. We propose a model, satisfying available experimental data on HSV-1 and -2 (21, 32, 49), that solves this paradox (**Figure 4*b***). The viral strategy utilizes a key basic virology difference between the surface of the virgin, virus-naïve viral target cell and the late-stage virus-secreting parental cell (32): The plasma membrane of virus-secreting parent cells contains multiple copies of the viral MLD-containing glycoproteins, identical to those exposed on the viral envelope, whereas the virus-naïve target cells exclusively contain host membrane glycoproteins. As shown in **Figure 4*b***, the excess of extra negative charges introduced by the parent cell–associated gC MLD results in enhanced electrostatic repulsive force (absent in virus-naïve cells), which is the basis for the selectivity that facilitates the detachment of progeny virus yet supports viral attachment to bona fide target cells.

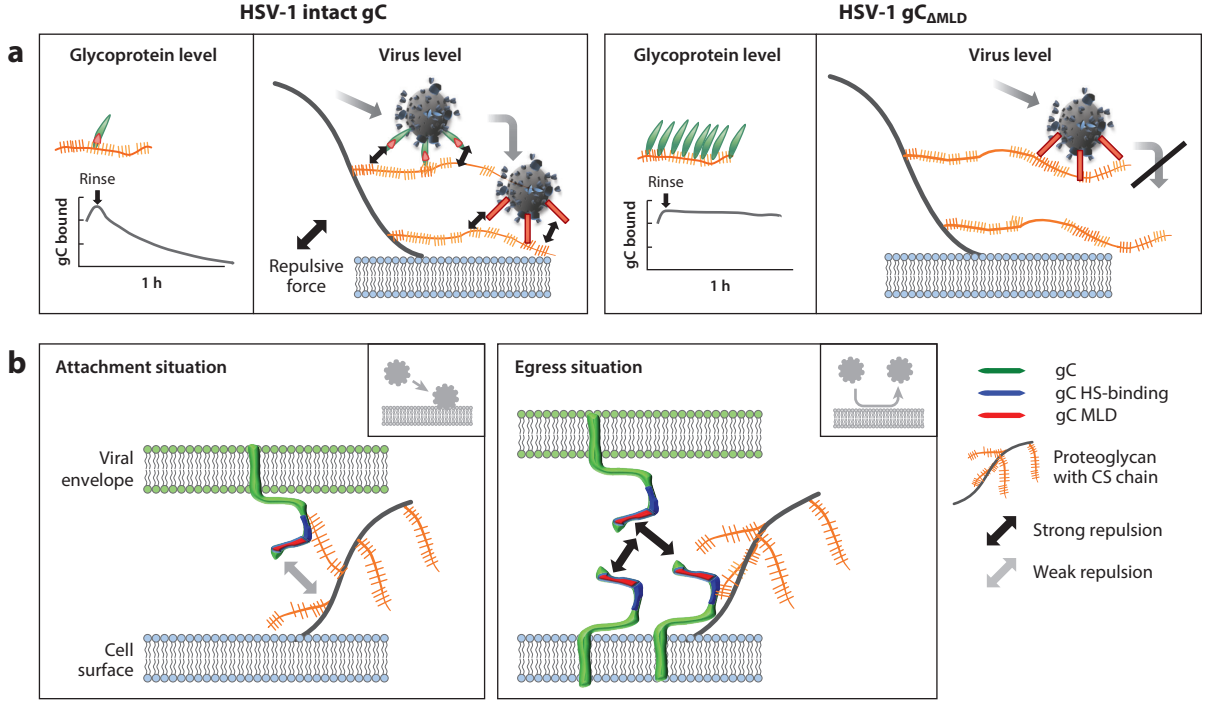


Figure 4

How viral MLDs control appropriate viral GAG binding during attachment and egress. (*a*) Viral interactions with cell surface GAGs: stoichiometrical regulation of HSV-1 gC binding to individual CS strands. At the gC level, there is only one gC molecule binding per CS strand despite the 15 possible binding sites for gC, whereas gC, lacking its MLD, saturates all 15 binding sites. This discrepancy is accompanied by a quantitative reduction of the dissociation rate (method described in 21). At the virus-cell level, the restricted stoichiometry of gC binding to CS limits the polyvalent binding of virus-associated gC to CS that interferes with viral navigation. In contrast, the polyvalent gC-CS interactions of mutagenized, gC$_{\Delta MLD}$-expressing HSV-1 retard viral glycocalyx navigation. All data are from Reference 21. (*b*) Difference between the attachment and egress situation. The attachment situation is HSV-1 approaching naïve target cells. The regulatory, electrostatic repulsive force between the NeuAc-terminated O-glycans of the gC MLD and the negative charges of the target GAG (see **Figures 3*c*** and panel *a*) balance the viral binding, thereby promoting viral migration from GAG chain to GAG chain to the cell surface (*inset*). The egress situation is progeny HSV-1, having egressed, diffusing toward the parent cell surface. Late in the infectious cycle, newly formed gC and other viral glycoproteins are found at the cell surface and may diffuse laterally. Owing to its GAG affinity, such surface gC will stick to cell proteoglycans, thereby presenting a complex of the gC-1 MLDs and the surface GAG receptors for any approaching progeny HSV-1. The resulting, increased repelling force (compared with the attachment situation) disarms surface GAG as an HSV-1 binder and acts to prevent readsorption of progeny virus. A similar HSV-2 mechanism is more complex, constituting a *trans*-phenomenon as the relevant MLD and the GAG-binding domain reside on separate viral glycoproteins (32). Abbreviations: CS, chondroitin sulfate; GAG, glycosaminoglycan; HS, heparan sulfate; HSV, herpes simplex virus; MLD, mucin-like domain; Neu-Ac, N-acetylneuraminic acid.

The MLD effect on viral release is impressive in quantitative terms. Compared with wild-type HSV-1, infection of permissive cells with mutagenized HSV-1, lacking the gC MLD, results in a twentyfold decrease in released virus (21). The decrease in released mutant virus may be explained by a corresponding increase of attached dead-end mutant virus to the parent cell (49). The MLD function resembles that of the influenza virus neuraminidase for preventing reattachment of progeny virus (50), although the influenza strategy is based on a receptor-destroying enzyme rather than the HSV-1 electrostatic strategy presented here. The clinical success of the neuraminidase inhibitor Tamiflu® in treatment of influenza virus infection supports a notion that reattachment of progeny virus may also be used as an antiviral target for GAG-binding viruses.

Role of O-Linked Glycans in Fusion/Entry of Attached Virus

Entry of the surface-associated virus particle into the cell takes place by fusion of the viral envelope with the plasma membrane, but for some viruses, fusion takes place only after uptake of the attached virus into an endosome in which acid pH triggers fusion between the envelope and the endosome wall (51). Much of our knowledge on the relationship between viral O-glycans and the entry of enveloped viruses also stems from studies on the prototype herpesvirus HSV-1. The basic HSV-1 machinery for entry includes four envelope glycoproteins, the above-mentioned GAG-binding glycoproteins gB and gD together with gH and gL (**Figure 3*b***), of which only gB is the direct fusion factor. Fusion glycoproteins of enveloped viruses, such as HSV-1 gB, share structural similarities, in particular a trimeric organization in its post-fusion form of three centrally localized rod-like α-helices, each surrounded by more hydrophilic parts (recent reviews in 52, 53).

All eight human herpesviruses have gB counterparts that share similarities in general structure and functional performance (43). Herpesvirus gB does not contain any MLDs but several solitary O-linked glycans; HSV-1 gB has as many as 17 MS-confirmed utilized O-glycan sites (9). Five of these sites (associated with threonines 169, 267, 268, 497, and 690) are highly conserved in gB of several human herpesviruses (9, 25) and concentrated to two small spots on the post-fusion gB trimer, situated in a waistline-like, less exposed region of gB, close to the stem region (see the crystal structure in **Figure 5*a***). Although no functions are identified as yet, we may speculate that these O-glycans are biologically relevant because their exact structural orientation and positions

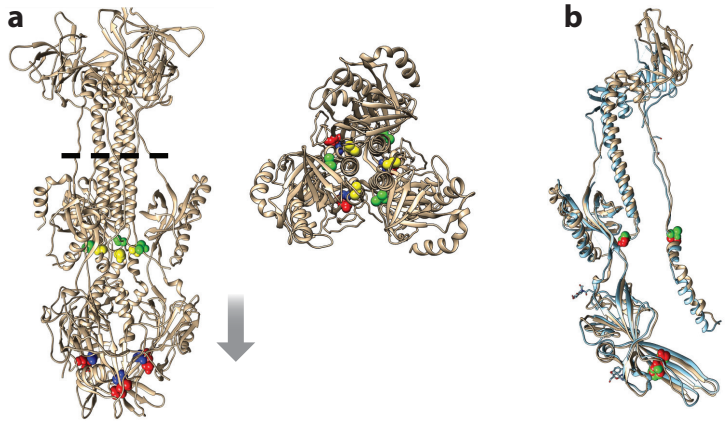


Figure 5

Localization of conserved O-linked glycans in the crystal structure of HSV-1 gB. (*a*, *left*) Side view of crystal structure of the native HSV-1 gB trimer in the post-fusion conformation. The arrow points at the viral envelope. The conserved Thr sites carrying confirmed O-linked glycans are depicted as colored ball structures in the gold ribbon structure of the gB trimer. Blue indicates Thr169, red Thr267 and Thr 268, green Thr497, and yellow Thr690. Structure data (PDB identifier 4BOM) are from Reference 55, confirmation of O-linked data are from Reference 9, and modeling was performed using the UCSF Chimera software (56). (*a*, *right*) Top view of the same structure truncated by the dashed line (see *left*) to emphasize a dense and narrow clustering of O-linked glycans around the central pore of the HSV-1 trimer. (*b*) Conformational homology between HSV-1 and EBV gB structural elements despite low amino acid sequence similarity. PDB structures of HSV-1 gB (*gold*) and EBV gB (*light blue*) were aligned using the UCSF Chimera software (56). The confirmed O-glycosylation sites of HSV-1 at Thr169, Thr267, Thr 268, Thr497, and Thr690 are depicted as red ball structures. Corresponding O-glycosylation sites in EBV gB (PDB identifier 3FVC; see 57) are indicated as green balls. Positional overlap is indicated by merged green and red balls. Abbreviations: EBV, Epstein-Barr virus; HSV-1, herpes simplex virus 1; PDB, Protein Data Bank; Thr, threonine.

in the 3D structure of gB are phylogenetically old. Thus, a comparison of the gB crystal structures derived from HSV-1 and Epstein-Barr virus (EBV)—α- and γ-herpesvirus, respectively—reveals a spatial congruence at the angstrom level of the localization of the five conserved glycosylation sites of EBV and HSV-1 gB (**Figure 5*b***). This appears even more remarkable, considering that the viral ancestor of α- and γ-herpesviruses separated approximately 200 million years ago (54), generating a less than 17% amino acid sequence homology between HSV-1 gB and EBV gB. Thus, the enigmatic role of the five O-glycan positions for gB function has obviously remained essential during herpesvirus evolution over the last hundreds of million years.

Other HSV-1 gB O-glycans are directly coupled to activation of the gB fusion activity at viral uptake. Thus, in addition to other binding targets at the cell surface, gB may also bind to the paired immunoglobulin-like type 2 receptor α (PILRα) (58). The normal functions of PILRα and its match, PILRβ, are negative (PILRα) or positive (PILRβ) regulatory effects in innate immunity (59, 60). PILRα binds to NeuAc-terminated glycans of its normal ligand, CD99, provided that the CD99 target amino acid sequence contains the appropriate O-glycan (60). HSV-1 gB binding to PILRα is strictly dependent on O-glycan sites at T53 and T480. Both sites were confirmed to be occupied (9) but not resolved in the crystal structure (**Figure 5**). The gB interaction with target cell PILRα primes HSV-1 entry via a PILRα-specific pathway (61), and the best inhibitor of the gB-PILRα binding and entry priming is a gB-derived heptapeptide (residues 50–56), O-glycosylated with sialyl-Tn (structure in **Figure 1*a***) at Thr-53 (60), opening perspectives for rational design of inhibitors with therapeutic potential (61). Interestingly, gB of another α-herpesvirus, varicella zoster virus (VZV), uses myelin-associated glycoprotein, a constituent of glial cells, as an entry receptor, a phenomenon partly responsible for the VZV neurotropism (62) via binding to NeuAc on both N- and O-glycans of VZV gB (63).

Also for paramyxoviruses and hepatitis C virus (HCV) scattered O-glycans of viral envelope proteins regulate viral fusion and uptake. Paramyxovirus specifies two envelope proteins of which one (denoted HN, H, or G dependent on paramyxovirus type) is an attachment protein whereas the other glycoprotein (denoted F) is a classical fusion protein (64). Studies on two paramyxovirus representatives, Hendra and Nipah viruses from the subfamily Henipaviruses, revealed that presence/absence of particular O-glycosylation sites of the stalk domain (connecting the *trans*-envelope region with the globular receptor-binding head) of the attachment G protein modulates the fusogenic capacity of adjacent fusion F glycoproteins in the viral envelope (65). It appears that the O-linked and N-linked glycans at specific positions of the G stalk region regulate the magnitude of fusion induction of F, as manipulation of the glycosylation sites of G may result in either hyper- or hypoactivation of the fusion capacity dependent on the particular pattern of occupied glycosylation sites of the G stem region. Owing to overall similarities, this may also be applicable for other viruses of the Paramyxovirus family (65).

The envelope of HCV contains at least six sites for O-linked glycosylation that are equipped with O-glycans of various sizes, some of which are sialylated pentasaccharides (20). Elimination of these O-glycans results in an impaired ability of HCV pseudovirus to be taken up by permissive cells, probably due to interference with E2 binding to cell surface CD81, an entry receptor of HCV (66).

O-LINKED GLYCANS AND VIRULENCE MECHANISMS

Ebolavirus

The O-glycans of the MLD, easily accessible at an exposed position of the Ebola virus (EBOV) glycoprotein (67), appear to be important virulence factors. Thus, EBOV strain virulence covaries with their ability to induce detachment of human endothelial cells (68, 69). These effects are

strictly dependent on O-glycans on particular sites in a short stretch of the glycoprotein MLD, whose O-glycosylation is initiated by GalNAc-T1 rather than the other GalNAc-Ts investigated (70). This role of GalNAc-T1 is interesting because design of specific inhibitors of individual GalNAc-Ts is now possible (71), suggesting that a future GalNAc-T1 inhibitor may be of use for treatment of EBOV vascular effects in patients.

Severe Acute Respiratory Syndrome Coronavirus 2

The envelope glycoprotein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), designated S, contains several O-glycan sites (72), but the access for GalNAc-Ts to some of these sites is restricted owing to steric constraints in the S trimer (73, 74). Circumstantial evidence, based on inhibitory studies with O-glycosylated synthetic peptides, suggests that small O-glycans, e.g., sialyl-Tn (**Figure 1*a***), of the S RBD may be involved in viral receptor-binding (75). Moreover, one specific NeuAc-terminated O-glycan of the SARS-CoV-2 glycoprotein regulates an adjacent, functionally critical proteolytic site (105).

Self: self matter, e.g., proteins, is tolerated by the acquired immune response

O-glycopeptide epitope: a nonself, glycoprotein-derived glycopeptide of 5–7 amino acids with O-linked GalNAc; binds to specific antibodies

O-LINKED GLYCANS, ANTIVIRAL IMMUNITY, AND VACCINES

O-Linked Glycans in B Cell Immunity

The relationship between B cell immunology and O-glycans of viral glycoproteins is a dichotomy: On one hand, owing to their immunological self status, large O-glycans, similar to N-glycans, of a viral glycoprotein may function as a virus-promoting factor by offering physical shielding from neutralizing antibodies (2, 76–78). But on the other hand, for sufficiently small O-glycans, the innermost GalNAc may act in the opposite way, i.e., by constituting an immunologically equally active constituent as any of the amino acid parts of a composite glycopeptide B cell epitope (26, 79). Such O-glycopeptide epitopes can either be constant (ubiquitously glycosylated), i.e., acting as B cell targets in most infected patients, or variable (selectively glycosylated), i.e., individual patients may present different O-glycopeptide glycoforms of the same peptide backbone to the immune response resulting in distinct specificities of such antibodies in a patient-dependent manner (**Figure 6*a*,*b***).

The prototype constant O-glycopeptide epitope concerns HSV-2 and is based on one O-monoglycosylated gG-2 heptapeptide stretch of the envelope glycoprotein gG, containing clustered as well as solitary O-linked glycans (3, 83, 84). This epitope reacts with immunoglobulin G (IgG) antibodies in more than 70% of HSV-2-infected individuals (79) (**Figure 6*a***). These antibodies have a remarkably narrow specificity. Thus, the identity and position of each of the eight units forming the target epitope (seven amino acids and the inner GalNAc) is indispensable for antibody binding, and any change results in loss of antibody binding (79). However, the inner GalNAc unit can be extended to a trisaccharide without loss of antibody binding (**Figure 6*a***), suggesting that shielding epitopes from antibodies requires large or clustered O-glycans.

Variable O-glycopeptide epitopes reside in peptide stretches that are addressed only by sparsely expressed human GalNAc-Ts (**Figure 1*b***), e.g., in particular tissues or individuals. The prototype target is an MLD peptide stretch of the major immunogenic EBV envelope glycoprotein gp350 that displays different occupancy patterns for four O-glycan sites, apparently in a patient-dependent manner (**Figure 6*b***). Hence, different patients selectively develop antibodies to different glycoforms of this O-glycopeptide epitope (85). This may reflect that differences between individual patients in the tissue signatures of gp350 O-glycan patterns are mirrored in epitope presentation to the B cell response in each patient. Thus, one and the same short viral MLD peptide sequence can give rise to several serologically active but not cross-reactive epitopes, further emphasizing the narrow specificities of IgG antibodies to O-glycopeptide epitopes.

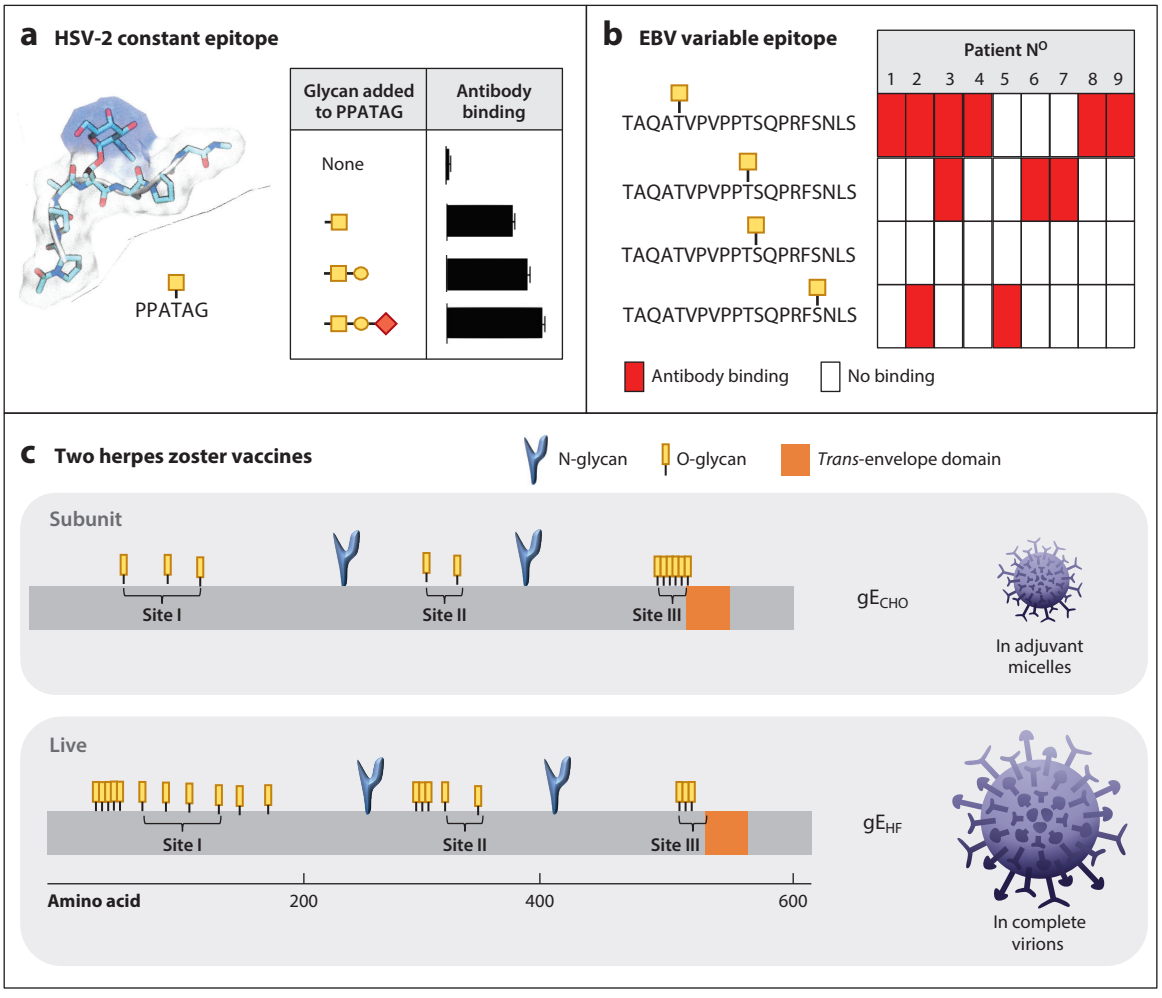


Figure 6

The immunoreactivity of constant and variable O-glycopeptide epitopes and their relevance for subunit vaccine function. (*a*) HSV-2 gG constant epitope. The minimal nonself structure of a broadly reactive glycopeptide epitope (^{501}Pro to ^{507}Gly) of the HSV-2 glycoprotein gG is shown. The solvent-accessible surface of the glycopeptide 3D model is in light gray for the peptide and light blue for the GalNAc component (see 26). The gG PPATAG is a part of a broad target for frequently expressed GalNAc-Ts (**Figure 1*b***), implying a ubiquitous O-glycosylation at this position. A moderate elongation of O-glycan is permitted, but no antibody binding in the absence of inner GalNAc. Data are from References 26, 79, and 80. Figure adapted with permission from Reference 26. (*b*) EBV gp350 variable epitope. The serological responses of 20 EBV-positive patients to four mono-glycosylated glycovariants of the same synthetic peptide (amino acids 841–860 of gp350, an EBV-specified envelope glycoprotein) are indicated. Data are from Reference 26. (*c*) Two herpes zoster vaccines. Different O-glycan signatures of VZV-specified glycoprotein gE, as it is expressed in the two different currently used shingles vaccines, are indicated. The Zostavax® live vaccine consists of a large dose of attenuated but yet infectious virus particles produced in human fibroblasts, containing more than ten glycoprotein species encoded by VZV, including gE. The subunit vaccine Shingrix® is based entirely on recombinant gE (transmembrane region genetically excised), produced in CHO cells (81, 82). The depicted micellar organization of Shingrix is hypothetical. All sites verified to be occupied with O-glycans (occupation rate ~10–100%) are indicated. Three major clusters of O-glycan-modulated epitopes (I–III) reacting with sera from patients recovered from VZV infection are indicated. Data are from References 25 and 80. Abbreviations: CHO, Chinese hamster ovary; EBV, Epstein-Barr virus; GalNAc, N-acetylgalactosamine; GalNAc-T, GalNAc transferase; HF, human fibroblast; HSV-2, herpes simplex virus 2; VSV, varicella zoster virus.

O-Glycopeptide Epitopes and Viral Subunit Vaccines

The complex O-glycan immunobiology, presented above, may help explain the efficiency of viral subunit vaccines, not least the newly introduced herpes zoster (HZ) vaccine, based on glycoprotein gE. HZ is a reactivation of latent VZV [residing in dorsal ganglia after the ubiquitous childhood varicella (86)], with a lifetime incidence of about 25% (87). Two HZ vaccines are currently marketed (**Figure 6*c***): an attenuated live vaccine (Zostavax®, Merck) (88) and a subunit vaccine (HZ subunit vaccine Shingrix®, Glaxo Smith-Kline Biologicals), based on the single VZV glycoprotein E (gE) (81). Zostavax results in a relatively modest and temporary protection (60–70%) against HZ and postherpetic neuralgia, decreasing with age (88). Surprisingly, Shingrix provides a much higher protection efficacy (above 97%) with a negligible age-dependent decrease in immunity (81). Recently, a head-to head comparison of IgG responses to the two vaccines revealed that, in addition to providing enhanced T cell responses, Shingrix was superior in inducing high avidity, neutralizing antibodies of long duration to VZV gE (89).

One possibility is that the high efficacy of Shingrix compared with Zostavax reflects differences in the nature of their gE presentation. Thus, gE of Zostavax results from vaccine virus replication in human fibroblasts (designated gE_{HF} below), whereas the Shingrix gE (gE_{CHO}) is recombinantly produced in Chinese hamster ovary (CHO) cells (26). A comparison of the patterns of occupied and vacant O-glycan sites of gE from human fibroblasts and CHO cells, respectively, revealed a much higher number of occupied O-glycosylation gE sites as well as differences in site occupation patterns in gE_{HF} (Zostavax situation) compared with gE_{CHO} (Shingrix situation) (25, 80) (**Figure 6*c***).

Therefore, synthetic variants of all possible gE O-glycopeptide epitopes reflecting both vaccine situations were used as targets in a serological analysis of VZV-positive sera from HZ-recovered subjects, revealing two differences between the Shingrix and Zostavax situations (80). First, the human natural B cell response to epitope cluster I (**Figure 6*c***) is mainly directed to nonglycosylated epitopes that are exposed by gE_{CHO}, owing to its lower O-glycan occupancy, but hidden in gE_{HF} by additional, obviously epitope-shielding O-linked glycans. Second, the antibody reactivity to epitope cluster III involves variable O-glycopeptide epitopes that differ from patient to patient (analogous to EBV gp350) (**Figure 6*b***). Much of this wide, patient-variable spectrum of epitope glycoforms is represented by the different glycoforms of gE_{CHO} (**Figure 6*c***), but not in the more extensively glycosylated gE_{HF} (25, 80). Only direct immunization studies can establish to what extent this difference contributes to the high efficacy of Shingrix. Although a role here for other acquired immunity effectors must not be excluded, the versatile epitope pattern presented by gE_{CHO} appears more adapted, compared with gE_{HF}, for rising the high avidity protective antibodies proposed by Schmid et al. (89).

Functional studies on HCV (66, 90) support the notion of manipulation of O-glycan sites as a more general option for subunit vaccine development. Other possible candidates for this option include several emerging viruses representing several families, exemplified by EBOV and Crimean-Congo virus, that are equipped with MLD-containing envelope glycoproteins targeted by the immune response (91, 92). The O-glycans of the SARS-CoV-2 glycoproteins, although possibly engaged in epitope shielding (93), appear to be a minor component of unclear significance for vaccine development (72).

Live vaccine: an attenuated, yet replicative, variant of a pathogenic virus that induces immunity to the pathogen

Subunit vaccine: formulation of a viral glycoprotein, inducing immunity without involving any viral replication

Human fibroblast: embryonal diploid cell originally isolated in 1963 and widely used for production of viral live vaccines ells

Chinese hamster ovary (CHO) cell: immortalized cell line suitable for large-scale production of recombinant proteins, including vaccines

O-Linked Glycans in T Cell and Innate Immunity

T cell immunity is an important cornerstone in the acquired immune response, essential for clearing viral infections (94). The major histocompatibility complex class I and class II molecules are key factors here as they present peptides at the cell surface to $CD8^+$ and $CD4^+$ T cells, respectively

(reviewed in 95). In certain tumors, short, nonself O-glycans (e.g., Tn in **Figure 1*a***) are enriched in cell glycoproteins (13), and proteasome-generated O-glycopeptides thereof become nonself T cell antigens constituting antitumor response targets (96). However, in spite of the great significance of the T cell response for clearing of viral infections, there is so far no evidence that O-glycans in T cell epitopes should play a similar role in the immune response to viral infections (97), although bulky MLDs of viral glycoproteins may interfere with antigen presentation by $CD8^+$ T cells (98).

Nonself: foreign nonself structures may induce, e.g., an antibody response

Selectins: family of carbohydrate-binding proteins of the inner blood vessel (endothelial) wall on leukocytes or on platelets

However, O-glycans of viral envelope glycoproteins may be engaged in the innate immune response by activating a novel, extremely early response, induced in the HSV-2-infected mucosal surface in a mouse model (99). Specifically, clustered O-glycans of the gG-2 MLD, exposed from HSV-2 envelopes, induce CXCR3 chemokines resulting in an antiviral response that is dependent on neutrophils but seemingly independent on established early virus sensing systems (99).

VIRUS-INDUCED SELECTIN LIGANDS OF O-GLYCAN NATURE

Leukocytes, Selectins, and Their Ligands: Biological Impact

Interactions between the endothelial selectin (E-selectin) and its specific ligand, residing at the leukocyte surface, are the activation signals for specially primed leukocytes to cross the endothelial wall and migrate to, e.g., peripheral inflammatory tissue (100, 101). The E-selectin ligand at the leukocyte surface is a glycoprotein-associated O-glycan, e.g., the sLex glycan epitope (structure in **Figure 1*a***). Uncontrolled leukocyte travel across the endothelium is normally prevented because by default, selectin ligand (sLex and others) synthesis is strictly kept switched off. Switch on takes place only when leukocytes are recruited for leaving the blood vessel. Activation is based on transient transcriptional activation of critical *FUT* genes encoding glycosyltransferases that are a rate limiting-step for sLex formation (reviewed in 102) (**Figure 1*c***). Consequently, only appropriately activated leukocytes express the sLex that enables interactions with the E-selectin, the prerequisite for crossing the endothelial wall.

Virus-Induced Selectin Ligands and Their Functions

The selectin-dependent endothelial transmigration is exploited maliciously for tissue invasion by tumors and viruses, not least human T-lymphotropic virus type 1 (HTLV-1), the causative of adult T cell leukemia (103). This disease is characterized by extravascular infiltration of leukemia cells into various organs, including the skin (22). The HTLV-1 protein, Tax, interferes with the strong suppression of transcription of the critical *FUT*7 gene that encodes one rate-limiting factor for sLex synthesis (103). This induces surface expression of sLex in the leukemic cells, a condition strictly associated with skin infiltration (22).

Like HTLV-1, human herpesviruses, including HSV-1, VZV, and cytomegalovirus, may also interfere with normal repression of host gene products of relevance for sLex synthesis (23, 24, 104), i.e., adding a fucose in the α(1–3) linkage to sLex precursor (**Figure 1*c***). HTLV-1 may activate only *FUT*7, whereas herpesvirus may also activate other related human *FUT* genes. However, more studies are needed to confirm whether herpesvirus-induced selectin ligands on virus-containing cells play a similar role in colonization as for HTLV-1.

CONCLUDING REMARKS AND PERSPECTIVES

Recent advances have revealed that the O-glycan modifications of viral envelope glycoproteins are highly relevant for mechanisms related to several stages of viral infection of target cells, as well as for their virulence, and manipulation of the antiviral immune response. This may open new leads

for development of antiviral intervention and prophylactic means. As a future perspective, the heterogeneity of the MLD O-glycan occupancy patterns is dependent on the particular combination of human GalNAc-Ts expressed in a given viral parent tissue. Thus, the resulting glycovirological fingerprint is probably of relevance for the host specificity as well as for the target tissue range, not least of all GAG-binding viruses. Hence, the parent cell O-glycosylation status, varying between different tissues, may constitute a pathogenetic factor by its potential for influence on viral tropism. Conceivably, the next few years of exploration of these enigmatic (until recently) constituents of viral glycoproteins will mean a harvest time in terms of better handling of the threats from enveloped viruses.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We apologize to the many contributors of the field whose work was not directly cited due to space limitations. The authors are indebted to Dr. Yara Abidine, Department of Clinical Microbiology, Umeå University, for valuable input to the models presented in **Figure 3**. S.O. is grateful for long-term cooperation and discussions with Drs. Henrik Clausen, Ulla Mandel, Eric P. Bennett, Hans H. Wandall, and Ieva Bagdonaite of the Copenhagen Center for Glycomics. This work was supported by grants to M.B. from the Knut and Alice Wallenberg Foundation through the Wallenberg Centre for Molecular Medicine and the Swedish Research Council 2017-04029 and 2020-06242, and to T.B. from Swedish Medical Research Council 2018-03918 and 2021-06386, and Sahlgrenska University Hospital grant ALFGBG-965849 (2022–2024).

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