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Review

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Review

Global aspects of viral glycosylation

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Abstract

Enveloped viruses encompass some of the most common human pathogens causing infections of different severity, ranging from no or very few symptoms to lethal disease as seen with the viral hemorrhagic fevers. All enveloped viruses possess an envelope membrane derived from the host cell, modified with often heavily glycosylated virally encoded glycoproteins important for infectivity, viral particle formation and immune evasion. While N-linked glycosylation of viral envelope proteins is well characterized with respect to location, structure and site occupancy, information on mucin-type O-glycosylation of these proteins is less comprehensive. Studies on viral glycosylation are often limited to analysis of recombinant proteins that in most cases are produced in cell lines with a glycosylation capacity different from the capacity of the host cells. The glycosylation pattern of the produced recombinant glycoproteins might therefore be different from the pattern on native viral proteins. In this review, we provide a historical perspective on analysis of viral glycosylation, and summarize known roles of glycans in the biology of enveloped human viruses. In addition, we describe how to overcome the analytical limitations by using a global approach based on mass spectrometry to identify viral O-glycosylation in virus-infected cell lysates using the complex enveloped virus herpes simplex virus type 1 as a model. We underscore that glycans often pay important contributions to overall protein structure, function and immune recognition, and that glycans represent a crucial determinant for vaccine design. High throughput analysis of glycosylation on relevant glycoprotein formulations, as well as data compilation and sharing is therefore important to identify consensus glycosylation patterns for translational applications.

Key words: enveloped viruses, herpesvirus, mass spectrometry, mucin type O-glycosylation, viral glycans

Introduction

Enveloped viruses are well adapted pathogens infecting a great variety of hosts from protozoans to mammals that hijack the host cell's molecular machinery for reproduction (International Committee on Taxonomy of Viruses 2012; Buchmann and Holmes 2015). They have evolved together with their hosts and developed various means to counteract the host's defense mechanisms (Christiaansen et al. 2015; Beachboard and Horner 2016; Schuren et al. 2016). A characteristic feature of these viruses is a cell membrane-derived envelope modified with virally encoded proteins glycosylated by the host glycosylation apparatus as they pass through the secretory pathway. While some viral glycoproteins are glycosylated at relatively few sites, others are densely glycosylated with N-linked and O-linked glycans. Prominent examples include the Ebola virus glycoprotein modified by a very high content of O-linked glycans, and the HIV-1 glycoprotein gp160 that is glycosylated by the addition of multiple N-linked glycans (Jeffers et al. 2002; Pabst et al. 2012; Yang W et al. 2014). Although specific functional roles have been assigned to distinct glycosites of multiple viruses (Goffard et al. 2005; Falkowska et al. 2007; Helle et al. 2010; Wang et al. 2013, 2017;

Lennemann et al. 2014; Bradel-Tretheway et al. 2015; Luo et al. 2015; Orlova et al. 2015; Suenaga et al. 2015; Stone et al. 2016; Wu et al. 2017), it is generally believed that the high levels of glycosylation serve as a protective shield from the host's immune system (Francica et al. 2010; Helle et al. 2011; Sommerstein et al. 2015; Behrens et al. 2016; Gram et al. 2016; Walls et al. 2016). The other major role of glycosylation occurs during virus entry where glycans on the host cell represent viral receptors interacting with carbohydrate binding proteins on the viral surface. A prominent example is the influenza viruses that attach to surface glycans residing on cellular glycoproteins (Air 2014). Hemagglutinins (HA) of human influenza viruses use α2,6-linked sialic acid moieties, while those of avian viruses bind preferentially to α2,3-linked sialic acids (Air 2014). Apart from influenza virus, several other nonenveloped human viruses, including adenoviruses, reoviruses and rotaviruses, use sialic acid-containing oligosaccharides as cell receptors (Stencel-Baerenwald et al. 2014). Sialic acid is not the only glycan that can be used by viruses for cell attachment. For example, flaviviruses, human respiratory syncytial virus and some of the herpesviruses use proteoglycans as a point of attachment to cells, whereas core fucosylation is suggested to play a role in HBV entry into hepatoma cells (Jolly and Sattentau 2013; Takamatsu et al. 2016). The important aspects of host glycans as receptors for viral infectivity will not be reviewed here as they are covered elsewhere (Jolly and Sattentau 2013; Stencel-Baerenwald et al. 2014; Kim et al. 2017). Instead, we will present the available information on glycosylation of human enveloped viruses. We will discuss general functions of glycosylation in various aspects of the viral life cycle and interaction with the immune system. In addition, we will discuss human herpesvirus glycosylation in more detail and include conclusions emerging from our recent global O-glycoproteomic analyses.

N- and O-linked glycosylation of proteins

Glycosylation of viral envelope glycoproteins depends on the host glycosylation machinery. Viral glycoproteins, like cellular proteins, possess signal peptides directing them to the secretory pathway. Proteins targeted to the secretory pathway can be decorated with different kinds of post-translational modifications en route to the cell surface or another cellular compartment. Glycosylation is one of the most widespread and versatile modifications of proteins and is classified as N-, O- and C-linked, named after the acceptor amino acid atom to which the sugar moiety is attached. While there is only one type of N-linked glycosylation in terms of initiation, many types of O-glycosylation exist, classified based on the identity of initiating monosaccharides (Stanley 2011; Moremen et al. 2012). Most types of O-glycosylation are carried out in the secretory pathway, but cytosolic and nuclear proteins can also be glycosylated with *N*-acetylglucosamine (GlcNAc) by a cytosolic glycosyltransferase (Moremen et al. 2012; Yang and Qian 2017). Some types of O-glycosylation are specific to distinct classes of proteins or domains (Moremen et al. 2012; Haltom and Jafar-Nejad 2015; Larsen et al. 2017). In this review the term O-glycosylation will refer to GalNAc- or mucin-type O-glycosylation, which is one of the most widespread forms of protein O-glycosylation (Bennett et al. 2012). N-linked glycosylation of proteins is acquired cotranslationally as they fold in the endoplasmic reticulum (Rothman and Lodish 1977). The initial glycan moiety is comprised of nine mannose (Man), three glucose (Glc) and two GlcNAc residues that are transferred on proteins from a dolichol-phosphate-linked precursor to asparagine residues within NXS/T consensus sequons (X—all amino acids except P) (Robbins et al. 1977; Bause 1983; Kornfeld and Kornfeld 1985). The initial glycan structure is trimmed to $Man_8GlcNAc_2$ in the endoplasmic reticulum, with intermediates assisting in protein quality control (Hammond et al. 1994; Helenius and Aebi 2004). In the Golgi apparatus, the glycans can be further trimmed, elongated and branched by a differentially expressed set of glycosidases and glycosyltransferases to yield hybrid or complex-type *N*-glycans (Hunt et al. 1978; Rabouille et al. 1995; Schachter 2000). As proteins pass through the Golgi apparatus, they can also be modified with mucin-type *O*-glycans, initiated by a family of 20 GalNAc-transferases that add the initial GalNAc monosaccharide to Ser, Thr and possibly Tyr residues (Rottger et al. 1998; Halim et al. 2011; Bennett et al. 2012). The GalNAc-transferases exhibit somewhat overlapping, but also distinct substrate specificities and the expression of the isoenzymes is regulated in a tissue- and differentiation-specific manner (Sutherlin et al. 1997; Wandall et al. 1997, 2007; Mandel et al. 1999; Young et al. 2003; Tian and Ten Hagen 2006). Since there are no conserved protein sequence motifs for general or isoform-specific O-glycosylation, it is much more difficult to predict this modification (Gerken et al. 2011). However, prediction algorithms exist and are rapidly improving due to the vast increase in the number of identified O-glycosites using proteome-wide strategies. Nevertheless, experimental evidence is still required for reliable site identification (Steentoft et al. 2013). O-linked glycans can be further elongated by competing glycosyltransferases to form up to eight core structures, which adds to the heterogeneity of O-glycosylation and impedes its analysis (Brockhausen et al. 1990; Yeh et al. 1999; Dalziel et al. 2001; Schachter 2000). Although many types of protein glycosylation exist, N- and mucin-type O-linked glycosylation are the most studied in viral research. These types of glycosylation will also be the focus in the following sections.

Methods for analysis of viral glycosylation

Historically, herpesviruses and togaviruses were some of the first viruses investigated for modification with carbohydrates, which led to important findings regarding general aspects of viral glycosylation. Early methods of analysis were based on monitoring the incorporation of radioactively labeled monosaccharides and amino acids into newly synthesized viral proteins during infection (Keller et al. 1970; Spear and Roizman 1970; Kim et al. 1976). It was thereby established that viral protein glycosylation took place at the cellular membranes and not the cytoplasmic compartment (Spear and Roizman 1970). Early glycoprofiling experiments utilized pronase digestion of labeled glycoproteins followed by gel filtration, which allowed separation of the short glycopeptides, bearing different glycan moieties (Honess and Roizman 1975; Schwarz et al. 1977). Moreover, differentially labeled sugars were used to provide insight into the putative composition of individual glycan structures (Sefton 1975). An important conclusion based on such experiments was that the extent of sugar incorporation into individual viral proteins differed depending on the infected cell type (Keller et al. 1970), establishing that viruses are dependent on the host glycosylation machinery. Use of glycosylation inhibitors, such as tunicamycin, 2-deoxy-D-glucose and glucosamine, provided additional insights into regulation of glycan synthesis, its impact on viral replication, and showed that glycosylation of viral proteins is critical for infectivity and cell–cell fusion (Knowles and Person 1976; Leavitt et al. 1977; Olofsson and Lycke 1980; Herrler and Compans 1983; Lambert and Pons 1983; Mann et al. 1983). Studies in cell lines deficient for specific glycosyltransferases or intracellular transport inhibitors provided additional means for investigating biological consequences of disrupted

glycan synthesis and maturation, respectively (Campadelli-Fiume et al. 1982; Serafini-Cessi, Dall'Olio, Scannavini, Campadelli-Fiume et al. 1983; Edwardson 1984).

Alongside numerous studies addressing viral *N*-glycan composition and function, it was discovered that viral envelope glycoproteins could also be modified with O-linked glycans (Olofsson et al. 1981; Shida and Dales 1981; Niemann et al. 1982; Gruber and Levine 1985; Montalvo et al. 1985; Gong et al. 1987; Lundstrom et al. 1991). As for human proteins, the Golgi apparatus was identified as the site of viral O-glycosylation (Johnson and Spear 1983; Locker et al. 1992). Historically it was presumed that few viruses were O-glycosylated and the function of this modification remained undetermined for some time (Feldmann et al. 1991; Bernstein et al. 1994). One of the first functions of viral O-glycosylation was discovered in vaccinia virus, where it has been demonstrated that the hemagglutinating activity of glycoprotein HA was entirely dependent on O-linked glycans (Shida and Dales 1981). A similar carbohydrate-dependent function was described for rubella virus, where treatment with a mix of glycosidases removing all glycans resulted in inhibition of hemagglutination (Ho-Terry and Cohen 1984).

Development of new biochemical methods facilitated the isolation and analysis of viral glycoproteins and glycans. Use of plant lectins or sera from immunized animals as well as vaccinated patients facilitated purification of viral envelope proteins and enabled analysis of glycans at the individual protein level (Eisenberg et al. 1979; Wenske et al. 1982; Friedrichs and Grose 1984; Respess et al. 1984; Montalvo et al. 1985). Introduction of chemical glycan release from the peptides or sequential enzymatic deglycosylation enabled more precise characterization of viral glycan size and composition compared to pronase digests (Burke and Keegstra 1979; Rasilo and Renkonen 1979), and led to determination of type and structure of N- and O-linked glycans for many viruses (Pesonen 1979; Pesonen, Kuismanen et al. 1982; Pesonen, Ronnholm et al. 1982; Niemann et al. 1984). The subsequent introduction of reverse phase HPLC further facilitated glycopeptide analysis, allowing separation of larger glycopeptides, generated by digestion of proteins with proteases of defined specificity, such as trypsin. Subsequent enzymatic glycan release enabled *N*-glycan analysis on isolated glycopeptides (Rosner and Robbins 1982; Cohen et al. 1983; Hsieh et al. 1983), and demonstrated site-specific glycan microheterogeneity in different hosts (Hsieh et al. 1983). Development and advancement of mass spectrometry-based applications had a large impact on the analysis of glycans, which was quickly adopted in the virology field. More recent advances in mass spectrometry-based glycoprofiling and glycoproteomics of enveloped viruses are discussed in the following sections.

Glycoprofiling of viruses using mass spectrometry

Modern mass spectrometry-based methods of analysis enable robust characterization of N- or O-linked glycans in complex biological samples with unprecedented sensitivity and resolution. It has become routine practice in the characterization of recombinant therapeutics as well as vaccine candidates (Xie et al. 2011; Dubayle et al. 2015; Jacob et al. 2015). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is one of the most commonly used tools in modern day glycan analysis. Combining MALDI-MS with lectin microarray enables more reliable characterization of glycan structures, where lectin binding profiles can provide additional information on the glycosidic linkage (Mechref et al. 2003; Lei et al. 2015). Moreover, tandem MALDI-MS or tandem electrospray ionization MS allows fragmentation of selected precursor ions, enabling more detailed characterization of structures of a given composition (Mechref et al. 2003; Ritchie et al. 2010). The techniques were soon adapted for analysis of *N*-glycans on recombinant viral proteins and mature viral particles.

Applying mass spectrometry-based analysis to viral glycans released from mature viral particles illustrated the heterogeneity of *N*-glycan structures present on a single protein. For example, detailed studies on Dengue virions revealed enormous heterogeneity of *N*-glycan structures modifying two putative sites of Dengue virus glycoprotein, with 19 distinct structures identified (Lei et al. 2015). In addition, MS-based glycoprofiling demonstrated distinct glycosylation patterns in different host species for Chikungunya virus (Lancaster et al. 2016), confirming earlier observations on togaviruses (Hsieh et al. 1983).

MALDI-MS has also been used to compare glycan profiles of recombinantly expressed viral proteins and proteins isolated from native viruses. Comparison of *N*-glycan profiles on recombinant HIV-1 gp120 monomers and gp120/gp41 trimers, present on native viruses, revealed that while monomeric recombinant protein contained a high proportion of complex-type glycans, native trimers with preserved protein quaternary structure predominantly contained high-mannose type carbohydrates (Doores, Bonomelli et al. 2010; Bonomelli et al. 2011). The high-mannose type glycans consistently found in monomeric and trimeric formulations are part of the intrinsic mannose patch that contains densely spaced underprocessed glycans (Bonomelli et al. 2011). In contrast, analysis of dimeric viral glycoprotein of Hendra virus suggests that oligomerization does not always impede glycan processing (Bowden et al. 2010). Viral protein architecture, however, is not the only requirement for recapitulation of physiologically relevant glycosylation, as the repertoire of glycosyltransferases naturally also plays an important role. For example, *N*-glycans on gp120 on pseudovirions from the cell line HEK293T mainly carried α2,3-linked sialic acids, while *N*-glycans on gp120 from virions derived from peripheral blood mononuclear cells predominantly carried α2,6-linked sialic acids (Pritchard, Harvey et al. 2015). Such differences might have huge implications on immunogenicity of recombinant vaccine antigens, given that some broadly neutralizing antibodies to HIV-1 recognize glycan-containing epitopes (Doores and Burton 2010; Mouquet et al. 2012; McCoy and Burton 2017). Therefore, both antigen formulations and production cell lines must be considered very carefully.

Another concern when analyzing recombinant viral proteins as the source of information for viral glycosylation, is the occasional need for artificial formulations of recombinant viral proteins that often lack natural processing and oligomerization signals when not assembled and presented in the viral context (Bowden et al. 2008; Ritchie et al. 2010). For example, it has been shown that lack of proteolytic cleavage of the membrane-truncated soluble HIV-1 envelope glycoprotein (gp140) resulted in aberrant glycosylation that altered the conformation of the trimer (AlSalmi et al. 2015). Ebola virus glycoprotein represents another example of a highly elaborate protein complex assembly. It exists as three distinct species due to transcriptional stuttering—sGP, GP and ssGP—two of which are secreted (Lee and Saphire 2009). The membrane bound GP is proteolytically processed to GP1 and GP2, and forms a trimer at the cell surface (Lee and Saphire 2009). Nevertheless, formulations of GPs that differ from the natural viral context have been pursued for

glycoanalysis. *N*-glycan analysis was performed for soluble fragment of GP1 and sGP. The GP1 exhibited a mix of complex, hybrid and high-mannose structures, while sGP carried a higher proportion of processed structures (Ritchie et al. 2010). A more recent glycoprofiling of stabilized monomeric GP1,2 constructs from five different strains of Ebola virus confirmed the presence of heterogeneous and mostly complex-type *N*-glycans (Collar et al. 2016). This is in contrast to a highly variable *O*-glycan pattern in the different strains (Collar et al. 2016). A few other recombinant glycoproteins from Nipah virus and Machupo virus were expressed in their monomeric forms and reported to predominantly carry complex-type *N*-glycans (Bowden et al. 2008, 2009). In conclusion, it is difficult to predict glycosylation patterns of native oligomeric protein complexes based on studies of recombinant monomeric proteins. It is, however, sometimes the only solution because of difficulties in expression of correctly folded and authentic protein complexes.

MALDI-MS can also be applied for *O*-glycan analysis (Canis et al. 2010; Franc et al. 2013); however, it has not been widely used in the virology field and only a few examples of O-glycoprofiling of viral glycoproteins using MALDI-MS exist (Schmitt et al. 1999; Collar et al. 2016). It can probably be explained by lower interest in viral O-glycosylation as opposed to N-glycosylation, primarily due to poorly characterized functions of viral *O*-glycans and the presumption that most viral glycoproteins are not heavily O-glycosylated. In addition, more sophisticated methods allowing simultaneous determination of modified amino acids have rapidly taken over, which have in the recent years been widely used for analysis of viral O-glycosylation.

Glycoproteomics of viruses

While glycoprofiling studies provide important information on distribution and composition of different glycan structures present on a given protein, it lacks the information on individual glycosite location, occupancy and glycan structure heterogeneity. The recent development of instruments equipped with ECD or ETD MS2 fragmentation has allowed for simultaneous determination of the peptide sequence and the position to which the carbohydrate moiety is attached (Levery et al. 2015). When relevant reporter glycan oxonium ions are present and it is easy to predict the glycosylation position within peptides, HCD MS2 fragmentation can also be used (Wuhrer et al. 2007). Interfacing the mass spectrometer with capillary liquid chromatography enables separation of complex proteome-scale glycopeptide mixtures and allows identification of thousands of glycopeptides in a single run. At the single protein level, it is now possible to determine the exact position, structure heterogeneity, and site occupancy for each individual glycosite (Wuhrer et al. 2007). Both N- and O-glycosylation can be analyzed by tandem MS. Biosynthetic features of N-glycosylation enable relatively easy identification and quantification of deglycosylated sites by MS2 sequencing. Due to asparagine deamidation during enzymatic *N*-glycan removal, the site occupancy can be determined by calculating the intensity ratio of naked (Asn) and deglycosylated (Asp) peptides (Pabst et al. 2012; An et al. 2015). However, the more appropriate methodology includes carrying out the reaction using heavy-oxygen water (O^{18}–H_2O), which discriminates deglycosylated sites from spontaneous Asn deamidation (Palmisano et al. 2012; Cao et al. 2017). It is also possible to evaluate the overall distribution of N-glycosite occupancy on intact glycoproteins by metabolically simplifying the glycans to homogeneous structures (Struwe et al. 2017). For O-linked glycans, complete *O*-glycan removal does not result in chemical peptide modification and cannot be used for identification of glycosylated amino acid positions (Levery et al. 2015). ETD and ECD MS2 techniques allow analysis of intact *O*-glycopeptides by fragmentation of the peptide backbone without the loss of *O*-glycan modification, however, it is of limited use for determining site occupancy (Wuhrer et al. 2007; Sihlbom et al. 2009; Zauner et al. 2012). Approximations are yet often made by comparing intensities of nonmodified and glycosylated peptides (Brautigam et al. 2013; Stansell et al. 2015). The relative quantitation of both N- and O-glycosylated peptides is not as accurate due to different ionization efficiencies of nonglycosylated peptides and peptides carrying complex glycans. Dependent on the complexity of the glycans, glycopeptides generally exhibit poorer ionization compared to peptides (Stavenhagen et al. 2013). The analysis of complex proteins therefore often requires enrichment with hydrophilic interaction chromatography or specific lectins, which is particularly relevant for proteome-wide applications (Bunkenborg et al. 2004; Zielinska et al. 2010; Khatri et al. 2014; Levery et al. 2015). However, information regarding site occupancy needs to be sacrificed.

N-glycoproteomics

Tandem mass spectrometry has been widely used for characterization of N-glycosylation sites on viral proteins, with respect to individual site occupancy status (macroheterogeneity) and site-specific structural diversity (microheterogeneity), as these are important features that can affect protein–protein interactions and immunogenicity. Comprehensive glycoproteomic analysis has, for example, mapped N-linked glycosylation of seasonal influenza A virus H3N2 HA, identifying more than 90 % site occupancy of all putative N-linked glycan sites (An et al. 2015). Moreover, the globular head glycosites, associated with host immune receptor interaction, were strictly high-mannose type (An et al. 2015). In a separate study on the highly pathogenic H5N1 influenza A virus, it was also shown that all potential N-glycosites were consistently occupied between several different strains (Blake et al. 2009), suggesting that N-glycosylation of HA is conserved between the different isolates of the same virus subtype, with a high occupancy of potential N-glycosites. In a similar way, it has been demonstrated that all predicted *N*-glycan sequons were utilized in Hepatitis C virus E2 and Murray Valley encephalitis virus NS1 (Blitvich et al. 2001; Iacob et al. 2008), where the majority of HCV E2 glycosites were modified with high-mannose type glycans (Iacob et al. 2008). On the densely glycosylated HIV-1 envelope glycoprotein several strategies have been employed to characterize the nature and location of the many glycans, with up to 27 mostly highly occupied N-glycosites identified, some of which were exclusively high-mannose type (Pabst et al. 2012; Go et al. 2013; Yang W et al. 2014). Tandem mass spectrometry has also been used for addressing differences in cell-type specific glycosylation and influence of protein conformation. Out of convenience, soluble HIV-1 gp120 or gp140 preparations are often analyzed. HIV-1 gp120 produced in CHO and 293 T cells had very similar occupancy, degree of fucosylation, sialylation, and glycan maturation, with a larger share of glycosites predominantly carrying hybrid and complex-type *N*-glycans (Go et al. 2013). The positions of some of the exclusively high-mannose *N*-glycans were located within the intrinsic mannose patch of gp120 (Go et al. 2013; Behrens et al. 2016). In contrast, a much higher proportion of the N-glycosites on gp140 expressed in CHO, recombinant trimers, or those on gp120 purified from native virions were modified with high-mannose type *N*-glycans (Pabst et al. 2012; Behrens et al. 2016;

Panico et al. 2016; Go et al. 2017). This again signifies the importance of analyzing the native protein conformation for generation of relevant glycosylation patterns. Glycosylation in different cell lines was also investigated for Hendra virus recombinant glycoprotein G, where all seven potential *N*-glycan sites were occupied in HeLa cells (Colgrave et al. 2012). In contrast, only four sites were N-glycosylated in HEK293, although the degree of glycan maturation was similar in both cell lines (Colgrave et al. 2012). To summarize the results obtained from various N-glycoprofiling and glycoproteomic studies, it seems that most of the putative N-glycosylation sequons are glycosylated with high occupancy on viral proteins. However, the site occupancy of viral protein N-glycosylation can vary in different producer cell lines. Moreover, high-mannose type *N*-glycans may constitute a substantial, if not the major, proportion of viral N-glycosites, particularly when native protein structure and oligomerization is taken into account. Thus, results obtained from analysis of recombinant monomeric proteins should be interpreted with caution also when considering the occupancy of individual N-glycosylation sites as well as complexity of glycan structures as discussed above.

O-glycoproteomics

For decades very little information has been available regarding site-specific O-glycosylation of viral proteins. Some of the first described virus-derived site-specific *O*-glycans were on isoform M of HBV surface antigen purified from patient-derived viral particles (Schmitt et al. 1999). The O-glycosylation site was identified by combining MALDI analysis of exoglycosidase-treated glycopeptide and Edman sequencing of the underlying peptide. The position of the glycan attachment site was deduced by carboxypeptidase digestion and confirmed by collision-induced dissociation tandem mass spectrometry, representing some of the early glycoproteomic experiments of viral proteins (Schmitt et al. 1999). The recent advances in mass spectrometry-based proteomics have resulted in numerous studies addressing O-glycosylation of individual viral glycoproteins. *O*-glycoproteomic analyses have been performed for several recombinant or isolated viral glycoproteins, including HIV-1 gp120, influenza A virus HA1, HCV E2, HSV-1 gC and Hendra virus glycoprotein G (Colgrave et al. 2012; Brautigam et al. 2013; Go et al. 2013; Yang W et al. 2014; Norden et al. 2015; Stansell et al. 2015). Recombinantly expressed gp120 and HA1 were glycosylated at a single position each, which in both cases was modified with core 1 or core 2 elongated *O*-glycans (Stansell et al. 2015). Interestingly, site occupancy was much lower in recombinant gp140 undergoing proteolytic cleavage to gp120, assuming equivalent ionization efficiencies of nonmodified and glycosylated peptides. Recombinant gp140 possessed shorter less sialylated, predominantly core 1 *O*-glycans, again underlining the importance of native protein conformation for analogous studies (Stansell et al. 2015). In contrast, gp120 purified from T-cell derived virions was devoid of the single site found glycosylated in recombinantly expressed gp120 (Stansell et al. 2015). This, however, might be related to the viral strain used, as the single site was reported to be glycosylated in a separate study using virions from a different HIV-1 strain (Yang W et al. 2014). In addition, treatment of passaged or plasma-derived HIV-1 virions with antibodies against O-linked carbohydrate structures resulted in inhibition of cell entry, and virus neutralization (Hansen et al. 1990, 1991), suggesting that HIV-1 gp120 can indeed be O-glycosylated in vivo. Comparison of recombinant gp120 O-glycosylation in two different cell lines revealed predominant core 1 O-glycosylation in CHO cells, compared to core 1, core 2 and core 4 in 293 T cells (Go et al. 2013). In a similar manner, Hendra virus glycoprotein G expressed in HeLa and HEK293 cells, differed considerably with different numbers of O-glycosites identified and carrying different core structures (Colgrave et al. 2012). Recombinant HCV E2 was O-glycosylated at six positions, with predominantly core 1 and core 2 *O*-glycan structures (Brautigam et al. 2013). More than 80 % occupancy was estimated for five of the six sites, whereas one site had very low occupancy. Moreover, a high level of structural heterogeneity was observed for the *O*-glycans localized at the individual sites, with up to 14 different structures identified (Brautigam et al. 2013). A recent study on O-linked glycosylation of HSV-1 mucin-like protein gC provided some insight into *O*-glycan synthesis, suggesting that the eleven O-glycosites were added in an orderly fashion, before elongation took place (Norden et al. 2015). These studies underscore the high heterogeneity of *O*-glycan structures, which are both cell type and protein specific, and the need for careful selection of candidates and expression cell lines for clinical applications. Moreover, comprehensive analysis of immune responses mounted by these different structures would be highly beneficial.

Global *O*-glycoproteomics

While it is clear that single protein-targeted mass spectrometry approaches can provide comprehensive information on single site occupancy and structure heterogeneity, we lack robust methods of analysis for site-specific O-glycosylation in complex or proteome-wide samples. To solve this problem, we recently introduced a method for globally mapping O-glycosylation sites in glycoengineered cell lines lacking O-linked glycan elongation, which is based on *Vicia villosa* lectin (VVA) affinity enrichment of simple glycopeptides coupled to tandem mass spectrometry (Steentoft et al. 2011). The method is also applicable for analysis of wild type cells, predominantly expressing core 1 O-glycans, by using peanut agglutinin (PNA) enrichment of desialylated glycopeptides (Yang Z et al. 2014). We have applied these methods in the analysis of O-glycosylation of HSV-1 infected human fibroblasts by performing a sequential enrichment with PNA and VVA, thus reporting the first comprehensive viral *O*-glycoproteome (Bagdonaite et al. 2015). This approach provides several clear advantages: first of all, it allows simultaneous analysis of all viral glycoproteins expressed in an infected cell. Secondly, the strategy takes into account the endogenous glycosylation of a permissive cell, dictated by the repertoire of glycosyltransferases, as well as native conformations of proteins and the cytopathic effects of viral infection. Irrelevant cell lines are often chosen for recombinant expression of viral proteins. The glycosylation obtained in these cells lines does not always reflect the glycosylation pattern in a natural host.

Using herpesviruses as a model system, we have applied the same method for defining the *O*-glycoproteomes of other members of *Herpesviridae* family—HSV-2, VZV, HCMV and EBV (Bagdonaite et al. 2016; Iversen et al. 2016). The wide occurrence, associated complications and shortage of prophylactic measures make herpesviruses a relevant model system to analyze *O*-glycans and their importance in viral life cycle (Vazquez et al. 2001; Cohen et al. 2006; Adjei et al. 2008; Kramer et al. 2008; Oxman 2010; Shiley and Blumberg 2010; Lopo et al. 2011; Sauerbrei et al. 2011; Levine et al. 2012; van Rijckevorsel et al. 2012; Astuto et al. 2013; Conde-Glez et al. 2013; Fishman 2013; Gorfinkel et al. 2013; Odland et al. 2013; Pembrey et al. 2013; Rowe et al. 2013; Awasthi and Friedman 2014; Bradley et al. 2014; Fu et al. 2014; Sabugo et al. 2014; Sili et al. 2014; Chen et al. 2015; Cohen 2015; Korndewal et al. 2015;

Shaiegan et al. 2015). In addition, the large proteomes of herpesviruses highlight the benefits of global viral *O*-glycoproteomics. Human herpesviruses encode seven to 12 glycoproteins associated with the viral particle; however, many more viral proteins possess signal peptides and transit through the host secretory pathway. Some of them have previously been investigated for glycan modifications in focused studies. N-linked glycans have been identified on viral envelope glycoproteins from all 8 human herpesviruses (Wenske et al. 1982; Edson and Thorley-Lawson 1983; Friedrichs and Grose 1984; Serafini-Cessi et al. 1984, 1985, 1989; Montalvo et al. 1985; Montalvo and Grose 1986, 1987; Gong et al. 1987; Britt and Vugler 1989; Gong and Kieff 1990; Okuno et al. 1990, 1992; Foa-Tomasi et al. 1992; Nolan and Morgan 1995; Pfeiffer et al. 1995; Hata et al. 1996; Mukai et al. 1997; Chandran et al. 1998; Pertel et al. 1998; Huber and Compton 1999; Li et al. 1999; Zhu et al. 1999; Baghian et al. 2000; Skrincosky et al. 2000; Wu et al. 2000; Maresova et al. 2000; Theiler and Compton 2002; Koyano et al. 2003; Paulsen et al. 2005; Yamagishi et al. 2008; Gore and Hutt-Fletcher 2009; Luo et al. 2015), where individual glycoproteins have been demonstrated to exhibit variable extent and pattern of glycan chain maturation (Wenske et al. 1982; Edson and Thorley-Lawson 1983; Friedrichs and Grose 1984; Serafini-Cessi et al. 1984, 1985, 1989; Montalvo et al. 1985; Montalvo and Grose 1986, 1987; Britt and Vugler 1989; Gong and Kieff 1990; Okuno et al. 1990, 1992; Huber and Compton 1999; Maresova et al. 2000; Theiler and Compton 2002; Yamagishi et al. 2008). A relatively smaller proportion of envelope glycoproteins of herpesviruses have been investigated in terms of O-glycosylation, and in some of these envelope proteins *O*-glycans have been detected by biochemical assays (Serafini-Cessi, Dall'Olio, Scannavini, Costanzo et al. 1983; Montalvo et al. 1985; Gong et al. 1987; Montalvo and Grose 1987; Serafini-Cessi et al. 1988, 1989; Britt and Vugler 1989; Kari et al. 1992; Yao et al. 1993; Nolan and Morgan 1995; Borza and Hutt-Fletcher 1998; Cardinali et al. 1998; Lake et al. 1998; Peng et al. 1998; Torrisi et al. 1999; Zhu et al. 1999; Wu et al. 2000; Theiler and Compton 2002; Xiao et al. 2007). Only a few of these proteins have merited more thorough investigation with most attention devoted to proteins containing mucin-like domains. HSV-1 attachment factor gC was the first envelope glycoprotein described to carry *O*-glycans, acquiring distinct structures in different cell types (Olofsson et al. 1981, 1983; Dall'Olio et al. 1985; Lundstrom et al. 1987), and specific O-glycosites have recently been mapped to the mucin-like region (Bagdonaite et al. 2015; Norden et al. 2015). Furthermore, the HSV-2 and VZV orthologs were also found to be O-glycosylated (Zezulak and Spear 1983; Bagdonaite et al. 2016). Similarly, other mucin-like region-containing proteins such as HSV-1 gI, HSV-2 gG, EBV gp150 and gp350 have been shown to accommodate high density of O-glycosylation, and the types of *O*-glycan structures were identified for some of these proteins (Serafini-Cessi et al. 1985, 1989; Nolan and Morgan 1995; Borza and Hutt-Fletcher 1998; Norberg et al. 2007). The conserved viral fusion effector gB has been shown or predicted to be O-glycosylated in all herpesvirus subfamilies (Serafini-Cessi, Dall'Olio, Scannavini, Costanzo et al. 1983; Gong et al. 1987; Montalvo and Grose 1987; Britt and Vugler 1989). The era of proteome-wide mass spectrometry-based applications allowed robust characterization of viral *O*-glycoproteomes (Bagdonaite et al. 2015, 2016; Iversen et al. 2016). The characterizations confirmed the identity of the majority of previously described *O*-glycoproteins of herpesviruses, and provided a tremendous expansion of site-specific O-glycosylation (Bagdonaite et al. 2015, 2016; Iversen et al. 2016). While GalNAc-type O-glycosylation is often associated with dense glycosylation in mucin-like regions, it is also abundantly found in isolation or small clusters in human proteins (Steentoft et al. 2013), which is more difficult to predict. In agreement with this, we have demonstrated ample presence of isolated *O*-glycan sites on viral glycoproteins of HSV-1, HSV-2, VZV, HCMV and EBV by glycoproteomic approaches (Bagdonaite et al. 2015, 2016; Iversen et al. 2016). Location of O-glycosites identified via proteome-wide MS/MS approaches with respect to protein structural features suggests possible involvement in the protein–protein interactions (Bagdonaite et al. 2015, 2016), as exemplified in subsequent sections. Large scale glycoproteomic analyses of human herpesviruses of varying phylogeny (HSV-1, HSV-2, VZV, HCMV and EBV) have made it possible to compare the O-glycosite patterns in homologous proteins (Bagdonaite et al. 2015, 2016; Iversen et al. 2016). Comparison of O-glycosite conservation between alphaherpesviruses HSV-1 and HSV-2 suggests that sequence homology is an important determinant for O-glycosylation in closely related viruses (Figure 1A and F). Isolated homologous glycosites were mainly situated on highly homologous peptide stretches, whereas densely spaced glycosites in Pro/Ser/Thr-rich regions were glycosylated irrespective of low sequence identity, as expected (Bagdonaite et al. 2016). Several glycoproteins are homologous between all herpesviruses, including gB, gH, gL, gM and gN, of which gB, gH and gL comprise the conserved cell entry machinery (McGeoch et al. 2006). We identified a large number of O-glycosites on HSV-1 fusogenic effector gB, and predicted that a number of O-glycosites could be conserved in most, if not all, human herpesviruses (Figure 1A–C) (Bagdonaite et al. 2016). Based on multiple sequence alignments across investigated herpesvirus family members, enrichment of O-glycosylation was found in the extreme N-terminus of gB regardless of the underlying considerable sequence variation between different herpesviruses. This suggests that glycosylation patches are less dependent on the underlying sequence, and might serve a glycan specific function, such as protection of the N-terminal exposed region of gB from proteolytic degradation or immune recognition. In contrast, conserved single glycosites were predominantly found between HSV-1, HSV-2, and, to a smaller extent, VZV, and suggest that they mainly exert subfamily-specific functions. The conserved protein gH, which is another essential component of the fusion machinery, was found glycosylated in four out of five investigated viruses. Although no clear conserved pattern of glycosylation was observed, the O-glycosites were predominantly localized to the two exposed N-terminal domains involved in interaction with other viral proteins (Figure 1D and E) (Bagdonaite et al. 2016).

In summary, global O-glycoproteomics of viruses open up possibilities to rapidly "scan" the proteome of viruses for O-glycan modifications. Although the occupancy and the relevance of the individual glycan sites are still unknown, the information can be used to follow up by complimentary techniques at individual protein and glycosite level. It can be applied to any human virus of interest; given relevant propagation systems are available. The method, of course, has its limitations, such as a limited number of glycoforms that can be captured, as well as the availability of protein sequences in the databases, which is challenging when analyzing emerging or poorly annotated viruses, as well as clinical isolates. Another aim for the future is to make the results broadly available to the scientific community not only by means of publishing, but also by inclusion into public protein databases. Ideally, a virus database compiling structural data, sequence variability, available glycomic and glycoproteomic data as well as antigenic sites could be created to advance basic and applied research in virology. If sufficient experimental data is compiled, machine learning bioinformatic techniques could

be applied to predict glycosylation patterns of emerging viral strains within distinct virus species or even families.

Roles of glycosylation in virus biology

Glycans on viral envelope glycoproteins play important roles in virus biology, with specific functions identified in various stages of viral infection (Table I and Figure 2). As previously mentioned, glycosylation is generally required for progeny formation and infectivity for many viruses (Knowles and Person 1976; Leavitt et al. 1977; Olofsson and Lycke 1980; Herrler and Compans 1983; Lambert and Pons 1983; Mann et al. 1983; Kuhn et al. 1988; Li et al. 2006; Reszka et al. 2010; Mathys and Balzarini 2015; Shrivastava-Ranjan et al. 2016; Mossenta et al. 2017). In some cases, mature *N*-glycans in particular are necessary for proper viral particle formation and release (Datema et al. 1984). As in human proteins, N-linked glycosylation is critical for proper folding of viral proteins (Land and Braakman 2001; Zai et al. 2013; Wu et al. 2017), and, in some cases, viral protein secretion (Hu et al. 1995; Lu et al. 1997; Yoshii et al. 2013; Mossenta et al. 2017). Drugs targeting glycoprotein maturation and exhibiting a favorable therapeutic ratio can therefore be used to combat viral infections, where no other therapeutics are available (Miller et al. 2012). Most importantly, N- and O-linked glycans shield immunodominant epitopes from immune recognition (Francica et al. 2010; Helle et al. 2011; Sommerstein et al. 2015; Behrens et al. 2016; Gram et al. 2016; Walls et al. 2016). In addition to general functions, specific types of glycans on viral glycoproteins often affect receptor binding and cell fusion (Lin et al. 2003; Lozach et al. 2003; Gramberg et al. 2005; Davis et al. 2006; Miller et al. 2008; Chen et al. 2014; Phoenix et al. 2016; Wang et al. 2016). Moreover, it is becoming increasingly clear that distinct glycans play separate roles in various stages of the viral cycle (Goffard et al. 2005; Falkowska et al. 2007; Helle et al. 2010; Wang et al. 2013, 2017; Lennemann et al. 2014; Bradel-Tretheway et al. 2015; Luo et al. 2015; Orlova et al. 2015; Wu et al. 2017). Some site-specific functions of viral glycosylation have been assigned for both N- and O-linked glycans mostly by site-directed mutagenesis studies. Identified roles of glycans within various stages of the viral cycle will be discussed in the following sections.

Attachment and entry

A number of viruses, such as HCV, Dengue virus, Ebola virus, SARS coronavirus, West Nile virus, Rift Valley fever virus and Japanese encephalitis virus use N-linked glycans as attachment and entry receptors by interacting with cellular lectins DC-SIGN, L-SIGN, LSECtin, ASGP-R or mannose receptor (Becker et al. 1995; Lin et al. 2003; Lozach et al. 2003; Gramberg et al. 2005; Davis et al. 2006; Miller et al. 2008; Chen et al. 2014; Phoenix et al. 2016; Wang et al. 2016). Mature *N*-glycans have been suggested to influence Lassa virus binding to cell entry receptor α-dystroglycan (Shrivastava-Ranjan et al. 2016). In addition, specific *N*-glycans on HIV-1 have been shown to affect CD4 receptor binding on T cells or regulate coreceptor usage (Nakayama et al. 1998; Pollakis et al. 2001; Francois and Balzarini 2011; Lombardi et al. 2015). Systemic functional analysis of HIV-1 gp160 potential N-glycosylation sites has identified a number of additional glycosites affecting infectivity (Wang et al. 2013). In addition, a certain *N*-glycan on HIV-1 gp41 was shown to have a consistent effect in several viral strains (Mathys and Balzarini 2014). Specific sialylated *N*-glycans on VZV gB have been identified that are important for interaction with myelin-associated glycoprotein and crucial for cell–cell fusion (Suenaga et al. 2015), and sialylation of virions in general was shown to affect entry of HSV-1 (Teuton and Brandt 2007). Similarly, specific N-linked glycans on HSV-2 gB influenced viral entry (Luo et al. 2015). An N-glycosylation site on IAV HA has also been shown to affect viral entry indirectly by modulating the avidity and specificity for sialosides (Wu et al. 2017). Finally, distinct putative N-glycosylation site mutations on HCV E1 and E2 resulted in diminished entry and altered CD81 binding (Goffard et al. 2005; Falkowska et al. 2007; Helle et al. 2010).

In contrast to the rather well documented roles of N-linked glycans, there are very few known examples, where specific *O*-glycans participate in virus–host interaction. Among these, two sialylated O-glycans on HSV-1 gB have been identified that determine binding to cellular receptor paired immunoglobulin-like type 2 receptor α residing on immune cells (Wang et al. 2009; Arii et al. 2010; Bagdonaite et al. 2015). In addition, HSV-1 O-glycans are involved in a few other aspects of viral binding to the host cell, as deletion of the densely O-glycosylated region of attachment factor gC affects both the binding affinity to the cell surface, and the release of progeny virus via modulation of interactions with cell surface glycosaminoglycans (Altgarde et al. 2015). Another example includes the carbohydrate-dependent binding of filoviruses to the macrophage galactose lectin that is known to recognize GalNAc-*O*-glycans (Takada et al. 2004). In hepatitis C virus, the mutation of several putative O-glycosites has also been shown to decrease HCV E2 affinity for CD81, suggesting that *O*-glycans might be of a more general importance in mediating interaction with host cells (Falkowska et al. 2007). *O*-glycoproteomic analysis of Hendra virus glycoprotein G has recently sprouted the first, to the best of our knowledge, systematic functional analysis of known O-glycosylation sites, revealing a multitude of functions including attachment and entry to host cells (Colgrave et al. 2012; Stone et al. 2016). Importantly, most of the functions could also be identified in analogous O-glycosites of a closely related Nipah virus (Stone et al. 2016). Interestingly, only in Nipah virus a single O-glycosite significantly affected Ephrin B2 receptor binding (Stone et al. 2016). This is an important example of conserved *O*-glycan function between closely related viruses, and presents intriguing possibilities in the light of recently identified consistent O-glycosylation patterns of several herpesviruses. Besides confirming the HSV-1 O-glycosites involved in binding PILRα in vitro, a few other O-glycosites residing in protein regions expected to influence HSV-1 attachment via cell entry receptors nectin-1 and HVEM (Carfi et al. 2001; Krummenacher et al. 2005; Heldwein et al. 2006; Di Giovine et al. 2011; Gallagher et al. 2014), or VZV attachment to receptor insulin degrading enzyme (Berarducci et al. 2010) were identified (Bagdonaite et al. 2015, 2016). This is another example how MS-based approaches combined with structural knowledge can help narrow down a list of O-glycosite candidates for focused studies. In conclusion, glycans on viral entry proteins are widely used for modulation of receptor binding and entry, with both N-linked and O-linked glycans having the capacity to affect the interaction (Figure 2, top left panel). In some cases, though, it seems to be an effect of conformational stability, rather than direct interaction (Stone et al. 2016).

Assembly and exit

Specific N-glycosites have been identified, that are important for glycoprotein secretion and viral particle formation in some viruses, including Zika virus, hepatitis viruses B and C (HBV and HCV),

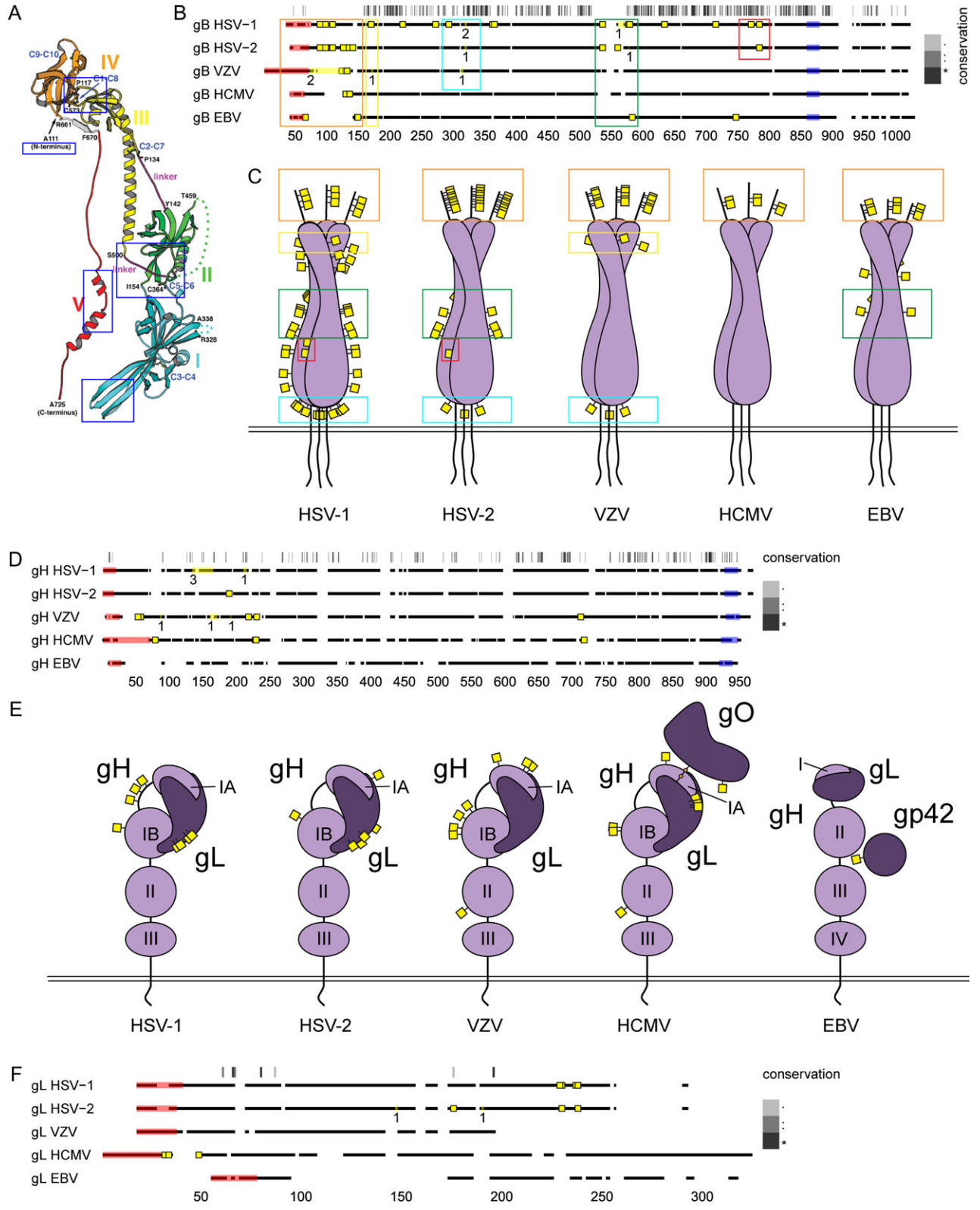

Fig. 1. O-glycosylation of herpesvirus conserved fusion machinery. (**A**), Crystal structure representation of HSV-1 gB monomer. From "Heldwein EE Lou H Bender FC Cohen GH Eisenberg RJ Harrison S 2006. Crystal structure of glycoprotein B from herpes simplex virus 1. Science, 313:217–220". Reprinted with permission from AAAS. Blue boxes mark the parts of the molecule where *O*-glycans are consistently found between at least two investigated herpesviruses. Modified with permission from the authors. (**B**), (**D**) and (**F**), Conservation of O-linked glycosylation sites on homologous envelope glycoproteins of human herpesviruses (from Bagdonaite et al., 2016). Reprinted with permission. © 2008 The American Society for Biochemistry and Molecular Biology. All rights reserved. Clustal Omega server was used to align amino acid sequences of gB (**B**), gH (**D**) and gL (**F**) between HSV-1 (Bagdonaite et al. 2015), HSV-2 (Iversen et al. 2016),

HSV-2 and HIV-1 (Fournillier et al. 2001; Falkowska et al. 2007; Helle et al. 2010; Ito et al. 2010; Wang et al. 2013; Luo et al. 2015; Mossenta et al. 2017). A few N-glycosites on influenza A virus neuraminidase have been shown to affect protein activity and viral particle release (Wu et al. 2017). Also, it is well documented that N-linked glycosylation of alphaherpesvirus envelope proteins is required for trafficking to the cell surface and viral particle egress (Norrild and Pedersen 1982; Montalvo et al. 1985; Luo et al. 2015; Suenaga et al. 2015). In particular, complex-type *N*-glycans are required for robust egress (Serafini-Cessi, Dall'Olio, Scannavini, Campadelli-Fiume et al. 1983). In varicella zoster virus the role of potential O-glycosylation sites has also been addressed by site-directed mutagenesis. Several glycosites were found to affect cell surface expression of the protein. However, such an approach is too laborious for analysis of all potential O-glycosylation sites, given the lack of conserved sequence motifs for O-glycosylation (Suenaga et al. 2015). As a more targeted approach, site-directed mutagenesis of some of the O-glycosites on Hendra virus glycoprotein G, identified via an O-glycoproteomic approach, affected the incorporation of glycoprotein F into pseudotyped virions. This suggests that the *O*-glycans might be required for protein–protein interaction (Stone et al. 2016). Similarly, in HIV gp120 O-glycosylation of a conserved threonine that is crucial for the association with gp41 has recently been shown to enhance infectivity and incorporation of gp120 into virions, though the virus maintains normal functionality in the absence of the *O*-glycan (Termini et al. 2017).

In conclusion, manipulation of glycans or glycosylation sites seems to affect both viruses that use direct budding and those using exocytosis. It is therefore likely that glycans affect many different aspects of viral particle formation (Figure 2, bottom right panel), possibly through an indirect consequence of disrupted trafficking of glycoproteins that are required for virus formation (Stone et al. 2016; Mossenta et al. 2017).

Viral spread and innate immunity

Different viruses employ various spread mechanisms both systemically and within the site of infection, including cell–cell fusion, transmigration and neuroinvasion. Viruses often take advantage of components of the innate immune system to target the cell type of interest at distant locations. Glycans play an important role in these mechanisms (Figure 2, bottom left panel). In the deadly Hendra virus the globular head region of glycoprotein G is important for binding to the entry receptors Ephrin B2/B3. Mutation of N-glycosylation sites in the globular head of glycoprotein G increased cell–cell fusion, whereas removal of a glycosite in the stalk region of Hendra glycoprotein G abolished cell–cell fusion (Bradel-Tretheway et al. 2015). In case of *O*-glycans, however, removal of distinct O-glycosites within a cluster in the stalk region resulted in both hypo- and hyperfusogenic phenotypes (Stone et al. 2016), and neither N- nor O-glycosite mutants exhibited impaired receptor binding (Bradel-Tretheway et al. 2015; Stone et al. 2016). This suggests that glycans influence other aspects of membrane fusion; possibly by affecting the thermodynamics of conformational changes. The impact of *N*-glycans in viral spread has also been demonstrated in in vivo models. Interestingly, the glycosylation status of West Nile virus glycoproteins has been associated with neuroinvasiveness, where abolishment of glycosites led to attenuation (Whiteman et al. 2010). Similarly, loss of a N-glycosite on Ross River virus glycoprotein E1 was associated with milder pathogenesis and increased virus clearance in a disease mouse model (Nelson et al. 2016). Finally, specific N-glycosylation patterns on both HA and NA on avian influenza A virus are required for efficient transmission in mammalian models (Park et al. 2016). A more direct role of glycans emerges from the viral exploitation of innate immune receptors as a helping hand during establishing the viral infection. Such innate immune receptors and other secreted glycan binding proteins present on infected host cells or antigen presenting cells are naturally the first line of defense against invading pathogens. For instance, secreted C-type lectins, ficolin-2 and mannose-binding lectin, inhibit HCV entry into hepatocytes (Brown et al. 2010; Zhao et al. 2014). However, these carbohydrate binding proteins can convey both protective and deleterious effects during viral infection, which are exploited by viruses to enter host cells. For example, secreted galectin-1 promotes entry of Nipah virus into endothelial cells by bridging viral and cellular glycans, when the glycan binding proteins are present during initial phases of infection. In contrast, galectin-1 inhibits viral spread, when added postinfection (Garner et al. 2015). Galectin-1 has also been shown to promote HIV-1 infection by crosslinking the viral particles and cell entry receptors, whereas influenza virus infection was inhibited (St-Pierre et al. 2011; Yang et al. 2011). As already mentioned, several viruses specifically use C-type lectin receptors for entry or transmission. Ebola virus utilizes an array of different C-type lectins, such as L-SIGN, DC-SIGN and LSECtin, for entry into various cell types (Lennemann et al. 2014). HIV-1 uses DC-SIGN on dendritic cells for migration to T cells (Hong et al. 2002). Although it is not entirely clear how the balance between antigen presentation and transmission is maintained, it has been proposed that homogeneous high-mannose type *N*-glycans enhance antigen presentation and virus degradation compared to intrinsically heterogeneous gp120 N-glycosylation (van Montfort et al. 2011). Interestingly, HIV-1 has also been shown to exploit sialic-acid binding lectin Siglec-1 for more efficient entry into macrophages, likewise promoting the existence of complex-type *N*-glycans on viral particles (Zou et al. 2011). In a similar context, we have demonstrated,

VZV (Bagdonaite et al. 2016), HCMV (Bagdonaite et al. 2016) and EBV (Bagdonaite et al. 2016). Protein backbones are depicted as broken black lines, where spaces represent gaps in the alignment. Individual alignments were drawn to scale (indicated below each graph). Sequence conservation is indicated above the aligned sequences for each set, and is represented by a greyscale barcode that maps to the clustal alignment score, as shown in the legend. In brief, for the clustal alignment score, an asterisk indicates positions with fully conserved residues, a colon indicates conservation of amino acids with strongly similar properties, whereas a period indicates conservation of amino acids with weakly similar properties. Predicted signal peptides and transmembrane regions are shaded in pink and blue, respectively. Unambiguous O-glycosylation sites are shown as yellow squares, whereas ambiguous sites are marked as yellow lines within the protein backbone, where the number below indicates the number of glycosites. An ambiguous O-glycosylation site from our previous publication (Bagdonaite et al. 2015, HSV-1 gB 109–123 (HexHexNAc)) was omitted from the graph, as we cannot exclude the possibility it could be part of an elongated structure on an adjacent site. Reference strain sequences were used for HSV-2, VZV and EBV due to incomplete or unavailable annotation of investigated strains. HSV-1—human herpes simplex virus type 1 (strain 17), HSV-2—human herpes simplex virus type 2 (strain HG52), VZV—varicella-zoster virus (strain Dumas), HCMV—human cytomegalovirus (strain Towne), EBV—Epstein-Barr virus (strain AG876). (**C**) and (**E**) Cartoon depiction of HSV-1 gB trimers (**C**) or gH–gL complexes and accessory proteins (**E**) of the five herpesviruses. O-glycosylation sites are shown as yellow squares. (**B**) and (**C**) Colored boxes mark association with herpesvirus gB domains as defined in (**A**).

Table I. Glycosylation of viral proteins of human eveloped viruses

Abbr.	Virus name	Family	Genus	Genome	N-glycosylated[a]	O-glycosylated[a]	Binding/entry/ haemagglutination/infectivity[a]	Spread[a]	Formation/release/ yield/protein transport[a]	Immune response[a]	Protein–protein interaction and other functions[a]
LASV	Lassa virus	*Arenaviridae*	*Arenavirus*	(−)ssRNA			Shrivastava-Ranjan et al. (2016)			Sommerstein et al. (2015)	
MACV	Machupo virus	*Arenaviridae*	*Arenavirus*	(−)ssRNA	Bowden et al. (2009);						
INKV	Inkoo virus	*Bunyaviridae*	*Orthobunyavirus*	(−)ssRNA	Pesonen, Ronnholm et al. (1982)						
RVFV	Rift Valley fever virus	*Bunyaviridae*	*Phlebovirus*	(−)ssRNA	Phoenix et al. (2016)		Phoenix et al. (2016)				
UUKV	Uukuniemi virus	*Bunyaviridae*	*Phlebovirus*	(−)ssRNA	Pesonen, Kuismanen et al. (1982)						
HCoV	Human coronavirus	*Coronaviridae*	*Alphacoronavirus*	(+)ssRNA						Walls et al. (2016)	
MHV	Mouse hepatitis virus[b]	*Coronaviridae*	*Betacoronavirus*	(+)ssRNA	Niemann et al. (1982)	Niemann et al. (1982); Niemann et al. (1984); Locker et al. (1992)			Niemann et al. (1982)		
SARS-CoV	SARS-related human coronavirus	*Coronaviridae*	*Betacoronavirus*	(+)ssRNA			Gramberg et al. (2005)				
EBOV	Ebola virus	*Filoviridae*	*Ebolavirus*	(−)ssRNA	Jeffers et al. (2002); Ritchie et al. (2010); Collar et al. (2016)	Jeffers et al. (2002); Ritchie et al. (2010); Collar et al. (2016)	Lin et al. (2003); Takada et al. (2004); Gramberg et al. (2005); Lennemann et al. (2014)		Wang et al. (2017)	Dowling et al. (2007); Francica et al. (2010); Noyori et al. (2013); Lennemann et al. (2014)	
MARV	Marburg virus	*Filoviridae*	*Marburgvirus*	(−)ssRNA	Feldmann et al. (1991)	Feldmann et al. (1991)	Becker et al. (1995); Gramberg et al. (2005)			Noyori et al. (2013)	
DENV	Dengue virus	*Flaviviridae*	*Flavivirus*	(+)ssRNA	Miller et al. (2008); Dubayle et al. (2015); Lei et al. (2015)		Davis et al. (2006); Miller et al. (2008)			Rouvinski et al. (2015)	
JEV	Japanese encephalitis virus	*Flaviviridae*	*Flavivirus*	(+)ssRNA	Zai et al. (2013)		Wang et al. (2016)		Zai et al. (2013)		
MVEV	Murray Valley encephalitis virus	*Flaviviridae*	*Flavivirus*	(+)ssRNA	Blitvich et al. (2001)						
TBEV	Tick-borne encephalitis virus	*Flaviviridae*	*Flavivirus*	(+)ssRNA	Yoshii et al. (2013)				Yoshii et al. (2013)		
WNV	West Nile virus	*Flaviviridae*	*Flavivirus*	(+)ssRNA			Davis et al. (2006)	Whiteman et al. (2010)	Li et al. (2006)		
ZIKV	Zika virus	*Flaviviridae*	*Flavivirus*	(+)ssRNA			Mossenta et al. (2017)		Mossenta et al. (2017)		
HCV	Hepatitis C virus	*Flaviviridae*	*Hepacivirus*	(+)ssRNA	Iacob et al. (2008)	Brautigam et al. (2013)	Lozach et al. (2003); Goffard et al. (2005); Falkowska et al. (2007); Helle et al. (2010); Chen et al. (2014)		Fournillier et al. (2001); Goffard et al. (2005); Helle et al. (2010); Reszka et al. (2010); Orlova et al. (2015)	Fournillier et al. (2001); Falkowska et al. (2007); Liu et al. (2007); Brown et al. (2010); Helle et al. (2010); Anjum et al. (2013); Zhao et al. (2014); Li et al. (2016)	Meunier et al. (1999); Goffard et al. (2005); Orlova et al. (2015)
HBV	Hepatitis B virus	*Hepadnaviridae*	*Orthohepadnavirus*	dsDNA-RT	Schmitt et al. (1999)	Schmitt et al. (1999)			Lu et al. (1997); Ito et al. (2010); Julithe et al. (2014)	Julithe et al. (2014); Hyakumura et al. (2015)	

HCMV (HHV-5)	Human cytomegalovirus (human herpesvirus 5)	*Herpesviridae*	*Cytomegalovirus*	dsDNA	Kim et al. (1976)[c]; Britt and Vugler (1989); Kaye et al. (1992); Al-Barazi and Colberg-Poley (1996); Jones et al. (1996); Margulies et al. (1996); Maidji et al. (1998); Huber and Compton (1999); Mullberg et al. (1999); Rehm et al. (2001); Gewurz et al. (2002); Theiler and Compton (2002); Griffin et al. (2005); Margulies and Gibson (2007); Lin et al. (2008); Stanton et al. (2010); Engel et al. (2011); Gabaev et al. (2014); Geyer et al. (2014); Cavaletto et al. (2015)	Britt and Vugler (1989); Kari et al. (1992); Theiler and Compton (2002); Gabaev et al. (2014); Geyer et al. (2014); Bagdonaite et al. (2016)				Kropff et al. (2012)	
EBV (HHV-4)	Epstein-Barr virus (human herpesvirus 4)	*Herpesviridae*	*Lymphocryptovirus*	dsDNA	Edson and Thorley-Lawson (1983); Gong et al. (1987); Serafini-Cessi et al. (1989); Gong and Kieff (1990); Nolan and Morgan (1995); Paulsen et al. (2005); de Turenne-Tessier and Ooka (2007); Gore and Hutt-Fletcher (2009)	Gong et al. (1987)[c]; Serafini-Cessi et al. (1989); Nolan and Morgan (1995); Borza and Hutt-Fletcher (1998); de Turenne-Tessier and Ooka (2007); Xiao et al. (2007); Bagdonaite et al. (2016)				D'Arrigo et al. (2013); Gram et al. (2016)	
KSHV (HHV-8)	Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8)	*Herpesviridae*	*Rhadinovirus*	dsDNA	Chandran et al. (1998)[c]; Pertel et al. (1998); Li et al. (1999); Zhu et al. (1999); Baghian et al. (2000); Wu et al. (2000); Chung et al. (2002); Koyano et al. (2003); Dela Cruz et al. (2004); Meads and Medveczky (2004); Dela Cruz et al. (2009); Wu et al. (2015)	Zhu et al. (1999); Wu et al. (2000)					Dela Cruz et al. (2004); Wu et al. (2015)[d]
HHV-6	Human herpesvirus 6	*Herpesviridae*	*Roseolovirus*	dsDNA	Okuno et al. (1990); Foa-Tomasi et al. (1992); Okuno et al. (1992); Pfeiffer et al. (1995)[c]	Cardinali et al. (1998); Torrisi et al. (1999)					
HHV-7	Human herpesvirus 7	*Herpesviridae*	*Roseolovirus*	dsDNA	Hata et al. (1996); Mukai et al. (1997)[c]; Skrincosky et al. (2000); Glosson et al. (2010)						Glosson et al. (2010)[d]
HSV-1 (HHV-1)	Herpes simplex virus type 1 (human herpesvirus 1)	*Herpesviridae*	*Simplexvirus*	dsDNA	Keller et al. (1970)[c]; Spear and Roizman (1970)[c]; Honess and Roizman (1975)[c]; Eisenberg et al. (1979); Wenske et al. (1982); Cohen et al. (1983); Respess et al. (1984); Serafini-Cessi et al. (1984)	Keller et al. (1970)[c]; Olofsson et al. (1981); Serafini-Cessi, Dall'Olio, Scannavini, Costanzo et al. (1983); Olofsson et al. (1983); Dall'Olio et al. (1985); Lundstrom et al. (1987); Serafini-Cessi et al. (1988);	Kuhn et al. (1988); Teuton and Brandt (2007); Wang et al. (2009); Altgarde et al. (2015)	Knowles and Person (1976); Campadelli-Fiume et al. (1982); Serafini-Cessi,	Olofsson and Lycke (1980); Norrild and Pedersen (1982); Serafini-Cessi, Dall'Olio, Scannavini,	Sodora et al. (1989)	

Continued

Table I. Continued

Abbr.	Virus name	Family	Genus	Genome	N-glycosylated[a]	O-glycosylated[a]	Binding/entry/ haemagglutination/infectivity[a]	Spread[a]	Formation/release/ yield/protein transport[a]	Immune response[a]	Protein–protein interaction and other functions[a]
						Peng et al. (1998); Norberg et al. (2007)[c]; Bagdonaite et al. (2015); Norden et al. (2015)		Dall'Olio, Scannavini, Campadelli-Fiume et al. (1983)	Campadelli-Fiume et al. (1983); Altgarde et al. (2015)		
HSV-2 (HHV-2)	Herpes simplex virus type 2 (human herpesvirus 2)	*Herpesviridae*	*Simplexvirus*	dsDNA	Cohen et al. (1983); Zezulak and Spear (1983); Serafini-Cessi et al. (1985)	Zezulak and Spear (1983); Serafini-Cessi et al. (1985); Serafini-Cessi et al. (1988); Iversen et al. (2016)	Luo et al. (2015)	Luo et al. (2015)	Luo et al. (2015)	Clo et al. (2012); Iversen et al. (2016)	
VZV (HHV-3)	Varicella-zoster virus (human herpesvirus 3)	*Herpesviridae*	*Varicellovirus*	dsDNA	Friedrichs and Grose (1984); Montalvo et al. (1985); Montalvo and Grose (1986); Montalvo and Grose (1987); Yao et al. (1993); Maresova et al. (2000); Yamagishi et al. (2008)	Montalvo et al. (1985); Montalvo and Grose (1987); Yao et al. (1993); Bagdonaite et al. (2016)		Suenaga et al. (2015)	Montalvo et al. (1985)		
FLUAV	Influenza A virus	*Orthomyxoviridae*	*Influenzavirus A*	(−)ssRNA	Schwarz et al. (1977)[c]; Edwardson (1984); Blake et al. (2009); An et al. (2015)	Stansell et al. (2015)	Park et al. (2016); Wu et al. (2017)		Edwardson (1984); Wu et al. (2017)	Skehel et al. (1984); Yang et al. (2011); Wu et al. (2017)	
HeV	Hendra virus	*Paramyxoviridae*	*Henipavirus*	(−)ssRNA	Bowden et al. (2010); Colgrave et al. (2012)	Colgrave et al. (2012)	Bradel-Tretheway et al. (2015); Stone et al. (2016)	Bradel-Tretheway et al. (2015); Stone et al. (2016)	Bradel-Tretheway et al. (2015); Stone et al. (2016)	Bradel-Tretheway et al. (2015)	Bradel-Tretheway et al. (2015); Stone et al. (2016)
NiV	Nipah virus	*Paramyxoviridae*	*Henipavirus*	(−)ssRNA	Bowden et al. (2008)		Stone et al. (2016)	Bradel-Tretheway et al. (2015); Stone et al. (2016)		Aguilar et al. (2006); Garner et al. (2015)	Stone et al. (2016)
MeV	Measles virus	*Paramyxoviridae*	*Morbillivirus*	(−)ssRNA	Hu et al. (1995)				Hu et al. (1995)		
HRSV	Human respiratory syncitial virus	*Paramyxoviridae*	*Pneumovirus*	(−)ssRNA	Lambert and Pons (1983); Gruber and Levine (1985)	Gruber and Levine (1985)[c]		Lambert and Pons (1983)	Lambert and Pons (1983)		
MuV	Mumps virus	*Paramyxoviridae*	*Rubulavirus*	(−)ssRNA	Herrler and Compans (1983)				Herrler and Compans (1983)		
VACV	Vaccinia virus	*Poxviridae*	*Orthopoxvirus*	dsDNA	Shida and Dales (1981)	Shida and Dales (1981)	Shida and Dales (1981)				
HIV-1		*Retroviridae*	*Lentivirus*	ssRNA-RT							

	Human immunodeficiency virus 1				Bernstein et al. (1994); Doores, Bonomelli et al. (2010); Bonomelli et al. (2011); Pabst et al. (2012); Go et al. (2013); Yang W et al. (2014); AlSalmi et al. (2015); Pritchard, Harvey et al. (2015); Behrens et al. (2016); Panico et al. (2016); Cao et al. (2017); Go et al. (2017); Struwe et al. (2017)	Bernstein et al. (1994); Go et al. (2013); Yang Z et al. (2014); Stansell et al. (2015)	Nakayama et al. (1998); Pollakis et al. (2001); Hong et al. (2002); Lin et al. (2003); Francois and Balzarini (2011); Zou et al. (2011); Wang et al. (2013); Mathys and Balzarini (2014); Lombardi et al. (2015); Mathys and Balzarini (2015); Termini et al. (2017)	Mathys and Balzarini (2014); Mathys and Balzarini (2015)	Mathys and Balzarini (2014); Mathys and Balzarini (2015)	Sanders et al. (2002); Wei et al. (2003); Wang et al. (2013); Doores and Burton (2010); St-Pierre et al. (2011); van Montfort et al. (2011); Pritchard, Spencer et al. (2015); Behrens et al. (2016); Lee et al. (2016)
CHIKV	Chikungunya virus	*Togaviridae*	*Alphavirus*	(+)ssRNA	Lancaster et al. (2016)					
RRV	Ross River virus	*Togaviridae*	*Alphavirus*	(+)ssRNA				Nelson et al. (2016)		
SFV	Semliki Forest virus	*Togaviridae*	*Alphavirus*	(+)ssRNA	Pesonen (1979); Rasilo and Renkonen (1979)					
SINV	Sindbis virus	*Togaviridae*	*Alphavirus*	(+)ssRNA	Sefton (1975)[c]; Burke and Keegstra (1979); Hsieh et al. (1983)			Mann et al. (1983)	Leavitt et al. (1977); Datema et al. (1984)	
RUBV	Rubella virus	*Togaviridae*	*Rubivirus*	(+)ssRNA	Ho-Terry and Cohen (1984); Hobman et al. (1991); Lundstrom et al. (1991)	Ho-Terry and Cohen (1984)[c]; Lundstrom et al. (1991)	Ho-Terry and Cohen (1984)			Hobman et al. (1991)

[a]Literature references are indicated as first author last name and year.

[b]Nonhuman infecting virus historically used as model for investigating betacoronaviruses.

[c]Indirect evidence.

[d]Other functions.

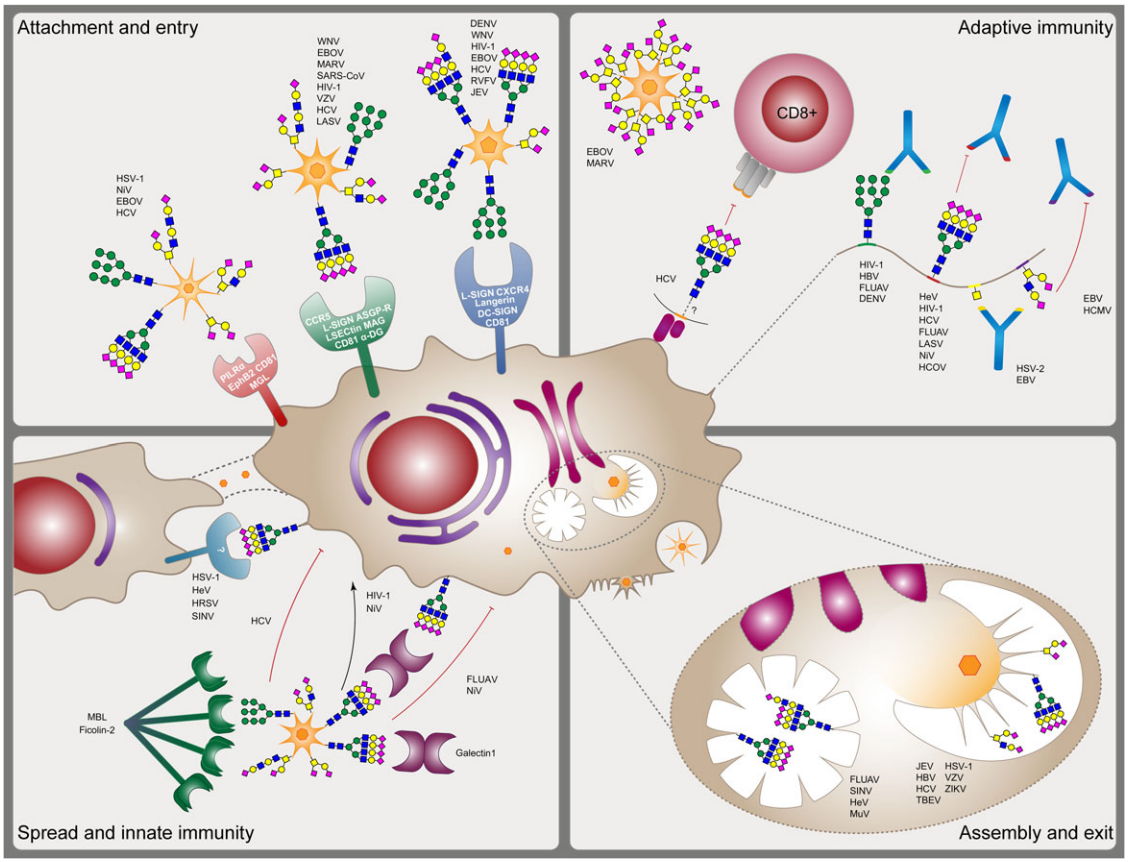

Fig. 2. Roles of glycosylation in the biology of enveloped viruses. The cartoon depicts functions of glycosylation described in the literature to affect various stages of the infectious cycle of enveloped viruses (Table I). The glycan chains presented on the generic viral particles are of illustrative manner and do not represent the glycoprofiles of individual viruses. Likewise, the glycan structures shown to interact with specific glycan binding molecules represent the most likely type of structure (high mannose or complex type) based on known carbohydrate binding specificities, unless specified otherwise in individual studies defining the interactions or glycoprofiling/glycoproteomic studies of the viruses in question. In the bottom right panel, the viruses are grouped according to the predominant mode of exit within the individual families. Virus abbreviations as in Table I. Monosaccharide symbols follow the SNFG (Symbol Nomenclature for Glycans) system (PMID 26,543,186, Glycobiology 25: 1323–1324, 2015).

that very early chemokine-mediated immune response to HSV-2 infection of the vaginal mucosa in mice relies on the presence of elongated O-linked glycans on the viral particles (Iversen et al. 2016). Although truncation of *O*-glycans might benefit the virus in escaping the early recognition by the host, elongated *O*-glycans are essential for viral replication (Bagdonaite et al. 2015; Iversen et al. 2016). Various carbohydrate structures from many bacteria and parastic worms are known to elicit robust innate immune responses via toll-like and other receptors (Rabinovich and Toscano 2009; Prasanphanich et al. 2013). It is only conceivable that analogous carbohydrate-mediated mechanisms could exist for early detection of viral infections, but have not been described in detail.

Adaptive immunity

During evolution, the immune system has developed redundant and fine-tuned mechanisms to identify and eliminate invading pathogens, including viruses. In turn, viruses continuously adapted to counteract or take advantage of the host's defense mechanisms, which is an ongoing battle. Glycans on viral glycoproteins have dual roles in virus–host interplay (Figure 2, top right panel). On one hand, glycans can be part of the immune determinants themselves, but on the other hand glycans can mask the antigenic protein epitopes from recognition (Alexander and Elder 1984). Accumulating structural evidence suggests that glycans cover large surface areas of viral envelope proteins providing steric hindrance and physical shielding of vulnerable sites (Lee et al. 2016; Walls et al. 2016; Beniac and Booth 2017). Both N- and O-linked glycans can mask immunogenic B-cell epitopes, and, in some cases also determine or influence immunogenicity (Sodora et al. 1989; Hobman et al. 1991; Fournillier et al. 2001; Falkowska et al. 2007; Helle et al. 2010; Wang et al. 2013; Bradel-Tretheway et al. 2015). *N*-glycans covering structured domains and functional regions often confer protection from neutralization (Helle et al. 2010; Wang et al. 2013; Bradel-Tretheway et al. 2015; Wu et al. 2017). As an example, systemic functional analysis of N-glycosites in HCV revealed that protective N-linked glycans were mainly situated around the CD81 binding site on the HCV envelope glycoprotein E2 (Helle et al. 2010). In other cases, removal of specific N-linked glycans can alter the local antigenic landscape and broaden the immune response rather than simply uncovering underlying epitopes (Fournillier et al. 2001; Wang et al. 2013). In contrast, mutation of seemingly unrelated N-glycosites can reduce the sensitivity to neutralization at distant epitopes (Wang et al. 2013), highlighting the role of *N*-glycans on maintaining protein structure. In conclusion, mutation of potential N-glycosylation sites can result in unpredicted effects on protein

conformation and molecular dynamics, which differentially affect neutralization by antibodies, and are not explained solely by epitope recovery. Site-specific N-glycosylation has also been reported to affect cellular immunity to HCV E1, where deletion of specific glycans enhanced recognition by cytotoxic T cells (Liu et al. 2007).

Dense O-glycosylation of viral proteins has been widely described to provide "bulk" shielding from host immunity, and protect from antibody neutralization (Machiels et al. 2011; Kropff et al. 2012). Besides shielding select immunodominant epitopes (Machiels et al. 2011), highly O-glycosylated mucin-like domains, such as those found in Ebola and Marburg viruses, have been suggested to physically hinder the interaction between virus-infected cells and immune cells (Francica et al. 2010; Noyori et al. 2013). Conversely, the deletion of the Ebola virus mucin-like domain decreased the immune response in mice, although it did not have an effect on immunogenicity in vitro (Dowling et al. 2007). Finally, *O*-glycans not only shield, but in some cases can be presented as part of neo-epitopes when elongation of the glycans is prevented. As an example, several O-glycosylated immunodominant B-cell epitopes have been reported for HSV-2 and EBV, by probing *O*-glycopeptides with sera of infected patients, suggesting that the glycan moiety can be recognized by B-cells (Clo et al. 2012; D'Arrigo et al. 2013).

In the context of epitope shielding and immune recognition of viruses, it might be of importance that RNA viruses, such as influenza virus, HIV-1 and HCV, intrinsically have a very high mutation rate, thus minimizing the effect of adaptive immune response (Sanjuan et al. 2010; Anjum et al. 2013; Lauring et al. 2013; Lynch et al. 2015). Early on it was observed that the highest amino acid substitution rates occurred in antigenic regions of envelope glycoproteins, including such mutations that changed the number and position of glycosylation sites (Krystal et al. 1983; Skehel et al. 1984; Wei et al. 2003). Due to high density of glycans in such regions, some of the carbohydrate chains are not fully processed resulting in exposure of immature structures as seen in the mannose patch of HIV gp120 (Doores, Bonomelli et al. 2010). Paradoxically, such underprocessed structures are recognized by the immune system, giving rise to broad neutralizing antibodies (bnAbs), with the classical example being 2G12 (Sanders et al. 2002; Doores and Burton 2010). In addition, highly conserved glycosylation sites that are directly involved in binding to cell entry receptors or viral interaction partners often become targeted by bnAbs (Rouvinski et al. 2015). Interestingly, it has been demonstrated, that the recognition by such bnAbs is largely unaffected by deletion of individual *N*-glycans within the gp120 mannose patch, mimicking the consequences of intrinsic mutagenesis (Pritchard, Spencer et al. 2015). In some cases, however, mutations at distant glycosites may have subtle effects on epitope recognition (Behrens et al. 2016). Similarly, antigenicity of the HBV surface antigen isoform S did not suffer from introduction of additional N-glycosylation sites, and even resulted in stronger and more persistent immune responses (Hyakumura et al. 2015). In contrast, arenaviruses represent very well adapted pathogens where glycan shield density inversely correlates with potency of neutralizing antibodies (Sommerstein et al. 2015). However, in other instances, over-glycosylation of important functional regions of viral proteins result in better shielding from the immune system at a cost of binding affinity and virus production (Aguilar et al. 2006; Julithe et al. 2014; Lennemann et al. 2014; Lynch et al. 2015). As a compromise, variable glycosite occupancy can both fulfill the requirement for receptor binding via the nonglycosylated protein, and provide protection via the glycosylated counterpart (Julithe et al. 2014).

Being the components of viral particles exposed to the extracellular environment, viral envelope glycoproteins serve as major targets for vaccine development; however, protein sequence variability within and across strains, high mutation rate, as well as enormous heterogeneity of glycan structures makes it very difficult to identify immunogens evoking universal reactivity. Various expression systems are used for production of subunit vaccine candidates, such as bacteria, yeast, plant, insect and mammalian cell lines (Cox 2012; Kushnir et al. 2012; Redkiewicz et al. 2014). While some glycosylation types can be replicated in insect and mammalian cells, yeast cells will not carry out mucin type O-glycosylation and proteins produced in bacterial cells will also lack *N*-glycans. Even mammalian expression systems may lack the required glycosyltransferase repertoire to glycosylate relevant sites and build up relevant structures. Although there are many successful examples of vaccines produced in aforementioned systems, there is quite a number of infectious diseases that still lack vaccine coverage due to failure of vaccine candidates to induce adequate and lasting immune responses (Grimm and Ackerman 2013). Therefore, cell lines closer to the natural host cell type should be explored to more accurately reproduce the overall protein structure, modifications, and exposed antigenic sites for the primed immune system to be able to neutralize the naturally encountered antigen. Recently, efforts are being made to identify consensus glycosylation patterns, as well as production platforms leading to elicitation of desired immune responses and pathogen neutralization (Li et al. 2016; Go et al. 2017). In contrast to fast mutating RNA viruses, DNA viruses, including herpesviruses, have relatively stable genomes and rely on other means for counteracting the host's immune system (Sanjuan et al. 2010). It is therefore conceivable, that vaccine development should be less challenging for DNA viruses. However, most of herpesvirus-targeted subunit vaccines have failed so far. A recently developed HSV-2 vaccine lacking the main neutralizing antibody target is, however, showing great promise in mice. This vaccine evokes production of non-neutralizing antibodies to other envelope glycoproteins on infected cells, stimulating cellular NK cell immunity through engagement of Fcγ receptors (Petro et al. 2015). In conclusion, glycans on viral envelope glycoproteins have a tremendous impact on recognition by the host, and glycosylation heterogeneity makes it very difficult to identify universal vaccine candidates. While experimental evidence is key, development of accurate bioinformatic tools to predict glycosylation and likely mutation patterns would be of big value for vaccine research. This should become possible once a substantial number of viral strains are sequenced and analyzed for glycan modifications in native contexts.

Roles of glycosylation on viral protein complex formation

While N-linked glycans situated on structural domains often shield functionally important protein regions from antibody neutralization, stem region *N*-glycans can affect glycoprotein stability and protein–protein interactions. For example, the mutations in the N-glycosite localized to the stem region of the Hendra virus glycoprotein G affected the conformation of the molecule, leading to increased oligomerization and interaction with its viral binding partner (Bradel-Tretheway et al. 2015). Moreover, the stalk mutation had similar effects in a closely related Nipah virus (Bradel-Tretheway et al. 2015). In Hepatitis C Virus, disruption of specific N-glycosites in the two highly N-glycosylated envelope glycoproteins E1 and E2 results in formation of unproductive E1–E2 heterodimers (Meunier

et al. 1999; Goffard et al. 2005; Orlova et al. 2015), whereas specific O-glycosites on Hendra virus protein G have been shown to affect association with protein F (Stone et al. 2016), suggesting both types of glycans can carry out similar functions. Similarly, O-glycosites have been found in regions of varicella zoster virus gE, important for interaction with partner gI (Bagdonaite et al. 2016), but the glycan specific functions are yet to be uncovered.

Induction of host glycosylation machinery

In addition to hijacking host glycosylation machinery for modification of viral proteins, certain viruses induce changes in the expression levels of host glycosyltransferases. For example, it is documented that herpesviruses induce expression of host fucosyltransferases leading to expression of sLe^x or Le^y antigens (Nystrom et al. 2007; Norden et al. 2013, 2017). In addition, HSV-1 and HSV-2 infection lead to changes in gene expression related to glycosphingolipid synthesis (Miyaji et al. 2016). Also, in the case of HCV, it has been demonstrated that the total glycoprofile of HCV-infected hepatoma cells was shifted towards more fucosylated, sialylated, and in general complex *N*-glycans (Xiang et al. 2017). Another example includes HBV that has been shown to upregulate mannosidases, which supports increased glycan processing (Hu et al. 2016). In the case of influenza A virus, induction of the isoform, GalNAc-T3, has been detected in infected respiratory epithelial cells, possibly increasing O-glycosylation of airway mucins (Nakamura et al. 2015). The changes in expression levels of host carbohydrate active enzymes can either hint towards requirements for specific structures for functionality, or highlight the demand for increased glycosylation capacity to support viral glycoprotein glycosylation.

Perspectives

It has long been known that glycosylation of viral envelope proteins is essential for infectivity and affects immune recognition. Surprisingly, few clinical applications have been developed using this knowledge, and glycosylation has rarely been considered in vaccine design. However, awareness is increasing and more and more studies are addressing the need of relevant glycosylation in vaccine formulations (Li et al. 2016; Go et al. 2017). The rapidly evolving analytical methods now allow precise characterization of the location and structure of both N- and O-linked glycosylation on viruses. Likewise, the advancement of glycobiology and gene editing techniques offers ample opportunities to investigate the functional roles of glycan structures and specific sites. Unreliable prediction of O-glycosylation have discouraged site-specific analysis of O-glycosylation in viruses, but now the structural information obtained by high throughput glycoproteomic approaches as well as bioinformatic analysis of glycosylation patterns can serve as a resource for defining targets for such studies. In addition, the knowledge base can be used for addressing the role of O-glycosylation for antigenicity and immunogenicity of subunit vaccine candidates. Currently it is difficult to obtain site occupancy information at a proteome-wide scale, but soon this will be possible with optimized targeted glycoproteomics strategies. Accumulating evidence suggest that O-glysosylation of viruses play important roles in various aspects of virus biology. The advent of readily available and robust genome engineering tools provides opportunities to investigate these findings in more detail and map the structural requirements for O-glycan structures on the virus and potential interaction partners on epithelial cells by using glycoengineered cell libraries (Radhakrishnan et al. 2014; Yang et al. 2015). The challenge is, however, to apply such knowledge for clinical purposes. Since viral glycosylation is orchestrated by the capacity of the host cell, general glycosylation inhibitors are of limited use due to toxicity. It is therefore important to identify structural determinants of combinatorial self- and nonself-nature for safe targeting of viral glycans and boosting antiviral immunity.

Conclusions

Enveloped viruses have long been known to hijack the host cell's glycosylation machinery for glycosylation of viral proteins. The development and advancement of mass spectrometry techniques have paid a tremendous contribution to the characterization of precise location and structure of glycans decorating the viral envelope proteins, and revealed substantial heterogeneity of glycan structures and site occupancy. Moreover, glycan density, as well as *N*- to *O*-glycan ratio, varies greatly among different classes of viruses, with some being highly glycosylated, and others having only a few glycan chains. Accumulating site-directed mutagenesis data suggests that individual glycosites play diverse important roles in virus biology. Moreover, glycans heavily contribute to the overall structure of the glycoprotein, epitope accessibility, and, in some cases, directly affect immune recognition. While there are some examples where carbohydrate moieties directly participate in interactions, in many cases disrupted biological functions are results of impaired protein structure, localization or function. Failure to develop universal vaccines for multiple viruses such as HIV-1, HCV and HSV suggest that comprehensive knowledge of structure and immunogenicity of viral envelope proteins in relevant biological systems is needed to achieve desired immune responses. While the location and function of N-linked glycans have been very well characterized for many viruses, discovery of O-glycosites has been neglected due to the nature of *O*-glycan synthesis and analytical difficulties. We have developed techniques to globally address site-specific O-glycosylation of enveloped viruses and revealed unprecedented magnitude of O-glycosylation in herpesviruses. We argue that the contribution of *O*-glycans needs to be addressed for vaccine development in order to have a complete picture of post-translational modifications affecting the protein function and immunogenicity. It is hoped that the data summarized in this review will ignite more interest in viral O-glycosylation, and give rise to focused studies leading to a better understanding of virus biology and improved means to prevent and combat the associated infections.

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Conflict of interest statement

None declared.

Abbreviations

GlcNAc, N-acetylglucosamine; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; PNA, peanut agglutinin; VVA, Vicia villosa lectin (VVA).

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