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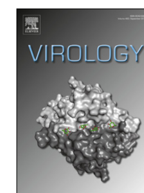

Contribution of N-linked glycans on HSV-2 gB to cell–cell fusion and viral entry

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ABSTRACT

HSV-2 is the major cause of genital herpes and its infection increases the risk of HIV-1 acquisition and transmission. HSV-2 glycoprotein B together with glycoproteins D, H and L are indispensable for viral entry, of which gB, as a class III fusogen, plays an essential role. HSV-2 gB has seven potential N-linked glycosylation (N-CHO) sites, but their significance has yet to be determined. For the first time, we systematically analyzed the contributions of N-linked glycans on gB to cell–cell fusion and viral entry. Our results demonstrated that, of the seven potential N-CHO sites on gB, mutation at N390, N483 or N668 decreased cell–cell fusion and viral entry, while mutation at N133 mainly affected protein expression and the production of infectious virus particles by blocking the transport of gB from the endoplasmic reticulum to Golgi. Our findings highlight the significance of N-linked glycans on HSV-2 gB expression and function.

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Introduction

Herpes simplex virus type 2 (HSV-2), a member of the family Herpesviridae, is a large enveloped double-stranded DNA virus. HSV-2 mainly infects epithelial cells and causes genital herpes. It also infects immuocytes and neuronal cells, leading to encephalitis and disseminated diseases that affect other organ systems. As a human pathogen with extreme prevalence, HSV-2 can establish a life-long latent infection in sacral ganglia, resulting in about 16% human adults living with a HSV-2 latency worldwide (Looker et al., 2008). Given that the prevalence of HIV-1 and HSV-2 co-infection and that HSV-2 infection can facilitate HIV-1 acquisition and accelerate the progress of AIDS, HSV-2 remains a threat to human health worldwide (Huang et al., 2012a; Martinelli et al., 2011; Tan et al., 2013; Zhu et al., 2009).

HSV-1/2 is proposed to enter host cells by fusion with plasma or endosomal membranes depending on the cell types infected (Heldwein and Krummenacher, 2008). During either entry pathway, membrane fusion is mediated by four viral glycoproteins named gB, gD, gH, gL (Muggeridge, 2000; Turner et al., 1998) and a gD-specific cellular receptor (HVEM, Nectin-1/Nectin-2 or 3-O sulfated heparin sulfate) (Campadelli-Fiume et al., 2000). The current fusion model for HSV-1/2 is generally accepted as follows. Firstly, binding of gD to one of its receptors on cell surface induces conformational changes in gD (Krummenacher et al., 2005). Subsequently, the conformation altered gD sequentially recruits and binds to gB and gH/gL heterodimer, resulting in the activation of gH/gL (Atanasiu et al., 2007; Lazear et al., 2008). Meanwhile, gB brings viral envelope and host cell membrane together by direct insertion into cell membrane through its internal fusion loops (Hannah et al., 2009), which further facilitates the interaction between gB and gH/gL heterodimer (Atanasiu et al., 2010b). Activated gH/gL heterodimer upregulates the fusogenic activity of gB, resulting in the execution of fusion between viral envelope and cell membrane (Atanasiu et al., 2010a).

As a class III fusion glycoprotein with seven potential N-linked glycosylation sites, HSV-1 and 2 gB cannot only facilitate viral infection by promoting fusion, but also mediate virus attachment through interactions with HSPG or DC-SIGN (de Jong et al., 2008; Herold et al., 1994; Laquerre et al., 1998). For instance, gB has been reported to play a predominant role in mediating the attachment of HSV-2 to target cells (Cheshenko and Herold, 2002). Due to its importance in HSV-1/2 infection, gB has been extensively

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investigated in relation to its roles in viral entry, with particular focus on the functional regions (Bender et al., 2007; Chowdary and Heldwein, 2010; Li et al., 2006) and amino acids involved (Arii et al., 2010b; Connolly and Longnecker, 2012; Fan et al., 2002; Uchida et al., 2010; Wang et al., 2009). Although HSV-2 gB-specific receptor has not been identified, the involvement of several HSV-1 gB-specific cellular receptors (PILR-α, MAG and NMHC-IIA) in viral infection (Arii et al., 2009, 2010a; Satoh et al., 2008; Suenaga et al., 2010) suggests the existence of HSV-2 putative receptors.

It has been widely reported that removal of certain glycans from a wide range of viral glycoproteins has severe impacts on the viruses by altering protein expression or/and biological activities. For instance, N-linked glycans on the glycoproteins of HIV-1, influenza virus, West Nile virus, Ebola virus, respiratory syncytial virus and Nipah virus, have been shown to be important for the fusogenicity and infectivity of the viruses (Aguilar et al., 2006; Fenouillet et al., 1993; Terence et al., 2006; Vigerust and Shepherd, 2007). N-linked glycans on the glycoproteins of West Nile virus and Dengue virus have been reported to be required for viral replication (Fan et al., 2014; Shirato et al., 2004). In addition, envelope associated N-linked glycans also play roles in viral virulence, antigenicity, immune evasion (Biering et al., 2012; Binley et al., 2010; Kobayashi and Suzuki, 2012; Vu et al., 2011; Yuste et al., 2008) and sensitivity to carbohydrate-binding agents (Balzarini, 2007; Huang et al., 2011). In addition, gB of HSV-1 has been shown to be barely detectable on the plasma membrane of infected cells in the presence of a glycosylation inhibitor (Glorioso et al., 1983). However, more detailed studies on the significance of glycosylation in both HSV-1 and HSV-2 gBs are currently lacking.

In the current study, we systematically investigated the contribution of N-linked glycans to gB expression, cell–cell fusion and viral entry.

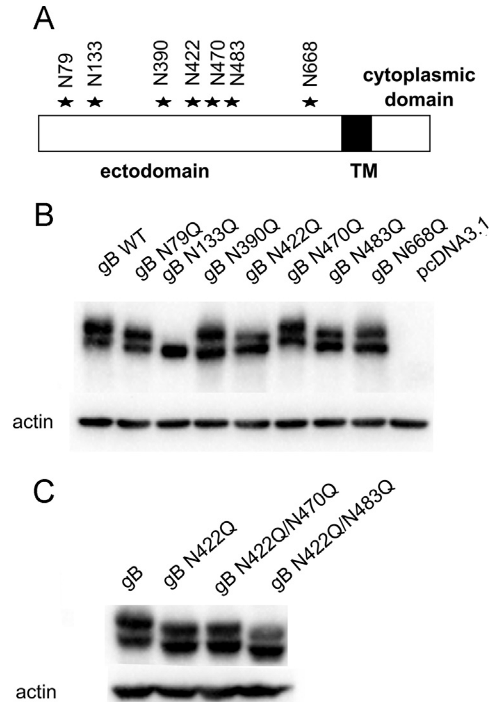


Fig. 1. Expression and glycosylation analysis of HSV-2 gB glycan mutants. (A) Schematic of N-linked glycosylation sites on gB. HSV-2 gB is composed of an ectodomain, a transmembrane domain and a cytoplasmic domain, of which the ectodomain contains seven potential N-linked glycosylation sites. Asterisks represent these potential N-linked glycosylation sites on gB. (B and C) Expression of gB mutants. CHO cells were transfected with plasmids encoding WT gB or mutants. 48 h post-transfection, total expression of gB was analyzed by Western blot using anti-HSV-2 PAB13979 with actin as a loading control. One representative experiment out of three is shown.

Results

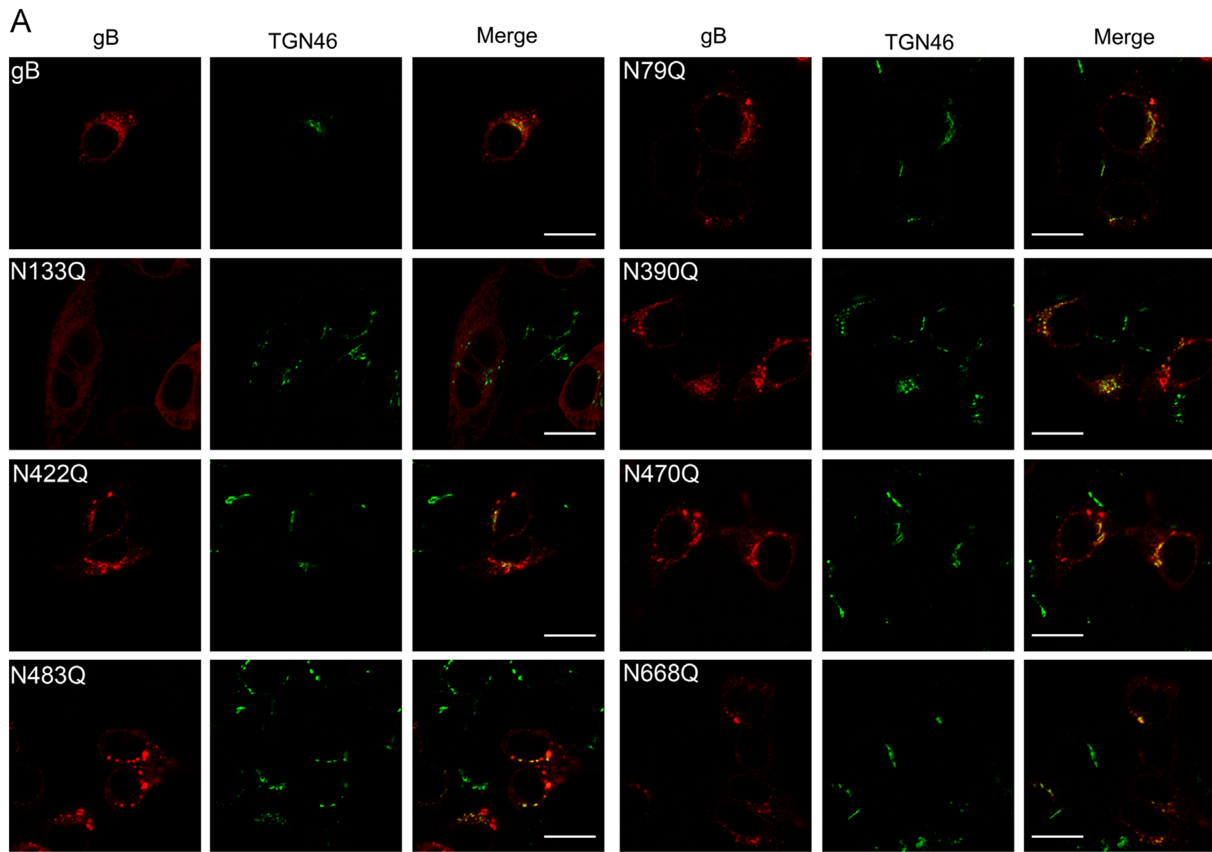
Influence of N-CHO site mutation on gB expression and electrophoretic mobility

HSV-2 gB has seven putative N-linked glycosylation sites (Asn-X-Ser/Thr, of which X represents an amino acid except proline) at positions N79, N133, N390, N422, N470, N483 and N668 (Fig. 1A). We constructed Asn-linked carbohydrate (N-CHO site) (Sodora et al., 1991a) mutants by introducing a conservative first-site amino acid substitution from Asn to Gln, termed N79Q, N133Q, N390Q, N422Q, N470Q, N483Q and N668Q, respectively. Wild type (WT) and mutated gB constructs were transfected into CHO cells for expression. Following gel analyses (Fig. 1B), N-CHO site mutation had little effect on gB expression, while most of the gB mutants except N470Q migrated slightly faster than WT gB, indicating that these mutated gBs were smaller in molecular weight than WT gB. These results together implied that six out of the seven putative N-linked glycosylation sites, including N79, N133, N390, N422, N483 and N668, were likely indeed glycosylated in gB, whereas N470 was an exception since N470Q mutation did not change the molecular weight of gB. It is known that HSV-1 gB has only six potential N-linked glycosylation sites at the ectodomain. The corresponding site of 470NAT472 in HSV-1 gB is a non-glycosylation site (NPT) because proline would interrupt the formation of a turn or a loop conformation or destabilize the conformation which allows sugars to be added to the asparagine residue within the N-X-T/S sequence (Bause, 1983). To further analyze the glycosylation state of N470 in HSV-2 gB, two dual site mutants, termed N422Q/N470Q and N422Q/N483Q, were constructed. As seen in Fig. 1C, N422Q/N483Q had a slightly greater mobility than N422Q/N470Q while the later had the same mobility as N422Q, further suggesting that the N470 in HSV-2 gB is highly unlikely glycosylated.

HSV-1 gB is initially expressed as a 110-kDa high-mannose precursor followed by rapid maturation to a 120-kDa form (Sommer and Courtney, 1991). In agreement, we observed two forms of gB in transfected cells for WT gB and most of the mutants (Fig. 1B). In contrast, only the premature form of gB was detected in N133Q-transfected cells (Fig. 1B), suggesting that N133Q were not processed to the mature form. It has been reported that expression of viral membrane glycoproteins including HSV-2 gB seems to follow a classic exocytotic pathway (Doms et al., 1993; Maggioni and Braakman, 2005; Papworth et al., 1997). Newly synthesized proteins are glycosylated in the endoplasmic reticulum (ER) with a high mannose core attached to the asparagine of the conserved motif N-X-S/T and then processed to the mature form by trimming and remodeling the oligosaccharide during transit through ER and Golgi (Rosnoblet et al., 2013). N133Q likely failed to be transported to the Golgi apparatus.

Influence of N-CHO site mutation on the transport of gB to the Golgi apparatus and the cell surface

It is known that glycoproteins are matured in the Golgi and sorted to their final destinations in the trans-Golgi network (TGN) (Roth, 2002). Since N133Q was unlikely to be transported to the Golgi apparatus, we asked whether N-CHO site mutation affected the intracellular trafficking of gB. To test this, co-localization of WT or mutated gB with TGN46, a marker of TGN, was analyzed. When observed by confocal microscopy, gB was present diffusely in the cytoplasm and only a fraction of gB co-localized with TGN46 in gB high-expressing cells, making the gB-TGN46 co-localization less representative than that in gB low-expressing cells. In addition, we



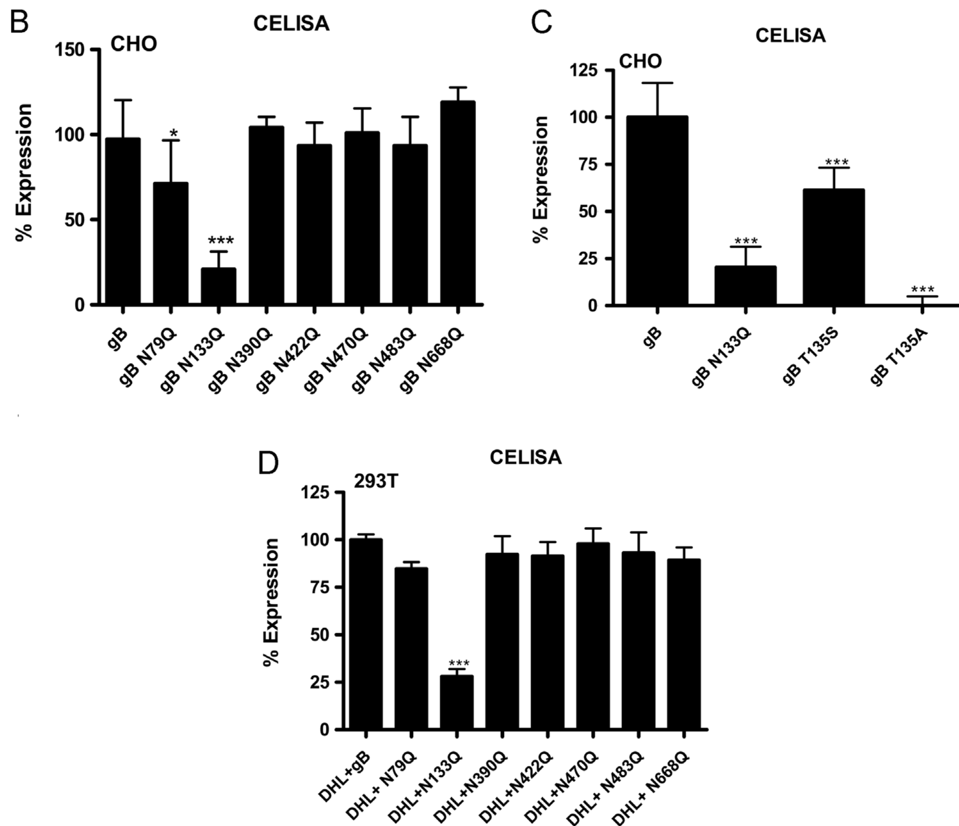


Fig. 2. Subcellular localization and cell surface expression of gB glycan mutants. (A) HeLa cells were transfected with plasmids encoding WT or mutated gB for 24 h. After fixation, cells were co-stained with anti-gB (red) antibody and antibody against TGN46 (green) specifically recognizing TGN. Co-localization of gB and the TGN was analyzed by confocal microscopy. Representative confocal images from three independent experiments are shown. Scale bars in all panels represent 20 μm. (B, C and D) Cell surface expression of gB mutants. CHO cells were transfected with plasmids encoding WT or mutated gB (B and C) or 293T cells were transfected with plasmids expressing gD, gH, gL and WT or mutated gB (D). 24 h post-transfection, cells were fixed with 4% paraformaldehyde and incubated with anti-HSV-2 PAB13979 (CHO) or anti-gB sera (293T) to detect the cell surface expression of gB. Cells transfected with empty vector were used as the negative control. The expression of gB mutants was normalized to that of WT gB. Data shown are mean ± SD of three independent experiments with each condition performed in triplicate. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

observed that over expression of gB would lead to vacuolation of cytoplasm and apoptosis of the transfected cells. Therefore, we analyzed the co-localization of gB with TGN46 in gB low-expressing cells to better understand the impact of N-CHO site mutations on gB intracellular trafficking. As shown in Fig. 2A, WT gB and most of the mutants co-localized with TGN46 in transfected cells, whereas N133Q had no apparent co-localization with TGN46. These results indicated that N-CHO site mutation at N133 likely impaired the trafficking of gB from the ER to the Golgi, consequently preventing the maturation of gB.

Since only fully matured viral membrane glycoproteins are transported to and displayed on the cell surface, N-CHO site mutation might affected the transport of gB to the cell surface. Therefore, CELISA was subsequently carried out to assess whether N-CHO site mutation affected the expression of gB on cell surface. As shown in Fig. 2B, mutation at N390, N422, N470, N483 or N668 had little impact on the cell surface expression of gB, whereas N79Q or N133Q mutation reduced the expression of gB to ~72% or ~21% of WT, respectively. To confirm that the sharp decrease of cell surface expression of N133Q was glycan specific rather than due to amino acid change, we constructed mutants T135A and T135S by third-site substitution from Thr to Ala or Ser. As shown in Fig. 2C, cell surface expression of glycan mutant T135A was below the detection limit, while cell surface expression of glycan-retained mutant T135S was reduced to about 65% of WT gB. Taken together, these results indicate that the decreased expression of N133Q was probably due to the N-CHO site mutation at N133, although we cannot completely rule out the possibility that amino acid substitution might also affect its cell surface expression to some extent. Similar effects of N-CHO site mutation on cell surface expression of gB were also observed on 293T cells transiently co-expressing gD, gH and gL (Fig. 2D).

Impact of gB N-CHO site mutation on cell–cell fusion

gB together with gD, gH and gL are indispensable for HSV-2 infection and spread. We assessed the impact of gB N-CHO site mutation on cell–cell fusion using a quantitative fusion assay. As shown in Fig. 3A, five glycan mutants including N79Q, N133Q, N390Q, N483Q and N668Q exhibited decreased fusion activities. The fusion activities of N390Q, N483Q and N668Q decreased to ~40–50% of WT gB regardless of target cells expressing HVEM or Nectin-1. Fusion mediated by N79Q or N133Q also decreased, and in particular, N133Q almost completely lost its fusion activity, which was highly likely due to the extremely low level of expression on cell surface resulted from the blockage of its intracellular transport. In contrast, N422Q and N470Q had little effect on cell–cell fusion. These data together indicate that N-linked glycans at position N390, N483 and N668 may contribute to cell–cell fusion. To confirm the notion that the impairments caused by N390Q, N483Q and N668Q were mainly due to N-CHO site mutation, we constructed mutants by introducing a third-site substitution from Thr/Ser to Ala or Ser/Thr. The impact of these mutations on the fusion activity of gB was examined. As shown in Fig. 3B, mutants T392A and S485A, with similar expression level of WT gB on cell surface (Fig. S1), had the same effects on cell–cell fusion as N390Q and N483Q, decreasing the fusion activities to 40–50% of WT gB. T670A showed a slightly decreased level of cell surface expression, and a similar fusion activity compared with N668Q. Compared with the corresponding glycan mutants, the expression levels and fusion activities of glycan-retained mutants T392S, S485T and T670S were similar to those of WT gB, except that T392S showed a slightly decreased cell–cell fusion activity. In general, our results indicated that N-linked glycans at positions N390, N483 and N668 are necessary for the optimal fusion activity of gB.

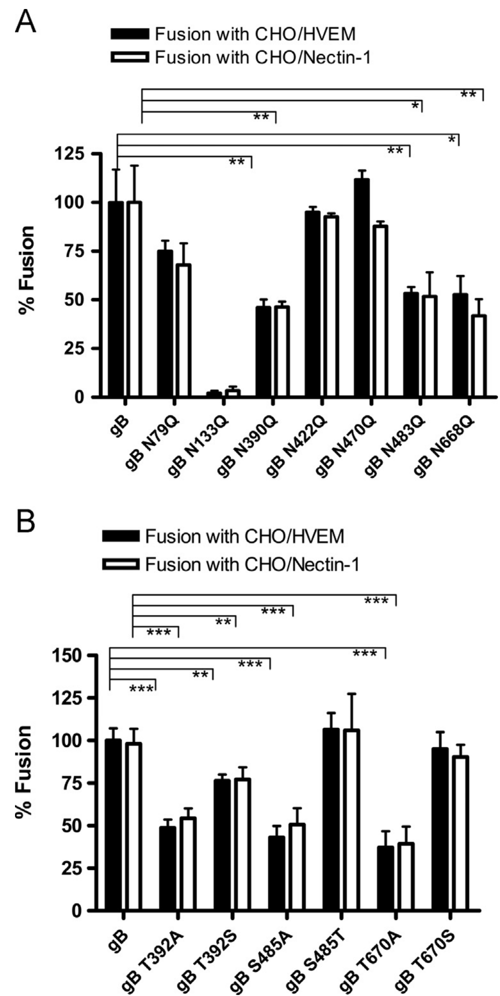


Fig. 3. Fusion mediated by gB glycan mutants. (A and B) Effector cells (QT6) were infected with vTF1.1 expressing T7 RNA polymerase followed by transfection with plasmids encoding gD, gH, gL and WT gB or mutants. Target cells (CHO) were transfected with the plasmid encoding luciferase under the control of a T7 promoter and the plasmid encoding HVEM or Nectin-1. 24 h post-tranfection, effector and target cells were mixed and co-cultured at 37 °C for 9 h before luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in triplicate. $^{*}p \le 0.05$, $^{**}p \le 0.01$.

Impact of gB N-CHO site mutation on viral entry

Having demonstrated that mutation of certain potential glycosylation sites on gB reduced cell–cell fusion, we further investigated whether such impact could be reproduced in HSV-2 entry. HSV-2 and HSV-1 share 82% identity at the amino acid level, and gB is the most conserved protein in herpesvirus family functioning in a similar way in both HSV-2 and HSV-1 entry (Akhtar and Shukla, 2009; Shukla and Spear, 2001). Due to the unavailability of HSV-2 gB null virus, HSV-1 gB null virus was used instead in the subsequent experiments, and such alternative strategy has been generally accepted (Li et al., 2006). To generate complemented viruses, BHK-21 cells transiently expressing WT or mutated gB were subsequently infected with HSV-1 gB null virus. The infectivity of complemented progeny viruses was examined in Vero cells. As shown in Fig. 4A, N390Q, N483Q and N668Q decreased viral entry to ~59%, ~79% and ~44% of WT gB, respectively. N79Q also demonstrated reduced activity in the viral entry assay, while N133Q complemented viruses almost lost entry activity because of the lack of matured gB in transfected cells. In accordance with the results from fusion assay, N422Q and N470Q did not affect HSV-2 entry. Similar results were obtained in viral complement assay performed in HeLa cells (Fig. 4B). In agreement, gB mutants with decreased level of

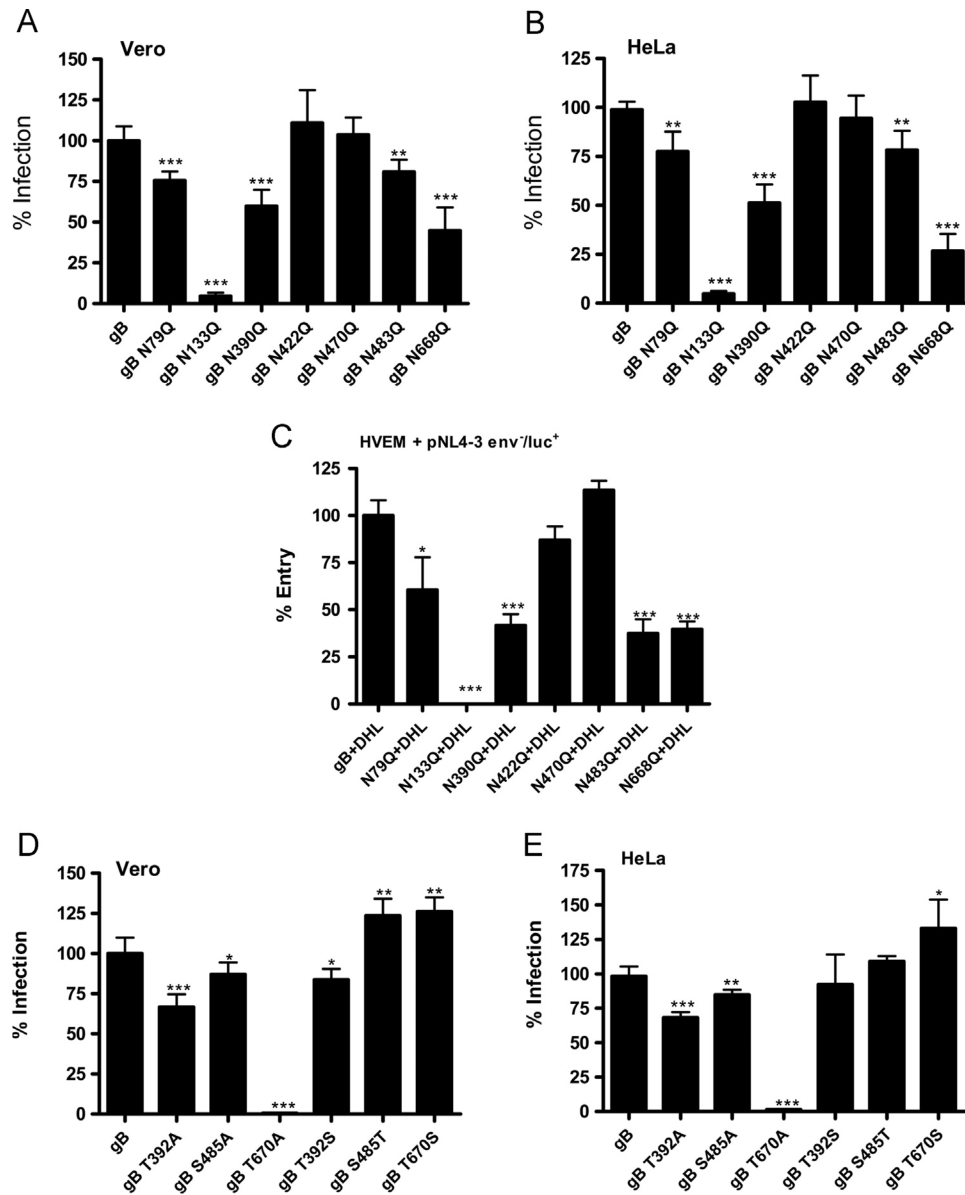


Fig. 4. Entry mediated by gB glycan mutants. BHK-21 cells were transfected with plasmids encoding WT gB or mutants, followed by infection with HSV-1 ΔgB Luc/GFP virus to produce phenotypically complemented viruses. Vero (A and D) and HeLa (B and E) cells pre-seeded in 96-well plates were infected with 50 μl/well of complemented viruses at 37 °C for 24 h, lysed and luciferase activity was measured. (C) HIV-1 pseudovirus bearing HVEM were obtained by co-transfecting 293T cells with pNL4-3 env-luc+ and plasmid encoding HVEM. HIV-1 particles were harvested 48 h post-transfection and incubated with 293T cells transiently co-expressing gD, gH, gL and WT gB or mutants at 37 °C for 24 h. Cells were lysed and luciferase activity was measured. Infection or entry mediated by gB mutants was normalized to that of WT gB. Data shown are mean ± SD of three independent experiments with each condition performed in triplicate. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

cell–cell fusion also had impaired capability in viral entry. Of note, the impact of N483Q on viral entry was less severe than that on cell–cell fusion. Unlike fusion assay using only four glycoproteins gD, gH, gL and gB, the complemented virus infection system involves more viral components, some of which may assist viral entry and compensate the impairments caused by gB N-CHO site mutation to some extent. To exclude such interference, we further adopted a HIV-1 Env-pseudotyped virus system to assess the impact of N-CHO site mutation at N390, N483 or N668 on the fusion between viral envelope and cell membrane, using pNL4-3 env-/luc+ virus bearing HVEM to infect 293T cells transiently co-expressing gD, gH, gL and WT or mutated gB. As shown in Fig. 4C, consistent with the findings in fusion assay, the three mutants all impaired the capability of gB in mediating viral entry.

We subsequently used the glycan mutants T392A, S485A and T670A and the glycan-retained mutants T392S, S485T and T670S to analyze the contribution of N-linked glycans at N390, N483 and N668 to HSV-2 entry. As shown in Fig. 4D and E, the complemented virus with T392S, S485T or T670S kept comparable infectivity with that of WT gB-complemented virus. T392A and S485A had the same effects on viral entry as N390Q and N483Q, respectively. Of interest, unlike N668Q, T670A-complemented virus almost lost the infectivity. It has been reported that a mutation of gB, which was five residues away from T670, abolished the fusion activity and viral infectivity of HSV-2 because of a defect in oligomerization (Li et al., 2006). We therefore conducted immunofluorescence assay to analyze whether T670A could be recognized by DL16, a oligomer-specific monoclonal antibody (Bender et al., 2005). As shown in Fig. 5, compared with WT gB, T670A was stained normally with gB anti-sera but poorly with DL16, implying that T670A mutation highly likely affected the oligomerization of gB. In addition, N390Q, N483Q, N668Q and T392S were stained normally with both gB anti-sera and DL16, indicating that these mutations

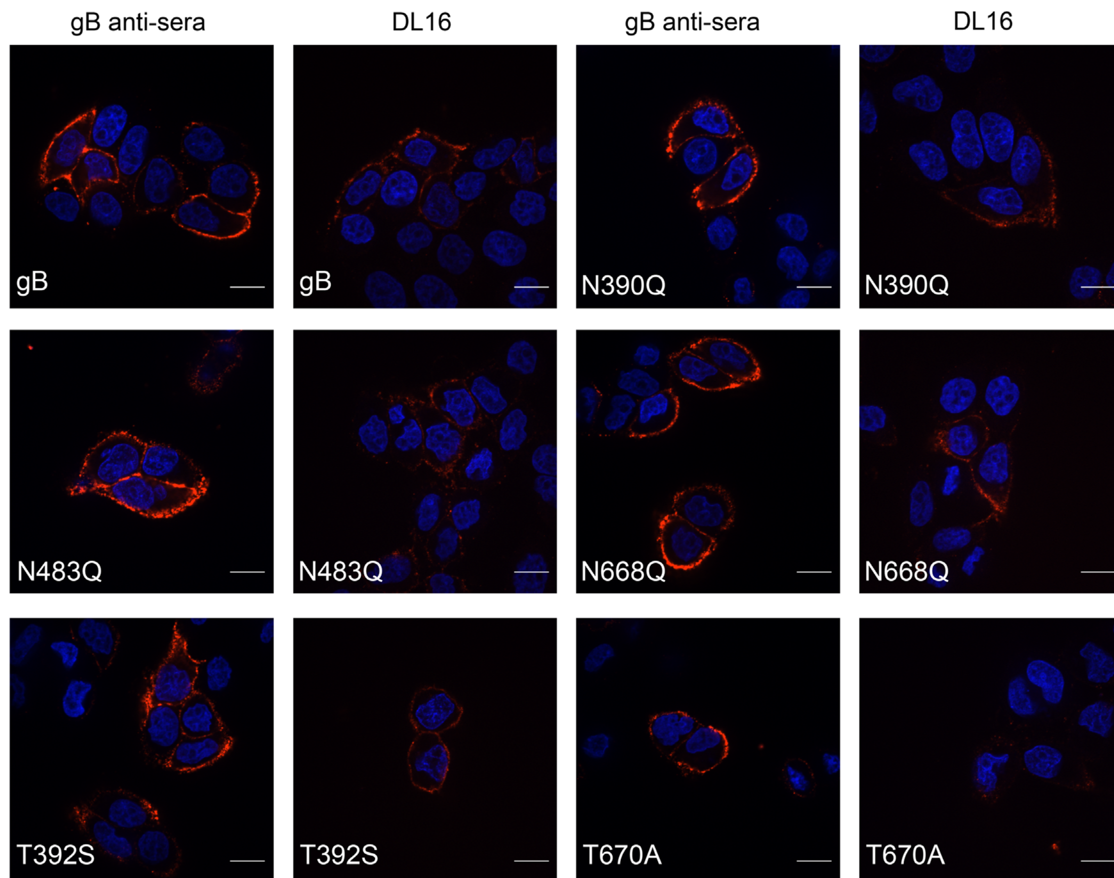


Fig. 5. Immunofluorescence analysis of gB glycan mutants. HeLa cells were transfected with plasmids encoding WT or mutated gB for 24 h. After blocking with 3% BSA in PBS for 30 min at room temperature, the transfected cells were incubated with gB anti-sera or DL16 at 4 °C for 1 h, followed by a fixation with 4% paraformaldehyde. Cells were subsequently incubated with Cy3-conjugated goat anti-mouse IgG. Fluorescence was examined under confocal microscopy. Representative confocal images from three independent experiments are shown. Scale bars in all panels represent 21 μm.

unlikely affected gB oligomerization. These results informed that N-CHO site mutation at N390, N483 or N668 decreased viral entry, reinforcing our conclusion that N-linked glycans at N390, N483 and N668 contribute to the optimal function of gB.

Discussion

The entry of HSV-2 into target cells is a complex process, which remains to be further elucidated. HSV-2 gB is a glycosylated protein critical for virus–cell fusion, but little is known regarding the significance of N-linked glycans in the function of gB in herpesvirus family. In this study, we systematically investigated the importance of N-linked glycans on gB. Our results have demonstrated that, of the seven potential N-linked glycosylation sites on gB, N-CHO site mutation at N133 affect the intracellular trafficking and protein maturation of gB, while N-linked glycans at N390, N483 and N668 are necessary for optimal fusion activity of gB and viral entry.

Previous studies have shown that HSV-1 gB has two forms of monomer in infected cells due to posttranslational processing (Sommer and Courtney, 1991). We observed two forms for WT gB and most of the glycan mutants but only one for glycan mutant at N133 regardless of the cell types used for gB expression, implying a defect in the maturation of gB N133Q mutant. By analyzing the co-localization between gB and a TGN marker, we observed that gB N133Q mutant did not co-localize with TGN, suggesting that lack of glycosylation at N133 might affect the intracellular trafficking of gB by blocking the exit of gB from the ER. Moreover, in accordance with a previous report that incomplete glycosylation led to the reduction of gB on the cell membrane (Glorioso et al., 1983), our results revealed that N-CHO site mutation at N79 or N133 decreased the expression of gB on cell surface. In particular, the expression of gB with N-CHO site mutation at N133 decreased to ~20% of WT, resulting in extremely low activities of fusion and viral entry mediated by gB. It has been reported that single glycan deletion could cause impairment on protein expression and viral infectivity. For example, the glycan deletion of influenza virus HA at N81 reduced the protein expression because of misfolding (Hebert et al., 1997), while deglycosylation at N284 of SIV gp120 resulted in a lethal defect in protein processing, consequently rendering the virus non-infectious (Pikora et al., 2005). In our study, while most of the gB glycan mutants displayed similar cell surface expression levels to that of WT, N-CHO site mutation at N133 severely affected the maturation of gB and its expression on cell surface. It is believed that newly synthesized proteins including HSV-1/2 gB are processed to the mature form in Golgi (Doms et al., 1993) and fully matured gB is subsequently transported to cell surface or accumulated in TGN or TGN-derived vesicles (Beitia Ortiz de Zarate et al., 2004, 2007; Calistri et al., 2007) where HSV-1 acquired the second envelope (Sugimoto et al., 2008; Turcotte et al., 2005; Wisner and Johnson, 2004). Therefore, N-CHO site mutation at N133 dramatically affected the production of infectious HSV-2 particles likely by preventing gB from entering Golgi for protein maturation.

Considering that glycans at N390, N483 and N668 contribute to gB-mediated membrane fusion, we modeled the locations of N-linked glycans based on the crystal structure of HSV-1 gB (Heldwein et al., 2006; Stampfer et al., 2010) because the crystal

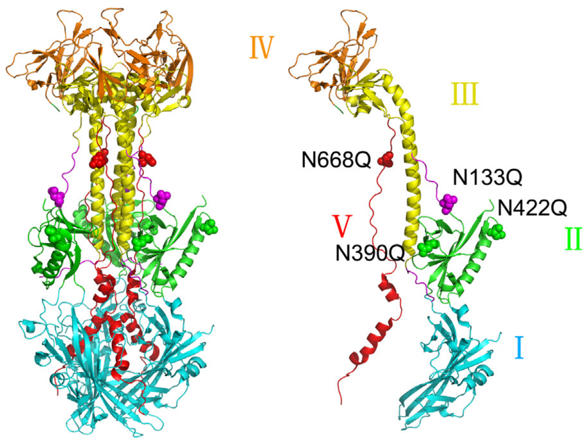


Fig. 6. The location of potential N-linked glycosylation sites on gB. The three dimensional structure of HSV-1 gB (PDB ID: 2GUM) was generated by using PyMOL and labeled with different colors to distinguish the functional domains. Positions of N133, N390, N422 and N668 are shown in the context of the crystal structure of gB. N390 and N422 (green) lie in domain II in which the binding site of the gH/gL heterodimer is located, while N668 (red) lies in the C-terminal arm of domain V which packs against the coils of domain III (yellow) to form a coil-arm trimer. N133 (purple) is located at the linker segment between domain II and domain III.

structure of HSV-2 gB has not been solved (Fig. 6). According to the crystal structure of HSV-1 gB, the glycosylation motif 390NLT392 is located in the domain II. It was reported that insertion of two additional amino acids in the position between residues 395 and 396 of HSV-2 gB abolished cell–cell fusion and viral entry (Li et al., 2006), suggesting the importance of this region in domain II for gB function. The domain II of HSV-1 gB contains a functional region important for the interaction between gB and gH as well as for cell–cell fusion (Atanasiu et al., 2010b; Bender et al., 2007). However, conformation independent neutralizing antibody targeting residues 391 to 410 of HSV-1 gB (corresponding to residues 382 to 402 of HSV-2 gB) only moderately decreased the fusion activity of gB (Atanasiu et al., 2010b), implying that these residues may not directly interact with gH but are possibly to be involved in the formation of a conformation for gB–gH interaction. In agreement, we found that N-CHO site mutation at N390 moderately impaired cell–cell fusion. Blocking glycosylation at N390 possibly impaired the function of gB in fusion by weakening the interaction between gB and gH/gL. It is known that glycosylation plays important roles in maintaining the structure of viral glycoproteins. For instance, N-glycans on HSV-1 gD have been reported to be important for maintaining the proper structure of gD protein (Sodora et al., 1991a; Sodora et al., 1991b). In addition, removal of N-glycans on the variable region 1 of HIV-1 impacted gp120-gp41 interaction by allosterically modulating the distal gp120-gp41 association site (Drummer et al., 2013). In addition, removal of N-glycans proximal to the CD4 binding region of HIV-1 gp120 affected its steric configuration and consequently impaired viral infectivity (Huang et al., 2012b). Moreover, oligosaccharides adjacent to the receptor binding site of influenza virus HA has also been shown to affect the affinity of HA to neuraminic acid-containing receptors (Ohuchi et al., 1997a). The motif 668NIT670, belonging to domain V, is located in the C-terminal arm of gB trimeric coil-arm complex, which is arranged in an anti-parallel manner and resembles the six-helix bundles of class I fusogen, thought to provide a driving force for its refolding from a pre-fusion to a post-fusion conformation (Melikyan et al., 2000; Russell et al., 2001). In this regard, three arm amino acids of HSV-1 gB, including I671, H681 and F683, have been reported to be critical for maintaining the coil-arm trimer in an anti-parallel manner (Connolly and Longnecker, 2012). Oligosaccharides in the stem region of influenza virus HA were reported to maintain HA in a metastable form required for fusion activity (Ohuchi et al., 1997b). Similarly, N-CHO site mutation at N668 of HSV-2 gB might destabilize the post-fusion conformation of the coil-arm trimer or weaken the interaction between residues in the coil and the arm, consequently reducing the activity of fusion. Unlike N390 and N668, the motif 483NAS485 is located in a linker between domain II and domain III, not included in the crystal structure of HSV-1 gB. Insertion of five additional amino acids in this linker region of HSV-1 gB did not affect its cell surface expression but resulted in decreased activity in cell–cell fusion and viral entry (Lin and Spear, 2007), implying the importance of this region on the function of gB.

In our study, data from both cell–cell fusion system and HIV-1 Env-pseudotyped virus system indicate that glycans at N390, N483 and N668 are involved in the process of cell/virus–cell fusion. In agreement, N-CHO site mutation at N390, N483 or N668 was demonstrated to impair the entry efficiency of the complemented progeny viruses, although the impact of N-CHO site mutation at N483 on viral entry was less apparent than that in cell–cell fusion. It is likely that other HSV-1/2 components, which are dispensable but involved in the process of HSV-1/2 infection, may partially compensate the impairment of virus–cell fusion caused by gB N-CHO site mutation. For instance, gC has been shown to share the same capability as gB in viral absorption (Laquerre et al., 1998). In addition, HSV-1 gK, UL20, gM, gH, gB and PILRα can assemble a complex to regulate virus–cell fusion (Chouljenko et al., 2009, 2010; Chowdhury et al., 2013; Kim et al., 2013). The glycoproteins dispensable for membrane fusion might enhance fusion efficiency by strengthening or stabilizing the association of the fusion core machinery or by interacting with gB directly to modulate its fusion activity. Of note, N-CHO site mutation at N668 modestly decreased vial entry whereas its counterpart T670A completely abolished viral entry. A mutation, only five amino acids away from T670, was reported to abolish cell–cell fusion and viral entry by negatively affecting the oligomerization of gB (Li et al., 2006), indicating that this region might be involved in oligomerization. In our study, the fluorescence intensity of T670A stained with the oligomer-specific monoclonal antibody DL16 was apparently lower than that of WT gB or N668Q, implying that substitution of T670 with an alanine likely affected the oligomerization of gB or destabilized the gB trimer in addition to affecting the glycosylation at 668NIT670. Unexpectedly, mutant T392S demonstrated a negative impact on gB function. One possible explanation is that the substitution of Thr with Ser might decrease the glycosylation efficiency since previous studies by others have reported that the Asn-X-Thr sequon was glycosylated 2–3 fold more than the Asn-X-Ser sequon (Bause and Legler, 1981; Kasturi et al., 1995). Nevertheless, we cannot rule out the possibility that this region is sensitive to amino acid substitutions.

In conclusion, we have demonstrated for the first time that, of the seven potential N-linked glycans on gB, N79 and N133 are critical for protein expression on the cell surface, of which N133 was essential for intracellular trafficking of gB from ER to TGN, while N390, N483 and N668 are involved in cell–cell fusion and viral entry. Taken together, our results highlight the contribution of N-linked glycans to gB expression and function.

Materials and methods

Cells, viruses, plasmids and antibodies

QT6, Vero, VgB (Vero cells stably expressing HSV-1 gB), HeLa, BHK-21, 293T and CHO cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The VgB cell line was a gift from Professors Patricia Spear and Richard Longnecke, and used to propagate the HSV-1 gB null virus. Genes encoding gB, gD, gH and gL were amplified from HSV-2 strain G obtained from LGC Standards. gB sequence was deposited in the GenBank database

(accession no. KP143740). Genes encoding Nectin-1 and HVEM were amplified from the cDNAs of HeLa and human PBMCs, respectively. Amplified genes were cloned into pcDNA3.1(+) (Invitrogen). gB mutants were generated by site-directed mutagenesis and all programmed mutations were confirmed by DNA sequencing. pHSV-1F BAC Luc/GFP (Li et al., 2011) was used as a template to construct pHSV-1F BAC ΔgB Luc/GFP through homologous recombination. Recombinant vaccinia virus vTF1.1 expressing T7 RNA polymerase was previously described (Hu et al., 2000).

Polyclonal antibody against HSV-2 strain G (PAB13979) was from Abnova. Mouse monoclonal antibody against HSV-1/2 gB (ab6506) and Alexa Fluor 488-conjugated donkey anti-sheep IgG were from Abcam. Sheep polyclonal antibody against TGN46 was from AbD Serotec. Mouse monoclonal antibody against actin and HRP-conjugated mouse anti-goat IgG were from Santa Cruz. HRP-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-mouse IgG were from Boster. Alexa Fluor 555-conjugated donkey anti-mouse IgG was from Beyotime. Anti-gB sera were prepared by immunizing mice three times with 50 μg gB expressing plasmid DNA at two-week intervals according to a previous report (Kuklin et al., 1997). DL16, a gB oligomer-specific monoclonal antibody, was kindly provided by Professors Gary H Cohen and Roselyn J Eisenberg.

Construction of pHSV-1F BAC ΔgB Luc/GFP and preparation of HSV-1 gB null virus

To obtain HSV-1 gB null virus, gB encoding gene was knocked out from the HSV-1 genome constructed previously (Li et al., 2011; Sharan et al., 2009). In brief, pHSV-1F BAC Luc/GFP was first introduced into *E.coli* GS1783 (kindly provided by Dr. Klaus Osterrieder) by electroporation (2.5 kV, 25 μF capacitance and 200 Ω resistance). The *E.coli* culture was incubated at 42 °C for 15 min to activate the λ red recombinant system carried in *E.coli* GS1783, and thereafter, a linear DNA encoding Kan^r expression cassette with a 40 bp homology at each end to that of ul27 was introduced to completely substitute the target gene through the activated λ red recombinant system. Recombinant clones were subsequently screened by growing on LB plates containing kannamycin (30 μg/ml) and verified by PCR and sequencing. The ul27 knockout BAC DNA was transfected into VgB cells by electroporation (220 V and 950 μF capacitance) to propagate HSV-1 ΔgB Luc/GFP virus. After plaque formation on VgB cells, the plaque was collected to produce virus stocks.

Western blot

CHO cells pre-seeded in 6-well plates were transfected with 4 μg plasmid encoding WT or mutated gB or pcDNA3.1(+) using Lipofetamine 2000 (Invetrogen). 48 h post-transfection, cells were harvested and lysed with lysis buffer in the presence of protease inhibitor cocktail (Roche). Debris and nuclei were removed by centrifugation. Protein samples were resolved by 6% SDS-PAGE and transferred onto PVDF membrane using the Bio-Rad Microassay System. The PVDF membrane was blocked with TBS-T plus 5% non-fat milk for 2 h followed by incubation with HSV-2 polyclonal antibody PAB13979 at a dilution of 1:5000 for 2 h at room temperature. After three washes with TBS-T, the membrane was incubated with HRP-conjugated mouse anti-goat IgG at a dilution of 1:8000 for another 1 h at room temperature. Following five washes, immunoreactive bands were visualized after development using ECL chemiluminescent substrate (Beyotime).

Immunofluorescence and confocal microscopy

HeLa cells seeded on 35 mm glass bottom culture dishes were transfected with 1 μg plasmids encoding gB WT or mutants per well using Lipofectamine 2000 (Invitrogen) and cultured for 24 h at 37 °C. Transfected cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS both for 15 min at room temperature. Following three washes with PBS, cells were blocked with 3% BSA in PBS for 1 h at room temperature and then incubated with mouse monoclonal antibody against HSV-1/2 gB (ab6506) at a dilution of 1:100 for 1 h at room temperature. After three washes, cells were incubated with Alexa Fluor 555-conjugated donkey anti-mouse IgG at a dilution of 1:500 for 1 h at room temperature. For Golgi organelle labeling, TGN was stained with sheep polyclonal antibody against TGN46 at a dilution of 1:250. Thereafter, the cells were incubated with Alexa Fluor 488-conjugated donkey anti-sheep IgG at a dilution of 1:200. Cell nuclei were stained with DAPI. After extensive washes, stained cells were analyzed under Nikon A1 MP confocal microscope equipped with a 100× oil immersion objective. Image sequences were processed and analyzed using NIS-elements Viewer software (Nikon).

Immunofluorescence of cell surface staining

Labeling conditions were similar to those used in immunofluorescence and confocal microscopy assay, except that the transfected cells were first blocked with 3% BSA in PBS for 30 min at room temperature and then incubated with antibodies against gB for 1 h at 4 °C, followed by a fixation with 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, cells were incubated with Cy3-conjugated goat against mouse secondary antibody for 1 h at room temperature. Cell nuclei were stained with DAPI. Stained cells were analyzed under confocal microscope (UltraView VoX; Perkin-Elmer Life and Analytical Sciences) equipped with a 60× oil immersion objective. Image sequences were processed and analyzed using Volocity software (Perkin-Elmer).

CELISA

The assay was performed as previously described with some modifications (Manoj et al., 2004). Briefly, CHO cells growing in 96-well plates were first transfected with 0.2 μg plasmids of gB, gB mutant or pcDNA3.1(+) per well using Lipofectamine 2000 (Invitrogen) and cultured for 24 h at 37 °C. Cells in plates were fixed in 4% paraformaldehyde for 15 min. After three rinses with PBS, cells were incubated with HSV-2 polyclonal antibody PAB13979 for 1 h followed by incubation with HRP-conjugated mouse anti-goat secondary antibody for 1 h at room temperature (both 1:5000 diluted in 3% BSA). Cells were extensively rinsed and the color reaction was developed using the TMB liquid substrate system for ELISA (Sigma) and stopped by the addition of 2 N H_2SO_4. The OD values were subsequently read in an ELISA plate reader (Tecan) using a test wavelength of 450 nm and a reference wavelength of 570 nm.

For CELISA, 293T cells pre-seeded in 96-well plates were transfected with plasmids encoding gD, gH, gL and WT gB or gB mutants or empty vector (40 ng, 40 ng, 40 ng, and 80 ng, respectively) for 24 h. Cells were fixed in 4% paraformaldehyde and incubated with anti-gB sera (1:500 diluted in 3% BSA), followed by incubation with HRP-conjugated goat anti-mouse IgG (1:5000 diluted in 3% BSA) at room temperature. Subsequently, the color reaction was developed and OD values were read as described above.

Quantitative fusion assay

Quantitative fusion assay was conducted as previously described with some modifications (Hu et al., 2000; Manoj et al., 2004). Briefly, effector QT6 cells pre-seeded in 96-well plates were first infected with vTF1.1 (MOI=3) for 1 h at 37 °C followed by transfection with plasmids encoding gD, gH, gL and gB or a gB mutant or pcDNA3.1(+) (20 ng, 20 ng, 20 ng, and 40 ng, respectively). Target CHO cells pre-seeded in 6-well plates were transfected with plasmids expressing HVEM or Nectin-1 and a T7 promoter-controlled reporter gene luciferase (1 μg, 1 μg and 3 μg, respectively). 4 h post-transfection, the culture for QT6 was changed to growth medium containing rifampicin (100 μg/ml) while the culture for CHO was changed to growth medium. After overnight incubation, target cells were resuspended by trypsine treatment and co-cultured for 9 h at 37 °C with effector cells at the ratio of 1:1 in DMEM supplemented with 10% FBS, rifampicin (100 μg/ml) and cytosine arabinoside (100 nM). Subsequently, cells were lysed and luciferase activity was measured using a luminometer (Promega).

Viral complementation assay

To determine the effect of N-CHO site mutation on viral entry, complementation assay was performed as previously described with modifications (Connolly and Longnecker, 2012; Manoj et al., 2004). In brief, BHK-21 cells growing in 6-well plates were transfected with 4 μg plasmids expressing WT gB or mutants or pcDNA3.1(+) using Lipofectamine 2000 (Invitrogen). 24 h post-transfection, cells were incubated with HSV-1 gB null virus (HSV-1 ΔgB Luc/GFP) for 2 h at 37 °C (MOI=1). Virus inocula were removed and the unpenetrated viruses were inactivated by exposure of the cells to 0.1 mol/L citrate buffer (pH 3.0) for 1 min. After three washes with PBS, cells were cultured in growth medium (1 ml/well) for 24 h at 37 °C. To harvest the virus, BHK-21 cells were lysed through three freeze-thaw cycles and cell debris was removed by centrifugation. Subsequently, Vero and HeLa cells growing in 96-well plates were infected with 50 μl/well of HSV-1 gB null virus stock complemented with HSV-2 gB WT or mutants for 24 h (Manoj et al., 2004). Cells were subsequently washed, lysed and luciferase activity was detected. Virus entry was assessed by quantifying the expression of luciferase.

Statistical analysis

Analyses were performed with GraphPad Prism 4.00 software (GraphPad). Values were given as the mean of triplicates ± standard deviation (SD) unless otherwise specified. Statistical analysis was performed using the Student's *t*-test. A p value < 0.05 was considered statistically significant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.04.005.

References

Aguilar, H.C., Matreyek, K.A., Filone, C.M., Hashimi, S.T., Levroney, E.L., Negrete, O.A., Bertolotti-Ciarlet, A., Choi, D.Y., McHardy, I., Fulcher, J.A., Su, S.V., Wolf, M.C., Kohatsu, L., Baum, L.G., Lee, B., 2006. N-glycans on nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. J. Virol. 80 (10), 4878–4889.

Akhtar, J., Shukla, D., 2009. Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. FEBS J. 276 (24), 7228–7236.

Arii, J., Goto, H., Suenaga, T., Oyama, M., Kozuka-Hata, H., Imai, T., Minowa, A., Akashi, H., Arase, H., Kawaoka, Y., Kawaguchi, Y., 2010a. Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. Nature 467 (7317), 859–862.

Arii, J., Uema, M., Morimoto, T., Sagara, H., Akashi, H., Ono, E., Arase, H., Kawaguchi, Y., 2009. Entry of herpes simplex virus 1 and other alphaherpesviruses via the paired immunoglobulin-like type 2 receptor α. J. Virol. 83 (9), 4520–4527.

Arii, J., Wang, J., Morimoto, T., Suenaga, T., Akashi, H., Arase, H., Kawaguchi, Y., 2010b. A single-amino-acid substitution in herpes simplex virus 1 envelope glycoprotein B at a site required for binding to the paired immunoglobulin-like type 2 receptor {alpha} (PILR{alpha}) abrogates PILR{alpha}-dependent viral entry and reduces pathogenesis. J. Virol. 84 (20), 10773–10783.

Atanasiu, D., Saw, W.T., Cohen, G.H., Eisenberg, R.J., 2010a. Cascade of events governing cell–cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. J. Virol. 84 (23), 12292–12299.

Atanasiu, D., Whitbeck, J.C., Cairns, T.M., Reilly, B., Cohen, G.H., Eisenberg, R.J., 2007. Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion. Proc. Natl. Acad. Sci. USA 104 (47), 18718–18723.

Atanasiu, D., Whitbeck, J.C., de Leon, M.P., Lou, H., Hannah, B.P., Cohen, G.H., Eisenberg, R.J., 2010b. Bimolecular complementation defines functional regions of herpes simplex virus gB that are involved with gH/gL as a necessary step leading to cell fusion. J. Virol. 84 (8), 3825–3834.

Balzarini, J., 2007. Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. Nat. Rev. Microbiol. 5 (8), 583–597.

Bause, E., 1983. Structural requirements of N-glycosylation of proteins. Studies with proline peptides as conformational probes. Biochem. J. 209 (2), 331–336.

Bause, E., Legler, G., 1981. The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the N-glycosylation step during glycoprotein biosynthesis. Biochem. J. 195 (3), 639–644.

Beitia Ortiz de Zarate, I., Cantero-Aguilar, L., Longo, M., Berlioz-Torrent, C., Rozenberg, F., 2007. Contribution of endocytic motifs in the cytoplasmic tail of herpes simplex virus type 1 glycoprotein B to virus replication and cell–cell fusion. J. Virol. 81 (24), 13889–13903.

Beitia Ortiz de Zarate, I., Kaelin, K., Rozenberg, F., 2004. Effects of mutations in the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B on intracellular transport and infectivity. J. Virol. 78 (3), 1540–1551.

Bender, F.C., Samanta, M., Heldwein, E.E., de Leon, M.P., Bilman, E., Lou, H., Whitbeck, J.C., Eisenberg, R.J., Cohen, G.H., 2007. Antigenic and mutational analyses of herpes simplex virus glycoprotein B reveal four functional regions. J. Virol. 81 (8), 3827–3841.

Bender, F.C., Whitbeck, J.C., Lou, H., Cohen, G.H., Eisenberg, R.J., 2005. Herpes simplex virus glycoprotein B binds to cell surfaces independently of heparan sulfate and blocks virus entry. J. Virol. 79 (18), 11588–11597.

Biering, S.B., Huang, A., Vu, A.T., Robinson, L.R., Bradel-Tretheway, B., Choi, E., Lee, B., Aguilar, H.C., 2012. N-glycans on the nipah virus attachment glycoprotein modulate fusion and viral entry as they protect against antibody neutralization. J. Virol. 86 (22), 11991–12002.

Binley, J.M., Ban, Y.-E.A., Crooks, E.T., Eggink, D., Osawa, K., Schief, W.R., Sanders, R. W., 2010. Role of complex carbohydrates in human immunodeficiency virus type 1 infection and resistance to antibody neutralization. J. Virol. 84 (11), 5637–5655.

Calistri, A., Sette, P., Salata, C., Cancellotti, E., Forghieri, C., Comin, A., Göttlinger, H., Campadelli-Fiume, G., Palù, G., Parolin, C., 2007. Intracellular trafficking and maturation of herpes simplex virus type 1 gB and virus egress require functional biogenesis of multivesicular bodies. J. Virol. 81 (20), 11468–11478.

Campadelli-Fiume, G., Cocchi, F., Menotti, L., Lopez, M., 2000. The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. Rev. Med. Virol. 10 (5), 305–319.

Cheshenko, N., Herold, B.C., 2002. Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread. J. Gen. Virol. 83 (Pt 9), 2247–2255.

Chouljenko, V.N., Iyer, A.V., Chowdhury, S., Chouljenko, D.V., Kousoulas, K.G., 2009. The amino terminus of herpes simplex virus type 1 glycoprotein *K* (gK) modulates gB-mediated virus-induced cell fusion and virion egress. J. Virol. 83 (23), 12301–12313.

Chouljenko, V.N., Iyer, A.V., Chowdhury, S., Kim, J., Kousoulas, K.G., 2010. The herpes simplex virus type 1 UL20 protein and the amino terminus of glycoprotein *K* (gK) physically interact with gB. J. Virol. 84 (17), 8596–8606.

Chowdary, T.K., Heldwein, E.E., 2010. Syncytial phenotype of C-terminally truncated herpes simplex virus type 1 gB is associated with diminished membrane interactions. J. Virol. 84 (10), 4923–4935.

Chowdhury, S., Chouljenko, V.N., Naderi, M., Kousoulas, K.G., 2013. The amino terminus of herpes simplex virus 1 glycoprotein K is required for virion entry via the paired immunoglobulin-like type-2 receptor alpha. J. Virol. 87 (6), 3305–3313.

Connolly, S.A., Longnecker, R., 2012. Residues within the C-terminal arm of the herpes simplex virus type 1 glycoprotein B ectodomain contribute to its refolding during the fusion step of virus entry. J. Virol. 86 (12), 6386–6393.

de Jong, M.A.W.P., de Witte, L., Bolmstedt, A., van Kooyk, Y., Geijtenbeek, T.B.H., 2008. Dendritic cells mediate herpes simplex virus infection and transmission through the C-type lectin DC-SIGN. J. Gen. Virol. 89 (Pt 10), 2398–2409.

Doms, R.W., Lamb, R.A., Rose, J.K., Helenius, A., 1993. Folding and assembly of viral membrane proteins. Virology 193 (2), 545–562.

Drummer, H.E., Hill, M.K., Maerz, A.L., Wood, S., Ramsland, P.A., Mak, J., Poumbourios, P., 2013. Allosteric Modulation of the HIV-1 gp120-gp41 association site by adjacent gp120 variable region 1 (V1) N-glycans linked to neutralization sensitivity. PLoS Pathog. 9 (4), e1003218.

Fan, J., Liu, Y., Yuan, Z., 2014. Critical role of dengue virus NS1 protein in viral replication. Virol. Sin. 29 (3), 162–169.

Fan, Z., Grantham, M.L., Smith, M.S., Anderson, E.S., Cardelli, J.A., Muggeridge, M.I., 2002. Truncation of herpes simplex virus type 2 glycoprotein B increases its cell surface expression and activity in cell–cell fusion, but these properties are unrelated. J. Virol. 76 (18), 9271–9283.

Fenouillet, E., Jones, I., Powell, B., Schmitt, D., Kieny, M.P., Gluckman, J.C., 1993. Functional role of the glycan cluster of the human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) ectodomain. J. Virol. 67 (1), 150–160.

Glorioso, J., Szczesiul, M.S., Marlin, S.D., Levine, M., 1983. Inhibition of glycosylation of herpes simplex virus glycoproteins: identification of antigenic and immunogenic partially glycosylated glycopeptides on the cell surface membrane. Virology 126 (1), 1–18.

Hannah, B.P., Cairns, T.M., Bender, F.C., Whitbeck, J.C., Lou, H., Eisenberg, R.J., Cohen, G.H., 2009. Herpes simplex virus glycoprotein B associates with target membranes via its fusion loops. J. Virol. 83 (13), 6825–6836.

Hebert, D.N., Zhang, J.X., Chen, W., Foellmer, B., Helenius, A., 1997. The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. J. Cell Biol. 139 (3), 613–623.

Heldwein, E.E., Krummenacher, C., 2008. Entry of herpesviruses into mammalian cells. Cell Mol. Life Sci. 65 (11), 1653–1668.

Heldwein, E.E., Lou, H., Bender, F.C., Cohen, G.H., Eisenberg, R.J., Harrison, S.C., 2006. Crystal structure of glycoprotein B from herpes simplex virus 1. Science 313 (5784), 217–220.

Herold, B.C., Visalli, R.J., Susmarski, N., Brandt, C.R., Spear, P.G., 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J. Gen. Virol. 75 (Pt 6), 1211–1222.

Hu, Q.-X., Barry, A.P., Wang, Z.-X., Connolly, S.M., Peiper, S.C., Greenberg, M.L., 2000. Evolution of the human immunodeficiency virus type 1 envelope during infection reveals molecular corollaries of specificity for coreceptor utilization and AIDS pathogenesis. J. Virol. 74 (24), 11858–11872.

Huang, W., Hu, K., Luo, S., Zhang, M., Li, C., Jin, W., Liu, Y., Griffin, G.E., Shattock, R.J., Hu, Q., 2012a. Herpes simplex virus type 2 infection of human epithelial cells induces CXCL9 expression and CD4+ T cell migration via activation of p38-CCAAT/enhancer-binding protein-β pathway. J. Immunol. 188 (12), 6247–6257.

Huang, X., Jin, W., Griffin, G.E., Shattock, R.J., Hu, Q., 2011. Removal of two high-mannose N-linked glycans on gp120 renders human immunodeficiency virus 1 largely resistant to the carbohydrate-binding agent griffithsin. J. Gen. Virol. 92 (Pt 10), 2367–2373.

Huang, X., Jin, W., Hu, K., Luo, S., Du, T., Griffin, G.E., Shattock, R.J., Hu, Q., 2012b. Highly conserved HIV-1 gp120 glycans proximal to CD4-binding region affect viral infectivity and neutralizing antibody induction. Virology 423 (1), 97–106.

Kasturi, L., Eshleman, J.R., Wunner, W.H., Shakin-Eshleman, S.H., 1995. The hydroxy amino acid in an Asn-X-Ser/Thr sequon can influence N-linked core glycosylation efficiency and the level of expression of a cell surface glycoprotein. J. Biol. Chem. 270 (24), 14756–14761.

Kim, I.-J., Chouljenko, V.N., Walker, J.D., Kousoulas, K.G., 2013. Herpes simplex virus type 1 (HSV-1) glycoprotein *M* (gM) and the membrane associated protein UL11 are required for virus-induced cell fusion and efficient virus entry. J. Virol. 87 (14), 8029–8037.

Kobayashi, Y., Suzuki, Y., 2012. Evidence for N-Glycan shielding of antigenic sites during evolution of human influenza A virus hemagglutinin. J. Virol. 86 (7), 3446–3451.

Krummenacher, C., Supekar, V.M., Whitbeck, J.C., Lazear, E., Connolly, S.A., Eisenberg, R.J., Cohen, G.H., Wiley, D.C., Carfi, A., 2005. Structure of unliganded HSV gD reveals a mechanism for receptor-mediated activation of virus entry. EMBO J. 24 (23), 4144–4153.

Kuklin, N., Daheshia, M., Karem, K., Manickan, E., Rouse, B.T., 1997. Induction of mucosal immunity against herpes simplex virus by plasmid DNA immunization. J. Virol. 71 (4), 3138–3145.

Laquerre, S., Argnani, R., Anderson, D.B., Zucchini, S., Manservigi, R., Glorioso, J.C., 1998. Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. J. Virol. 72 (7), 6119–6130.

Lazear, E., Carfi, A., Whitbeck, J.C., Cairns, T.M., Krummenacher, C., Cohen, G.H., Eisenberg, R.J., 2008. Engineered disulfide bonds in herpes simplex virus type 1 gD separate receptor binding from fusion initiation and viral entry. J. Virol. 82 (2), 700–709.

Li, W., Minova-Foster, T.J., Norton, D.D., Muggeridge, M.I., 2006. Identification of functional domains in herpes simplex Virus 2 glycoprotein B. J. Virol. 80 (8), 3792–3800.

Li, Y., Wang, S., Zhu, H., Zheng, C., 2011. Cloning of the herpes simplex virus type 1 genome as a novel luciferase-tagged infectious bacterial artificial chromosome. Arch. Virol. 156 (12), 2267–2272.

Lin, E., Spear, P.G., 2007. Random linker-insertion mutagenesis to identify functional domains of herpes simplex virus type 1 glycoprotein B. Proc. Natl. Acad. Sci. USA 104 (32), 13140–13145.

Looker, K.J., Garnett, G.P., Schmid, G.P., 2008. An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. Bull. World Health Organ. 86 (10), 805–812, A.

Maggioni, C., Braakman, I., 2005. Synthesis and quality control of viral membrane proteins. Curr. Top. Microbiol. Immunol. 285, 175–198.

Manoj, S., Jogger, C.R., Myscofski, D., Yoon, M., Spear, P.G., 2004. Mutations in herpes simplex virus glycoprotein D that prevent cell entry via nectins and alter cell tropism. Proc. Natl. Acad. Sci. USA 101 (34), 12414–12421.

Martinelli, E., Tharinger, H., Frank, I., Arthos, J., Piatak Jr., M., Lifson, J.D., Blanchard, J., Gettie, A., Robbiani, M., 2011. HSV-2 infection of dendritic cells amplifies a highly susceptible HIV-1 cell target. PLoS Pathog. 7 (6), e1002109.

Melikyan, G.B., Markosyan, R.M., Hemmati, H., Delmedico, M.K., Lambert, D.M., Cohen, F.S., 2000. Evidence that the transition of HIV-1 Gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J. Cell Biol. 151 (2), 413–424.

Muggeridge, M.I., 2000. Characterization of cell–cell fusion mediated by herpes simplex virus 2 glycoproteins gB, gD, gH and gL in transfected cells. J. Gen. Virol. 81 (Pt 8), 2017–2027.

Ohuchi, M., Ohuchi, R., Feldmann, A., Klenk, H.D., 1997a. Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. J. Virol. 71 (11), 8377–8384.

Ohuchi, R., Ohuchi, M., Garten, W., Klenk, H.D., 1997b. Oligosaccharides in the stem region maintain the influenza virus hemagglutinin in the metastable form required for fusion activity. J. Virol. 71 (5), 3719–3725.

Papworth, M.A., Van Dijk, A.A., Benyon, G.R., Allen, T.D., Arrand, J.R., Mackett, M., 1997. The processing, transport and heterologous expression of Epstein-Barr virus gp110. J. Gen. Virol. 78 (Pt 9), 2179–2189.

Pikora, C., Wittish, C., Desrosiers, R.C., 2005. Identification of two N-linked glycosylation sites within the core of the simian immunodeficiency virus glycoprotein whose removal enhances sensitivity to soluble CD4. J Virol. 79 (19), 12575–12583.

Rosnoblet, C., Peanne, R., Legrand, D., Foulquier, F., 2013. Glycosylation disorders of membrane trafficking. Glycoconj J. 30 (1), 23–31.

Roth, J., 2002. Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions. Chem. Rev. 102 (2), 285–303.

Russell, C.J., Jardetzky, T.S., Lamb, R.A., 2001. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. EMBO J. 20 (15), 4024–4034.

Satoh, T., Arii, J., Suenaga, T., Wang, J., Kogure, A., Uehori, J., Arase, N., Shiratori, I., Tanaka, S., Kawaguchi, Y., Spear, P.G., Lanier, L.L., Arase, H., 2008. PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B. Cell 132 (6), 935–944.

Sharan, S.K., Thomason, L.C., Kuznetsov, S.G., Court, D.L., 2009. Recombineering: a homologous recombination-based method of genetic engineering. Nat. Protoc. 4 (2), 206–223.

Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H., Takashima, I., 2004. Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. J. Gen. Virol. 85 (Pt 12), 3637–3645.

Shukla, D., Spear, P.G., 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J. Clin. Invest. 108 (4), 503–510.

Sodora, D.L., Cohen, G.H., Muggeridge, M.I., Eisenberg, R.J., 1991a. Absence of asparagine-linked oligosaccharides from glycoprotein D of herpes simplex virus type 1 results in a structurally altered but biologically active protein. J. Virol. 65 (8), 4424–4431.

Sodora, D.L., Eisenberg, R.J., Cohen, G.H., 1991b. Characterization of a recombinant herpes simplex virus which expresses a glycoprotein D lacking asparagine-linked oligosaccharides. J. Virol. 65 (8), 4432–4441.

Sommer, M., Courtney, R.J., 1991. Differential rates of processing and transport of herpes simplex virus type 1 glycoproteins gB and gC. J Virol. 65 (1), 520–525.

Stampfer, S.D., Lou, H., Cohen, G.H., Eisenberg, R.J., Heldwein, E.E., 2010. Structural basis of local, pH-dependent conformational changes in glycoprotein B from herpes simplex virus type 1. J. Virol. 84 (24), 12924–12933.

Suenaga, T., Satoh, T., Somboonthum, P., Kawaguchi, Y., Mori, Y., Arase, H., 2010. Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses. Proc. Natl. Acad. Sci. USA 107 (2), 866–871.

Sugimoto, K., Uema, M., Sagara, H., Tanaka, M., Sata, T., Hashimoto, Y., Kawaguchi, Y., 2008. Simultaneous tracking of capsid, tegument, and envelope protein localization in living cells infected with triply fluorescent herpes simplex Virus 1. J. Virol. 82 (11), 5198–5211.

Tan, D.H., Murphy, K., Shah, P., Walmsley, S.L., 2013. Herpes simplex virus type 2 and HIV disease progression: a systematic review of observational studies. BMC Infect. Dis. 13, 502.

Terence, P., McDonalda, C.E.J., Lia, Ping, Rixona, Helen W. McL., Browna, Gaie, Aitkenc, James D., MacLellana, Kirsty, Sugruea, Richard J., 2006. Evidence that maturation of the N-linked glycans of the respiratory syncytial virus (RSV) glycoproteins is required for virus-mediated cell fusion: the effect of α-mannosidase inhibitors on RSV infectivity. Virology 350 (2), 289–301.

Turcotte, S., Letellier, J., Lippé, R., 2005. Herpes simplex virus type 1 capsids transit by the trans-golgi network, where viral glycoproteins accumulate independently of capsid egress. J. Virol. 79 (14), 8847–8860.

Turner, A., Bruun, B., Minson, T., Browne, H., 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J. Virol. 72 (1), 873–875.

Uchida, H., Chan, J., Goins, W.F., Grandi, P., Kumagai, I., Cohen, J.B., Glorioso, J.C., 2010. A double mutation in glycoprotein gB compensates for ineffective gD-dependent initiation of herpes simplex virus Type 1 infection. J. Virol. 84 (23), 12200–12209.

Vigerust, D.J., Shepherd, V.L., 2007. Virus glycosylation: role in virulence and immune interactions. Trends Microbiol. 15 (5), 211–218.

Vu, H.L., Kwon, B., Yoon, K.J., Laegreid, W.W., Pattnaik, A.K., Osorio, F.A., 2011. Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. J. Virol. 85 (11), 5555–5564.

Wang, J., Fan, Q., Satoh, T., Arii, J., Lanier, L.L., Spear, P.G., Kawaguchi, Y., Arase, H., 2009. Binding of herpes simplex virus glycoprotein B (gB) to paired immunoglobulin-like type 2 Receptor {alpha} depends on specific sialylated O-Linked Glycans on gB. J. Virol. 83 (24), 13042–13045.

Wisner, T.W., Johnson, D.C., 2004. Redistribution of cellular and herpes simplex virus proteins from the trans-golgi network to cell junctions without enveloped capsids. J. Virol. 78 (21), 11519–11535.

Yuste, E., Bixby, J., Lifson, J., Sato, S., Johnson, W., Desrosiers, R., 2008. Glycosylation of gp41 of simian immunodeficiency virus shields epitopes that can be targets for neutralizing antibodies. J. Virol. 82 (24), 12472–12486.

Zhu, J., Hladik, F., Woodward, A., Klock, A., Peng, T., Johnston, C., Remington, M., Magaret, A., Koelle, D.M., Wald, A., Corey, L., 2009. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. Nat. Med. 15 (8), 886–892.