Entry of Alphaherpesviruses

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Abstract

Alphaherpesviruses are enveloped viruses that enter cells by fusing the viral membrane with a host cell membrane, either within an endocytic vesicle or at the plasma membrane. This entry event is mediated by a set of essential entry glycoproteins, including glycoprotein D (gD), gHgL, and gB. gHgL and gB are conserved among herpesviruses, but gD is unique to the alphaherpesviruses and is not encoded by all alphaherpesviruses. gD is a receptor-binding protein, the heterodimer gHgL serves as a fusion regulator, and gB is a class III viral fusion protein. Sequential interactions among these glycoproteins are thought to trigger the virus to fuse at the right place and time. Structural studies of these glycoproteins from multiple alphaherpesviruses has enabled the design and interpretation of functional studies. The structures of gD in a receptor-bound and in an unliganded form reveal a conformational change in the C terminus of the gD ectodomain upon receptor binding that may serve as a signal for fusion. By mapping neutralizing antibodies to the gHgL structures and constructing interspecies chimeric forms of gHgL, interaction sites for both gD and gB on gHgL have been proposed. A comparison of the postfusion

structure of gB and an alternative conformation of gB visualized using cryo-electron tomography suggests that gB undergoes substantial refolding to execute membrane fusion. Although these structures have provided excellent insights into the entry mechanism, many questions remain about how these viruses coordinate the interactions and conformational changes required for entry.

Introduction

As the first step of infection, alphaherpesvirus entry requires the coordinated action of several glycoproteins on the viral surface. Unlike many other viruses, herpesviruses encode receptor-binding and membrane fusion functions on separate proteins. These proteins must cooperate to mediate fusion of the viral and cellular membranes at the right time and place, either at the plasma membrane or within an endosome.

Entry proceeds through three basic steps: initial attachment, entry receptor binding, and membrane fusion (Figure 1A). Initial attachment to a cell is mediated typically by glycoprotein C (gC) and/or gB binding to cell surface proteoglycans (Herold et al., 1994; Herold et al., 1991; Laquerre et al., 1998; Rux et al., 2002). This attachment is reversible and does not trigger membrane fusion or virus entry. Although the attachment enhances infection, it is not required for entry because cells lacking heparan sulfate can be infected, albeit with reduced efficiency (Gruenheid et al., 1993).

After attachment, membrane fusion and virus entry require a set of core entry glycoproteins (Figure 1B). All herpesviruses encode gH, gL, and gB and most alphaherpesviruses also encode gD, with the notable exception of varicella zoster virus (VZV). These four glycoproteins are essential for infection and they are sufficient to mediate cell-cell fusion of receptor-bearing cells expressing these glycoproteins (Browne et al., 2001; Cole and Grose, 2003; Muggeridge, 2000; Pertel et al., 2001; Turner et al., 1998). In the current model of alphaherpesviral entry into cells (Figure 1B), gD binding to an entry receptor triggers a conformational change that transmits a signal to the gHgL

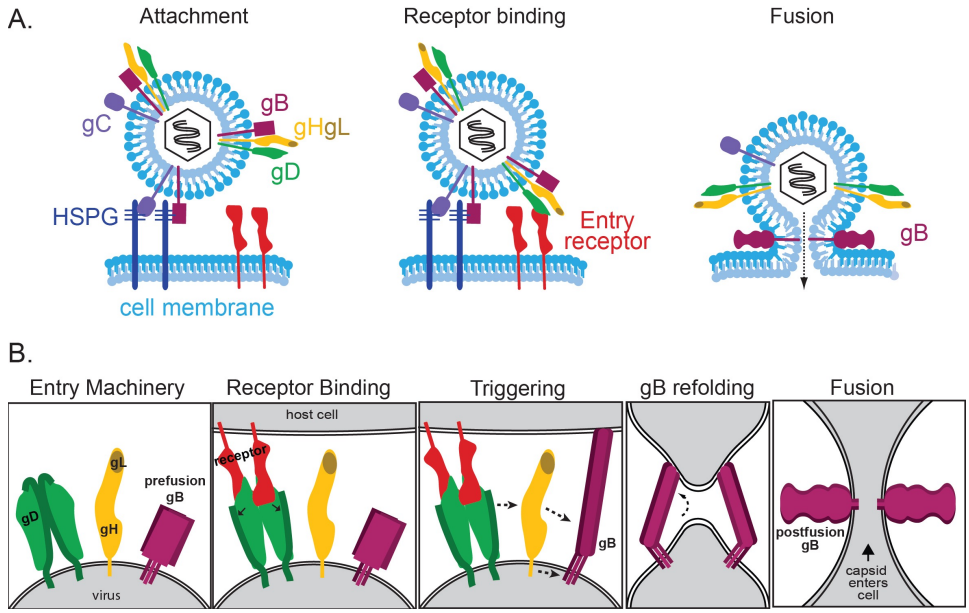

Figure 1. Mechanism of alphaherpesvirus entry. (A) Using HSV as a model for virus entry, heparan sulfate proteoglycans (HSPG), entry receptors, and viral glycoproteins gB, gD, gHgL, and gB are shown. Virus attaches to cells by binding to HSPG via gC and/or gB. Although this attachment step enhances entry, it is not required for fusion. Attachment is followed by the engagement of entry receptors. With the exception of VZV, this receptor-binding is mediated by gD. Functional gD receptors include members of the nectin family, HVEM, and 3-O-sulfonated heparan sulfate. In the current model of entry, gD-receptor binding signals gHgL to trigger gB to mediate fusion. gB inserts into the host cell membrane and refolds to fuse the viral and cellular membranes, allowing the viral capsid and genome to enter the host cell. (B) The core entry glycoproteins that are necessary and sufficient for fusion include the receptor-binding protein gD (green), the gHgL heterodimer (yellow), and the trimeric fusion protein gB (purple). Although VZV does not encode a gD homolog, it does encode gHgL and gB. Prior to receptor binding, the C-terminus of the gD ectodomain occludes the receptor-binding site. gD binding to either the HVEM or nectin-1 receptor (red) displaces the gD C-terminus and transmits a signal to activate gHgL. This signal may come directly from the gD C-terminal region or as a result the C-terminal repositioning to expose another interaction site on gD. gHgL then transmits this signal to trigger the fusion protein gB. The interaction with gB may be mediated by the gHgL ectodomain and/or the gH CT. Upon triggering, gB refolds from a prefusion conformation to insert its fusion loops into the host cell membrane. The orientation of the fusion loops with respect to the viral membrane in the prefusion form of gB is unresolved. gB then collapses back on itself, bringing the fusion loops embedded the cell membrane into proximity with the gB TM, thereby fusing the viral and cell membranes. Most likely, more than one gB trimer must be triggered to create a fusion pore through which the viral capsid can enter the cell, hence two trimers are shown in the later panels.

heterodimer. gHgL serves as a regulator of fusion, interacting with both the gD-receptor complex and the viral fusion protein, gB. Through an undefined mechanism, gHgL triggers the metastable gB to undergo an extensive refolding event, inserting its hydrophobic fusion loops into the host cell membrane and then folding back on itself to bring the viral and cellular membranes together. Fusion of these membranes creates a lipid pore that permits the viral genome to enter the cell.

Structures of each of the core entry glycoproteins from multiple alphaherpesviruses have been determined. These structures have advanced our understanding of the viral entry mechanism greatly. Using these structures as a framework, this article will review our current knowledge of alphaherpesvirus entry, relating functional studies to the structures and identifying current areas of interest.

Glycoprotein D: Receptor binding

For most alphaherpesviruses, gD is the receptor-binding protein and one of the core viral glycoproteins necessary to carry out membrane fusion and cell-cell spread. Two exceptions are VZV, which lacks gD and may instead rely on other glycoproteins for cell surface attachment (see below) (Duus et al., 1995; Maresova et al., 2001; Oliver et al., 2016), and pseudorabies virus (PRV), for which gD is essential for entry but not cell-cell spread (Ch'ng et al., 2007; Klupp and Mettenleiter, 1999; Klupp et al., 2000). Interestingly, with the right selection pressure, replication-competent gD deletion mutants can be generated *in vitro* for PRV and bovine herpes virus type 1 (BHV-1) (Schmidt et al., 2001; Schroder and Keil, 1999). These mutant lab strains possess compensatory mutations in gB and gH that circumvent the need for gD; however, in wild-type alphaherpesviruses other than VZV, the binding of gD to specific cellular receptors is required for viral entry. Viral infection can be inhibited by blocking virus-receptor interaction with soluble versions of gD or the gD receptor ectodomains (Johnson et al., 1990; Montgomery et al., 1996; Nicola et al., 1997; Warner et al., 1998; Whitbeck et al., 1997). In addition, antibodies generated against gD or the gD receptors are potent virus

neutralizers and block membrane fusion (Atanasiu et al., 2018; Cairns et al., 2017; Cocchi et al., 1998; Fuller and Spear, 1987; Highlander et al., 1987; Krummenacher et al., 2000; Lazear et al., 2012; Montgomery et al., 1996; Saw et al., 2015; Whitbeck et al., 2001). Many neutralizing monoclonal antibodies (MAbs) against gD have been generated that block the binding of one or both receptors (Krummenacher et al., 1998; Lee et al., 2013; Nicola et al., 1998; Whitbeck et al., 1999). Additional neutralizing MAbs that do not block receptor binding have been hypothesized to interfere with the ability of gD to activate gHgL (Atanasiu et al., 2016; Atanasiu et al., 2018; Gallagher et al., 2013; Lazear et al., 2012).

gD receptors

Three classes of gD entry receptors have been identified, and gD has been co-crystallized with two of these receptors (Figure 2A, B). The first receptor class consists of the nectin and nectin-like proteins, which are immunoglobulin (Ig) superfamily members, each having one variable-like (V-like) and two constant-like (C-like) Ig domains (Nakanishi and Takai, 2004). Nectin proteins function as homophilic adhesion molecules and are widely distributed on the surface of most human tissues, including epithelial and neuronal cells, recognized targets for alphaherpesviral infection (Cocchi et al., 1998; Galen et al., 2006; Mata et al., 2001; Mizoguchi et al., 2002; Richart et al., 2003; Takahashi et al., 1999; Warner et al., 1998). The use of nectin-1 as a cell surface receptor is common among alphaherpesviruses. HSV-1, HSV-2, PRV, BHV-1, and herpes B virus all can use nectin-1 for entry (Fan and Longnecker, 2012; Milne et al., 2001; Ono et al., 2004; Warner et al., 1998). HSV and PRV gD bind to nectin-1 with high affinity while the relatively low affinity of BHV-1 for nectin-1 suggests either that low affinity is sufficient for entry or that BHV uses another receptor in its natural host (Connolly et al., 2001; Milne et al., 2001). Nectin-2 has a more limited scope as an alphaherpesvirus receptor and can mediate the entry of PRV, HSV-2, and certain HSV-1 mutants (Lopez et al., 2000; Spear et al., 2006; Warner et al., 1998). Poliovirus receptor (nectin-like molecule 5) permits entry of PRV, BHV-1, and herpes B virus, but not HSV (Fan and Longnecker, 2012; Warner et al., 1998). Under selective pressure, HSV can acquire gain-of-

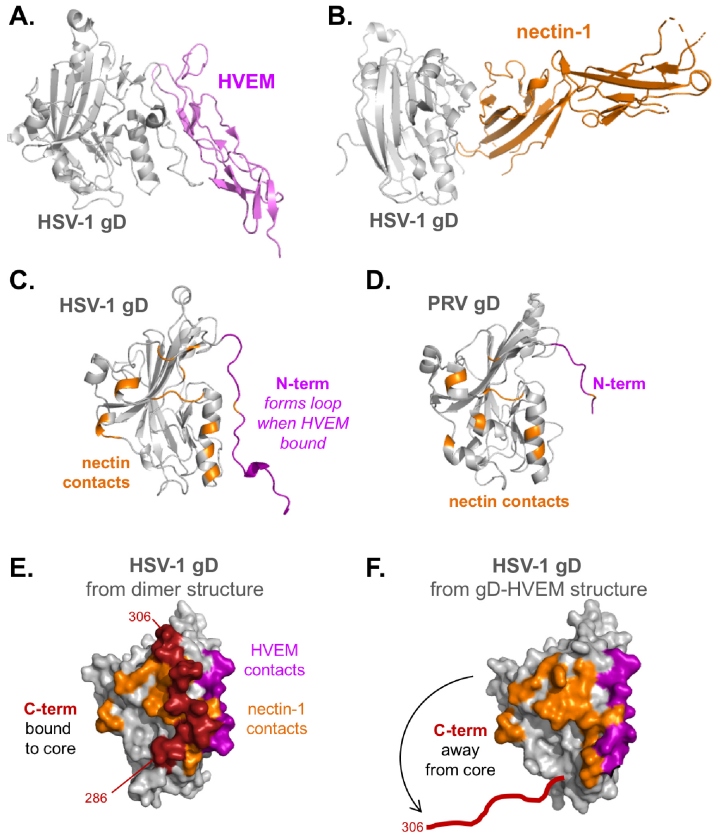

Figure 2. gD structures. (A) The crystal structure of gD (gray) bound to HVEM receptor (purple) is shown (PDB 1JMA) (Carfi et al., 2001). All of the HVEM contacts are contained within the N-terminal loop of gD. (B) The crystal structure of gD bound to nectin-1 receptor (orange) is shown (PDB 3SKU) (Di Giovine et al., 2011). HVEM and nectin-1 bind to the same face of gD. Nectin-1 binding prevents formation of the gD N-terminal loop, thus providing a structural basis for why HVEM and nectin-1 cannot bind to gD simultaneously. (C) The crystal structure of gD alone, not bound to receptor (PDB 1L2G) (Carfi et al., 2001). The nectin-1 contact residues (orange) and HVEM binding site (purple) are shown. The C-terminal region is not present in this structure. (D) The crystal structure of PRV gD alone (PDB 5X5V) (Li et al., 2017b), with nectin-1 contacts colored as in part C. The N-terminus of PRV gD is shorter than that of HSV-1 gD, providing a structural explanation for the failure of HVEM to serve as a PRV entry receptor. (E). Surface rendering of HSV-1 gD alone from a mutant with a stabilized C-terminus (PDB 2C36) (Krummenacher et al., 2005). The C-terminus of the gD ectodomain was stabilized by the addition of a cysteine at residue 307 that resulted in a disulfide-linked gD dimer. The C-terminus of the gD ectodomain (residues 285-306, red) is shown, as well as the contact residues for HVEM (purple) and nectin-1 (orange). The position of the C-terminus occludes both the HVEM and nectin-1 binding sites, providing a structural basis for the increase in affinity for both HVEM and nectin-1 when this portion of the C-terminus is deleted from soluble forms of the gD ectodomain. (F). Surface rendering of HSV-1 gD from the gD-HVEM co-crystal (PDB 1JMA) (Carfi et al., 2001). The contact residues for HVEM (purple) and nectin-1 (orange) are shown. Binding of either receptor would require the C-terminus to move, as depicted by the red tail extending from gD. Movement of the C-terminus may serve as a signal for triggering the downstream fusion events.

function mutations (not only in gD, but also in gB) that allow it to use other nectins for entry (nectins 2 through 4) (Cocchi et al., 2004b; Uchida et al., 2010).

The second class of gD receptors consists of the herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor superfamily, containing four characteristic cysteine-rich domains (CRD) (Carfi et al., 2001; Locksley et al., 2001). HVEM is used as a receptor by HSV-1 and HSV-2, but not by the other alphaherpesviruses including PRV, BHV-1, and herpes B virus. HVEM is expressed primarily on lymphocytes, a cell type that is not a primary target for HSV infection (Montgomery et al., 1996; Raftery et al., 1999; Whitbeck et al., 1997). The affinity of HSV gD for HVEM is the same as it is for nectin-1 (Krummenacher et al., 1998; Willis et al., 1998). Interestingly, despite the fact that a single mutation in the HVEM binding site on gD can abrogate HVEM usage without preventing nectin-1 usage (Yoon et al., 2003), all of the 49 clinical isolates tested were able to use both HVEM and nectin-1 as a receptor (Krummenacher et al., 2004). This finding suggests a selective pressure to retain HVEM usage.

The third class of gD receptor, 3-O-sulfonated derivatives of heparan sulfate (3-OST HS), is generated by D-glucosaminyl-3-O-sulfotransferase-3 modification of heparan sulfate and can mediate the entry of HSV-1 (Shukla et al., 1999; Tiwari et al., 2007). 3-OST HS is present in neurons of the mouse trigeminal ganglion, but its contribution to HSV infection is yet unclear (Lawrence et al., 2007).

The relative importance of the receptors to HSV infection has been studied using mice knocked-out for one or two of the receptors. Intercranial infection of knock-out mice suggests that nectin-1 is required for infection and progression to encephalitis (Kopp et al., 2009). Intercranial infection in the absence of HVEM progressed as in wild-type mice, whereas mice lacking nectin-1 did not show disease. Similarly, when knock-out mice were infected intravaginally in the absence of nectin-1, the virus did not spread to the sensory ganglia, but

infection in the absence of HVEM was normal (Taylor et al., 2007). Mice knocked-out for both HVEM and nectin-1 were resistant to infection of the vaginal epithelium, indicating that either receptor alone is sufficient for infection of epithelial cells. The role of HVEM in ocular infection is more complex. Corneal infection of the knockout mice revealed that the loss of either nectin-1 or HVEM attenuated HSV-1 infection of the cornea (Karaba et al., 2011), although infection with HSV-2 was not dependent on HVEM (Karaba et al., 2012). Further studies suggest that the contribution of HVEM to ocular disease is due to its immune modulatory role, rather than a direct role in virus entry (Edwards et al., 2015; Edwards and Longnecker, 2017).

gD and receptor structures

The crystal structures of gD from HSV-1 (Carfi et al., 2001; Krummenacher et al., 2005), HSV-2 (Lee et al., 2013), and PRV (Li et al., 2017a) reveal a highly conserved domain organization (Figure 2C, D). The core of gD consists of a V-like Ig fold flanked by long N- and C-terminal extensions. Structures of gD co-crystallized with the receptors nectin-1 and HVEM have also been resolved, and gD binds to the membrane-distal N-terminal regions of both receptors (Figure 2A, B) (Carfi et al., 2001; Di Giovine et al., 2011; Li et al., 2017a; Lu et al., 2014). The structure of HVEM is similar to that of other members of the tumor necrosis factor receptor family and is composed of four CRDs of approximately 40 residues each (Carfi et al., 2001). Although amino acids in both CRD1 and CRD2 of HVEM contact HSV gD, residue Y23 in CRD1 represents a "hot-spot" for gD binding while CRD2 is required mainly due to its effect on the presentation of the binding site on CRD1 (Carfi et al., 2001; Connolly et al., 2002; Whitbeck et al., 2001).

Nectin-1 and HVEM bind to HSV gD at distinct sites on the same face of gD (Carfi et al., 2001; Di Giovine et al., 2011; Krummenacher et al., 1998; Lu et al., 2014; Whitbeck et al., 1999). When HVEM is bound to gD, the gD N-terminus forms a hairpin loop and this loop comprises the entire HVEM-binding site (Figure 2A). Antibodies against a peptide corresponding to this N-terminal gD loop neutralize viral infectivity (Cohen et al., 1984). The N-terminal sequences

of PRV and BHV-1 gD diverge from that of HSV-1 and HSV-2 gD, most likely because HSV gD uses this stretch for HVEM binding, whereas the others do not. In the absence of HVEM, the N-terminal gD residues do not form a hairpin and are disordered (Figure 2C) (Carfi et al., 2001). Therefore, the formation of the hairpin loop represents a conformational change in the gD N-terminus that must occur for HVEM to bind gD (Lazear et al., 2014). PRV gD contains a shortened N-terminal extension, as compared to HSV gD, that is incapable of forming this hairpin (Figure 2D), coinciding with the inability of PRV gD to bind HVEM (Connolly et al., 2001). HVEM and nectin-1 cannot bind to gD simultaneously because the N-terminal hairpin loop formed via HVEM binding partially occludes the nectin-1 binding site (Figure 2A. B) (Di Giovine et al., 2011; Krummenacher et al., 2005). Comparison of the nectin-1 binding sites on HSV and PRV gD reveal a similar footprint on the core domain of gD (Figure 2C, D). The conservation of the nectin-1 binding site between HSV and PRV suggests that this binding site is conserved between most gD homologues.

3-OST HS has not been co-crystallized with gD, however two sulfate ions found in the gD structure may represent the site of 3-OST HS binding, including one ion in a basic cavity on gD that is close to the gD/HVEM interface (Carfi et al., 2001). Further evidence that the 3-OST HS and HVEM binding sites on gD may overlap is that mutations in the N-terminus of gD impact the usage of HVEM and 3-OST HS similarly (Yoon et al., 2003).

The C-terminal region of the gD ectodomain plays an important role in receptor binding and gD function. The structure of a dimeric form of HSV-1 gD, created by the addition of a disulfide bond at the end of the ectodomain, revealed that the C-terminus of the gD ectodomain lies against the gD core, overlapping the nectin-1 binding site and occupying the same space as the gD N-terminal loop residues that form the HVEM binding site (Figure 2E) (Di Giovine et al., 2011; Krummenacher et al., 2005; Lu et al., 2014). The binding of either HVEM or nectin-1 would require the displacement of this C-terminus away from the gD core (Figure 2F). To validate the position of the C-terminus in this structure, an additional gD mutant with a disulfide bond engineered to lock the C-terminus

against the gD core was crystallized (Krummenacher et al., 2005). As would be predicted, although this mutant was antigenically intact, it was unable to bind either receptor. Conversely, mutations in HSV and PRV gD that either delete the C-terminal tail or prevent its association with the gD core enhance binding to both nectin−1 and HVEM by 10-50 fold, due to an increase in the rate of complex formation (k_{on}) (Krummenacher et al., 1999; Krummenacher et al., 2005; Rux et al., 1998; Willis et al., 1998) (Li et al., 2017a). Without the gD C-terminal tail bound to the gD core, the nectin-1 binding site is available for receptor binding and the N-terminal hairpin loop can more readily form to permit HVEM binding. So, although the C-terminal tail of gD does not contain any receptor-contacting residues, this region influences gD-receptor binding.

Transmitting a signal for fusion

gD binding to receptor plays a greater role in virus entry than simply tethering the virus to the cell. gD homologs are not interchangeable, even when they bind the same nectin-1 receptor (Bohm et al., 2016; Fan et al., 2014). The C-terminus of gD appears to play a dual role in fusion by both inhibiting fusion when covering the receptor binding site and promoting fusion after receptor binding (Figure 1B). By creating a panel of gD mutants with engineered disulfide bonds that lock the C-terminus to the gD core at different points, a mutant was created that retained the ability to bind the receptors but failed to promote fusion (Lazear et al., 2008). This result suggests that the displacement of the C-terminus does more than just permit receptor binding. Moreover, an engineered gD mutant designed to force the displacement the C-terminal tail is able to mediate fusion at a low level in the absence of gD receptors (Gallagher et al., 2013), suggesting that displacement of this C-terminal tail is sufficient to trigger the downstream events required for fusion.

A region within the C-terminal tail (residues 261-285) of HSV gD has been termed the "profusion domain" due to its requirement for virus entry (Cocchi et al., 2004a; Zago et al., 2004). Since removal of the profusion domain inhibits virus entry without preventing gD-receptor binding, this region was proposed to serve as a binding site for gHgL and/or gB (Cocchi et al., 2004a; Gianni et al.,

2009). Indeed, antibodies that bind at the profusion domain inhibit cell-cell fusion and virus spread (Du et al., 2017; Lazear et al., 2012; Saw et al., 2015). However, the epitopes of several other anti-gD antibodies that neutralize virus without blocking gD/receptor binding map to regions outside the profusion domain (residues 54 and 67) (Atanasiu et al., 2018; Lazear et al., 2012). Both these neutralizing antibodies and those that bind to the profusion domain were shown to block the interaction between soluble gD and gHgL using surface plasmon resonance (SPR) (Cairns et al., 2019). The epitopes of these antibodies therefore outline a potential gHgL binding region on gD that spans a large region on the face adjacent to, but distinct from, the receptor binding site. In fact, both receptor (either HVEM or nectin-1) and gHgL were able to bind gD simultaneously (Cairns et al., 2019). It is currently unknown if, in the context of the virus, gD must disengage from receptor before its interaction with gHgL in order for the fusion cascade to proceed.

Vaccine potential

gD is a highly immunogenic protein and a prime target for vaccine development (Awasthi et al., 2014; Awasthi et al., 2015; Hook et al., 2018). Humans naturally infected with HSV-1, HSV-2, or both serotypes generate strong antibody responses against gD, that can be type-common or type-specific (Cairns et al., 2015; Cairns et al., 2014). In these individuals, the dominant immune response that results in virus-neutralizing antibodies is due to gD or a combination of gD and gB. Anti-gD antibodies generated from natural infection correspond to three distinct gD epitopes, two involved in receptor binding and one involved in gHgL binding, and correlate with total antibody neutralization activity (Cairns et al., 2014).

Retargeting HSV for oncolytic therapy

Defining the receptor-binding sites on gD has stimulated attempts to detarget HSV from its natural receptors and retarget the virus to novel receptors. By inserting the appropriate natural ligands into the N-terminus of gD, HSV has been retargeted to use the interleukin-13 receptor or urokinase plasminogen activator receptor for entry, instead of nectin-1 or HVEM (Zhou and Roizman,

2006, 2007; Zhou et al., 2002). HSV also has been retargeted to use human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) for entry by mutating the native HVEM or nectin-1 binding sites in gD and inserting receptor-specific single-chain antibodies into gD (Menotti et al., 2008; Tuzmen et al., 2020; Uchida et al., 2010). Supplementing these gD mutations with additional manipulations in other glycoproteins that impact entry, including gHgL, gB, and gK, may further strengthen the oncolytic potential for HSV (Campadelli-Fiume et al., 2016; Okubo et al., 2016; Petrovic et al., 2018). How the insertion of novel ligands into gD successfully recapitulates the triggering that normally occurs when wild-type gD binds a natural receptor is unclear.

Glycoprotein H and glycoprotein L: Fusion regulators

gH and gL form a heterodimer that is essential for both virus-cell and cell-cell fusion (Babic et al., 1996; Desai et al., 1988). This heterodimer is a target of antibodies that neutralize virus or inhibit cell-cell spread (Birlea et al., 2013; Buckmaster et al., 1984; Cairns et al., 2006; Gompels et al., 1991; Montalvo and Grose, 1986; Peng et al., 1998b; Rodriguez et al., 1993; Showalter et al., 1981; Xing et al., 2015). Although its requirement for herpesvirus entry has long been known, its precise role remains a mystery.

gH is a type I transmembrane protein, whereas gL is not membrane-anchored and, in alphaherpesviruses, associates non-covalently with the gH ectodomain. On mature virions and on the surface of HSV-infected cells, gH and gL are found together in a stable 1:1 complex (Peng et al., 1998a). gL expressed by itself is released into the media as a soluble protein (Dubin and Jiang, 1995; Hutchinson et al., 1992; Peng et al., 1998a). In contrast, for most alphaherpesviruses, gH expressed by itself is not transported to the cell surface and remains unfolded and trapped in the ER (Foa-Tomasi et al., 1991; Hutchinson et al., 1992; Klyachkin et al., 2006; Roberts et al., 1991). gH is able to be transported to the cell surface and incorporated into the virion only when co-expressed with gL (Dubin and Jiang, 1995; Hutchinson et al., 1992; Peng et al., 1998a; Roop et al., 1993; Wu et al., 2001). The exception to this rule is

PRV gH, which can be transported and incorporated into virions without gL (Klupp et al., 1997).

Although gL shares little amino acid identity across herpesviruses, gH is more conserved in sequence, and both are required for virus-cell and cell-cell fusion (Duus and Grose, 1996; Duus et al., 1995). Initially, gL was thought to be merely a chaperone or a scaffold protein for gH. In fact, VZV gL contains a putative ER targeting signal at its N terminus instead of the typical signal sequence, a trait shared with ER chaperone proteins. Furthermore, when VZV gH was co-expressed with gE or gI, gH was transported out of the ER and to the cell surface in the absence of gL (Duus et al., 1995). However, this gH was not processed completely and was most likely non-functional. Likewise, a mutant form of HSV-2 gH (with the N-terminal 29 residues of the mature protein deleted) was transported to the cell surface in the absence of gL but was non-functional (Cairns et al., 2007). Co-expression with gL restored function to this gH mutant and these findings suggest that gL plays a role beyond the transport of gH. In support of this concept, several HSV antibodies specific to gL have been isolated that inhibit cell-cell fusion and virus spread (Cairns et al., 2006; Novotny et al., 1996).

gHgL is not a viral fusion protein

Early on, gHgL was speculated to be a viral fusogen, i.e., an active participant in the mixing of virus and cellular membranes. Several labs identified potential fusion peptides within HSV-1 gH based on sequence analysis (Galdiero et al., 2005; Galdiero et al., 2007; Galdiero et al., 2006; Gianni et al., 2005a; Gianni et al., 2005b; Lopper and Compton, 2004). HSV-1 virions inactivated by neutralizing anti-gH antibodies or through the deletion of gH were reported to attach to cells and form a fusion bridge (Fuller and Lee, 1992). Although these viruses were unable to expand the bridge to allow entry, partial infectivity could be restored to gH-null virions if a fusogenic agent was added to the cell medium (Babic et al., 1996; Forrester et al., 1992; Roop et al., 1993). In VZV, gHgL alone, produced from transfected cells, mediated low levels of cell-cell fusion (Duus et al., 1995).

The proposed viral fusogen role for gHgL was refuted when the first structure of gHgL was determined (Chowdary et al., 2010). This structure of a soluble form of the HSV-2 gH ectodomain in complex with gL did not resemble any known fusogen. Although many helices within the core of gH formed helical bundles, the trimeric hairpin bundle characteristic of fusion proteins was absent from the gHgL structure. In fact, all of the previously hypothesized fusion peptides and heptad repeats were buried in the core of the protein and were unlikely to promote any significant conformational changes (Chowdary et al., 2010; Xing et al., 2015).

gHgL structures

The structure of HSV-2 gHgL revealed an extensive interaction between the two proteins that explained their interdependence for folding, transport, and function. gHgL has the overall shape of a "boot" (Figure 3A), which fits with earlier cryo-electron tomography studies of HSV-1 virions (Grunewald et al., 2003). gH itself is split into three domains: H1, which encompasses the N-terminus and the gL-binding domain; H2, the helical central domain; and H3, the C-terminal β-sandwich "toe" of the boot and the domain closest to the transmembrane anchor (TM). The sequences of gH domain H1 and of gL vary substantially among herpesviruses, probably due to their co-evolution together as a tight-knit binding pair (Chowdary et al., 2010). The N-terminal domain of gH lacks a folded core and requires gL for proper folding and stabilization.

The crystal structure of a soluble form of VZV gHgL was also solved and bears a striking resemblance to that of HSV-2 (Figure 3B) (Xing et al., 2015). In addition, the structure of a core fragment of PRV gH (lacking gL and H1) was solved in complex with a FAb (Figure 3C) (Backovic et al., 2010; Vallbracht et al., 2019). Both the VZV and PRV structures adopt very similar folds to that of HSV-2, especially in domains H2 and H3, which are the most highly conserved in their amino acid sequence. However, whereas HSV-2 and VZV gHgL are boot-shaped, the PRV gH C-terminal fragment adopts a more cylindrical shape, in which the H2 and H3 domains are aligned in the same plane. The

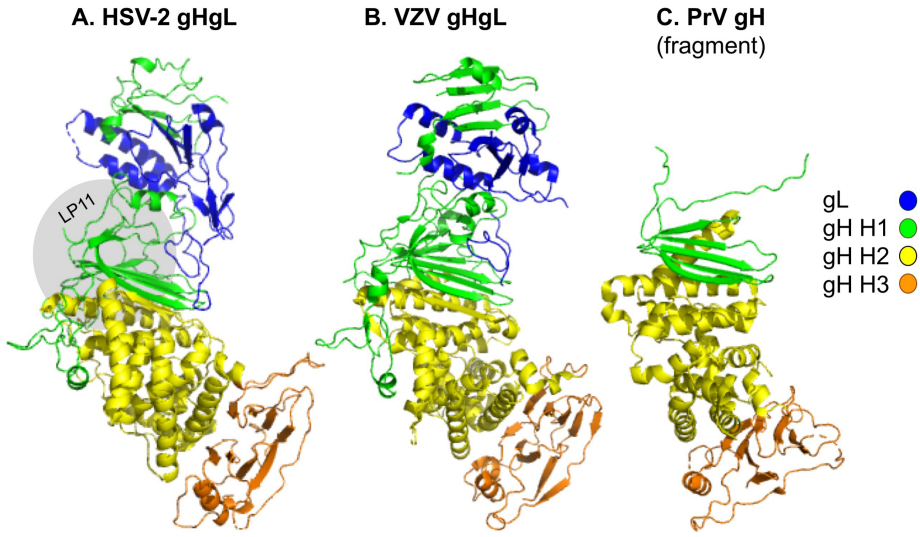

Figure 3. gHgL structures. (A) The crystal structure of the HSV-2 gHgL heterodimer (PDB 3M1C) (Chowdary et al., 2010). The structure is colored to define gL (blue) and three domains of gH, including the gL-interacting domain H1 (green), the central helical domain H2 (yellow) and the C-terminal β-sandwich domain H3 (orange). The gH domains are designated as originally defined in the HSV-2 gHgL structure. The tight association of gL with the N-terminal domain of gH provide a structural explanation for the requirement of gL for proper gH folding. The epitope for the neutralizing MAb LP11 (shaded gray) maps to domains H1 and H2, on the back of the heterodimer, and highlights a possible gB binding site. (B) The crystal structure of the VZV gHgL heterodimer (PDB 4XHJ) (Xing et al., 2015). The domains are colored as in part A. Both the HSV-2 and VZV structures adopt a boot shape. (C) The crystal structure of a fragment of PRV gH (PDB 2XQY) (Backovic et al., 2010). The domains are colored as in part A. The N-terminus of gH was absent from the protein crystallized and the core fragment of gH was co-crystallized with a Fab.

cylindrical shape of PRV gH more closely resembles that of the gammaherpesvirus EBV gHgL heterodimer (Matsuura et al., 2010). This difference in shape among alphaherpesvirus gHgL (boot vs. cylinder) may be due to the PRV structure lacking gL or being bound to a FAb or may it indicate that flexibility exists between the domains.

The PRV gH structure highlighted the existence of an extended "flap" in domain H3, near where the ectodomain would transition to the TM of the protein and encounter the viral membrane (Backovic et al., 2010). This flap covers a conserved hydrophobic patch and the movement of this flap through isomerization of conserved disulfide bonds was hypothesized to expose the hydrophobic patch and allow it to interact with the lipid membrane (Backovic et al., 2010). In contrast, mutational analysis of this region in both PRV and VZV suggests that this "flap" needs to be rigid, not flexible (Fuchs et al., 2012; Vleck et al., 2011). Nevertheless, mutations in gH that abrogate or impair fusion typically map to domain H3 (Cairns et al., 2005; Galdiero et al., 1997; Jackson et al., 2010), highlighting its importance in membrane fusion and entry. This domain is also the most highly conserved, which implies a functional importance.

The gH TM and short cytoplasmic tail (CT) also contribute to gHgL function. Although a soluble form of the HSV gHgL ectodomain is able to trigger fusion of cells expressing gB, gD, and a gD receptor, the efficiency of fusion is lower, suggesting a role for the gH TM and/or CT in this process (Atanasiu et al., 2010a). Indeed, replacement or deletion of certain residues within the gH CT of HSV inhibit fusion (Browne et al., 1996; Jackson et al., 2010; Rogalin and Heldwein, 2015; Silverman and Heldwein, 2013; Wilson et al., 1994). gHgL anchored using a heterologous TM or a GPI-anchor is nonfunctional (Harman et al., 2002; Jones and Geraghty, 2004). In PRV, removal of the gH CT results in decreased membrane fusion activity, while deletion of the TM completely abolishes function (Vallbracht et al., 2018). Conversely, mutations in the VZV gH CT have been shown to enhance fusion (Yang et al., 2014).

gHgL regulates viral fusion

The crystal structure and the functional data indicate that gHgL is a fusion regulator. The core entry glycoproteins from HSV-1 and HSV-2 can be functionally swapped in the context of cell-cell fusion of receptor-bearing cells transfected with these four glycoproteins (Atanasiu et al., 2016; Muggeridge, 2000). These experiments demonstrate that the rate of cell-cell fusion is higher

for HSV-2 than HSV-1 and that the gHgL serotype is a rate-limiting factor (Atanasiu et al., 2016). Similarly, several HSV-1 gH insertion mutants display a slow-fusing phenotype (Jackson et al., 2010). The current model proposes that, upon receiving a signal from gD after receptor binding, gHgL regulates the transition of the gB fusion protein from a prefusion to a fusion-activated state (Chowdary et al., 2010). HSV gHgL and gB interact only when gD and a gD receptor are also present (Atanasiu et al., 2007) (see below), suggesting that gB and gHgL are not normally associated and only come into contact when fusion is triggered. In addition, the CT of HSV gH has been implicated in regulating fusion through an interaction with the gB cytodomain (Cooper et al., 2018; Rogalin and Heldwein, 2015). All current models of how gHgL regulates the fusogenic activity of gB involve a direct interaction between the three proteins, but capturing this multi-protein complex has proven challenging.

gHgL as a receptor-binding protein

In beta- and gammaherpesviruses, gHgL, either alone or in complex with other viral proteins, binds to cellular receptors (Chen et al., 2018; Chen et al., 2019; Chesnokova and Hutt-Fletcher, 2011; Chesnokova et al., 2009; Hahn et al., 2012; Nishimura and Mori, 2019; Santoro et al., 2003; Wang and Shenk, 2005). Although most alphaherpesviruses use gD as a receptor binding protein, gHgL may bind its own cellular ligand(s) as well during entry. In VZV, which lacks gD, cell-cell fusion mediated by gB and gHgL is reduced when av integrin expression is knocked down with siRNAs or when these integrins are bound by antibodies, suggesting an interaction between the viral glycoproteins and integrins (Yang et al., 2016). Interestingly, the canonical RGD motif and the disintegrin-like domain, both shown to bind integrins, are absent from VZV gB and gHgL, suggesting that any potential interaction between integrins and the VZV core entry glycoproteins would occur through an as yet unidentified motif

Three different types of integrins have been shown to bind to HSV gH: αvβ3, αvβ6, and αvβ8 integrins (Gianni et al., 2013; Parry et al., 2005). αvβ3 integrin binds HSV gHgL with low affinity and is proposed to serve as a "routing factor" because its silencing altered the route of virus infection but did not inhibit

infection (Gianni and Campadelli-Fiume, 2012; Parry et al., 2005). Both αvβ6 and αvβ8 integrins bind gHgL with high affinity and contribute to the attachment of virus to the cell surface although they are less important for viral attachment than heparin sulfate (Gianni et al., 2013). Furthermore, the integrins could not substitute for the gD receptor nectin-1. αvβ6 and αvβ8 integrins have been proposed to act as additional HSV receptors that influence the pathway of HSV entry. The integrins may act as additional "triggers" for gHgL, to determine when and where to signal gB to mediate fusion.

Glycoprotein B: The viral fusogen

Viral fusogens are proteins that execute the final membrane fusion step of viral entry by inserting into a target cell membrane and refolding to bring the viral and cell membranes together (White et al., 2008). Fusogens are initially present in a metastable prefusion conformation that, upon triggering, rearranges into an extended form that inserts hydrophobic residues into the target cell membrane. This extended conformation then refolds into a stable hairpin-like postfusion conformation to bring the membrane-inserted region into proximity with the membrane anchor of the protein, thereby facilitating fusion of the two membranes.

Crystal structures of the gB ectodomain

gB is conserved in all herpesviruses and required for viral entry (Cooper and Heldwein, 2015). Crystal structures of the ectodomains of several gB homologs have been determined, including those from the alphaherpesviruses HSV-1 (Cooper et al., 2018; Heldwein et al., 2006) and PRV (Li et al., 2017b; Vallbracht et al., 2017), as well as from the betaherpesvirus cytomegalovirus (CMV) (Burke and Heldwein, 2015; Chandramouli et al., 2015) and the gammaherpesvirus Epstein-Barr virus (Backovic et al., 2009). These structures provide the strongest evidence that gB is a viral fusogen. gB shares structural similarity with the fusogens from rhabdoviruses (Baquero et al., 2015; Belot et al., 2020; Roche et al., 2006; Yang et al., 2020), baculovirus (Kadlec et al., 2008), and Thogotovirus (Peng et al., 2017) (Figure 4). This structural similarity is remarkable given a lack of sequence similarity among these viral fusogens.

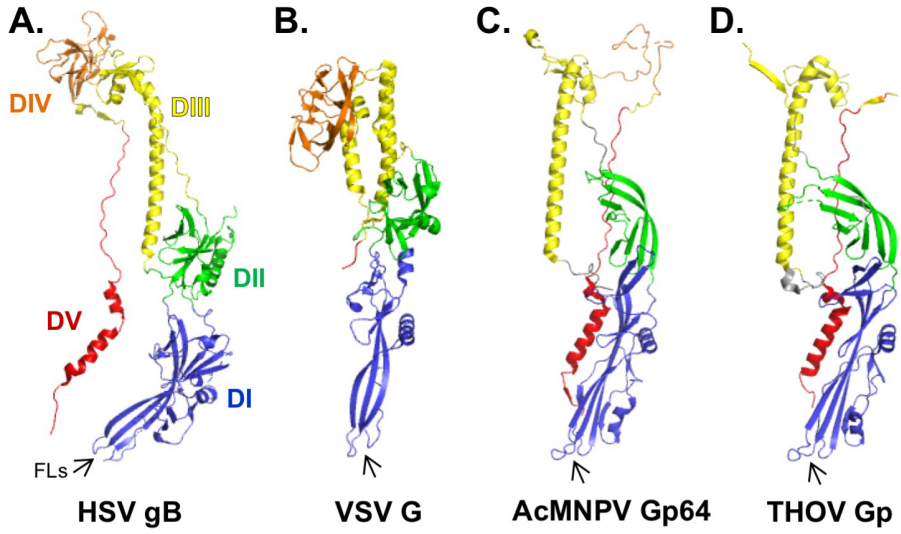

Figure 4. Class III fusogens. (A) The crystal structure of the HSV-1 gB monomeric ectodomain is shown (PDB 2GUM) with each structural domain (DI-DV) colored (Heldwein et al., 2006). The location of the fusion loops (FLs) is indicated with an arrow. The crystal structures of rhabdovirus VSV G (B) (PDB 512M) (Roche et al., 2006), baculovirus Autographa californica nucleopolyhedrovirus Gp64 (C) (PDB 3DUZ) (Kadlec et al., 2008), and Thogotovirus Gp (D) (PDB 5XEA) (Peng et al., 2017) monomeric ectodomains are shown in ribbon diagram, with domains colored as in part A. The VSV G structure is missing domain V, while domain IV was unresolved in Thogotovirus Gp. All four fusion proteins are positioned with their fusion loops pointing down (arrows).

Together, these proteins represent class III of fusogens (Backovic and Jardetzky, 2009). Structures of both the prefusion and postfusion forms of the rhabdovirus fusion protein G from vesicular stomatitis virus (VSV) and rabies virus have been determined, an accomplishment facilitated by the ability of G to undergo reversible, pH-dependent conformational changes (Roche et al., 2007; Yang et al., 2020). Structural similarity to G indicates that the gB crystal structures represent the postfusion conformation.

gB is a trimeric type I transmembrane protein, with a large ectodomain, a single transmembrane domain (TM), and a cytoplasmic tail domain (CT). The gB ectodomain is organized into five domains (Figure 5A) (Heldwein et al.,

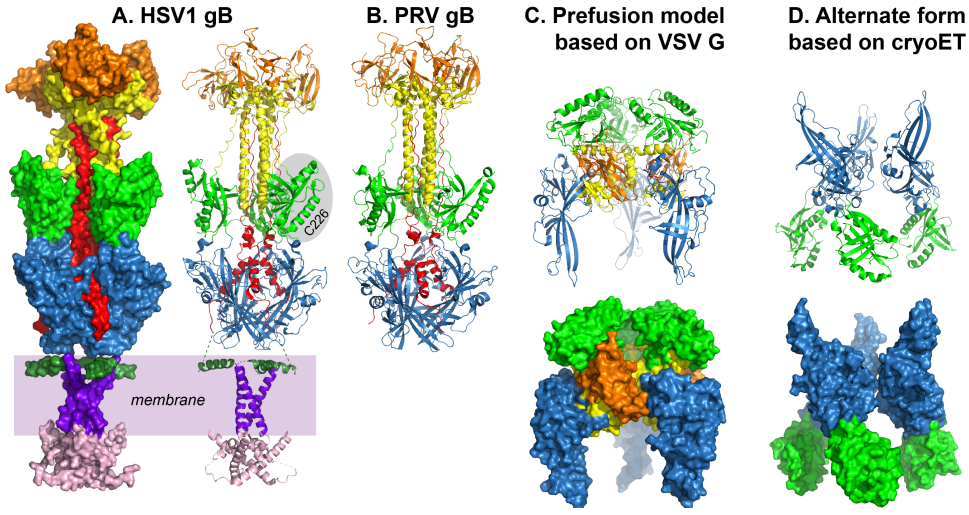

Figure 5. gB structures. (A) The crystal structure of full-length HSV-1 gB is shown, with surface rendering on the left and a ribbon diagram on the right (PDB 5V2S) (Cooper et al., 2018). This structure represents the postfusion conformation and the presumed position of the membrane is indicated by a purple box. Five domains of the ectodomain are colored, including domains I (blue), II (light green), III (yellow), IV (orange), and V (red). C-terminal to the ectodomain, the membrane-proximal region (MPR, dark green), a single-pass transmembrane domain (TM, purple) and cytoplasmic domain (CT, pink) are shown. The fusion loops are located at the tip of domain I, in contact with the membrane. Domain V extends the length of the molecule and packs against the central coiled-coil of domain III before descending into the MPR. The MPR lies parallel to the membrane and the single-pass TM forms an inverted teepee beneath the structure. The CT has trimeric contacts that may influence the activation of gB. The epitope for the neutralizing MAb C226 (shaded gray) maps to domain II (Atanasiu et al., 2010b) and highlights a possible binding site for gHgL. (B) The crystal structure of PRV gB ectodomain is shown in ribbon diagram, with domains colored as in part A (PDB 6ESC) (Vallbracht et al., 2017). The structural similarity between HSV-1 and PRV gB is apparent. (C) A working model of prefusion HSV-1 gB is shown, as a ribbon diagram at the top and surface rendering at the bottom (Gallagher et al., 2014). This model was generated by fitting the domains from the postfusion gB structure to the prefusion crystal structure of VSV G (PBD 2J6J) (Roche et al., 2007). The model presumes that the gB prefusion-to-postfusion conformational change is comprised primarily of tertiary structure rearrangements. The model predicts a break in the extended coil of domain III and domain V is absent because it is not present in the VSV G structure. (D) A model of an alternative conformation of gB, based on cryoET, is shown (Zeev-Ben-Mordehai et al., 2016). Fitting domains I and II from the postfusion gB structure into this form orients the fusion loops away from the membrane. Domains III-V from the postfusion structure were not placed into this form and the structure would not accommodate the intact extended coil of domain III.

2006). Domain I, the "fusion domain", is located at the base of the ectodomain, near the membrane. This domain contains a pleckstrin-homology domain along with two adjacent beta-hairpin extensions that present hydrophobic residues at their tips that can insert into membranes ("fusion loops") (Falanga et al., 2012). Mutational studies demonstrate that the hydrophobic residues in the fusion loops are essential for the gB fusogenic function (Backovic et al., 2007; Hannah et al., 2009; Oliver et al., 2009; Vallbracht et al., 2017). Structural domain II contains a second pleckstrin-homology domain.

The core of gB consists of a long central helix (domain III). In the gB trimer, three helices twist around one another to form an extended coiled-coil (Figure 5A). An extended polypeptide in domain V spans the length of the molecule and packs against the coiled-coil. Domain V from one protomer packs against domain III of two other protomers in an anti-parallel orientation. The packing of domain V against the central coil is analogous to a six-helix bundle, a structural feature that is present in the postfusion form of class I fusogens. Formation of this very stable, low-energy structure has been proposed to contribute energy for membrane fusion (Melikyan et al., 2000). Mutations that are predicted to disrupt the interaction between domains III and V reduce both the rate of viral entry and the extent of cell-cell fusion (Connolly and Longnecker, 2012; Fan et al., 2017). In addition, peptides that include sequences from domain III or V have been shown to inhibit viral entry (Akkarawongsa et al., 2009), supporting the notion that these regions are exposed at some point during conformational rearrangements and come together as gB folds into its final postfusion conformation.

The C terminus of domain V threads through domain I (Figure 5A). In the full-length protein, domain V would then lead into the membrane proximal region (MPR), TM and CT. The close juxtaposition of the C terminus of domain V and the fusion loops of domain I in the postfusion form of gB explains how folding to the postfusion form could bring the two membrane-anchored portions of the protein into proximity, similarly to the postfusion forms of fusogens from other classes (Harrison, 2015).

Transmembrane and cytoplasmic domains of gB

For gB crystallization, removal of the MPR, TM, and CT to create a soluble ectodomain presumably resulted in spontaneous folding into a postfusion form. The TM and/or CT appear to be critical for maintaining the prefusion form of gB, as has been observed for some class I fusogens (Yin et al., 2005; Yin et al., 2006). This postfusion gB structure is remarkably stable. Mutants designed to capture an alternate conformation of gB, by substituting the TM with a trimeric domain or by adding mutations known to prevent fusion, still adopt the postfusion form (Silverman et al., 2010; Vitu et al., 2013).

The crystal structure of the full-length gB, including the MPR, TM, and CT, was determined recently (Figure 5A, B) (Cooper et al., 2018). The ectodomain of this full-length molecule adopted a postfusion conformation, likely due to detergent solubilization. The MPR lies beneath the ectodomain, parallel to the membrane, the TM forms an inverted teepee, and the CT forms a trimeric pedestal. The gB CT is significantly longer than the CT of other class III fusogens, and mutational studies indicate that it plays a crucial role in fusion. Some mutations in the CT inhibit fusion (Cai et al., 1988; Wanas et al., 1999) whereas others can enhance fusion and/or impart a syncytial phenotype (Baghian et al., 1993; Diakidi-Kosta et al., 2003; Engel et al., 1993; Fan et al., 2002; Foster et al., 2001; Gage et al., 1993; Heineman and Hall, 2002; Muggeridge et al., 2004; Oliver et al., 2013; Silverman et al., 2012). Mapping known gB mutations to this gB CT structure suggests that this structure is present in the prefusion form of the protein. The structural and mutagenic data together suggest that the CT stabilizes the prefusion form of gB, interacting with the membrane and acting as a clamp to control the refolding of the protein. The CTs of other fusion proteins impart a similar regulation of fusion function (Waning et al., 2004; Wyss et al., 2005).

Alternate conformations of the gB ectodomain

For gB, only the structure of the postfusion form is known. Computational homology models of the prefusion gB have been generated using the prefusion

structure of VSV G (Figure 5C) (Backovic et al., 2009; Gallagher et al., 2014). These models predict that the extended helix of the domain III core is partially unfolded in the prefusion form while domain V could not be modeled because it is absent from the VSV G structure.

Recently, cryo-electron tomography (cryoET) reconstructions of membrane-anchored HSV-1 gB by two research groups have revealed alternative compact forms of gB with trimeric symmetry (Fontana et al., 2017; Zeev-Ben-Mordehai et al., 2016). In the first study, gB was visualized on the surface of extracellular vesicles produced in cells overexpressing HSV-1 gB (Zeev-Ben-Mordehai et al., 2016). Two distinct conformations of gB were detected, one that matched the known postfusion form and the other, a more compact, novel trimeric form, which could represent prefusion gB or an intermediate form between pre- and postfusion. Fitting domains I and II from the postfusion gB structure into the compact form positioned domain I distal to the membrane, with the fusion loops facing away from the membrane (Figure 5D). Interestingly, this fitting resulted in the distances between the fusion loops of each gB protomer being similar to the distances between fusion loops in the prefusion VSV G. Domains III-V did not fit into the model, potentially due to structural rearrangements in those domains.

The second cryoET study used landmarks to orient gB during imaging of full-length gB on vesicles, including large insertions of fluorescence proteins at known sites in gB and antibodies with known binding sites on gB (Fontana et al., 2017). Proper folding of gB on these vesicles was confirmed using a panel of antibodies specific for conformational epitopes, suggesting that this gB is in the prefusion conformation. Using these landmarks, the computational model of HSV gB based on prefusion VSV G (Gallagher et al., 2014) was fit into the structure with the fusion loops oriented towards the membrane (Figure 5C). A recent cryoET study of CMV gB also oriented the fusion loops towards the membrane (Si et al., 2018). Although these cryoET studies propose different orientations of gB with respect to the membrane, the studies all reveal alternate membrane-anchored forms of gB that, at heights of 8-10 nm, are

significantly shorter than the 16-18-nm postfusion form. Both models also predict extensive conformational changes in domains III and V during the transition from a prefusion to the postfusion conformation.

While comparisons to the prefusion VSV G structure provide a valuable working model for the prefusion gB structure, the prefusion form of gB likely differs from that of VSV G. Alphaherpesvirus entry can occur at neutral pH and requires triggering by other entry glycoproteins, whereas triggering of conformational changes in VSV G is pH-dependent, reversible, and does not require other viral glycoproteins (Ferlin et al., 2014; Roche et al., 2008). Interestingly, exposure to low pH has been shown to cause antigenic changes in HSV-1 and HSV-2 gB, some of which are reversible (Cairns et al., 2011; Dollery et al., 2010a; Dollery et al., 2011; Muggeridge, 2012; Nicola, 2016; Siekavizza-Robles et al., 2010; Weed et al., 2018). Ultimately, a detailed understanding of the prefusion form of gB, including the organization of domains III and V, will require determination of the structure at atomic resolution.

Functional regions across gB

HSV gB elicits neutralizing antibodies (Cairns et al., 2015; Cairns et al., 2014), but all of the antibodies that have been tested can bind to the postfusion conformation (Bender et al., 2007), suggesting that these MAbs do not inhibit fusion by trapping a prefusion conformation. Neutralizing MAbs map to different domains within the gB ectodomain, indicating that multiple regions of gB are critical for proper refolding during fusion and/or interactions with other glycoproteins. Likewise, insertion mutations in each of the gB domains can prevent fusion function (Lin and Spear, 2007). By mapping the epitopes for the neutralizing antibodies, four distinct functional regions (FR) on the surface of HSV gB were defined (Bender et al., 2007). FR1 lies at the base of the postfusion structure and includes the fusion loops and regions of both domain I and V. FR2 maps to domain II and may overlap a gHgL interaction site (see below). FR3 is located on the gB crown and includes regions of domain III and IV. Finally, FR4 maps to the N-terminal region of gB that was not resolved in

the structure and includes the binding site for heparan sulfate proteoglycan (HSPG) (Laquerre et al., 1998).

Receptors for gB

In addition to its role as the fusogen, HSV gB binds several host surface molecules. While gB can bind to HSPG, this interaction is not essential for virus entry. HSV gB can also bind to paired immunoglobulin-like type 2 receptor-α (PILRα), an immune regulator (Fan et al., 2009; Satoh et al., 2008; Wang et al., 2009). PILRα is not required for HSV entry into all cells; however, its expression in the absence of gD receptors can mediate virus entry into cells and low levels of cell-cell fusion. Interestingly, gD must be present for PILRα-mediated fusion to occur. The clinical importance of PILRα has yet to be determined; however, it may contribute to entry into retinal pigment epithelial cells (Shukla et al., 2009) and replication on murine cornea (Arii et al., 2010b). Two additional gB receptors that enhance HSV entry have been described, including myelin-associated glycoprotein (MAG) (Suenaga et al., 2010) and myosin-9 (also called non-muscle myosin heavy chain IIA, NMMHC-IIA) (Arii et al., 2010a). MAG also binds to VZV gB and can enhance VZV entry into cells (Suenaga et al., 2010), although it is not required for infection. The ability of receptors to trigger fusion by binding to gB diverges from the current model of entry, in which a signal for fusion is transmitted to gB by gHgL. Future structural studies of gB complexed with these receptors may reveal a mechanism of activation.

Viral accessory proteins for gB

Although transfection of gD-receptor-bearing cells with the core HSV entry glycoproteins gD, gH, gL, and gB is sufficient for cell-cell fusion, other virally encoded proteins influence fusion in the context of the viral entry. For example, while certain mutations in the gB CT impart a syncytial (syn) phenotype (Cooper et al., 2018; Gage et al., 1993; Silverman et al., 2012), syn mutations also map to other viral genes, including gK, UL20, and UL24 (Dolter et al., 1994; Leiva-Torres et al., 2010). gK and UL20 interact both with gB and with each other (Chouljenko et al., 2010). Although gK is not required for cell-cell

fusion, deletion of the N terminus of gK inhibits entry into neurons in culture and spread to the mouse trigeminal ganglia after ocular infection (Jambunathan et al., 2015; Musarrat et al., 2018). Additional viral proteins also contribute to fusion in potentially complex ways. For example, UL21 is required for the syncytial phenotype of gB syn mutants, but not gK, UL20, or UL24 syn mutants (Sarfo et al., 2017). Other proteins that also have been reported to influence fusion include gE, gI, gM, gN, UL11, UL16, and UL45 (Dollery et al., 2010b; El Kasmi and Lippe, 2015; Haanes et al., 1994; Han et al., 2012; Kim et al., 2013).

Interactions among entry glycoproteins

Detecting interactions

HSV entry glycoproteins can be chemically crosslinked, indicating their proximity on the viral envelope (Handler et al., 1996); however, formation of a stable entry complex prior to entry is unlikely because individual glycoprotein deletions do not disrupt glycoprotein incorporation into the virion (Rodger et al., 2001). HSV gD is reported to co-immunoprecipitate (coIP) with both gHgL and gB (Gianni et al., 2009), and, in one study, the coIP of gD with gHgL was dependent on the presence of receptor (Perez-Romero et al., 2005). A direct association of purified soluble forms of gHgL and gB in the presence of liposomes has be detected at low pH (Cairns et al., 2011). Lateral interactions among gB trimers also have been observed by EM of soluble gB bound to liposomes (Maurer et al., 2013).

Direct interactions among the essential HSV entry glycoproteins have also been examined using bimolecular fluorescence complementation (BiFC), a technique in which two inactive halves of a fluorescent protein, for example, green fluorescent protein (GFP), are fused onto separate glycoproteins. Interaction between the glycoproteins brings the two halves into proximity such that the active fluorescent protein forms. Using BiFC, interactions between all of the essential HSV entry glycoproteins (gD/gB, gD/gHgL, gHgL/gB) have been detected in two independent studies (Atanasiu et al., 2007; Avitabile et al., 2007). In one study, the interaction of gHgL with gB was dependent on the

presence of both gD and its receptor, suggesting a cascade of interactions starting with gD/receptor and ending with gHgL/gB (Atanasiu et al., 2007). Interestingly, although each BiFC construct was functional individually, coexpression of complementary gD and gH BiFC constructs inhibited fusion, suggesting that the gD/gHgL interaction may need to be transient. This was further supported by recent SPR experiments showing that gH/gL binds to gD rapidly and also rapidly dissociates from it (Cairns et al., 2019).

Mapping interactions

Putative interaction sites on the glycoproteins can be mapped by blocking their interactions with neutralizing antibodies that bind to defined epitopes. Using SPR, MAbs that block gD-gHgL binding were used to map the gHgL binding site on gD to a large region adjacent to, but separate from, the receptor binding domain (Cairns et al., 2019). Using BiFC, the gHgL interaction site on gB was mapped to gB domain II, overlapping the epitope for the MAb C226 in FR2 (Figure 5) (Atanasiu et al., 2010b). Similarly, the gB interaction site on gHgL was mapped to a conserved groove in domains H1/H2 of gHgL, overlapping the epitope for the MAb LP11 (Figure 3) (Chowdary et al., 2010).

Interaction sites on glycoproteins can also be mapped by creating chimeric entry glycoproteins that carry regions from non-complementing viral species. By coexpressing various combinations of HSV-1 and saimiriine herpesvirus 1 (SaHV-1) entry glycoproteins, a species-specific functional interaction between gD and gHgL was demonstrated (Fan et al., 2014). By generating gHgL chimeras encoding segments of HSV-1 and SaHV-1 sequence, a gD interaction site within gHgL was mapped to domains H1 and the N terminus of H2, in the membrane-distal region of the gH ectodomain (Fan et al., 2015). Consistent with a model that gD interacts with the N terminus of gHgL, an N-terminally truncated form of HSV gHgL can mediate gD-independent cell-cell fusion (Atanasiu et al., 2013). Taken together, these studies suggest gD may trigger a conformational change in the N-terminus of gHgL.

Species-specific interactions also have been used to map gHgL-gB interactions. Chimeric constructs combining gHgL sequences from HSV-1 and PRV were used to map a species-specific gHgL-gB interaction site to domain H3 at the C terminus of the gHgL ectodomain (Bohm et al., 2016). Interestingly, these chimeric gHgL studies and the BiFC approach identified distinct sites on gHgL that contribute to the gB interaction, in domains H3 and H1/H2, respectively. A potential explanation for this discrepancy is that, in the BiFC studies, MAb binding to H1/H2 may alter the presentation of an interaction site in H3. Conversely, in the chimera studies, domain H3 may influence the presentation of an interaction site in H1/H2.

Defining interactions among the herpesvirus glycoproteins has been challenging, likely due to low-affinity or transient interactions. Using chimeric constructs to map functional interaction sites may reveal domains of a protein that are required for an interaction, but this approach does not exclude the possibility that other regions of the protein also contribute to the interaction. Using neutralizing antibodies to map interactions is complicated by the fact that antibody binding may alter the protein structure and/or block an interaction by steric hindrance. Neither approach can identify the specific residues that mediate glycoprotein interactions. Accurate mapping of HSV glycoprotein interaction sites awaits future studies, in which structures of entry complexes determined by cryoET or x-ray crystallography can be verified using biochemical assays.

Routes of entry

Due, in part, to compelling electron micrographs showing alphaherpesvirus particles fusing directly with the plasma membrane (Granzow et al., 1997), the entry of alphaherpesviruses for a long time was thought to occur at the plasma membrane at a neutral pH. Upon subsequent reassessment, the route of entry into cells for alphaherpesviruses was shown to be dependent on cell type, as for beta- and gammaherpesviruses (Compton et al., 1992; Hutt-Fletcher, 2007; Ryckman et al., 2006). For example, HSV enters keratinocytes, human epithelial HeLa cells, and Chinese hamster ovary (CHO) cells in a pH-

dependent manner via endocytosis (Nicola et al., 2005; Nicola et al., 2003; Nicola and Straus, 2004), whereas HSV fuses directly with the plasma membrane during entry into neurons, keratinocytes, human epithelial Hep2 cells, and Vero cells (Fuller and Spear, 1987; Koyama and Uchida, 1987; Nicola et al., 2005; Rahn et al., 2011; Sodeik et al., 1997; Wittels and Spear, 1991). Similarly, equine herpesvirus (EHV) enters CHO cells and endothelial cells via endocytosis but enters rabbit kidney RK13 cells by fusion at the plasma membrane (Frampton et al., 2007; Hasebe et al., 2009; Van de Walle et al., 2008).

Why endocytosis is required for entry into some cells but not into others is unknown. For entry via endocytosis, the low pH environment of the endosome appears to serve as a trigger for fusion, since agents that disrupt endosomal pH prevent entry (Nicola et al., 2005; Nicola et al., 2003). Low pH can affect gB conformation and oligomeric state, in both reversible and irreversible manners (Cairns et al., 2011; Dollery et al., 2010a; Dollery et al., 2011; Muggeridge, 2012; Weed et al., 2018). Interestingly, there is evidence that gC may selectively facilitate low-pH entry by regulating conformational changes in gB (Komala Sari et al., 2020). VSV G, another class III fusion protein, also undergoes reversible pH-triggered conformational changes (Roche et al., 2007), raising the question of whether gB is triggered similarly to VSV G. Nevertheless, endocytosis appears to contribute more to entry than simply an exposure to low pH, because exposure of HSV to acidic pH fails to trigger the fusion of bound virus with the plasma membrane (Walker et al., 2015). In addition, in some cases, HSV-1 can enter cells via endocytosis without a low pH requirement (Milne et al., 2005).

Discussion

Recent work, driven in large part by structural studies of the core entry machinery, has improved our understanding of the stepwise mechanism for alphaherpesvirus entry into cells (Figure 1B). The identification of entry receptors has shown that nectin-1 serves as a common receptor that is critical for infection. The biological relevance of the receptors that bind to gB, the HSV

receptor HVEM, and the potential entry receptors for VZV (Chen et al., 2004; Suenaga et al., 2015; Suenaga et al., 2010; Yang et al., 2016) is yet unclear. The comparison of several gD structures indicates that the C terminus of the gD ectodomain shifts upon receptor binding. Future studies will determine how this shift exposes a gHgL interaction site as well as map the precise location of this site, be it within the gD C terminus or within the gD core.

gHgL structures from three different alphaherpesviruses indicated that this essential entry glycoprotein complex is not a viral fusogen and allowed the design of structure-based experiments to dissect its function and sites of interaction. How the gHgL conveys a fusion signal from gD (and/or receptor) to gB has not yet been determined. gHgL could, potentially, adopt multiple conformations, including a native, pre-triggered conformation, a gD-activated conformation, and a post-triggered conformation.

To date, all of the gB variants isolated for crystallographic studies, including those containing intact TM and CT, adopted the postfusion conformation (Cooper et al., 2018; Vitu et al., 2013). CryoET of membrane-associated gB reveals compact forms of gB with trimeric symmetry that may represent a prefusion or an alternative conformation (Fontana et al., 2017; Zeev-Ben-Mordehai et al., 2016). These compact structures cannot accommodate the extended coil of domain III, seen in the postfusion form, indicating that gB undergoes extensive conformational rearrangement to mediate membrane fusion. Structural and mutagenic data suggest that the gB CT acts as a clamp, regulating gB activation.

Future trends

Despite recent advances, many questions regarding the entry of alphaherpesviruses remain. Structural approaches, including both crystallography and cryoET, will continue to prove indispensable for addressing the remaining questions in alphaherpesvirus entry. CryoET in particular is being employed more frequently to examine the structures of individual proteins. Results from these structural studies will provide the basis for

hypotheses that can be tested using mutagenesis and functional assays of entry and membrane fusion.

What do the complexes of gD/gHgL and gHgL/gB look like? What surfaces of the proteins serve as contact sites? Stable association of these complexes will be required for structural studies, a notable disadvantage for the alphaherpesviruses compared to other herpesviruses (Ciferri et al., 2015; Sathiyamoorthy et al., 2016). Identification of antibodies and/or mutations that stabilize potentially transient conformations of these proteins may facilitate structural studies of the complexes.

Why is gHgL required for the entry of herpesviruses? Viruses from most other families use a single protein to mediate both receptor-binding and membrane fusion. Paramyxoviruses encode separate receptor-binding and fusion proteins; however, they do not require an additional protein to serve as a "bridge" between the receptor-binding protein and fusion protein. How exactly does gHgL trigger gB? Although a purified form of the gHgL ectodomain can trigger fusion at low levels (Atanasiu et al., 2010a), mutational studies indicate that the gH TM and short CT domains are critical for function (Browne et al., 1996; Harman et al., 2002; Wilson et al., 1994). Do interactions between the gH CT and gB CT influence gB triggering (Cooper et al., 2018; Rogalin and Heldwein, 2015; Silverman and Heldwein, 2013)? In addition, does gHgL have any direct impact on the cellular or viral membranes at the site of fusion? What additional roles does gHgL play during VZV entry?

What conformational changes are required for and/or triggered by complex formation among the entry glycoproteins? Does gHgL adopt multiple distinct conformations as it interacts with gD and gB? What does the prefusion form of gB look like and where are the fusion peptides positioned in this form? What intermediate conformation(s) does gB fold into as it transitions from its prefusion to postfusion form? Can an extended form of gB, embedded in both the cellular and viral membranes, be captured? Defining the conformational changes required for entry may highlight regions susceptible to inhibition by

small molecules, offering tools to trap intermediate conformations for further study and potentially providing a basis for the development of antiviral drugs that inhibit virus entry.

Web resources

Structures of the alphaherpesvirus entry glycoproteins can be accessed from the protein data bank (https://www.rcsb.org). These crystal structures include unliganded HSV-1 gD (1L2G, 2C3A, and 2C36) (Carfi et al., 2001; Krummenacher et al., 2005), HSV-1 gD bound to HVEM (1JMA) (Carfi et al., 2001), HSV-1 gD bound to nectin-1 (3SKU and 3U82) (Di Giovine et al., 2011; Zhang et al., 2011), HSV-2 gD (4MYV), HSV-2 gD bound to nectin-1 (4MYW) (Lu et al., 2014), PRV gD (5X5V), PRV gD bound to nectin-1 (5X5W) (Li et al., 2017a), HSV-2 gHgL (3M1C) (Chowdary et al., 2010), PRV gH (2XQY) (Backovic et al., 2010), VZV gHgL (4XHJ) (Xing et al., 2015), postfusion forms of HSV-1 gB (2GUM, 2NWF, and 5V2S) (Cooper et al., 2018; Heldwein et al., 2006; Stampfer et al., 2010), and PRV gB (6ESC and 5YS6) (Li et al., 2017b; Vallbracht et al., 2017). A model of membrane-anchored HSV-1 gB generated using cryoET also is available (5FZ2) (Zeev-Ben-Mordehai et al., 2016).

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