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Review

Entry of herpesviruses into mammalian cells

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Abstract. The mechanism that herpesviruses use to enter cells is one of the most complex viral entry mechanisms studied so far. This complexity seems to mount as new participants, both cellular receptors and viral glycoproteins, are identified. Recent structural work on entry glycoproteins gD and gB from herpes simplex virus (HSV) 1 has illuminated the functional roles of these glycoproteins in the process of entry. In doing so, it provided information on the mechanism of two critical steps of HSV entry: receptor-mediated activation and membrane fusion. Remarkably, it is becoming clear that herpesviruses have a lot in common with other, simpler viruses.

Keywords. Viral entry, glycoprotein, structural study, receptor, membrane fusion.

Introduction

Herpesviruses are a large and diverse family of enveloped viruses composed of three subgroups, alpha, beta, and gamma. Virions consist of a large, double-stranded DNA genome packaged into an icosahedral capsid, which, in turn, is encased in a layer of proteins called tegument and an envelope composed of a large number of glycoproteins embedded in a lipid bilayer.

Herpesviruses can infect a diverse range of hosts but only eight herpesviruses are known to infect humans. These human herpesviruses include the alphaherpesviruses, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV); the betaherpesviruses, cytomegalovirus (CMV) and human herpesviruses 6 and 7 (HHV-6 and HHV-7); and the gammaherpesviruses, Epstein-Barr virus (EBV) and human herpesvirus 8 or Kaposi's sarcoma herpesvirus (HHV8/KSHV). All herpesviruses infect their hosts for life; they establish latency in cells whereby the virus lies dormant until reactivated – awakened – by an environmental cue. Herpesviruses cause a number of diseases ranging from those that are fairly non-threatening, such as oral and genital sores, to more serious conditions, such as cancer and encephalitis.

General features of herpesvirus entry

Like other enveloped viruses, to enter a target cell, herpesviruses must fuse their lipid membrane – envelope – with the lipid membrane of the cell. But unlike most other enveloped viruses, which use one or two glycoproteins to effect entry, herpesviruses require at least three conserved glycoproteins plus, in some cases, an additional receptor-binding glycoprotein (Fig. 1). Although herpesviruses vary in the type of cell receptors to which they bind, they use a

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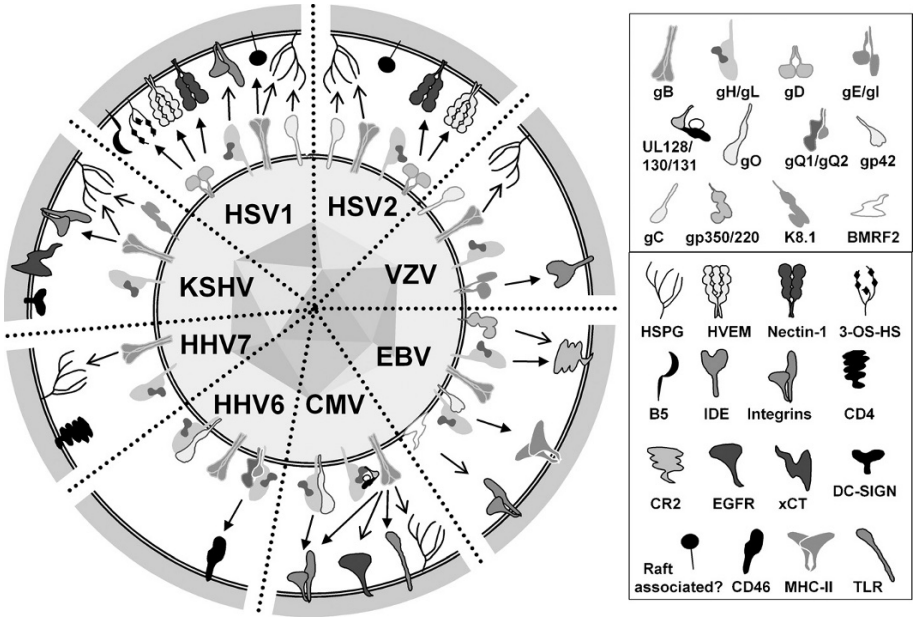

Figure 1. Viral glycoproteins (inner circle), cell surface molecules (outer circle), and interactions involved in entry of human herpesviruses. Interactions resulting in initial attachment are indicated by open arrowheads, and interactions with entry receptors, by filled arrowheads. For entry to occur, all the documented interactions shown in this summary cartoon do not necessarily take place on the same cell or at the same time.

remarkably conserved fusion mechanism. This review will provide an overview of the field of herpesvirus entry with emphasis on recent discoveries and the outlook for future work.

Entry of alphaherpesviruses

HSV-1 and HSV-2 most commonly cause oral and genital lesions, respectively, but can also cause keratitis and encephalitis. VZV causes skin lesions during primary infection (chicken pox) and zoster (shingles) upon reactivation. Neurotropic alphaherpesviruses establish latency in sensory neurons of the host and replicate in epithelial cells during primary infection and reactivation [1]. Virions first attach to target cells using glycoprotein C (gC) to bind the heparan sulfate moieties of the cell surface proteoglycans (HSPGs) [2, 3]. gC is not essential for entry, and in its absence, gB can mediate the attachment step by binding HSPGs [4]. In HSV, mutations of the putative HSPG-binding sites of gC and gB abolish the high-affinity interaction with HSPG and decrease the efficiency of entry [5, 6]. The envelope glycoprotein D (gD) is specific to alphaherpesviruses and is essential for entry of most of them [7]. Binding of a cell surface receptor to gD is a necessary step for entry of HSV-1, HSV-2, and multiple animal alphaherpesviruses. The most widely used gD receptors belong to the nectin family. Nectin-1 is used by human HSV-1 and HSV-2 as well as by porcine pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1) [8]. Nectin-2 is a receptor for PRV and some mutant forms of HSV-1 and HSV-2 [9]. Human nectin-like-5, the poliovirus receptor, is used by PRV and BHV-1 [8]. In addition to nectins, two other unrelated gD receptors allow entry of HSV. First, herpesvirus entry mediator (HVEM), which belongs to the tumor necrosis factor receptor family, is used by HSV-1 and HSV-2 [10]. Second, heparan sulfate specifically modified by 3-O-sulfotransferases, 3-O-HS, can mediate entry of HSV-1 [11]. These various molecules bind gD independently and do not act as co-receptors during entry. Nectin-1 and HVEM are both used by all the tested clinical strains of HSV-1 and HSV-2, regardless of the origins of the virus (e.g. oral and genital lesions, encephalitis, and disseminated herpes of neonates) [12]. This observation suggests that both nectin-1 and HVEM are involved in HSV infection and pathogenesis in humans.

In the host, alphaherpesviruses disseminate mostly by spreading from cell to cell. During HSV spread, the requirements for the gD interaction with its receptor parallel those involved in entry of free virions [13–15]. In contrast, gD is required for entry of the wild-type PRV particle but is dispensable for spread [16, 17]. In the case of VZV, which does not have gD, glycoprotein E (gE) acts as the essential receptor-binding glycoprotein. The human insulin degrading enzyme (IDE) is an entry receptor for VZV and binds the gE ectodomain to mediate entry into cultured cells [18, 19]. VZV remains strongly cell associated and mostly spreads directly from cell to cell. HSV also expresses gE that, while enhancing HSV cell-to-cell spread, is not required for entry of free virions [20, 21]. Interestingly, gD and gE are thought to derive from duplicated genes [22], and both structures are organized around an IgV fold [23, 24].

The core fusion machinery of alphaherpesviruses, as of all herpesviruses, is composed of gB and gH/gL. gL, which lacks a transmembrane region, forms a hetero-

dimer with gH [25, 26]. In HSV, gL is required for expression of gH on the cell surface [26] and is also involved in gH function [27]. For function, alphaherpesvirus gB and gH/gL need a receptor-binding protein, gD or gE, along with a cognate receptor. In the case of HSV, gD, gB, gH/gL, and a gD receptor are necessary and sufficient to cause cell fusion [28–30]. Additional proteins have been reported to intervene during alphaherpesvirus entry. On the viral side, gM [31, 32] and gK [33, 34] modulate fusion of HSV and PRV. On the cellular side, a lipid-raft-associated receptor has been proposed for HSV gB [35, 36], and integrin αVβ3 has been shown to bind directly to gH/gL [37]. An orphan receptor (named B5) has also been shown to enhance HSV entry into porcine cells [38]. However, the precise roles of these receptors in entry remain to be defined.

Entry of betaherpesviruses

Three human betaherpesviruses, CMV, HHV-6, and HHV-7, cause multiorgan infections in immunocompromised patients following reactivation of latent virus. As the major envelope constituent, gB is involved both in attachment to cells, by binding HSPG, and in fusion [39]. In the case of CMV, gB is proteolytically processed to adopt the SU-TM topology of receptor-binding/fusion proteins found on other families of viruses [40]. CMV gB binds to various integrins, via a conserved disintegrin-like domain, or to epidermal growth factor receptor (EGFR) to promote entry [41–43], and gH/gL can bind to integrins [44]. Both gB and gH/gL are required for CMV entry, although in at least one report, cell-cell fusion was observed in the presence of gH/gL alone, without gB [45]. gH and gL associate with a large, heavily glycosylated viral glycoprotein gO to facilitate CMV entry into fibroblasts [46]. Recently a number of genes that are absent from the genome of laboratory-acclimated strains of CMV were found to be important for entry into endothelial and epithelial cells [47–51]. Three of these proteins, UL128, UL130, and UL131, bind gH/gL to form a quinary complex that functions in entry into these cells [52]. These accessory proteins may promote a transient interaction between gH/gL and gB to induce membrane fusion [53].

In HHV-6, gH/gL is associated with either gO or gQ1/gQ2 [54, 55]. Complexes containing gO or gQ1/gQ2 are mutually exclusive, and only gH/gL-gQ1/gQ2 interacts with CD46, a candidate entry receptor for HHV-6 [56]. Molecular interactions during entry of HHV-7 are not defined, but this virus may use CD4 as a receptor to enter lymphocytes [57]. However, HHV-7 entry follows the general rules of herpesvirus entry by using gB to bind HSPG for cell attachment [58] and requiring gH/gL and gB for fusion [59]. In the majority of cases, the viral proteins associated with betaherpesvirus gH/gL do not yet have a defined role in fusion, but may enhance fusion efficiency, select entry pathways, and/or determine cellular tropism.

Entry of gammaherpesviruses

Infections by two human gammaherpesviruses, EBV and KSHV, have been linked with several human tumors. EBV is associated with nasopharyngeal carcinoma and endemic Burkitt's lymphoma; it is also implicated in Hodgkin's disease and gastric carcinoma. KSHV has been implicated in the pathogenesis of Kaposi's sarcoma (hence, its name), primary effusion lymphoma, and some forms of multicentric Castleman's disease.

EBV can enter B cells and epithelial cells. The most abundant EBV glycoprotein is gp350/220, a heavily glycosylated protein containing three β barrel domains within its ectodomain [60]. Attachment of the virus to the surface of B cells is mediated by gp350/220 binding to the complement receptor type 2 (CR2/CD21). CR2 binds to a glycan-free surface in the N-terminal portion of gp350/220 [60, 61]. Although not required for entry, this interaction activates several signaling pathways and may be important for infection [62, 63]. In contrast to B cells, CR2 levels on epithelial cells are very low, suggesting that another cell protein mediates EBV binding. It has been shown that gH/gL binds to a cellular ligand [64]. Additionally, glycoprotein BMRF2 binds integrins (β1, α3, α5, αV) and is important for entry into polarized epithelial cells, even though it is unclear whether this interaction is required specifically for attachment or for later signaling events [65, 66].

As with all other herpesviruses, fusion of EBV with epithelial cells requires gB and gH/gL. However, fusion of EBV with B cells requires an additional receptor-binding protein, gp42, that forms a complex with gH/gL [67, 68]. gp42 binds MHC class II molecules to allow entry into B cells [69, 70]. In contrast, gp42 has an inhibitory effect on entry into epithelial cells where only gH/gL plus gB are required [71, 72]. Thus, EBV has two kinds of complexes, the ternary gp42/gH/gL and binary gH/gL. Only ternary complexes can mediate entry into B cells, while only binary complexes allow entry into epithelial cells. As a receptor-binding protein and a partner for gH/gL, gp42 provides the means for controlling the cell tropism of EBV. Because virions released by B cells bear little gp42 (which remains bound by MHC II in the cells), they preferably infect epithelial cells, whereas virions produced in epithelial cells carry more gp42 associated with gH/gL and thus these virions preferentially target B cells [68, 73]. This elegant mechanism likely ensures a persistent infection of the host.

KSHV can enter B cells, macrophages, epithelial cells, and fibroblasts, among others. Initial attachment of KSHV to the cell surface is mediated by gB, which binds HSPG and integrins [74, 75]. In addition, the envelope protein K8.1, a positional homolog of EBV gp350/220, binds HSPG as well [76, 77]. Recently, xCT, the light chain of the human cystine/glutamate transporter system x_C^-, has been identified as an entry receptor for KSHV [78]. Which KSHV glycoprotein binds xCT is not yet known but it is likely to be gB or gH/gL because both are necessary and sufficient for KSHV entry [78, 79]. Alternatively, during infection of dendritic cells and macrophages, DC-SIGN may act as a receptor for KSHV [80]. As a lectin, DC-SIGN probably interacts with carbohydrates on viral glycoproteins, but its specific viral ligand has not yet been identified.

Entry pathways: endocytosis and fusion at the plasma membrane

Herpesviruses of all three families follow different entry routes according to the type of cell they infect. What dictates the choice between endocytosis and fusion at the plasma membrane is, for the most part, unclear. For alphaherpesviruses, fusion of HSV at the plasma membrane was long considered the sole route of infection and is well documented in Vero and Hep2 cells [81, 82]. This route of entry of HSV was also directly observed in primary sensory neurons by electron microscopy (EM) [83]. EM was also used to observe fusion of PRV at the plasma membrane of various cell types [84, 85]. However, recent studies showed that HSV is endocytosed in many other cell types [86, 87]. One possibility is that gD receptors may play a role in inducing internalization of the virions. In non-permissive mouse melanoma B78H1 cells, HSV virions can attach to the cell surface, but internalization requires expression of HVEM or nectin-1 [86]. Furthermore, a mutant HSV enters CHO cells via endocytosis when nectin-1 is used, but fuses at the plasma membrane when nectin-2 is used [88]. Additionally, other cellular and viral factors may act in combination with gD and its receptors to direct the virus to an endocytic pathway.

Endocytic entry of HSV is pH dependent in some cells and pH independent in others [86, 87, 89]. For example, endosomal acidification is required for fusion after endocytosis of HSV in epithelial cells and keratinocytes [87]. Why low pH is required in some cells and not others remains a mystery, especially given that the same glycoproteins (gB, gD, gH/gL) are required for both entry pathways [89]. A gD receptor is always needed, and low pH cannot act as a substitute to trigger the fusion machinery [89]. *In vitro*, acidic pH favors association of virions with liposomes in the presence of a soluble form of the receptor HVEM [90]. Thus, in some cells, both receptor and low pH may be needed to trigger conformational changes in gB and/or gH/gL.

In the betaherpesvirus family, CMV fuses with the plasma membrane of fibroblasts but is endocytosed in endothelial and retinal epithelial cells where fusion requires low pH [47, 53, 91]. The two different pathways have different glycoprotein requirements: the gH/gL/gO complex mediates entry into fibroblasts whereas the gH/gL/UL128/UL130/UL131 complex is necessary for infection of endothelial cells [53]. Thus, viral accessory glycoproteins may be involved in specifying the entry pathway [47]. Among gammaherpesviruses, EBV is endocytosed during entry into B cells but not epithelial cells [63, 92], and KSHV infects fibroblasts by endocytosis [93].

Whatever the entry pathway, gB, gH/gL, and, in the case of most alphaherpesviruses, gD are required for entry. Exactly how these four proteins function remains unclear. Recent structural work on the ectodomains of gD and gB from HSV-1 has illuminated the role of these glycoproteins in the entry process. This new information provides a springboard for working out the mechanism of two critical steps of HSV entry: receptor-mediated activation and membrane fusion.

Receptor-mediated activation of entry by gD

In the past decade, the role of gD as a receptor-binding protein of alphaherpesviruses has been extensively characterized, mostly for HSV-1, HSV-2, and PRV. Here, we will focus on HSV-1 gD, which has been studied in more details.

Soon after gD was identified as an essential component of HSV entry [94], it was proposed to act as a receptor-binding protein. Specifically, when cells are engineered to express gD, they become resistant to infection by the mechanism of interference [95, 96]. Furthermore, soluble forms of gD bind to the cell surface and block HSV entry [97, 98]. To date, three unrelated cell surface molecules have been identified as entry receptors for gD. The first receptor is HVEM (also known as HveA or TNFRSF14), a tumor necrosis factor family member found mostly on lymphocytes where it modulates activation by binding to B and T lymphocyte attenuator (BTLA) or LIGHT [10]. The second receptor is an immunoglobulin superfamily member named nectin-1 (HveC, PRR1, CD111) [8]. Nectin-1 is a cell adhesion molecule found at adherens junctions between epithelial cells and at neuronal synapses where it forms homo- or heterodimers with other nectins. The third receptor is

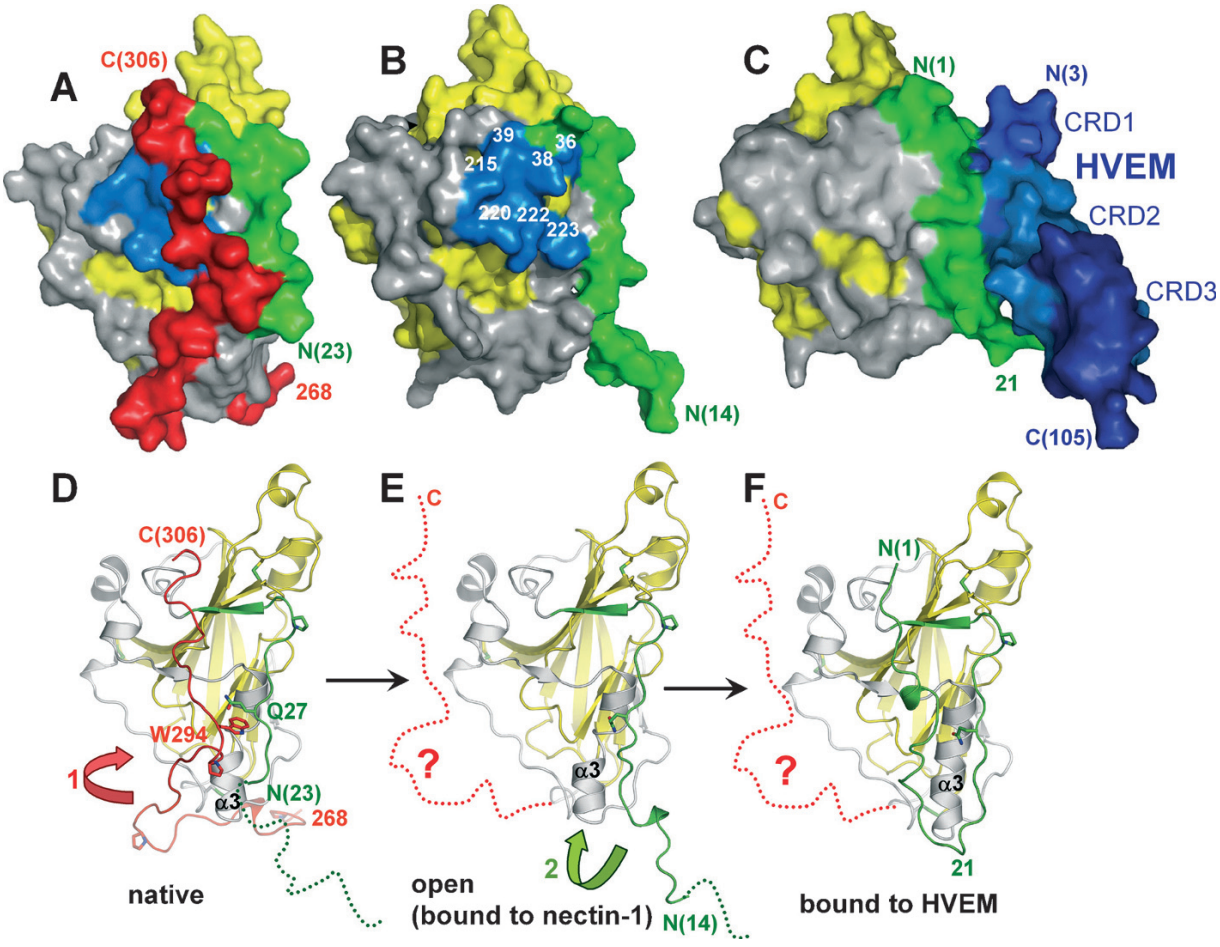

Figure 2. Structure of the HSV-1 gD ectodomain. The conformations adopted by gD before and after it binds to HVEM are illustrated by three independent structures [24, 110]. (*A*, *D*) Full ectodomain prior to receptor binding. Half of the $gD306t_{307C}$ dimer is shown. The C terminus (red aa 268–306) folds around the Ig core (yellow aa 55–185) and contacts the N terminus (green aa 23–36). (*B*, *E*) gD285 alone. The N terminus is extended and flexible. The gD285 construct lacks residues 286–306 and the remaining flexible C terminus was not solved past amino acid 255. The surface involved in nectin-1 binding is colored blue. (*C*, *F*) gD285 bound to HVEM. The N terminus (aa 1–36, green) forms a loop that contacts HVEM. The first 105 residues of HVEM are shown and colored in shades of blue according to CRD1, 2 and 3. For clarity, HVEM has been omitted in panel F. The position of the C terminus of gD bound to HVEM or nectin-1 is unknown. Dotted lines are hypothetical. Arrows 1 and 2 indicate conformational changes affecting the C terminus and then the N terminus.

a type of heparan sulfate, 3-OS-HS, specifically modified by 3-O-sulfotransferases [11]. Furthermore, nectin-2 may be used by HSV-2 and mutant forms of HSV-1 [9]. A recent study using vaginal infections of nectin-1 and HVEM knockout mice helped differentiate the roles of these two receptors in the pathogenesis and spread of HSV-2, highlighting their critical roles as well as the large redundancy between them and, possibly, other receptors [99]. Nevertheless, determining the role that each receptor plays in human pathogenesis remains a challenge because of the broad expression of these molecules and their overlapping activity as HSV receptors.

gD binding to its receptors

The mature HSV-1 gD is a 369-amino-acid protein, with a 316-amino-acid ectodomain. Crystal structures of a truncated form of the gD ectodomain (gD285) alone and bound to the ectodomain of gD receptor HVEM revealed that the glycoprotein has a V-like immunoglobulin (IgV) core, which may have been acquired from a cellular gene to serve as a backbone (Fig. 2E, F) [24]. Large N-terminal and C-terminal extensions wrapped around the central IgV core are involved in receptor binding and conformational changes.

Binding of gD to its receptor HVEM is now well understood. The HVEM-binding site of gD is unusual because it is limited to the N-terminal hairpin of gD, residues 1–32 (Fig. 2C, F). This N-terminal hairpin is buttressed by a long α helix α3, which, in turn, is supported by three β strands from the IgV core. Three sets of interactions are critical at the gD/HVEM interface [100]. The first is an intermolecular anti-

parallel β sheet, with gD and HVEM each contributing one short strand. This effectively adds a strand to a two-stranded β sheet of HVEM resulting in a three-stranded intermolecular β sheet. At the center of the interface, the second set involves HVEM Y23 that inserts into a pocket encircled by the N-terminal loop of gD. The final set consists of hydrogen bonds between gD N15 and HVEM T76 and S24. Collectively, these interactions bury a significant accessible surface area and ensure stability of the complex. gD proteins, from several strains of HSV-1, that contain mutations in the HVEM-binding site cannot bind HVEM, and the corresponding viruses can no longer use HVEM as an entry receptor [101, 102]. The N-terminal hairpin that composes the HVEM-binding site is conformationally flexible. In the crystals of unliganded gD285, the hairpin is unbent at residue 21 such that residues 1–21 extend away from the protein core; moreover, residues 1–15 are disordered indicating a high degree of flexibility (Fig. 2B, E) [24]. Thus, HVEM contributes to the formation or stabilization of the N terminus of gD.

Interestingly, gD shares an overlapping binding site on HVEM with one of its natural cellular ligands, BTLA. The structure of the BTLA/HVEM complex was recently determined [103]. Despite differences in primary sequence and structure, BTLA and gD bind HVEM in a similar manner, with HVEM Y23 and an intermolecular β sheet playing critical roles in both complexes.

The second gD receptor, nectin-1, binds gD in a region that is distinct from the N-terminal hairpin that interacts with HVEM. For example, deletion of the N-terminal hairpin of gD does not significantly affect nectin-1 binding. No direct structural information is yet available on the gD/nectin-1 interaction. Mutagenesis studies have pinpointed several residues from the α3 helix and the N- and C-terminal extensions (Fig. 2B). The gD residue most critical for gD/nectin-1 interaction, identified to date, is Y38. Mutation of this residue to alanine (Y38A) prevents usage of nectin-1 [104]. Additional residues involved in nectin-1 binding include D215, R222, and F223 [105]. These four residues map to a contiguous area on the surface of gD (Fig. 2B). In the structure of the gD/HVEM complex, this putative binding site is partially occluded by the N-terminal hairpin (Fig. 2C). Binding of nectin-1 is abolished if the hairpin is locked in place. This can be achieved by generating an intramolecular N terminal disulfide bond, as in the double gD mutant Y38C-A3C [104]. Therefore, to allow gD to bind nectin-1, the hairpin must be unfolded. These observations suggest that the N-terminus of gD assumes different conformations to allow it to bind different receptors. Moreover, binding of HVEM and nectin-1 to gD must be mutually exclusive.

The ectodomain of nectin-1 consists of three Ig domains, and the binding site for gD is located entirely in the first Ig domain that is most distal from the membrane. Soluble gD prevents nectin-1-mediated cell aggregation and disrupts already formed cell aggregates suggesting that gD competes with natural ligands of nectin-1 and prevents nectin-1 *trans*-interaction [106]. Therefore, for gD to bind nectin-1 engaged at intercellular junctions, natural nectin/nectin contacts must be disrupted.

Binding of the third, non-protein gD receptor, modified HS (3-O-HS), is the least well studied. The specific positioning of sulfate groups added by 3-O-sulfotransferases on HS suggests that gD has a well-defined 3-O-HS binding site. Although its precise location is unknown, according to mutagenesis data, the 3-O-HS binding site likely overlaps the binding site for HVEM but not that for nectin-1. For example, mutations and N-terminal deletions in gD that impair viral entry via HVEM also impair viral entry via 3-O-HS [107]. Two positively charged pockets on gD, observed in the crystal structure of the gD/HVEM complex, could potentially accommodate sulfate groups of 3-O-HS [24]. One such pocket lies close to the N-terminal hairpin and thus the HVEM-binding site. More detailed understanding of the mechanism of 3-O-HS binding by gD requires further studies.

Activation of gD

Despite structural differences and different interactions with gD, binding of the three receptors described above leads to the same endpoint: membrane fusion. Because gD does not display structural characteristics of a fusion protein, it is thought that its interaction with a receptor triggers a cascade of events leading to membrane fusion. Determining the structure of gD in its native conformation, that is, prior to receptor binding, was essential for understanding how this trigger may act at the atomic level.

Studies with soluble truncated forms of gD have shown that residues 1–260 are sufficient for receptor binding but not for promoting membrane fusion. Further, mutants carrying insertions or substitutions in the C terminus are not functional in entry even though the C terminus is not necessary for interactions with either receptor. These observations led to the hypothesis that residues within the C terminus, residues 261–316, are important for triggering membrane fusion [108, 109].

The structures of gD285 alone and in complex with receptor reveal conformational changes in the N terminus associated with HVEM binding [24]. Unfortunately, they do not show the location of the C

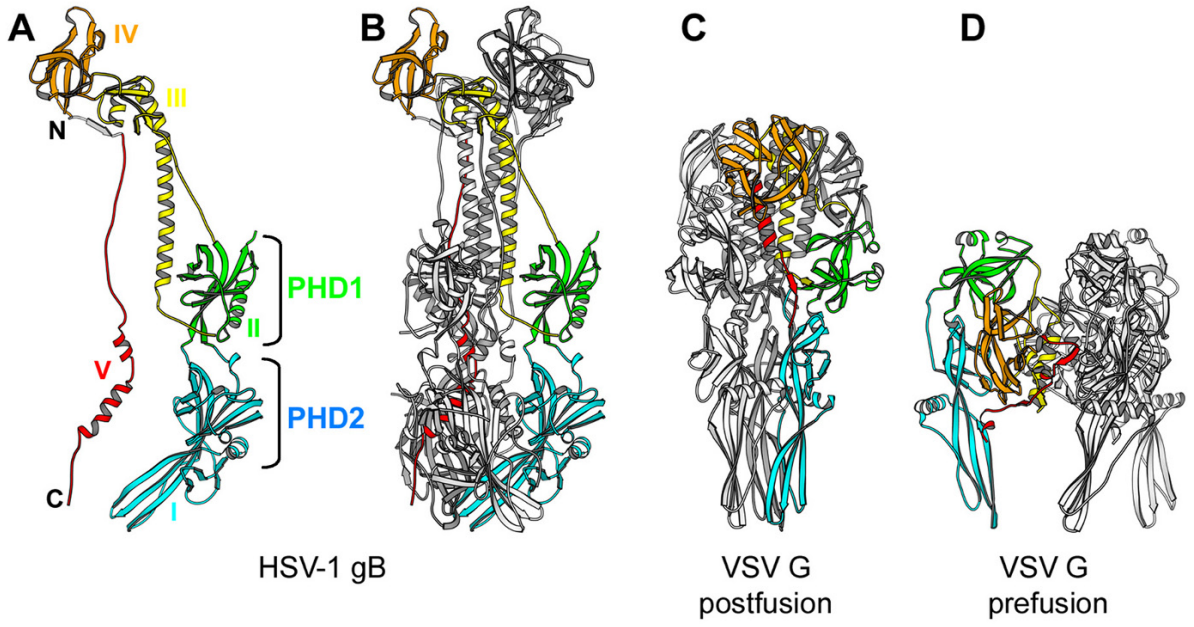

Figure 3. HSV-1 gB ectodomain [122]. A single protomer (*A*) and a trimer (*B*) are shown side by side. The N and C termini and individual domains are labeled. The plekstrin homology (PHD) domains are marked with brackets and labeled. VSV G ectodomain in post-fusion (*C*) [126] and pre-fusion (*D*) conformation [127]. Orientation of the latter was chosen for clarity.

terminus because residues past amino acid 259 are disordered in both structures. To stabilize the C terminus of the ectodomain, a construct was designed in which a cysteine residue was added at position 307. The resulting protein gD306t$_{307Cys}$ formed a disulfide-linked dimer that likely reflects the gD dimer that can be cross-linked on the viral envelope [110, 111]. The engineered intermolecular disulfide bond probably mimics the transmembrane region of gD that may stabilize the gD dimer on the viral envelope. Indeed, immunological and functional data indicate that gD306t$_{307Cys}$ has characteristics similar to that of gD on the viral envelope [110].

The structure of the disulfide-linked dimer gD306t$_{307Cys}$ revealed the location of the C terminus in the ectodomain of unbound gD. This segment of gD wraps around the IgV core and makes contacts with the N terminus and the α3 helix (Fig. 2D). This effectively brings the C terminus into proximity with the nectin-1- and HVEM-binding sites. Although HVEM and nectin-1 have non-overlapping binding sites on gD, the C terminus directly precludes access to gD for either receptor (Fig. 2A). Specifically, the C-terminus partially masks the nectin-1-binding site and prevents access to key residues, such as Y38, R222, and F223. In the case of HVEM, the C terminus prevents the formation of the N-terminal hairpin. In the structure of gD306t$_{307Cys}$, residues 289–306 of the C terminus occupy the same location as residues 1–16 of the N-terminal hairpin in the gD/HVEM complex. Because the two conformations of gD are mutually exclusive, the HVEM-binding site cannot form unless the C terminus moves out of the way. Assuming that the HVEM and the 3-O-HS binding sites are similar, the native C terminus would prevent binding of this latter receptor as well. Thus, binding of any of the three gD receptors requires that the C terminus be pushed aside. Consistent with this hypothesis, a mutant form of gD with a disulfide bond that locks the C terminus in its native conformation, gD316(A37C-V302C), is unable to bind HVEM and nectin-1 [110, 112]. Conversely, mutations in the C terminus or deletion of this entire region result in a 50- to 100-fold increase in affinity of gD for HVEM and nectin-1. This increase is caused by a faster rate of complex formation rather than by the stabilization of the gD/receptor complex [113, 114].

Although the flexibility of the C terminus is required for gD binding to receptors, it is not sufficient for its function in entry. One prominent structural feature of the gD306t$_{307Cys}$ structure is the configuration of residues W294 and P291 that insert into the pocket created by the N terminus and the α3 helix. Mutational analysis of W294 revealed the crucial role of this residue in maintaining the native structure of gD [110]. Namely, mutant gD(W294A) binds HVEM and nectin-1 with increased affinity, similar to that of a truncated form of gD, lacking residues past amino acid 285. Importantly, a gD-null virus cannot be functionally complemented with full-length gD(W294A), in-

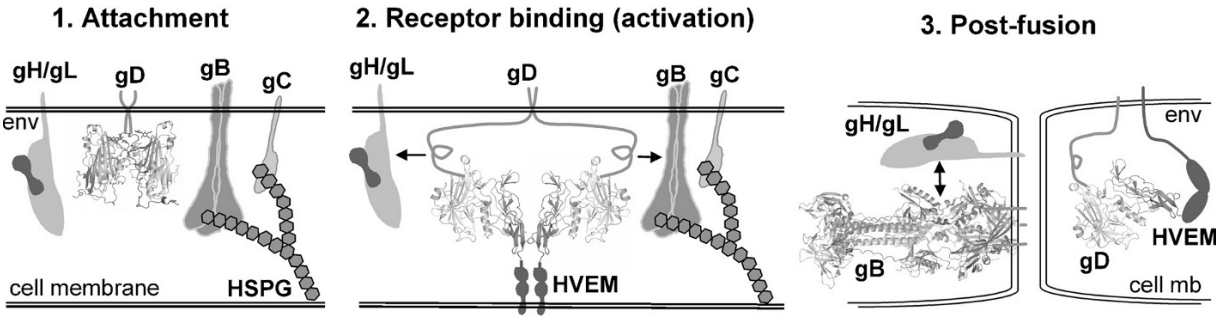

Figure 4. Illustration of steps during HSV entry. Structures of gD and gB have been placed in the context of HSV entry to illustrate molecular changes and interactions during attachment, receptor binding, and the post-fusion state. The relative position of each protein is hypothetical. The gB and gD crystallographic structures are drawn to scale relative to each other.

dicating that 'excessive' flexibility of the C terminus is deleterious for gD function [110]. This suggests that the displacement of the gD C-terminus has to occur at the time of receptor binding to properly activate the fusion machinery. Alternatively, the destabilization of the C terminus, as in the case with gD(W294A), may lead to a premature irreversible activation of membrane fusion. The gD triggering system is likely shared among alphaherpesviruses because the PxxW motif is conserved among a number of animal alphaherpesviruses, including PRV and BHV-1, which also use nectins as receptors [8].

What happens after the trigger has been activated by the receptor? It is likely that an activation signal is transmitted to gB and gH/gL, which results in conformational changes in these proteins thus leading to membrane fusion. A proline-rich region (PRR) of gD between residues 260 and 285 likely plays a crucial role in this signaling [115]. Upon receptor binding, this segment may become exposed to contact the fusion machinery. Alternatively, it may function as a hinge to expose a still unidentified region of gD that can then interact with the fusion proteins.

Recently, it was proposed that the activation of the fusion machinery of herpesviruses bears resemblance to that of gamma-retroviruses [116]. In Friend and Moloney murine leukemia viruses, (Fr-MLV and Mo-MLV), a PRR connects the receptor-binding domain (RBD) and the fusogenic domain of the viral fusion glycoprotein Env. Binding of receptor to the RBD triggers the conformational change in the fusogenic part of the protein in a manner that is dependent on PRR. The gD core where receptors bind is also followed by a PRR in the C-terminal extension, the position of which is affected by receptor binding. Interestingly, gD like the MLV RBD has an IgV core with long loops [117]. Thus, whether the receptor binding and fusogenic functions are located on one protein, as in MLV Env, or on separate proteins, as in herpesviruses, there are mechanistic similarities in the activation of the fusogenic machinery triggered by receptor binding.

The role of gB in herpesvirus entry

gB is the most highly conserved entry glycoprotein in herpesviruses and is required for viral entry. The first indication that gB is likely involved in membrane fusion came from the observation that point and truncation mutants within the cytoplasmic domain of gB display a striking syncytial phenotype [118]. Although wild-type gB elicits little to no cell-cell fusion, mutants that induce rampant formation of syncytia occur with considerable frequency. Although necessary, gB alone is insufficient for either viral entry or for virus-free cell-cell fusion. Most herpesviruses require at least gH/gL in addition to gB for both processes [119, 120]. The only exception to this rule was reported for EBV [121]. There, fusion of epithelial cells was mediated by a mutant form of gB that had a truncated cytoplasmic tail and occurred in the absence of gH/gL.

The recently determined crystal structure of the ectodomain of HSV-1 gB represented a significant breakthrough in understanding the role of gB in entry [122]. The structure revealed the location of residues 111–725, nearly the entire ectodomain (Fig. 3 A, B). Trimeric gB is a spike with the approximate dimensions of 85×80×160 Å, consistent with previous EM studies [123]. Each protomer consists of five distinct domains (Fig. 3 A) and has a complex architecture whereby the polypeptide chain traverses the entire length of the ectodomain three times.

Unexpectedly, gB contains two pleckstrin homology (PH) domains arranged in tandem. One is composed of the entire domain II, and the other, of the upper subdomain of domain I (Fig. 3 A). In cytoplasmic signaling pathways, proteins with this fold serve as scaffolds for phosphoinositide binding and function to

target these proteins to various cellular compartments [124, 125].

Although PH domains have never been seen in extracellular proteins, it is tempting to speculate that in gB, they may function to bind lipids, although certainly not phosphoinositides, which are found only inside cells. Alternatively, the PH domains in gB may bind molecules other than lipids. Several other binding modules, collectively referred to as PH-like domains, adopt folds very similar to that of PH domains [125]. These bind peptides in different locations, and it has been argued that the PH-like fold may represent a particularly stable structural scaffold that displays various ligand-binding surfaces that can be used for different functions. Therefore, it is plausible that the PH domains in gB serve as protein interaction sites.

Remarkably, HSV-1 gB possesses an unexpected and striking structural homology with glycoprotein G from vesicular stomatitis virus (VSV), a member of the rhabdovirus family, despite lack of any primary sequence similarity. Recently, the structures of the VSV G ectodomain in both its pre- and post-fusion states have been determined (Fig. 3C, D) [126, 127]. The structure of the gB ectodomain (Fig. 3B) has obvious similarities with the extended post-fusion (Fig. 3C) but not the folded-in pre-fusion structure of VSV G (Fig. 3D). It is therefore likely that the available structure of gB represents the post-fusion conformation. Although the VSV G ectodomain is smaller and more compact than gB, the corresponding individual domains are structurally homologous, and their spatial arrangement is remarkably similar. G, the sole glycoprotein on the surface of VSV virions, functions as a fusogen by itself. Although gB does not act alone, its resemblance to G strongly implicates gB as the effector of fusion in herpesviruses and suggests that aspects of the fusion mechanisms proposed for other viral fusion proteins may apply to gB.

Viral fusion proteins undergo conformational changes that bring the viral envelope and the cell membrane so close that they fuse. Currently, two classes of viral fusion machinery are recognized: class I, used, for example, by influenza and retroviruses, and class II, used by flaviviruses and alphaviruses. Class I fusion proteins are trimers of hairpins that contain a central α-helical coiled coil and N-terminally located fusion peptides – hydrophobic stretches that can insert into membranes [128–130]. Class II fusion proteins are composed of β structures with internally located fusion loops [131, 132]. Remarkably, these architecturally distinct classes both use the same 'fold-back' principle: they bring regions embedded in two pre-fusion membranes toward one end of a rod-shaped molecule in the post-fusion state. This conformational change is thought to drive the fusion of the viral and cell membranes [133].

HSV gB and VSV G are clearly distinct from class I and class II fusion proteins, but they share common features with both classes. As in class I fusion proteins, the post-fusion trimer has a central α-helical core. But the fusion loops located at the tips of the two elongated β hairpins in each protomer are remarkably similar to class II fusion proteins. Therefore, together, gB and G combine the features of class I and class II fusion proteins and define a third, new class [134].

VSV G contains an internal bipartite fusion peptide in the form of loops at the tips of two adjacent β hairpins. One of the internal fusion loops has been previously identified by membrane photolabeling experiments [135]. Four hydrophobic residues, two from each loop, are fully exposed at the tip of VSV G and could penetrate the outer layer of the membrane by about 8.5 Å. In gB, two analogous loops at the tip of domain I contain several hydrophobic residues, including a phenylalanine and a tryptophan. This tip could insert into a lipid bilayer in the same way that the fusion loops of class II fusion glycoproteins do [133]. Nevertheless, the conformation of the fusion loops seen in the gB structure appears suboptimal for membrane insertion such that local conformational changes are necessary to expose more hydrophobic residues. Interestingly, several hydrophobic residues, W174, Y179, and A261, in the two loops appear important for cell-cell fusion as shown by mutagenesis [136]. However, further studies are required to establish whether these loops, indeed, insert into the lipid bilayer.

Interestingly, analogous putative fusion loops in EBV gB are highly hydrophobic [137]. This higher hydrophobicity may explain the ability of the soluble EBV gB ectodomain, corresponding to the crystallized HSV-1 gB, to form rosettes [137], which resemble those formed by several class I and class II fusion proteins, such as influenza hemagglutinin [138] and Semliki Forest virus E1 protein [132]. The rosettes, as observed by EM, are disrupted by detergent, which supports the idea that they are held together by interactions between hydrophobic residues at the tips of the putative fusion loops. Furthermore, when hydrophobic residues in these loops of EBV are mutated to match their counterparts in HSV-1 gB, the mutant EBV gB ectodomain no longer forms rosettes. Furthermore, this mutant can no longer mediate cell-cell fusion in the absence of EBV gH/gL [139]. It is tempting to speculate that the higher hydrophobicity of the fusion loops in EBV gB increases its intrinsic fusogenic capacity and allows it to mediate fusion in the absence of gH/gL. This observation prompted the speculation that in herpesviruses where gB has less hydrophobic loops, other regions of gB or, perhaps,

gH/gL might be needed to satisfy energetic requirements for membrane insertion [137].

The pre-fusion form of gB, unlike that of VSV G, has not yet been characterized. Some clues about this form come from random linker-insertion mutagenesis of HSV-1 gB [140]. This study suggests that the conformation of gB expressed on the cell surface is different from the conformation observed in crystals of the soluble ectodomain. The most informative mutants were those that were expressed on the cell surface but showed reduced cell-cell fusion and viral entry activities. In two such mutants, the insertions are in domain I and cannot be sterically accommodated in the cavity defined in the known gB structure, thus strongly arguing that the determined gB structure is different from the native, pre-fusion structure. In addition, insertions that fall into the junction between domains III and V may not be tolerated in the post-fusion form because their presence would force several hydrophobic residues anchored into the protein core to become exposed. Based on the VSV G model, this junction in gB may serve as a hinge involved in the reorientation of several domains during the fusogenic transition [140].

The results of these mutagenesis studies suggest that the prefusion structures of gB and VSV G are similar. However, it is important to note that the two proteins have significant structural differences that complicate direct modeling of a pre-fusion conformation of gB. The two proteins also differ functionally. Unlike VSV G, which binds receptor and accomplishes fusion, gB alone is not sufficient for viral entry; all herpesviruses also require the gH/gL complex. Moreover, the trigger for HSV fusion is, at least in part, the rearrangement of gD induced by receptor binding. In VSV, fusion is triggered by low pH, and the conformational change between the pre-fusion and the post-fusion states is pH dependent and reversible. In contrast, it is currently unclear to what extent, if at all, the conformation and the function of gB are dependent on pH. For example, the gB ectodomain retains the same overall structure in the crystals obtained under low and high pH [122]. Thus, direct structural information on the pre-fusion state of gB is needed to understand the fusion-promoting conformational rearrangements in this protein.

gH/gL and its role in viral entry

The gH/gL complex is highly conserved among herpesviruses and is absolutely required for viral entry and cell-cell fusion, along with gB. Two reports do, however, mention that gH/gL, from HCMV and VZV, when transfected into cells can induce formation of syncytia in the absence of any other viral proteins [45, 141]. gH/gL is also a major target of virus-neutralizing antibodies [142]. gH is an 838-amino-acid glycoprotein with a large ectodomain and a single transmembrane anchor; gL is a 224-amino-acid glycoprotein lacking a transmembrane region. Residues involved in binding of gH and gL lie between residues 19–323 of gH and residues 20–161 of gL [142, 143]. It has been hypothesized that gL is required for correct folding and trafficking of gH because, in its absence, gH is retained in the endoplasmic reticulum and is not incorporated into the viral envelope. Conversely, gL is secreted from cells in the absence of gH; however, gL may have a role beyond simply working as a chaperone for gH. A recent study identified a mutant of HSV-2 gH that can be transported to the cell surface in the absence of gL, yet requires gL for cell-cell fusion and infectivity [27]. Nevertheless, even in this case, the functional role of gL may still be simply to ensure correct folding of gH, as some epitopes on the gH mutant are not formed in the absence of gL.

The precise role of gH/gL in fusion remains uncertain. Several reports suggest that gH may be a fusion effector sharing common features with class I fusion glycoproteins. No fewer than five putative fusion peptides have been proposed in HSV gH [144–146], and three heptad repeats have been identified in HSV and CMV gH [147–149]. Putative fusion peptides interact with and promote the fusion of liposomes *in vitro* while the heptad-repeat peptides block cell-cell fusion and viral infectivity [145, 146, 148, 149]. However, in the absence of direct structural information, these data lack an appropriate context and do not provide an adequate functional interpretation. Clearly, further structural and functional investigations are needed to clarify the role of gH/gL in fusion.

Glycoprotein interactions during entry

Very little is known about how herpesvirus glycoproteins interact during entry. In the case of HSV, the interaction of gD with one of its cellular receptors is an early step of the process leading to fusion. Receptor-mediated release of the gD C terminus, described in detail earlier, would allow it to contact another HSV glycoprotein involved in entry (Fig. 4). Several pieces of evidence suggest that gD interacts with gH/gL next, while gB is contacted later, possibly through its peripheral PH-like domain. First, in EBV, the receptor-binding gp42 – functionally analogous to HSV gD – works in complex with gH/gL [68, 71]. Second, passaging a gL-null variant of PRV in cell culture gives rise to a functional mutant virus that uses a gDgH

chimeric protein instead of gD, gH, and gL [150]. Third, a defect in cell-cell spread of a gD-null mutant of BHV-1 can be compensated by a single-amino-acid substitution in gH [151]. Finally, in cell-cell fusion experiments, hemifusion, an intermediate step during membrane fusion, was sometimes observed in the presence of gD, gH/gL, and a gD receptor and did not require gB [152]. Taken together, these data indicate possible interactions between gD and gH during entry. After activation, HSV membrane fusion requires gB and gH/gL. Two non-exclusive possibilities can be imagined: gB and gH/gL can act sequentially or they can form a fusogenic complex. At present there is evidence for both mechanisms. Recently, bimolecular complementation was used to detect interactions between gH and gB, each C-terminally tagged with two complementary halves of enhanced yellow fluorescent protein, in the context of cell-cell fusion. In cells bearing a gD receptor and transfected with gB and gH, fluorescent syncytia were observed upon triggering with soluble gD thus suggesting formation of a multiprotein complex containing gB bound to gH/gL [153]. In a similar cell expression system utilizing enhanced green fluorescent protein, the presence of gD was shown to bring gB and gH together [154]. These two studies favor a model involving a fusogenic complex. A different study suggested that gB and gH/gL glycoproteins may act sequentially to accomplish fusion [152]. In a cell fusion assay, expression of gD, a gD receptor, and gH/gL led to membrane hemifusion, where only the outer leaflets of the lipid bilayers were mixed. In contrast, expression of gD, a gD receptor, and gB was insufficient for hemifusion. Nevertheless, complete fusion was observed only when gB, gD, and gH/gL were present. Conceivably, fusion requires the coordinated and stepwise activity of gB and gH/gH within an assembled fusion complex.

The picture of protein interactions is even more complicated in betaherpesviruses. As described earlier, gH and gL associate with other glycoproteins to form the complexes gH/gL/gO and gH/gL/UL128/UL130/UL131 of HCMV, and gH/gL/gO and gH/gL/gQ1/gQ2 of HHV-6 (Fig. 1). How these complexes work with gB in fusion is unknown for any betaherpesvirus.

In EBV, receptor-binding gp42, which is required for B cell entry, forms a complex with gH/gL [67]. The crystal structure of gp42 bound to HLA-DR1 [155] revealed that gp42 has a lectin-like core with an N-terminal extension. The N-terminal region of gp42, disordered in the crystal structure, is involved in binding gH/gL [70]. Deletions in the N-terminal region of gp42 result in mutant proteins displaying reduced membrane fusion activity [72]. Furthermore, a synthetic peptide that spans region 36–81 of gp42 can bind gH/gL with wild-type affinity and compete for gp42 binding in B cell fusion [72]. Moreover, it can inhibit fusion in epithelial cells, where gp42 is not required, by either inhibiting binding of gH/gL to its receptor or by preventing a fusogenic conformational change in gH/gL [72]. Binding of HLA-DR1 may trigger fusion of B cells by relieving inhibition of gH/gL by the N terminus of gp42.

Conclusions

The entry mechanism of herpesviruses is undoubtedly complex. Recent structural work has shed light on the functional roles of two participants, gD and gB. Additionally, these studies reveal that, despite their complexity, herpesviruses share much in common with other, simpler viruses. Specifically, gB resembles VSV G, while the activation mechanism used by gD has parallels with gamma-retroviruses. Still, many puzzling questions remain. More work is necessary to reveal the structure and function of gH/gL, clarify the role of gB, and decipher the temporal and spatial interactions between these proteins that lead to membrane fusion.

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