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The structural basis of herpesvirus entry

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Abstract

Herpesviruses are ubiquitous, double-stranded DNA, enveloped viruses that establish lifelong infections and cause a range of diseases. Entry into host cells requires viral binding to specific receptors followed by the coordinated action of multiple viral entry glycoproteins to trigger membrane fusion. Although the core fusion machinery is conserved for all herpesviruses, each species uses distinct receptors and receptor-binding glycoproteins. Structural studies of the prototypical herpesviruses herpes simplex virus 1 (HSV-1), HSV-2, human cytomegalovirus (HCMV), and Epstein–Barr virus (EBV) entry glycoproteins have defined the interaction sites for glycoprotein complexes and receptors, as well as revealed conformational changes that occur upon receptor binding. Recent crystallography and electron microscopy studies have refined our model of herpesvirus entry into cells, clarifying both the conserved and unique features. In this Review, we discuss recent insights into herpesvirus entry by analyzing the structures of entry glycoproteins, including the diverse receptor-binding glycoproteins (HSV-1 gD, EBV gp42, and HCMV gH–gL–gO trimer and gH–gL–UL128–UL130–UL131A pentamer, as well gH–gL and the fusion protein gB, which are conserved in all herpesviruses.

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Recent crystallography and electron microscopy studies have refined our model of herpesvirus entry into cells. In this Review, Connolly, Jardetzky and Longnecker discuss recent insights into herpesvirus entry by analyzing the structures of entry glycoproteins, including the diverse receptor-binding glycoproteins and conserved fusion proteins.

Introduction

The *Herpesviridae* are a family of large, double-stranded DNA, enveloped viruses that cause a range of diseases. The nine human herpesviruses include herpes simplex virus 1

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

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(HSV-1), HSV-2, varicella zoster virus (VZV), human cytomegalovirus (HCMV), human herpesvirus 6A (HHV6A), HHV6B, Epstein–Barr virus (EBV), HHV7, and Kaposi's sarcoma herpesvirus (KSHV). Herpesvirus infections are ubiquitous and establish lifelong latency in infected hosts.

Despite infecting a variety of cell types, entry into host cells occurs through a conserved mechanism. This Review will focus on structural studies of entry for prototypical viruses from each subfamily: HSV-1 and HSV-2 from the *Alphaherpesvirinae*, HCMV from the *Betaherpesvirinae*, and EBV from the *Gammaherpesvirinae*. Extensive structural studies of these three viruses have been performed and the structures of multiple entry glycoproteins have been resolved for each virus allowing general features of herpesviruses to be discerned as well as virus specific features.

All three herpesviruses infect a large percentage of the human population. HSV-1 and HSV-2 most commonly cause mucocutaneous lesions in the oral or genital regions, but infection also can cause encephalitis or meningitis under rare circumstances. HCMV infection during childhood usually is asymptomatic; however, primary infection later in life can result in serious complications for fetal development in pregnant women, for transplant recipients, and for immune-compromised people. EBV typically causes infectious mononucleosis, but infection also can result in malignancies, including Burkitt's and Hodgkin's lymphoma. An understanding of the entry mechanisms for these viruses may provide a basis for the design of antiviral drug candidates and/or subunit vaccines.

This Review explains the mechanism of herpesvirus entry into cells, as outlined in multiple structural studies of the entry glycoproteins required by each representative virus. The structures of the distinct receptor-binding proteins from each virus will be compared, including HSV-1 glycoprotein D (gD), EBV glycoprotein 42 (gp42), and the HCMV gH–gL–gO trimer and gH–gL–UL128–UL130–UL131A pentamer. Structures of the conserved entry glycoproteins gH–gL and the fusion protein gB, examined using electron microscopy (EM) and crystallography, also are described.

These structural studies are linked to our understanding of the conformational changes and glycoprotein interactions required for entry to provide insight into how the fusion machinery drives virus entry into cells. These studies have broad implications because they provide targets to develop efficacious vaccines to prevent herpesvirus infections and associated disease.

Entry mechanism

Herpesvirus entry into cells requires the coordinated interaction of multiple glycoproteins on the surface of the virion. The initial attachment of a virus to a host cell tethers the virus to the cell, but does not trigger entry. This attachment is mediated by multiple viral glycoproteins and a variety of binding receptors. This Review focuses on the required entry events that occur after this attachment, including interactions among the glycoproteins and entry receptors that trigger membrane fusion and virus entry.

For herpesviruses, entry receptor binding and membrane fusion functions are performed by multifunctional viral glycoproteins[1-3]. The heterodimer gH–gL and the viral fusion protein gB represent a core set of entry glycoproteins that is required for all herpesviruses. By contrast, different herpesvirus subfamilies use distinct viral glycoprotein combinations to bind to various entry receptors. Even within a single virus species, different receptor-binding glycoprotein complexes may be required to mediate entry into different cell types.

In the current model of entry, binding to an entry receptor triggers conformational changes in the viral glycoproteins that signal to gB, the fusion protein, to execute membrane fusion (Fig. 1). Interactions among some of the entry glycoproteins, especially interactions with gB, have been difficult to capture, potentially because they are transient and/or low affinity.

For HSV-1, gD serves the receptor-binding function, as described below (Table 1). gD binding to receptor prompts an interaction between gD and gH–gL. gH–gL serves as a regulator of fusion that transmits a signal to gB[4]. Upon triggering, gB undergoes a conformational change that results in insertion into the host cell membrane followed by refolding to bring the cell and viral membranes together. The refolding of multiple gB trimers creates a pore in the membrane, allowing the viral capsid to enter the cellular cytoplasm and to be transported to the nucleus.

EBV enters cells in a similar manner, except that gp42 serves as the receptor-binding protein for EBV entry into B cells, instead of gD. gp42 forms a stable complex with gH–gL and binding of the gp42–gH–gL complex to receptor signals gB to mediate fusion. By contrast, for EBV entry into epithelial cells, gH–gL binds to receptor directly before signaling gB to trigger fusion.

For HCMV, receptor binding is mediated by two distinct complexes: a trimeric complex including gO and gH–gL or a pentameric complex including UL128, UL130, UL131A, and gH–gL. As for HSV-1 and EBV, binding of the HCMV trimer or pentamer to receptor transmits a signal to gB to trigger fusion.

Cell tropism

Herpesviruses exhibit broad cell tropism and the routes of entry can depend on the cell type and/or the viral determinants. During infection, HSV-1 and HSV-2 typically infect epithelial cells and neurons; however, the virus can infect a wide range of cells, including fibroblasts and lymphocytes. Entry into epithelial cells occurs by low pH-dependent fusion with the endosomal membrane, whereas entry into neurons occurs by fusion at the plasma membrane[5]. HCMV also infects a variety of cells, including epithelial cells, endothelial cells, fibroblasts, and leukocytes[6]. Entry into epithelial and endothelial cells requires endocytosis and low pH[7], whereas fusion with fibroblasts occurs at the plasma membrane[8]. EBV mainly infects B cells and epithelial cells, but can infect other cells such as monocytes[9]. B cell entry occurs after endocytosis in a pH-independent manner, whereas epithelial cell entry occurs after fusion with the plasma membrane[10]. Despite adapting to infect a broad range of host cells, herpesviruses share a common entry mechanism with conserved core fusion machinery (gH–gL and gB) and divergent receptor-binding proteins.

Receptor-binding protein structures

Herpesviruses encode distinct receptor-binding proteins that influence cell tropism, but are not conserved among virus subfamilies, including gD for HSV-1 and HSV-2; gp42 for EBV; and gO and UL128–UL130–UL131A for HCMV (Table 1). Crystal structures of gD and gp42 have been resolved alone and in complex with their receptors[11-15]. A structure of gp42 bound to gH–gL has also been determined[16]. A complex of gH–gL with UL128–UL130–UL131A has been crystalized[17] and a complex of gH–gL with gO has been visualized at low resolution by EM[18]. Receptors bound to both HCMV gH–gL complexes also have been observed by EM[19,20]. Although many binding receptors have been identified for these viruses and structures such as EBV gp350 (ref. [21]) have been determined, this Review focuses on validated entry receptors that have been examined in structural studies.

HSV gD

gD serves as the required receptor-binding protein for most alphaherpesviruses, with the notable exception of VZV, which lacks gD[22]. gD binds to three classes of receptors, including nectins, herpesvirus entry mediator (HVEM), and a modified form of heparan sulfate. Nectin-1, a cell adhesion protein, functions as an entry receptor for most alphaherpesviruses and is expressed on a variety of tissues, including neurons, a crucial cell type for alphaherpesviruses[23]. Nectin-2 mediates entry of some HSV-1 and HSV-2 strains and the related poliovirus receptor can serve as an entry receptor for other alphaherpesviruses[24,25]. HVEM, a member of the tumor necrosis factor receptor (TNFR) family, is expressed primarily on immune cells and functions as a receptor for HSV-1 and HSV-2, but not other alphaherpesviruses[26]. 3-O-sulfonated derivatives of heparan sulfate (3-OST HS) also can serve as entry receptors for HSV-1[27].

Crystal structures of gD from HSV-1 (refs. [12,13]), HSV-2 (ref. [28]), and pseudorabies virus (PRV) (ref. [29]) show that the gD core adopts an immunoglobulin (Ig) fold flanked by amino-terminal and carboxyl-terminal extensions (Fig. 2). Structures of gD bound to its receptor reveal that gD binds to the membrane-distal portion of both HVEM and nectin-1[11,13,29,30]. HVEM binds to an N-terminal loop on HSV-1 gD that is stabilized only in the presence of HVEM[13]. In the absence of receptor, the gD N-terminus is disordered. This gD N-terminus is not conserved in bovine herpesvirus (BHV) and PRV, consistent with the inability of gD from these alphaherpesviruses to bind HVEM[31]. Nectin-1 binds to gD at a distinct but overlapping site, adjacent to the HVEM binding residues[11,13,30,32,33]. 3-OST HS also binds to gD and, although it has not been crystalized, mutational analysis suggests that the 3-OST HS binding site on gD also overlaps with that of HVEM[27,34].

The structure of a dimeric form of HSV-1 gD, artificially stabilized by an engineered intermolecular disulfide bond at the C-terminus of the gD ectodomain, shows that the C-terminal gD extension packs against the gD core in the absence of receptor and occludes both the nectin-1 and HVEM binding sites[11,12,30] (Fig. 2). Thus, a conformational change must occur upon receptor binding to displace this C-terminal gD extension. In fact, truncation of the C-terminus enhances gD binding to both HVEM and nectin-1 (refs. [35,36]). This conformational change may serve as a downstream signal for fusion. A panel of gD mutants designed to lock the C-terminal extension to the gD core at different sites

successfully generated a mutant that retains the ability to bind to receptor but fails to trigger fusion, demonstrating that movement of the gD C-terminal extension contributes the fusion signal beyond simply permitting receptor binding[37]. The C-terminus of the gD ectodomain may serve as an interaction site for gH–gL or its movement may expose a gH–gL interaction site on gD (see below).

EBV gp42

Although EBV entry into epithelial cells requires only gH–gL and gB, gp42 serves as a required receptor-binding protein for EBV entry into B cells. gp42 binds to human leukocyte antigen type II (HLA class II), a member of the C-type lectin family. Crystal structures have been determined for gp42 alone, gp42 bound to HLA class II, and gp42 complexed with gH–gL plus an anti-gH antibody E1D1 (refs. [14-16]) (Fig. 3). Using EM, the complex of gp42–gH–gL–HLA also has been visualized[38].

gp42 is a type II membrane protein that must be cleaved at the N-terminal transmembrane (TM) domain to generate a functional soluble form[39]. The C-terminal domain of gp42 adopts a C-type lectin domain (CTLD) fold and binds to the HLA class II receptor[14]. When complexed with gH–gL, the N-terminus of gp42 extends across gH, interacting with three gH domains[16] (Fig. 3). Peptides derived from the gp42 N-terminal region can bind to gH–gL and inhibit entry into both B cells and epithelial cells[40], suggesting that these peptides may occlude a binding site on gH–gL for the epithelial receptor (see below). The gp42 N-terminus anchors the gp42 C-terminal domain to gH–gL, however the interaction between the gp42 C-terminal domain and gH–gL is not extensive. gH–gL contacts three residues within a hydrophobic pocket (HP) in the gp42 C-terminal domain, located at the canonical CTLD ligand binding site. Mutations in this gp42 HP inhibit fusion without preventing gH–gL or HLA binding[41], suggesting a functional role for the HP. The binding of gp42 to gH–gL positions the HLA binding site near the middle of the gH–gL–gp42 complex, in contrast to the arrangement seen for HCMV gH–gL complexes (see below).

EM reconstructions of gp42 simultaneously bound to HLA and gH–gL reveal multiple conformations consisting of open and closed forms[38]. gp42 bridges HLA and gH–gL, creating an approximately parallel orientation of gH–gL and HLA in the closed conformation and a highly variable orientation in open conformations. The TM anchors of gH (linked to the virus) and HLA (linked to the cell) would be located on the same side of this complex in the closed conformation, suggesting that binding to HLA may impact the arrangement of the juxtaposed membranes during fusion.

HCMV gO and UL128–UL130–UL131A

HCMV entry into all cells, including fibroblast, epithelial, and endothelial cells, requires gH–gL in a trimeric complex with gO[20,42]. Infection of epithelial and endothelial cells also requires gH–gL to form a pentameric complex with UL128, UL130, and UL131A[7,43]. Nearly all of the gH–gL in the virion is complexed with either gO or UL128–UL130–UL131A (ref. [44]) and the formation of these complexes is mutually exclusive. Residue C144 in gL forms a disulfide bond with UL128 in the pentamer and with gO in the trimer[18].

The gH–gL complexes bind to different receptors, although all of these have not been identified yet. The trimer binds to platelet-derived growth factor receptor α (PDGFRα) to mediate entry into fibroblasts, whereas the pentamer binds to neuropilin-2 (Nrp2) to mediate entry into epithelial and endothelial cells[19,20,45]. OR14I1 was identified recently as an additional epithelial cell receptor that mediates HCMV pentamer-dependent attachment and infection[46] and CD147 also was found to promote pentamer-dependent entry, although direct binding did not occur[47]. In addition, the trimer is thought to interact with another, as yet unidentified receptor, on epithelial and endothelial cells[48].

The crystal structure of pentamer bound to an antibody shows that UL128–UL130–UL131A assemble onto the N-terminus of gH–gL, docking at an N-terminal extension of gL that is conserved in betaherpesviruses but not present in other subfamilies[17] (Fig. 4a). The limited interface between UL128–UL130–UL131A and gH–gL permits flexibility, which was observed experimentally[17]. Although the gH–gL–gO trimer has not been determined using crystallography, the trimer has been visualized using EM. Similar to UL128–UL130–UL131A, gO also docks at the tip of gH–gL (Fig. 4b) [18,20]. This N-terminal gH–gL docking site differs from the gp42-docking site at the middle of gH–gL, suggesting that distinct arrangements of receptor complexes are capable of activating gH–gL between the subfamilies.

Low-resolution structures of the trimer bound to PDGFRα[20] and the pentamer bound to Nrp2 receptor[19] have been visualized using EM (Fig. 4c-d). The receptors appear to interact nearly exclusively with gO or UL128–UL130–UL131A. Crosslinking analysis confirms that Nrp2 contacts residues in gL, UL128, UL130, and UL131A[19].

gH and gL

The gH–gL heterodimer is an essential component of the fusion machinery that is conserved among all herpesviruses. gH–gL is proposed to regulate fusion by interacting with gB. gH–gL serves a receptor-binding function for many herpesviruses; however, receptor-binding by gH–gL does not appear to be required for most alphaherpesviruses. Instead, gH–gL is proposed to relay a receptor-binding signal from gD to gB in alphaherpesviruses. For some herpesviruses, gH–gL alone acts directly as a receptor-binding complex, as is observed for EBV entry into epithelial cells and VZV entry (Table 1). In other instances, gH–gL forms a stable complex with other viral proteins to bind to entry receptors, as described above for EBV and HCMV.

EBV entry into epithelial cells is mediated by gH–gL binding directly to ephrin receptor tyrosine kinase A2 (EphA2)[49]. Similarly, KSHV entry is mediated by gH–gL binding to EphA2 or EphA4 (refs. [50-52]). Soluble gp42 is able to block EphA2-mediated fusion[49] and the ability of gp42 N-terminal peptides to block EBV entry into epithelial cells suggests that the epithelial receptor binding may overlap with the gp42 binding site on gH–gL or alternatively with a site that is crucial for gB activation in epithelial cell entry[16,40]. The E1D1 antibody, which binds exclusively to gL at a distinct site at the distal end of gH–gL, partially inhibits epithelial cell entry but not B cell entry, suggesting that E1D1 also inhibits epithelial receptor binding or post-receptor binding activation (Fig. 3c)[16,53,54]. These findings indicate that multiple regions of gH–gL are important for epithelial cell entry.

VZV gH–gL is proposed to interact with integrins, as cell–cell fusion can be inhibited by adding an anti-αV integrin antibody or knocking down αV integrin expression[55]. Interestingly, although not required for fusion, HSV gH–gL also binds to αvβ3, αvβ6, and αvβ8 integrins[56,57].

Crystal structures of gH–gL have been resolved for HSV-2 (ref. [58]), VZV[59], PRV[60], EBV[61], and HCMV[17]. The gH–gL ectodomain is comprised of four domains that adopt a boot-shaped organization for HSV-2 and VZV, and a more rod-like shape for PRV and EBV (Fig. 5). The overall architecture of HCMV gH–gL is intermediate to both forms.

gH contains a C-terminal TM domain that anchors the complex to the membrane. gL lacks a TM anchor. The gH–gL structure can be described in four progressive domains, although specific domain designations between the gH–gL structures can differ[62]. Domain I is the most membrane distal region, comprised of gL and the N-terminus of gH. Domain II includes β-sheets and α-helices in a parallel arrangement. The N-terminal domains of gH are the least conserved in sequence, consistent with this region interacting with other species-specific viral proteins (see above). The two membrane proximal domains are more conserved, including the helices of domain III and β-sandwich of domain IV.

Neutralizing monoclonal antibodies (nAbs) map to several regions of gH–gL (Fig. 5), consistent with a requirement for gH–gL to interact with multiple partners during virus entry, including receptors, receptor-binding proteins, and gB. In HSV-1, selection of nAb resistance mutations indicates that the nAb LP11 binds to domain II of gH–gL, whereas the nAb 52S binds to domain III on the opposite face of the complex[58,63]. In HCMV, EM reconstructions show the nAbs 3G16 and 13H11 binding to distinct sites on gH–gL, neither of which interfere with the docking of gO or UL128–UL130–UL131A (refs. [20,64]). 3G16 binds at the membrane proximal end of gH–gL, whereas 13H11 binds at the middle of gH–gL. In EBV, two nAbs (CL40 and CL59) inhibit entry into all cell types and also map to separate sites on gH–gL[65]. A crystal structure of CL40 bound to gH–gL shows this nAb at the middle of gH–gL, overlapping the site of the gp42 C-terminal domain, in a location analogous to that of 13H11. This CL40 binding site partially overlaps with the binding site for another nAb (AMMO1) which blocks entry into both B cells and epithelial cells[66]. By contrast, an EM reconstruction of CL59 bound to gH–gL places this nAb at domains III and IV, in a position analogous to 3G16 (ref. [65]).

The gH cytoplasmic tail (CT) domain is short, ranging from 8 residues in EBV to 19 residues in PRV, but it contributes substantially to entry. Deletion or mutation of gH CT residues inhibits fusion for multiple species[67-74] and enhances fusion for VZV[72]. When the gH–gL ectodomain is expressed in cells as a soluble protein or as a glycosylphosphatidylinositol-anchored form, cell–cell fusion either fails[73,75,76] or occurs at only low levels[4]. Some mutations in the gH CT inhibit fusion without detectable changes to gH–gL ectodomain conformation or expression[69,71,74], suggesting that an intracellular interaction, potentially between the CTs of gH and gB, may contribute to fusion regulation [71,77]. Some EBV gH CT truncation mutants result in decreased gH–gL binding to gp42, suggesting that the gH CT may also impact the gH ectodomain[74].

The fusion protein glycoprotein B

gB is the viral fusion protein, the glycoprotein responsible for inserting into the host cell membrane and refolding to drive fusion of the viral envelope and cell membrane. Viral fusion proteins are membrane-anchored glycoproteins that initially fold into a prefusion conformation[78]. Upon triggering by receptor binding and/or exposure to acidic pH, fusion proteins undergo a conformational change to insert hydrophobic residues into the host membrane and then fold back on themselves to bring the two membranes together.

Crystal structures of gB from all three herpesvirus subfamilies have been resolved, including from HSV-1 (ref. [77,79]), PRV[80,81], VZV[82], HCMV[83,84], and EBV[85] (Fig. 6a-c). These gB homologs adopt similar structures. Unexpectedly, gB resembles the fusion proteins from rhabdovirus[86,87], baculovirus[88], and thogotovirus[89], despite a lack of sequence homology between gB and the fusion proteins of these unrelated viruses. Together, these fusion proteins comprise the ‘class III’ fusion proteins[90].

gB is a trimeric single-pass transmembrane protein with five extracellular domains. nAbs map to multiple sites in the gB ectodomain[83,91-93] and mutations in each of the gB domains can prevent fusion[94,95], suggesting that multiple regions of gB participate in the conformational changes required for fusion and/or interactions with the proteins that trigger gB. The formation of a fusion pore likely requires multiple gB trimers. Lateral interactions among gB trimers have been observed by EM and may contribute to the expansion of the fusion pore[96].

Receptors that bind to gB and mediate entry have been reported, such as paired immunoglobulin-like type 2 receptor-α[97], myelin-associated glycoprotein[98], and myosin-9[99]. Triggering fusion by binding to gB directly departs from the current model of virus entry and future structural studies of these receptors may explain how they enhance entry.

Postfusion gB structure

Crystal structures of the rhabdovirus vesicular stomatitis virus (VSV) fusion protein G have been resolved in both the prefusion[100] and postfusion[86] conformations. All of the gB crystal structures resolved thus far resemble the postfusion form of VSV G. Removal of the gB TM and CT regions appears to destabilize gB, preventing the crystallization of a prefusion form[77,79]. In fact, gB folds into a postfusion form even when mutations that reduce fusion are introduced[101-103], when the TM is substituted[101,102], or when the membrane is disrupted with detergent[77,79]. Antibodies specific for the prefusion conformation that might facilitate stabilization of a prefusion form have not been identified. All the antibodies that have been characterized, including neutralizing antibodies, appear to be able to bind to the postfusion form[91,93].

Domain I lies at the base of the molecule in the postfusion structure and contains two fusion loops with hydrophobic residues (Fig. 6a-c). These fusion loops are thought to insert into the host cell membrane during fusion[104] and mutations within the loops demonstrate that they are required for fusion function[80,105-107]. Domain II contains a pleckstrin-homology

domain. Extended linker regions that lead into and out of domains I and II may allow for large-scale conformational rearrangements during fusion. Domain III forms an extended trimer of helices that comprises the core of the structure. Domain IV forms a crown at the top of the structure. Domain V extends down the length of the molecule, packing against the helices of domain III in an antiparallel orientation that is reminiscent of the hallmark 'six-helix bundle' (6HB) structure present in the postfusion form of class I fusion proteins[108]. The formation of a stable 6HB structure during fusion is thought to contribute energy for fusion pore formation[109]. Mutations in domain V of gB designed to weaken its packing against domain III result in reduced fusion and a small plaque phenotype[110,111], suggesting that the interaction of domain III and V may also contribute to the energetics of fusion.

As seen for class I fusion proteins[112,113], the CT domain of gB regulates fusion. The mechanism for this regulation is unclear; however, inter-protomer interactions among the CTs or between the CTs and the membrane may stabilize the prefusion state of the gB ectodomain, preventing premature activation[77]. Mutations in the gB CT can inhibit or enhance fusion, depending on the specific mutation[114-119]. The crystal structure of HSV-1 gB including its TM and CT shows that the CT forms a trimer that interacts with the membrane (Fig. 6a)[77]. The gB CT also may interact with the gH CT (see below)[71]. Among class III fusion proteins, fusion regulation by the CT may be unique to the herpesviruses because the VSV and baculovirus fusion protein CT domains are substantially shorter than the gB CT[120].

Prefusion gB models

Computational homology models of the prefusion gB of HSV and EBV have been created based on the prefusion VSV G crystal structure[85,121]. These models suggest that gB undergoes substantial refolding to transition to postfusion.

More recently, alternate conformations of full-length gB anchored in a membrane have been studied using cryoelectron tomography (cryoET) (Fig. 6d-f)[103,122-124]. These cryoET reconstructions reveal compact trimers that are shorter than postfusion gB (8–12 nm versus 16–18 nm in height). These structures may represent prefusion or intermediate conformations of gB. When gB domains from the crystal structures were fit into the cryoET reconstructions, the resulting models orient the fusion loops towards the membrane[103,123,124]. The postfusion conformation of domain III cannot be fit into the cryoET reconstructions, suggesting that transition from this alternate compact form of gB to the postfusion form requires substantial structural rearrangement.

Glycoprotein interactions

Interaction between HSV-1 and HSV-2 gD and gH–gL

Although complexes of gH–gL and the receptor-binding proteins of HCMV and EBV have been examined structurally, a complex between the alphaherpesvirus gD and gH–gL has not been visualized. Biochemical and genetic studies suggest that they interact, perhaps transiently and/or with low affinity. gD and gH–gL can be crosslinked in the virion[125] and

they are reported to co-precipitate[126,127]. An interaction between gD and gH–gL has been observed using bimolecular fluorescence complementation (BiFC)[128,129] and stabilization of the gD–gH–gL complex by the BiFC tags inhibits fusion, suggesting that the interaction between gD and gH–gL may need to be transient[128,129]. A species-specific functional interaction between gD and gH–gL was shown by coexpressing combinations of entry glycoproteins from the non-complementing species HSV-1 and saimiriine herpesvirus 1 (SaHV1)[130]. gH–gL chimeras including segments of HSV-1 and SaHV1 mapped the gD interaction site to gH domains DI and DII (Fig. 5)[131]. This membrane-distal site on gH–gL coincides with the location of the receptor-binding proteins for EBV and HCMV, suggesting gD may trigger a conformational change in the N-terminus of gH–gL. In fact, an N-terminally truncated form of HSV gH–gL can mediate cell–cell fusion in the absence of gD[132].

Most recently, the binding of HSV-2 gD to gH–gL was demonstrated by surface plasmon resonance (SPR) using purified soluble forms of gD and gH–gL[133]. Presentation of gD by some antibodies allowed gH–gL binding, whereas other antibodies occluded the gH–gL interaction site. These data defined the gH–gL interaction site on one face of gD, distinct from the receptor-binding face. The ability of an anti-gD nAb (MC2) to block fusion without blocking receptor binding or gH–gL binding indicates that an additional site on gD is required for function. The fast on-rate and off-rate observed suggests that the interaction between gD and gH–gL is transient.

Interactions between gB and gH–gL

Using cryoET, an interaction between gB and a linear structure, presumed to be gH–gL, was visualized on HCMV virions[124]. The linear structure was frequently associated with a compact conformation of gB, which may represent prefusion gB, but not with the postfusion gB present in the membrane. Exclusive association with the prefusion form of gB is consistent with gH–gL stabilizing gB prior to the receptor-binding step that triggers fusion. Whether this gB complex forms with gH–gL trimers or pentamers, and how these proteins interact to coordinate viral entry, remains to be determined. This low-resolution structure suggests that gH–gL and gB may interact at a region adjacent to the membrane. A close association of the TM domains is consistent with a proposed interaction between the gH and gB CT domains[71,77]. The gB CT may function as a clamp, preventing premature triggering of gB. In an alternate model, gH is proposed to act as a wedge, potentially triggering gB upon receptor-binding by disrupting gB CT interprotomer interactions and/or gB CT interactions with the membrane[77]. The proposed stabilizing and wedge functions of gH–gL are not mutually exclusive. In additional support for a functional interaction between the gH and gB CT domains, the substitution of EBV gB CT residues with those of rhesus lymphocryptovirus (rhLCV) confers partial fusion function with rhLCV gH–gL[74].

The ectodomains of gB and gH–gL also are proposed to interact. An interaction between gB and gH–gL detected using BiFC was blocked by nAbs that bind to the gB and gH–gL ectodomains[58,134], including nAb LP11 (Fig. 5A). Furthermore, gB and gH–gL from HSV-1 or HCMV can mediate low levels of entry when expressed on opposing membranes, as can a soluble form of the HSV-2 gH–gL ectodomain[4,135].

Conclusions and perspectives

The complexity of herpesvirus entry into cells results from the broad range of receptor usage and cell tropism, and the requirement for multiple glycoproteins to mediate membrane fusion. The extensive structural studies described here clarify our understanding of the stepwise mechanism of virus entry and lay the groundwork for the development of antiviral interventions. The only herpesviruses vaccines currently available include a live attenuated VZV vaccine[136] and a VZV gE subunit vaccine[137]. Current antiviral medications for herpesviruses, such as acyclovir, target virus replication, rather than the viral entry glycoproteins. Understanding the structural details of herpesvirus entry may aid the design of better vaccines, inhibitors, or neutralizing antibodies that target receptor binding, interactions among the entry glycoproteins, and/or the refolding of gB. The requirement of gH–gL and gB for all herpesviruses means that discovery of an inhibitor of one species could serve as a model for analogous inhibitors for other species. The entry glycoproteins generate neutralizing antibodies and the structural determination of the binding sites for these antibodies also provides candidate sites for the design of inhibitors.

Advances in EM imaging have provided 3D reconstructions of prefusion or intermediate conformations of gB[103,122-124], as well gB interacting with gH–gL[124]. For all of the herpesviruses, atomic resolution of the prefusion gB conformation and the gH–gL–gB complex remains to be determined. For HSV-1, the interaction between gD and gH–gL has not been visualized using any structural approach. For EBV, the structure of gH–gL bound to EphA2 and the complex of gp42–HLA–gH–gL at atomic resolution remain unknown. For HCMV, the structures of the gH–gL–gO trimer and the gH–gL complexes bound to PDGFRα or Nrp2 receptors at atomic resolution have not been determined.

Although biochemical and genetic approaches have provided models for the functional interactions among entry glycoproteins, future crystallography and cryoET studies will continue to identify the interaction sites and conformational changes required for virus-induced membrane fusion. These studies may be assisted by the use of antibodies and/or mutations that stabilize the complexes and/or transient conformations. Additional receptors for these viruses may be identified and characterized using structural methods as well.

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Glossary terms

Enveloped viruses

Viruses with an outer layer consisting of a lipid bilayer, in which the viral glycoproteins responsible for mediating virus entry into cells are embedded.

Conformational change

A change in protein structure made possible by the intrinsic flexibility of the protein that can be triggered by environmental factors, such as binding to a receptor or another glycoprotein.

Cell tropism
The specific cell type(s) that support the replication of different viruses

Entry receptors
Molecules present in host cells that bind directly to viruses and mediate virus entry into the cell.

Neutralizing monoclonal antibody (nAb)
An antibody that binds to a virus particle and prevents infection, typically by preventing virus entry into the cell.

Fusion loops
Short stretches of hydrophobic residues within a fusion protein that insert into the host cell membrane during the fusion event.

Electron tomography
Method to produce high-resolution 3D models of molecules by reconstructing a series of 2D electron microscopy images taken from multiple angles.

Crystal structure
Structural model based on x-ray diffraction of a crystal that often permits atomic resolution for protein structures.

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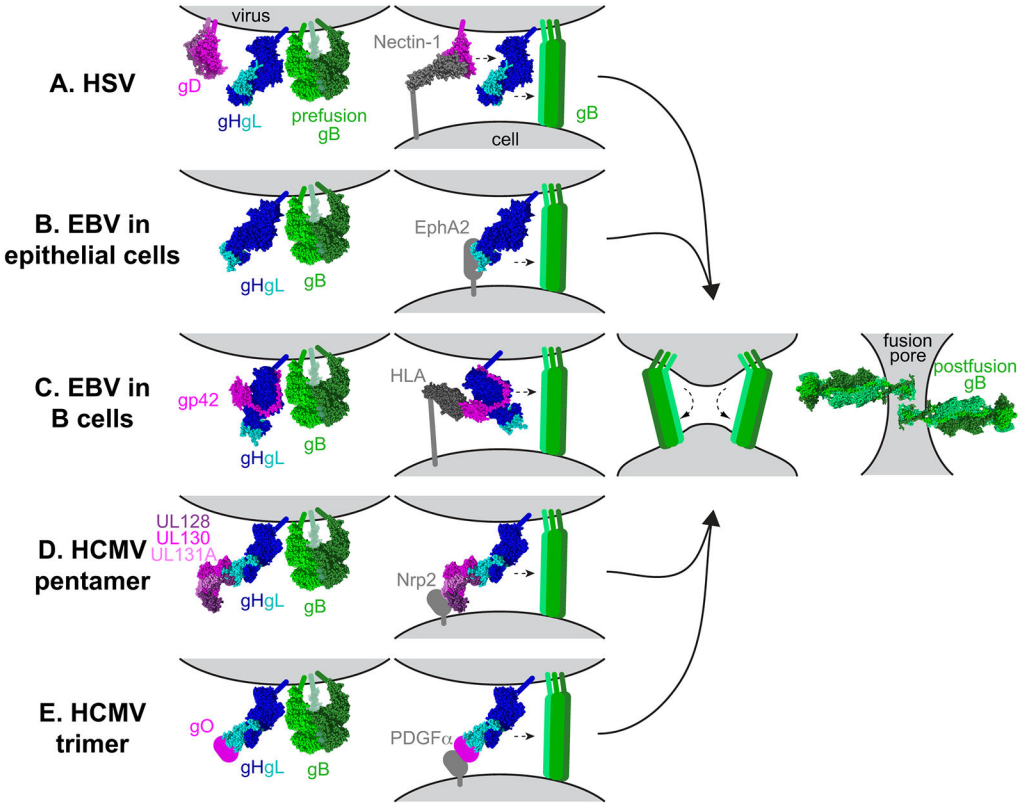

Fig. 1. Model of the herpesvirus entry mechanism.

al Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) fuse with a host cell at the plasma or endosomal membrane. The glycoprotein D (gD) dimer (pink), gH–gL heterodimer (dark and light blue), and gB trimer (green) are necessary and sufficient for entry (column 1). gD binds to one of several entry receptors, including nectin-1 (grey, column 2). Receptor binding displaces the carboxyl-terminus of the gD ectodomain and transmits a signal to gH–gL (small arrow). gH–gL activates the fusion protein gB (small arrow) to insert hydrophobic fusion loops into the cell membrane. bl Epstein–Barr virus (EBV) fuses with the plasma membrane of an epithelial cell. gH–gL (blue) and gB (green) are sufficient for fusion (column 1). The binding of gH–gL to EphA2 (grey) triggers gB to insert into the host cell (column 2). cl EBV fusion with B cells occurs in the endosome. A complex of gp42 (pink) and gH–gL (blue) binds to human leukocyte antigen (HLA) class II (grey). The binding triggers gB and may impact membrane orientation (column 2). dl Human cytomegalovirus (HCMV) entry into epithelial and endothelial cells occurs after endocytosis and requires a pentamer complex of gH–gL (blue) bound to UL128–UL130–UL131A (shades of pink) (column 1). Pentamer binding to Neuropilin-2 (Nrp2) triggers gB (column 2). el HCMV entry into all cells requires a trimer complex comprised of gO and gH–gL (column 1). In fibroblasts, the trimer binds to platelet-derived growth factor receptor α (PDGFRα) and triggers gB at the plasma membrane (column 2). The reason that the trimer is required for entry into epithelial and endothelial cells is unclear currently. After inserting into the target cell membrane, gB folds back on itself (column 3). Fusion most likely requires more than one gB trimer to be triggered. The other entry glycoproteins are removed for the figure for

clarity. As gB refolds into its postfusion conformation, the viral and cell membranes are fused, creating a fusion pore, through which the viral capsid can enter the cell (column 4).

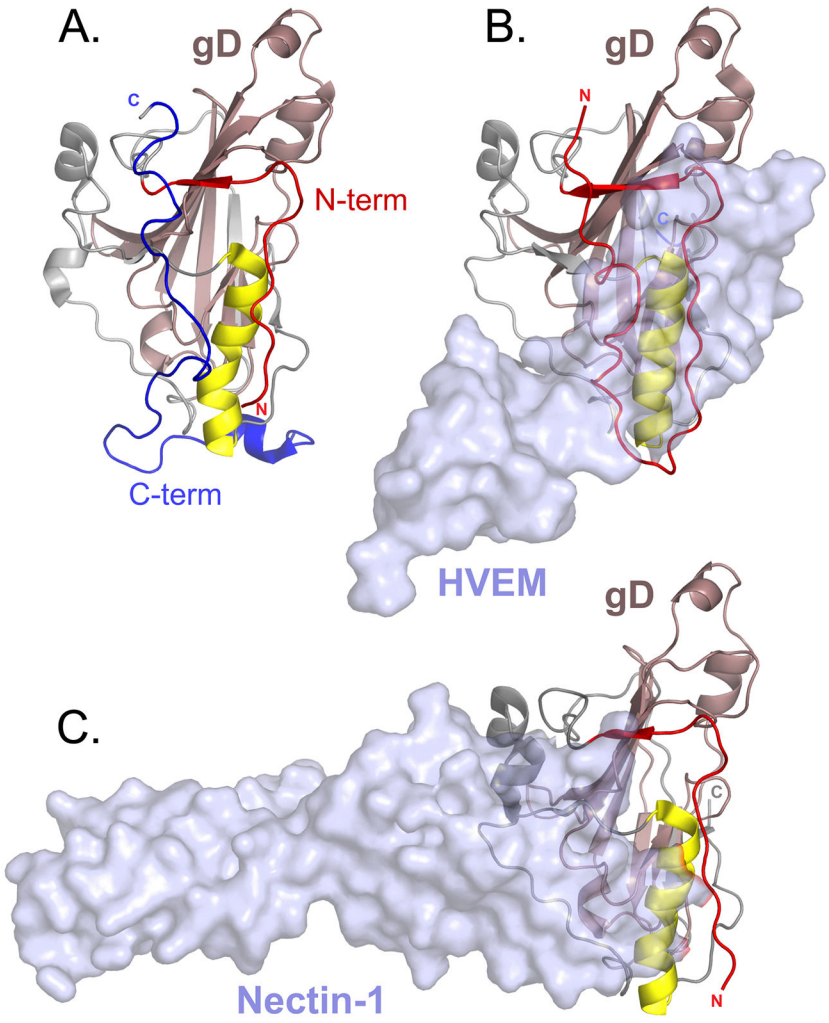

Fig. 2. Herpes simplex virus 1 glycoprotein D crystal structures.

a| A monomer from a dimeric form of glycoprotein D (gD) that was stabilized by introducing a disulfide bond at the carboxyl-terminus of the ectodomain (Protein Data Bank (PDB) ID 2C36)[12]. gD is oriented to show the receptor binding face. The core immunoglobulin fold of gD (pale pink) is flanked by amino-terminal (red) and C-terminal (blue) extensions. A α-helix (yellow) that supports the receptor binding site is shown and the N-termini and C-termini are marked. b| Herpesvirus entry mediator (HVEM) receptor (transparent surface rendering) bound to gD (PDB ID 1JMA)[13]. The N-terminus of gD (red) forms a loop that contains all of the contact residues for HVEM. c| Nectin-1 receptor (transparent surface rendering) bound to gD (PDB ID 3SKU)[11]. The HVEM and nectin-1 binding sites overlap. Binding of either HVEM or nectin-1 would displace the gD C-terminus.

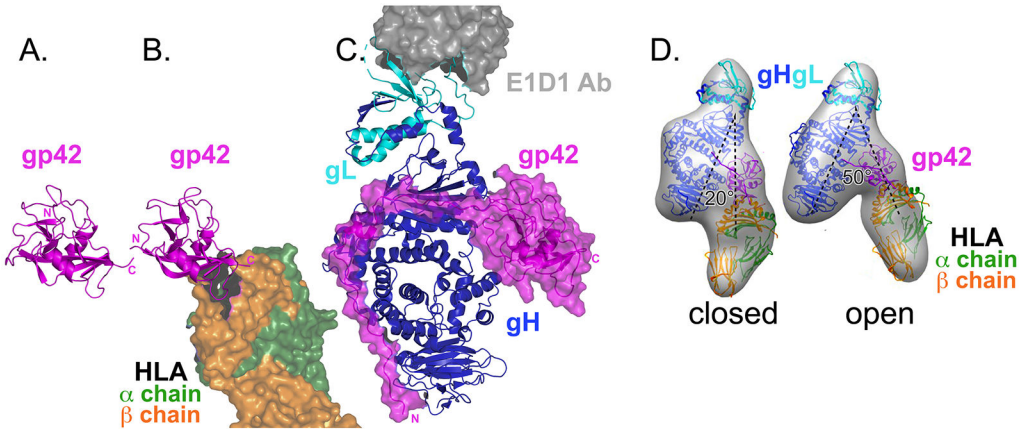

Fig. 3. Epstein–Barr virus glycoprotein 42 structures.

a| Crystal structure of glycoprotein 42 (gp42) alone (pink ribbons). The carboxyl-terminal C-type lectin domain (CTLD) is shown (Protein Data Bank (PDB) ID 3FD4)[15]. b| Crystal structure of gp42 (pink) bound to the human leukocyte antigen (HLA) class II receptor (green and orange surface rendering, partly shown) (PDB ID 1KG0)[14]. HLA binds to the gp42 CTLD and the gp42 amino-terminus is not resolved. HLA binding does not change the gp42 conformation drastically. c| Crystal structure of gp42–glycoprotein H (gH)–gL–E1D1 complex (PDB ID 5T1D)[16]. Although the CTLD of gp42 (pink ribbon with surface rendering) contacts gH–gL (blue and cyan ribbons, respectively), the majority of contacts lie in the gp42 N-terminal extension that extends down the length of gH–gL. Peptides from this gp42 N-terminus can inhibit entry into cells. E1D1 (grey surface rendering, partly shown) is a monoclonal antibody that binds to gL and partially neutralizes entry into epithelial cells but not B cells. d| Electron microscopy reconstructions of gp42–gH–gL–HLA complexes (grey surface). Fit inside the densities are the crystal structures of gH-gL (blue and cyan), gp42 (pink), and HLA (green and orange). Two conformations were observed: closed and open. In the closed conformation, HLA and gH–gL are arranged in a more parallel orientation than in the open conformation. Panel d is modified from ref. [38].

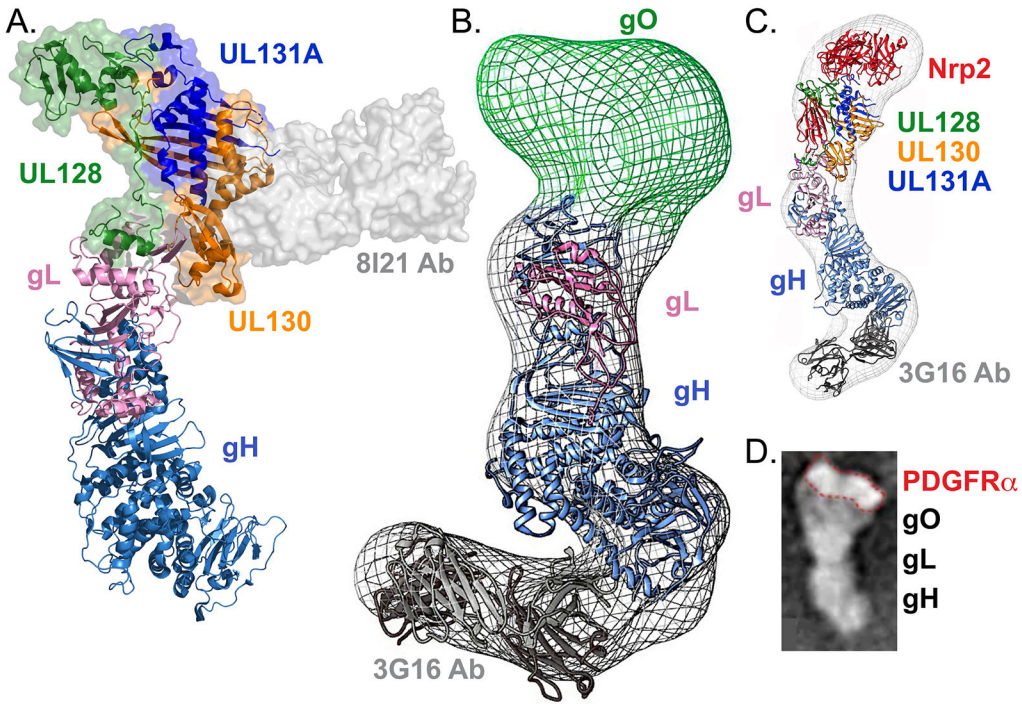

Fig. 4. Structures of human cytomegalovirus glycoprotein H–glycoprotein L complexes.

a| Crystal structure of pentamer bound to mAb 8I21 (grey surface rendering). UL128 (green), UL130 (orange), and UL131A (dark blue) assemble on the distal tip of glycoprotein H (gH)–gL (light blue and pink, respectively), contacting an amino-terminal extension of gL that is unique to betaherpesviruses (Protein Data Bank (PDB) ID 5V0B)[17]. b| Electron microscopy (EM) reconstruction of gH–gL–gO trimer bound to monoclonal antibody (mAb) 3G16 (Electron Microscopy Data Bank entry EMD-6431). The gH–gL (blue and pink) and mAb (grey) structures have been fit inside the density. gO (green) maps to the distal end of gH–gL, in a position analogous to UL128–UL130–UL131A. c| EM reconstruction of pentamer (colored as in A) bound to mAb 3G16 (grey) and Neuropilin-2 (Nrp2) receptor (red) (EMD-8884). Crystal structures have been fit inside the EM density and Nrp2 maps to the distal tip of the complex, contacting the UL128–UL130–UL131A–gL portion of the complex. d| EM image of trimer bound to the receptor platelet-derived growth factor receptor α (PDGFRα). A comparison of gH–gL–gO with and without receptor demonstrates that the receptor binds at the gO side of the complex, outlined in red. Panel b is modified from ref. [64] Panel c is modified from ref. [19]. Panel d is modified from ref. [20].

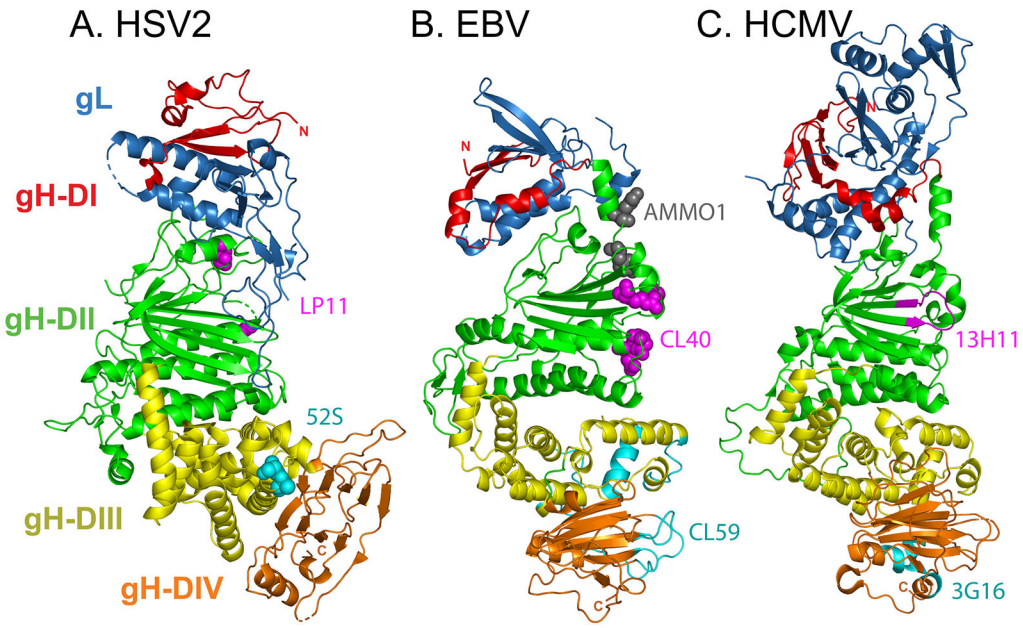

Fig. 5. Crystal structures of the glycoprotein H–glycoprotein L complex.

a| Herpes simplex virus 2 (HSV-2) glycoprotein H (gH)–gL (Protein Data Bank (PDB) ID 3M1C)[58]. Four progressive gH domains (DI–DIV) and gL are shown. gH DI (red) is intimately associated with gL (blue). gL requires gH for proper folding and anchoring to the membrane. DII (green) includes parallel β-sheets and helices. DIII (yellow) is mostly helical and DIV (orange) includes a β-sandwich. Although domain designations between the gH–gL structures can differ, specifically for distinction between DII and DIII, this figure uses the domain designations identified for Epstein–Barr virus (EBV) gH–gL. The carboxyl-terminus would extent from DIV into the transmembrane (TM) domain. The overall complex adopts a boot-like shape. Substitutions at HSV-1 gH residues 168 or 329 (magenta spheres) prevent binding of the neutralizing monoclonal antibody (nAb) LP11. On the opposite face, substitution mutations at gH residues 536 or 537 (cyan spheres) prevent binding of the nAb 52S (ref. [63]). b| EBV gH–gL (PDB 3PHF)[61]. The domains are colored as in part a and they adopt a more linear orientation. Co-crystallization revealed that nAb CL40 contacts gH residues 184, 239, 243, 284, and 286 (magenta spheres) and overlaps the binding site for the gp42 C-terminal domain. Electron microscopy reconstruction of bound nAb CL59 shows that this nAb binds to a distinct site within DIII and DIV (cyan ribbons; gH residues 406–415, 456–468, 494–503, 568–577, 623–626, and 645–656)[65]. The CL40 epitope partially overlaps with the nAb AMMO1 binding site (grey spheres; gH residues 73 and 76)[66]. c| Human cytomegalovirus (HCMV) gH–gL (PDB ID 5VOC) [17]. Domains are colored as in part a. HCMV gL includes an amino-terminal extension that is absent from the other structures. Hydrogen deuterium exchange coupled to mass spectroscopy maps nAb 13H11 binding to gH residues 238–247 (magenta ribbons) and nAb 3G16 binding to gH residues 677–684 and 705–708 (cyan ribbons)[64].

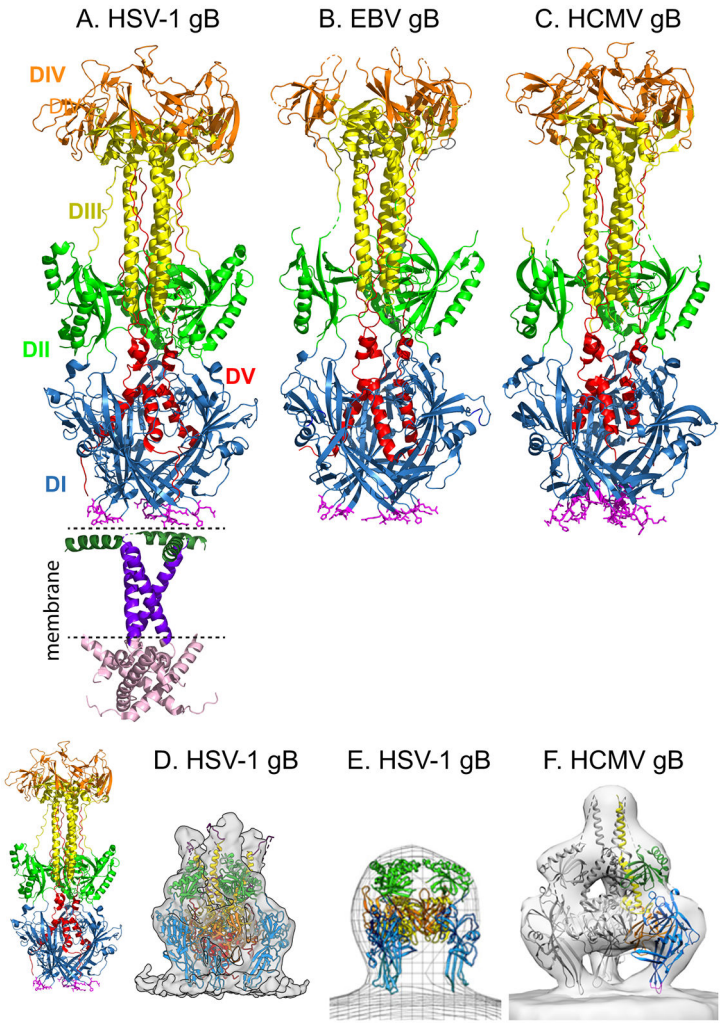

Fig. 6. Glycoprotein B structures.

a| Crystal structure of full-length postfusion herpes simplex virus 1 (HSV-1) glycoprotein B (gB) (Protein Data Bank (PDB) ID 5V2S)[77]. The trimeric ectodomain is comprised of five domains (DI–DV). DI (blue) contains hydrophobic fusion loops (magenta sticks) that insert into the host cell membrane. DIII (yellow) includes an extended central trimeric coiled-coil, against which DV (red) packs in an anti-parallel orientation, as DV proceeds through DI, towards the transmembrane (TM) domain. The membrane proximal region (dark green), TM (dark purple), and cytoplasmic (pink) domains are shown. b| Crystal structure of postfusion Epstein–Barr (EBV) gB ectodomain (PDB 3FVC) [85]. c| Crystal structure of postfusion human cytomegalovirus (HCMV) gB ectodomain (PDB ID 5CXF)[84]. For parts b and c, the domains are colored as in part a. For crystallization, residues in the fusion loops of EBV and HCMV gB were replaced with HSV-1 residues. d| Cryoelectron tomography (cryoET) reconstruction of the compact conformation of a mutant form of HSV-1 gB, 12 nm in height. Density fitting of the gB domains from the postfusion gB structure is shown. Domains are colored as in part a. The fusion loops of DI (light blue) are oriented towards the membrane. For comparison, the ectodomain of postfusion HSV-1 gB is shown at a similar scale on the left, at 16 nm in height. e| Alternative cryoET reconstruction of a compact

form of HSV-1 gB, 9 nm in height. A pseudoatomic model of prefusion gB[121], based on the prefusion structure of vesicular stomatitis virus protein G (VSV G) (PDB ID 5I2S)[100], was fit into the 3D electron microscopy (EM) reconstruction. Domains are colored as in part a. DV is excluded from the model. fl CryoET reconstruction of human cytomegalovirus (HCMV) gB (Electron Microscopy Data Bank entry EMD-9328), 13 nm in height. Domains from a single protomer are colored as in part a. Domain fitting using the postfusion HCMV structure placed DI (blue) near the membrane, with the fusion loops (magenta) oriented towards the membrane. As the EM reconstruction cannot accommodate the DIII postfusion conformation, the DIII fit was modeled on VSV G. DV is excluded from the model. Part d is modified from ref. [103]. Part e is modified from ref. [123]. Part f is modified from ref. [124].

TABLE 1:

Herpesvirus receptor-binding glycoproteins and receptors

Subfamily	Virus	Receptor-binding proteins	Receptors
Alpha	Herpes simplex viruses 1 and 2	gD	Herpesvirus entry mediator (HVEM), Nectin-1 and 3-O-sulfated heparan sulfate
	Varicella zoster virus	gH–gL	Integrins
Beta	Human cytomegalovirus	gH–gL–UL128–UL130–UL131A (pentamer)	Neuropilin-2 (Nrp2)
		gH–gL–gO (trimer)	Platelet-derived growth factor receptor α (PDGFRα)
	Human herpesviruses 6A and 6B	gH–gL–gQ1–gQ2	CD46 (for HHV6A) CD134 (for HHV6B)
		gH–gL–gO	Unknown
Gamma	Epstein–Barr virus	gH–gL–gp42	HLA class II
		gH–gL	EphA2
	Kaposi’s sarcoma herpesvirus	gH–gL	EphA2 and EphA4