

Forces and Structures of the Herpes Simplex Virus (HSV) Entry Mechanism

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Abstract: This paper discusses physical and structural aspects of the mechanisms HSV uses for membrane fusion. Calculations show that *Herpes simplex virus* glycoprotein D has such avidity for its receptors that it can hold the virion against the plasma membrane of a neuron strongly enough for glycoprotein B to disrupt both leaflets of the bilayer. The strong electric field generated by the cell potential across perforations at this disruption would break the hydrogen bonds securing the gB fusion loops, leading to fusion of the plasma and viral membranes. This mechanism agrees with the high stability of the tall trimeric spike structure of gB and is consistent with the probable existence of a more compact initial conformation that would allow it to closely approach the plasma membrane. The release of the fusion domains by disruption of hydrogen bonds is shared with the endocytotic entry pathway where, for some cell types not punctured by gB, the virus is able to induce inward forces that cause endocytosis and the fusion loops are released by acidification. The puncture:fusion mechanism requires low critical strain or high tissue strain, matching primary tropism of neural processes at the vermillion border. In support of this mechanism, this paper proposes a functional superstructure of the antigens essential to entry and reviews its consistency with the experimental evidence.

Keywords: *Herpes simplex virus*; critical stress; membrane fusion; entry mechanism.

Introduction

Herpes simplex virus 1 and 2 (HSV) virions infect cells via specific interactions between viral glycoproteins and cell-surface receptors that lead to the virion envelope fusing with either an endosome or the plasma membrane, to create a fusion pore through which the tegument proteins and genome-containing capsid are released into the cytoplasm. There are three glycoprotein antigens essential to entry, gB, gD and gHgL, a heterodimer.¹⁻⁶ These are illustrated in Fig. 1. The viral fusogen is gB, whose stable ectodomain structure is a 16 nm long trimeric spike^{7, 8} with three movable arms incorporating lipophilic domains⁸ called fusion loops, that are held away from the solvent by hydrogen bonds but associate with lipid membranes once released.⁹ Viral fusogens usually produce membrane fusion by coupling it to a large free energy change during conformational rearrangements from an initial metastable structure¹⁰⁻¹² but although a compact form of gB has been computationally reconstructed,¹³ it is known to be much less stable than the spike configuration¹⁴ and has proven

impossible to crystallize.¹⁵ Nevertheless, for the physical and structural reasons in Sections 1 and 3 respectively, this paper generally assumes that the compact form of gB does exist. Two of the other essential HSV glycoproteins form an interwoven heterodimer, gHgL¹⁶ that has a putative binding domain for gB¹⁷ halfway along its 11 nm ectodomain. This paper shows how gHgL could sterically stabilize the compact form of gB, only binding to it once destabilized.

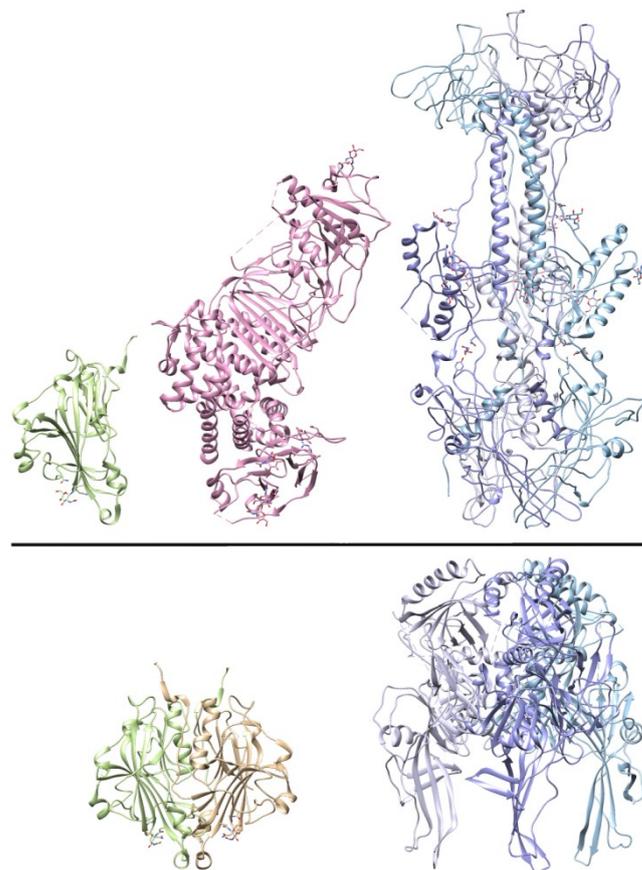


Figure 1. Structured ectodomains of the essential HSV glycoproteins. gD (green) binds at least three entry receptors despite its small size. gHgL (pink) is a heterodimer. gB (grey, blue & cyan) is the fusogen. Below the 20 nm scale bar, upon which the antigens are vertically centred, are shown dimeric gD and the compact form of gB.

The fourth essential glycoprotein, gD, has an ectodomain approximately 6 nm in size,¹⁸ and binds at least three cell-surface receptors. The binding domains for these receptors are initially obstructed by a largely disordered loop, with a profusion domain (PFD) essential to entry,¹⁹⁻²² that leads to the gD transmembrane domain. This chain is displaced by either of the main gD receptors, Nectin (Nectin-1 or Nectin-2) or HVEM (Herpes Virus Entry Mediator),^{23, 24} or by binding 3-O-sulfated heparan sulfate (3OSHS),²⁵⁻²⁷ after which it induces a structural transition in gHgL that enhances its affinity for gB²⁸ and then remains bound to gHgL and gB in a supercomplex.²⁸⁻³⁰ The gD loop is at least 37 residues long,³¹ approximately 13.32 nm.³² This estimation only accounts for residues 270–306 of the ectodomain though; the transmembrane region does not actually begin for another 35 residues, at position 341.³³

Although gD is essential for entry, an HSV virion with the other glycoproteins present as normal can still enter a cell

with all but one or two out of approximately 335 gD glycoproteins missing³⁴ or capped by antibody.³⁵ This paper calculates that the avidity of the cellular receptors for HSV gD^{36, 37} would hold gB strongly enough against the plasma membrane of a neuron to allow gB to produce membrane fusion by transiently puncturing it. The membrane potential of a live cell across such a puncture would make an electric field strong enough to disrupt hydrogen bonds. In common with the low pH on the endocytotic entry pathway to epithelial cells, this disruption of hydrogen bonds would release the gB fusion loops.

On tough cells with high critical strains or stresses an initial failure of gB to puncture the membrane does not necessarily stop infection because on some non-neuronal cell types the virus is able to induce cellular forces that produce endocytosis, by the interaction of gHgL with $\alpha\beta6$ or $\alpha\beta8$ integrins.³⁸ On this entry pathway endosome acidification releases the gB fusion loops instead. In this way the hypothesis agrees with the respective entry pathways found for neurons and epithelial cells, with entry at the plasma membrane for neurons, and endocytotic entry for tougher cells like keratinocytes.³⁹ However, there is a common mechanism for the secondary activation of gB; the release of its fusion domains by the disruption of hydrogen bonds.

In this paper I quantitatively review the interactions of the gD, gHgL and gB ectodomains and discuss how they induce the fusion of the viral membrane with the plasma membrane or an endosomal membrane, allowing capsid entry.

1. Physics of HSV entry

1.1. Targeted membrane rupture

In order for areal strain to rupture a membrane its thermal undulations must first be smoothed out by a low stress before the critical stress required for rupture is applied.⁴⁰ This pre-tensioning is achieved by the binding sequence of the HSV glycoproteins: Firstly, gB and the non-essential glycoprotein gC adhere to the cell surface via heparan sulfate.⁴¹ The multiplicity of these low affinity interaction sites generates a high overall avidity while allowing the virion to roll around on the cell surface,⁴² exploiting cell movement and bulk tissue strain to gradually bind more tightly. In this way gHgL can bind an $\alpha\beta3$ integrin,⁴³ routing the virus to cholesterol-rich rafts,⁴⁴ and HSV-1 gB may consolidate or constrain this localization by binding to a receptor called PILR α .⁴⁵⁻⁴⁷ Once the virion envelope is close enough to the plasma membrane, gD is able to bind HVEM, Nectin or 3-O-sulfated heparan sulfate, releasing its profusion domain which binds gHgL and gB.²⁴ The association of the gD profusion domain with gB and gHgL is known to greatly increase the probability of membrane fusion and virion entry.^{29, 30} We see later that the free gD profusion domain likely causes gHgL to release gB from an initial compact conformation. In this way it maximizes the local strain of the plasma membrane because the presence of

the gD chain guarantees that the plasma membrane is already adjacent and under tension.

The binding energy of each gD receptor would do a small amount of work pre-tensioning the virus against the plasma membrane. Membrane tension would be ratcheted up by each binding event because of the remarkably small size of gD relative to gB, even in its proposed compact form,¹³ and because forces on gD would be transmitted to gB both via their common anchor point in the virion envelope⁴⁸ and via the hinged^{49, 50} c-terminal chain, or loop, of gD, once attached to gB.²⁸⁻³⁰ gD has recently been shown to trans bind to the side of Nectin-1,^{31, 51} to its cis dimerization site;⁵² this allows Nectin to bind gD while lying flat in between the two membranes, pulling them to within 6-8 nm of each other.¹⁵ The ectodomain of HVEM is a similar size to nectin-1,^{15, 26} so binding these receptors pulls the virion envelope about as close to the plasma membrane as is realistically possible.

Apart from the main gD receptors, Nectin-1 and HVEM, HSV-1 gD has another binding site, for 3-O-sulfated heparan sulfate (3OSHS)²⁷. When covalently linked to the short but specific saccharide sequence of 3-O-sulfated heparan sulfate that binds HSV-1 gD,^{27, 53, 54} the Syndecan 1 & 2 proteoglycans are full entry receptors for HSV-1.²⁵ Syndecans have a strong functional homology with Nectin: They are also involved in cell adhesion,⁵⁵ do not protrude far from the plasma membrane, because of protease cleavage sites in their extracellular domains,⁵³ and can also transduce signals to the cell interior via phosphorylation sites on their cytoplasmic domains.⁵⁶ Furthermore, the binding site for 3OSHS on HSV-1 gD is next to the HVEM binding site,²⁶ consistent with 3OSHS displacing the gD linker in the same way as HVEM or Nectin.²⁵ All of the above characteristics match the requirements for 3OSHS-Syndecan to function in the proposed entry mechanism in exactly the same way as HVEM or Nectin: The short 3OSHS motif, covalently bonded to a securely anchored Syndecan, displaces the gD linker upon binding and has enough affinity to support plasma membrane disruption by gB.

Any two of these binding sites, let alone three or four on two or more gD antigens,¹⁵ would generate an exceptionally high avidity. A lower estimate for the strength of the interaction is the work attainable from gD binding single instances of its receptors: The avidities of multiple gD protomers for HVEM, 3OSHS, and Nectin-1 can be calculated using the dissociation constants for gD:HVEM, 3.2 μM ,^{21, 22, 57} gD:3OSHS, 2.0 μM ,²⁷ and gD:Nectin-1, 17.1 nM.³¹ Transforming the overall dissociation constants via $\Delta_r G = -k_B T \ln K_D$ gives the binding energies shown in Table 1.

The minimum distance that gB would have to move in order to disrupt both leaflets of the cell membrane would be the width of the lipid bilayer, approximately 5 nm. For two gD protomers to remain attached to two Nectin-1 molecules over this distance the transmissible force would be 31 pN. This force acting over the three points of contact of the crown of gB with the membrane, each approximately $2.4 \times$

1.2 nm ellipses $2.26 \times 10^{-18} \text{ m}^2$ in area, implies the maximum pressure exertable by gB in this case is 4.52 MPa. This pressure easily exceeds the 3 MPa critical stress required to rupture a lipid bilayer,⁴⁰ and is comparable to the ultimate tensile strength of skin, 20 MPa.⁵⁸ The real stress is likely to be higher during the configurational rearrangement of gB, so these figures are lower estimates. The calculations mean the multipartite interaction between gD and its receptors would definitely hold the virion against the plasma membrane strongly enough to allow the gB spike to puncture it.

Number of gD:HVEM	Avidity as kD / M	Avidity as E / zJ	Pressure P / Mpa
1	$3.2e-06$	54	1.60
2	$1.0e-11$	109	3.20
3	$3.3e-17$	163	4.80
gD:3OSHS	kD / M	E / zJ	P / MPa
1	$2.0e-06$	56	1.66
2	$4.0e-12$	113	3.32
3	$6.0e-18$	169	4.98
gD:Nectin	kD / M	E / zJ	P / Mpa
1	$1.7e-08$	77	2.26
2	$2.9e-16$	153	4.52
3	$5.0e-24$	230	6.78

Table 1. Avidities of gD binding its receptors translated to pressures exerted by gB. Two or more complexes of gD with its receptors are usually necessary to allow disruption of both membrane leaflets by gB at 3 MPa, according to the calculations given in the main text.

The targeting of the virus to cholesterol-rich rafts by gHgL binding an $\alpha V\beta 3$ integrin^{43, 44} and possibly by HSV-1 gB binding PILR α ,^{45-47, 59} would also make a short, forceful movement more likely to achieve membrane puncture, because although membranes with a high percentage of cholesterol have high critical stress, they have low critical strain – the lowest local fractional change in area which causes rupture.⁴⁰ For example, at 28% cholesterol the critical strain is 0.051 and the critical stress is 2.52 MPa whereas at 78% cholesterol these values are 0.021 and 4.94 MPa.⁴⁰ The stress gB can exert is calculated to exceed both critical stresses but a lower critical strain would assist it in puncturing the membrane. The likely activation of gB from its compact to its tall form by the profusion domain released when gD binds either HVEM or Nectin,²⁸⁻³⁰ means that gB is not activated until the plasma membrane of the cell is adjacent and held tightly against the virion by the binding of the viral antigens with their receptors. These aspects of the puncture mechanism are illustrated schematically in Fig. 2. Cell movement and virion surfing on filopodia,⁶⁰ as well as bulk tissue distortion, would help the virus to tauten the section of cell membrane to which it had bound, by breaking and reforming binding interactions. For example, most HSV infections are orolabial HSV-1 infections at the vermillion border of the mouth,⁶¹ which is highly stretched and compressed during eating and speech. High tissue strain evidently assists HSV to establish bridgehead infections in cells that have tougher membranes than neurons because tensioning the section of bound membrane would also assist

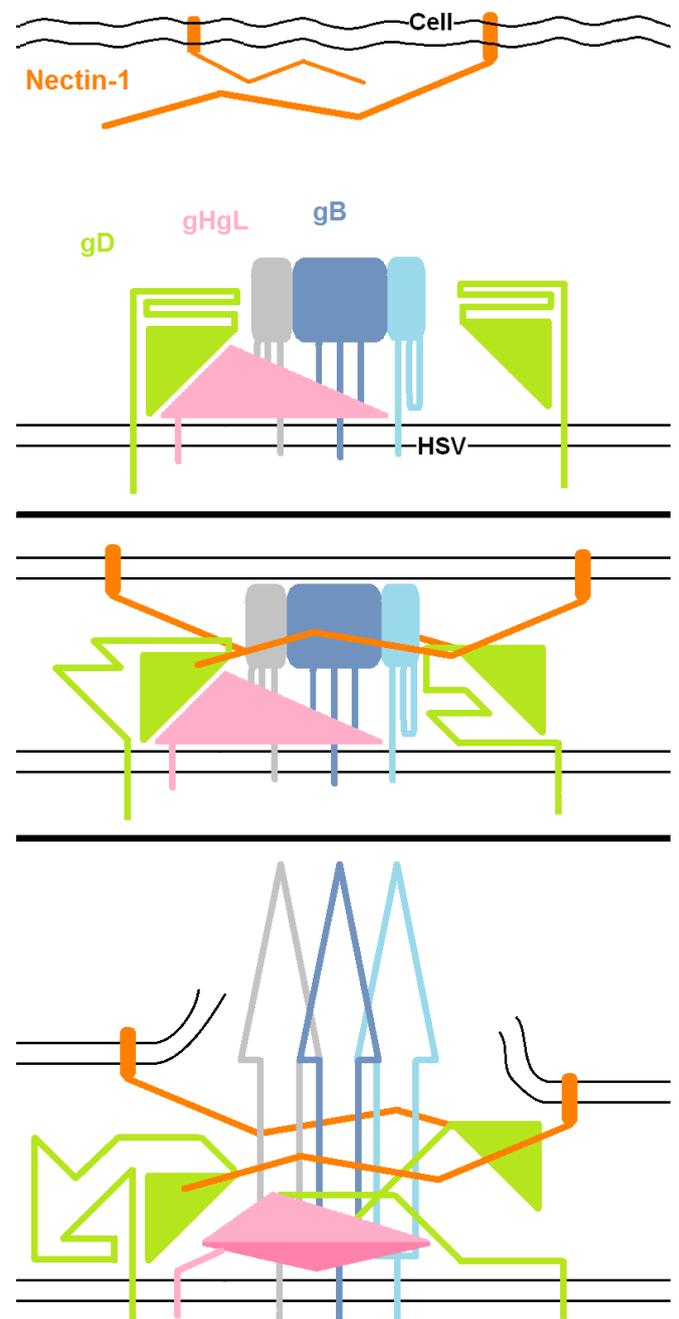


Figure 2. Schematic diagram of plasma membrane puncture by the essential HSV glycoproteins, leading to membrane fusion. Panel 1: None of the glycoproteins are bound to each other but steric interactions between them, and their anchorage to transmembrane and intravirion domains, hold down gD against gHgL, preventing it from releasing gB from a high-energy metastable configuration. Panel 2: The binding of Nectin or HVEM to gD ensures the plasma membrane is adjacent and under tension and releases the disordered gD loop. Panel 3: When the profusion domain of a gD loop binds gHgL it triggers a conformational change in gHgL that then releases gB from its compact form, puncturing the plasma membrane. The binding interaction of gD protomers with HVEM, Nectin and/or 3-O Sulfated Heparan Sulfate is sufficient to hold the virion against the plasma membrane strongly enough for gB to puncture it. In the figure two gD protomers each bind Nectin. For clarity only one gHgL of three is shown.

$\alpha V\beta 6/\alpha V\beta 8$ integrin-mediated endocytotic entry,³⁸ once it becomes endosomal. Moreover, for entry at the plasma membrane of a neuron, if gB did not initially puncture it when springing up, then due to the complexation of gD with gB these forces could cause enough stress to do this subsequently. This point also applies if a compact form of gB does not exist. A related consideration for infection of

epithelial tissue is that tissue distortion also breaks tight junctions between epithelial cells, which HSV virions could not otherwise traverse.⁶²

Apart from ensuring it has closely and strongly bound the plasma membrane before activating gB, the binding of the essential glycoproteins to their cellular receptors also induces a strengthening of the cytoskeleton by triggering the Phosphoinositide 3 kinase (PI3K) signaling cascade,^{6, 63, 64} this results in the phosphorylation of cofilin, deactivating its depolymerization of F-actin.⁶⁵ This pathway is mediated by extracellular signal-regulated kinase (ERK)⁶⁵ and the activation of Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1)⁶⁵⁻⁶⁷. Artificially inhibiting these kinases allows cofilin to degrade actin and results in reduced infectivity.⁶⁵ HSV-induced inhibition of actin depolymerization before entry strengthens the cytoskeleton and this would assist gB in puncturing the plasma membrane by providing a stiffer backing to push it against. A dense cytoskeleton would also prevent the cell from resealing the puncture itself by exocytotic fusion of lysosomes.⁶⁸

1.2. High electric field at membrane puncture would release the gB fusion domains

The stable structure of the HSV fusion glycoprotein gB is a sturdy trimeric spike with a 16 nm tall ectodomain^{7, 8} and two lipophilic fusion loop domains per protomer held in place by hydrogen bonds.⁶⁹ These bonds are disrupted by reduced pH in endosomes.⁶⁹ If gB were to transiently puncture the plasma membrane however, a very strong electric field would be produced – the 50 mV membrane potential of a live cell dropped across the 5 nm thickness of the bilayer is a field strength of 10 MV.m⁻¹. This electric field would also disrupt the hydrogen bonds and release the fusion loops, putting gB in its membrane fusion conformation as a direct physical consequence of puncturing the membrane. The lipophilic fusogen domains⁸ are in proximity to the envelope of the virion rather than further out,⁹ so it is possible that many other hydrogen bonds would be disrupted too, promoting the secondary structure to reform in the midst of the damaged bilayer. An alternative way to describe this process would be to say that, as protons are many times more mobile than other ions, the pH of the immediate locality would experience a sharp dip before any steady state ion current was established, and that this would disrupt the hydrogen bonds. In any case, completion of fusion is known to require another conformational change in gB,^{70, 71} its fusion domains must arc outwards to facilitate the initial expansion of the fusion pore.^{8, 9}

1.3. The active forces triggered by the virus are all cell-retrograde

The active cellular forces mentioned above are triggered by the interaction of the essential HSV glycoproteins with their cellular receptors. One example is the main receptor for HSV, Nectin-1. The structural relation of Nectin-1 to the cell is particularly strong, as its cytosolic side is tethered

straight to the actin cytoskeleton via afadin.⁷² Even as a static anchor-point this marks its suitability for gD to bind to. However, gD binding Nectin-1 also induces its active internalization by the cell.³⁶ Similarly, binding of HVEM by gD induces its internalization.⁷³ Doses of cytochalasins B or D, which prevent polymerization of actin filaments,⁷⁴ reversibly inhibit HSV-1 entry to HEp-2 cells.⁷⁵ It is not yet known whether HSV-1 also induces Syndecan internalisation though.

Apart from inducing HVEM internalization, gD also competes with an endogenous ligand called LIGHT³⁷ (Lymphotoxin-like Inducible protein that competes with Glycoprotein D for Herpes virus entry on T cells) that would otherwise induce endocytotic pathways in epithelia via myosin II regulatory light chain (MLC) phosphorylation.⁷⁶ Thus gD binding HVEM also prevents the membrane from actively retreating.

Triggering of the internalization of the main gD receptors from the plasma membrane may be achieved by the binding of gD alone^{36, 73} but might be assisted by the interaction of gB with PILR α , because this promotes HSV-1 entry at the plasma membrane.⁷⁷ For Nectin-1 the triggering event(s) may be the displacement by gD of other cis-bound Nectin-1 molecules,³⁶ or the displacement of the α V β 3 integrin that also cis-binds the Nectin-1 ectodomain.^{36, 78} Apart from gD binding 3OSHS, Nectin-1 and HVEM, the other two essential glycoproteins also form complexes with receptors capable of transducing signals to the cell, gHgL with α V β 3 and gB with PILR α . Cytoskeletal rearrangements are also known to be triggered by ligands binding to integrins.^{43, 79}

Another important response to the HSV envelope binding is that the cell is induced to display a further raft-associated^{80, 81} receptor for gB, Non-muscle Myosin Heavy Chain IIA (NMHC-IIA).⁸² NMHC-IIA is usually intracellular,⁸³ but can also be expressed in normal function on the cell surface^{82, 84} where it contributes to tissue architecture by controlled cell adhesion and active cell migration.⁸³ Another name for NMHC-IIA is myosin 9, and this and myosin 6 are the only myosins known to walk towards the minus end of actin filaments (out of eighteen known classes). This retrograde transport, which in non-muscle cells is towards the interior, means that NMHC-IIA will actively pull gB towards the interior of these cells once triggered.

For cell lines without much membrane cholesterol, or with incorrect localization of α V β 3, entry at the plasma membrane (by puncture:fusion), would be less likely. On some cell types though, the above forces would be induced, and the pull from Nectin-1 and HVEM via gD, and from NMHC-IIA/myosin 9 could pull the tall form of gB through the plasma membrane even if it had failed to do this when springing up from its compact form. This point would also apply to entry at the plasma membrane if the compact form of gB does not exist. In the total absence of entry at the plasma membrane on tough cells though, the interaction of gHgL with the α V β 6 or α V β 8 integrins,³⁸ if present as for

epithelial cells, can still mediate entry via various forms of endocytosis.^{6, 85-87}

Once an endosome was detached from the membrane any forces exerted would generally move the entire endosome rather than pull the virion closer in to the membrane. The absence of membrane potential across an entirely intracellular endosome would require endosome acidification to reveal the gB fusion loops^{88,89} in place of the transient pH drop at the plasma membrane puncture point described above. Fusion with an endosomal membrane could still be influenced by membrane composition and fluctuations in mechanical stress inside the cell, which could explain the delay between endocytosis and capsid entry to cytoplasm¹⁸ and the dependencies of fusion on ultraviolet light⁹⁰ or temperature⁹¹ and cell type.^{6, 85, 86, 92}

2. Disrupting HSV entry

2.1. Reduced membrane tension

The adhesion of gC to cell surface heparan sulfate is not absolutely required for virion entry but nevertheless greatly enhances its efficiency.⁹³ According to the mechanism outlined above this is because it also plays a part in pre-tensioning the plasma membrane when it binds to its receptors. In this way, HSV-1 entry is significantly enhanced by the association of gC and gB with cell surface heparan sulfate.⁹⁴

HSV entry into Vero and Hep2 cells occurs at the plasma membrane,^{86, 95-97} but cholesterol-sequestering methylcyclodextrin inhibits HSV entry into Vero cells.⁹⁸ In the context of the puncture:fusion hypothesis this is because the membrane is then less brittle, with a higher critical strain. Entry to keratinocytes also requires cholesterol.⁹⁹ Finally, avidity-induced tensioning is consistent with the facts that soluble gB ectodomain binds cells and blocks entry,⁹⁸ and that monoclonal antibodies for gD, gB and gH all block viral penetration into the cell.¹⁰⁰⁻¹⁰²

In the absence of tissue strain on cultured cells, or due to reduced membrane tension, the molecular entry mechanism would occasionally stall at the state of hemifusion, where only the outer leaflets of the envelope and cell membrane are fused. Hemifusion cannot arise solely by apposition of the envelope bilayer with a cellular membrane because of strong repulsion due to hydration at gaps less than 2-3 nm¹⁰³ that prevents direct contact,^{11, 104} making membrane disruption by gB necessary for fusion and pore formation. The gB receptors PILR α and NMHC-IIA both enhance infectivity^{45,82} and may also function in this by their physical presence lowering the local critical stress of the cellular bilayer. This additional possibility is most evident from the lability of the display of NMHC-IIA.^{82,83}

2.2. Disruption of mechanism

There are a range of results in the literature using soluble gD.^{20, 105-108} The truncated gD construct only includes the

gD ectodomain and only mediates fusion if added after virions have bound to the cell surface.¹⁰⁷ In this situation it still activates gHgL and hence gB only after it is next to the plasma membrane, and can even attach gB to the cytoskeleton, via gB-gDt-Nectin-Affadin, so this entry is expected. However, soluble gD added before virion attachment prevents infection rather than promoting it.¹⁰⁶ In this scenario the opportunity to pre-tension the compact form of gB against the plasma membrane is totally lost: After binding an initial cellular receptor, gD would release its profusion domain too early, activating gB as soon as it was in the vicinity and before it was close enough to puncture the plasma membrane during its conformational rearrangement.

Apart from the disruption of force transmission between gD and gB, the cytoskeletal strengthening that the virus induces can also be disabled: The Cytochalasins B and D, which prevent polymerization of actin into microfilaments,⁷⁴ reversibly inhibit HSV-1 entry to HEp-2 cells.⁷⁵ This follows from the dismantling of the cytoskeleton that gB could otherwise push into and against to puncture the plasma membrane, which is necessary because it has very little surface tension of its own.¹⁰⁹

3. Structures of HSV entry

3.1. Deduction of a gB:gHgL:gD superstructure

Viral fusogens usually have a compact, metastable conformation that stores energy required for membrane fusion.¹⁰⁻¹² Although no such conformation for HSV gB has been crystallized, it now appears likely that it does actually exist but is just very unstable.^{14, 110} Indeed, it has even been possible to reconstruct its probable structure by analogy with VSV G.¹³

Given only that fusion-competent gB is usually in a compact high-energy form somewhat similar to the recent reconstruction¹³, and that this is so unstable,^{14, 110} it would seem natural to think that gHgL initially binds gB, keeping it compact, until gD destabilizes the complex and gB springs up and fuses the membranes. However, this would disagree with the evidence that gHgL is not initially bound to gB and only up-regulates gB function and binds to it having been activated itself by receptor-bound gD.^{29, 30, 111} Instead, these latter facts suggest that gHgL only constrains the compact form of gB sterically, without binding it. This motivated me to look for a possible metastable superstructure of gHgL that could cage gB. Because gHgL had been said not to form a trimer¹⁵ the only plausible superstructure seemed to be a claw of three separate gHgL protomers reaching up around the compact form of gB. This arrangement does not work though. However, an unstable tri-fold superstructure of gHgL could be stabilized by steric interactions, without strong enough binding interactions to make it a trimer on its own. Reasoning that to surround gB the size of a gap would have to be as large as possible, I projected to construct any possible tri-fold gHgL structure by maximizing the apparent

cross-sectional area of its ectodomain then rotating two copies by 120 degrees in opposite directions and translating these to bring glycosylated residues close together without clashing any bonds. This arrangement worked very well, generating the gHgL triangle shown in Figs. 3 & S1. This triangle is conjectured to be an unstable, high-energy configuration that can only be held in a metastable superstructure by steric interactions with the other essential glycoproteins. Three remarkable aspects to this structure are immediately apparent – Firstly, gB fits in the center perfectly as Fig. 3 illustrates. Secondly, one side of the gHgL triangle is very flat and has some glycosylated residues. Thirdly, there are three wedge-shaped spaces on the more irregular side that are also glycosylated.

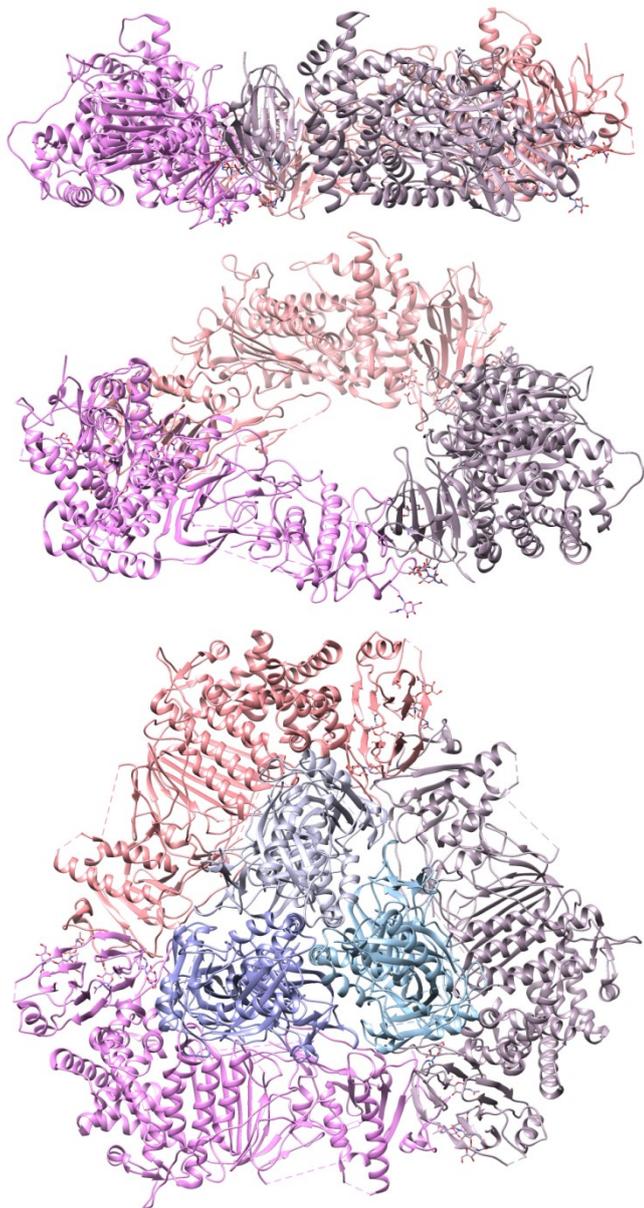


Figure 3. gHgL triangle. Three tessellated gHgL ectodomains; no stable binding interaction is implied: This is not a trimer. Side view: The lower face is very flat and displays glycosylated residues. Oblique angle view: Each side of the triangle has a glycosylated wedge-shaped gap. Top view: The structure is slightly interlocked like an impossible triangle. The wider, compact form of gB fits exactly inside the central gap, despite the fact that the triangle was pre-aligned without reference to it. gHgL is shown in magenta, pink and purple.

Two features of the gHgL triangle strongly indicate it is a native structure: Firstly, the central space of the gHgL triangle fits gB exactly. Secondly, its glycosylated side is extremely flat. This flatness of its glycosylated side indicates that it faces the virion membrane, which does not contradict the finding that gHgL does not bind to membranes,¹⁷ because it may be held down by something else. Structural alignment indicates that this is the function of gD: Single gD protomers fit in each of the three glycosylated spaces on the top of the gHgL triangle, with their n-acetyl-d-glucosamine (NAG) residues aligned between two gHgL NAG residues. In this position each gD pushes back against its partner protomer behind it while strongly anchored by its transmembrane region,⁴⁸ thereby holding down each side of the triangular gHgL structure. gD

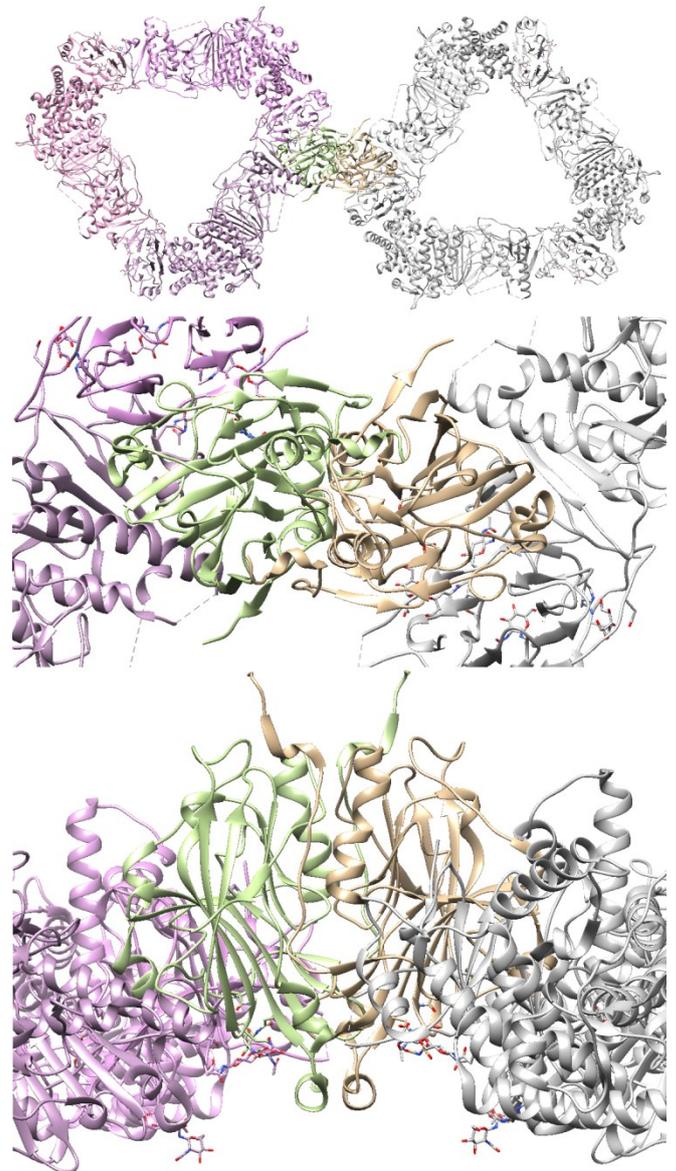


Figure 4. gD sterically locks the edges of gHgL triangles. Top: These superstructures tessellate hexagonally but leeway in the gD position may also allow pentagonal units. Zoom: The spaces in the gHgL triangle edges are the right size and shape for gD protomers. Interlocking in this way brings glycosylated residues together. Side view: Each gD pushes back against its partner locking the superstructure behind it and is tightly anchored to its transmembrane region until its linker is displaced. It is remarkable that the receptor binding regions are on the exposed top-half of the more crowded side of gD. One gHgL triangle is shown in silver.

is known to be dimeric on the virion surface.¹¹² The configuration is illustrated in Figs. 4 & 5. It is possible to align gD to the space on gHgL using symmetry considerations: The back-to-back symmetry required for tessellation of these units constrains the axis of rotational symmetry of each gD dimer to be perpendicular to the local plane of the membrane, somewhat reducing the configurational space of possible orientations. Nevertheless, the flexibility of NAG alignment suggests there is enough leeway in the gD angle relative to gHgL to allow the units to tessellate in either hexagonal or pentagonal arrangements in order to accommodate the curvature of the virion surface. With gD in place, the unstructured region on top of gHgL¹⁷ might also contribute to stabilizing the gHgL triangle. It is

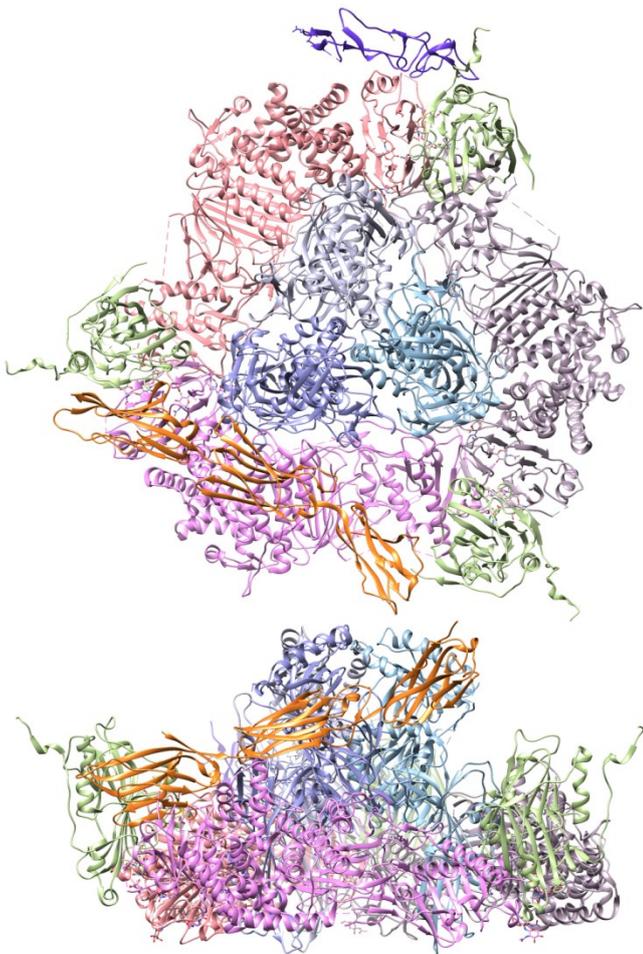


Figure 5. gD locks down gHgL around compact gB. The HSV receptors, HVEM (purple), Nectin (orange) and 3OSHS-Syndecan-2 bind gD without impinging on the rest of the superstructure (3OSHS is not shown but binds gD in a similar position to HVEM and Nectin). The bound gD structures were superposed with the unbound structures shown. No variation of the gD position was necessary. In binding the three gD molecules, its receptors displace the gD linkers, which allows the N-terminal gD domains to move away from each gHgL, destabilizing the superstructure shown here.

possible that this part of gHgL only locks each protomer to its neighbor when gD is clamping it down. Thus there is no question that the free gHgL ectodomain would ever form a trimer – this triangular configuration is dependent on being held down on the membrane by its anchor-points, by three gD molecules and possibly also by the presence of the gB trimer at its center. Both steric and binding interactions that would immediately fall apart in solution could become quite

strong locks once metastably anchored in such a superstructure. For example, in the gD dimer the loop holding an alpha helix of the other protomer could twist apart in solution, but in its native state the steric constraint of being anchored to the virion membrane would prevent this from happening until pulled upwards from one side by HVEM or Nectin as Fig. 5. indicates.

When displaced by HVEM or Nectin, the gD loop could disrupt the weak association of the glycosylated residues on the underside of gHgL with the virion membrane by binding its profusion domain to the top surface of gHgL, inducing it to slightly arch away from the virion membrane and escaping any weak, short-range, attraction to it. This type of positional activation of gHgL is likely for the reasons set out in the next paragraph, which also suggest the gD PFD binds gHgL fusion region 1 (FR1). The gD loop is easily long enough to reach this binding region and still loop back to its transmembrane domain in the vicinity: The ordered structure of the gD ectodomain shown in the figures stops at residue 255 making a chain of 86 residues before the transmembrane domain begins at residue 341. This loop would be 31 nm long and thereby reach up to 15 nm from the anchor-point when released, easily within range of binding regions on gHgL about 7 nm to the left of each gD protomer. These scales are illustrated by Fig. S2.

There is an important feature of the proposed triangular gHgL structure not yet mentioned: Aligning the crystal structures in this way results in the chain leading to the transmembrane region of each protomer pointing upwards, which would be away from the membrane. These residues are close to the gap available to gD and are visible in Figs. 3, 4 & 5. In the initial, high-energy, superstructure these sections of each gHgL protomer, or its linker, would be strained to bend round through the edge of the central gap in the triangle to meet its transmembrane region. If gD primes gHgL to leave the membrane, this conformational strain would store the energy required to lift it away from the weak attraction between its glycosylated residues and the membrane, and would flip up the top side of each gHgL protomer towards gB. For this to happen the residual attraction of gHgL to membrane would have to be too weak to stably bind to it, which is consistent with the finding that it does not bind membrane.¹⁷ There are three reasons for proposing that gHgL moves like this: Firstly, the bulky corner of each gHgL protomer would knock into the compact gB structure, contributing to its destabilization. Secondly, the FR1 binding region for gB is near this corner of gHgL,^{15, 19} and adjusting the orientation of gHgL in this way does indeed align FR1 with a likely binding site near the base of the tall gB structure, as shown in Fig. 6. Thirdly, when all three gHgL protomers have flipped upwards, their fully open configuration seen in Fig. 6 would allow the three downward-pointing beta-hairpin loops of the compact form of gB to move anti-clockwise and open outwards, permitting gB to spring into its tall form.

A spring-loaded movement of gHgL triggered by gD was modelled by pivoting each gHgL protomer about Leucine 790, highlighted in green in Fig. 6, on the anchored chain of gHgL. This point is chosen because it is the first residue in the linker after a set of beta sheets, and is about mid-height in the crystal structure. FR1 appears to join a complementary region near the base of gB. If the PFD²⁸ on the gD loop were to cause a structural transition in gHgL by binding to FR1 then in remaining attached there it could also enhance the avidity of FR1 for gB. These ideas are fully consistent with the evidence that gHgL only binds gB in the presence of receptor-bound gD,^{29, 30} that the gD C-terminal chain is essential to entry,¹⁹⁻²² that it induces a structural transition in gHgL that enhances its affinity for gB²⁸ and

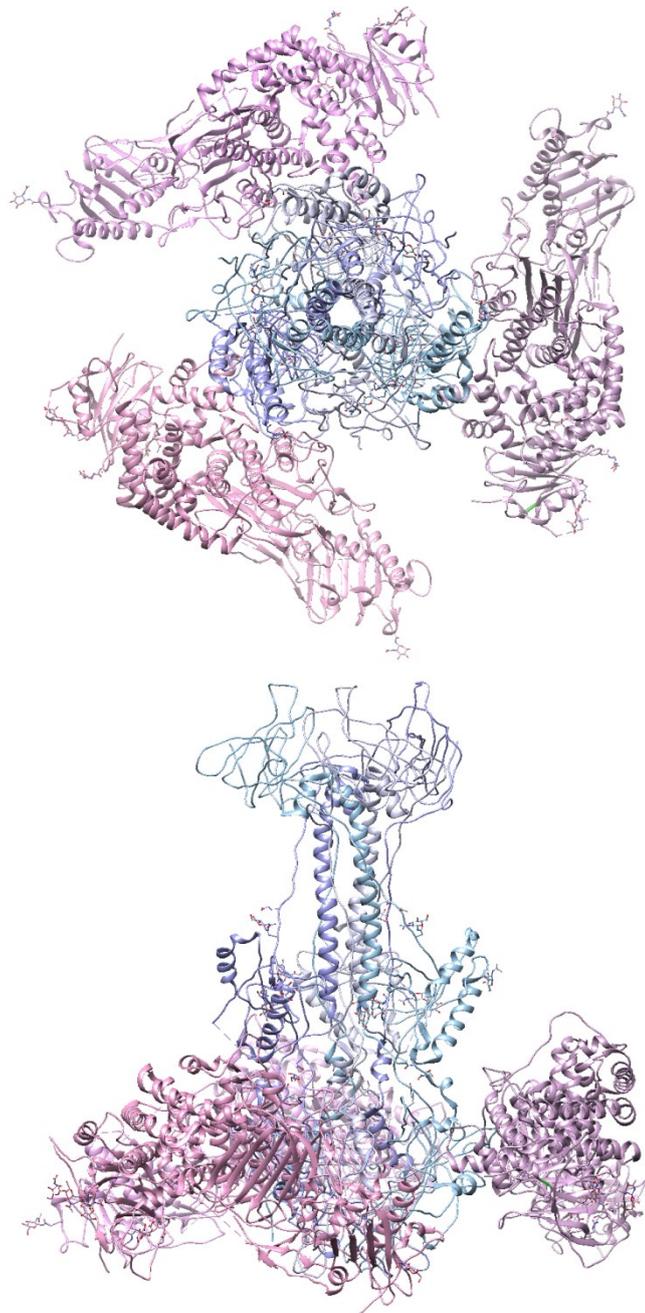


Figure 6. gHgL binds to the tall form of gB. This open structure, now a super-complex due to the putative gHgL FR1 binding domain visible at lower right, does not restrict the gB fusion loops. The gD profusion domains would be clamped between the gB and gHgL binding domains. gHgL Leucine 790 is highlighted in green.

remains bound to gHgL and gB in a supercomplex,²⁸⁻³⁰ and that the function of gB is regulated by activation of gHgL by receptor-bound gD.¹¹¹ With gHgL bound to it in this way, the tall conformation of gB is supported without its fusion domains being blocked in, which is consistent with the discussion in earlier sections. Finally, gD is very well anchored as we would expect for this mechanism: The intravirion domain of gD binds directly to the capsid, or via tegument protein VP22.¹¹³

Apart from the active cellular forces induced by the HSV antigens initially binding their receptors for endocytotic entry, puncturing the cell membrane also induces similar active responses.^{68, 114} Therefore there is also a possibility that the gD PFD could transmit cellular forces on HVEM and/or Nectin to the tall form of gB, having already punctured the plasma membrane. The gD loop is integrally bonded to both its ectodomain bound to the gD receptors and to the membrane-spanning domain of gD attached to the capsid,^{113, 115} so is ideally suited to transmit force. The PFD would also be pulling up on the gHgL transmembrane region and might even be able to pull it out to locally invert the virion envelope. This would clearly promote fusion of the virion and plasma membranes to a great extent.

This superstructure has probably not been identified experimentally yet because it is so large and unstable: Cross-linking studies would have discarded all groups of superstructures from initial centrifugations and the sample preparation required for electron micrographs would disrupt the superstructure. Super-resolution optical techniques require fluorophore labelling that would also disrupt it; for example a recent dSTORM study¹¹⁵ used the strongly neutralising LP2 antibody¹¹⁶ that would displace the profusion domain from gD.³⁵ Of all these techniques, super-resolution microscopy using a non-neutralising FAB for gHgL may stand the best chance of verifying an initial triangular configuration for it in future studies. The structure could then be qualified as a native structure after optical examination by assaying the same virions for infectivity.

3.2. The virion surface

Electron tomography of cryogenically frozen HSV virions indicates they are 225 nm in diameter and have 600–750 spikes on the surface.¹¹⁷ The surface spikes were not very well resolved compared to the reconstruction of the capsid. Assuming the preparation and freezing process disrupts any superstructures then the spikes counted would only generally represent the larger glycoproteins, gB and gHgL. We can estimate what proportion these are of the spikes counted using the single-molecule fluorescence determination of the gD copy number per virion at 335 * / 0.7.¹¹⁸ This would indicate the number of superstructures originally on the surface was approximately 112, making the expected number of gB and gHgL spikes 448. This is approximately two-thirds of the spikes counted. The agreement of these estimations allows us to estimate the proportion of the virion surface occupied by the

gB:gHgL:gD superstructures. A virion of 225 nm diameter would have a surface area of $1.59 \times 10^{-13} \text{ m}^2$. The distance between adjacent centers of the superstructures is 15 nm, implying the triangular space they each occupy has a width of 22.5 nm and a base of 26 nm. Thus the area needed by each superstructure is $2.93 \times 10^{-16} \text{ m}^2$ and the area required for 112 of them is approximately $3.28 \times 10^{-14} \text{ m}^2$, 20.6% of the total surface area of the virion. Accounting for the variation in gD copy number this would be 14.4% - 20.6% - 29.4% of the virion surface. These seem quite low percentages but the virion surface does usually accommodate many other types of glycoproteins, including UL32, UL49A, gM, gC, gE, gI, gJ, and gK, so a surface area fraction of 14 - 29% for the gB:gHgL:gD superstructure is probably realistic.

Structural alignments

The structural alignments presented in the figures, made using UCSF Chimera,¹¹⁹ are not computationally optimized and should only be viewed as detailed cartoons showing symmetries and approximate tessellations. Because the initial metastable superstructure is conjectured only to be locked by steric interactions, rather than binding interactions, future optimization by energy minimization would have to allow for potential energy storage by anti-binding, and for the strains and forces on transmembrane regions. This level of modelling is beyond our resources, but no like-charged residues are adjacent in the tessellations.

The structures used for alignment and figures were as follows; codes are PDB ID references: gB ectodomain, compact form.¹³ gB ectodomain, tall form: 3NW8 & gB ectodomain, fusogenic form: 3NWF.⁶⁹ gHgL ectodomain: 3M1C.¹⁷ gD ectodomain, no disordered loop: 1L2G & gD ectodomain bound to HVEM: 1JMA.²⁶ gD ectodomain bound to Nectin-1: 3U82.³¹

Summary of the full membrane-fusion hypothesis

Binding a cell-surface receptor displaces the gD loop whose profusion domain binds to gHgL FR1, triggering a spring-loaded movement of gHgL from a metastable membrane-associated conformation that was sterically stabilizing a compact form of gB. This directed movement of gHgL brings FR1 into contact with a binding region on the tall form of gB, where its avidity is enhanced by the gD profusion domain clamped between them. In this way a sterically metastable superstructure of gD, gHgL and gB is pre-tensioned against the most brittle areas of the plasma membrane by receptor binding before gB is activated from its compact to its tall conformation, during which it would often puncture the plasma membrane. This would generate a brief and local drop in pH due to the membrane potential and the high mobility of protons in water. The drop in pH would trigger another slight conformational rearrangement of gB that releases its fusion domains, allowing them to embed in both leaflets of the plasma membrane, mediating its fusion with the virion envelope. Formation of the full

fusion pore follows. Membrane puncture and fusion would be assisted by bulk tissue strain further tensioning gB against the plasma membrane and by virion-induced strengthening of the cytoskeleton. On some tougher cell types the virion induces its endocytosis having failed to puncture the plasma membrane by the activation of gB. In this alternative entry pathway the virion then remains in a pre-tensioned state in an endosome until mechanical fluctuations may eventually induce fusion. The activation energy for endosomal entry would be lowered by vesicular acidification revealing the fusion loops of gB.

Conclusions

This paper presents various physical considerations relevant to HSV entry and its disruption, and proposes a functional, sterically metastable, superstructure for the essential glycoproteins. The main conceptual result is that gD binds its receptors strongly enough to hold gB against the plasma membrane with enough force to puncture it during its activation. The principal targets of HSV are neurons because there it can maintain latent infection for the full life of the organism. On less brittle cells gB cannot puncture the membrane in the first instance but on some cell types retrograde forces induced in the cell endocytose the virion. This leads to entry when endosome acidification breaks the hydrogen bonds securing the fusion loops of gB. In the optimized entry pathway to neurons, this paper has argued that these hydrogen bonds would also be broken when the activated fusogen is suddenly exposed to a high local electric field after transiently puncturing the live plasma membrane, leading to membrane fusion.

Overall, there is now a physical and structural basis for the result that a single gD can support HSV entry.^{34, 35} Physically, gD has enough receptor avidity to assist gB in puncturing a brittle plasma membrane by holding it against the fusogen during its rearrangement. Structurally, we have seen that only one gD is required to hold down each gHgL heterodimer sterically stabilizing the compact form of gB.

Publication Notes

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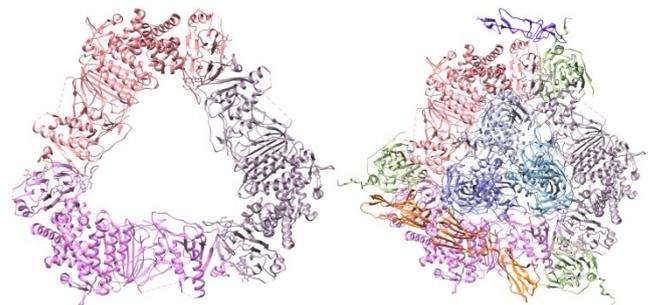
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Synopsis: Three gHgL heterodimers, each held down by gD, sterically stabilize a compact form of the fusogen gB. Once all three gD glycoproteins bind cellular receptors, holding the plasma membrane taut and directly adjacent, the displacement of the gD linker regions allows the gHgL triangle to open. The hairpin loops of gB are then free to rotate anti-clockwise, whereupon the central fusogen springs up into the plasma membrane, bolstered by virally-induced internalization of the gD receptors, to produce membrane fusion. On some cell types other virally induced forces can endocytose the virion before the full activation of the superstructure.

Supporting Information

Additional figures showing the gHgL triangle (S1) and the approximate scale of the disordered gD linker (S2):

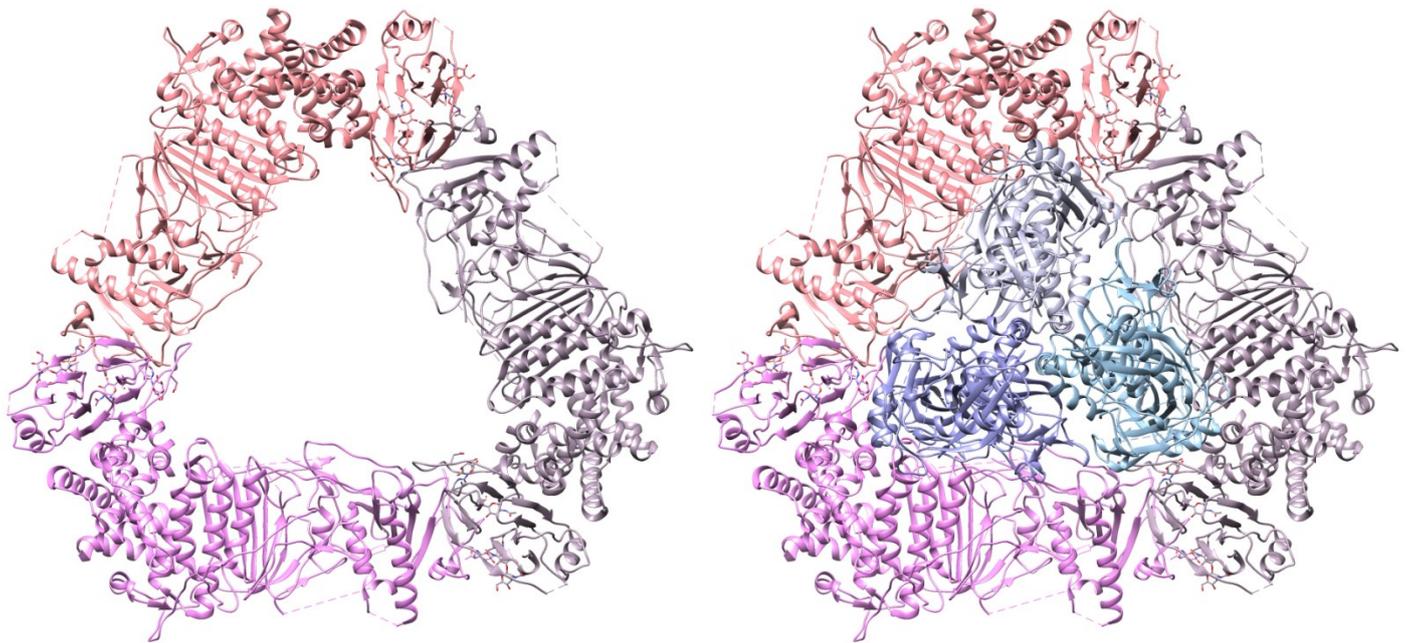


Figure S1. Triangle of three tessellated gHgL heterodimers. Left: Without gB. Right: Surrounding the compact form of the gB trimer.

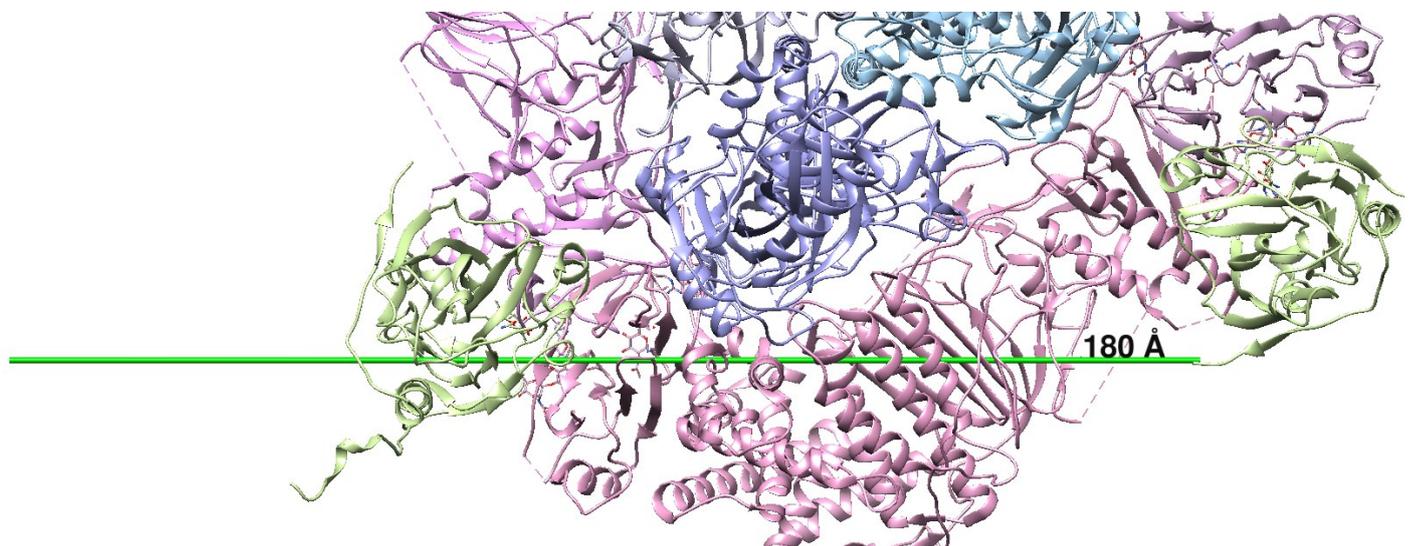


Figure S2. Scale of gD linker. A lower estimate for the scale of the gD linker, given that it is disordered. It easily reaches the putative binding regions on the lefthand gHgL with enough slack to double back to the transmembrane region of gD.