Chapter 3
Class III Viral Membrane Fusion Proteins

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Abstract Members of class III of viral fusion proteins share common structural features and molecular architecture, although they belong to evolutionary distant viruses and carry no sequence homology. Based of the experimentally determined three-dimensional structures of their ectodomains, glycoprotein B (gB) of herpesviruses, G protein of rhabdoviruses and glycoprotein 64 (gp64) of baculoviruses have been identified as class III fusion proteins. The structures are proposed to represent post-fusion conformations, and they reveal trimeric, elongated, rod-like molecules, with each protomer being composed of five domains. Sequences which interact with target membranes and form the fusion peptides are located in two loops found at one end of the molecule. Class III fusion proteins are embedded in viral envelope with the principal function of catalyzing fusion of viral and cellular membranes, an event that is essential for infection to occur. In addition, they have been implicated in processes such as attachment to target cells and viral maturation. G protein is the only class III fusion protein for which structures of both pre- and post-fusion states have been determined, shedding light on the mechanism involved in the conformational change and membrane fusion. Whether similar structural organization of class III fusion proteins translates into a common mechanism involved in carrying out membrane fusion remains to be investigated.

3.1 Introduction

3.1.1 Definition of Class III Fusion Proteins

Viral membrane fusion proteins have been grouped into three classes (class I, II and III) based on their key structural features. The proteins described in this chapter, the members of class III fusion proteins, have been identified recently. Despite the lack of sequence conservation, they share a common three dimensional organization distinct from the one found in class I and II type fusion proteins. The unique structural features of class III fusion proteins include: (1) presence of five domains composed of both α- and β- secondary structure elements, unlike the predominantly helical class I, or class II proteins, made mostly of β-sheets; (2) a common secondary structure topology in which each domain is an insertion into another domain (described in detail in Section 3.2.2); (3) an elongated, centrally located α-helix, that forms a trimeric coiled-coil.

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T. Dittmar, K.S. Zänker (eds.), *Cell Fusion in Health and Disease*, Advances in Experimental Medicine and Biology 714, DOI 10.1007/978-94-007-0782-5_3, © Springer Science+Business Media B.V. 2011

3.1.2 Viruses Encoding Class III Fusion Proteins

Based on the experimentally determined structures, viral membrane proteins identified as class III fusion proteins are: glycoprotein B (gB) of herpesviruses (Herpes Simplex virus type 1 (HSV-1) and Epstein-Barr virus (EBV)), protein G of Vesicular Stomatitis virus (VSV) and glycoprotein 64 (gp64) of Baculoviruses.

Herpesviridae are a large family of double-stranded DNA viruses, counting more than 200 members that infect humans and a wide range of invertebrates and vertebrates [1]. They are notable human pathogens. HSV-1 infections cause benign epithelial labial lesions although they can result in life-threatening conditions such as encephalitis. EBV is an oncogenic herpesvirus associated with development of malignancies such as Burkitt's and Hodgkin's lymphoma, and nasopharyngeal carcinoma [2]. Herpesviruses carry a large linear genome of 100–200 Kb, which is packed in an icosahedral capsid. The nucleocapsid is immersed in a protein rich matrix called tegument, which is enveloped by a lipid bilayer decorated with gB spikes and a dozen of other surface glycoproteins. Herpesvirus virions appear as quasispherical particles in electron micrographs, with a diameter of 120–260 nm, depending on the thickness of the tegument.

VSV belongs to the *Rhabdoviridae* family of the negative-strand RNA viruses, which infect plants, insects, and a variety of animals, from which the virus can be transmitted to humans (rabies being the best known human pathogen) [3]. The virions of Rhabdoviruses form bullet-shaped 180 nm × 75 nm particles. The single RNA molecule forming the genome is ∼11 Kb long, and is coiled in a tight complex with a nucleocapsid protein, giving rise to a helical ribonucleoprotein structure that is surrounded by the cell-derived membrane. Unlike herpesviruses, which contain a range of proteins embedded in the membrane, the sole type of spike present in the envelope of rhabdoviruses is made of the protein G.

Members of the family *Baculoviridae* are double-stranded DNA viruses, whose circular genome ranges in size from 90 to 160 Kb. Baculoviruses infect insects, and have been found in more than 500 insect species. Their genome is packed with a nucleoprotein core into a capsid, which is surrounded by a membrane giving rise to rod-shaped virions, 250–300 nm long and 30–60 nm wide. The major surface glycoprotein associated with the envelope is gp64.

Viruses encoding class III type fusion proteins are evolutionarily distant, and they differ markedly in shape, size, virion structure and genome organization. Their fusion proteins show no sequence conservation, however they share a common structural architecture as described below. Whether the structural homology translates into a shared mechanism that these proteins utilize to cause membrane fusion is the topic of ongoing investigations.

3.1.3 The Multiple Roles of Class III Fusion Proteins

Herpesvirus gB, rhabdovirus G and Baculovirus gp64 share a common role of catalyzing fusion of viral and cellular membranes. The fusion event allows viral entry into the cells, and is essential for infection to occur. Rhabdoviruses and budded virions of Baculoviruses enter cells by endocytosis, where the low pH environment of the endosome triggers fusion of the viral envelope and endosomal membrane, resulting in the release of viral genome into the cytosol [4, 5]. Herpesviruses enter cells primarily by fusion of the viral and plasma membrane, although endocytosis, both pH-dependent and pH-independent, has been reported as a mode of entry when certain herpesviruses are presented with specific cell types (reviewed in detail in [6]). It is worth noting that while gp64 and G are necessary and sufficient to carry our membrane merger, herpesviruses, in addition to gB, require presence of a non-covalent hetero-dimeric complex made of glycoproteins H and L (gH/gL). It has been proposed that gH/gL mediates hemifusion of the outer lipids leaflets, while gB is required for resolution of the hemifusion intermediate [7].

Class III type fusion proteins are involved in processes other than fusion, suggesting that these proteins evolved to execute multiple functions. There are numerous reports implicating class III fusion proteins in intracellular processes such as viral maturation and egress of virus from the infected cells. For example, HSV-1 gB promotes fusion between the virion and outer nuclear envelope during the virion trafficking from the cell nucleus to cytosol [8]. The carboxy-terminal domain (CTD) of the human cytomegalovirus (HCMV) gB is critical for viral maturation [9], while the CTD of EBV and HHV-8 gB are required for lytic replication and production of infectious particles [10, 11]. gp64 is essential for efficient virion budding as well [12].

Some class III fusion proteins also mediate virus binding to cells. gB of herpesviruses allows the virus to attach to the heparan sulfate moieties expressed on target cells [13], and HSV-1 gB in addition associates with paired immunoglobulin-like type 2 receptor (PILR) alpha [14]. The latter interaction is required for infection, even though HSV-1 has another receptor binding protein gD, which binds to herpes virus entry mediator (HVEM) and Nectin-1 cellular receptors. HCMV gB interacts with the epidermal growth factor receptor, which serves as one of its entry receptors [15, 16], while HHV-8 gB binds to integrin $\alpha_3\beta_1$ [17]. A receptor for entry of Baculoviruses has not been identified, but gp64 has been reported to bind to a cellular receptor [18], with the putative receptor binding domain being located in the N-terminal region of the gp64 ectodomain [19]. Phosphatidylserine was believed to serve as a receptor for VSV G for a long time, but this has been recently disputed [20, 21]. There is also no clear consensus which cellular factors act as receptors for the rabies virus, a human rhabdovirus. Gangliosides, phospholipids, nicotinic acetylcholine receptors are among the ones proposed (reviewed in [22]).

3.2 Structural Features of Class III Viral Fusion Proteins

3.2.1 Global Structural Organization of Class III Fusion Proteins

Before introduction of class III type proteins, all viral fusion proteins had been categorized as class I or II fusion proteins [23–25]. Class I proteins, the best characterized being influenza virus hemagglutinin, contain functionally critical α-helices, and in the post-fusion conformation contain a prominent trimeric α-helical coiled coil [26]. They are present as trimers in both pre- and post-fusion states. The fusion protein E of tick borne encephalitis virus was the first viral protein, whose structure was shown to be radically different [27] from any described class I fusion protein, prompting the definition of a new class of fusion proteins (class II). Class II fusion proteins are mostly made of β-sheets, and unlike class I proteins, which remain trimeric during the conformational change from pre- to a post-fusion state, class II proteins are present as pre-fusion dimers that convert to post-fusion trimers.

Class III fusion proteins are transmembrane proteins, composed of a large ectodomain, followed by a single transmembrane region and a smaller cytosolic domain (C-terminal domain or CTD). The X-ray structures of the ectodomains of HSV-1 gB [28], EBV gB [29], VSV G [30, 31] and Baculovirus gp64 [32] became available in the past four years. VSV G is the only class III protein for which the structures of both pre-fusion and post-fusion (low-pH) states have been solved. Based on the structural homology of gB and gp64 with the latter form of G, their conformations were proposed to represent post-fusion states as well. Since there is currently limited functional data (see Section 3.4.2) and no experimental structural data describing the pre-fusion forms of gB and gp64, the following section will focus on the description of the putative post-fusion forms of the class III fusion proteins.

Crystallized ectodomains of all class III type proteins form post-fusion trimers (VSV G is a trimer in pre-fusion state as well). There is a centrally located > 40 residue long helix, that forms a coil (reminiscent of the coiled coils found in class I fusion proteins), and around which the three protomers wrap in a left-handed twist, forming an elongated rod-like molecule (Fig. 3.1). In addition to

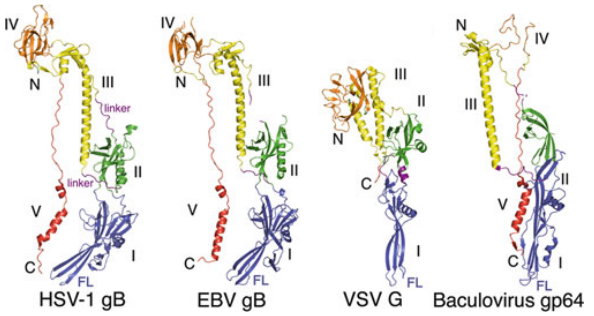

Fig. 3.1 Structures of class III fusion proteins. Three-dimensional, X-ray structures of the post-fusion, monomeric ectodomains of HSV-1 gB, EBV gB, VSV G and Baculovirus gp64 are shown. Domains are colored as in Fig. 3.1, and are labeled with roman numbers I to V. N and C are used to designate the amino and carboxy termini of the crystallized ectodomains. Linkers are colored in *purple* in all four panels. FL is used to designate the "fusion loops". The cartoon representations were generated in Pymol [71], using the following Protein Databank identifiers: 2GUM for HSV-1 gB, 3FVC for EBV gB, 2CMZ for VSV G and 3DUZ for Baculovirus gp64

the helical secondary structure elements, class III proteins contain β-sheets, the mixture of α and β structures giving rise to the distinct molecular architecture.

Ectodomains of class III fusion proteins share a similar secondary structure topology and three dimensional arrangement (Fig. 3.1). Yet, there are significant differences in the size of the ectodomains and their individual domains. G and gp64 ectodomains are more compact (~450 residues), compared to the gB ectodomain which contains more than 700 residues. Correspondingly, the G and gp64 crystallized spikes are shorter (12.5 and 15 nm, respectively), while gB trimers spikes are ~16 nm long. Conservation of the core structures suggests a common evolutionary origin, while the differences in the size and complexity of some domains indicate that class III proteins might have evolved to carry out functions specific to the viruses they belong to.

3.2.2 Domain Organization of Class III Fusion Proteins

Ectodomains of class III fusion proteins are composed of five domains. Domain I or the "fusion domain" contains a bipartite fusion peptide, composed of two loops, which carry the residues that insert into target membranes. In the post-fusion conformation, the fusion loops are exposed and located at one end of the rod-like trimer, in close proximity of the ectodomain C-terminus (domain V) (Fig. 3.1), which proceeds the transmembrane region (the latter not being part of the crystallized constructs). The whole domain I is inserted in between two β-strands of domain II (Fig. 3.2), which has a plekstrin homology (PH) fold. PH domains serve as binding surfaces for phospholipids and for protein ligands [33, 34], indicating that domain II of class III fusion proteins may interact with other proteins involved in the fusion process (for example, with the gH/gL complex or receptor binding proteins in herpesviruses). Domain II is embedded within domain III, which contains the prominent central α-helix contributing to most of the trimerization contacts. Domain III is inserted into domain IV (Fig. 3.2), located on the opposite end of the molecule from domain I and fusion loops (Fig. 3.1). Domain IV is formed of β-sheets, and exhibits the highest variability in size and structural arrangement (in gp64 it is largely disordered). Domain V is an extended segment of a polypeptide chain, that runs along the long side of the trimeric molecule, and in gB and gp64 inserts into the crevice formed by two other protomers, thereby contributing to an extensive trimerization surface. The VSV

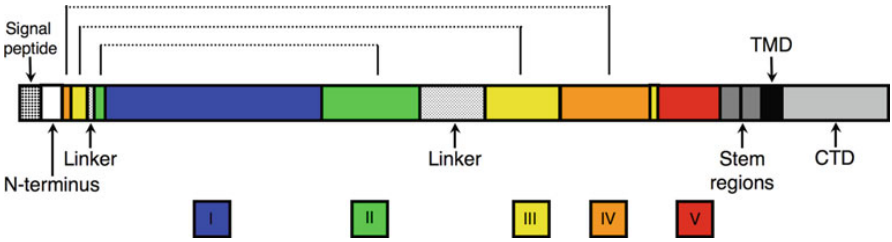

Fig. 3.2 Schematic representation of the domain organization of class III fusion proteins. The sequence of EBV gB is shown as an example. Other class III fusion proteins follow a similar global domain arrangement, although with different domain sizes and boundaries. *Brackets* shown on *top* are used to illustrate the insertion of domain I (*blue*) into domain II (*green*), which is on its own embedded in domain III (*yellow*), that is inserted in domain IV (*orange*). Domain V, which leads to the membrane-proximal stem regions, is shown in *red*. TMD and CTD designate transmembrane domain and C-terminal domain, respectively. The N-terminal region of gB, which is flexible and was not resolved in the HSV-1 and EBV structures, is shown as a *white* box and labeled as "N-terminus". Linkers are flexible regions that connect domains, and are believed to be important for conformational changes

G ectodomain lacks most of domain V, as it has been removed by proteolytic cleavage, employed to cleave the protein from the virion surface. More detailed description of the gB, gp64 and G domain structures, sizes and boundaries is reported in [35].

3.3 Functionally Important Regions of Class III Fusion Proteins

3.3.1 Fusion Loops

Fusion peptides are defined as stretches of polypeptide chain that interact with target membranes, allowing the fusion protein to be anchored in both membranes, the viral envelope via the transmembrane domain and the target membrane through the fusion peptide. Further structural changes, which occur in the fusion protein intermediate that bridges the two membranes, are proposed to bring the membranes in close apposition, resulting in their merger. Fusion peptides are secluded in the pre-fusion conformation, and become exposed to interact with the target membrane upon receiving an activating fusion trigger. Class I proteins have fusion peptides that are typically located at the N-terminus of a subunit, while the fusion peptide of class II proteins is internal and located in a loop between two β-strands. Fusion peptides of class I and II proteins are highly conserved sequences, rich in small, apolar residues (A, G, L, I), which have a high propensity to insert into lipid bilayers (reviewed in [36]).

The fusion peptide of VSV G has been located by mutagenesis [37, 38], and the structure of G ectodomain pre-fusion state [31] revealed two internal loops, located at the end of the trimer, and in a "pointy" conformation, similar to what has been observed in the fusion peptides of class II fusion proteins. The bipartite fusion peptide of G is composed of residues W^{72}, Y^{73}, Y^{116} and A^{117}. Mutagenesis studies demonstrated that the aromatic residues W^{72}, Y^{73}, Y^{116} are critical for the ability of G to mediate fusion, while A^{117} is less important [39]. Direct association of the region carrying the fusion peptide of G with lipid bilayers was observed by photolabeling studies [40].

Loops in gB, which are structurally analogous to the fusion loops of G, were proposed to form gB putative fusion peptide [28]. Mutagenesis studies confirmed that substitutions of some of these residues (shown as underlined) in HSV-1 gB (V**W**FGHR**Y**$^{173–179}$ and RVE**A**F**HR**Y$^{258–265}$) [41] and EBV gB (G**WY**A$^{111–114}$, G**WLIW**TY$^{192–198}$) [42] abolished the ability of gB to mediate fusion, supporting the idea that these loops play an important role in fusion. Finally, mutations of the hydrophobic

as well as charged residues (shown in bold) in the HSV-1 gB fusion loops diminished the binding of the resulting recombinant proteins to cells and naked liposomes, demonstrating that the proposed regions of gB interact with membranes directly and constitute the gB fusion peptide [43].

The fusion peptide of EBV gB contains more hydrophobic residues than HSV-1 gB, consistent with the tendency of the EBV gB recombinant ectodomains to form rosette structures [44], typically found in post-fusion preparations of class I and II fusion proteins. Rosette formation in the latter cases is driven by hydrophobic interactions of the exposed fusion loops, and indeed EBV gB ectodomains form simple trimers, which could be crystallized, only when its residues $WY^{112–113}$ and $WLIW^{193–196}$ are substituted by the analogous residues, HR and RVEA, found in HSV-1 gB.

Residues in gp64, forming loops analogous to the fusion peptide of G and gB, are $GGSLDPNT^{79–86}$ and $NNNHFA^{149–154}$ [32]. Substitutions of the hydrophobic, but also polar residues within the fusion loops (shown as underlined) result in the loss of ability of the gp64 variants to induce syncytium formation [32].

Unlike fusion peptides of class I and II fusion proteins, the bipartite fusion peptides of class III proteins are not conserved sequences. They however locate to the structurally homologous loops, which are found at the end of the spike molecule, and contain aromatic, polar residues (such as W and Y) and histidine residues, which are often found at membrane interfaces [45]. This indicates that while class III protein fusion loops may not be inserting deeply into the lipid bilayer (as peptides of class I and II proteins abundant in residues such as A, I, L, and G), they may associate with membranes in a more superficial manner, sufficient enough to destabilize the integrity of lipid bilayers and promote fusion.

3.3.2 Membrane-Proximal (Stem) Regions and C-Terminal Domain (CTD)

Membrane-proximal or stem regions of gB and G are around 40 residue long segments that precede the transmembrane domain. They are rich in hydrophobic residues, indicating that they may interact with membranes and play a role in fusion. Deletions made in the stem region of VSV G cause profound decrease in cell:cell fusion and reduce virus infectivity [46]. Grafting of the G stem regions onto heterologous fusion proteins enhances their fusion activity, and stem regions together with the CTD and the transmembrane domain (i.e. lacking the ectodomain) are sufficient to mediate fusion penetration, supporting the idea that the G stem regions have inherent fusogenic potential [47]. The last 12 residues of the G stem were also shown to be necessary for efficient virus assembly, possibly because they promote virus release by destabilizing membranes at the sites of budding [48].

The involvement of gB stems in fusion has not been systematically investigated. It is however curious that the gB proteins that have a lower content of hydrophobic and aromatic residues in their fusion loops (indicating weaker propensity to interact with membranes), have higher abundance of such residues in their membrane-proximal regions [44]. It is possible that fusion loops and stem regions complement each other in destabilizing the membranes, but this hypothesis remains to be tested.

The CTD of gB modulates the fusion activity of the ectodomain, resulting in some cases in hyperfusion or null fusion phenotypes, and demonstrating the existence of cross-talk between the gB ectodomain and its CTD through the membrane. For example, a truncation in the CTD of EBV gB results in a protein which can cause fusion in the absence of the gH/gL complex [49], and deletions in the CTD of HSV-2 gB can yield protein variants with dramatically enhanced cell fusion activity [50]. Experiments using the recombinant CTD of HSV-1 gB demonstrated that CTD associates with membranes, preferentially binding to the negatively charged lipid heads [51]. The CTD truncations that caused a hyperfusion phenotype bound poorly to artificial liposomes, suggesting that a stable CTD interaction with lipid bilayer may serve as a negative regulator of fusion.

The stem region and CTD of gp64 (around 20 and 7 residues, respectively) are shorter than those of gB and G. Deletion of the CTD only moderately decreases production of infectious virus, and has a more dramatic effect on budding efficiency, suggesting involvement of the CTD in viral maturation [12].

3.4 Molecular Basis of the Class III Type Proteins Fusion Mechanism

3.4.1 Activation Triggers for Class III Viral Fusogens

Herpesviruses enter cells through fusion of the viral and plasma membranes, in a process that is triggered by binding of a viral surface protein to a specific host-cell encoded receptor. This interaction is essential for entry as it provides an activation signal for the fusion machinery composed of gB and the gH/gL complex. Herpes simplex viruses utilize gD to bind to Nectin-1 or HVEM receptor (reviewed in [13]), while gp42 of EBV interacts with MHC class II expressed on B lymphocytes, the target cells for EBV [52]. Both gD and gp42 are membrane anchored proteins, however their soluble, recombinant ectodomains activate fusion in virus-free cell:cell fusion assays as well [53, 54]. gD and gp42 have been proposed to undergo conformational changes upon binding to cellular receptors [55, 56], suggesting that the structural alterations may provide the activation signal to the gB-gH/gL fusion machinery. How the fusion trigger is transferred to the fusion machinery, and to which of its components is not understood. pH has also been suggested to serve as a fusion trigger in cases where herpesviruses enter cells by endocytosis [57, 58].

The G fusion protein of rhabdoviruses and gp64 of Baculoviruses are activated by exposure to the acidic pH of the endosome, which occurs after the virus is endocytosed by target cells. G and gp64 have a unique capability of undergoing a reversible conformational change [59–61], and both proteins can be reactivated to induce fusion after acidification [60, 62]. This is in contrast to all other known fusion proteins, where the post-fusion state is the more thermodynamically stable one, regardless of pH, and whose conformational rearrangement is irreversible. It has been proposed that the reversible conformational change may serve to maintain the fusion proteins in a fusion-competent state after they pass through the acidic Golgi compartment, during protein trafficking to the cell surface.

3.4.2 Structural Rearrangements of Class III Fusion Proteins

VSV G is the only class III fusion protein whose structure has been solved for both the pre-fusion and post-fusion states [30, 31]. During its conformational change, which is triggered by exposure to the acidic pH of the endosome, individual domains of G relocate in a manner that results in conversion of a more compact pre-fusion (8.5 nm) to an extended post-fusion trimer (12 nm). Unlike class I and II fusion proteins, in which individual domains undergo significant refolding, the domains of G mostly retain their structure. The domain repositioning is rather a consequence of structural alterations occurring in flexible linker regions that connect domains. As a result, domain I, which carries the fusion loops, relocates 16 nm from one to the opposite side of the molecule (reviewed in detail in [22, 35]). The conformational change is proposed to be a consequence of the pH-induced protonation and deprotonation of key histidine residues, leading to a loss of a network of interactions that hold domain I and membrane-proximal regions together, thus initiation domain I repositioning.

Baculovirus gp64 contains a number of conserved histidine residues, and a similar mechanism driving the conformational change has been proposed [32]. Interestingly, in addition to the conserved intra-molecular disulfide bridges, gp64 contains an inter-chain disulfide bridge (Cys^{26}-Cys^{372}) which is located at the top of the central helix, connecting domains III of two protomers. It could be expected

that the inter-molecular disulfide imposes restrictions on domain movement and reorganization, thus being important for the fusion mechanism. Elimination of the inter-chain disulfide however was shown not to affect fusion and virion budding. However, the gp64 variant lacking the disulfide bond cannot rescue a gp64null bacmid [63], suggesting a still unknown role of the inter-chain disulfide in viral infectivity.

As mentioned in Section 3.4.1, the post-fusion, low-pH form of G and gp64 can be reactivated to adopt a fusion-component state by exposing the protein to elevated pH. Whether gB undergoes a similar reversible conformational change is not clear, and data suggesting the ability of gB to adopt different conformational states has become available just recently. Structural changes in murid herpesvirus gB were detected upon exposure to low pH [64], as well as in HSV-1 gB [65]. In the latter case, the observed conformational changes in the antigenic structure were reversible, and could be detected in purified, recombinant gB material as well. pH-dependent entry of herpesviruses via endocytosis has been reported for certain herpesviruses in combination with specific target cell types, for example in the case of HSV-1 entry into human epithelial cells [58]. Whether gB undergoes a conformational change during receptor-mediated entry, which is the predominant entry pathway of herpesviruses, still needs to be determined.

Unlike Baculoviruses and rhabdoviruses which have a single fusogen protein, herpesviruses have evolved a more complex machinery that in addition to gB contains the gH/gL complex. The structure of the gH/gL ectodomain from HSV-1, EBV and pseudorabies have become available recently [66–68], and revealed a protein with a fold that does not resemble any known fusion protein. Functional data show that while the gH/gL complex mediates hemifusion, gB is required for fusion to proceed to formation of a fusion pore thus allowing capsid entry [7]. gH/gL and gB form transient complexes, but only when gD (as membrane-anchored or soluble, recombinant ectodomain) is added, indicating that the fusion complex assembles only when gH/gL, gB or both components of the fusion machinery receive an activating signal from the receptor-binding protein gD [69]. Finally, by using a panel of monoclonal antibodies that bind to distinct domains of gB, it was suggested that the fusion process begins with the insertion of gB fusion loops into target membrane, followed by a gB–gH/gL interaction, and eventually fusion [70]. What the molecular mechanism of these events and what kind of structural changes, if any, occur in gB and gH/gL to drive fusion, are some of the questions that need to be answered.

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