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Structural and Mechanistic Insights into the Tropism of Epstein-Barr Virus

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Epstein-Barr virus (EBV) is the prototypical γ-herpesvirus and an obligate human pathogen that infects mainly epithelial cells and B cells, which can result in malignancies. EBV infects these target cells by fusing with the viral and cellular lipid bilayer membranes using multiple viral factors and host receptor(s) thus exhibiting a unique complexity in its entry machinery. To enter epithelial cells, EBV requires minimally the conserved core fusion machinery comprised of the glycoproteins gH/gL acting as the receptor-binding complex and gB as the fusogen. EBV can enter B cells using gp42, which binds tightly to gH/gL and interacts with host HLA class II, activating fusion. Previously, we published the individual crystal structures of EBV entry factors, such as gH/gL and gp42, the EBV/host receptor complex, gp42/HLA-DR1, and the fusion protein EBV gB in a postfusion conformation, which allowed us to identify structural determinants and regions critical for receptor-binding and membrane fusion. Recently, we reported different low resolution models of the EBV B cell entry triggering complex (gHgL/gp42/HLA class II) in "open" and "closed" states based on negative-stain single particle electron microscopy, which provide further mechanistic insights. This review summarizes the current knowledge of these key players in EBV entry and how their structures impact receptor-binding and the triggering of gB-mediated fusion.

INTRODUCTION

Included in the nine members of the ***Herpesviridae*** that infect humans is Epstein-Barr virus (EBV), which causes infectious mononucleosis in adolescents and the vast majority of humans have a lifelong persistent latent infection. Establishment of lifelong persistence is a hallmark of herpesvirus infections that can be associated with serious disease such as malignancies of B lymphocytes, as in Burkitt and Hodgkin lymphoma, or epithelial cells, such as nasopharyngeal carcinoma and certain gastric carcinomas. However, EBV is also associated with T/natural killer-cell lymphoproliferative disorders and a variety of lymphoproliferative disorders observed in immunosuppressed individuals such as post-transplant or HIV/AIDS patients (Longnecker et al., 2013).

Herpesviruses feature approximately a dozen envelope-glycoproteins, but the conserved core fusion machinery consists of three key players - glycoprotein B (gB) and the heterodimeric complex gH/gL accompanied by additional non-conserved receptor-binding proteins. While the highly-conserved fusion machinery indicates that these proteins function with a similar mechanism during herpesvirus fusion, the receptor-binding proteins are virus- and host cell type-specific. The receptor-binding proteins include gD for *α-**herpesvirinae*** (except varicella-zoster virus VZV), gO or UL128/130/131A for cytomegalovirus (β-herpesvirus) and its functional homolog gp42 for EBV (γ-herpesvirus), establishing the distinct cell tropism of individual herpesviruses (Adler, 2015; Spear and Longnecker, 2003). The purpose of this review is to summarize the state of the research about the entry routes utilized by EBV to mediate fusion of the virion envelope with a cellular membrane focusing on the key players and how their structure imparts receptor-binding and fusion function during herpesvirus entry. We also compare the entry mechanism of EBV to of other members of the ***Herpesviridae*** family.

EPSTEIN-BARR VIRUS FUSION AND ENTRY

Although entry is executed by the conserved core fusion machinery, herpesviruses utilize a variety of different entry routes. EBV entry is a prime example since it enters epithelial cells by direct fusion with the plasma membrane but must be endocytosed prior to membrane fusion for B cell infection (Miller and Hutt-Fletcher, 1992; Nemerow and Cooper, 1984). EBV utilizes five glycoproteins for efficient B cell entry, which includes the attachment protein gp350/220, the receptor-binding protein gp42 and the core fusion machinery gH/gL and gB. In contrast, for epithelial cell infection the attachment proteins gp350/220 and/or BMRF2 are all that is required to complement the core fusion machinery (Longnecker et al., 2013). The abundant glycoprotein gp350/220 binds complement receptor 2 (CR2/CD21) or CD35, alternatively tethering EBV to its host cells in an attachment step that is not essential for entry but increases the efficiency of infection, similar to herpes simplex virus (HSV) gC (Janz et al., 2000; Langeland et al., 1990; Ogembo et al., 2013). The multispan membrane protein BMRF2 is thought to bind integrin α5β1, tethering EBV to polarized epi-

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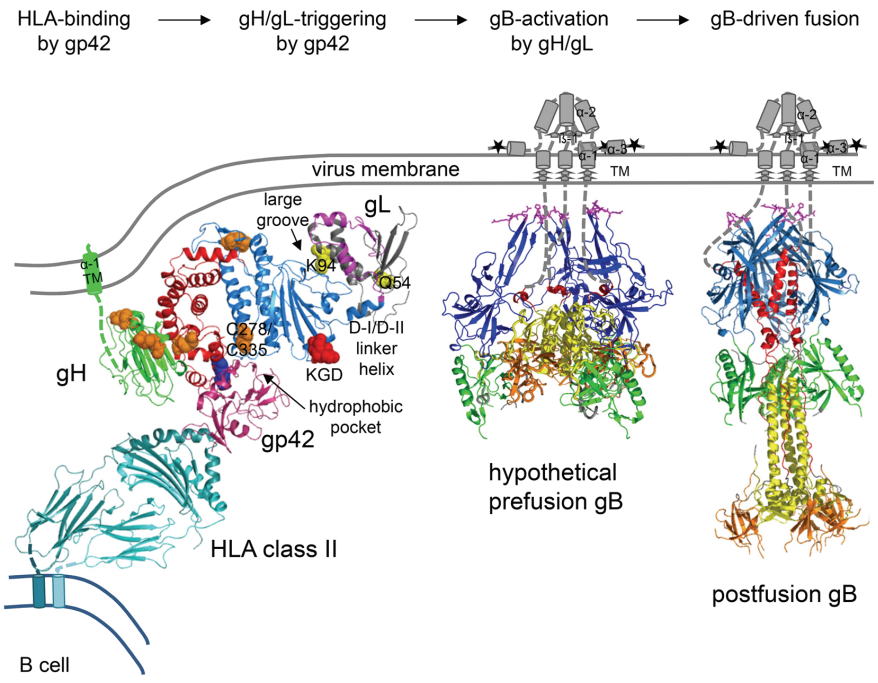

Fig. 1. Model of EBV entry into B lymphocytes. First, gp42 binds to its B lymphocyte receptor HLA class II, which cause a widening of the hydrophobic pocket within gp42. The conformational change within gp42 might be transmitted to gH/gL, which allows the interaction with the fusogen gB. In this model, D-I of gH/gL includes the residues Q54/K94 (yellow spheres), which are supposed to be involved in gB interactions and are directed towards a model of prefusion gB. The interaction of the tripartite complex gH/gL/gp42 with gB triggers the conformational transition to postfusion gB, which drives fusion with B cell membranes. The structural view of EBV gH/gL shows the disulfide bonds (orange spheres), gp42-binding region (blue spheres) and the KGD-motif (red spheres). TM is transmembrane region, α is α-helix and β is β-strand. The protein secondary structure prediction for the CTDs of EBV gB and gH were designed using JPred4 (Drozdetskiy et al., 2015). The structural views of prefusion and postfusion gB (PDB ID: 3FVC) (Backovic et al., 2009) as well as the complex composed of gH/gL (3PHF) (Matsuura et al., 2010) and gp42-HLA-DR1 (1KG0) (Mullen et al., 2002; Sathiyamoorthy et al., 2014) were generated using The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

thelial cells (Tugizov et al., 2003). Whereas binding of gp42 to its B cell receptor human leukocyte antigen (HLA) class II activates gH/gL, which in turn triggers gB-mediated membrane fusion (Fig. 1), the gp42 interaction with gH/gL blocks epithelial cell fusion and entry (Borza and Hutt-Fletcher, 2002; Chen et 2012). Thus, the highly adapted tropism of EBV is established by the prevalence of either the heterodimeric complex gH/gL or the tripartite complex gH/gL/gp42 on EBV virions. For virus produced in B cells, HLA class II regulates the amount of gp42 on the budding virions by sequestrating it during its maturation pathway, thereby modulating the ratio of the epithelial cell-tropic complex (gH/gL) and the B cell-tropic complex (gH/gL/gp42), reducing the latter. Since epithelial cells lack HLA class II, virions produced from these cells infect B cells more readily since they are enriched in the tripartite gH/gL/gp42 complex essential for B cell infection (Borza and Hutt-Fletcher, 2002; Wang et al., 1998). The receptor-binding activation step is initiated by either the interaction of gH with integrins such as αvβ5, αvβ6 or αvβ8 on epithelial cells (Fig. 2) or binding of gp42 to its B cell receptor HLA class II. It is likely that this receptor-binding event activates gH/gL to interact with gB in a homotypic manner (EBV gH/gL can trigger only EBV gB), triggering the conformational change to the postfusion form of gB and thereby mediating fusion (Fig. 1-2) (Connolly et al., 2011; Stampfer and Heldwein, 2012).

STRUCTURAL FEATURES OF THE CORE ENTRY MACHINERY

The crystal structures for the core fusion machinery consisting of gB, gH/gL and gp42 shed light on the herpesvirus-induced membrane fusion process.

Structural features of the fusogen gB

The crystal structure of EBV gB in its post-fusion conformation, with its fusion peptides close to its transmembrane domains, revealed 16 nm spike-like homotrimers formed by individual subunits that adopt an elongated conformation divided into five domains. The similarity to the postfusion conformation of the vesicular stomatitis virus G protein, along with other structural features that distinguish these proteins from previously established class I and II viral fusogens, led to their new classification as class III viral fusion proteins (Backovic et al., 2009; Heldwein et al., 2006; Roche et al., 2006; Steven and Spear, 2006). HSV-1, human cytomegalovirus (HCMV) and EBV gB share strikingly similar postfusion structures but the diverse domain orientations suggest species-specific functional adaptations for the EBV glycoproteins (Backovic et al., 2009; Burke and Heldwein, 2015; Chandramouli et al., 2015; Heldwein et al., 2006). Moreover, the crystal structure revealed that the trimeric form of gB features a long C-terminal arm, which is arranged in an antiparallel orientation to the coiled-coil core (Backovic et al., 2009; Connolly and Longnecker, 2012). A functional study of HSV gB showed that this arm-region is important for fusion suggesting that the formation of the coil-arm complex mediates the conformational change to the postfusion form of gB simito class I fusogens (Connolly and Longnecker, 2012). Surprisingly, the hydrophobic fusion loops of EBV gB, which are typical features of class I/II viral fusogens, cannot be exchanged with the more hydrophilic fusion loops of HSV gB. Further, it has been noted that there is a virus-specific correlation between the hydrophobicity of the gB fusion loops and membrane proximal region (Backovic et al., 2007a; 2007b; Zago et al., 2012).

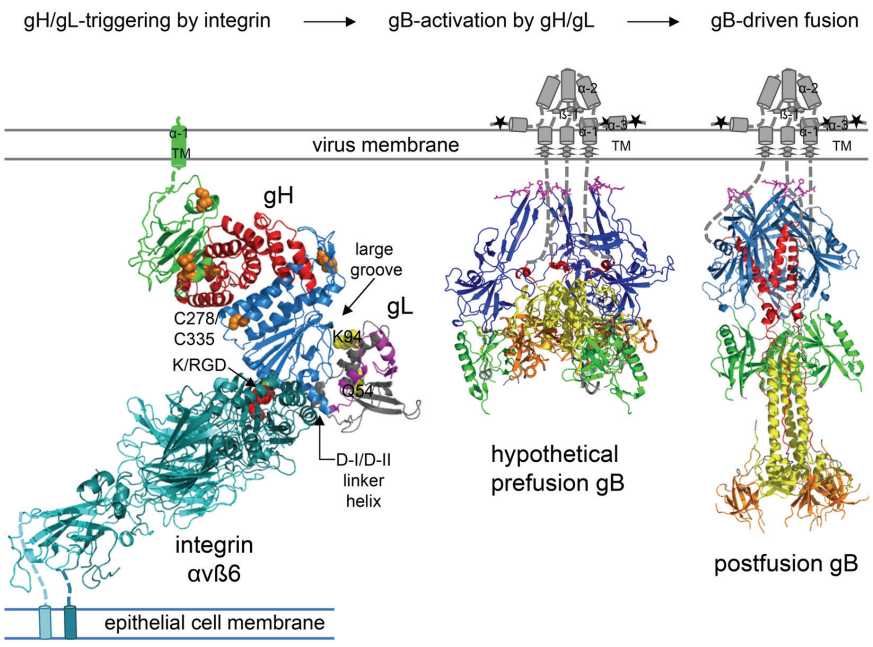

Fig. 2. Model of EBV entry into epithelial cells. First, gH binds to its epithelial cell receptor integrin αvβ6 causing a conformational change within the large groove of gH/gL, which might allow the interaction with the fusogen gB. This interaction triggers the conformational transition to postfusion gB, which drives fusion with the plasma membrane. The K/RGD-motif was used to model integrin binding to gH/gL based on the structure of αvβ6/TGF-β3 (4UM9). The structural views of integrin αvβ6 (4UM9) (Dong et al., 2014), prefusion and postfusion gB (3FVC) (Backovic et al., 2009) were generated using The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

Structural features of the heterodimeric complex gH/gL

The structures of EBV, HSV-2 and VZV gH/gL as well as pseudorabies virus (PrV) gH indicated that gH/gL has no features in common with known fusogens (Backovic et al., 2010; Chowdary et al., 2010; Matsuura et al., 2010; Xing et al., 2015). EBV gH/gL and PrV gH are characterized by an elongated rod-like shape, whereas HSV-2 and VZV gH/gL have a boot-like overall conformation (Backovic et al., 2010; Chowdary et al., 2010; Matsuura et al., 2010). The strikingly similar structure of gH/gL, despite the low amino acid conservation, led to the partition into four structural domains with varied domain interfaces due to divergent inter-domain packing angles (Backovic et al., 2010; Matsuura et al., 2010). Mutagenesis studies of EBV and PrV gH found that these domain interfaces and their flexibility are important for gH/gL function during fusion (Böhm et al., 2015; Chen et al., 2013; Möhl et al., 2015; Wu et al., 2005). The intimate interaction of gL with the N-terminus of gH forms domain I (D-I) and only a single helix, known as the D-I/D-II-linker-helix, connects D-I with the rest of gH forming a large inter-domain groove (Matsuura et al., 2010). Interestingly, an alanine-scanning mutagenesis approach indicates that the linker-helix is important for gB-mediated fusion with both epithelial and B cells (Figs. 1 and 2) (Omerovic et al., 2005). The gH homologs feature a striking pattern of disulfide bonds (DB) distributed over the surface of gH, except for one unpaired cysteine (C153) in the large D-I/D-II-groove. The highly conserved DB of D-III is buried suggesting a stabilizing function. Compatible with this hypothesis, we found that this DB forms interactions with surrounding amino acids which ensure the cell surface expression of PrV and EBV gH/gL and thus is important for HSV, PrV and EBV gH/gL function during fusion (Cairns et al., 2005; Möhl et al., 2015; Schröter et al., 2014).

Structural features of the tropism determinant gp42

In contrast to gB and gH/gL, the type II single pass membrane protein gp42 is unique to EBV and closely-related lymphocryptoviruses. The overall structure of gp42 is formed by a C-type lectin domain (CTLD) and a flexible, extended N-terminus extending away from the CTLD, which is tied to the core by a DB (Mullen et al., 2002). Despite the characteristic CTLD within gp42, the HLA class II binding site is distinct from the canonical binding site of lectin and natural killer receptor ligands (Mullen et al., 2002; Spear and Longnecker, 2003). Interestingly, the CTLD contains a hydrophobic pocket at this canonical site, which is located next to the HLA-binding site and experiences a slight conformational change in its loop, widening the pocket after HLA receptor-binding, which could be important for activating entry (Kirschner et al., 2009). The conformational change within the hydrophobic pocket is thought to be involved in activation of the gB-mediated fusion process, but also requires the simultaneously tight binding to gH/gL via the flexible N-terminus (residues 36-81) (Kirschner et al., 2007; 2009; Liu et al., 2010; Mullen et al., 2002; Silva et al., 2004).

gH/gL ALSO ACTS AS A DETERMINANT FOR EBV CELL TROPISM

Besides the presumed role of gH/gL in triggering fusion, gH/gL is also an important determinant of the cell tropism for some herpesviruses such as EBV (Borza and Hutt-Fletcher, 2002; Chen et al., 2012; Hutt-Fletcher and Chesnokova, 2010; Möhl et al., 2014), HCMV (Adler, 2015; Revello and Gerna, 2010; Zhou et al., 2015) and human herpesvirus 6 (Jasirwan et al., 2014; Mori, 2009; Tang et al., 2014). EBV has evolved specific adaptations for the epithelial and B cell infection, such as a KGD-motif and the DB C278/C335 of D-II of gH. The bifunctional KGD-binding motif, an exposed loop, enables the competitive binding of gH to integrins on epithelial cells (Fig. 1) or to gp42, which in turn binds to HLA class II (Fig. 2) (Chen et al., 2012; Hutt-Fletcher and Chesnokova, 2010; Matsuura et al., 2010; Sathiyamoorthy et al., 2014). The exposed DB C278/C335 functions as an epithelial cell receptor-specific determi-

nant for the highly-adapted tropism of EBV. This DB tightens the syntaxin-like bundle causing a local rigidity necessary for epithelial cell receptor binding by gH/gL, whereas a larger perturbation of this region also disrupts the B cell fusion activity suggesting a disturbance in the formation of the B cell entry triggering complex facilitated by the interaction of gH and gp42 (Möhl et al., 2014; Sathiyamoorthy et al., 2014). Compatible with these observations, negative-stain electron microscopy studies of the EBV B cell entry triggering complex composed of gH/gL/gp42 with HLA class II described a distinct binding re- for the gp42 CTLD. This gH-binding region is formed by the D-II/D-III-interface, defining this as an important site for gH-mediated cell tropism of EBV (Sathiyamoorthy et al., 2014). Previous studies implicated the KGD-motif, which is adjacent to the D-I/D-II-interface, in gp42-binding (Chen et al., 2012; Sathiyamoorthy et al., 2014). Distinct engagement of gp42-binding sites within gH/gL may occur to mediate the observed open-closed conformational transition to activate the gB-mediated fusion process. It has been suggested that the binding of gH/gL to its epithelial cell receptor integrin causes a conformational change within the large D-I/D-II-groove (Chesnokova and Hutt-Fletcher, 2011). It is possible that structural rearrangements within the D-I/D-II-interface or even interactions across the large groove are triggered by binding of gH/gL to its epithelial cell receptor (Chen et al., 2013; Chesnokova and Hutt-Fletcher, 2011).

INTERACTION OF gB AND gH/gL

It is thought that the gH/gL D-I and D-I/D-II-interface are involved in gB-binding and activation (Plate et al., 2009; Xing et al., 2015). In support of this possibility, a previous study established that the gL residues glutamine (Q) 54 and lysine (K) 94 are involved in the EBV-specific engagement and activation of gB by gH/gL during herpesvirus fusion due to a species-specific dependence between gB and gL (Figs. 1 and 2) (Omerovic and Longnecker, 2007; Plate et al., 2009; 2011). For HSV-1 gH/gL, it was shown that the neutralizing antibody LP11 inhibits gH/gL-binding to gB using bimolecular fluorescence complementation (Chowdary et al., 2010), potentially identifying gB interacting surface. Based on LP11-resistant viruses and insertion mutants that prevent the LP11-binding, the LP11-epitope was mapped to the aspartic acid 168 and arginine 329, as well as the residues 201-326 (Chowdary et al., 2010; Galdiero et al., 1997; Gompels et al., 1991). The recently published crystal structure of VZV gH/gL revealed that residues corresponding to the LP11-epitope on HSV gH/gL and the gB-binding residues of EBV gL area also engaged by the neutralizing antibody Fab-RC/94, suggesting that this surface is near the VZV gH/gL-binding site for gB activation (Xing et al., 2015).

To further map these interactions on gB, the species-specific dependence between gB and gL was studied by a panel of chimeric gB proteins of EBV and the closely-related Rh-LCV using bimolecular complementation. These studies indicated that EBV gB residues 450-800 (corresponding to 456-807 in Rh-LCV gB) are necessary for binding to gH/gL (Plate et al., 2011). The hyperfusogenic EBV gB mutant gB843 showed that the cytoplasmic tail of gB is not necessary for gH/gL binding using proximity ligation assay (Chen et al., 2014). Moreover, it was shown that neutralizing antibodies against the fusion loops of HSV gB prevented binding to gH/gL using bimolecular complementation. This data suggest that this region, including residues 670-725, is important for the gB-gH/gL-interaction during fusion (Atanasiu et al., 2010). Interestingly, the corresponding region within HCMV gB is heavily glycosylated, which suggests a shielding function to prevent immune recognition and neutralization (Burke and Heldwein, 2015).

THE CYTOPLASMIC/INTRAVIRAL DOMAIN OF gB AND gH/gL

While the principal function of the ectodomain of herpesvirus gH/gL and gB is well characterized, the function of the cytoplasmic/intraviral tail domains (CTD) of gB and gH/gL remains incompletely understood. Compared to the CTD of EBV gH, which only has 8 amino acids, gB has a much longer CTD comprising 104 amino acids. Recent findings suggest that the CTD is also involved in keeping gB in an inactive metastable prefusion state by functioning as a clamp (Chen et al., 2014; Rogalin and Heldwein, 2015). These findings are supported by previous gB mutagenesis studies, which confirm that the CTD of gB carries distinct regulatory regions involved in virion transport and regulating fusion activity (Garcia et al., 2013; Haan et al., 2001; Lee and Longnecker, 1997). Furthermore, a comprehensive library study of EBV gB CTD truncation mutants explored that the length of the gB tail is not directly related to fusion function (Garcia et al., 2013). Compatible with this observation, the CTD of EBV or HSV gB may regulate the energy requirement for fusion activity (Chen et al., 2014; Rogalin and Heldwein, 2015). Interestingly, the gH CTD differentially regulates the fusion activity among herpesviruses since mutagenesis or deletion studies of HSV and VZV gH indicate that the CTD may positively or negatively regulate fusion function (Harman et al., 2002; Rogalin and Heldwein, 2015; Silverman et al., 2012; Yang et al., 2014). Since the gH tail is very short with close proximity to the membrane, it may function as “inside-out” signal by affecting the extracellular conformation of gH/gL as has been reported in fusion proteins of other viruses (Waning et al., 2004). Another function for the CTD of gB is to regulate the trafficking of the protein. EBV gB is localized mainly in the endoplasmic reticulum/nuclear membrane with little gB expression on the plasma membrane or a mature viral particle (Gong and Kieff, 1990).

CONCLUSION

In summary, the structures are known for all of the essential EBV receptor-binding proteins including gH/gL and gp42, as well as postfusion gB. The prefusion structure is not known for gB for any members of the herpesvirus family. The B cell entry triggering complex without gB has been reported (Sathiyamoorthy et al., 2014) and provides a basis for comparison with models of the epithelial cell entry triggering complex. Similarities in the overall architectures for these two triggering complexes further our understanding of how gH/gL may activate fusion and provide a basis for designing experiments to examine the interactions required to activate gB.

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