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Review article

REVIEW ARTICLE

Epithelial cell infection by Epstein–Barr virus

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One sentence summary: Overall the mechanism of EBV entry into B cells and epithelial cells is becoming clearer; numerous receptors have been implicated in this process and may also be involved in additional processes of EBV entry, transport, and replication.

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ABSTRACT

Epstein-Barr Virus (EBV) is etiologically associated with multiple human malignancies including Burkitt lymphoma and Hodgkin disease as well as nasopharyngeal and gastric carcinoma. Entry of EBV into target cells is essential for virus to cause disease and is mediated by multiple viral envelope glycoproteins and cell surface associated receptors. The target cells of EBV include B cells and epithelial cells. The nature and mechanism of EBV entry into these cell types are different, requiring different glycoprotein complexes to bind to specific receptors on the target cells. Compared to the B cell entry mechanism, the overall mechanism of EBV entry into epithelial cells is less well known. Numerous receptors have been implicated in this process and may also be involved in additional processes of EBV entry, transport, and replication. This review summarizes EBV glycoproteins, host receptors, signal molecules and transport machinery that are being used in the epithelial cell entry process and also provides a broad view for related herpesvirus entry mechanisms.

Keywords: Epstein–Barr virus; gamma-herpesviruses; epithelial cells

Epstein–Barr virus (EBV) belongs to the herpesviridae family and specifically to gamma-herpesvirus subfamily. EBV infects >90% of the total population and there is no vaccine available (Longnecker, Kieff and Cohen 2013). EBV infection in childhood is generally asymptomatic; however, infection during adolescence or as an adult may result in mononucleosis, also called the kissing disease. EBV was the first identified human oncogenic herpesvirus and the infection is associated with Burkitt lymphoma and Hodgkin disease (B lymphocyte origin) as well as nasopharyngeal and gastric carcinoma (epithelial cell origin), reflecting the cell tropism of EBV (Burkitt 1961; Burkitt and O'Conor 1961; Epstein, Achong and Barr 1964; Gunven *et al.* 1970). The virus was found in nasopharyngeal carcinoma (NPC) in 1970 (Gunven *et al.* 1970; zur Hausen *et al.* 1970; Nonoyama and Pagano 1973; Wolf, zur Hausen and Becker 1973). The risk of NPC is higher for those with elevated anti-EBV DNase antibodies or anti-EBV VCA (viral capsid antigen) IgA and even higher when both antibodies are elevated indicating the importance of EBV infection in disease development (Henle *et al.* 1977). EBV is also present in gastric carcinoma. EBV is also commonly detected in gastric carcinoma with ∼9% being EBV positive (Burke *et al.* 1990; Murphy *et al.* 2009). EBV anti-VCA and anti-EBNA antibody titers are higher in persons with dysplasia on gastric biopsy, suggesting that EBV reactivation could be related to an early phase of gastric carcinoma (De Paschale and Clerici 2012). EBV may also occasionally infect other cell types such as T/natural killer cells (Isobe *et al.* 2004; Coleman *et al.* 2015).

The EBV life cycle starts with salivary transmission of shed virus from an infected person. The virus then replicates in or is transcytozed across epithelial cells and the virus then spreads to naive B cells (Yao, Rickinson and Epstein 1985; Tugizov, Herrera and Palefsky 2013). Lifelong latency is established in memory B cells (Babcock *et al.* 1998). In terminally differentiated plasma cells, the lytic virus life cycle is reactivated (Laichalk and Thorley-Lawson 2005). Epithelial cell infection *in vitro* usually results in active replication and lysis of the cells. EBV DNA can be routinely detected in epithelial cells of the oropharynx from acute infectious mononucleosis patients,

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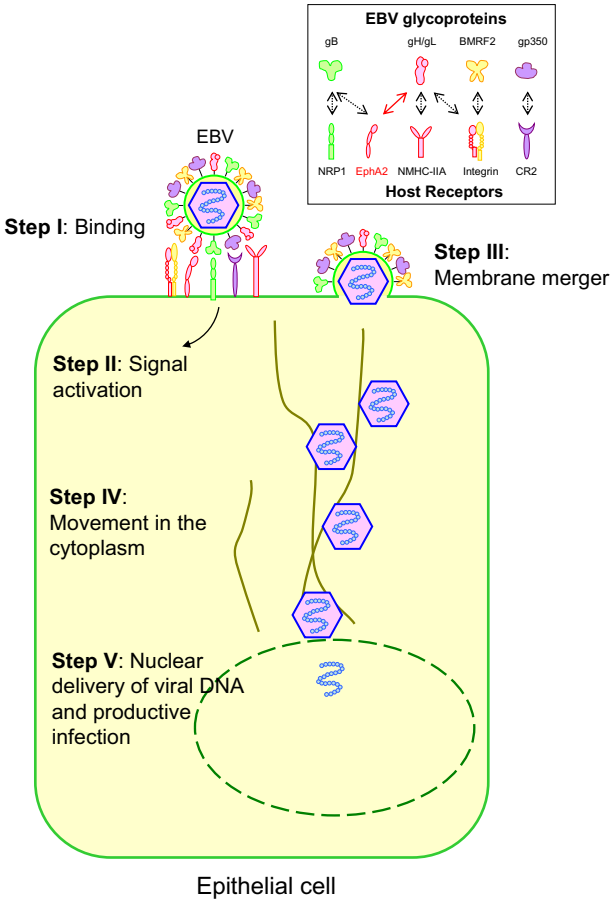

Figure 1. Model illustrating the steps of EBV infection of epithelial cells. In the first step (Step I), EBV binds to target cells using variable host cell surface receptors and multiple viral envelope glycoproteins. In some cases, binding of EBV virions can induce signaling pathway activation (Step II). After binding to the cell surface receptors, (either by direct membrane fusion or fusion with the endosomal membrane) (Step III), the viral capsid is then transported in the cytosol to the nuclear periphery (Step IV). Once at a nuclear pore, the viral genome is released into the nucleus through a nuclear pore (Step V). Integrins ($\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha v\beta 8$), NRP1 and NMHC-IIA interact with corresponding glycoproteins indicated as black-dashed arrows. EphA2 binds to both gH/gL (red solid arrow) and gB (black-dashed arrow). EphA2 is the most important entry receptor for EBV epithelial cell infection and is therefore indicated in red.

suggesting that *in vivo*, EBV replicates lytically in epithelial cells (Sixbey *et al.* 1983). Normal nasopharyngeal epithelial cells are not readily permissive for latent EBV infection. Instead, infection typically results in growth arrest (Tsang *et al.* 2012). However, overexpression of cyclin D1 and/or Bmi-1 as well as the inactivation of p16 can overcome the growth arrest to support stable and latent EBV infection in nasopharyngeal epithelial cells (Tsang *et al.* 2010, 2012, 2014; Yip *et al.* 2013)

EBV INFECTION OF TARGET CELLS

EBV infection of target epithelial cells is a complex multistep process (Fig. 1). In the first step (Step I), EBV binds to target cells using variable host cell surface receptors and multiple viral envelope glycoproteins. In some cases, binding of EBV virions can induce signaling pathway activation (Step II). After binding to the cell surface receptors, the viral and host membranes merge (either by direct membrane fusion or fusion with the endosomal membrane) (Step III). The viral capsid is then transported in the cytosol to the nuclear periphery (Step IV). Once at a nuclear pore, the viral genome is released into the nucleus through a nuclear pore (Step V).

EBV ENTRY STEP I: EBV BINDING TO TARGET CELLS INVOLVES MULTIPLE VIRAL ENVELOP GLYCOPROTEINS AND HOST CELL SURFACE RECEPTORS

Initiation of EBV infection of two major target cells, B lymphocytes and epithelial cells, is substantially different, involving different viral envelope glycoproteins and cell receptors for entry into each cell type. Entry into B cells occurs via endocytosis followed by fusion of the viral membrane with the membrane of the endocytic vesicle (Miller and Hutt-Fletcher 1992). Entry into epithelial cells occurs through direct fusion of the viral membrane with the host cell plasma membrane (Miller and Hutt-Fletcher 1992), although some studies suggest that entry of epithelial cells by EBV is via lipid raft-dependent endocytosis and macropinocytosis (Wang *et al.* 2015). It is likely that both pathways of entry, endocytosis or direct fusion, are used to infect cells, but what pathway is used is dependent on a variety of factors including expression of relevant EBV receptors.

The entry process of EBV into B cells and the receptors involved in this process are well studied compared to the entry process of epithelial cells; the B cell receptor was identified very early, allowing for extensive functional and structural studies of the B cell entry complex (Spriggs *et al.* 1996; Mullen *et al.* 2002; Connolly *et al.* 2011; Sathiyamoorthy *et al.* 2014; Sathiyamoorthy *et al.* 2016). Thus, this review will focus on EBV epithelial cell infection to provide a comprehensive review of what is currently understood regarding the process of EBV epithelial cell entry.

EBV GLYCOPROTEINS IMPORTANT FOR VIRAL ENTRY

The core fusion machinery for EBV includes glycoproteins gB and the gH/gL complex, which are required for both B cell fusion and epithelial cell fusion (Connolly *et al.* 2011). gB is a class III viral fusogen that activates membrane fusion of virus and host cell membranes. The crystal structure of EBV gB without its transmembrane or cytoplasmic domains was identified in 2009 in a presumed post-fusion form. It is a 16 nm spike-like trimer composed of five domains (Backovic, Longnecker and Jardetzky 2009).

While gB activates fusion, gH/gL regulates fusion; upon binding to a host cell receptor, it is thought to trigger the conformational change of gB from pre-fusion to post-fusion form, resulting in membrane fusion (Connolly *et al.* 2011; Gallagher *et al.* 2014). The crystal structure of EBV gH/gL is an elongated rod-like shape ~100 Å in length and 30–60 Å in width. gH/gL is divided into four major domains, with domain I composed of gL and the N terminus of gH (1–66). The rest of gH (66–672) folds into three sequential globular domains (Matsuura *et al.* 2010). There is a large groove between domain I (D-I) and domain II (D-II) of gH/gL, which may be important for epithelial cell receptor binding (Chen, Jardetzky and Longnecker 2013). Previous mutagenesis studies, as well as studies performed by the Hutt-Fletcher laboratory, identified gH/gL mutations that decreased epithelial cell fusion but did not alter B cell fusion, indicating that gH/gL is also an important determinant for EBV cell tropism.

These mutants are R152A (D-II), disulfide bond C278/C335 (D-II), as well as mutations located in D-V (Wu, Borza and Hutt-Fletcher 2005; Chen, Jardetzky and Longnecker 2013; Mohl *et al.* 2014). These mutants may provide new tools to study differences in the mechanism of epithelial cell and B cell fusion.

There are several anti-gH/gL monoclonal antibodies that target different regions of gH/gL that have been tested in fusion including CL40, CL59 and E1D1 (Fig. 2) (Molesworth *et al.* 2000). CL40 binds to a site occupied by the gp42 receptor-binding domain (D-II). CL59 binds to the C-terminal domain IV of gH. E1D1, however, engages a distinct surface of gH/gL compared to CL59 and CL40: the tip of D-I (Sathiyamoorthy *et al.* 2016; Sathiyamoorthy *et al.* 2017). Previously, it was reported that all three mAbs block epithelial but not B cell infection (Molesworth *et al.* 2000; Chesnokova and Hutt-Fletcher 2011). In a more recent study, CL40 and CL59 were shown to block membrane fusion with both B cells and epithelial cells (Sathiyamoorthy *et al.* 2017). Interestingly, E1D1 selectively inhibits epithelial cell fusion but not B cell fusion (Sathiyamoorthy *et al.* 2016). One explanation for this phenomenon is that the tip of D-I binds to the epithelial cell receptor, but not to gp42.

A human monoclonal anti-gH/gL antibody, AMMO1, was recently isolated from EBV-infected humans. AMMO1 binds opposite to the large groove formed by both gH and gL at the D-I/D-II interface. It also binds on the same side as the gp42 binding region, partially overlapping the CL40 binding site. AMMO1 potently neutralizes infection of both B cells and epithelial cells (Snijder *et al.* 2018). These data together indicate that there are multiple functional regions on gH/gL that may bind to different host receptors and participate in EBV epithelial cell infection.

gB and gH/gL are sufficient for epithelial cell fusion. An additional glycoprotein, gp42, is required for B cell fusion and binds to HLA class II (Li, Turk and Hutt-Fletcher 1995; Wang and Hutt-Fletcher 1998; Shaw *et al.* 2010). Virus generated in B cells expresses less gp42 since gp42 is sequestered and degraded in HLA class II-positive B cells. Virus generated in epithelial cells expresses more gp42, which inhibits epithelial cell entry and fusion by binding to gH/gL. Thus, gp42 is the tropism switch for EBV infection (Borza and Hutt-Fletcher 2002; Kirschner *et al.* 2006).

Interestingly, soluble gp42 can inhibit viral fusion with epithelial cells. This suggests that the gp42 binding site on gH/gL is an important site for epithelial cell entry (Borza *et al.* 2004; Kirschner *et al.* 2006). Further mutational studies of the integrin-binding KGD motif on gH/gL have shown the mutation of the KGD motif to AAA decreases fusion with both epithelial cells and B cells and reduces gH/gL binding to both epithelial cells and gp42 (Chen *et al.* 2012). These results indicated that the KGD motif is a bifunctional region for both epithelial cell and B cell fusion.

HOST CELL PROTEINS IMPORTANT FOR VIRUS ATTACHMENT (A) AND VIRUS ENTRY (B) IN EPITHELIAL CELLS

Unlike B cell infection, which is initiated by attachment of gp350, the most abundant EBV glycoprotein in virions, to the complement receptor type 2 (CR2) or CD35(Fingeroth *et al.* 1984; Nemerow *et al.* 1987; Ogembo *et al.* 2013), EBV uses different glycoproteins for attachment to epithelial cells depending on cell types and expression of CR2. EBV can use gp350 for attachment to CR2-positive epithelial cells (Nemerow *et al.* 1987) (Fig. 1). In CR2-negative epithelial cells, EBV may use multispanning transmembrane envelope protein BMRF-2 (Tugizov, Berline and Palefsky 2003) to attach to integrin $\alpha v\beta 1$ or it may use gH/gL to attach to integrin $\alpha v\beta 6$ or $\alpha v\beta 8$ (Fig. 1). It has also been reported that BMRF-2 can form a complex with the type II membrane protein BDLF2, which participates in rearrangement of cellular actin to increase intercellular contacts and thereby promote virus cell-to-cell spreading (Loesing *et al.* 2009).

Several host receptors important for EBV epithelial cell infection have been identified, including multiple integrins, neuropilin-1, non-muscle myosin heavy chain IIA (NMHC-IIA) and the recently identified primary entry receptor: ephrin receptor A2 (EphA2).

INTEGRINS AS EBV ATTACHMENT/TETHERING RECEPTORS

The receptor for EBV B cell infection was identified in 1996 by a gp42 ligand-binding screen. However, the first EBV epithelial cell receptor was not identified until 2009 (by the Hutt-Fletcher lab) due to less efficient EBV infection of epithelial cells compared to B cells (Spriggs *et al.* 1996; Chesnokova, Nishimura and Hutt-Fletcher 2009; Chesnokova and Hutt-Fletcher 2011). Integrins are widely used by many viruses, including herpesviruses, for virus attachment, virus endocytosis and cellular activation, which facilitates virus entry into host cells (Stewart and Nemerow 2007). It was proposed that EBV may also use integrins as epithelial cell entry receptors because an integrin-binding KGD motif was identified in gH/gL (Chesnokova, Nishimura and Hutt-Fletcher 2009; Chen *et al.* 2012). EBV gH/gL can bind to integrin $\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha v\beta 8$ (Fig. 1). Soluble integrin (a peptide including residues 184–196 of gH that contains the KGD motif) and the natural ligands of integrins such as fibronectin and vitronectin can all partially reduce EBV binding and infection (Chesnokova, Nishimura and Hutt-Fletcher 2009; Chesnokova and Hutt-Fletcher 2011). Together, these results indicated that integrins play a role in EBV infection.

However, in more recent studies, integrin αv knockout HEK293 cells were generated using the CRISPR-Cas9 system and no difference between WT HEK293 and integrin αv knockout HEK293 cells was found for viral infection and fusion activity indicating that $\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha v\beta 8$ integrins are not primary EBV entry receptor(s) in HEK293 cells but likely act as a tethering receptor (Chen *et al.* 2018). There have been similar studies in the context of KSHV infection that identified entry mechanisms independent of integrins $\alpha 3\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$ (TerBush *et al.* 2018) even though integrins were also thought to be the primary entry receptor for KSHV (Garrigues *et al.* 2008).

NEUROPILIN-1(NRP1) AS AN EBV ENTRY FACTOR FOR EPITHELIAL CELLS

The neuropilins (NRPs) are multifunctional proteins that play important roles in development, immunity and cancer. NRPs were initially found to be expressed in neuronal cells and play a role in axonal growth and guidance (Takagi *et al.* 1987; Schwarz *et al.* 2008).

NRP1 has diverse functions in different cell types and has a particular role in signaling by enhancing the activity of receptor tyrosine kinases (RTKs) as a co-receptor for class III semaphorins and multiple growth factors (Zachary 2011). NRP1 is also involved in human T-cell lymphotropic virus type 1 entry (Ghez *et al.* 2006; Lambert *et al.* 2009). Many growth factors and

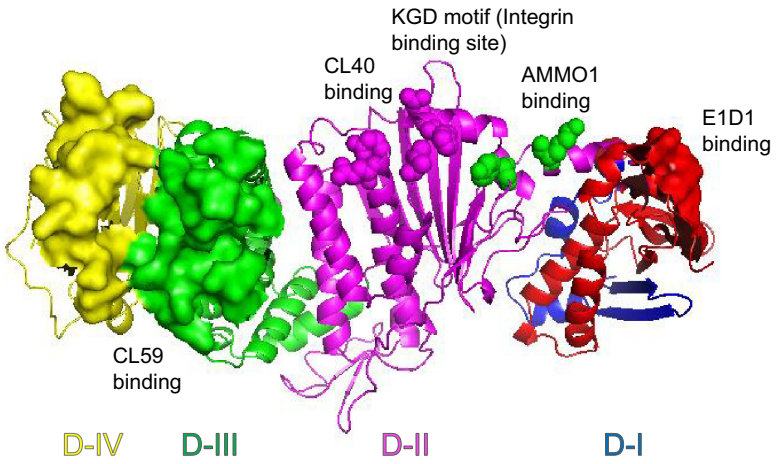

Figure 2. Different anti-gH/gL antibodies target different regions of gH/gL and inhibit EBV epithelial cell infection. The structure of gH/gL is shown as cartoon (Matsuura *et al.* 2010; Connolly *et al.* 2011). gH consists of four domains: D-I (blue), D-II (magenta), D-III (green) and D-IV (yellow). gL is colored red and interacts with gH in D-I. The E1D1 antibody binding region on gH/gL is shown as red surface representation in D-I gL (gL 27–33, gL 72–79 and gL 127–131; D-I is also the potential EphA2 binding region). The most important AMMO1 antibody-binding residues on EBV gH/gL are represented by green spheres in D-I (gH K73 and gH Y76). The CL40 binding amino acids on gH/gL are represented by magenta spheres (gH R184, gH H239, gH V243, gH D284 and gH E286). The CL59 antibody binding regions on gH/gL are shown as green and yellow surface representations in D-III and D-IV (gH406–415, gH456–468, gH494–503, gH623–626 and gH645–656).

other signaling molecules bind to NRPs through a carboxy (C)-terminal basic sequence motif (C-end Rule or CendR motif) (Pang *et al.* 2014). NRP1 can bind to peptides containing a CendR motif, which has a consensus sequence R/K/XXR/K for internalization (Pang *et al.* 2014). EBV gB is highly conserved within the herpesvirus family and a number of gB homologs contain the cleavage motif R-X-K/R-R recognized by the cellular protease furin (Sorem and Longnecker 2009). This cleavage site could also be a potential cryptic C-end Rule (CendR) motif.

It is hypothesized that NRP1 can serve as an entry factor for EBV gB infection (Wang *et al.* 2015). The interaction of gB with NRP1 was examined by an *in vitro* binding assay. It was found that NPR1 directly interacts with EBV gB23–431 (Fig. 1). A CendR motif-deletion mutant (gB23–427) had decreased interaction with NRP1. Further analysis showed that deletion of both gB23–88 and gB428–431 abolished the interaction between NRP1 and gB, indicating that both regions are important for binding. Knockdown of NRP1 or treatment with soluble NRP1 decreased EBV infection to ~50% of control infection. Overexpression of NRP1 significantly increased the efficiency of EBV infection. Interestingly, the role of NRP2 was opposite to that of NRP1 reducing infection.

Previously, our lab reported that an EBV gB deletion mutant lacking the furin cleavage motif was expressed well in cell culture but was not cleaved. The fusion activity was reduced by 52% in epithelial cells and 28% in B cells compared to WT gB (Sorem, Jardetzky and Longnecker 2009). This data supports NRP1 as an epithelial cell entry factor for gB since the furin cleavage site is also the NRP1 binding site (Sorem, Jardetzky and Longnecker 2009). Oral squamous cell carcinoma (OSCC) is the most common subset (90%) of oral cancer with a global incidence of 275 000 cases annually. It results from the outgrowth of the mucosal epithelium. It has been shown that that EBV DNA, mRNAs and EBV proteins were expressed in the majority of OSCC cells (Shamaa *et al.* 2008; Sinevici and O'Sullivan 2016). Interestingly, NRP1 is also overexpressed in OSCC (Chu *et al.* 2014). This may explain the reason that EBV infection is associated with an increased risk of OSCC (She *et al.* 2017).

Since NPR1 is the gB receptor, the level of gB on EBV virion is related to the infection efficiency.

EBV virions that express high levels of gB (gB^{high}) infect target cells more efficiently than virions that express lower levels of gB (gB^{low}) (Neuhierl *et al.* 2002). Interestingly, gB^{high} can also infect cells that are normally resistant to EBV infection (Neuhierl *et al.* 2002). Previous studies identified EBV gB truncations or point mutations in the carboxy-terminal tail that have higher cell-surface expression of gB, allowing cell-cell fusion independent of other viral proteins (McShane and Longnecker 2004). It was proposed that this gH/gL-independent fusion was due to the increased gB cell surface expression. However, more recent studies using a comprehensive library of mutants with truncations of the C-terminal cytoplasmic tail domain (CTD) of EBV gB found that the higher level of gB cell surface expression did not correlate with higher fusion activity (Garcia, Chen and Longnecker 2013). More recent studies have shown that the gB CTD may also participate in fusion by maintaining gB in an inactive pre-fusion form prior to activation by receptor binding (Chen *et al.* 2014). One possibility is that mutation of the EBV gB CTD may release this restriction and cause gB to more readily change to a post-fusion conformation. In regard to identification of NRP1 as an EBV entry factor that specifically interacts with gB (Wang *et al.* 2015), there may be other explanations for its role in EBV infection that may include that the EBV gB CTD mutant may adopt a conformation that interacts better with NRP1 to facilitate fusion.

NON-MUSCLE MYOSIN HEAVY CHAIN IIA (NMHC-IIA) MEDIATES EPSTEIN–BARR VIRUS INFECTION OF NASOPHARYNGEAL EPITHELIAL CELLS

The identification of NMHC-IIA as EBV receptor comes from the differential infection efficiency on different NPEC culture. The major obstacle to identify factors that are important for EBV infection of NPECs is the inefficiency of EBV infection for primary or immortalized NPECs (Tsang *et al.* 2014). During the optimization of growth of the immortalized NPECs, the Zeng lab found 10-fold higher density of cells can cause the formation of 'sphere-like cells' (SLCs) compared to monolayer growth. EBV infection of these SLCs is increased ~10-fold when compared to

the same cells when grown as monolayer cells. Interestingly, in EBV-associated nasopharyngeal carcinoma, there are cancer stem-like cells (CSCs) that have the ability to self-renew, differentiate and sustain propagation. They are also chemo-resistant and can form spheres similar to these SLCs in anchorage-independent environments (Lun, Cheung and Lo 2014). As previously mentioned, there are four antibodies targeting different domains of gH/gL that can inhibit epithelial cell fusion, indicating that gH/gL may interact with more than one host factor for efficient infection. To identify host factors that may play an important role in this increased infection, a myc-tagged gH/gL pull-down assay was performed using EBV-infected SLC lysates followed by liquid chromatography-tandem MS (LC-MS/MS) proteomic analysis. The 250 kDa NMHC-IIA was identified to be the gH/gL binding protein (Fig. 1). This result was also confirmed by co-immunoprecipitation (Wang *et al.* 2015).

NMHC-IIA is an actin-binding protein that has actin cross-linking and contractile properties and is regulated by the phosphorylation of its light and heavy chains (Vicente-Manzanares *et al.* 2009). It has been shown that NMHC-IIA is important for many virus infections including porcine reproductive and respiratory syndrome virus, herpes simplex virus-1 and thrombocytopenia syndrome virus (Arii *et al.* 2010; Sun *et al.* 2014; Gao *et al.* 2016).

NMHC-IIA is mainly located in the cell cytoplasm in normal cultured cells. Using immunofluorescence staining and membrane fractionation methods, it was found that there is aggregated NMHC-IIA in apical surfaces of SLCs, which is associated with gH/gL (Wang *et al.* 2015). Knockdown of NMHC-IIA and NMHC-IIA antibody blocking resulted in both reduced EBV binding and SLC infection, but with no change for adenovirus infection (Wang *et al.* 2015). Overexpression of NMHC-IIA in the cytoplasm did not increase EBV infection. Infection was only increased when NMHC-IIA was redistributed to the cell membrane (Wang *et al.* 2015). Thus, the increased EBV infection efficiency is due to NMHC-IIA and gH/gL localization on the cell surface. However, the mechanism of how NMHC-IIA is redistributed is not known. Interestingly, herpes simplex virus 1 (HSV-1) was also reported to utilize NMHC-IIA as an entry co-receptor associating with gB, indicating that HSV-1 may use a similar mechanism for entry and infection (Arii *et al.* 2010).

EPHRIN RECEPTOR A2 (EPHA2) AS THE EBV EPITHELIAL CELL ENTRY RECEPTOR

Previous results by the Hutt-Fletcher laboratory demonstrated that integrins $\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha v\beta 8$ are host binding factors for EBV gH/gL (Chesnokova and Hutt-Fletcher 2011). However, after complete knockout of integrin αv in HEK293 cells using the CRISPR–Cas9 system, there was no difference between WT and integrin αv knockout cells in fusion or infection. These results indicated that integrins are not major host factors for EBV infection (Chen *et al.* 2018). To identify the major receptor for EBV epithelial cell fusion, a novel and more rapid approach using readily available RNA-seq databases was used to identify potential epithelial cell receptors (Chen *et al.* 2018). Potential receptors were determined by the ratio of membrane protein RNAs that were only expressed in permissive epithelial cells to that of B cells that are non-permissive for epithelial fusion from high to low. EphA2 was ranked as the number one candidate through this analysis. EphA2 belongs to the largest receptor tyrosine kinase (RTK) family, with 14 known human members that play roles in boundary formation, cell migration, axon guidance, synapse formation, angiogenesis, proliferation and cell differentiation (Park, Son and Zhou 2013; Kania and Klein 2016). EphA2 is also a receptor for Kaposi's sarcoma-associated herpesvirus (KSHV), another human gammaherpesvirus (Hahn *et al.* 2012). It has also been shown that other pathogens, including the hepatitis C virus, *Cryptococcus neoformans,* and the fungal pathogen *Candida albicans* use EphA2 as entry factors (Lupberger *et al.* 2011; Aaron *et al.* 2018; Swidergall *et al.* 2018). Overexpression of EphA2 but not EphA4 can promote the fusion and infection of EBV in HEK293 cells. Knockout of EphA2 can reduce EBV fusion and infection by up to 90% and 80%, respectively, and rescue of infection or fusion in EphA2 knockout cells is readily observed with overexpression of EphA2 but not EphA4 (Chen *et al.* 2018). Using label-free surface plasmon resonance (SPR) binding studies, we also confirmed that EphA2 but not EphA4 specifically bound to EBV gH/gL through the EphA2 extracellular domain (Chen *et al.* 2018) (Fig. 1).

Previous KSHV studies indicated that EphA2 regulates clathrin-mediated KSHV endocytosis through its kinase domain (Dutta *et al.* 2013). Results with EBV demonstrated that EphA2 kinase activity is not required for fusion activity, likely due to the different routes of entry between EBV and KSHV (Chen *et al.* 2018).

Interestingly, the Zeng laboratory also identified EphA2 as an EBV epithelial cell receptor using a different approach. It was found that EGF pre-treatment greatly increases EBV infection (Wang *et al.* 2015). To identify the genes that were upregulated after EGF treatment, an integrated approach using microarray and RNA interference screen analyses was used to identify plasma membrane proteins that were highly induced after EGF treatment. The membrane proteins AREG, NT5E, EPHA2, F3, EGFL5 and DCBLD2 were highly induced in two EGF-treated NPEC lines. However, only knockdown of EphA2 resulted in decreased EBV infection. This result is similar to what was found using CRISPR–Cas9 and overexpression of EphA2 in EBV epithelial cell infection. It was also found that soluble EphA2 protein, EphA2 antibodies, ephrinA1 (a soluble EphA2 ligand) and the EphA2 inhibitor 2,5-dimethylpyrrolyl benzoic acid derivative all efficiently inhibited EBV epithelial cell infection (Zhang *et al.* 2018).

The binding region of EphA2 on EBV gH/gL is not known. However, the binding region for gH/gL on EphA2 has been identified. EphA2 is a membrane protein with four different ectodomain regions including a ligand binding domain (LBD), a cysteine rich region (CYS) and two fibronectin regions (FBN). Interestingly, EphA2 binds to both EBV gB and EBV gH/gL at nM levels. The binding region for gH/gL is the LBD and the binding regions for EBV gB are the LBD and FBN (Zhang *et al.* 2018). Thus, the results of both laboratories are consistent and complementary.

EBV ENTRY STEP II: SIGNALING PATHWAY ACTIVATED BY BINDING OF THE EBV VIRUS PARTICLE TO EPITHELIAL CELLS

Virus interaction with cellular receptors often activates intracellular signaling pathways that consequently facilitate virus uptake. Multiple members of the herpesvirus family have been shown to activate such pathways. Previous studies of HSV showed that early virus–cell interactions at the plasma membrane may induce rapid phosphorylation of focal adhesion kinase (FAK) in several human target cells important for

HSV entry post-binding (Cheshenko *et al.* 2005). KSHV interacts with cell surface integrin $\alpha 3\beta 1$ of human endothelial cells and fibroblasts and activates the FAK that is immediately downstream in the outside-in signaling pathway by integrins, leading to the activation of several downstream signaling molecules (Krishnan *et al.* 2006). EBV has two glycoproteins including BMRF2 (Xiao *et al.* 2007) and gH (Chesnokova, Nishimura and Hutt-Fletcher 2009) that have integrin binding motifs and can bind to integrins. Upon ligand binding to integrins, downstream signaling pathways are activated resulting in the recruitment of adaptor proteins that regulate many cellular activities (Giancotti and Ruoslahti 1999). There is direct interaction between integrin cytoplasmic tails and specific actin-binding proteins (Morse, Brahme and Calderwood 2014). Thus, integrins may regulate actin dynamics. Inhibition of Src, ROCK, Rho and p38/MAPK, which are all involved in integrin signal transduction in SVKCR2 epithelial cells, decreases transcription from incoming virus genomes, indicating the role of integrin signaling in EBV infection (Valencia and Hutt-Fletcher 2012).

EphA2 is the entry receptor for both KSHV and EBV. Both KSHV and the natural EphA2 ligand ephrinA1 recombinantly expressed and fused to Fc (ephrinA1-Fc) increased EphA2 phosphorylation. Overexpression of full-length EphA2, but not EphA2ΔICMycHis (EphA2 without the intracellular kinase domain), enhanced KSHV infection by >70% indicating the importance of the EphA2 kinase domain (Hahn *et al.* 2012). The kinase domain of EphA2 is responsible for its downstream signaling. KSHV infection activates EphA2 and, in turn, EphA2 associates with phosphorylated c-Cbl, myosin IIA, FAK, Src and PI3-K as well as clathrin and its adaptor AP2 and effector Epsin-15 proteins (Dutta *et al.* 2013). EphA2 knockdown significantly reduced these signal inductions, virus internalization and gene expression (Dutta *et al.* 2013). For EBV, the kinase activity is not important for fusion, since the EphA2 kinase-dead mutants that were mutated in the EphA2 kinase domain have the same fusion activity as WT EphA2 (Chen *et al.* 2018). It has also been shown that upon overexpression of WT EphA2 (EphA2WT) or intracellular domain truncation of EphA2 (EphA2ΔIC) in HNE1 cell knockout of EphA2, there is no difference in EBV infection, again indicating that the EphA2 intracellular domain is dispensable for EBV internalization (Zhang *et al.* 2018). The different requirement of EphA2 kinase activity for EBV and KSHV is probably due to the different routes of entry for these two viruses.

Moreover, cell-free EBV binding to NRP1 activates NRP1-dependent epidermal growth factor receptor (EGFR) signaling pathways as well as its downstream signaling components AKT and ERK (Wang *et al.* 2015). As a co-receptor of RTKs, NRP1 enhances the affinity of multiple growth factors to RTKs, such as EGF, HGF, VEGF, PIGF and PDGF-BB, and thus augments RTK signaling (Zachary 2011). Knockdown of NRP1 partially suppressed the phosphorylation of EGFR, AKT and ERK activated by EBV infection, suggesting that NRP1 was associated with EBV activation of EGFR/AKT and EGFR/ERK pathways.

In addition to cell-free virus infection, cell-to-cell contact is a more efficient mode of EBV infection of diverse human epithelial cells (Imai, Nishikawa and Takada 1998). Epidermal growth factor (EGF) increases cell-to-cell infection of EBV from infected Akata cells to uninfected HNE1 cells. This effect is partially dependent on the expression of NRP1 (Wang *et al.* 2015), confirming that NRP1 is also important for cell-to-cell contact-mediated infection that involves the downstream signaling components AKT and ERK.

EBV ENTRY STEP III: MERGER OF THE VIRAL AND HOST MEMBRANES

After gH/gL binding to a host receptor and induction of host cell signaling pathways, gH/gL may regulate fusion through interactions with gB. Using chimeric gL molecules composed of EBV and rhesus lymphocryptovirus sequences, a species-specific functional interaction between gH/gL and gB was mapped to EBV gL residues 54 and 94 and regions from 456 to 807 on EBV gB (Plate *et al.* 2009; Plate *et al.* 2011). The EBV fusion protein, gB, has surprising structural homology to the post-fusion form of vesicular stomatitis virus glycoprotein G (VSV G), the sole fusion protein of VSV that is necessary and sufficient for cell entry (Backovic, Longnecker and Jardetzky 2009). It is proposed that EBV gB may undergo large conformational changes from pre-fusion form to post-fusion form to bring the host cell membrane and viral membrane together. However, there is lack of the EBV gB pre-fusion structure and evidence of EBV gB refolding transition during fusion. A recent study of HSV gB in which fluorescent proteins (FP) were genetically inserted throughout the gB ectodomain revealed that CFP and YFP dual-labeled HSV gB had a significantly different FRET signal than the construct containing CFP alone thus allowing the monitoring of gB conformations (Gallagher *et al.* 2014).

The fusion loops of gB are crucial for membrane fusion and are located in Domain I, which is close to the expected location of the transmembrane region in the crystal structure (Backovic, Jardetzky and Longnecker 2007; Backovic, Longnecker and Jardetzky 2009). During the fusion process, the fusion loops are inserted into the cell membrane. gB then refolds to a post-fusion conformation, thereby driving the merger of the viral and host membranes. Mutation of the putative gB fusion loops WY (112–113) and WLIW (193–196) to alanine or insertion mutations in all five of the gB domains decreased EBV gB fusion in both epithelial and B cells, consistent with the hypothesis that EBV gB undergoes a large conformational change to facilitate membrane fusion (Backovic, Jardetzky and Longnecker 2007).

EBV ENTRY STEP IV AND V: TRANSPORT OF THE VIRUS PARTICLE IN THE CYTOSOL TO THE NUCLEAR PERIPHERY AND PRODUCTIVE INFECTION

Like other herpesviruses, EBV replicates in the nucleus of target cells (Hammerschmidt and Sugden 2013). Thus, EBV must transit from the membrane to the nucleus through the cytosol. Transport of virus particles is different for B cells and epithelial cells based on the sensitivity of B cell infection to the effects of chlorpromazine and actin remodeling inhibitors whereas epithelial cell infection is not altered (Borza *et al.* 2004; Valencia and Hutt-Fletcher 2012). This difference in inhibitor sensitivity is due to the different routes of B cell and epithelial cell infection: endocytosis (B cells) versus direct fusion of the membrane (epithelial cells) (Nemerow and Cooper 1984; Miller and Hutt-Fletcher 1992). As discussed earlier, lipid raft-dependent endocytosis and macropinocytosis of epithelial cell infection has also been reported (Wang *et al.* 2015). Endocytosis provides a mechanism through which viruses can pass through the actin cortex by exploiting the intrinsic migratory properties of endocytic vesicles (Grove and Marsh 2011). The transport of virus in B cells is more efficient compared to epithelial cells since virus endocytozed by the B cell is protected in the vesicle before fusion out

of the vesicle. Delivery of EBV DNA into the infected cell nucleus peaks at 4 hours without being degraded. However, virus DNA is lost following internalization into epithelial cells, which reaches its peak at 15–30 minutes and then starts to degrade (Valencia and Hutt-Fletcher 2012).

Leupeptin, a serine protease inhibitor, stabilizes viral DNA in epithelial cells but has no effect on transcription of the viral genome. Wortmannin or LY294002 pre-treatment, which both inhibit class III phosphoinositide 3-kinase (PI3K) and reduce autophagy, can greatly increase the transcription of the virus genome. These results indicate that viral DNA loss in epithelial cells is due to shuttling of the entire particle to a degradative compartment rather than premature uncoating of virus and subsequent exposure of DNA to digestion in the cytoplasm (Valencia and Hutt-Fletcher 2012).

Nuclear delivery of viral DNA and infection of other herpesviruses usually requires the microtubule network. For EBV epithelial cell infection, both the actin and the microtubule networks are required. Reagents that disrupt actin remodeling and microtubules reduce transcription of the incoming virus genome (Valencia and Hutt-Fletcher 2012).

While comparing efficiency of virus binding and infection of CR2-positive and CR2-negative epithelial cells, it was found that virus binding is five times lower in CR2-negative cells. Virus transport is 100 times less in CR2-negative cells (Borza *et al.* 2004). Thus, CR2 may play a role in virus transport after cross-linking by EBV and this effect might occur through its cytoplasmic tail domain binding to actin nucleator formin FHOS/FHOD (Valencia and Hutt-Fletcher 2012). However, the infection rate remained the same when CR2-negative epithelial cells were transfected with either WT CR2 or CR2 lacking the cytoplasmic domain, indicating that the cytoplasmic domain of CR2 is not important (Valencia and Hutt-Fletcher 2012).

Additionally, EBV can traverse polarized human oral epithelial cells without causing productive infection. This process occurs bidirectionally from both the apical to the basolateral membranes (initial EBV infection) or vice versa (EBV secretion into saliva) (Tugizov, Herrera and Palefsky 2013). Inhibitors of macropinocytosis can reduce apical to basolateral virus transcytosis (Tugizov, Herrera and Palefsky 2013). Inhibitors of caveolin can also greatly reduce the basolateral entry (Tugizov, Herrera and Palefsky 2013). EBV infects oropharyngeal cells at their apical surface by direct cell-to-cell contact with infected lymphocytes. Cell-free EBV virions enter at the basolateral membrane of the epithelial cell lines HSC-3 ^{sort}, Detroit ^{sort} and OCO cells (Tugizov, Berline and Palefsky 2003). BMRF-2 and integrins are all expressed at the basolateral membranes of polarized cells, where virion attachment occurs. Anti-EphA2 but not anti-EphA4 antibody can inhibit EBV infection of Detroit 562 cells (Chen *et al.* 2018). Previous studies showed that in normal oral mucosa, immunostaining of EphA2 was detected in the basal cells and parabasal cells (Shao *et al.* 2008). Thus, EBV may use BMRF-2 and integrins for attachment and EphA2 for virus entry on the basolateral membrane.

In summary, EBV is an excellent model to study viral entry into different host cell types because it requires the coordination of multiple cellular molecules. The nature and mechanism of EBV entry is different for B cells and epithelial cells, requiring different glycoprotein complexes to bind to specific receptors on target cells. Overall the mechanism of EBV entry into epithelial cells is becoming clearer. Numerous receptors have been implicated in this process and may also be involved in additional processes of EBV entry, transport and replication.

EphA2 is the most important entry receptor for epithelial cells since infection of EphA2 knockout HEK 293T cells by EBV is reduced by 85% (Chen *et al.* 2018). It has been shown that EphA2 may bind gH/gL through its LBD (Chen *et al.* 2018; Zhang *et al.* 2018); however, the detailed binding region on EBV gH/gL is not known. For EBV, the intracellular kinase activity is not important for fusion (Chen *et al.* 2018). Further research such as CryoEM of gH/gL interacting with EphA2 might be helpful to determine the exact binding interactions of EBV gH/gL with EphA2. Moreover, comparative studies of EBV epithelial cell triggering complexes and B cell triggering complexes will be useful to understand how infection of these two cell types is orchestrated by EBV fusion glycoproteins.

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