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Review

Epstein-Barr virus infection mechanisms

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Abstract

Epstein-Barr virus (EBV) infection occurs by distinct mechanisms across different cell types. EBV infection of B cells *in vitro* minimally requires 5 viral glycoproteins and 2 cellular proteins. By contrast, infection of epithelial cells requires a minimum of 3 viral glycoproteins, which are capable of interacting with one or more of 3 different cellular proteins. The full complement of proteins involved in entry into all cell types capable of being infected *in vivo* is unknown. This review discusses the events that occur when the virus is delivered into the cytoplasm of a cell, the players known to be involved in these events, and the ways in which these players are thought to function.

Key words Glycoproteins, complement receptor type 2, HLA class II, integrins, entry

In the 50 years since Epstein-Barr virus (EBV) was discovered, several lines of evidence have established that the major route of infection is probably oral transmission of cell-free or cell-associated virus. This mode of transmission puts the virus in a position from which it might directly infect epithelial cells[1] or B lymphocytes, be picked up by dendritic cells[2], or transcytose into the lymphoid tissues of the Waldeyer's ring[3]. Determining the route the virus takes, or predominantly takes, at the organismal level is difficult. What is easier to approach experimentally is how EBV uses viral and cellular proteins to breach cell membranes and deliver its tegumented capsid into the cytoplasm of a cell.

Essentially all work in this area has focused on the two major target cells of EBV, B lymphocytes and epithelial cells, that are principally, though not exclusively, involved in EBV-associated tumors. Primary B cells are readily obtained and information gathered about B cell entry probably more faithfully reflects what happens *in vivo*. Epithelial cell entry is more complicated, as most studies are limited to cell lines that have undergone variable changes in cell culture affecting both cell surface proteins and cytoskeletal behavior. A framework for understanding entry into both cell types is, however, emerging.

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Proteins Involved in Entry

Efficient entry of EBV into a B cell minimally requires 5 viral envelope glycoproteins and 3 cellular proteins[4]. Attachment is mediated by an interaction between EBV glycoprotein gp350 and the complement receptor type 2 (CR2/CD21). The virus binds to a site in the 2 amino-terminal short consensus repeats of CR2, very close to the binding site of the natural ligand of CR2, the C3dg fragment of complement. The attachment site on gp350 is a single glycan-free patch in the amino-terminus of the glycoprotein. The virus is subsequently endocytosed into a low pH compartment, where fusion occurs as a result of the activity of the 3 proteins that are components of what is now known as the "core fusion machinery"[5]. This machinery, thought to be common to all herpesviruses, consists of a homotrimer of glycoprotein gB and a heterodimer of glycoproteins gH and gL[6]. Both gB and gH are membrane-anchored, type I membrane proteins, whereas gL has only a signal peptide that is cleaved. The fifth viral glycoprotein that EBV requires for B cell entry is gp42, which can be anchored by a signal peptide but which functions more efficiently in its cleaved form[7]. gp42 binds directly to gH[8] and, in doing so, turns dimeric gHgL into a trimeric gHgLgp42 complex. Glycoprotein gp42 also interacts with human leukocyte antigen (HLA) class II, and this interaction activates the core fusion machinery.

Several attachment proteins have been proposed to play a role in epithelial cell infection. Some epithelial cells in culture express at least low levels of CR2[9], and cells engineered to express high levels of CR2 can be as readily infected as B cells[10,11]. Although difficulties with available antibodies preclude identification of CR2 *in vivo*, tonsil and adenoid epithelial cells express CR2 mRNA[12,13], suggesting that the protein may also be relevant to infection in this context. A multispan viral membrane protein encoded by the *bmrf2* open reading

frame has additionally been implicated in attachment. The BMRF2 protein includes an RGD sequence that can bind β1 integrins, and blocking this interaction inhibits infection of polarized epithelial cells[14,15]. EBV gH carries a KGD sequence that binds αvβ5, αvβ6, or αvβ8 integrins[16,17] and is capable of mediating very high affinity virus binding, although use of gH for attachment, rather than just fusion, reduces the efficiency of infection[11]. The presence of gp42 occludes the KGD sequence[11,18], but the amount of gp42 in the virion are lower than the amount of gHgL, leaving more heterodimers without a third partner and hence able to access integrins. Low affinity but saturable binding can also be detected on hTert-immortalized, normal oral keratinocytes (unpublished data), and preliminary data suggest that the highly glycosylated virus membrane protein gp150 may be involved[19]. However, it is not yet clear whether this represents a productive interaction.

Fusion with an epithelial cell is also somewhat different from fusion with a B cell. Unlike fusion with a B cell, fusion with at least some epithelial cells occurs at neutral pH and does not appear to require endocytosis[20]. Even more strikingly, epithelial cells lack constitutive expression of HLA class II, which renders gp42 incapable of participating in the fusion process. Instead, the interaction of dimeric gHgL complexes with any of the 3 αv integrins to which they can bind replaces the trigger provided by the interaction of gp42 with HLA class II[16]. The use of dimeric complexes to initiate epithelial cell fusion and trimeric complexes to initiate B cell fusion provides the virus with a mechanism by which to switch its tropism alternately between B cells and epithelial cells[21]. Trimeric gp42 complexes are reduced in number in a B cell, as an interaction with HLA class II in the secretory pathway can target them to the peptide loading compartment, which is rich in proteases. In an epithelial cell, the absence of HLA class II allows for a relative enrichment of trimeric complexes. The increase in gp42 is only 2-fold to 4-fold but can render epithelial-derived virus as much as 100-fold more infectious for a B cell than B-cell-derived virus. The reverse phenotype is less pronounced, with B-cell-derived virus being approximately 5-fold more infectious for an epithelial cell than epithelial-derived virus. Whether this means that fewer dimeric complexes are needed to be engaged to trigger fusion with an epithelial cell compare with trimeric complexes that are needed to be engaged to trigger fusion with a B cell, or simply that levels of gp42 are close to limiting in B-cell-derived virus, is not clear.

The structures of all the proteins involved in fusion of the EBV virion with B cells and epithelial cells have been solved, and gp42 has been crystallized in both its liganded and unliganded states[22-25]. The ectodomain of homotrimeric gB has a remarkable similarity to vesicular stomatitis G protein and baculovirus gp64, proteins that have been grouped together as class III fusogens, and the general consensus is that gB is probably the final executor of fusion. gB is a 5-domain structure with a central α-helical coiled coil, which is seen in many viral fusion proteins. The crystal structure is thought to represent the post-fusion conformation of the protein. So-called fusion loops are probably close to the transmembrane region in domain I. These loops are hydrophobic residues that, when mutated, render the protein incapable of mediating fusion[26].

The structure of the gHgL ectodomain, in contrast, resembles no known fusion protein, and the heterodimer is now generally considered a regulator, rather than an executor, of fusion[27]. It is a cylindrical, 4-domain complex. Domain I, which lies furthest from the membrane, is built from the amino-terminal sequences of gH and the entire cleaved gL. The KGD sequence, which interacts with epithelial integrins, is part of an exposed loop in domain II. Glycoprotein gp42 has a carboxy-terminal, C-type lectin domain that binds to HLA class II, and the amino-terminus of the protein, which was not resolved in the crystal structure, contains the region that binds gH[28]. Binding of gp42 to gHgL is also in part dependent on the KGD motif[18].

Possible Mechanisms of Fusion

In the emerging model for fusion of all herpesviruses, a signal is transmitted directly to gHgL by a coreceptor or entry mediator such as αvβ5, β6, or β8, or indirectly via a coreceptor-binding protein such as gp42. The gHgL heterodimer then transmits the signal on to gB. The signal can be transmitted when the proteins are expressed in cis, which is of course how they are found in virus, but also when they are expressed in trans, such as when a gH-null virus is used to infect a cell expressing gHgL[29]. This suggests that the interactions between the ectodomains are critical. When gB is activated, it is thought to insert its fusion loops into the target cell membrane and undergo a dramatic conformational change, pulling viral and cellular membranes together and driving fusion[6]. The occurrence of such a conformational change is supported by an altered proteolytic digestion of gB when the virus is incubated with soluble integrins capable of triggering fusion[29].

Comparison of the liganded and unliganded gp42 structures indicates that more subtle conformational changes, which widen a functionally important hydrophobic pocket in the protein[30], are induced by binding to HLA class II[24]. These changes are probably relevant to gp42's role in triggering membrane fusion with a B cell. Conformational changes at the domain I/domain II interface of gHgL are also suggested to be functionally relevant[23]. In support of this, fluorescence of environmentally sensitive fluorescent probes, coupled to a single unpaired cysteine residue at the interface in a soluble form of gHgL, increased and shifted wavelength when the labeled molecules were incubated with the soluble integrins that trigger epithelial fusion[16]. In addition, mutations that would be expected to alter the flexibility of the region reduced fusion with epithelial cells, though, perhaps curiously, not fusion with B cells[31]. Mutations in a flap-like structure in domain IV close to the membrane, which is conserved in all herpesvirus gHgL dimers crystalized to date[32,33], also affect fusion with both B cells and epithelial cells, though not always in the same way[8,34]. The structure is also targeted in EBV by a monoclonal antibody that can completely neutralize infection of epithelial cells but not B cells[16,34]. These differences between fusion with B cells and epithelial cells are intriguing given that fusion, no matter how triggered, ultimately involves the same 3 players. Indeed, the KGD motif, so important to triggering fusion with epithelial cells, is also important both to gp42 binding to gHgL and to fusion with B cells[18]. The differences are, however, in keeping with the observation

that B cell infection requires endocytosis, though not low pH, whereas epithelial cell infection can occur at the cell surface. Thus, there may still be cell-specific factors, important to fusion, that have yet to be uncovered.

Post-Fusion Events

Most of the work done to explore virus entry has focused on events at or close to the time at which virus leaves the cell surface and begins its movement toward the nucleus. Relatively little is known about subsequent events or about how events at the cell surface may influence them. Comparisons of the ability of the virion to deliver DNA into the nucleus suggest that the process is much less efficient in an epithelial cell than in a B cell, even when B-cell receptors, CR2 and HLA class II, are exogenously expressed[35,36]. This may reflect the nature of a cultured epithelial cell rather than the reality of what happens *in vivo* but may also reflect the different routes that the virus appears to take. Uptake into a transport vesicle may be an intrinsically more efficient way to infect. Post-fusion events, though challenging to explore, clearly need much more study.

Conclusions

We can probably be reasonably confident that the EBV proteins most essential for entry have been identified, and this is considerably important to vaccine development. We have at least some understanding of how they may operate. There is still, however, a great deal that we do not understand overall about the entry mechanisms used by the virus. It has been now almost 40 years since the first hint of one cellular protein important to B cell entry was reported[37]. The thought that so many cellular proteins might be involved had not been conceived. Despite the strides that have been made since, it seems probable that there are more players yet to be identified. This may not be the case for B cells, but seems likely for epithelial cells for T cells, natural killer cells, and for muscle cells—all of which can be targeted by EBV under some circumstances. As EBV is implicated in the development of an increasing number of diseases and syndromes, entry remains a critical area for study.

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