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A soluble form of Epstein-Barr virus gH/gL inhibits EBV-induced membrane fusion and does not function in fusion

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ABSTRACT

We investigated whether soluble EBV gH/gL (sgH/gL) functions in fusion and made a series of truncations of gH/gL domains based on the gH/gL crystal structure. We found sgH/gL failed to mediate cell–cell fusion both when co-expressed with the other entry glycoproteins and when added exogenously to fusion assays. Interestingly, sgH/gL inhibited cell–cell fusion in a dose dependent manner when co-expressed. sgH/gL from HSV was unable to inhibit EBV fusion, suggesting the inhibition was specific to EBV gH/gL. sgH/gL stably binds gp42, but not gB nor gH/gL. The domain mutants, DI/gL, DI-II/gL and DI-II-III/gL were unable to bind gp42. Instead, DI-II/gL, DI-II-III/gL and sgH/gL but not DI/gL decreased the expression of gp42, resulting in decreased overall fusion. Overall, our results suggest that domain IV may be required for proper folding and the transmembrane domain and cytoplasmic tail of EBV gH/gL are required for the most efficient fusion.

Introduction

Epstein-Barr virus (EBV), a member of the herpesvirus family, is transmitted orally and infects the majority of the adult population. Infant and young childhood infections are asymptomatic or subclinical whereas adulthood infections typically result in infectious mononucleosis (Rickinson and Kieff, 2007). Significantly, EBV is associated with lymphoid malignancies as well as epithelial malignancies, for which immunodeficient patients are at particular risk (Bieging et al., 2010; Longnecker, 1998; Rickinson and Kieff, 2007; Takada, 2001; Thompson and Kurzrock, 2004; Wei and Sham, 2005).

Members of the herpesvirus family are large enveloped DNA viruses, which use a conserved set of glycoproteins for the complex process of viral fusion with the host cell membrane either at the plasma membrane or following endocytosis (Connolly et al., 2011). The majority of herpesviruses use two or more receptors to dictate cell tropism and spread. EBV uses different glycoprotein and receptor combinations to infect oral epithelial cells than it does to infect B cells where it establishes latency until reactivation. In B cells, HLA class II receptor binding by gH/gL/gp42 complexes triggers conformational changes that result in activation of gB-dependent fusion between the viral envelope and a cellular membrane (Haan et al., 2000; Li et al., 1997; McShane et al., 2003; Spriggs et al., 1996). In epithelial cells, which typically lack HLA class II, binding to an epithelial receptor, such as integrins by gH/gL, presumably triggers conformational changes that result in activation of gB and fusion (Chen et al., 2012; Chesnokova and Hutt-Fletcher, 2011; Chesnokova et al., 2009). EBV virions exiting B cells primarily contain the bipartite complex gH/gL and infect epithelial cells efficiently, whereas virions exiting epithelial cells primarily contain the tripartite complex gH/gL/gp42 and infect B cells efficiently (Borza and Hutt-Fletcher, 2002). When added exogenously, sgp42 enhanced B cell fusion but inhibited epithelial fusion (Kirschner et al., 2006). The cytoplasmic domains for both gp42 and the HSV functional homolog, gD, are not required for cell–cell fusion, since the soluble forms of both gp42 and gD are functional in fusion (Atanasiu et al., 2010; Sorem et al., 2009).

The recently solved x-ray crystallographic structures of gH/gL from EBV, and HSV-2 as well as the partial gH structure of psuedorabies virus (PRV) suggest striking conservation of gH/gL within the herpesvirus family (Backovic et al., 2010; Chowdary et al., 2010; Matsuura et al., 2010). The EBV structure suggests four sequential semiautonomous domains: Domain I, II, III and IV (DI, DII, DIII and DIV) (Matsuura et al., 2010). EBV gL forms a stable heterodimer with the N-terminal residues of gH to form DI. gL is an integral part of the DI structure and is required for the export of gH to the cellular membrane (Pulford et al., 1995; Yaswen et al., 1993). DI is composed of mixed parallel/antiparallel

Abbreviations: EBV, Epstein Barr Virus; cELISA, cell enzyme-linked immunosorbent assay; a.a., amino acid; PCR, polymerase chain reaction; ug, microgram; V, volts; hr, hour

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β sheets (five strands from gL and two strands from gH) supported by a layer of three helices. The three helix layer of DI forms a wall of charged and polar residues which may interact with other viral proteins involved in the core fusion machinery. Between DI and DII there is a single α-helix linker which has been proposed to act as a "hinge". DII is composed of an eight stranded anti-parallel β sheet ("picket fence") followed by an anti-parallel five helix bundle. Projecting from the picket fence is a prominent KGD loop that is implicated in binding gp42, as well as a gH/gL epithelial receptor, presumably integrin αvβ6, αvβ8 or αvβ5 (Chen et al., 2012; Chesnokova et al., 2009). DIII consists of a total of nine helices, the first five of which spiral down DII and the last four form a distinct subdomain bundle. DIV consists of two connected anti-parallel β sheets forming a "β-sandwich". Elucidation of the gH/gL structure provides a basis for deletion and mutagenesis studies aimed at understanding the function of gH in the core fusion machinery.

The N-terminal residues as well as the C-terminal residues of EBV gH/gL have been implicated in promoting membrane fusion. When EBV gL residues 54 and 94 were replaced with those of rhesus lymphocryptovirus (Rh-LCV) gL, the mutant gL showed wild-type expression but decreased fusion activity. Fusion could be restored by replacing EBV gB with Rh gB, suggesting gL interacts with gB in a species-specific manner to activate membrane fusion (Plate et al., 2009). Interestingly, a single substitution in the C-terminal Domain IV, G594A, resulted in complete abrogation of fusion with both B cells and epithelial cells, whereas the substitution E595A reduced fusion with epithelial cells but greatly enhanced fusion with B cells (Wu and Hutt-Fletcher, 2007).

Mutational studies of herpes simplex virus type 1 (HSV-1) gH have been more extensive and suggest that the transmembrane domain as well as the cytoplasmic tail of gH/gL plays an important role in membrane fusion. Harman et al. found that gH lacking the authentic transmembrane or cytoplasmic tail was unable to mediate cell–cell fusion (Harman et al., 2002). In addition, a conserved glycine residue at position 812 within the transmembrane domain was found to be crucial for fusion (Harman et al., 2002). Interestingly, fusion is unaffected by deletion of the final six residues of the gH cytoplasmic tail (residues 832 to 838), however further deletions decreased polykaryocyte formation by a syncytial HSV strain (Browne et al., 1996; Wilson et al., 1994). Maintenance of the valine at position 831 within the serine–valine–proline motif of the cytoplasmic tail was of particular importance for fusion (Wilson et al., 1994). The addition of a linker insertion at residue 824, which borders the junction between the transmembrane and the cytoplasmic tail, completely abrogated fusion (Jackson et al., 2010). gD-glycophosphatidylinositol (gDgpi), in which the transmembrane and cytoplasmic tail of gD is replaced with a gpi linkage, promoted fusion to near wild-type levels (Jones and Geraghty, 2004). However, gHgpi and gBgpi were unable to promote fusion, suggesting glycoprotein specific transmembrane and cytoplasmic tails are required for gH and gB. In contrast, more recent work has shown that purified HSV-2 sgH/gL protein, lacking the transmembrane and cytoplasmic tail, can induce a modest (2.1% of wild type) level of fusion of nectin-1-bearing C10 cells expressing gB and gD (Atanasiu et al., 2010). Interestingly, EBV sgH/gL purified from insect cells does not promote fusion when added exogenously (Kirschner et al., 2006).

To rigorously rule out that the source of the sgH/gL is not playing any role in the observed functional differences between the HSV and EBV studies, we expressed and tested sgH/gL in mammalian cells a in cell–cell fusion assay. We generated a soluble form of gH/gL and either co-expressed it with the other entry glycoproteins or added it exogenously to the core fusion machinery. Our results indicate that sgH/gL does not function efficiently in fusion and instead inhibits fusion. We generated domain deletion mutants of gH to try to map the region responsible for inhibition of fusion. We found only full length sgH/gL was able to bind gp42, suggesting DIV of gH is required for gp42 binding. Surprisingly, however, we found DI-II/gL, DI-II-III/gL and the full length sgH/gL all reduce gp42 expression levels. Overall, our results support previous observations that anchoring of gH in the membrane is essential for most efficient fusion.

Results

Construction of gH/gL FLAG-tagged mutants

Recent x-ray crystallography results suggest that gH/gL is composed of four semi-autonomous domains: DI, DII, DIII and DIV. To study whether sgH/gL could mediate fusion, we constructed soluble C-terminal deletion variants of gH, incorporating the FLAG epitope at the C-terminus of each (Fig. 1). Mutant sgH (consisting of the full ectodomain: DI-II-III-IV) was truncated at amino acid 679, immediately upstream of the transmembrane domain and cytoplasmic tail which was replaced with the FLAG tag followed by a stop codon. Mutants DI-II-III, DI-II and DI were truncated at residues 529, 344 and 65 respectively, and C-terminally tagged with the FLAG epitope followed by a stop codon. We chose to add the FLAG epitope at the C-terminus of the truncations to allow us to readily monitor expression of the proteins and verify they were full length.

Expression and secretion of soluble gH/gL domain mutants

To determine whether the FLAG-tagged gH mutants were expressed and produced as soluble forms, plasmids encoding each of the mutants together with gL were transfected into CHO-K1 cells and protein expression was analyzed 48 h post-transfection by Western Blot. We found that all of the mutants were expressed in cellular extracts at the approximate expected molecular weight (DI, 7 kDa; DI-II, 38 kDa; DI-II-III, 58 kDa; sgH 74 kDa), as determined by probing with an anti-FLAG antibody (Fig. 2A). However, only DI and sgH were detected as distinct bands in cell culture supernatants (Fig. 2B). DI, DI-II, DI-II-III, as well as sgH all tended to form higher molecular weight aggregates in cell lysates (intracellularly) and in culture supernatants (extracellularly), although DI to a lesser extent. The aggregates (indicated by an arrow, Fig. 2B) could not be resolved by changing

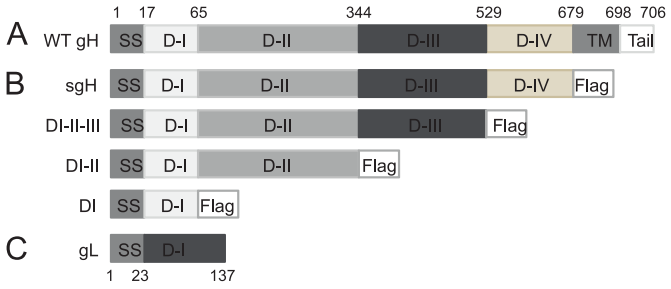

Fig. 1. Schematic representation of wild type gH, FLAG tagged sgH, DI-II-III, DI-II and DI gH mutants, and gL. (A) Approximate location of gH functional domains are shown including the signal sequence (residues 1–17), DI (residues 18–65), DII (residues 66–344), DIII (residues 345–529), DIV (residues 530–679), transmembrane domain (residues 680–698) and the cytoplasmic tail (residues 699–706). (B) FLAG tagged mutants: sgH, DI-II-III, DI-II, DI are all C-terminally tagged with the FLAG epitope followed by a stop codon, as indicated. None of the mutants contain the transmembrane domain nor the cytoplasmic tail of gH. (C) Wild type gL includes the signal sequence (residues 1–23) followed by a single domain (residues 24–137).