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The fusion loops and membrane proximal region of Epstein-Barr virus glycoprotein B (gB) can function in the context of herpes simplex virus 1 gB when substituted individually but not in combination

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Abstract

Among the herpesvirus glycoprotein B (gB) fusion proteins, the hydrophobic content of fusion loops and membrane proximal regions (MPR) are inversely correlated with each other. We examined the functional importance of the hydrophobicity of these regions by replacing them in herpes simplex virus type 1 gB with corresponding regions from Epstein-Barr virus gB. We show that fusion activity is dependent on the structural context in which the specific loops and MPR sequences exist, rather than a simple hydrophobic relationship.

Keywords

Herpes simplex virus; Epstein-Barr virus; glycoprotein B; fusion; entry

The core fusion machinery of the human herpesviruses is composed of three glycoproteins, gB and the heterodimer gH/gL. In herpes simplex virus (HSV) types 1 and 2, these three glycoproteins work in association with a receptor binding glycoprotein, gD, to facilitate the entry of the virus into the cells. The current model of HSV fusion suggests that binding of gD to one of its receptors transmits a signal to gH/gL and gB to trigger fusion (Connolly et al., 2011; Eisenberg et al., 2012; Heldwein and Krummenacher, 2008). Several classes of proteins can serve as entry receptors for gD, including nectin-1 (Geraghty et al., 1998) and nectin-2 (Warner et al., 1998), cell adhesion molecules of the immunoglobulin superfamily present on neurons and other cells; herpes virus entry mediator (HVEM) (Montgomery et al., 1996), a member of the TNF receptor family; and 3-O-sulfated heparan sulfate (Shukla et al., 1999). A comparison of the crystal structures of gD in unliganded (Krummenacher et al., 2005) and receptor-bound (Carfi et al., 2001; Di Giovine et al., 2011) forms suggests receptor binding triggers gD by displacing the C-terminus of the gD ectodomain. A working model suggests that gD transmits this signal to gH/gL which then activates gB to mediate fusion (Atanasiu et al., 2010). Consistent with this model, the crystal structures of HSV-2

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gH/gL (Chowdary et al., 2010), EBV gH/gL (Matsuura et al., 2010), and PrV gH (Backovic et al., 2010) do not resemble known fusion proteins. In contrast, the crystal structures of HSV-1 and Epstein-Barr virus (EBV) gB (Backovic et al., 2009; Heldwein et al., 2006) reveal that gB is a class III fusogen, suggesting that this protein is responsible for executing fusion of the virus membrane with the host cell membrane.

HSV-1 gB is a 904 amino acid protein that is highly conserved in herpesviruses and exhibits 29% amino acid identity with EBV gB (Backovic et al., 2009). The gB ectodomain shares structural homology with the vesicular stomatitis virus (VSV) G protein (Heldwein et al., 2006; Roche et al., 2006; Steven and Spear, 2006), another class III fusogen which mediates attachment to and fusion with the host cell. VSV G contains a bipartite fusion peptide in the form of loops at the tips of two adjacent β hairpins and analogous loops are seen in both HSV and EBV gB. These fusion loops are highly hydrophobic in EBV gB (Fig. 1A). Mutation of the EBV gB hydrophobic residues in either fusion loop to the corresponding HSV-1 gB residues causes loss of fusion activity (Backovic et al., 2007), suggesting that the hydrophobicity of the fusion loops is required for EBV gB function. The fusion loops in HSV-1 gB (Fig. 1A) have a lower hydrophobic content. However, mutagenesis studies show that hydrophobic residues in both loops also are critical in fusion (Hannah et al., 2009; Hannah et al., 2007). These observations illustrate the importance of the hydrophobicity of the loops in membrane interaction and fusion promotion.

Previous studies have shown that the membrane proximal region (MPR) of VSV G contributes to fusion (Jeetendra et al., 2003) and may interact directly with membranes (Jeetendra et al., 2002). The gB MPR lies downstream of the solved crystal structures of HSV-1 and EBV gB (Backovic et al., 2009; Heldwein et al., 2006). Mutagenesis studies demonstrate that the gB MPR also contributes to gB function in fusion. Insertions in the MPR of HSV-1 gB at residues 725, 730, 732, or 742 eliminate fusion function with only modest effects on cell-surface expression (Lin and Spear, 2007). Similarly, insertions in the MPR of EBV gB at residues 675, 703, or 717 result in a loss of fusion function (Reimer et al., 2009). These EBV gB MPR insertion mutants were expressed on the cell surface, but exhibited altered glycosylation patterns.

Analysis of the MPRs of alpha and gamma human herpesviruses demonstrates an inverse correlation between the hydrophobicity of the gB MPRs when compared to the fusion loops (Backovic et al., 2007). As the hydrophobicity of the loops decreases, there is a corresponding increase in the hydrophobicity of the MPRs. Specifically, the HSV-1 gB MPR is more hydrophobic on average than the EBV gB MPR, but the inverse is true of their respective fusion loops. Using the Kyte-Doolittle hydropathy scale (Kyte and Doolittle, 1982), which assigns increasing values to residues as their hydrophobicity increases, the sum of the hydrophobicity scores for the HSV-1 and EBV MPRs shown in Fig. 1 are 52 and 18 respectively, whereas the sum hydrophobicity scores for the HSV-1 and EBV fusion loops shown are -12 and -2 respectively.

To investigate the significance of the hydrophobicity of gB loops and MPRs, we constructed a panel of chimeric proteins by replacing the fusion loops, MPR, transmembrane domain (TM), and/or cytoplasmic tail (CT) of HSV-1 gB with those of EBV gB. We used sequence alignment (Fig. 1A) and structural data (Fig. 1B) (Backovic et al., 2009; Heldwein et al., 2006) to design the HSV/EBV gB chimeras. To address whether the hydrophobicity of the fusion loops is important for function, the chimeras CH1, CH2, CH3 were created by replacing the HSV-1 gB loops (VWFGHRY¹⁷³⁻¹⁷⁹ and RVEAFHRY²⁵⁸⁻²⁶⁵) with the more hydrophobic EBV residues (IYNGWYA¹⁰⁸⁻¹¹⁴ and WLIWTYRT¹⁹³⁻²⁰⁰) (Fig. 1C). To investigate if the hydrophobicity of the fusion loops is “balanced” by the hydrophobicity of the MPR, two additional sets of chimeras were created (Fig. 1C). Chimeras CH4, CH5, and

CH6 swap either the entire C-terminal region starting at the MPR, the MPR and TM only, or the MPR alone, respectively. Chimeras CH4.2, CH5.2, and CH6.2 add both of the EBV gB fusion loops to the three chimeras containing the C-terminal EBV gB regions.

Expression of each of the chimeras was assessed by cell-based ELISA (Geraghty et al., 2000) using cells transfected with plasmids encoding the gB constructs. HSV-1 gD, gH, gL and T7 polymerase were co-expressed in the cells so that expression of gB would be assessed in the same conditions used for cell fusion assays (below). The live intact cells were incubated with an anti-gB polyclonal antibody (PAb) R74. The results for CH1 show that replacing HSV-1 gB fusion loop 1 with the corresponding EBV sequence did not inhibit cell surface expression. On the contrary, a slight increase in cell surface expression was detected (Fig 2A). Swapping HSV-1 gB fusion loop 2 with the corresponding EBV sequence caused a decrease in expression for CH2 (Fig. 2A). CH3, which contains both EBV gB loops, was expressed at an intermediate level (Fig. 2A), suggesting that the addition of EBV gB fusion loop 1 partly restores the cell surface expression lost by insertion of EBV gB fusion loop 2. The ratio of monoclonal antibody (MAb) to PAb R74 binding to these chimeras indicated that the binding of MAbs II-105 and I-1-7 (Para et al., 1985) to these chimeras was essentially equivalent to that observed for wild-type gB (data not shown), suggesting that the differences in cell surface detection among these three chimeras is unlikely to be explained by an alteration of conformation that specifically affects R74 PAb binding. These MAbs were chosen because they bind HSV-1 gB robustly and have been shown to recognize other HSV-1 gB mutants (Connolly and Longnecker, 2012; Lin and Spear, 2007).

The chimeras CH4 and CH4.2 were not detectable on the cell surface (Fig. 2A). This is in accord with low surface expression of EBV gB, which is retained primarily in the nuclear and endoplasmic reticulum due to a retention signal in the cytoplasmic tail (Fig. 1A) (Lee, 1999). Unexpectedly, the chimeras CH5 and CH5.2 also were poorly expressed on the surface, indicating that the transmembrane domain may govern cell surface expression of gB in a virus-specific manner since the CH6 and CH6.2 chimeras, which contain the HSV-1 gB TM, were expressed at levels comparable to wild-type gB. Western blot analysis of whole cell lysates from these transfected cells indicated that all of the chimeras were expressed in the cells (Fig. 2B). Thus, a lack of surface expression was due to failure of processing and transport to the surface.

A virus-free cell-cell fusion assay (Pertel et al., 2001) was employed to test the ability of these gB chimeras to mediate fusion with cells expressing the gD receptor nectin-1. CHO effector cells were transfected with plasmids encoding wild-type HSV-1 gB or a gB chimera in combination with HSV-1 gD, gH, and gL (the other viral glycoproteins of the fusion machinery) and T7 RNA polymerase. These effector cells were mixed with target CHO cells stably expressing nectin-1 and transfected with a plasmid encoding luciferase under control of the T7 promoter. After 18 h, luciferase activity was quantified as a measure of cell fusion activity (Fig 3).

Chimeras containing one or both EBV gB fusion loops (CH1, CH2, or CH3) showed fusion levels ranging from 67 to 76% of wild-type gB activity. This indicates that HSV-1 gB can tolerate an increase in the hydrophobicity of its fusion loops. Interestingly, the inverse is not true. Previously, when the EBV gB fusion loop 1 residues WY¹¹²⁻¹¹³ and fusion loop 2 residues WLIW¹⁹³⁻¹⁹⁶ were replaced with the less hydrophobic corresponding residues of the HSV-1 gB fusion loops, fusion function was lost (Backovic et al., 2007), however that mutant protein was able to adopt the putative postfusion conformation when expressed in a soluble form (Backovic et al., 2009).

Chimeras swapping the MPR and TM CH4, CH4.2, CH5, and CH5.2 failed to mediate fusion. This was expected given the low levels of cell surface expression of these four constructs. CH6 mediated fusion at wild-type levels, indicating that the EBV MPR is functional in the context of the HSV-1 gB. Unexpectedly, although CH6.2 was expressed at levels comparable to wild-type gB, it failed to mediate significant levels of cell-cell fusion, showing only 17% of wild-type gB fusion activity. Thus, although the EBV fusion loops are functional in the context of HSV-1 gB (see CH3) and the EBV MPR is functional in the context of HSV-1 gB (see CH6), the combination of the EBV fusion loops and MPR in the context of HSV-1 gB significantly reduces fusion function (see CH6.2). Although we did not expect any of the chimeras to function with the EBV entry glycoproteins, we tested the chimeras for epithelial cell fusion with EBV gH/gL coexpressed and B cell fusion with gH/gL and gp42 coexpressed and found no fusion activity with either cell type despite robust fusion with controls (data not shown).

Substitution of the hydrophobic residues of EBV gB fusion loops with the corresponding and less hydrophobic residues from HSV-1 gB was previously shown to result in a loss of fusion function (Backovic et al., 2007). We hypothesized that replacing the HSV-1 gB fusion loops with the more hydrophobic fusion loops of EBV gB might enhance fusion by increasing the membrane interaction of gB. However, HSV-1 gB carrying the EBV fusion loops (CH3) demonstrated no enhancement of fusion activity but rather a slight reduction. The results indicate that although the hydrophobicity of the fusion loops is important to gB function (Backovic et al., 2007; Hannah et al., 2009; Hannah et al., 2007), an increase in hydrophobicity is permitted (CH3). Since our previous studies identified an inverse correlation within the amino acid sequence between the hydrophobicity of the gB fusion loops and the gB MPR for multiple gB homologs (Backovic et al., 2007), we designed HSV/EBV gB chimeras to investigate a potential functional interaction or interdependence between the gB fusion loops and MPR. The chimeras were created to investigate if these two structural elements work together in the fusion process. In the postfusion conformation, the gB fusion loops are believed to insert into the membrane adjacent to the gB MPR and TM (Fig. 1B). This arrangement may imply a functional interaction between the fusion loops and MPR during the execution of fusion. Unexpectedly, we found that although the EBV gB MPR was functional in the context of wild-type HSV-1 gB (CH6), gB function was lost when both the HSV gB fusion loops and MPR were replaced with EBV sequence (CH6.2). The inability of the CH6.2 chimera to mediate fusion is not likely due to a loss of interaction with the other HSV-1 entry glycoproteins due to local effects of the domain swaps, since CH3 and CH6 (which contain the relevant EBV domains present in CH6.2) were functional with HSV gD and gH/gL. HSV-1 gB retains function when its fusion loops are made more hydrophobic (CH3) or when its MPR is made less hydrophobic (CH6), however concurrent changes in both regions are not tolerated (CH6.2). Thus any functional interaction between the fusion loops and MPR must be governed by more than just an inverse hydrophobic relationship. The context of the gB sequence in which these regions exist, that of HSV-1 or EBV gB, determines whether a fusion loop-MPR combination is functional. This requirement for a proper context and combination of fusion loops and MPRs for gB function may be due to interactions that occur in prefusion gB or folding intermediates of gB, structures that have yet to be solved.

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Fig. 1.

(A) Sequence alignment of gB domains including the fusion loops and the C-terminal region encompassing the membrane proximal region (MPR, red), the transmembrane region (TM, green) and cytoplasmic tail (CT, blue). Identical residues are shaded and hydrophobic residues are in bold. Alignment was generated using the ESPript program. Arrows indicate predicted hydrophobic regions in HSV-1 gB (above) and EBV gB (below). The EBV gB retention signal is boxed. (B) The HSV-1 and EBV gB putative postfusion trimeric structures are colored by domain (Backovic et al., 2009; Heldwein et al., 2006). Fusion loops (magenta) and the N-terminal regions of the MPRs (gray spheres, HSV-1 gB residues 717-725 and EBV gB residues 672-679) are shown. The majority of the MPR residues were absent from the gB constructs crystallized. For crystallization, the fusion loops of EBV gB were replaced with those of HSV-1 gB. Images were created using MacPyMol. (C) Stick diagram of the chimeric gB constructs. Sequences from HSV-1 gB are represented by empty boxes, and the sequences from EBV gB are represented by gray boxes. Chimera names are on the left. Numbers indicate the EBV residues introduced into the HSV-1 backbone. In CH4 and CH4.2, HSV-1 gB residues 717-904 were replaced with the corresponding EBV

residues 672-857. In CH5 and CH5.2, HSV-1 gB residues 717-795 were replaced with EBV residues 672-753. In CH6 and CH6.2, HSV-1 gB residues 717-770 were replaced with EBV residues 672-728.

Fig. 2. Expression of the gB chimeras

(A) Cell surface expression. CHO-K1 cells were transfected in 6-well plates with plasmids encoding T7 RNA polymerase, gD, gH, gL and either gB or empty vector, as in Fig. 3. Cells were detached 7 hours post-transfection and plated in triplicate in a 96-well plate. After 18 h of incubation, the cells were washed with phosphate-buffered saline (PBS) and a cell-based ELISA was performed with the anti-gB serum R74. After PAb binding, the cells were washed, fixed, and incubated with biotinylated goat anti-rabbit IgG (Sigma), followed by streptavidin-horseradish peroxidase (streptavidin-HRP, GE Healthcare) and an HRP substrate (BioFX). Optical densities at 380 nm for wild-type gB ranged from 1.7 to 1.15. Negative control values (empty vector replacing gB) ranged from 0.19 to 0.35 and were subtracted from each experimental value. Results are presented as a percentage of wild-type gB expression. The means and standard deviations for 3 independent experiments are shown. Gray bars indicate chimeras containing EBV fusion loops. Striped bars represent data for chimeras containing the C-terminal regions of EBV gB Results for chimeras containing EBV gB fusion loops (gray), EBV gB C-terminal regions (stripes), or both (gray with stripes) are shown. (B) Total cellular expression. Western blots showing the relative amounts of wild-type and chimeric gB expression in CHO cells. CHO cells were transfected as above and lysed and proteins were separated by SDS-PAGE under denaturing conditions. Proteins were blotted on nitrocellulose and probed for gB using PAb R74. Molecular weight markers are indicated in kilodaltons.

Fig. 3.

Cell-cell fusion of gB chimeras. CHO-K1 cells were transfected as in Fig. 2A. Cells were detached at 7 hours post-transfection and mixed with a population of CHO-K1 cells stably expressing the nectin-1 receptor and transfected with a plasmid encoding luciferase under a T7 promoter. This mixed population of cells was then plated in triplicate in a 96-well plate. After 18 h, luciferase activity was measured to quantify the level of cell-cell fusion. Values of the negative control were subtracted from each experimental value and the results are presented as a percentage of fusion activity with wild-type gB. Negative controls ranged from 28,796 to 62,509, whereas uncorrected values for wild-type gB ranged from 133,495 to 1,464,790. The means and standard deviations for three independent experiments are shown.