Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread

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Heparan sulfate moieties serve as receptors for initial binding of herpes simplex virus types 1 and 2 (HSV-1 and -2) to cells. Deletion of HSV-1 glycoprotein C (gC-1) but not HSV-2 gC (gC-2) results in virions with reduced specific binding activity (virus particles bound per cell) and specific infectivity (p.f.u. per particle), suggesting that for HSV-1, but not HSV-2, gC plays a major role in mediating virus attachment. To test the hypothesis that glycoprotein B (gB), the other heparin-binding glycoprotein, mediates HSV-2 attachment, HSV-2 viruses deleted in gB-2 alone or deleted in both gB-2 and gC-2 were constructed. These viruses were grown on complementing or non-complementing cells and were compared with parental HSV-2(G) or a gC-2-deleted HSV-2 mutant (with respect to ability to bind and infect cells). At equivalent input concentrations of purified virions, significantly fewer gB-2-deleted virions bound to cells compared to parental HSV-2(G) or virus grown on complementing cells. In addition, viruses deleted in gB-2 were non-infectious. No immediate early proteins were detected in cells infected with gB-2-deleted virus harvested from non-complementing Vero cells, whereas these proteins were readily detected 4 h post-infection in cells infected with virus grown on complementing cells or with parental viruses. Viruses deleted in gB-2 failed to spread cell to cell, as evidenced by the inability to form plaques. Together these studies demonstrate that gB-2 plays a key role in mediating HSV-2 attachment and is required for entry and cell-to-cell spread. This glycoprotein is an important target for development of novel antiviral drugs.

Introduction

Entry of alphaherpesviruses into cells requires binding of virus to receptors on the cell surface and fusion of the virion envelope with the cell plasma membrane (Spear et al., 2000). This process is complex and presumably involves multiple interactions between viral envelope glycoproteins and cell surface components (Spear, 1993; Spear et al., 2000). For herpes simplex viruses (HSV), the first step is mediated by binding of envelope glycoproteins C and/or B (gC and gB, respectively) to cell surface heparan sulfate. Evidence for this includes the following observations. First, cells that are devoid of heparan sulfate (but not other glycosaminoglycans) because of enzymatic treatment or genetic mutation are markedly less susceptible to HSV but are unaltered in their susceptibility to other unrelated viruses (Shieh et al., 1992; WuDunn & Spear, 1989). Second, soluble heparin, which is structurally similar to heparan sulfate, inhibits virus binding (Gerber et al., 1995; Herold et al., 1991; WuDunn & Spear, 1989), whereas the structurally less similar glycosaminoglycan chondroitin sulfate fails to inhibit binding or infection. Third, gC and gB bind selectively and independently to heparin–Sepharose columns under physiological conditions (Gerber et al., 1995; Herold et al., 1991; Tal-Singer et al., 1995).

However, important serotype differences in the relative contribution of gC towards virus attachment have emerged. For most strains examined, deletion of HSV-1 gC (designated gC-1" viruses) markedly reduces virus binding (Herold et al., 1991; Immergluck et al., 1998; Laquere et al., 1998; Tal-Singer et al., 1995; Griffiths et al., 1998). In contrast, deletion of gC-2 does not result in loss of specific binding activity (particles bound per cell) or specific infectivity (p.f.u. per particle) when compared with the parental wild-type virus, HSV-2(G) (Gerber et al., 1995). Moreover, while gC-1" mutants show a marked lag in the kinetics of virus penetration, the gC-2" virus does...
not. Together, these results indicate that, in contrast to other alphaherpesviruses, the gC homologue does not play a major role in mediating attachment for HSV-2. This challenges the assumption that homologous glycoproteins play identical roles for HSV-1 and -2 and underscores the need to independently study both serotypes.

The finding that the deletion of gC-2 does not result in any loss in specific binding activity or infectivity suggests that gB-2, the other heparin-binding glycoprotein, mediates HSV-2 attachment. To directly test this hypothesis, a gB-2 deletion virus (gB-2<sup>−</sup>) and a doubly deleted virus (gB-2<sup>−</sup> × gC-2<sup>−</sup>) were constructed and characterized with respect to binding, entry and cell-to-cell spread.

**Methods**

**Cells and viruses.** Vero cells were obtained from the ATCC. The VgB2 cell line, which carries a functional gene for gB-2, was used for the propagation of gB-2 deletion viruses. The VgB2 cell line was constructed by transfection of Vero cells with a plasmid containing the gB-2 gene (BamHI–EcoRV fragment) under the control of the viral thymidine kinase promoter and a neomycin resistance marker. Transformed cells were selected for the ability to grow in the presence of geneticin and were shown to express gB-2 after infection with an HSV-1 gB-1 deletion virus, KO82. VgB2 cells were passaged in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum and 500 µg/ml geneticin. The wild-type virus strains HSV-2(G) and gC-2<sup>−</sup> (Gerber et al., 1995) served as parental strains for construction of the gB-2 deleted (gB-2<sup>−</sup>) and doubly deleted (gB-2<sup>−</sup> × gC-2<sup>−</sup>) viruses, respectively. Construction of these viruses is described below. HSV-2(G) and gC-2<sup>−</sup> were propagated on Vero cells; HSV-2(G) gB-2<sup>−</sup> and gB-2<sup>−</sup> × gC-2<sup>−</sup> were propagated on complementing VgB2 cells in the presence of 50 µg/ml hygromycin. One passage of the gB-2<sup>−</sup> viruses through Vero cells yielded gB-2-negative virions.

**Construction and isolation of gB-2<sup>−</sup> and gB-2<sup>−</sup> × gC-2<sup>−</sup> viruses.** VgB2 cells were co-transfected with HSV-2(G) or gC-2<sup>−</sup> viral DNA and DNA from an engineered plasmid, designated pBEH-GH1, which contains the gB-2 gene (UL27) disrupted at the unique SmaI site by the hygromycin/enhanced green fluorescent protein (EGFP) fusion protein under the control of the immediate early promoter of human cytomegalovirus (HCMV) constructed from pHygGFP (Clontech). This dual functional marker vector allows for drug selection with the ability to identify positive transfectants using GFP as a fluorescent reporter. Co-transfections were performed using the Effectene Transfection reagent (Qiagen). Recombinants were selected on VgB2 cells for ability to grow in the presence of 800 µg/ml hygromycin and expression of EGFP. Selected recombinants were plaque-purified three times and subsequently working stocks of two clones, designated HSV-2(G) gB-2<sup>−</sup> and gC-2<sup>−</sup> × gB-2<sup>−</sup>, respectively, were selected for further analysis.

To confirm that the gB-2<sup>−</sup> viruses contain the EGFP–hygromycin cassette disrupting the gB-2 gene, viral DNA was purified, digested with HindIII and separated by agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose and hybridized to the Nol fragment of UL27, which differentiates intact gB-2 (177 kb) from disrupted gB-2 (10 kb) or to a probe containing the EGFP–hygromycin cassette. The lack of gB and gC expression was confirmed by Western blot analysis using a monoclonal antisera to gB or gC (1123 and 1125, Goodwin Institute). Blots were subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse IgG and developed using the ECL kit (Du'Pont), as described previously (Qie et al., 1999).

**Purification and quantification of virus.** Virions were purified from Vero or VgB2 cells on dextran gradients, as described previously (Herold et al., 1991). For gB-2<sup>−</sup> and gB-2<sup>−</sup> × gC-2<sup>−</sup> viruses, which produce non-infectious progeny on permissive cells, Vero cells were inoculated at a m.o.i. of 5 p.f.u. per cell for gB-2<sup>−</sup> and 10–15 p.f.u. per cell for gB-2<sup>−</sup> × gC-2<sup>−</sup> virus and purified on dextran gradients 18 h post-infection (p.i.). The m.o.i. refers to the titre on complementing VgB2 cells of virus stocks produced on the complementing cell line. Titres of the purified virus were determined by plaque assays on Vero and VgB2 cells. The number of virus particles was determined by comparing the amounts of VP5 or gD by densitometric scanning with slight modification of methods described previously (Tal-Singer et al., 1995; Qie et al., 1999; Gerber et al., 1995). Dilutions of each virion preparation were solubilized and polypeptides were fractionated by SDS–PAGE. Gels were Coomassie blue- or silver-stained and the relative purity of preparations compared. The proteins were transferred to PVDF (Perkin-Elmer) by Western transfer in 20 mM Tris, 150 mM glycine and 20% methanol and probed with monoclonal antibodies (mAbs) 1103 (anti-gD, Goodwin Institute) or 10-H44 (anti-VP5, Fitzgerald Industries International). Both the Coomassie blue-stained gels (VP5 band) and Western blots (gD) were scanned and analysed using the GELDOC 2000 system (Bio-Rad) linked to an IBM PC and the relative number of virus particles determined. The purity of the virus preparations from host cell proteins was also examined by probing the Western blots with a mAb for cellular β-actin (AC-15, Sigma).

**Virus binding assays.** Cells were grown in 6-well dishes, pre-cooled to 4 °C, blocked in 3% BSA for 30 min and then exposed to serial twofold dilutions of purified virus (representing relatively equivalent numbers of particles for each virus based on a Coomassie-stained gel scanned for VP5 and corresponding to a range of ~ 0.01–1 p.f.u. per cell for G) for 5 h at 4 °C. In pilot experiments, we found that binding reaches equilibrium after ~ 5 h at 4 °C. The unbound virus was removed by washing the wells three times with cold PBS. Cells were counted and harvested in 200 µl per well of buffer containing 20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.05% DOC and supplemented with complete protease inhibitors (Roche). The soluble fraction was separated by centrifugation at 16 000 g in a microcentrifuge for 10 min at 4 °C. Equal portions of the input and cell-bound virus (soluble fraction) were separated by SDS–PAGE and quantified by comparing relative virus particle numbers by densitometric scanning after Western blotting with anti-gD mAb (1103) as detailed above. Mock-exposed cells were scanned and the background subtracted. The blots were also probed with the anti-β-actin mAb (AC-15) to compare relative amounts of cellular proteins. To assess the ability of heparin or chondroitin sulfate C to inhibit binding, studies were conducted in the presence of these soluble glycosaminoglycans (Sigma).

**Immediate early gene expression.** Cells were infected with virus (m.o.i. of 0.1–10 p.f.u. per cell or equivalent particle numbers). At 4 h p.i., the cells were harvested in 200 µl per well of buffer containing 20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.05% DOC and supplemented with complete protease inhibitors. The soluble fraction was separated by centrifugation at 16 000 g. Proteins were separated by SDS–PAGE and ICP27 detected by immunoblotting with mAb 1113. The blots were scanned and analysed using the GELDOC 2000 system.

**Infectious centre assays.** Infectious centre assays were performed as described previously (Herold et al., 2000; Roller & Herold, 1997). Briefly, Vero or VgB2 cells (donor cells) were exposed to different viruses at 37 °C to allow entry (m.o.i. of 10 p.f.u. per cell based on titre on complementing cell line). Cells were washed with a low pH citrate buffer to inactivate residual extracellular virus 1–2 h after infection. Then, 4–5 h
after infection, the infected cells were detached with trypsin–EDTA, counted and ~ 100 cells plated onto duplicate monolayers of uninfected cells in the presence of medium containing pooled human IgG (Sigma). The pooled human IgG neutralizes infection by virus released into the medium.

Results

Construction of gB-2− and gB-2− × gC-2− viruses

gB-2− and gB-2− × gC-2− double deletion viruses were constructed from HSV-2(G) and gC-2−, respectively, and the genotype confirmed by Southern blotting, as described in Methods (data not shown). Purified preparations of the parental and gB-deletion viruses (purified from Vero or VgB2 cells) were analysed by comparing viral proteins on Coomassie blue- or silver-stained gels. The relative purity of each virus preparation was similar (data not shown). To analyse glycoprotein expression for each purified preparation of virus, the gels were transferred to PVDF by Western transfer and probed with mAbs to gB, gC or gD (Fig. 1). Lanes were loaded with 50 µl of purified virus for Western blotting with antiserum to gB and gC or with 5 µl for probing with antisera to gD [equivalent to ~ 5 × 106 p.f.u. and 5 × 104 p.f.u. of HSV-2(G), respectively]. Gels were run under non-reducing conditions for Western blotting with mAb to gB to visualize the various oligomeric forms of this glycoprotein; the other gels were run under reducing conditions.

No gB-2 was detected in lanes containing gB-2− or gB-2− × gC-2− grown on Vero cells, but gB-2 was visualized in virus purified from the complementing cell line. Notably, the gB-2 detected in HSV-2(G) or gC-2− virus was predominantly oligomeric, whereas the gB-2 detected in deletion viruses purified from complementing cells tended to be predominantly monomeric. The relative amount of gB-2 detected in purified gC-2− virus was greater than that observed for HSV-2(G), suggesting that, in the absence of gC-2, more gB-2 is incorporated into the viral envelope, possibly because more gB-2 protein is made. However, no reciprocal increase in the relative amount of gC-2 was observed in the viral envelope of the gB-2− virus. As anticipated, no gC-2 was detected in gC-2− or gB-2− × gC-2− viruses. The gC-2 band from HSV-2(G) and complemented gB-2− virus tended to run as a broad smear, but not from the non-complemented gB-2− virus. The significance of this is unclear.

Binding of gB-2− and gB-2− × gC-2− virus to cells

To determine the impact of deleting gB-2 on virus attachment, the binding of purified HSV-2(G), gC-2−, gB-2− or gB-2− × gC-2− viruses was compared. The gB-2− viruses were purified from both Vero and VgB2 cells as a control. Proteins from each purified virus preparation were separated by SDS–PAGE and the relative amount of VP5 quantified by optical density scanning of the VP5 band following staining
with Coomassie blue. Based on this initial quantification, serial
twofold dilutions (representing equivalent numbers of par-
ticles) of each virus were allowed to bind Vero cells for 5 h at
4 °C. Unbound virus was removed by washing the monolayers
three times with PBS (Gerber et al., 1995; Herold et al., 1991).
The input and bound viruses were then quantified in parallel by
scanning the gD band on Western blots prepared with the
serial dilutions of virus input and cell lysates of bound virus.
Blots were also probed for β-actin to compare the amount of
cell lysate loaded in each lane and to examine the purity of the
virus preparations. This approach allows for comparison of
binding activity in a more physiologic range (∼ 0:01–1 p.f.u.
per cell) and in the absence of radiolabelling.
Quantifying the virus input by scanning gels for VP5 or
blots for gD yielded similar results for relative virus particle
numbers (Fig. 2). The band intensities were in a linear range
(ν² = 0.97 and 0.95, respectively, by linear regression). A
densitometric scanning reading of 132 ± 8 units for gD
corresponds to an m.o.i. of 1 p.f.u. per cell for HSV-2(G).
Representative blots showing input and bound virus for HSV-
2(Δ) and gB-2− virus (purified on Vero cells) probed with anti-
gD and anti-β-actin mAbs. (a) Representative blots for the other four virus
preparations probed with anti-gD mAb. (b) Graphic representation of results obtained from three to four independent
experiments; error bars indicate standard deviation.

Fig. 3. Comparison of the binding of HSV-2(G), gC-2−, gB-2− and gB-2− x gC-2− viruses purified from complementing or non-
complementing cells. Twofold dilutions of purified virus [equivalent to an m.o.i. of ∼ 1, 0.5, 0.25, 0.12, 0.06 and 0.03 p.f.u.
per cell for HSV-2(G), lanes 1–6 of input, respectively] were added to Vero cells at 4 °C for 5 h as described in Methods. After
washing away unbound virus, cell lysates were prepared and equal portions of the input and cell-bound virus were separated
by SDS–PAGE and quantified by comparing relative virus particle numbers by densitometric scanning after Western blotting with
anti-gD mAb. The blots were also probed with anti-β-actin mAb. (a) Representative blots of HSV-2(G) and gB-2− virus purified
from Vero cells probed with both the anti-gD and anti-β-actin mAbs. (b) Representative blots for the other four virus
preparations probed with anti-gD mAb. (c) Graphic representation of results obtained from three to four independent
experiments; error bars indicate standard deviation.
Table 1. Effects of heparin and chondroitin sulfate on virus binding

Results are expressed as virus particles bound (% of input) and are the mean ± SD of three independent experiments.

<table>
<thead>
<tr>
<th>Virus</th>
<th>PBS</th>
<th>Heparin</th>
<th>Chondroitin sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2(G)</td>
<td>81.2 ± 5</td>
<td>13.3 ± 6</td>
<td>60 ± 19</td>
</tr>
<tr>
<td>gC-2−</td>
<td>74 ± 8</td>
<td>11 ± 6</td>
<td>60 ± 19</td>
</tr>
<tr>
<td>gB-2−</td>
<td>15.5 ± 8.5</td>
<td>0.81 ± 0.5</td>
<td>10 ± 6</td>
</tr>
</tbody>
</table>

detected in purified input virus but similar quantities were detected in the cell lysates. Blots for the other virus mutants probed with anti-gD are shown in Fig. 3(b). Three independent experiments for each virus were performed and the results are summarized in the accompanying graph (Fig. 3c). As anticipated, deletion of gC-2 alone does not result in any loss in binding activity, confirming studies published previously (Gerber et al., 1995). In contrast, deletion of gB-2 resulted in a marked reduction in virus binding activity. Complementation of the gB-2-deleted viruses by growth on VgB2 cells partially restored binding activity. Less than 5% of gB− × gC− virions bound to cells, although the input tested was lower because of difficulties in purifying as much double-negative virus. Using non-purified virus stocks, the gB-2− × gC-2− virus showed a greater reduction in specific binding activity compared with the gB-2− virus (data not shown).

Binding studies were also conducted in the presence of soluble heparin or chondroitin sulfate. Vero cells were exposed to equivalent particle numbers of purified virions based on densitometric scanning for 5 h at 4 °C in the absence or presence of 100 µg/ml of soluble heparin or chondroitin sulfate. Cell-bound virus was quantified as described above. Results for virus at an input equivalent to 1 p.f.u. per cell for HSV-2(G) are shown in Table 1. Several important observations can be made from the results obtained. First, heparin, but not chondroitin sulfate, inhibits binding of all three viruses [HSV-2(G), gC-2− and gB-2− viruses]. Second, the binding of gB-2− virus in the absence of heparin is comparable to that of HSV-2(G) in the presence of heparin. Together, these results support the notion that gB-2 plays the key role in mediating HSV-2 binding to cell surface heparan sulfate. In its absence, binding is markedly reduced and is presumably mediated by interactions between gC-2 and heparan sulfate.

**gB-2 is required for virus entry and cell-to-cell spread**

Studies with an HSV-1 gB-deleted virus (KO82) have shown that gB-1 is essential for infection in culture (Cai et al., 1988). Although KO82 shows similar binding kinetics and binding activity compared to parental HSV-1(KOS) (Cai et al., 1988; Herold et al., 1991), KO82 fails to form plaques or to synthesize virus-specific proteins following infection. To test experimentally whether gB-2 is also required for HSV-2 entry and plaque formation, several different strategies were adopted. First, expression of EGFP following infection with viruses harvested from VgB2 or Vero cells was used as a surrogate marker for entry and the ability to form plaques as a marker of cell-to-cell spread. Cells were infected with gB-2− virus harvested from VgB2 cells viewed under the fluorescence (a) or light (b) microscope; Vero cells infected with gB-2− virus harvested from VgB2 cells viewed under the fluorescence (c) or light (d) microscope; and Vero cells infected with gB-2− virus harvested from Vero cells viewed under the fluorescence (e) or light (f) microscope.

Fig. 4. EGFP expression and plaque morphology 48 h following infection of VgB2 or Vero cells with 5 p.f.u. of the cell culture or an equivalent number of virus particles of gB-2− virus harvested from non-complementing Vero cells. Shown are VgB2 cells infected with gB-2− virus harvested from VgB2 cells as viewed under the fluorescence (a) or light (b) microscope; Vero cells infected with gB-2− virus harvested from VgB2 cells viewed under the fluorescence (c) or light (d) microscope; and Vero cells infected with gB-2− virus harvested from Vero cells viewed under the fluorescence (e) or light (f) microscope.
Table 2. Relative specific infectivity (p.f.u. per relative particle number) of wild-type and deletion viruses harvested from Vero or VgB2 cells

Relative particle numbers were determined by comparing the amounts of VP5 or gD by densitometric scanning of several dilutions of purified virus after Coomassie staining or Western blotting, respectively. Titres were determined by plaque assay. Units were converted to yield a specific infectivity for HSV-2(G) on Vero cells equal to 0.05 p.f.u. per particle based on previous studies counting particles by electron microscopy (Gerber et al., 1995).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cells from which virus was purified</th>
<th>Vero p.f.u. per particle</th>
<th>VgB2 p.f.u. per particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2(G)</td>
<td>Vero</td>
<td>5 x 10^-2</td>
<td>6 x 10^-2</td>
</tr>
<tr>
<td>gC-2^-</td>
<td>Vero</td>
<td>1.3 x 10^-1</td>
<td>Not determined</td>
</tr>
<tr>
<td>gB-2^-</td>
<td>Vero</td>
<td>&lt; 3.8 x 10^-5</td>
<td>3.3 x 10^-2</td>
</tr>
<tr>
<td>gB-2^-</td>
<td>VgB2</td>
<td>9 x 10^-5</td>
<td>8.8 x 10^-3</td>
</tr>
<tr>
<td>gB-2^- x gC-2^-</td>
<td>VgB2</td>
<td>1.25 x 10^-4</td>
<td>8.3 x 10^-3</td>
</tr>
<tr>
<td>gB-2^- x gC-2^-</td>
<td>Vero</td>
<td>1.25 x 10^-4</td>
<td>2.1 x 10^-3</td>
</tr>
</tbody>
</table>

Fig. 5. ICP27 expression 4 h.p.i. Vero cells were infected with 0.1, 1 or 10 p.f.u. per cell of HSV-2(G) or equivalent particle numbers of gB-2^- viruses harvested from VgB2 or Vero cells. Cell lysates were prepared 4 h p.i., proteins separated by SDS–PAGE and expression of ICP27 examined by Western blotting. Results are representative of five independent experiments.
the plasma membrane of infected and uninfected cells. To further examine the role of gB-2 in cell-to-cell spread, an infectious centre assay was adapted (Roller & Herold, 1997). Vero or VgB2 cells, designated ‘donor cells’, were infected with virus for 1 h at 37 °C [m.o.i. of 5–10 p.f.u. per cell for HSV-2(G) or equivalent particle numbers for gB-2− and gB-2− × gC-2−]. The cells were then washed with a low pH citrate buffer to inactivate residual extracellular virus and detached with trypsin EDTA at 3–4 h p.i. to disperse the cells. Cells were counted and 50, 100 or 200 cells were then plated onto a fresh monolayer of uninfected ‘target cells’ in the presence of medium containing neutralizing antibody (pooled human IgG). Under these experimental conditions, cell-to-cell spread occurs; but released virus is neutralized by the IgG. Representative results with ~ 100 donor cells are shown in Table 3. HSV-2(G) forms similar numbers of plaques independent of the absence of gB-2, however, gC-2 can mediate virus attachment, and cell-to-cell spread.

Table 3. Number of plaques formed per well on a target cell monolayer following exposure to ~ 100 donor cells

<table>
<thead>
<tr>
<th>Cell type (donor/target)</th>
<th>HSV-2(G) gB-2−</th>
<th>gB-2− × gC-2−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero/Vero</td>
<td>71 ± 12</td>
<td>0</td>
</tr>
<tr>
<td>VgB2/VgB2</td>
<td>66 ± 69</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>Vero/VgB2</td>
<td>66 ± 66</td>
<td>0</td>
</tr>
<tr>
<td>VgB2/Vero</td>
<td>83 ± 10</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are representative of three independent experiments.

Discussion

These studies indicate that gB-2 is principally responsible for the binding of HSV-2 to cell surface heparan sulfate. This conclusion is supported by the finding that a gB-2− virus is significantly impaired in specific binding activity and that binding is restored when the virus is repaired by growth on complementing cells. The binding of the gB-2− virus was similar to that observed for wild-type virus when the competitor heparin was present. This suggests that gC-2, the other heparin-binding glycoprotein, contributes little to virus binding, at least under these experimental conditions. In the absence of gB-2, however, gC-2 can mediate virus attachment, albeit at markedly reduced levels, as supported by the observation that binding of gB-2− virus is further impaired and almost abolished when gC-2 is also deleted.

HSV-2, therefore, appears to be unique among alphaherpesviruses with respect to the role of its glycoproteins in virus attachment. For most strains of HSV-1 and the related animal herpesviruses, pseudorabies virus (PrV) and bovine herpes-virus-1 (BHV-1), binding is mediated principally by the interactions of gC homologues with cell surface heparan sulfate (Griffiths et al., 1998; Herold et al., 1991; Mettenleiter et al., 1990; Okazaki et al., 1991). Whether HSV-2 evolved differently and whether this difference contributes to cell and tissue tropism remains to be determined. It should be noted, however, that gB homologues for several of the human beta- and gammaherpesviruses, such as HCMV, human herpesvirus (HHV)-7 and HHV-8, bind heparan sulfate (Akula et al., 2001; Boyle & Compton, 1998; Secchiero et al., 1997).

HSV-1 viruses deleted of gC-1 are impaired in binding and kinetics of penetration but remain infectious, presumably because the heparan sulfate binding activity of gB-1 is sufficient to mediate virus binding (Herold et al., 1994). In contrast, in the absence of gC, the gB homologues for PrV and BHV-1 do not appear to mediate binding to heparan sulfate. PrV variants deleted in gC (PrV gC−) bind via a heparan sulfate-independent mechanism, suggesting that PrV gB does not productively interact with heparan sulfate. This is supported by the findings that PrV gC− strains are relatively heparin-resistant; only at concentrations of ~ 100 µg/ml does soluble heparin inhibit PrV gC− infection of Vero cells (Mettenleiter et al., 1990). Moreover, PrV gC− mutants infect wild-type or heparan sulfate-deficient mutant cells to a similar extent (Karger et al., 1995). PrV gB binds to heparin–Sepharose only if gC is also present in the viral envelope, possibly due to glycoprotein–glycoprotein interactions (Mettenleiter et al., 1990). These results may reflect the lack of a heparin-binding domain within the sequence of PrV gB; analysis of the linear amino acid sequence of PrV gB supports this notion. HSV-1 and -2 gB proteins are only ~ 50% homologous to PrV gB at the amino acid level (Mettenleiter & Spear, 1994; Spear, 1993). Another possible important difference is that PrV gB is proteolytically processed into subunits that remain linked via disulfide bonds, whereas HSV gB proteins are not (Mettenleiter & Spear, 1994). Similarly, BHV-1 gB, which is processed in a manner similar to PrV gB, also fails to mediate heparan sulfate binding, although isolated BHV-1 gB exhibits heparin-binding activity (Klupp et al., 1997).

Relatively high local concentrations of basic amino acids are characteristic of the heparin-binding domains of proteins. Analysis of the amino acid sequences at the N-termini of the HSV-1 and -2 forms of gB and gC reveals that there is a short lysine-rich region in gB and a longer lysine- and arginine-rich region in gC (Bzik et al., 1986; Dowbenko & Lasky, 1984; Frink et al., 1983; Stuve et al., 1987; Swain et al., 1988). Notably, the amino acid sequences of gB and gC are highly conserved between the two serotypes except at the N-termini and...
particular in these basic regions. Interestingly, gC-1 differs considerably from gC-2 in this region, including an inser- tional/deletional variation (Spear, 1993). If these basic regions mediate the binding to heparan sulfate, differences in sequences might contribute to differences in binding activity.

The observation that there are serotype differences in the relative contribution of gB and gC to virus binding is supported by known differences in epidemiology, cell tropism and sus- ceptibility to inhibitors of virus binding. For example, HSV-1 is more likely to cause oral labial infections and sporadic encephalitis, whereas HSV-2 commonly causes genital lesions. This epidemiological observation is supported by in vitro studies that show that HSV-1 binds to human synaptosomes and glial cells more efficiently than does HSV-2, whereas HSV-2 binds cervical cells more efficiently than does HSV-1 (Vahne et al., 1979, 1980). Neomycin, poly(t)-lysine and platelet factor 4 inhibit binding of HSV-1 but not HSV-2 (Campadelli-Fiume et al., 1990; Herold & Spear, 1994; Langeland et al., 1988, 1990; Oyan et al., 1993). Conversely, selectively O-desulfated heparins preferentially inhibit binding of HSV-2 (Herold et al., 1996). The observations that heparan sulfate is a common receptor for both serotypes but that the two preferentially bind different cell types and differ in susceptibility to select inhibitors of binding may be explained if gC-1 and gB-2 preferentially recognize distinct structural sequences of hepa- ran sulfate differentially expressed by different cells. Thus, genital tract epithelial cells may express heparan sulfate sequences preferentially recognized by gB-2, whereas oral mucosal cells may express sequences preferentially recognized by gC-1.

In addition to playing the major role in mediating HSV-2 attachment, gB-2 is also essential for penetration and cell-to-cell spread. Deletion of gB-2 results in non-infectious virions that fail to enter, as evidenced by an inability to detect any immediate early gene expression even at a relative m.o.i. of 10 p.f.u. per cell, and fail to laterally spread, as evidenced by an inability to form infectious centres on non-complementing cells. These essential functions in virus-mediated fusion events are not unique to HSV-2 as all of the gB homologues studied to date have been shown to play similar roles (Spear, 1993). Whether or how the heparan sulfate-binding activity of gB-2 contributes to its role in mediating these fusion events cannot be determined from these studies. For HSV-1, it was shown that deletion of the heparin-binding lysine-rich region (residues 68–76) resulted in viruses that, while infectious, exhibited impaired entry and cell-to-cell spread (Laquerre et al., 1998). Recombinant viruses carrying mutations in the putative heparin-binding domain of gB-2 are under construction to determine the contribution of heparan sulfate interactions towards entry and cell-to-cell spread.

Together these studies demonstrate a major role for gB-2 in HSV-2 binding and an essential role in entry and cell-to-cell spread. Thus, gB-2, along with the other essential envelope glycoproteins, gD and gH-gL, are important targets for development of novel antiviral therapies, including topical microbicides that might prevent sexual or perinatal trans- mission of HSV-2 by blocking virus entry.

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Role of gB-2 in binding and entry


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