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# Fusion activity of lipid-anchored envelope glycoproteins of herpes simplex virus type 1

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**Abstract**

Expression of the herpes simplex virus type 1 (HSV-1) glycoproteins gB, gD, gH, and gL is necessary and sufficient to cause cell fusion. To identify the requirements for a membrane-spanning domain in HSV-1 glycoprotein-induced cell fusion, we created gB, gD, and gH mutants with transmembrane and cytoplasmic domains replaced by a glycosylphosphatidylinositol (gpi)-addition sequence. The corresponding gBgpi, gDgpi, and gHgpi proteins were expressed with wild-type efficiency at the cell surface and were linked to the plasma membrane via a gpi anchor. The gDgpi mutant promoted cell fusion near wild-type gD levels when co-expressed with gB, gH, and gL in a cell-mixing fusion assay, indicating that the gD transmembrane and cytoplasmic domains were not required for fusion activity. A plasma membrane link was required for fusion because a gD mutant lacking a transmembrane and cytoplasmic domain was nonfunctional for fusion. The gDgpi mutant was also able to cooperate with wild-type gB, gH, and gL to form syncytia, albeit at a size smaller than those formed in the wild-type situation. The gBgpi and gHgpi mutants were unable to promote fusion when expressed with the other wild-type viral glycoproteins, highlighting the requirement of the specific transmembrane and cytoplasmic domains for gB and gH function.


*Keywords:* Glycosylphosphatidylinositol; Fusion; Syncytium; Nectin-1; Herpes simplex virus; Transmembrane domain; gB; gD; gH; Phosphatidylinositol phospholipase C

## Introduction

Virus-to-cell and cell-to-cell fusion induced by herpes simplex virus (HSV) require four envelope glycoproteins (gB, gD, gH, and gL) (Balan et al., 1994; Cai et al., 1988a, 1988b; Davis-Poynter et al., 1994; Forrester et al., 1992; Highlander et al., 1988; Huff et al., 1988; Hutchinson et al., 1992; Johnson and Ligas, 1988; Johnson et al., 1988; Ligas and Johnson, 1988; Roop et al., 1993; Wilson et al., 1994). Herpes simplex virus type 1 (HSV-1)-induced cell fusion can result in the formation of giant multinucleated cells, called syncytia, which are created by the fusion of infected cells with adjacent uninfected cells. Syncytia are observed in HSV-induced lesions and may represent a mechanism of cell killing during infection (Pertel and Spear, 1998; Spear, 1993). Syncytium formation can occur in cell culture in the absence of infectious virus by the expression of gB, gD, gH, and gL in cells that express a gD receptor (Klupp et al., 2000; Muggeridge, 2000; Pertel et al., 2001; Turner et al., 1998).

Cell fusion can also be detected by mixing cells expressing gB, gD, gH, and gL with cells expressing a gD receptor (Pertel et al., 2001). Expression of all four viral envelope glycoproteins is required for fusion to occur (Browne et al., 2001; Muggeridge, 2000; Pertel et al., 2001; Turner et al., 1998). The only cellular factor thus far identified to be required for fusion induced by HSV-1 envelope glycoproteins is a gD receptor (Pertel et al., 2001). Cell-surface glycosaminoglycans are not required for fusion (Browne et al., 2001; Pertel et al., 2001), despite the ability of gB to bind them (Herold et al., 1991, 1994). The binding of gD to receptor is critical for fusion and may initiate the fusion process. The details of what happens after gD binds receptor are poorly understood. Presumably, gD, gB, gH, and gL bind other cell-surface factors or interact with the apposing membrane to achieve fusion.

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The requirements for a specific transmembrane domain and cytoplasmic tail differ among the HSV-1 envelope glycoproteins. The specific transmembrane and cytoplasmic domains of gH are required for gH to function in fusion (Harman et al., 2002). Mutations in the cytoplasmic tail of gB can abolish or enhance fusion depending upon their location, whereas mutations in the cytoplasmic tail of gH can abolish fusion (Baghian and Kousoulas, 1993; Baghian et al., 1993; Cavalcoli et al., 1993; Diakidi-Kosta et al., 2003; Fan et al., 2002; Foster et al., 2001; Wilson et al., 1994). In contrast, gD can function when its cytoplasmic tail is deleted or its transmembrane domain and cytoplasmic tail are replaced by those from other transmembrane proteins (Cairns et al., 2003; Feenstra et al., 1990; Whiteley et al., 1999).

Many viruses encode a single protein capable of performing fusion. Some of these fusion proteins, called class I or type I fusion proteins, have many common characteristics (Colman and Lawrence, 2003). Examples of type I fusion proteins are influenza hemagglutinin (HA), human immunodeficiency virus gp160, and paramyxovirus F. None of the required four HSV-1 envelope glycoproteins has yet been found to possess all the characteristics of a type I fusion protein. However, because it appears that general aspects of fusion are conserved among most viruses, the characteristics of type I fusion proteins responsible for mediating fusion may be distributed among the four HSV envelope glycoproteins.

Type I fusion proteins with their transmembrane and cytoplasmic domains replaced by a glycosylphosphatidylinositol (gpi) linkage are nonfunctional for fusion (Kemble et al., 1994; Markosyan et al., 2000; Tong and Compans, 2000; Weiss and White, 1993; Zhou et al., 1997). In cell fusion assays, the influenza HA-gpi mediates the fusion of the outer leaflet of lipid membranes but without complete lipid bilayer fusion or mixture of cytoplasmic contents (Kemble et al., 1994; Melikyan et al., 1997; Markosyan et al., 2000). This partial and incomplete fusion is called hemifusion (Kemble et al., 1994). HA-gpi may actually form small fusion pores that do not enlarge sufficiently to allow fusion, suggesting that a membrane-spanning domain is required for the enlargement of fusion pores (Markosyan et al., 2000). Recently, a gpi-linked form of HSV-1 gD was unable to mediate cell fusion when expressed with wild-type gB, gH, and gL (Browne et al., 2003). The gDgpi mutant mediated virus-to-cell fusion because it complemented the entry defect of a gD minus isolate of HSV-1, albeit the gDgpi-containing virions entered cells at a rate slower than virions with wild-type gD (Browne et al., 2003). Although HSV-1 gD does not display many characteristics of a type I fusion protein, these results suggest that a membrane-spanning domain is required for gD to function in cell fusion and that gD may have a greater role in fusion than receptor binding.

Because investigations of gpi-linked type I fusion proteins indicate that a membrane-spanning domain may be necessary for the leverage or force required to form or enlarge fusion pores, we were interested in determining if membrane-spanning domains were required for fusion in the HSV-1 four glycoprotein fusion system. We removed the transmembrane domains and cytoplasmic tails from gB, gD, and gH and replaced them with the gpi-addition sequence from decay-accelerating factor (DAF). If a gpi-linked version of gD, gB, or gH was functional for fusion, then that particular glycoprotein is unlikely to be directly involved in the formation and enlargement of fusion pores in a manner analogous to a type I fusion protein. We show here that gDgpi, gBgpi, and gHgpi were expressed efficiently at the cell surface and were linked to the plasma membrane via a gpi anchor. Only gDgpi promoted fusion when co-expressed with the other wild-type proteins. The fusion activity of gDgpi was near wild-type gD levels in a cell-mixing fusion assay. However, the gDgpi mutant, although capable of inducing syncytium formation when expressed with gB, gH, and gL, formed smaller syncytia over time when compared to the wild-type situation.

## Results

### *Plasmids expressing gpi-linked versions of gD, gB, and gH*

To determine if a membrane-spanning domain and cytoplasmic tail were required for HSV-1 glycoprotein function, gpi-linked versions of the fusion glycoproteins were created. Proteins linked to the plasma membrane via a gpi anchor do not span the membrane but are tethered to the outer leaflet of the lipid bilayer. Fig. 1 is a diagram of versions of gB, gD, and gH with their transmembrane domains and cytoplasmic tails replaced by a gpi-addition sequence from decay-accelerating factor (DAF). The gDgpi mutant contains the entire predicted extracellular domain of gD fused to the gpi-addition sequence from DAF. It was suggested that gB may span the membrane three times because of the length of hydrophobic domains 1, 2, and 3 (Pellett et al.,

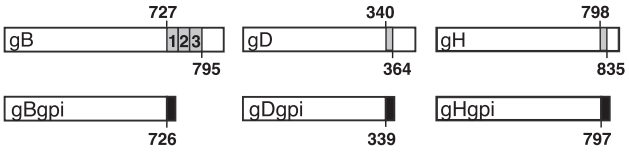


Fig. 1. Wild-type HSV-1 envelope glycoproteins and gpi-linked mutants. Areas shaded in gray represent predicted hydrophobic domains, some of which are transmembrane domains. Numbers above the individual glycoprotein indicate the amino acid number where domain begins. Numbers below glycoprotein indicate amino acid number where domain ends. The numbers, "1, 2, 3" represent the first, second, and third predicted hydrophobic domains of gB. Black box represents sequence added from DAF (the final 37 amino acids) that signals the addition of a gpi anchor. Number before black box indicates amino acid of glycoprotein fused to DAF sequence. Amino acid numbers include the signal sequence and correspond to the sequence in GenBank accession numbers E03113 (gB), L09243 (gD), X03896 (gH), and M31516 (DAF). Drawing is not to scale.

1985). Therefore, we placed the gpi-addition sequence in gBgpi just before hydrophobic domain 1 to ensure that gBgpi did not span the membrane. The gHgpi mutant contains the entire predicted extracellular domain fused to the DAF gpi-addition sequence. Because gL does not contain a membrane-spanning domain, no gpi-linked version was created.

*Cell-surface expression and PIPLC cleavage of gpi-linked mutants*

Phosphatidylinositol phospholipase C (PIPLC) cleaves gpi anchors and releases gpi-anchored proteins from the cell surface. As an indication of whether the predicted gpi-anchored versions of gB, gD, and gH were actually linked to the plasma membrane via a gpi anchor, Chinese hamster ovary K1 (CHO-K1) cells expressing the proteins were treated with PIPLC before CELISA or flow cytometry analysis. Both assays measure expression of cell-surface protein. The CELISA assay involved incubating live cells with PIPLC followed by primary anti-glycoprotein antibody, fixing the cells, and adding biotinylated secondary antibody, and a streptavidin-conjugated horseradish peroxidase. After the addition of substrate, horseradish peroxidase activity was measured as an indication of cell-surface protein expression (see Materials and Methods). CHO-K1 cells were transfected with plasmids expressing the wild-type or gpi-linked versions of the three envelope glycoproteins. The results in Fig. 2A demonstrate that the gDgpi mutant was expressed efficiently at the cell surface and that a significant fraction of the gDgpi was removed from the cell surface by PIPLC treatment although wild-type gD was unaffected. The gHgpi mutant was expressed efficiently at the cell surface when gL was co-expressed and gHgpi was sensitive to cleavage by PIPLC although wild-type gH was not (Fig. 2B). In the absence of gL, there was a small but reproducible increase in cell-surface expression of gHgpi over wild-type gH that was also PIPLC sensitive (Fig. 2B). The gBgpi mutant was expressed at the cell surface similarly to wild-type gB. Surprisingly, gBgpi was not sensitive to PIPLC cleavage in this assay (Fig. 2C). These results demonstrate that the gDgpi and gHgpi proteins were expressed at the cell surface and linked to the plasma membrane via a gpi tether as predicted.

It was normal to observe a percentage of gDgpi and gHgpi that was unable to be removed from the cell surface by PIPLC. Many studies have observed incomplete removal of well-known gpi-linked proteins from the cell surface by PIPLC treatment (Airas et al., 1997; Beghdadi-Rais et al., 1993; Caras et al., 1987, 1989; Crise et al., 1989; Davitz et al., 1986; Diamond et al., 1990; Kemble et al., 1993; Low, 1989; Low and Kincade, 1985; Skretting et al., 1999; Wang and Bergelson, 1999; Zhou et al., 1997). The degree to which any gpi-anchored protein is susceptible to PIPLC depends on the cell line used, potential modifications of the gpi anchor to render it resistant to

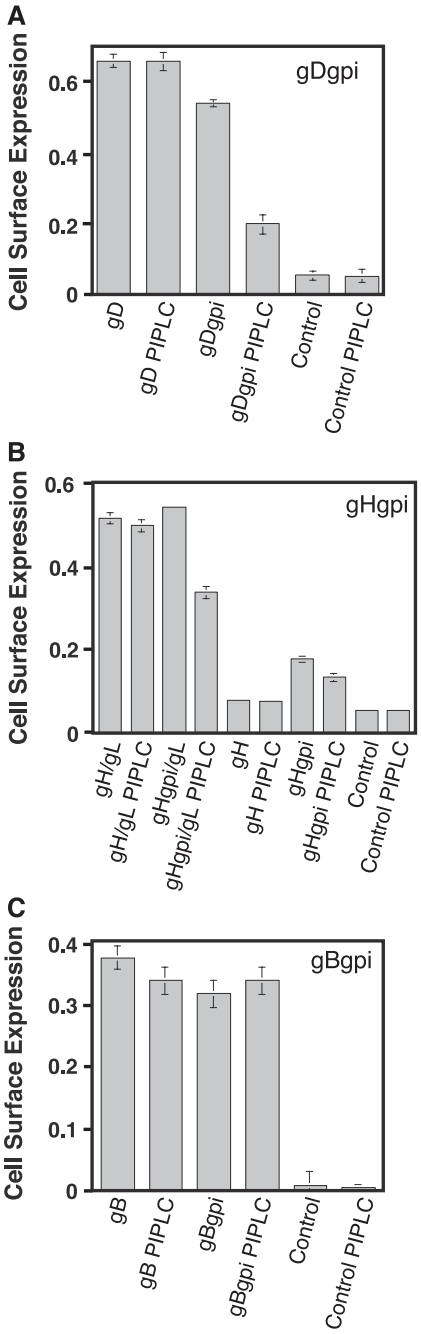


Fig. 2. Cell-surface expression and PIPLC sensitivity of gpi-linked glycoproteins using CELISA analysis. The envelope glycoprotein-expressing cells were treated with PIPLC or mock treated, incubated with a glycoprotein-specific antibody, followed by an antibody detection system. Mean results and standard deviations for a representative set of experiments are listed. Each experiment was performed a minimum of three times. The absence of error bars for mean values given is due to standard deviations too small to generate visible error bars. (A) CHO-K1 cells were transfected with plasmids expressing gD, gDgpi, and a control plasmid. Cell-surface expression of gD and gDgpi was detected using anti-gD serum R7. (B) CHO-K1 cells were transfected with plasmids expressing gH and gL, gH and control plasmid, gHgpi and gL, gHgpi and control plasmid, or control plasmid. The anti-gH antibody 52S-43 was used to detect cell-surface expression of gH and gHgpi. (C) CHO-K1 cells were transfected with plasmids expressing gB, gBgpi, or control plasmid. The anti-gB serum R74 was used to detect cell-surface expression of gB and gBgpi.

cleavage, the structure of the extracellular domain of the protein affecting access of PIPLC to the anchor, the processivity of the PIPLC, and level of protein expressed (Airas et al., 1997; Diamond et al., 1990; Low, 1989; Skretting et al., 1999). A major concern was that the "PIPLC-resistant" gDgpi failed to receive a gpi anchor and then would span the plasma membrane using the hydrophobic stretch in the DAF gpi-addition sequence. However, the hydrophobic domain contained in the DAF gpi-addition sequence is unable to act as a transmembrane domain for gD, and that sequence is not sufficient to confer plasma membrane association (Caras et al., 1989). Proteins with gpi-addition signals that are not processed to add a gpi anchor are degraded in the ER or are secreted into the extracellular space and do not have plasma membrane association (Caras et al., 1989; Field et al., 1994; Moran et al., 1991). Therefore, the gpi-anchored protein that remains on the cell surface after PIPLC incubation is not associated with the plasma membrane via the hydrophobic sequence in the gpi-addition signal. Versions of gDgpi nearly identical to the one described here have been extensively studied and shown to behave exactly as predicted for a gpi-linked protein (Beghdadi-Rais et al., 1993; Caras et al., 1987, 1989; Lisanti et al., 1989, 1990, 1991).

As a further indication of cell-surface expression and PIPLC sensitivity, CHO-K1 cells transiently expressing the wild-type or gpi-linked versions of gD, gH, and gB were incubated with PIPLC, washed, incubated with glycoprotein-specific polyclonal or monoclonal antibody followed by secondary FITC-conjugated antibodies, and analyzed by flow cytometry. Both the wild-type and gpi-linked versions of gH were co-expressed with gL. Expression of gDgpi and gHgpi was readily detectable at the cell surface in the absence of PIPLC treatment (Fig. 3). After PIPLC treatment, there was a significant reduction in surface expression of both gDgpi and gHgpi although the wild-type versions of gD and gH were unaffected by PIPLC treatment (Fig. 3). Cell-surface gBgpi was again resistant to cleavage by PIPLC using the flow cytometry analysis (Fig. 3).

One possible explanation for the lack of PIPLC cleavage is that the structure of the extracellular domain of gB blocked PIPLC access to the gpi anchor. To circumvent that potential problem, we attempted to detect the gpi anchor in a more direct manner. We labeled cells expressing gBgpi and gDgpi with $^{35}$S-methionine or with $^{3}$H-ethanolamine and immunoprecipitated the labeled cell lysates with specific antibodies to gB or gD. The gDgpi mutant was analyzed as a positive control because gDgpi was PIPLC sensitive and clearly gpi anchored. The gHgpi mutant was not analyzed because it was PIPLC sensitive and clearly gpi anchored. The $^{35}$S-methionine will label the polypeptide, and the $^{3}$H-ethanolamine will label the gpi anchor. The results in Fig. 4 show that gD, gB, and their corresponding gpi-linked versions were immunoprecipitated from transfected CHO-K1 cells labeled with $^{35}$S-methionine. The gBgpi and gDgpi mutants were labeled with the $^{3}$H-ethanolamine although the wild-type versions of the proteins were not (Fig. 4). The labeling of both gDgpi and gBgpi with $^{3}$H-ethanolamine

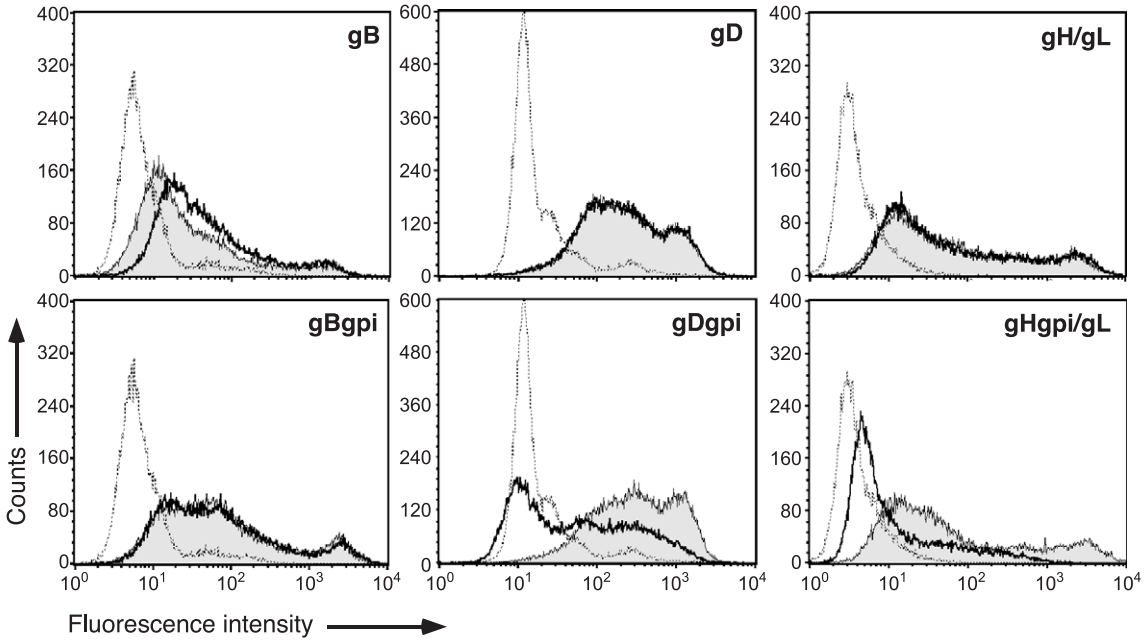


Fig. 3. Cell-surface expression and PIPLC sensitivity of gBgpi, gDgpi, and gHgpi using flow cytometry analysis. CHO-K1 cells were transfected with plasmids expressing wild-type versions of gB, gD, or gH, gpi-linked versions, or control plasmid (pCAGGS, dashed line open curve). Transfected CHO-K1 cells were treated with PIPLC (dark line open curves) or mock treated (shaded curves) and then processed for flow cytometry. Cell-surface gD and gDgpi expression was detected using anti-gD antiserum R7, the anti-gH antibody 52S-43 was used to detect cell-surface expression of gH and gHgpi, and R74 antiserum was used to detect cell-surface gB and gBgpi. The experiment was repeated three times and results from a representative experiment are shown.

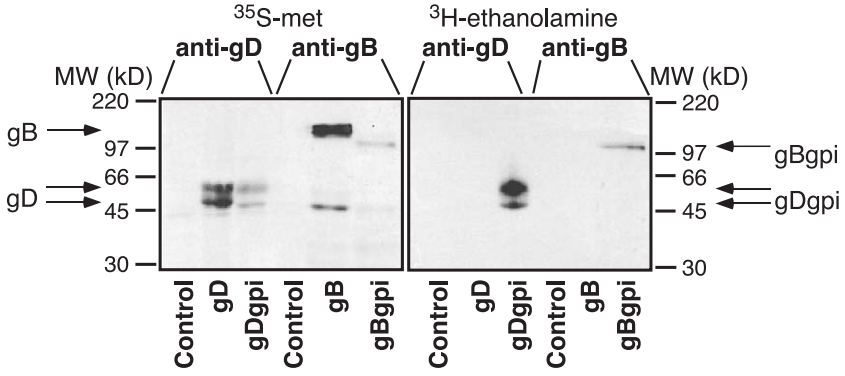


Fig. 4. Metabolic labeling and immunoprecipitation analysis to determine if the gBgpi mutant contains a gpi anchor. CHO-K1 cells were transfected with plasmids expressing gD, gDgpi, gB, gBgpi, or control plasmid (pCAGGS). One-half of each transfected population was labeled overnight with $^{35}$S-methionine/cysteine and the other half labeled with $^{3}$H-ethanolamine. The cell lysates were immunoprecipitated with either anti-gD antiserum R7 or the anti-gB polyclonal serum R74 and subjected to SDS-PAGE (10% gel).

provides evidence that the gDgpi and the gBgpi mutants possessed a gpi anchor. Because the immunoprecipitation results indicate that gBgpi contained a gpi anchor, the inability of PIPLC to remove gBgpi from the cell surface suggests that the structure of gBgpi may prevent access of PIPLC to the gpi anchor.

Interestingly, the level of gB expression appeared to be greater than that of gBgpi in the immunoprecipitation experiments (Fig. 4), yet the cell-surface expression of gB and gBgpi was approximately equivalent in another experiment (Fig. 3). The cytoplasmic tail of gB contains retrieval sequences that down-regulate expression of gB from the cell surface (Beitia Ortiz de Zarate et al., 2004; Brideau et al., 2000) and gB mutants lacking those sequences accumulate at the cell surface (Beitia Ortiz de Zarate et al., 2004). Because gBgpi lacks the cytoplasmic tail, there may be an accumulation of the mutant at the cell surface. Therefore, although the total levels of gBgpi expressed in cells may be reduced when compared to gB, cell-surface expression could be equivalent.

### *gDgpi binds a secreted form of nectin-1 with wild-type gD efficiency*

The binding of gD to a cell-surface receptor is critical for fusion (Ligas and Johnson, 1988; Pertel et al., 2001). Therefore, we tested the ability of the gDgpi mutant to bind the gD receptor nectin-1. We transfected CHO-K1 cells with plasmids expressing gD or gDgpi and then incubated the cells with a secreted form of nectin-1 (nectin-1:Fc). After the incubation, the live cells were washed and fixed before the addition of a detection system similar to that used for CELISA analysis (Materials and Methods). The results shown in Fig. 5A demonstrate that the nectin-1:Fc protein bound to cell-surface gD and gDgpi approximately equivalently. The CELISA analysis done in parallel with the nectin-1:Fc binding experiment demonstrates that there was approximately equivalent cell-surface expression of gD and gDgpi (Fig. 5B). Therefore, the removal of the gD transmembrane domain and cytoplasmic tail and corresponding addition of a gpi anchor did not interfere with the ability of gDgpi to bind nectin-1.

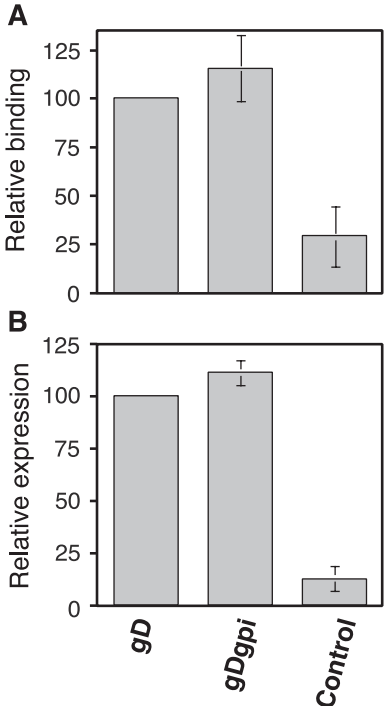


Fig. 5. Binding of nectin-1 to the surfaces of cells expressing wild-type gD or gDgpi. (A) CHO-K1 cells were transfected with plasmids expressing gD, gDgpi, or control plasmid. The cells were incubated with culture media containing a nectin-1/rabbit IgG Fc fusion protein (nectin-1:Fc), a biotinylated anti-rabbit IgG, and a streptavidin-conjugated horseradish peroxidase. Peroxidase activity was measured as an indication of nectin-1:Fc binding to the cell surface. The experiments were performed three times and the relative mean values plus standard deviations are shown. Incubation of the transfected cells with control supernatant yielded background levels of binding and is not shown. (B) Parallel analysis of cells transfected in A for cell-surface expression of gD and gDgpi using anti-gD antiserum R7. The transfected cells were processed for CELISA as described in Materials and methods.

*The gHgpi mutant binds gL*

Efficient expression of HSV-1 gH at the surface of the virion or at the cell surface requires the co-expression of gL and the formation of a gH-gL heterodimer (Hutchinson et al., 1992; Roop et al., 1993). Efficient cell-surface expression of gH and gHgpi was dependent upon gL co-expression (Fig. 2B), suggesting that gHgpi, like gH, associated with gL to reach the cell surface. To demonstrate more clearly that gHgpi formed a complex with gL, we conducted co-immunoprecipitation and Western blot analysis on cells co-expressing gHgpi and gL. CHO-K1 cells expressing either gH and an HA epitope-tagged version of gL or gHgpi and an HA-tagged version of gL were lysed and immunoprecipitated using an anti-gH monoclonal antibody. Western blot analysis of the immunoprecipitated proteins using an anti-HA monoclonal antibody clearly demonstrates that gL was co-immunoprecipitated with gH and gHgpi, indicating that gHgpi bound gL (Fig. 6).

*The gBgpi mutant binds dimer-dependent and conformation-dependent monoclonal antibodies*

The inability of PIPLC to cleave the gpi anchor of the gBgpi mutant may indicate that the gBgpi mutant was not folded properly. To address this possibility, we measured the cell-surface expression of gBgpi in CHO-K1 cells using well-characterized anti-gB monoclonal antibodies. The monoclonal antibodies H233, H352, H420, and H1783 bind to epitopes D2a, D3a, Dd5a, and Dd6, respectively, and the epitopes span the gB extracellular domain (Chapsal and Pereira, 1988; Pereira et al., 1989; Qadri et al., 1991). All four monoclonal antibodies recognize conformation-dependent domains on gB, and H420 and H1783 recognize gB dimers but not monomers (Chapsal and Pereira, 1988; Pereira et al., 1989; Qadri et al., 1991). CHO-K1 cells expressing gB, gBgpi, or control DNA were subjected to CELISA analysis with the four conformation-dependent antibodies as well as the conformation-independent antibody H1817 (epitope D1a) (Chapsal and Pereira, 1988; Pereira et al., 1989; Qadri et al., 1991). Cells expressing the gBgpi mutant bound all monoclonal antibodies approximately equivalently to wild-type gB with the exception of H352 and H420 which bound approximately 150% and 50% better, respectively, to gBgpi-expressing cells (Fig. 7). These results indicate that the gBgpi mutant formed a dimer and was not grossly misfolded when compared to wild-type gB. However, the lack of transmembrane and cytoplasmic domains in the gBgpi mutant rendered epitopes in the extracellular domain, D3a and Dd5a, more accessible to antibody binding when compared to wild-type gB.

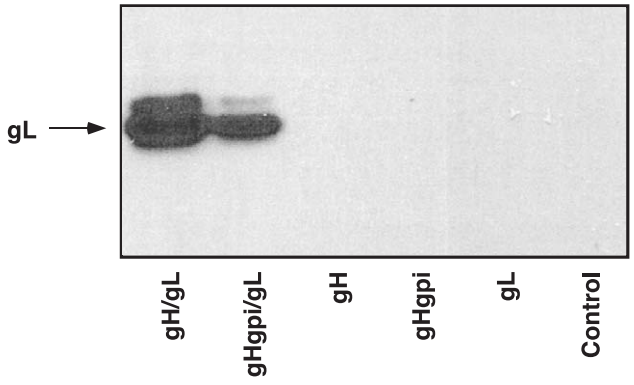


Fig. 6. Both gH and gHgpi bind gL. CHO-K1 cells expressing gH and gL, gHgpi and gL, gH, gHgpi, gL, or control cells were lysed and immunoprecipitated with the anti-gH monoclonal antibody 52S-43. The immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rat anti-HA monoclonal antibody and chemiluminescence detection system to detect the HA epitope-tagged gL.

*Fusion activity of gpi-linked mutant envelope glycoproteins*

To test the ability of the gpi-linked mutants to contribute to envelope glycoprotein-induced fusion, we performed two different cell fusion assays. The first was a cell-mixing assay in which we transfected CHO-K1 cells with a plasmid expressing T7 RNA polymerase and either plasmids expressing gB, gD, gH, and gL, or three of the wild-type envelope glycoproteins with one of the gpi-linked versions. We mixed those transfected cells with CHO-K1 cells transfected with a plasmid expressing the gD receptor nectin-1 and a plasmid containing the *E. coli lac z* gene under the control of the T7 promoter. CHO-K1 cells are normally resistant to HSV-1-induced fusion and must express a gD receptor to be capable of fusing with HSV-1 envelope glycoprotein-expressing cells (Pertel et al., 2001). β-Galactosidase (β-gal) activity will be detected only when the two transfected cell populations fuse, the cell contents mix, and the T7 polymerase enters the nucleus to activate β-gal expression. A similar cell-mixing fusion assay has been described (Pertel et al., 2001). The second fusion assay detects cell fusion by observing the formation of syncytia. Syncytia were formed when B78H1 cells over-expressing the gD receptor nectin-1 (B78H1-C10 cells) were transfected with envelope glycoprotein expression plasmids and monitored microscopically. B78H1 cells are normally resistant to fusion induced by HSV-1 and require ectopic expression of a gD receptor to form syncytia. A similar syncytium assay has been described (Connolly et al., 2003).

The results of the cell mixing experiments are shown in Fig. 8. The only gpi-linked version of an envelope glycoprotein that mediated detectable fusion was gDgpi. Cells expressing gB, gDgpi, gH, and gL fused nearly as well as cells expressing gB, gD, gH, and gL (Fig. 8B). There is a requirement for gD to be linked to the plasma membrane, however, because a secreted form of gD was unable to substitute for wild-type gD to mediate cell

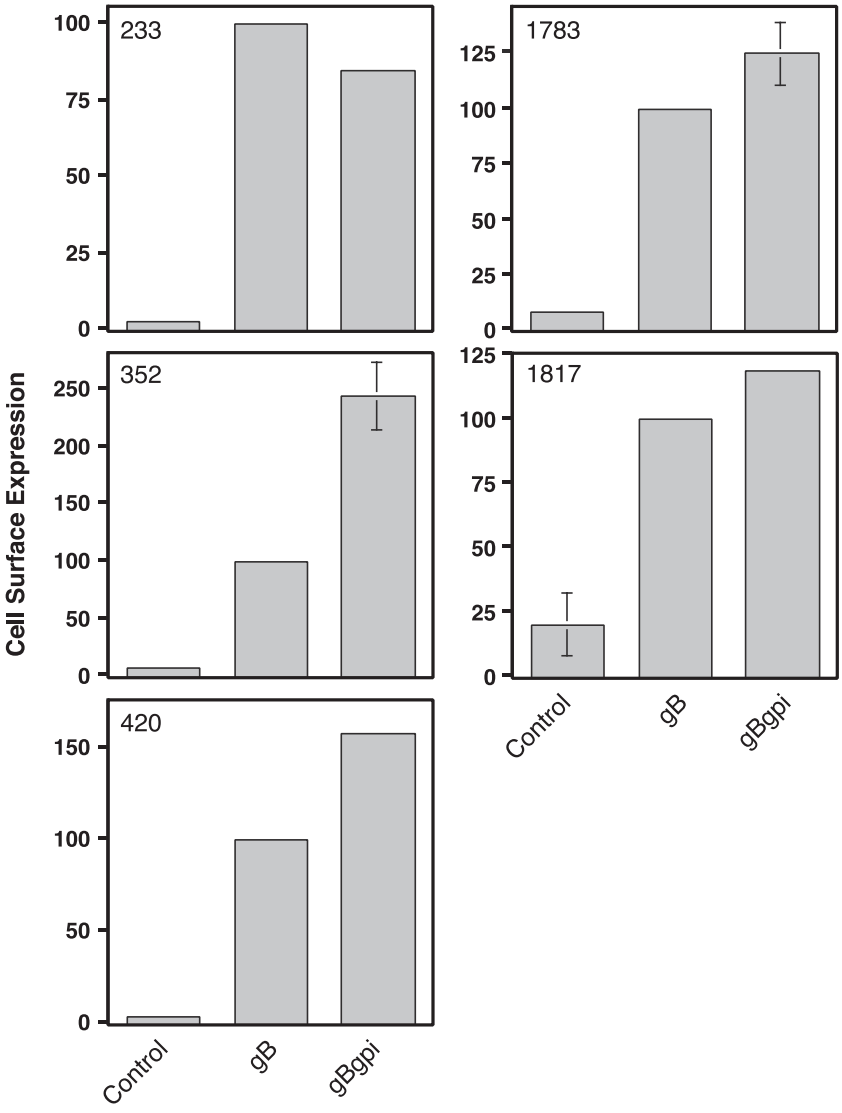


Fig. 7. The binding of gBgpi to conformation-dependent and dimer-dependent antibodies. CHO-K1 cells transfected to express gB, gBgpi, or a control plasmid were incubated separately with five different monoclonal antibodies. The monoclonal antibodies H233, H352, H420, and H1783 are conformation dependent. Antibodies H420 and H1783 bind only to gB dimers. The H1817 monoclonal antibody detects a linear epitope. The experiments were performed three times and the mean values plus standard deviations for the combined results are depicted. Within each experiment with a particular antibody, all values were made relative to the value obtained for the positive control (gB). The actual values for gB expressing cells ranged from 0.12 to 0.36 O.D. 370 nm. The absence of error bars for mean relative values given is due to standard deviations too small to generate visible error bars.

fusion (Fig. 8B). There was no detectable fusion in the experiments with cells expressing gBgpi, gD, gH, and gL or gB, gD, gHgpi, and gL (Figs. 8A and C, respectively) despite approximately equivalent cell-surface expression for gpi-linked mutants and wild-type glycoproteins (Figs. 8A and C).

The gpi-addition sequence in gBgpi was inserted immediately before hydrophobic domain 1 to ensure that gBgpi would not span the membrane (Fig. 1). However, hydrophobic domain 3 is sufficient for stable transmembrane anchoring whereas hydrophobic domains 1 and 2, either alone or together, are not capable of conferring stable membrane association (Rasile et al., 1993). The third hydrophobic domain may be the only membrane-spanning domain and the first two hydrophobic domains could contain sequences important for fusion activity. Thus, we created an additional gBgpi mutant, gBgpi2, where the gpi-addition sequence was placed just before the third hydrophobic domain. The gBgpi2 mutant contained hydrophobic domains 1 and 2, was expressed at the surface of the cell, and bound conformation-dependent antibodies similarly to wild-type gB (data not shown). Further, similarly to gBgpi, gBgpi2 was resistant to PIPLC cleavage and did not function to promote cell fusion (data not shown). The addition of hydrophobic domains 1 and 2 in the gBgpi2 mutant did not confer fusion activity.

The results for the syncytium formation assay are depicted in Fig. 9. B78H1-C10 cells were transfected with

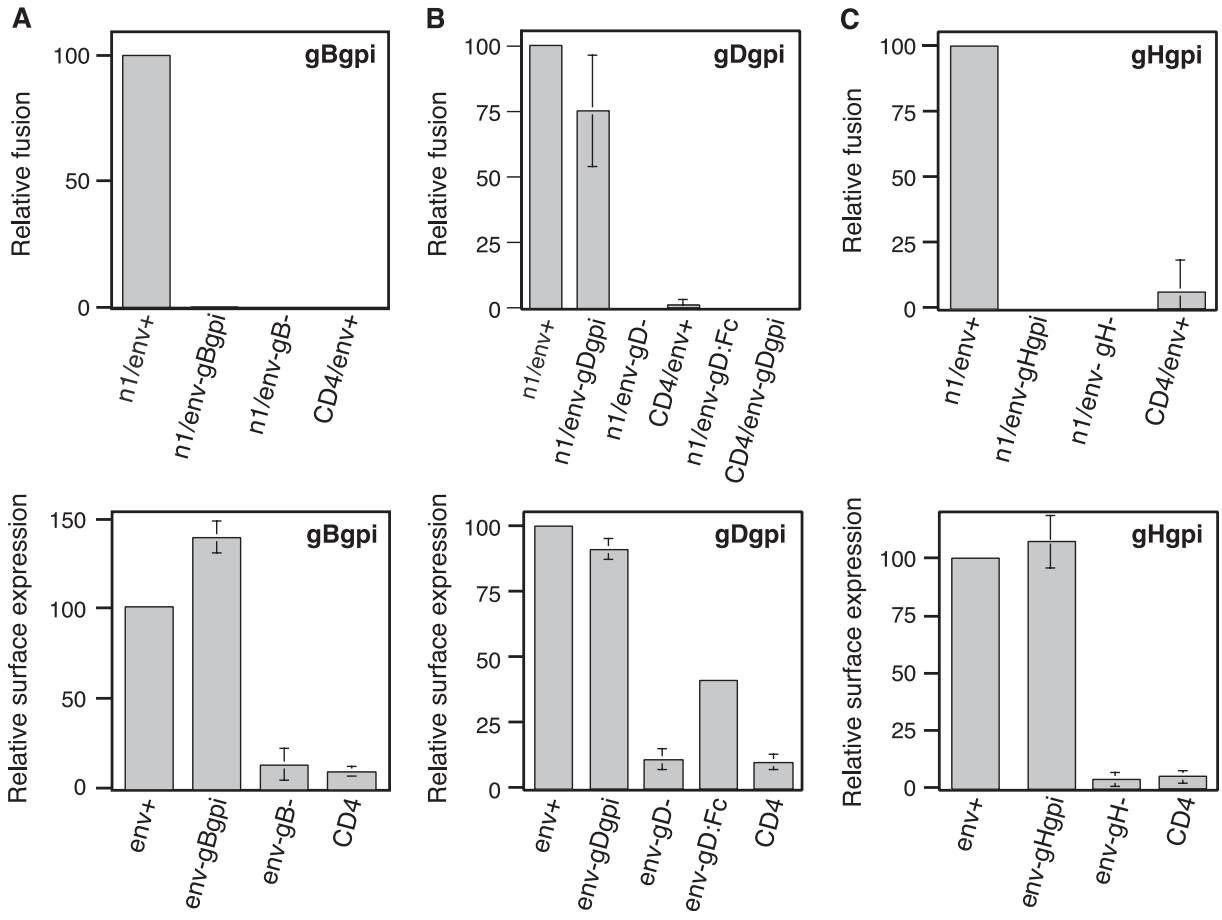


Fig. 8. Fusion activity of gpi-linked mutants in a cell-mixing fusion assay. The top graph shows the results of the fusion assay and the bottom graph represents cell-surface expression of envelope glycoproteins in the fusion assay. The fusion/CELISA experiments were performed at least three times and the mean values plus standard deviations for the combined results are depicted. Within each experiment, all values were made relative to the value obtained for the positive control (n1/env+ for the fusion assay and the env+ for the CELISA). (A) CHO-K1 cells were transfected with plasmids expressing gD, gH, gL, and T7 polymerase, including also a plasmid expressing gB (env+), gBgpi (env-gBgpi), or control plasmid (env-gB$^-$). Those transfected cells were mixed with CHO-K1 cells transfected with pG1NTT7β-gal and a plasmid expressing nectin-1 (n1) or CD4 (CD4). Values obtained for the positive control n1/env+ ranged between 0.225 and 0.447 O.D. 570 nm. Anti-gB monoclonal antibody H1817 was used to measure gB and gBgpi cell-surface expression. The values obtained for the positive control env+ transfection ranged between 0.098 and 0.129 O.D. 370 nm. (B) CHO-K1 cells were transfected as in A except that a plasmid expressing gDgpi (env-gDgpi), a secreted gD:Fc fusion protein (env-gD:Fc), or a control plasmid (env-gD$^-$) was substituted for wild type gD (env+) in the transfections. The values obtained for the positive control n1/env+ ranged 0.339–0.447 O.D. 570 nm. The anti-gD antiserum R7 was used in the CELISA analysis and the values obtained for the positive control env+ transfection ranged 0.222–0.570 O.D. 370 nm. The absence of error bars for relative values given is due to standard deviations too small to generate a visible error bar. (C) CHO-K1 cells were transfected as in A except that a plasmid expressing gHgpi (env-gHgpi) or a control plasmid (env-gH$^-$) was substituted for wild-type gH (env+) in the transfections. The values obtained for the positive control n1/env+ ranged 0.239–0.447 O.D. 570 nm. The anti-gH monoclonal antibody 52S-43 was used in the CELISA analysis and the values obtained for the positive control env+ transfection ranged 0.294–0.362 O.D. 370 nm.

wild-type versions of the envelope glycoproteins or with one of the gpi-linked versions substituted. The ability of each transfected cell population to form syncytia was examined microscopically at 24, 48, and 72 h. Syncytia were readily observed at 24 h for the transfections with plasmids expressing gB, gD, gH, and gL and for the transfections of plasmids expressing gB, gDgpi, gH, and gL (Fig. 9). The syncytia were approximately equal in size and number for the transfections expressing gB, gD, gH, and gL when compared to those expressing gB, gDgpi, gH, and gL at 24 h. Upon further incubation, the syncytia formed by the gB, gD, gH, and gL transfection recruited more cells, fused together with neighboring syncytia, and increased greatly in size. Interestingly, the syncytia formed by the gB, gDgpi, gH, and gL transfection increased in size over time but did not appear to increase in size similarly to the wild-type situation. At the 48- and 72-h time points, the syncytia formed in the transfections with the gDgpi mutant were clearly not as large as those formed in the transfections with the wild-type glycoproteins (Fig. 9). This small-syncytia phenotype in transfections with the gDgpi mutant was not a phenomenon related to the clonal nature of the B78H1-C10 cells because the phenotype was also observed in CHO-K1 cells expressing nectin-1, CHO HveC-1 cells (Geraghty et al., 1998), and an independently isolated B78H1 cell line expressing nectin-1, B78H1 CJ4E

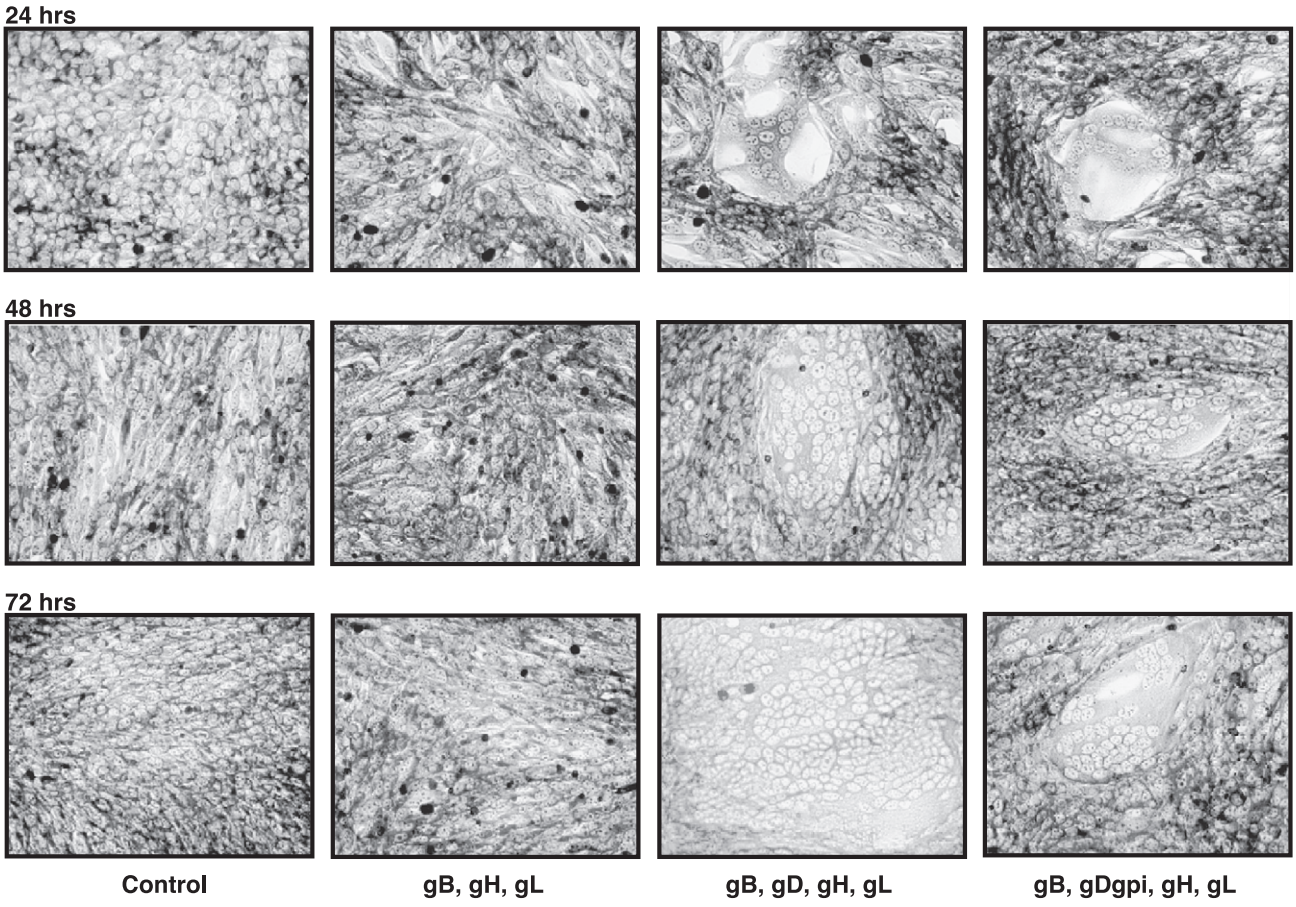


Fig. 9. Syncytium formation using wild-type envelope glycoproteins and gpi-linked mutants. B78H1-C10 cells were transfected with plasmids expressing gB, gD, gH, and gL (gB, gD, gH, gL), plasmids expressing gB, gDgpi, gH, and gL (gB, gDgpi, gH, gL), plasmids expressing gB, gH, gL, and control plasmid pCAGGS (gB, gH, gL), or control plasmid (Control). At 24, 48, or 72 h after transfection, the cells were fixed with methanol and stained with giemsa. Pictures of the cells were taken under identical conditions. Transfections of plasmids expressing gBgpi, gD, gH, and gL or gB, gD, gHgpi, and gL appeared identical to the results with the control plasmid and were not included.

cells (data not shown). The B78H1 CJ4E cells have similar virus entry characteristics to the B78H1-C10 cells (data not shown). Transfections including a gBgpi or gHgpi plasmid and transfections using B78H1 cells that did not express nectin-1 did not produce syncytia (data not shown). In conjunction with the results from the cell-mixing assay, these syncytium formation results demonstrate that the gDgpi mutant promoted cell fusion when co-expressed with gB, gH, and gL. The gBgpi and gHgpi mutants were unable to mediate fusion when co-expressed with gD, gH, and gL or gB, gD, and gL, respectively. Additionally, the syncytia formed using the gDgpi mutant were not as large as those with wild-type gD at time points after 24 h, indicating a subtle difference in syncytium formation activity.

A previously published report failed to detect fusion when a gDgpi mutant was expressed with wild-type gB, gH, and gL in a cell fusion assay (Browne et al., 2003). The assay involved expressing the envelope glycoproteins in 293T cells, overlaying with Vero cells, and counting the resulting syncytia. We performed the 293/Vero fusion assay with our wild-type envelope glycoprotein expression plasmids and the gDgpi mutant. In agreement with the previously published results, and in contrast with our results in Figs. 8 and 9, we were unable to detect the formation of syncytia in the transfections with the gDgpi mutant using the 293/Vero assay (data not shown). We were able to detect syncytium formation when all four wild-type envelope glycoproteins were expressed. The gDgpi mutant was expressed equivalently to wild-type gD in the 293T cells (data not shown). Therefore, there was a cell-line dependence for the detection of fusion involving the gDgpi mutant. One difference among the cell lines in the fusion assay was the level of nectin-1 expression. In flow cytometry experiments using an anti-nectin-1 monoclonal antibody, the mean fluorescence intensity of nectin-1 expression on the cells used in the syncytium assay in this report (B78H1-C10) was 35-fold higher than 293T cells and 15-fold higher than Vero cells (data not shown). Greater nectin-1 expression likely affords greater sensitivity in the fusion assay and could explain our ability to detect fusion mediated by gDgpi in two of the three fusion assays.

## Discussion

The results presented here indicate that HSV-1 gD does not require a transmembrane domain or cytoplasmic tail to mediate cell fusion when co-expressed with gB, gH, and gL. The gDgpi mutant was tethered to the outer leaflet of the plasma membrane, and therefore did not span the membrane, yet contributed to fusion activity. Some association with the plasma membrane was required, however, because a secreted form of gD lacking a transmembrane domain and cytoplasmic tail was unable to mediate fusion when expressed with gB, gH, and gL. The gBgpi and gHgpi mutants did not promote cell fusion indicating that the transmembrane domain and cytoplasmic tail were critical for gB and gH function, which is in agreement with other studies (Baghian et al., 1993; Diakidi-Kosta et al., 2003; Fan et al., 2002; Foster et al., 2001; Gage et al., 1993; Harman et al., 2002).

There are many possible explanations for why the gBgpi and gHgpi mutants did not function in cell fusion. An intriguing possibility is that the transmembrane and cytoplasmic domains of gB and gH are required to provide an anchor for interaction of their extracellular domains with the apposing membrane to form fusion pores and achieve full fusion. Under that hypothesis, the lack of a membrane-spanning domain would render gBgpi and gHgpi nonfunctional for fusion. An alternative explanation is that the mutants did not function because they were improperly folded. Some gpi-anchored proteins differ in folding from their wild-type versions (Maillard and Gaudin, 2002; Zhou et al., 1997), but we do not think the gBgpi or gHgpi proteins were entirely misfolded. The gBgpi mutant was recognized by conformation-dependent and dimer-dependent antibodies, suggesting it was not grossly misfolded. The gHgpi mutant bound a conformation-dependent anti-gH monoclonal antibody, bound gL, and was transported to the cell surface similarly to wild type gH; therefore, it is not obviously misfolded. Analysis of the fusion activity of gH cytoplasmic tail deletion mutants, however, has identified amino acids present in the cytoplasmic tail that are critical for gH to function in fusion (Harman et al., 2002). The lack of fusion observed with the gHgpi mutant could be due, on the whole or in part, to the absence of those amino acids.

It was surprising that gBgpi was not cleavable by PIPLC. Certain cells can modify gpi anchors such that they are not cleavable by PIPLC (Richier et al., 1992; Walter et al., 1990; Wong and Low, 1992). It is unlikely that the gpi anchor for only gBgpi (and not gDgpi and gHgpi) would be modified by CHO-K1 cells to render it resistant to PIPLC. To explore that potential problem, we attempted to cleave the gpi anchor with PIPLD. PIPLD has been used in instances where the gpi anchor has been modified to be resistant to PIPLC cleavage (Deeg and Davitz, 1995). We were unable to remove gBgpi from the cell surface with PIPLD (data not shown), suggesting that modification of the gpi anchor to prevent cleavage by PIPLC is not likely. Another possibility is that the structure of gBgpi prevented access of PIPLC to the gpi anchor. Certain mutants of the prion protein exhibit altered protein structure such that they block PIPLC access and subsequent cleavage of their gpi anchors (Narwa and Harris, 1999). Lastly, gBgpi may stay associated with the plasma membrane after the gpi anchor is cleaved and only appear resistant to PIPLC cleavage.

Although the gDgpi mutant clearly promoted cell fusion when expressed with gB, gH, and gL, there were two subtle differences in cell fusion between the mutant and wild-type glycoproteins. First, we were surprised that the syncytia formed with gDgpi were smaller than those formed with wild-type gD at 48 and 72 h, especially because syncytia formed at 24 h were identical in number and size. The difference in the size of syncytia at the later times may reflect a difference in the expression pattern of gDgpi vs. wild-type gD at the surface of a syncytium. Proteins that are gpi linked often display increased mobility in membranes compared to proteins with transmembrane domains. The gDgpi mutant may diffuse throughout the plasma membrane of a syncytial cell at a rate different from wild-type gD, gB, and gH/gL, and therefore be improperly localized to promote fusion. Such an effect may only be seen once syncytia attain a certain size, hence the initial syncytia are not affected. We do not believe the difference in syncytium formation for gDgpi and wild-type gD is simply the result of less cell-surface expression of gDgpi. We have always observed approximately equivalent expression at the cell surface between gpi-linked mutants and wild-type proteins (Fig. 2). Regardless, our results reveal a difference between the syncytium formation and cell-mixing assays for cell fusion. The cell-mixing assay probably involves the fusion of two or a few cells to generate β-gal activity. The syncytium formation assay clearly requires many cells (and even syncytia) to fuse together. The massive cell fusion required for syncytium formation may involve cytoskeletal or other elements to a greater extent than necessary in the fusion of few cells in the cell-mixing assay.

Secondly, the cell fusion assays performed in this report differed in their abilities to detect fusion involving gDgpi. The gDgpi mutant promoted cell fusion when expressed with gB, gH, and gL in two fusion assays where nectin-1 expression was relatively high (cell mixing, Fig. 8, and syncytium formation, Fig. 9) but no fusion was observed when gDgpi was expressed with gB, gH, and gL in cells with relatively low nectin-1 expression (293/Vero, data not shown). Although there are many potential factors differentially expressed among CHO-K1, B78H1, Vero, and 293 cells, the most relevant difference is likely to be nectin-1 expression. It is unlikely that the observed differences in cell fusion were due to nectin-1 binding because gD and gDgpi bound soluble nectin-1 equivalently (Fig. 5). However, because the CELISA assay used a secreted form of nectin-1 to measure binding, we cannot rule out the possibility that binding to a membrane-bound form of nectin-1 could differ between gD and gDgpi. Alternatively, post-receptor-binding

events necessary for fusion may require the formation of more gDgpi/nectin-1 complexes than are required for fusion with wild-type gD and nectin-1. Under that scenario, cell fusion might not be observed with gDgpi in situations where nectin-1 expression is limited. Lastly, the difference in mobility of gpi-linked proteins when compared to transmembrane proteins, as mentioned above, may make cell fusion less efficient in the relatively low nectin-1 expression system.

Since the gDgpi mutant functioned in fusion, it is unlikely that gD performs a role in fusion identical to type I fusion proteins because gpi-anchored type I fusion proteins do not mediate complete fusion (Kemble et al., 1994; Tong and Compans, 2000; Weiss and White, 1993; Zhou et al., 1997). The receptor binding and membrane fusion functions found in type I fusion proteins are likely spread among two, three, or all four of the HSV-1 envelope glycoproteins required for cell fusion. Our data are consistent with receptor binding as a role for gD in fusion. The binding of gD to receptor probably brings the membranes in close apposition, but gB or gH-gL likely interacts with the opposite membrane to mediate the lipid mixing and full fusion. Upon receptor binding, gD may transmit a signal to gB or gH-gL to adopt a fusion-active conformation and carry out the fusion process. This signal, however, cannot be transmitted through the gD transmembrane domain and cytoplasmic tail because the gDgpi mutant was competent to promote cell fusion.

## Materials and methods

### *Cell lines and antibodies*

CHO-K1 cells were provided by P. Spear (Northwestern University) and were grown in F12 media supplemented with 7% fetal bovine serum and pen/strep. B78H1-C10 cells (provided by P. Spear, Northwestern University) were grown in DMEM supplemented with 7% fetal bovine serum, pen/strep, and 500μg/ml G418. The antibody for nectin-1 was the mouse monoclonal CK6 (Krummenacher et al., 2000). Antibodies to HSV-1 glycoproteins were mouse monoclonal antibodies against HSV-1 gB, H233, H352, H420, and H1783 (Chapsal and Pereira, 1988; Pereira et al., 1990; Qadri et al., 1991) (purchased from Rumbaugh-Goodwin Institute for Cancer Research) as well as H1817 (Chapsal and Pereira, 1988; Pereira et al., 1990; Qadri et al., 1991) (purchased from Advanced Biotechnologies Incorporated #13-120-100); R74, a polyclonal anti-HSV-1 gB rabbit serum (P. Spear, Northwestern University); R7, a polyclonal rabbit anti-HSV-1 gD serum (G. Cohen and R. Eisenberg, University of Pennsylvania); and 52S-43, a mouse monoclonal to HSV-1 gH (P. Spear, Northwestern University). The rat anti-HA high-affinity antibody was purchased from Roche. The biotin- and FITC-conjugated secondary antibodies were α-mouse biotin, α-rabbit biotin, α-mouse FITC, and α-rabbit FITC (Sigma). The anti-rat conjugated to horseradish peroxidase was purchased from Roche.

### *Construction of expression vectors*

Wild-type plasmids expressing HSV-1-gB (pPEP98), HSV-1-gD (pPEP99), HSV-1-gH (pPEP100), and HSV-1-gL (pPEP101) were provided by P. Pertel (Northwestern University) and previously described (Pertel et al., 2001). Other previously described plasmids include the nectin-1 expression plasmid pCJ4 (Geraghty et al., 2000), CD4 expression plasmid pBG53 (Geraghty et al., 2001), T7 RNA polymerase plasmid pT7pol (provided by P. Pertel, Northwestern University) (Pertel et al., 2001), the plasmid expressing β-gal under the control of the T7 promoter, pG1NT7β-gal (provided by E. Berger, National Institutes of Health) (Nussbaum et al., 1994), and the plasmid expressing an HA-epitope tagged gL, pMN116 (provided by P. Spear Northwestern University) (Novotny et al., 1996).

The gpi mutants were created by amplifying the extracellular domain of the glycoprotein, amplifying the gpi-addition sequence of DAF, combining the two purified PCR products, and conducting PCR using the most 5′ and 3′ primers to yield the final full-length product. The gBgpi expression plasmid was constructed by amplifying pPEP98 with the primers HSVB1601 (5′ACGAGCTGACCCTG-TGGAACGAG) and gBgpiC (5′GTCGGCGTGGATGAC-CGTGTC). The plasmid pDAF-12 (provided by J. White, University of Virginia) (Kemble et al., 1993) was amplified with the primers HSVgBgpi (5′GACACGGTCATCCACG-CCGACCCAAATAAAGGAAGTGGAACC) and DAFC (5′CCAACCGAAGGAAAGATG). The two PCR products were gel purified, combined, and amplified with the primers HSVB1601 and DAFC. The final product was digested with restriction enzymes *Bst*EII and *Bgl*II. This product was ligated into pPEP98 digested with *Bst*EII and *Bgl*II. The gDgpi expression plasmid was constructed by amplifying pCJ3 (Geraghty et al., 2000) with the primers CD3prim (Geraghty et al., 2000) and gD1007C1 (5′GTTGTTCGGGGTGGCCGGGGGATG). The plasmid pDAF-12 was amplified using primers gDgpi (5′CCCCGGCCACCCCGAACAACCCAAATAAAGGA-CCCCGGCCACCCCGAACAACCCAAATAAAG-GAAGTGGAACC) and DAFC. The two PCR products were gel purified, combined, and amplified with primers CD3prim and DAFC. The resulting product was ligated into pCAGGS (provided by Y. Kawaoka, University of Wisconsin, Madison) (Kobasa et al., 1997) digested with *Sma*I. The gHgpi expression plasmid was constructed by amplifying pPEP100 with the primers HSVgH5 (5′ATGGGGAATGGTTTATGGTTCGTGG) and gHgpiC (5′GGGCTGCGTGTCAAAGGCTAG). The plasmid pDAF-12 was amplified using primers HSVgHgpi (5′CTAGCCTTTGACACGCAGCCCCCAAATAAAGGA-

CTAGCCTTTGACACGCAGCCCCCAAATAAAG-GAAGTGGAACC) and DAFC. The two PCR products were gel purified, combined, and amplified with primers HSVgH5 and DAFC. The resulting product was digested with *Bst*XI and ligated into pPEP100 digested with *Xho*I, treated with vent polymerase to blunt the *Xho*I overhang, and digested with *Bst*XI. The plasmid pBG37, expressing the secreted form of nectin-1, nectin-1:Fc, was constructed by amplifying the nectin-1α cDNA using the primers Prr113 (5′CGGGATCCGAATTCTGTGATATTGACCTCCACC) and BG1-2a (5′GCTCTAGAATGGCTCGGATGGGGCT-TGCG). The resulting product was digested with *Xba*I and *Bam*HI and ligated into a plasmid containing the rabbit IgG Fc region, pKZ374 (provided by J. Young, The Salk Institute), digested with *Xba*I and *Bam*HI, to form pBG36. The pBG36 was then digested with *Xho*I and *Not*I to remove the entire nectin-1:Fc fusion protein ORF. The nectin-1:Fc ORF was ligated into pcDNA3 digested with *Xho*I and *Not*I to create pBG37. To ensure appropriate construction, all newly created expression plasmids were verified by determining the DNA sequence (Davis Sequencing, Davis, CA).

### *Transfections*

In each well of a six-well plate, approximately 80% confluent CHO-K1 or B78H1-C10 cells were incubated with 1.5 μg of plasmid DNA and 5 μl of LipofectAMINE (GibcoBRL), according to the manufacturer's instructions. The cells were incubated with the transfection reagents for 6–8 h and the transfection media was replaced with F12 or DMEM media/20% fetal bovine serum.

### *CELISA assay*

The transfected cells were replated into 96-well dishes (approximately $4 \times 10^4$ cells per well) at 24 h post transfection. The next day, the cells were incubated with a primary antibody in 50 μl of PBS-ABC (phosphate-buffered saline supplemented with 0.9 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$) containing 3% bovine serum albumin (PBS-ABC/3% BSA). After 30 min at room temperature, the cells were washed three times with PBS-ABC and fixed with 100 μl of 2% formaldehyde–0.2% gluteraldehyde for 10 min at room temperature. The cells were washed three times with PBS-ABC/3% BSA, and incubated with a biotinylated secondary antibody in PBS-ABC/3% BSA for 30 min at room temperature. Following the secondary incubation, the cells were washed four times with PBS and incubated with AMDEX streptavidin-conjugated horseradish peroxidase (Amersham Biosciences) at a 1:15 000 dilution in 100 μl of PBS-ABC/3% BSA/0.1% Tween 20 for 30 min at room temperature. Following the tertiary incubation, the cells were washed four times with PBS/0.1% Tween 20 and incubated with 3,3′,5,5′-tetramethylbenzidine in phosphate-citrate buffer according to the manufacturer's instructions (Sigma). At various times after the addition of substrate, the plates were read at 370 nm in a VersaMax microplate reader (Molecular Devices). The anti-nectin-1 mouse monoclonal CK6 was used at 1:400 dilution; all anti-gB mouse monoclonals at 1:500; anti-gB R74 at 1:1000; anti-gD R7 at 1:2000; and anti-gH monoclonal antibody 52S-43 at 1:500. Biotin-conjugated anti-mouse and biotin-conjugated anti-rabbit secondary antibodies were used at a 1:500 dilution. To aid in quantitation using the CELISA assay, multiple readings of each sample were taken over time to ensure a linear relationship of HRP activity over time. Secondly, each assay was run with a set of six serial dilutions of recombinant HRP (USB) that was used to form a standard curve to ensure our conditions yielded readings in a linear range of HRP activity vs. amount of enzyme.

In the CELISA experiments using PIPLC, the transfected cells were incubated with 100 μl of Opti-MEM (GibcoBRL) or Opti-MEM with 0.2 units per ml PIPLC (Molecular Probes) before primary antibody incubation. After 3 h at 37 °C in a $CO_2$ incubator, the cells were washed three times with PBS-ABC. The primary anti-glycoprotein antibody, secondary antibody, tertiary reagent, and substrate were added as described above.

### *Nectin-1 binding assay*

To produce the nectin-1:Fc protein, CHO-K1 cells were transfected with pBG37. Because the nectin-1:Fc protein consists of the rabbit IgG Fc region replacing the transmembrane domain and cytoplasmic tail of nectin-1, cells expressing the hybrid protein secreted nectin-1:Fc into the culture medium. The cells were incubated in F12 medium containing 5% low-Ig calf serum, and 48 h later the culture supernatant was collected. The culture supernatant was clarified by low-speed centrifugation before use. For the binding assay, CHO-K1 cells were transfected with plasmids expressing gD, gDgpi, or a control plasmid (pCAGGS). After 24 h, the transfected cells were replated into 96-well dishes (approximately $4 \times 10^4$ cells per well). The next day, the cells were incubated with culture supernatants containing the nectin-1:Fc molecule or with control supernatants. After 30 min at room temperature, the cells were washed five times with PBS-ABC, fixed, incubated with secondary anti-rabbit biotin-conjugated antibody, tertiary reagent, and substrate, and analyzed by spectrometry as described above in the CELISA section.

### *Flow cytometry*

Two days post transfection, the cells were incubated with 1 ml of Opti-MEM or Opti-MEM with PIPLC at a concentration of 0.2 units/ml. After 3 h at 37 °C in a $CO_2$ incubator, the cells were washed three times with PBS. To remove the cells from the tissue culture dishes, the cells were rocked in a 37 °C incubator in a solution of PBS/4 mM EDTA. The cells were washed in FACS buffer (PBS/2% heat-inactivated fetal bovine serum) and then incubated for 10 min on ice in 100

μl of primary antibody diluted in FACS buffer. The cells were again washed in FACS buffer and then incubated for 10 min on ice in 100 μl of FITC-conjugated secondary antibody diluted in FACS buffer. The cells were washed in FACS buffer and stored on ice in a solution of FACS buffer/1.25 μg/ml propidium iodide before flow cytometry analysis. The antibodies used included anti-gB R74 at 1:400, anti-gD R7 at 1:400; and anti-gH 52S-43 at 1:150. Both FITC-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies were used at 1:100 dilutions.

*Immunoprecipitations*

CHO-K1 cells were transfected with plasmids expressing gD (pPEP99), gDgpi (pgDgpi), gB (pPEP98), gBgpi (pgBgpi), or control plasmid (pCAGGS). Twenty-four hours after transfection, the cells were labeled with 200 μCi/ml $^{35}$S Translabel (ICN) or 160 μCi/ml $^{3}$H-ethanolamine hydrochloride (Amersham) for 16 h. The cells were lysed on ice for 10 min in 1 ml of ice-cold lysis buffer containing 1% Triton X-100, 10 mM Tris–HCl, pH 7, 145 mM NaCl, 5 mM EDTA, and aprotinin, leupeptin, and pepstatin each at 10 μg/ml. The cell lysates were clarified by centrifugation for 10 min at 4 °C and then precleared with 75 μl of protein A/G (Pierce) for 1 h at 4 °C. At the same time, 75 μl of protein A/G was added to 2 μl of rabbit anti-HSV gD serum R7 or 2μl R74 HSV gB antiserum, in 1 ml of cold PBS and incubated at 4 °C for 1 h. The precleared lysates were incubated with the antibody-protein A/G for 1 h at 4 °C. The immune complexes were collected by centrifugation for 2 min at 4 °C. The complexes were washed five times with lysis buffer, boiled for 4 min in SDS-sample buffer containing 5% β-mercaptoethanol, and separated on a 10% SDS polyacrylamide gel. After electrophoresis, the gel was fixed in 30:10:60 methanol/acetone/water for 15 min. The gel was rinsed with water and soaked in Autofluor (National Diagnostics) for 20 min before being dried down and exposed to film.

*Co-immunoprecipitation/Western blot assay*

CHO-K1 cells were transfected with plasmids expressing gH (pPEP100), gH and gL (pPEP100 and pMN116), gHgpi (pgHgpi), gHgpi and gL (pgHgpi and pMN116), or control plasmid (pCAGGS). The cells were lysed on ice and immunoprecipitated as detailed in the section above. The antibody used for immunoprecipitation was the 52S-43 monoclonal anti-gH antibody (2 μl of ascites fluid). The complexes were boiled and run on a 10% SDS polyacrylamide gel as described in the section above. The gel was transferred to nitrocellulose and the blot was blocked in PBS with 5% dry milk, 2% BSA, and 0.1% Tween 20, and incubated with a rat high-affinity anti-HA antibody (1:2,500 dilution) in blocking buffer overnight at 4 °C. The blot was washed six times in PBS with 0.1% Tween 20, incubated with goat anti-rat IgG conjugated to horseradish peroxidase (1:1000 dilution) in blocking buffer for 1 h at room temperature, washed six times in PBS 0.1% Tween 20, and subjected to chemiluminescence detection according to the manufacturers instructions (Pierce).

*Cell mixing fusion assay*

The assay conditions used were as previously described (Pertel et al., 2001) with a few modifications. CHO-K1 effector cells were transfected with the plasmids expressing the HSV-1 fusion glycoproteins (gB, gD, gH, and gL) and T7 RNA polymerase. Each individual gpi-linked mutant was substituted singly for its wild-type counterpart. Target CHO-K1 cells were transfected with the plasmids expressing nectin-1 (pCJ4) and β-gal under control of the T7 promoter (pG1NT7β-gal). Twenty-four hours later, effector and target cells were mixed in a 1:1 ratio and co-cultivated for 18 h. β-gal activity was quantitated using the substrate CPRG (0.7 mg/ml in PBS with 0.5% NP40) and spectrometry. To aid in quantitation of this assay, multiple readings of each sample were taken to ensure a linear relationship of β-gal activity over time. Secondly, each assay was run with a set of six serial dilutions of recombinant β-gal (Roche) that was used to form a standard curve to ensure our conditions yielded readings in a linear range of β-gal activity vs. amount of enzyme.

*Syncytium formation assay*

B78H1-C10 cells were transfected with plasmids expressing gB (pPEP98), gD (pPEP99), gH (pPEP100), and gL (pPEP101). The gpi-linked versions of the glycoproteins were substituted singly for their wild-type counterparts. At 24, 48, and 72 h, the cells were fixed with methanol and stained with giemsa. The cells were examined on an Axiovert S100 inverted microscope at the same magnification and photographs taken using Axiovision 3 software (Zeiss) at the same exposure.

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