Analysis of the role of the membrane-spanning and cytoplasmic tail domains of herpes simplex virus type 1 glycoprotein D in membrane fusion

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Glycoprotein D (gD) of herpes simplex virus type 1 is a type 1 membrane protein in the virus envelope that binds to receptor molecules on the cell surface and which induces cell–cell fusion when co-expressed with gB, gH and gL. A chimeric gD molecule in which the membrane anchor and cytoplasmic tail domains were replaced with analogous regions from the human CD8 molecule was as competent as wild-type gD at mediating membrane fusion and virus entry. However, when gD was tethered to the membrane by means of a glycosylphosphatidylinositol (gpi)-anchor sequence, which binds only to the outer leaflet of the lipid bilayer, it was unable to function in cell–cell fusion assays. This chimera was incorporated into virions as efficiently as wild-type gD and yet virus particles containing gpi-linked gD entered cells more slowly than virions containing wild-type gD in their envelopes, suggesting that gD must be anchored in both leaflets of a lipid bilayer for it to function in both cell fusion and virus entry.
gpi-anchor sequences are known to associate only with the outer leaflet of the membrane lipid bilayer and a previous study has demonstrated that a gpi-linked HSV gD molecule can be released from the plasma membrane by treatment with phosphatidylinositol-specific phospholipase C (Lisanti et al., 1989). Constructs expressing these chimeric molecules were tested in transient fusion assays, the ability of these proteins to complement a gD-null virus was measured and the rates at which virions containing these modified forms of gD were able to enter cells were determined.

Sequences encoding the TM and cytoplasmic tail regions of the human CD8 molecule, corresponding to the C-terminal 53 residues, were derived from the plasmid pS84 (a gift from S. Munro, LMB, Cambridge, UK) and were fused in-frame to sequences encoding the gD ectodomain (aa 1–340) from HSV-1 strain Patton in the plasmid pCDNA3 (Invitrogen). The resulting construct was called pCDNAgDCD8CD8. The sequence encoding the gpi-anchor domain of decay-accelerating factor (DAF), which is composed of the C-terminal 37 aa residues, was derived from the plasmid gD1-DAF (Zurzolo et al., 1993) and was also fused in-frame with the sequence encoding the ectodomain of gD in a pCDNA3-based vector to generate pCDNA3gDDAF. Replacement of the authentic gD TM domain with that of either CD8 or DAF had no effect on the expression of these chimeric molecules on the plasma membrane of transfected cells (Fig. 1a).

To test whether these molecules could mediate cell fusion, we co-expressed them together with wild-type gHL and gB in 293T cells, as described by Harman et al. (2002), and scored the number of nuclei that were recruited into polykaryocytes after overlaying the transfectants with Vero cells. Under these conditions, no background syncytia were detected in untransfected controls and only polykaryocytes containing more than one nucleus were scored as positive. Table 1(a) shows that while gDCD8CD8 was as efficient as wild-type gD at mediating fusion, the gDDAF molecule was completely non-functional in this assay. These results suggest that, although no specific sequences in the TM or cytoplasmic tail domains of gD are required for fusion to occur, there appears to be an absolute requirement for the molecule to be anchored in both leaflets of the lipid bilayer.

To find whether the modified forms of gD could rescue a gD-null virus, a complementation assay was performed in which cells were transfected with the relevant expression constructs and superinfected with a gD-negative mutant, STZgD− (Rodger et al., 2001). The yield of infectious virions was determined by plaque assay on a gD-expressing helper cell line, gD+ (Rodger et al., 2001). Replacement of the gD TM and cytoplasmic domains with either analogous regions from CD8, or with the gpi-anchor sequence from DAF, had no effect on the ability of these molecules to complement the infectivity of a gD-negative virus. As shown in Table 1(b), both chimeras were as efficient as wild-type gD at rescuing infectivity. However, such rescue assays do not discriminate between the ability of pseudotyped progeny virions to enter cells at equivalent rates to wild-type HSV. As the virions are present for the 48 h duration of a plaque assay and because a gpi-anchored form of gD was non-functional in the fusion assay, it remained a possibility that virions containing this molecule may be less competent
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Table 1. (a) Ability of chimeric gD molecules to mediate cell fusion

<table>
<thead>
<tr>
<th>Construct expressed with gB, gH and gl.</th>
<th>Number of nuclei recruited into syncytia</th>
</tr>
</thead>
<tbody>
<tr>
<td>gD (wild-type)</td>
<td>2984</td>
</tr>
<tr>
<td>gDCD8CD8</td>
<td>3582</td>
</tr>
<tr>
<td>gDDAF</td>
<td>No syncytia detected</td>
</tr>
<tr>
<td>No DNA</td>
<td>No syncytia detected</td>
</tr>
</tbody>
</table>

(b) Complementation of a gD-null virus by gDCD8CD8 and gDDAF

293T cells were transfected with plasmids expressing chimeric gD molecules. After 24 h, cells were infected with 10 p.f.u. per cell of a gD-null virus, STZgD−, and after 1 h adsorption period, residual input inoculum was inactivated with a citrate wash (pH 3). At 24 h post-infection, cells were harvested and sonicated. Yields of infectious virus were determined by plaque assay on gD-positive cell monolayers.

<table>
<thead>
<tr>
<th>Plasmid transfected</th>
<th>Titre of progeny virus (p.f.u. ml−1)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDNA3 gD</td>
<td>1.9 × 10⁶</td>
</tr>
<tr>
<td>pCDNA3 gDCD8CD8</td>
<td>5.0 × 10⁶</td>
</tr>
<tr>
<td>pCDNA3 gDDAF</td>
<td>1.6 × 10⁶</td>
</tr>
<tr>
<td>No plasmid</td>
<td>3.6 × 10⁴</td>
</tr>
</tbody>
</table>

*These values are from duplicate transfection experiments.

than particles containing wild-type gD at entering cells. Therefore, we measured the rates at which the progeny virions from complementation experiments entered cells over a time-period of 90 min (Fig. 1b). In agreement with the results of cell fusion assays, a gD molecule containing the CD8 TM and cytoplasmic tail domains conferred entry kinetics that were equivalent to those mediated by the wild-type molecule, while virions containing gDDAF entered cells more slowly. These data confirm that there is a correlation between the phenotypes of mutated glycoprotein molecules in transient cell fusion assays and their ability to mediate virus entry (as has been reported previously by Harman et al., 2002), and that there are no requirements for specific sequences in the TM domain and tail of gD for either process to take place. They also imply that receptor binding by gD is not the only event required for efficient fusion to occur, as both chimeric molecules contain the receptor-binding regions of gD and yet the gDDAF molecule is compromised in its ability to mediate fusion.

However, a further possible interpretation of these findings is that pseudotyped virions containing gpi-linked gD enter cells more slowly because they incorporate less gD into their envelopes. To address this issue, we constructed a recombinant virus expressing gpi-linked gD so that we could determine whether purified virions contained wild-type amounts of the gDDAF molecule.

The open reading frame encoding gDDAF was isolated from plasmid pCDNA3gDDAF as a HindIII–XbaI fragment and ligated into the vector pINGHinc11gD (Whiteley et al., 1999). This contains HSV-1 sequences flanking the gD gene from nt 136 449 to 140 555, according to the numbering of nucleotides in the HSV-1 genome (Mceoch et al., 1988); the resulting construct was called pINGHincGdgpi. BHK cells were co-transfected, using the method of Chen & Okayama (1987), with pINGHincGdgpi and with DNA extracted from cells infected with a gD-negative derivative of HSV-1 strain SC16, ScgDdelZ (Whiteley et al., 1999). At 5 days after transfection, small plaques were observed and these were harvested and used to grow stocks of the recombinant virus, ScgDgpi. Stocks of this virus were assayed on a gD-helper cell line and extracellular virions were purified from infected BHK cells on Ficoll gradients, as described by Rodger et al. (2001). Numbers of virus particles were estimated by comparison with latex particles of known concentrations using negatively stained preparations, as described by Watson et al. (1963). The particle to infectivity ratio of ScgDgpi was estimated to be approximately 1000 : 1, whereas a preparation of purified wild-type SC16 virions had a ratio of 40 : 1.

The gD content of these virions was compared with that of wild-type virions by Western blotting serial twofold dilutions of equivalent numbers of virus particles using an anti-gD monoclonal antibody, LP14 (Minson et al., 1986). Parallel samples were immunoblotted with an antibody that recognizes the tegument protein VP16 as an internal control for loading equivalence; results are shown in Fig. 2. It is notable that the electrophoretic characteristics of gpi-linked gD are slightly different from the wild-type molecule and it appeared to migrate as a more diffuse species. The amounts of gD and VP16 in both wild-type and ScgDgpi virions were, therefore, semi-quantified by densitometric analysis of the Western blot using a Chemilmager 4000 (Flowgen). These data, which are also presented in Fig. 2, show that there are no significant differences between the amounts of gpi-linked gD and the amounts of wild-type gD that are incorporated into the virion envelope during assembly.

The finding that a gpi-anchored gD molecule is compromised in cell fusion and virus entry is similar to observations on influenza virus haemagglutinin (HA) and human immunodeficiency virus type 1 (HIV-1) gp160, both of which are unable to mediate fusion when anchored to the membrane by gpi sequences (Kemble et al., 1994; Weiss & White, 1993) and which can only induce a hemifusion state. Although, it is perhaps unwise to draw too close a parallel between HIV and influenza virus-mediated fusion and that induced by HSV-1, because the latter requires a combination of four proteins (gB, gD and gHL), it remains a possibility that gB, gHL and gDDAF cause hemifusion efficiently but that completion of fusion is slow.
This is not the first report of a gpi-anchored alphaherpesvirus gD molecule: Liang et al. (1995) produced a stably transfected cell line that expressed a gpi-anchored form of bovine herpes virus type 1 (BHV-1) gD. In agreement with our findings, they noted that gpi-anchored BHV-1 gD rescued the infectivity of a gD-negative virus. They also reported that a gD-null virus could enter cells expressing this hybrid molecule, implying that gDDAF, unlike wild-type gD, could act in trans to mediate the entry of virions lacking gD. However, these studies did not address the issue of whether gpi-anchored BHV-1 gD was less efficient than wild-type gD at mediating cell–cell fusion nor whether virions containing this chimera could enter cells at rates similar to those with wild-type gD in their envelopes; whether this is a common property of alphaherpesvirus gD molecules is, at present, unclear.

A further possible implication of the observations described in this report concerns the mechanism by which HSV glycoproteins assemble into virions during morphogenesis. It is believed that the virus acquires its envelope glycoproteins at a post-endoplasmic reticulum compartment (either the trans-Golgi network or an early endosome) (Enquist et al., 1998; Harley et al., 2001; Skepper et al., 2001; Whiteley et al., 1999), although the signals that direct incorporation of at least ten membrane proteins into the particle are not well understood. The results presented here show that gD assembles efficiently into virions in the absence of its authentic TM and cytoplasmic tail domains and that it is also incorporated when anchored in membranes by a gpi sequence. The gpi-anchor region of DAF has been shown to target the ectodomains of heterologous molecules to membrane microdomains, known as lipid rafts, which are enriched in cholesterol and sphingolipids (Friedrichson & Kurzchalia, 1998). And, as a gDDAF hybrid molecule is incorporated into virus particles, it is possible that such membrane microdomains may represent a site of HSV-1 glycoprotein accumulation during budding, as has been shown to be the case for the envelope proteins of measles and influenza viruses (Manie et al., 2000; Scheiffele et al., 1999).

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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