Characterization of herpes simplex virus type 1 recombinants with mutations in the cytoplasmic tail of glycoprotein H

H. M. Browne, B. C. Bruun and A. C. Minson

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

Herpes simplex virus (HSV) type 1 glycoprotein H is essential for fusion of virus envelopes with cellular membranes and for the fusion of an infected cell membrane with an uninfected neighbour. Previous studies have pointed to a requirement for certain amino acid residues of the cytoplasmic tail of gH in these processes. Results from transient transfection experiments suggested that the serine–valine–proline (SVP) motif in the cytoplasmic tail may be important for gH-mediated fusion. HSV recombinants expressing gH molecules with mutations in the cytoplasmic tail were constructed and analysed in terms of their abilities to fuse cellular membranes and to function in virus entry. Viruses containing a deletion of the SVP motif, or in which the valine residue of this triplet was replaced by alanine, entered cells less efficiently than wild-type virus and were unable to induce syncytium formation on Vero cells.

Infection of cells by herpes simplex virus (HSV) involves two distinct fusion processes; virus entry is believed to occur following the fusion of the virus envelope with the plasma membrane or an early endosome, while transfer of virus from an infected cell to an uninfected neighbour involves the fusion of adjacent cellular membranes containing virus glycoproteins. This process of cell-to-cell spread is normally a tightly regulated fusion event, but syncytial strains of HSV-1 induce extensive cell–cell fusion, presumably due to loss of control of this step. Such strains provide a useful model system for studying cell–cell fusion mechanisms. Both fusion of the virion envelope with the plasma membrane and fusion of an infected cell with its neighbour are mediated by virus-encoded membrane proteins, some of which are involved in both forms of fusion and others which are required only for cell-to-cell spread. This implies that although the two processes are in some ways analogous, there are likely to be differences in the precise molecular interactions involved. Glycoproteins B, D and the gH–gL complex are essential for both types of fusion, since they are essential for virus entry (Cai et al., 1988; Desai et al., 1988; Forrester et al., 1992; Ligas & Johnson, 1988; Roop et al., 1993) and deletion of these glycoprotein genes on a syncytial genetic background abolishes cell–cell fusion (Cai et al., 1988; Davis-Poynter et al., 1994; Ligas & Johnson, 1988). Glycoproteins E, I and M, however, are dispensable for virus entry yet are required for efficient polykaryocyte formation, at least in certain syncytial strains (Davis-Poynter et al., 1994).

Although gH is known to be essential for both entry and spread, it is as yet unclear how this molecule mediates these fusion events and it remains to be defined whether its function in the two processes is the same. Glycoprotein H is a 110 kDa glycoprotein, conserved in all herpesviruses, and is predicted to span the lipid bilayer by means of a single hydrophobic stretch of amino acids close to the C terminus (McGeoch & Davison, 1986). The external domain of the molecule is associated with the 40 kDa non-membrane-anchored gL (Hutchinson et al., 1992). The C-terminal tail of HSV-1 gH is composed of 14 amino acid residues, KVLRTSVPFFWRRE, which project either into the cytoplasm or towards the virus capsid. It has been reported (Wilson et al., 1994) that certain mutations in this C-terminal tail abolish the ability of a syncytial strain of HSV-1 to induce polykaryocyte formation, yet have no significant effect on the rate at which mutant gH-containing envelopes fuse with cellular membranes. These studies, based on a COS7 cell transient transfection and complementation system concluded that the SVP motif, and in particular the valine residue of the cytoplasmic tail of gH, are essential requirements for mediating syncytium formation and yet are not important for the fusion event which permits virus entry. The authors proposed that the two fusion processes require gH to act in different ways (Wilson et al., 1994).

Since the amounts of infectious virus produced in transient transfection systems are low and often not readily amenable to quantitative analysis, and since the levels of gH expressed in COS7 cells may not accurately reflect the amounts made during virus infection, we have extended these studies by transferring gH genes encoding mutations in the cytoplasmic tail into a gH-negative syncytial virus. We have examined the growth properties of these recombinants, their ability to

Author for correspondence: H. M. Browne.
Fax +44 1223 336 926. e-mail hb100@mole.bio.cam.ac.uk
induce polykaryocyte formation, their ability to penetrate cellular membranes and the amount of gH expressed in recombinant virus-infected cells and virions.

The wild-type and mutated gH cytoplasmic tail sequences that were introduced into recombinant viruses are listed below:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>CT4</th>
<th>CT5</th>
<th>CT7</th>
<th>CT8</th>
<th>CT9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>KVLRTSVPFFWRRE</td>
<td>KVLRTSVP</td>
<td>KVLRT</td>
<td>KVLRTSVA</td>
<td>KVLRTSAP</td>
</tr>
<tr>
<td>CT4</td>
<td>KVLRTSVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT5</td>
<td>KVLRT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT7</td>
<td>KVLRTSVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT8</td>
<td>KVLRTSAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT9</td>
<td>KVLRTAVP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plasmids encoding these molecules were all generated by mutagenic protocols described in Wilson et al. (1994). A wild-type gH gene and mutated genes were transferred from the COS7 cell expression vector, pSMH3, into a shuttle vector, pIMB52 (Browne et al., 1996), which contains HSV-1 sequences that flank the gH gene and a HpaI restriction site in place of the gH coding region. These clones were designated pIMPLWT and pIMPLCT4, CT5, CT7, CT8 and CT9 according to the sequence of the gH tail which they encode. These plasmids were cotransfected with DNA extracted from cells infected with a gH-negative virus, SZgH- pABang (Browne et al., 1996).

This virus is derived from HSV-1 strain SC16, lacks the entire gH coding region which has been replaced with a human cytomegalovirus immediate early promoter–lacZ cassette, and contains a syncytial mutation in the gB gene corresponding to that found in the Ang strain of HSV-1 (Balan et al., 1994). Ten μg SZgH- pABang DNA and 2.5 μg of pIMPLCT DNA were cotransfected according to the modified calcium phosphate precipitation protocol of Chen & Okayama (1987). Cotransfection progeny were screened for the presence of recombinant viruses by plating onto the gH-expressing cell line CR1 (Boursnell et al., 1996) in the presence of X-Gal. White-staining plaques were picked and cloned by limiting dilution. The CR1 cell line contains the gH coding sequence under the HSV-1 gD promoter and hence only expresses gH following infection with HSV. The region encoding the cytoplasmic tail of gH in each recombinant was amplified from infected cell DNA by PCR and confirmed by nucleotide sequencing. These mutant viruses were called CT4, CT5, CT7, CT8 and CT9 according to the designations of the mutated gH sequences. The transient transfection experiments described by Wilson et al. (1994) showed that deletion of the cytoplasmic tail of gH as far as the proline residue (CT4) had no effect on syncytium production, whereas a plasmid encoding a gH molecule with only the
KVLRT residues of the cytoplasmic tail (CT5) was unable to complement a gH-negative syncytial virus in a polykaryocyte-forming assay. The other three mutants which were included in this analysis, CT7, CT8 and CT9 contained alanine substitutions of each of the residues of the SVP motif and, of these, the SAP-containing sequence (CT8) was defective in supporting syncytium formation. The fusion phenotypes of the recombinant viruses containing these mutated gH genes were examined on Vero cell monolayers at high and low m.o.i. Vero cells infected at 10 p.f.u./cell with CT4, CT7 or CT9 recombinants showed an equivalent extent of syncytium formation to that seen in wild-type infections when examined 10 h after infection (data not shown). However, as shown in Fig. 1(a), mutant CT5 (in which the SVP motif is deleted) and CT8 (in which the valine is mutated to alanine) failed to induce polykaryocyte formation following high multiplicity infection. Plaques produced on Vero cells by CT4, CT7 and CT9 recombinants were indistinguishable in terms of size and syncytial phenotype from those produced by wild-type virus.

CT5 and CT8 viruses formed very small, non-syncytial plaques on Vero monolayers, with those formed by CT5 being almost undetectable by eye (Fig. 1b), yet both recombinants produced normal-sized plaques on the gH-expressing cell line CR1. These data, which point to a role for the SVP motif and in particular the central valine residue of gH in the fusion of cellular membranes during plaque formation, are entirely consistent with the results of Wilson et al. (1994) and support the idea that the cytoplasmic tail of gH is in some way important for the role of gH in mediating cell-cell fusion.

To examine the effects of these mutations on the fusion of virus envelopes with cellular membranes we measured the rates of penetration of CT5 and CT8 virions compared to virions containing wild-type gH. Approximately 250 p.f.u. of virus were adsorbed to CR1 cells which had been pre-chilled for 1 h at 4 °C. After 1 h, inoculum was removed, monolayers were washed twice and incubated at 37 °C. At various times after transferring the cells to 37 °C, unpenetrated virions were inactivated by an acid wash (135 mM-NaCl, 10 mM-KCl, 40 mM-citric acid pH 3) and returned to neutral pH by washing with PBS and PBS–MEM. Plaques were counted after 2 days and all the data points shown in Fig. 2 represent the mean values of duplicate samples. Fig. 2 shows that CT5 and CT8 virions penetrate cells less efficiently than virions containing wild-type gH molecules, implying that the SVP motif is important for gH function in mediating virus entry as well as being required for its role in cell–cell fusion. This conclusion differs from that reached by Wilson et al. (1994) who reported that CT5 and CT8 virions derived from a COS7–cell based transfection system were as competent as wild-type virions in their ability to penetrate cellular membranes.

It is not clear why amino acid residues in the cytoplasmic tail of gH are essential for efficient membrane fusion. One possibility is that the CT5 and CT8 mutations affect the stability of gH or its transport to the cell surface, and that the fusion phenotypes of these viruses reflect quantitative differences in amounts of gH present in cellular and viral membranes. We do not think this is the case, since gH was detected with equivalent efficiency on the cell surface of recombinant virus-infected cells by immunostaining with a pool of monoclonal antibodies directed to gH (Fig. 3a) and all recombinant virus-infected cells expressed wild-type levels of gH polypeptide (Fig. 3b). Virions containing the CT5 and CT8 forms of gH also appeared to contain wild-type amounts of gH polypeptide (Fig. 3c), as measured by Western blotting, implying that their entry deficiencies cannot be ascribed to quantitative differences in gH content. We accept, however, the limitations of both immunoblotting and immunofluorescence in detecting very small differences in the amounts of gH expressed by these viruses and cannot rule out the possibility that such minimal quantitative differences may lead to the fusion phenotypes observed in this study. The role of the cytoplasmic tail of gH during fusion remains to be defined, and it will be important to establish whether this short stretch
Fig. 3. (a) Cell surface expression of gH in recombinant-infected cells. Vero cells were infected for 18 h at 10 p.f.u./cell with WT, CT4, CT5, CT7, CT8 and CT9 recombinant viruses. Infected cells were fixed and analysed for gH expression by immunofluorescent staining with a pool of monoclonal antibodies to gH (LP11, 52S and 53S) using protocols described by Hutchinson et al. (1992). (b) Expression of gH polypeptide in recombinant-infected cells. Vero cells were infected for 18 h at 10 p.f.u./cell with WT, CT5 and CT8 recombinants. Lysates corresponding to 10^6 infected cells were electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose filters. gH polypeptides were detected by incubating Western blots with rabbit anti-gH polyclonal serum using methods previously described by Wilson et al. (1994). (c) Western blots of gH in CT5, CT8 and WT virions. Virions were pelleted at 40000 g for 1 h from infected cell supernatants after removing cellular debris by a low-speed spin (5000 g for 10 min). Samples of virions containing 10^{10} enveloped virions were subjected to PAGE and immunoblotting with polyclonal antiserum raised against gH.
of 14 amino acids is important for interactions between gH and other components of the fusion machinery such as other virion or cellular proteins or lipids. Although CT5 and CT8 show identical single-cycle growth kinetics to wild-type virus in BHK cells (data not shown), their defective fusion phenotypes in Vero cells and decreased rates of entry suggest that they may also show reduced pathogenesis in vivo.

This is not the first report of mutations in the cytoplasmic tail of a HSV-1 glycoprotein which affect membrane fusion and virus penetration; Gage et al. (1993) identified two discrete regions in the cytoplasmic tail of HSV-1 glycoprotein B, both of which were important for virus entry and syncytium production.

Although the data reported here support the conclusions derived from transient transfection experiments that the SVP motif in the cytoplasmic tail of gH is important for syncytium formation, we reach a different conclusion regarding its role in virus penetration when mutant forms of gH are expressed in recombinant viruses. These differences may reflect quantitative or temporal differences between the gH synthesized in transfected COS7 cells and the gH expressed during HSV-1 infection, and highlight both the usefulness and the limitations of transient assay systems for investigating the functions of HSV-1 glycoproteins.

This work was supported by the Wellcome Trust UK.

References


Received 29 April 1996; Accepted 24 June 1996