Complementation of a gI-deficient feline herpesvirus recombinant by allotopic expression of truncated gI derivatives

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The alphaherpesvirus glycoproteins gE and gI form a hetero-oligomeric complex involved in cell-to-cell transmission. The gI-deficient recombinant feline herpesvirus (FHV), FHV ΔgI-LZ, produces plaques that are only 15% the size of those of wild-type FHV. Here, we have complemented FHV ΔgI-LZ allotopically by expressing intact gI and C-terminally truncated gI derivatives from the thymidine kinase locus. The effect on gE–gI-mediated cell-to-cell spread was assessed by plaque assay employing computer-assisted image analysis (software available at http://www.androclus.vet.uu.nl/spotter/spotter.htm). Allotopic complementation with intact gI fully restored plaque size. Deletion of the C-terminal 11 residues of gI did not affect cell-to-cell spread, whereas deletion of the complete cytoplasmic tail reduced plaque size by only 35%. Mutants expressing gI166, roughly corresponding to the N-terminal half of the ectodomain, displayed a small-plaque phenotype. Nevertheless, their plaques were reproducibly larger than those of matched gI-deficient controls, indicating that the gE–gI166 hetero-oligomer, though crippled, is still able to mediate cell-to-cell spread. Our data demonstrate that plaque analysis provides a reliable and convenient tool to measure and quantitate gE–gI function in vitro.

Alphaherpesvirus entry is a process involving various envelope glycoproteins, such as gC, gB, the gH–gL hetero-oligomer and, in most cases, gD (Mettenleiter, 1994; Spear, 1993). Virions attach to the host cell through the sequential interaction of these glycoproteins with low and high affinity cell surface receptors. This ultimately results in the pH-independent fusion of the viral envelope and the plasma membrane and thus in the release of the nucleocapsid into the cytoplasm. There are indications of an alternative infection route via which virus can be transmitted directly from the infected cell to adjacent non-infected cells across cellular junctions. Whereas extracellular virus is readily accessible to neutralizing antibodies, cell-to-cell spread entails transfer of infectivity in a manner resistant to neutralization, both in vitro (Dingwell et al., 1994; Rebordosa et al., 1996; Zsak et al., 1992) and in vivo (Zsak et al., 1992). Cell-to-cell spread and the entry of extracellular virions seem to be related phenomena (both apparently rely on the presence of gB and gH–gL), yet they differ in a number of respects. For instance, in pseudorabies virus (PRV) and bovine herpesvirus (BHV), glycoprotein gD is essential for entry but dispensable for cell-to-cell spread (Liang et al., 1995; Peeters et al., 1992; Rauh & Mettenleiter, 1991). Conversely, the class I membrane glycoproteins gE and gI do not play a role in virus entry but are thought to be crucially involved in cell–cell transmission. For most alphaherpesviruses, including feline herpesvirus (FHV) (Mijnes & de Groot, unpublished), the loss of gE and/or gI has only a subtle effect on propagation in cultured cells. Attachment and entry are not affected and, under one-step growth conditions, the deletion mutants replicate at similar rates and to similar titres of extracellular infectivity to the wild-type virus. However, the mutants do commonly display a small-plaque phenotype (Balan et al., 1994; Dingwell et al., 1994; Rebordosa et al., 1996; Yoshitake et al., 1997; Zsak et al., 1992). gE and gI are important virulence factors. Viruses deficient in either glycoprotein are attenuated and their spread in the mucosa and in neuronal tissues is impaired (for references see Mijnes et al., 1997).

The mechanism of cell-to-cell spread is not understood at the molecular level. To gain more insight into the function of gE and gI, we studied their biosynthesis using FHV as a model. Like their homologues in other alphaherpesviruses, FHV gE and gI
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Fig. 1. (a) Schematic representation of the genomic structure of FHV-TK−/T7+/gIwt, FHV-TK−/T7+/gI− and the FHV∆gI-LZ derivatives allotopically expressing truncated gI proteins. The genes for UL24, thymidine kinase (TK), UL22, gD, gI, gE, T7 RNA polymerase (T7 RNA POL) and β-galactosidase (LacZ) are depicted as open boxes. The hatched boxes represent the cytomegalovirus immediately early promoter (CMV), the simian virus 40 polyadenylation signal (SV40) and the internal ribosomal entry site of encephalomyocarditis virus (IRES). Arrows indicate the direction of transcription. P, H, X and B indicate PmeI, HindIII, XhoI and BamHI restriction sites, respectively. (b) Expression of truncated gI proteins in FHV-infected cells. Monolayers of CRFK cells were infected with either FHV-TK−/gI+ (gI+), -gI∆Xb (∆Xb), -gI∆B (∆B), -gI∆M (∆M), -gI-152 (152) or FHV∆gI-LZ (∆gI). The cells were metabolically labelled from 7 to 8 h p.i. and lysed. Products precipitated by the Rα-gI serum were analysed in SDS–15% polyacrylamide gels. Differentially N-glycosylated forms of gI166 and gI152 are indicated by dots (see also Mijnes et al., 1997). (c) Maturation of gE. CRFK cells were infected with either wild-type FHV strain B927 (wt)
and gI assemble into a noncovalently linked hetero-oligomeric complex – presumably a dimer – shortly after their synthesis in the endoplasmic reticulum (ER) (Johnson & Feenstra, 1987; Kimura et al., 1997; Mijnes et al., 1996; Whealy et al., 1993; Whitbeck et al., 1996; Yao et al., 1993; Zuckermann et al., 1988). In fact, complex formation with gI is an absolute requirement for FHV gE to be released from the ER and to be transported along the exocytotic pathway. A gI derivative, gl166, truncated at Arg-166 and roughly corresponding to the N-terminal half of the ectodomain, can still form a transport-competent complex with gE. In contrast, gl152, only 14 residues shorter than gl166, binds to gE, but the resulting hetero-oligomeric complex is retained in the ER (Mijnes et al., 1997).

Consistent with observations made for other alphaherpesviruses (Balan et al., 1994; Dingwell et al., 1994; Mallory et al., 1997), the disruption of the FHV gI gene results in a small-plaque phenotype. The recombinant virus FHVΔgI-LZ, in which the gI gene (U7) was disrupted by marker insertion, produces plaques that, on average, are only 15% of the size of those of wild-type FHV (Mijnes et al., 1997). A revertant with a restored gI locus produced normal plaques, whereas two other FHVΔgI-LZ derivatives, expressing a tailless gl, gl169, and gl166 from the autologous U7 locus, produced plaques of intermediate size, i.e. 65% and 25% of the size of those of wild-type FHV, respectively. Intriguingly, the plaques of the gl166-expressing virus, though small, were still consistently larger than those of the parental FHVΔgI-LZ (Mijnes et al., 1997). These findings could be interpreted as indicating that a complex of gE and gl166 retains partial biological activity. However, it remained to be proven whether plaque size is a reliable measure for gE–gI function. One important caveat is that, in FHVΔgI-LZ, the disruption of U7 and the insertion of a lacZ expression cassette may not only have resulted in loss of gI but may also have affected the expression of surrounding genes. For example, FHV U6g, encoding the presumptive essential protein gD, is expressed through a structurally polycistronic mRNA which terminates downstream of the gI gene (Willemsen et al., 1995). Down-regulation of gD expression could well contribute to the small-plaque phenotype of FHVΔgI-LZ. Even partial restoration of the mutagenized locus, e.g. the exchange of the marker sequence for a truncated gI gene, could alleviate ‘nearest-neighbour effects’. Another concern was the possible occurrence of second-site revertants. Growth defects due to the loss or inactivation of a gene may be compensated for by mutations elsewhere in the viral genome (Cassady et al., 1998; Mohr & Gluzman, 1996). For example, gD-deficient mutants of PRV and BHV, upon propagation in cultured cells, gave rise to viruses that no longer required gD for entry via the extracellular route (Schmidt et al., 1997; Schröder et al., 1997). Similarly, during the isolation and propagation of recombinants expressing altered gI genes, mutant viruses harbouring second-site mutations that increase plaque size may have been selected for inadvertently. The obvious strategy against such mishaps is to compare the phenotypes of multiple, independently generated recombinant viruses.

Here, to validate and to extend our previous observations (Mijnes et al., 1997), we complemented gl-deficient recombinant FHVΔgI-LZ by inserting (truncated) gI genes not into the autologous locus but into a different genomic location. To this end, a set of FHVΔgI-LZ derivatives was constructed in which residues 353 to 696 of the TK (U23 TK; Nunberg et al., 1989) locus were substituted for by a gl expression cassette. In each case, the (truncated) gI gene was under the control of the immediate early cytomegalovirus promoter and provided with simian virus 40-derived intron and polyadenylation sequences (Fig. 1a). The recombinant viruses were generated as described by Nunberg et al. (1989). CRFK cells were co-transfected with genomic DNA of FHVΔgI-LZ and the appropriate transfer vectors, followed by selection of TK-deficient virus progeny using 100 µg thymidine 1-β-D-arabinofuranoside (AraT; Sigma)/ml culture medium. The recombinant viruses were plaque-purified three times prior to the preparation of high-titrated stocks. In each case, proper insertion of the expression cassette was confirmed by Southern blots analysis (not shown; for a more detailed description of the construction of each mutant virus, see Mijnes, 1999).

FHV-TK−/gl+ carries an intact gl I gene, whereas FHV-TK−/glAXb and FHV-TK−/glAB encode truncated gl proteins lacking the C-terminal 11 (gl137) and 75 (gl169) residues, respectively. FHV-TK−/gIAM #1 and #2 are independently generated recombinant viruses – i.e. originating from two separate transfection/recombination experiments – encoding gl proteins truncated at Arg-166, while the independently generated recombinants FHV-TK−/gl-152 #1 and #2 code for gl proteins truncated at Asp-152. To serve as a negative control, we constructed an FHVΔgI-LZ derivative, FHV-TK−/T7+/gl− expressing an irrelevant gene – the bacteriophage T7 RNA polymerase gene – from the U1 locus. In addition a related virus, FHV-TK−/T7+/gl166, was created with a similarly disrupted TK gene but with an intact U7 gI locus (Fig. 1a).

To test for proper expression of the gl derivatives and for maturation of gE, CRFK cells were infected at an m.o.i. of 10 p.f.u. per cell and metabolic labelling was performed from 7 to 8 h.p.i. Cells were harvested either immediately or after a 2 h chase and cell lysates were subjected to RIPA using the gI- and gE-specific antisera as described previously (Mijnes et al., 1997). As shown in Fig. 1(b), all gl derivatives were expressed.
Their expression levels, however, were increased approximately fivefold as compared to that of autologous gI (not shown). For FHV-TK−/gIΔXb and FHV-TK−/gIAB, the Ra-gI serum precipitated EndoH-sensitive products of 65 and 59 kDa, closely corresponding to the anticipated sizes. For gI166 and gI152, multiple bands were precipitated, most likely representing differentially glycosylated species; gI166 and gI152 each contain three N-linked glycosylation sites. Differential usage of these sites was also observed upon heterologous expression of these gI derivatives and in cells infected with recombinant FHV-gIΔAM (Mijnes et al., 1997). With the exception of gI152, all gI derivatives induced maturation of gE as evidenced by the conversion of the EndoH-sensitive 83 kDa form of gE into the EndoH-resistant 95 kDa species (Fig. 1c). In fact, gE maturation was more efficient than in cells infected with wild-type FHV, presumably as a result of the overexpression of the gI derivatives.

To determine the effect of the introduced mutations on cell-to-cell spread, plaque assays were performed in monolayers of CRFK cells grown in 35 mm diameter dishes as described previously (Mijnes et al., 1997). Plaques were stained immunohistochemically, and the monolayers were photographed and printed at a 2.5-fold magnification. The prints were converted into bitmap files using a Hewlett Packard ScanJet 4C, employing the software provided by the manufacturer, set at ‘sharp black and white photograph’ and 300 dots per inch. Alternatively and more conveniently, bitmap files of the monolayers were prepared by direct scanning of the dishes at 900 dots per inch using an Agfa Duoscan. The surface area occupied by each plaque was determined in arbitrary units by computer-assisted image analysis using the Spotter software; Spotter, a 32 bit application requiring Windows95 or higher or a WinNT platform, is a freeware program that can be obtained by downloading from http://www.androclus.vet.uunl/spotter/spotter.htm. This approach allowed us to accurately determine the sizes of large numbers of plaques. The results are based on at least four separate experiments; for each recombinant virus, 231 to 2128 plaques were analysed (Table 1).

It is important to note that inactivation of the TK gene also affects plaque size (Sanders et al., 1982). Indeed, a comparison of wild-type FHV strain B927 and FHVAgI-LZ with their respective TK-deficient counterparts FHV-TK−/T7+/gIwt and FHV-TK−/T7+/gI− revealed that the plaque sizes of the latter were, on average, reduced by 40% (Fig. 2a). It was therefore decided to use FHV-TK−/T7+/gIwt and FHV-TK−/T7+/gI− as positive and negative controls, respectively.

FHVAgI-LZ revertants, in which the U7 gI locus has been restored, produce normal-sized plaques (Mijnes et al., 1997). Whilst these findings demonstrate that the small-plaque phenotype of FHVAgI-LZ results from the engineered genetic modification – i.e. lacZ marker insertion into the gI locus – rather than from mutations elsewhere in the genome, they do not prove that the defect in cell-to-cell spread is due to the deficiency in gI. In principle, ‘nearest-neighbour effects’, exerted by the inserted lacZ expression cassette, could at least contribute to the small-plaque phenotype. However, as shown in Fig. 2(b), the defect in FHVAgI-LZ can also be complemented by allotopic expression of gI. gE–gI-mediated cell-to-cell spread was fully restored by the insertion of an active gI expression cassette into the U7TK locus; plaques produced by FHV-TK−/gI+ were indistinguishable from those of FHV-TK−/T7+/gImt. These findings were taken as formal evidence that the small-plaque phenotype of FHVAgI-LZ is solely caused by the loss of gI. Moreover, they provided a firm basis for further structure–function analysis of the gI protein using plaque size as a parameter.

gI proteins possess relatively large cytoplasmic domains, ranging from 54 to 92 residues in length. Conceivably, these may be important for gE–gI function, e.g. through their interaction with cytosolic proteins. Olson & Grose (1998) suggested that the cytoplasmic domain of gI of varicella-zoster virus (VZV) harbours signals for internalization and intra-cellular targeting of the gE–gI complex to the trans-Golgi network (TGN). Furthermore, residues within the cytoplasmic domain of gI proteins may become phosphorylated by host- and virus-encoded kinases (Ng et al., 1998; Yao & Grose, 1994). Our present data show that the deletion of the C-terminal 11 residues of FHV gI does not affect gE–gI-mediated spread, at least not in vitro: the plaque size of FHVAgI-LZ was restored to that of FHV-TK−/T7+/gImt by allotopic complementation with gIΔXb (Fig. 2b). Removal of the complete cytoplasmic domain of FHV gI had a significant yet relatively modest effect on gE–gI function: allotopic expression of a tailless gI protein, gI109, yielded large plaques that, on average, were only 35% reduced in size as compared to those of matched controls producing intact gI (Tukey test, P < 0.0005; Table 1 and Fig. 2b). Strikingly, an FHV recombinant expressing gI109 from the autologous U7 locus displayed an identical reduction in average plaque size as compared to wild-type FHV (Mijnes et al., 1997). Apparently, the cytoplasmic domain of gI is important but not essential for gE–gI-mediated spread. Interestingly, recent findings indicate that the cytoplasmic domain of gE rather than that of gI determines TGN localization of the gE–gI complex of VZV, PRV and herpes simplex virus (Alconada et al., 1996, 1998, 1999; Olson & Grose, 1997; Tirabassi & Enquist, 1998; Zhu et al., 1995). These observations have been confirmed for the FHV gE–gI complex in our laboratory (Mijnes, 1999).

Previously, we noted that an FHV recombinant, autotopically expressing a gI derivative truncated at Arg-166, produced plaques that were twofold larger than those of FHVAgI-LZ. This led us to speculate that a complex between gE and gI166 retains partial biological activity (Mijnes et al., 1997). However, alternative, more trivial explanations, could not be ruled out. For example, the difference in plaque size could also have been caused by second-site mutations acquired during the isolation or subsequent propagation of the re-
### Table 1. Statistical analysis of the effects of progressive C-terminal truncations in gI on average plaque size

<table>
<thead>
<tr>
<th>Recombinant FHV</th>
<th>No. plaques analysed</th>
<th>Relative plaque size*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7⁺/gIwt</td>
<td>1187</td>
<td>T7⁺/gIwt &gt; gI∆B, gIAM, gI-152, T7⁺/gl⁻</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>gI∆B</td>
<td>2128</td>
<td>gI∆B &gt; gIAM, gl-152, T7⁺/gl⁻</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>gIAM #1</td>
<td>231</td>
<td>gIAM #1 &lt; or &gt; gIAM #2</td>
<td>&gt; 0.9</td>
</tr>
<tr>
<td>gIAM #2</td>
<td>310</td>
<td>gIAM &gt; gl-152</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>gl-152 #1</td>
<td>455</td>
<td>gl-152 &lt; or &gt; T7⁺/gl⁻</td>
<td>&gt; 0.7</td>
</tr>
<tr>
<td>gl-152 #2</td>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7⁺/gl⁻</td>
<td>284</td>
<td></td>
<td></td>
</tr>
</tbody>
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*Data were subjected to the Tukey test of variants, with weights being the inverse of the variances of the means, to determine whether the average plaque size of a particular recombinant FHV is significantly larger (> or smaller (<) than those of other recombinant FHVs.

![Fig. 2.](a) The effect of deletion of the TK gene on plaque size. Monolayers of CRFK cells, grown in 35 mm diameter dishes, were infected with 50 to 100 p.f.u. of B927, FHV-TK⁻/T7⁺/gIwt (T7⁺/gIwt), FHV∆gI-LZ (∆gI) and FHV-TK⁻/T7⁺/gI⁻ (T7⁺/gl⁻). One hour p.i. a solid-phase overlay was applied and incubation was continued for 72 h at 37 °C. The monolayers were fixed with paraformaldehyde and stained immunohistochemically (Mijnes et al., 1997). The plaque sizes were measured using computer-assisted image analysis. The histogram shows the average plaque sizes relative to B927 (left panel) or FHV∆gI-LZ (right panel). (b) Plaque sizes of FHV recombinants allotopically expressing C-terminally truncated gI derivatives. Monolayers of CRFK cells, grown in 35 mm diameter dishes, were infected with 50 to 100 p.f.u. of FHV-TK⁻/T7⁺/gIwt (T7⁺/gIwt), FHV-TK⁻/gI⁺ (∆gI), -gI∆Xb (∆Xb), -gI∆B (∆B), -gI∆M (∆M) #1 and #2, -gI-152 (152) #1 and #2, FHV-TK⁻/gI⁻ (TK⁻/gI⁻) or FHV-TK⁻/T7⁺/gI⁻ (T7⁺/gl⁻). Plaques were visualized and measured as described above. The average plaque sizes were expressed relative to that of the positive control, FHV-TK⁻/T7⁺/gIwt. The histogram shows the combined results from four independent experiments. Standard deviations are indicated by bars.

combinant virus, or by the mere elimination of the lacZ expression cassette. Our present findings strongly support the notion that expression of gI⁺ can partially overcome the defect in cell-to-cell spread of FHV∆gI-LZ. Recombinant viruses, allotopically expressing gI⁺, displayed a small-plaque phenotype, yet on average, their plaques again were twofold larger than those of matched gI-deficient controls. These results were reproducible: experiments performed with the independently generated recombinant viruses FHV-TK⁻/gI⁺ yielded identical results (Fig. 2b). Moreover, the difference in plaque size between viruses that express gI⁺ and gI-deficient viruses was consistently found in
at least four separate experiments and was highly significant (P < 0.0005; Table 1). We therefore conclude that a complex between gE and gI, although severely crippled, is still partially functional and able to mediate some cell-to-cell transmission. Conversely, the complex between gE and gI, which is retained in the ER (Fig. 1c; Mijnes et al., 1997), does not seem to promote virus spread at all. Viruses expressing gI from the U1,23 locus produced minute plaques, similar to those of the negative control FHV-TK−/T7+/gI− and to those of FHV-TK−/gI−, a spontaneous TK-deficient mutant of FHVΔg-I-LZ (Fig. 2b). Presumably, gE–gI oligomers must be transported either to the TGN or the plasma membrane in order to be functional.

How do these findings bear on the role of gI in the complex? One option is that gI is a functionally important component, with the highly conserved N-terminal half of the ectodomain (Audonnet et al., 1990; Leung-Tack et al., 1994; McGeoch, 1990) as the most relevant part of the protein. It has been speculated that the gE–gI complex is a receptor-binding entity (Balan et al., 1994; Dingwell et al., 1994). If so, then the N terminus of gI is perhaps part of a ligand-binding site. Alternatively, the actual function may reside in gE, with gI having merely a supporting structural role. For example, gI could act as a chaperone assisting proper folding and intracellular transport of gE and/or protect gE against proteolysis, immunorecognition or denaturation. There are observations consistent with gI being a ‘supporting actor’. (i) gE appears to be essential for in vitro replication of VZV, whereas gI is dispensable (Mallory et al., 1997). (ii) For PRV, the deletion of gE has a greater impact on virulence in pigs than the deletion of gI (Kimman et al., 1992). If so, then the N terminus of gI is perhaps part of a ligand-binding site. Alternatively, the actual function may reside in gE, with gI having merely a supporting structural role. For example, gI could act as a chaperone assisting proper folding and intracellular transport of gE and/or protect gE against proteolysis, immunorecognition or denaturation. There are observations consistent with gI being a ‘supporting actor’. (i) gE appears to be essential for in vitro replication of VZV, whereas gI is dispensable (Mallory et al., 1997). (ii) For PRV, the deletion of gE has a greater impact on virulence in pigs than the deletion of gI (Kimman et al., 1992). If so, then the N terminus of gI is perhaps part of a ligand-binding site. Alternatively, the actual function may reside in gE, with gI having merely a supporting structural role. For example, gI could act as a chaperone assisting proper folding and intracellular transport of gE and/or protect gE against proteolysis, immunorecognition or denaturation. There are observations consistent with gI being a ‘supporting actor’. (i) gE appears to be essential for in vitro replication of VZV, whereas gI is dispensable (Mallory et al., 1997). (ii) For PRV, the deletion of gE has a greater impact on virulence in pigs than the deletion of gI (Kimman et al., 1992). If so, then the N terminus of gI is perhaps part of a ligand-binding site. Alternatively, the actual function may reside in gE, with gI having merely a supporting structural role. For example, gI could act as a chaperone assisting proper folding and intracellular transport of gE and/or protect gE against proteolysis, immunorecognition or denaturation. There are observations consistent with gI being a ‘supporting actor’. (i) gE appears to be essential for in vitro replication of VZV, whereas gI is dispensable (Mallory et al., 1997). (ii) For PRV, the deletion of gE has a greater impact on virulence in pigs than the deletion of gI (Kimman et al., 1992).

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References


