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), FHVΔgI-LZ, produces plaques that are only 15% the size of those of wild-type FHV. Here, we have complemented FHVΔgI-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 gI[166], 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–gI[166] 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 complicated process involving various envelope glycoproteins, such as gC, gB, the gH–gL hetero-oligomer and, in most cases, gD (Mettenleiter, 1994;

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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
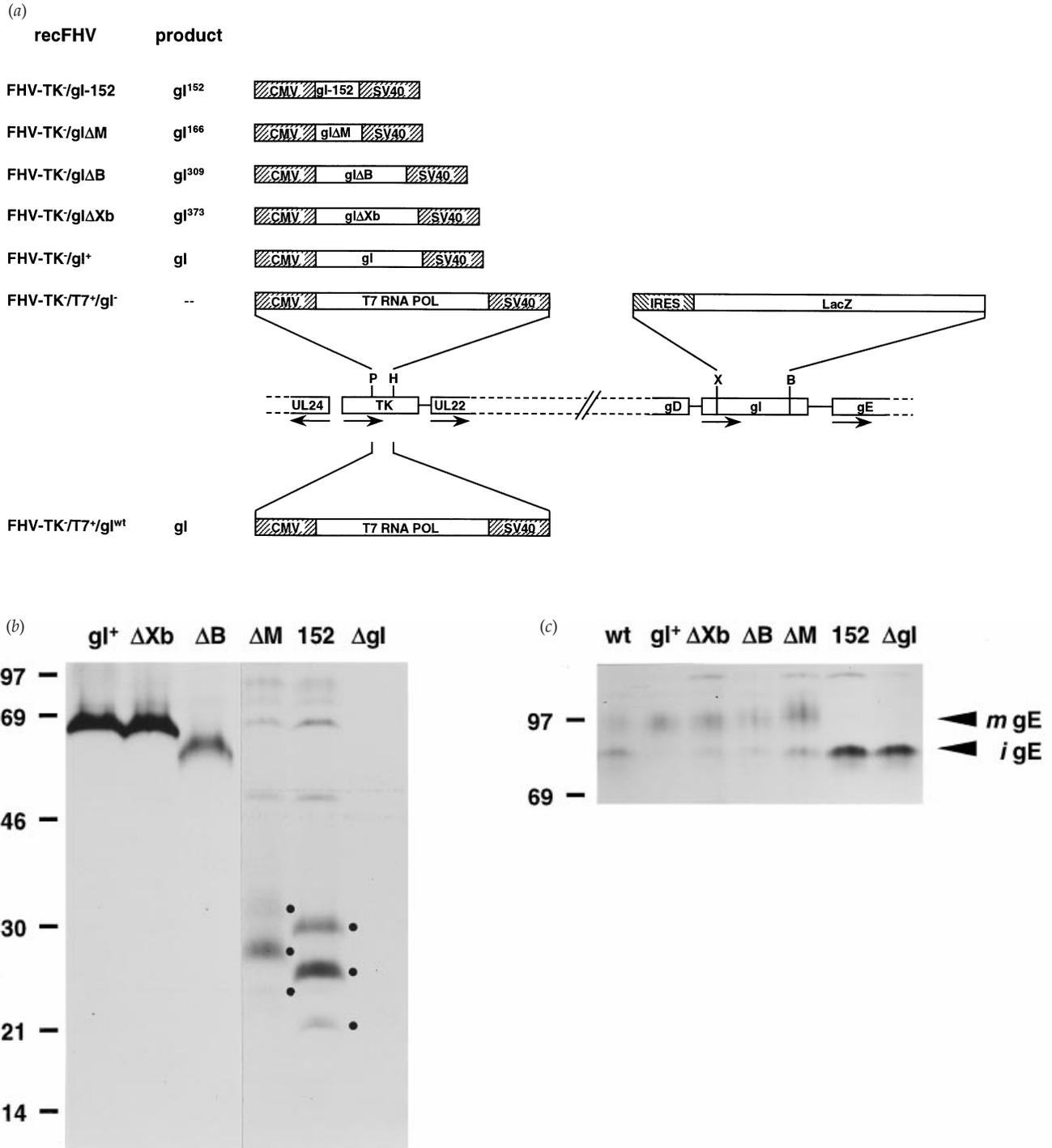
Fig. 1. (*a*) Schematic representation of the genomic structure of FHV-TK⁻/T7⁺/gl^wt, FHV-TK⁻/T7⁺/gl⁻ and the FHVΔgl-LZ derivatives allotopically expressing truncated gl proteins. The genes for U_L24, thymidine kinase (TK), U_L22, gD, gl, gE, T7 RNA polymerase (T7 RNA POL) and β-galactosidase (LacZ) 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 *Pme*I, *Hin*dIII, *Xho*I and *Bam*HI restriction sites, respectively. (*b*) Expression of truncated gl proteins in FHV-infected cells. Monolayers of CRFK cells were infected with either FHV-TK⁻/gl⁺ (gl⁺), -glΔXb (ΔXb), -glΔB (ΔB), -glΔM (ΔM), -gl-152 (152) or FHVΔgl-LZ (Δgl). The cells were metabolically labelled from 7 to 8 h p.i. and lysed. Products precipitated by the Ra-αgl serum were analysed in SDS–15% polyacrylamide gels. Differentially *N*-glycosylated forms of gl^166 and gl^152 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, gI^{166}, 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, gI^{152}, only 14 residues shorter than gI^{166}, binds to gE, but the resulting hetero-oligomeric complex is retained in the ER (Mijnes *et al.*, 1997).

Consistent with observations made for other alphaherpes-viruses (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 (U_S7) was disrupted through 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 tail-less gI, gI^{309}, and gI^{166} from the autologous U_S7 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 gI^{166}-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 gI^{166} 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 U_S7 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 U_S6, encoding the presumptive essential protein gD, is expressed through a structurally polycistronic mRNA which terminates downstream of the gI gene (Willemse *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 oc-currence 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 gener-ated recombinant viruses.

Here, to validate and to extend our previous observations (Mijnes *et al.*, 1997), we complemented gI-deficient recom-binant 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 (U_L23 TK; Nunberg *et al.*, 1989) locus were substituted for by a gI 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. 1*a*). 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-titred stocks. In each case, proper insertion of the expression cassette was confirmed by Southern blot analysis (not shown; for a more detailed description of the construction of each mutant virus, see Mijnes, 1999).

FHV-TK$^-$/gI$^+$ carries an intact gI gene, whereas FHV-TK$^-$/gIΔXb and FHV-TK$^-$/gIΔB encode truncated gI proteins lacking the C-terminal 11 (gI^{373}) and 75 (gI^{309}) residues, respectively. FHV-TK$^-$/gIΔM #1 and #2 are independently generated recombinant viruses – i.e. originating from two separate transfection/recombination experiments – encoding gI proteins truncated at Arg-166, while the independently generated recombinants FHV-TK$^-$/gI-152 #1 and #2 code for gI proteins truncated at Asp-152. To serve as a negative control, we constructed an FHVΔgI-LZ derivative, FHV-TK$^-$/T7$^+$/gI$^-$ expressing an irrelevant gene – the bacterio-phage T7 RNA polymerase gene – from the U_L23 locus. In addition a related virus, FHV-TK$^-$/T7$^+$/gI^{wt}, was created with a similarly disrupted TK gene but with an intact U_S7 gI locus (Fig. 1*a*).

To test for proper expression of the gI 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 gI derivatives were expressed.

or the FHV recombinants mentioned above, labelled from 7 to 8 h p.i. followed by a 2 h chase. Cell lysates were subjected to RIPA with Ra-αgE and precipitates were analysed in SDS–7·5 % polyacrylamide gels. Arrowheads indicate the immature (*ig*E) and the mature (*mg*E) gE species. Molecular sizes are in kilodaltons.

Their expression levels, however, were increased approximately fivefold as compared to that of autologous gI (not shown). For FHV-TK⁻/gIΔXb and FHV-TK⁻/gIΔB, the Ra-αgI serum precipitated EndoH-sensitive products of 65 and 59 kDa, closely corresponding to the anticipated sizes. For gI^{166} and gI^{152}, multiple bands were precipitated, most likely representing differentially glycosylated species; gI^{166} and gI^{152} 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-gIΔM (Mijnes *et al.*, 1997). With the exception of gI^{152}, 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. 1*c*). 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.uu.nl/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 FHVΔgI-LZ with their respective TK-deficient counterparts FHV-TK⁻/T7⁺/gI^{wt} and FHV-TK⁻/T7⁺/gI⁻ revealed that the plaque sizes of the latter were, on average, reduced by 40% (Fig. 2*a*). It was therefore decided to use FHV-TK⁻/T7⁺/gI^{wt} and FHV-TK⁻/T7⁺/gI⁻ as positive and negative controls, respectively.

FHVΔgI-LZ revertants, in which the U$_S$7 gI locus has been restored, produce normal-sized plaques (Mijnes *et al.*, 1997). Whilst these findings demonstrate that the small-plaque phenotype of FHVΔgI-LZ results from the engineered genetic modification – i.e. *lacZ* marker insertion into the gI locus – rather than from mutations elsewhere in the genome, they do not prove that the defect in cell-to-cell spread is due to the deficiency in gI. In principle, 'nearest-neighbour effects', exerted by the inserted *lacZ* expression cassette, could at least contribute to the small-plaque phenotype. However, as shown in Fig. 2(*b*), the defect in FHVΔgI-LZ can also be complemented by allotopic expression of gI. gE–gI-mediated cell-to-cell spread was fully restored by the insertion of an active gI expression cassette into the U$_L$23 TK locus; plaques produced by FHV-TK⁻/gI⁺ were indistinguishable from those of FHV-TK⁻/T7⁺/gI^{wt}. These findings were taken as formal evidence that the small-plaque phenotype of FHVΔgI-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 intracellular 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 FHVΔgI-LZ was restored to that of FHV-TK⁻/T7⁺/gI^{wt} by allotopic complementation with gIΔXb (Fig. 2*b*). Removal of the complete cytoplasmic domain of FHV gI had a significant yet relatively modest effect on gE–gI function: allotopic expression of a tail-less gI protein, gI^{309}, 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. 2*b*). Strikingly, an FHV recombinant expressing gI^{309} from the autologous U$_S$7 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 FHVΔgI-LZ. This led us to speculate that a complex between gE and gI^{166} 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

Recombinant FHV	No. plaques analysed	Relative plaque size*	P
T7$^+$/gI^{wt}	1187	T7$^+$/gI^{wt} > gIΔB, gIΔM, gI-152, T7$^+$/gI$^-$	< 0·0005
gIΔB	2128	gIΔB > gIΔM, gI-152, T7$^+$/gI$^-$	< 0·0005
gIΔM #1	231	gIΔM #1 <or> gIΔM #2	> 0·9
gIΔM #2	310	gIΔM > gI-152	< 0·0005
gI-152 #1	455	gI-152 <or> T7$^+$/gI$^-$	> 0·7
gI-152 #2	400		
T7$^+$/gI$^-$	284		

*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.
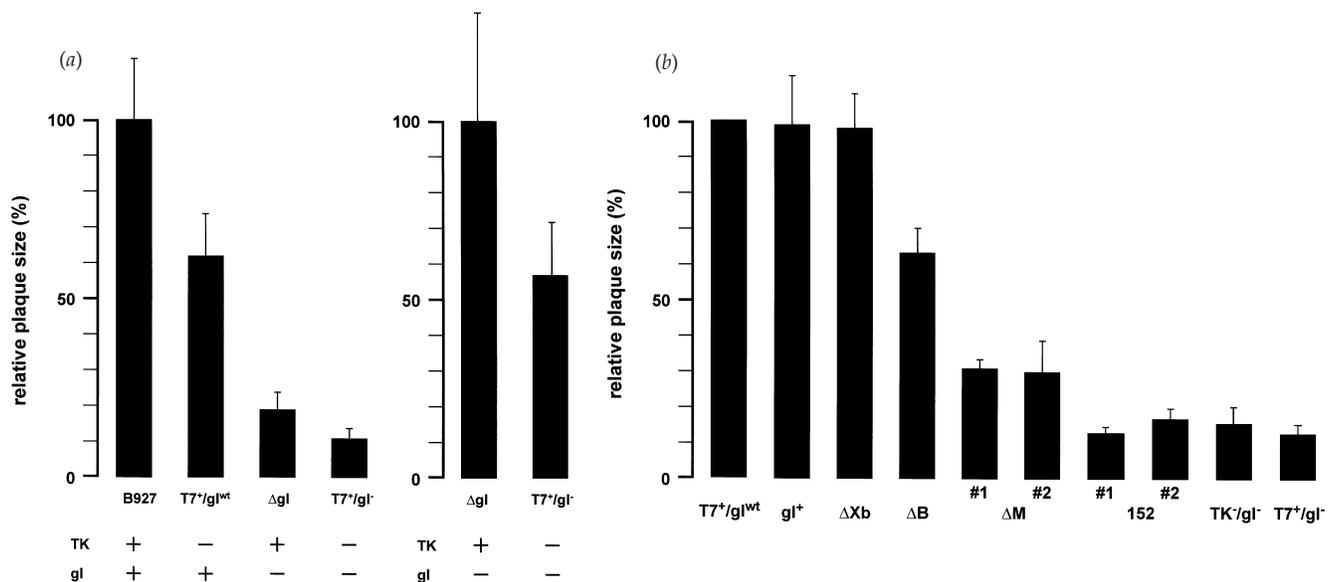
Fig. 2. (a) The effect of deletion of the TK gene on plaque size. Monolayers of CRFK cells, grown in 35 mm diameter dishes, were infected with 50 to 100 p.f.u. of B927, FHV-TK$^-$/T7$^+$/gI^{wt} (T7$^+$/gI^{wt}), FHVΔgI-LZ (ΔgI) and FHV-TK$^-$/T7$^+$/gI$^-$ (T7$^+$/gI$^-$). One hour p.i. a solid-phase 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 FHVΔgI-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$^+$/gI^{wt} (T7$^+$/gI^{wt}), FHV-TK$^-$/gI$^+$ (gI$^+$), -gIΔXb (ΔXb), -gIΔB (ΔB), -gIΔM (ΔM) #1 and #2, -gI-152 (152) #1 and #2, FHV-TK$^-$/gI$^-$ (TK$^-$/gI$^-$) or FHV-TK$^-$/T7$^+$/gI$^-$ (T7$^+$/gI$^-$). Plaques were visualized and measured as described above. The average plaque sizes were expressed relative to that of the positive control, FHV-TK$^-$/T7$^+$/gI^{wt}. 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^{166} can partially overcome the defect in cell-to-cell spread of FHVΔgI-LZ. Recombinant viruses, allotopically expressing gI^{166}, 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$^-$/gIΔM #1 and #2 yielded identical results (Fig. 2b). Moreover, the difference in plaque size between viruses that express gI^{166} 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[166], although severely crippled, is still partially functional and able to mediate some cell-to-cell transmission. Conversely, the complex between gE and gI[152], which is retained in the ER (Fig. 1c; Mijnes et al., 1997), does not seem to promote virus spread at all. Viruses expressing gI[152] from the U_L23 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 FHVΔgI-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). It is of note that both PRV and VZV gE are released from the ER and transported to the TGN and plasma membrane in the absence of gI (Alconada et al., 1996; Litwin et al., 1992; Mallory et al., 1997; Tirabassi & Enquist, 1998; Whealy et al., 1993; Yao et al., 1993), although complex formation enhances intracellular transport (Alconada et al., 1998; Whealy et al., 1993). (iii) A recombinant vaccinia virus (recVV), co-expressing PRV gE and gI from the TK locus, showed increased neurovirulence in mice as compared to other TK-deficient recVVs (Kost et al., 1989). This recombinant was also more virulent than recombinants expressing either gE or gI. However, the recVV producing only gE was still over tenfold more virulent than the one producing only gI as determined in an LD_{50} assay. Evidently, further studies are required to increase our knowledge of the structure and function of the gE–gI complex and its role in cell-to-cell spread. Our data demonstrate that the plaque assay provides a reliable and convenient tool to measure and quantify gE–gI function in vitro.

References

Alconada, A., Bauer, U. & Hoflack, B. (1996). A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network. *EMBO Journal* **15**, 6096–6110.

Alconada, A., Bauer, U., Baudoux, L., Piette, J. & Hoflack, B. (1998). Intracellular transport of the glycoproteins gE and gI of the varicella-zoster virus. *Journal of Biological Chemistry* **273**, 13430–13436.

Alconada, A., Bauer, U., Sodeik, B. & Hoflack, B. (1999). Intracellular traffic of herpes simplex virus glycoprotein E: characterization of the sorting signals required for its trans-Golgi network localization. *Journal of Virology* **73**, 377–387.

Audonnet, J.-C., Winslow, J., Allen, G. & Paoletti, E. (1990). Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. *Journal of General Virology* **71**, 2969–2978.

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H. & Minson, T. (1994). An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *Journal of General Virology* **75**, 1245–1258.

Cassady, K. A., Gross, M. & Roizman, B. (1998). The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha. *Journal of Virology* **72**, 7005–7011.

Dingwell, K. S., Brunetti, C. R., Hendricks, R. L., Tang, Q., Tang, M., Rainbow, A. J. & Johnson, D. C. (1994). Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *Journal of Virology* **68**, 834–845.

Johnson, D. C. & Feenstra, V. (1987). Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. *Journal of Virology* **61**, 2208–2216.

Kimman, T. G., de Wind, N., Oei-Lie, N., Pol, J. M. A., Berns, A. J. M. & Gielkens, A. L. J. (1992). Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. *Journal of General Virology* **73**, 243–251.

Kimura, H., Straus, S. E. & Williams, R. K. (1997). Varicella-zoster virus glycoproteins E and I expressed in insect cells form a heterodimer that requires the N-terminal domain of glycoprotein I. *Virology* **233**, 382–391.

Kost, T. A., Jones, E. V., Smith, K. M., Reed, A. P., Brown, A. L. & Miller, T. J. (1989). Biological evaluation of glycoproteins mapping to two distinct mRNAs within the *Bam*HI fragment 7 of pseudorabies virus: expression of the coding regions by vaccinia virus. *Virology* **171**, 365–376.

Leung-Tack, P., Audonnet, J. C. & Rivière, M. (1994). The complete DNA sequence and the genetic organization of the short unique region (US) of the bovine herpesvirus type 1 (ST strain). *Virology* **199**, 409–421.

Liang, X., Pyne, C., Li, Y., Babiuk, L. A. & Kowalski, J. (1995). Delineation of the essential function of bovine herpesvirus 1 gD: an indication for the modulatory role of gD in virus entry. *Virology* **207**, 429–441.

Litwin, V., Jackson, W. & Grose, C. (1992). Receptor properties of two varicella-zoster virus glycoproteins, gpI and gpIV, homologous to herpes simplex virus gE and gI. *Journal of Virology* **66**, 3643–3651.

McGeoch, D. J. (1990). Evolutionary relationships of virion glycoprotein genes in the S regions of alphaherpesvirus genomes. *Journal of General Virology* **71**, 2361–2367.

Mallory, S., Sommer, M. & Arvin, A. M. (1997). Mutational analysis of the role of glycoprotein I in varicella-zoster virus replication and its

effects on glycoprotein E conformation and trafficking. *Journal of Virology* **71**, 8279–8288.

Mettenleiter, T. C. (1994). Pseudorabies (Aujeszky's disease) virus: state of the art. August 1993. *Acta Veterinaria Hungarica* **42**, 153–177.

Mijnes, J. D. F. (1999). *Structure–function analysis of the feline herpesvirus virulence factors gE and gI*. PhD thesis, University of Utrecht, The Netherlands.

Mijnes, J. D. F., van der Horst, L. M., van Anken, E., Horzinek, M. C., Rottier, P. J. M. & de Groot, R. J. (1996). Biosynthesis of glycoproteins E and I of feline herpesvirus: gE–gI interaction is required for intracellular transport. *Journal of Virology* **70**, 5466–5475.

Mijnes, J. D. F., Lutters, B. C., Vlot, A. C., van Anken, E., Horzinek, M. C., Rottier, P. J. M. & de Groot, R. J. (1997). Structure–function analysis of the gE–gI complex of feline herpesvirus: mapping of gI domains required for gE–gI interaction, intracellular transport, and cell-to-cell spread. *Journal of Virology* **71**, 8397–8404.

Mohr, I. & Gluzman, Y. (1996). A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function. *EMBO Journal* **15**, 4759–4766.

Ng, T. I., Ogle, W. O. & Roizman, B. (1998). UL13 protein kinase of herpes simplex virus 1 complexes with glycoprotein E and mediates the phosphorylation of the viral Fc receptor: glycoproteins E and I. *Virology* **241**, 37–48.

Nunberg, J. H., Wright, D. K., Cole, G. E., Petrovskis, E. A., Post, L. E., Compton, T. & Gilbert, J. H. (1989). Identification of the thymidine kinase gene of feline herpesvirus: use of degenerate oligonucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs. *Journal of Virology* **63**, 3240–3249.

Olson, J. K. & Grose, C. (1997). Endocytosis and recycling of varicella-zoster virus Fc receptor glycoprotein gE: internalization mediated by a YXXL motif in the cytoplasmic tail. *Journal of Virology* **71**, 4042–4054.

Olson, J. K. & Grose, C. (1998). Complex formation facilitates endocytosis of the varicella-zoster virus gE:gI Fc receptor. *Journal of Virology* **72**, 1542–1551.

Peeters, B., de Wind, N., Hooisma, M., Wagenaar, F., Gielkens, A. & Moormann, R. (1992). Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. *Journal of Virology* **66**, 894–905.

Rauh, I. & Mettenleiter, T. C. (1991). Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. *Journal of Virology* **65**, 5348–5356.

Rebordosa, X., Piñol, J., Pérez Pons, J. A., Lloberas, J., Naval, J., Serra-Hartmann, X., Espuña, E. & Querol, E. (1996). Glycoprotein E of bovine herpesvirus type 1 is involved in virus transmission by direct cell-to-cell spread. *Virus Research* **45**, 59–68.

Sanders, P. G., Wilkie, N. M. & Davison, A. J. (1982). Thymidine kinase deletion mutants of herpes simplex virus type 1. *Journal of General Virology* **63**, 277–295.

Schmidt, J., Klupp, B. G., Karger, A. & Mettenleiter, T. C. (1997). Adaptability in herpesviruses: glycoprotein D-independent infectivity of pseudorabies virus. *Journal of Virology* **71**, 17–24.

Schröder, C., Linde, G., Fehler, F. & Keil, G. M. (1997). From essential to beneficial: glycoprotein D loses importance for replication of bovine herpesvirus 1 in cell culture. *Journal of Virology* **71**, 25–33.

Spear, P. G. (1993). Entry of alphaherpesviruses into cells. *Seminars in Virology* **4**, 167–180.

Tirabassi, R. S. & Enquist, L. W. (1998). Role of envelope protein gE endocytosis in the pseudorabies virus life cycle. *Journal of Virology* **72**, 4571–4579.

Whealy, M. E., Card, J. P., Robbins, A. K., Dubin, J. R., Rziha, H. J. & Enquist, L. W. (1993). Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. *Journal of Virology* **67**, 3786–3797.

Whitbeck, J. C., Knapp, A. C., Enquist, L. W., Lawrence, W. C. & Bello, L. J. (1996). Synthesis, processing, and oligomerization of bovine herpesvirus 1 gE and gI membrane proteins. *Journal of Virology* **70**, 7878–7884.

Willemse, M. J., Strijdveen, I. G., van Schooneveld, S. H., van den Berg, M. C. & Sondermeijer, P. J. (1995). Transcriptional analysis of the short segment of the feline herpesvirus type 1 genome and insertional mutagenesis of a unique reading frame. *Virology* **208**, 704–711.

Yao, Z. & Grose, C. (1994). Unusual phosphorylation sequence in the gpIV (gI) component of the varicella-zoster virus gpI–gpIV glycoprotein complex (VZV gE–gI complex). *Journal of Virology* **68**, 4204–4211.

Yao, Z., Jackson, W., Forghani, B. & Grose, C. (1993). Varicella-zoster virus glycoprotein gpI/gpIV receptor: expression, complex formation, and antigenicity within the vaccinia virus–T7 RNA polymerase transfection system. *Journal of Virology* **67**, 305–314.

Yoshitake, N., Xuan, X. & Otsuka, H. (1997). Identification and characterization of bovine herpesvirus-1 glycoproteins E and I. *Journal of General Virology* **78**, 1399–1403.

Zhu, Z., Gershon, M. D., Hao, Y., Ambron, R. T., Gabel, C. A. & Gershon, A. A. (1995). Envelopment of varicella-zoster virus: targeting of viral glycoproteins to the trans-Golgi network. *Journal of Virology* **69**, 7951–7959.

Zsak, L., Zuckermann, F., Sugg, N. & Ben Porat, T. (1992). Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. *Journal of Virology* **66**, 2316–2325.

Zuckermann, F. A., Mettenleiter, T. C., Schreurs, C., Sugg, N. & Ben Porat, T. (1988). Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. *Journal of Virology* **62**, 4622–4626.