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The Equine Herpesvirus 1 Glycoprotein gp21/22a, the Herpes Simplex Virus Type 1 gM Homolog, Is Involved in Virus Penetration and Cell-to-Cell Spread of Virions

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Experiments to analyze the function of the equine herpesvirus 1 (EHV-1) glycoprotein gM homolog were conducted. To this end, an Rk_{13} cell line (TCgM) that stably expressed EHV-1 gM was constructed. Proteins with apparent M_rs of 46,000 to 48,000 and 50,000 to 55,000 were detected in TCgM cells with specific anti-gM antibodies, and the gM protein pattern was indistinguishable from that in cells infected with EHV-1 strain RacL11. A viral mutant (L11ΔgM) bearing an *Escherichia coli lacZ* gene inserted into the EHV-1 strain RacL11 gM gene (open reading frame 52) was purified, and cells infected with L11ΔgM did not contain detectable gM. L11ΔgM exhibited approximately 100-fold lower titers and a more than 2-fold reduction in plaque size relative to wild-type EHV-1 when grown and titrated on noncomplementing cells. Viral titers were reduced only 10-fold when L11ΔgM was grown on the complementing cell line TCgM and titrated on noncomplementing cells. L11ΔgM also exhibited slower penetration kinetics compared with those of the parental EHV-1 RacL11. It is concluded that EHV-1 gM plays important roles in the penetration of virus into the target cell and in spread of EHV-1 from cell to cell.

Herpesvirus glycoproteins are crucially involved in the early stages of infection, in the release of virions from cells, and in the direct cell-to-cell spread of virions by fusion of neighboring cells (reviewed in reference 29). To date, 11 herpes simplex virus type 1 (HSV-1)-encoded glycoproteins have been identified and have been designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM. HSV-1 mutants lacking gC, gE, gG, gI, gJ, and gM are viable, indicating that these genes are dispensable for replication in cultured cells (reviewed in reference 26). Comparison of the HSV-1 and equine herpesvirus 1 (EHV-1) nucleotide sequences revealed that all of the known HSV-1 glycoproteins are conserved in EHV-1 (2, 4, 8, 10, 32–34). According to the current nomenclature, these glycoproteins are designated by the names of their HSV-1 homologs. Although it is known that EHV-1 gE and gI are not essential for growth in cell culture, the contributions of other EHV-1 glycoproteins to replication in cultured cells are not known (11). Six envelope glycoproteins of EHV-1 had been mapped by using a λgt11 expression library and monoclonal antibodies (MAbs) raised against purified EHV-1 (3). In addition, transcriptional and protein analyses have shown that the glycoproteins gB (19, 30), gC (18), gD (10), gG (8), gH (25), and gK (34) are expressed in EHV-1-infected cells. Glycoprotein gM (encoded by gene UL10 [6, 7]) is the most recent HSV-1 glycoprotein which has been analyzed in detail. It is the only reported nonessential glycoprotein which is conserved in all herpesviral subfamilies and has been described for human cytomegalovirus and the *Gammaherpesvirinae* members EHV-2, herpesvirus saimiri, and Epstein-Barr virus (1, 5, 16, 31). Like many herpesvirus glycoproteins, HSV-1 gM is present in virions and membranes of infected cells. HSV-1 mutants solely lacking gM grew to titers reduced approximately 10-fold relative to those of wild-type virus and showed a reduced virulence in a murine model (6, 17). The absence of functional gM obviated the syncytial phenotype of a viral gB mutant, suggesting that gM, like gB, plays a role in cell-to-cell fusion (9). The EHV-1 gM homolog (gp21/22a; referred to as EHV-1 gM in this report) was first described by Allen and Yeargan (3) and was shown to be a major constituent of the virus envelope. Further investigations revealed that gene 52, the gene homologous to HSV-1 UL10, encoded the 450-amino-acid EHV-1 gM polypeptide (23, 32). EHV-1 gM represents a multiple hydrophobic protein which contains six to eight transmembrane domains and has been reported to be present in infected cells and in purified virions as a 45,000-apparent-M_r protein (23, 32).

The goal of this study was to characterize the function of EHV-1 gM by constructing a cell line stably expressing EHV-1 gM and a gM-negative EHV-1 mutant. The results reported here indicate that the EHV-1 mutant lacking gM is impaired in entry and spread of virus in cultured cells.

Construction and characterization of a cell line expressing EHV-1 gM. Preliminary results had suggested that efficient purification of a gM-negative EHV-1 mutant required a complementing cell line expressing gM. The EHV-1 gM open reading frame was therefore amplified by a standard PCR (27) using a thermostable polymerase exhibiting 3′ proofreading activity (Pfu; Stratagene) and EHV-1 strain RacL11 (22) as a template. The 5′ primer (5′-atcggaattcATGGCACGACGGG GAGC-3′) contained an *Eco*RI site and the 3′ primer (5′-acgtctgcagTTACTATCGGTACTCCCGGCG-3′) contained a *Pst*I site to facilitate cloning. The resulting 1.37-kbp amplicon was cleaved with these restriction enzymes (Boehringer Mannheim) and cloned into pTZ18R (Pharmacia), and the recom-

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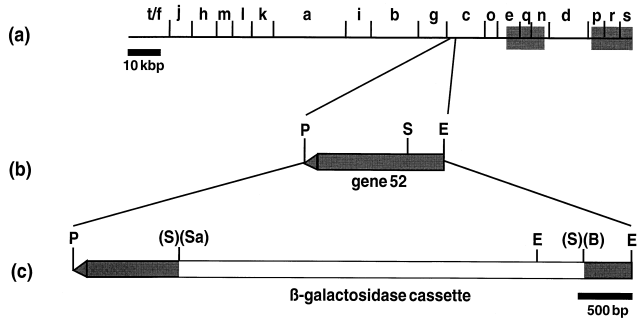

FIG. 1. (a) Schematic diagram showing the positions of *Bam*HI sites within the EHV-1 strain RacL11 genome. (b) Gene 52, the HSV-1 UL10 homolog, was amplified by PCR. The amplified product was cloned into pTZ18R (pPgM), pcDNAI/Amp (pCgM), or pSVK3 (pSVgM). (c) To disrupt gene 52, pSVgM was linearized at a unique *Sma*I site, and a *Sal*I-*Bam*HI fragment of pTT264A$^+$ (20) bearing the *E. coli lacZ* gene was cloned into pSVgM to yield plasmid pSVgMβ$^+$. P, *Pst*I; S, *Sma*I; E, *Eco*RI; Sa, *Sal*I; B, *Bam*HI.

binant plasmid was termed pPgM. The EHV-1 gM-encoding sequences were released from pPgM by cleavage with *Eco*RI and *Sph*I and cloned into pcDNAI/Amp (Invitrogen) under the control of the human cytomegalovirus immediate-early promoter, giving rise to plasmid pCgM (Fig. 1). The Rk_{13} cell line TCgM expressing EHV-1 gM was constructed by cotransfection of pCgM (1 to 10 μg) and pSV2-neo (1 μg) containing the gene encoding neomycin resistance under the control of the simian virus 40 early promoter (12). Transfected cells were grown in the presence of G-418 (Gibco-BRL) (800 μg/ml), and G-418-resistant colonies were cloned by limiting dilution. To verify the expression of EHV-1 gM by TCgM, cells were analyzed by indirect immunofluorescence with a flow cytometer (FACscan; Becton Dickinson). TCgM cells were fixed with 1% paraformaldehyde, permeabilized with digitonin (0.0025%), stained with the gM-specific MAb 13B2 (3) (kindly provided by G. P. Allen, Lexington, Ky.), and reacted with anti-mouse immunoglobulin G–fluorescein isothiocyanate conjugate (Sigma). Cells were then counterstained with propidium iodide (10^{-6} M) to distinguish intact from permeabilized cells. Analysis of the stained cells with a FACScan flow cytometer (22) indicated that in the presence of digitonin, 100% of the cells reacted with MAb 13B2. Thus, EHV-1 gM was expressed in the TCgM cell line (Fig. 2A). Cells that were not fixed and permeabilized with digitonin and that occluded the propidium iodide stain by virtue of their intact plasma membranes were not recognized by the 13B2 antibody (Fig. 2B). These data therefore suggested either that gM was not present on the cell surface or that gM was present on the TCgM cell surface and the epitope (located in the hydrophilic carboxy terminus of the gM molecule [21, 23]) was inaccessible to the 13B2 antibody.

To ensure that this observation was not an artifact of gM expression in the absence of other EHV-1 proteins, we performed experiments to determine if the epitope recognized by 13B2 was detectable in nonpermeabilized infected cells. Edmin337 cells were infected with 1 PFU of EHV-1 RacL11 per cell, reacted with MAb 13B2, and analyzed by flow cytometry. At 18 h postinfection (p.i.), almost 100% of cells showed a specific signal with MAb 13B2 after treatment with digitonin (Fig. 2C). By conventional and laser confocal microscopy it

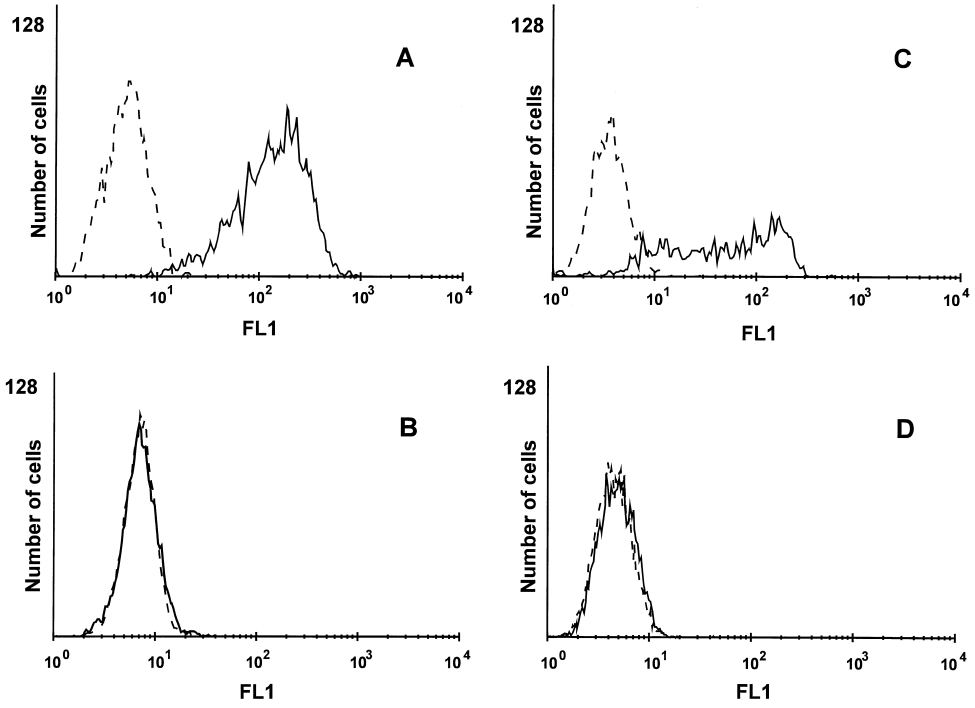

FIG. 2. Fluorocytometric analysis of TCgM and RacL11-infected Edmin337 cells harvested at 18 h p.i. with MAb 13B2. FL1, fluorescence intensity. The solid lines represent the histogram for transfected or infected cells, and the dotted lines represent the signal obtained for nontransfected or noninfected control cells. (A) Intracellular staining of EHV-1 gM produced by TCgM cells. Cells were fixed and treated with digitonin followed by reaction with MAb 13B2 and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody. (B) Analysis of EHV-1 gM on the surface of TCgM cells. Unfixed cells were reacted with the antibodies as described for panel A, and after the staining, cells were gated for no propidium iodide fluorescence. (C) Intracellular staining of EHV-1 gM in cells at 18 h after infection with RacL11. The fixation and staining of cells were performed as described for panel A. (D) Intact EHV-1-infected cells (gated for no propidium iodide fluorescence) were stained with MAb 13B2 at 18 h p.i. and then reacted with the fluorescein isothiocyanate conjugate.

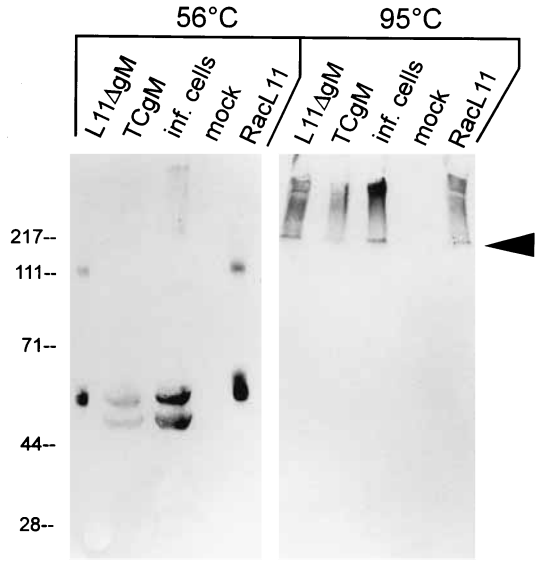

FIG. 3. Immunoblot analysis of TCgM lysates (TCgM), mock-infected cell lysates (mock), RacL11-infected cell lysates (inf. cells), purified RacL11 virions (RacL11), or purified L11ΔgM (L11ΔgM) virions produced on complementing TCgM cells. Samples were prepared in the presence of 2-mercaptoethanol and heated to either 56 or 95°C. Lysates were separated by SDS–10% PAGE, transferred to nitrocellulose, and incubated with the anti-gM antiserum. The origin of the separating gel is indicated by an arrowhead, and the M_rs of the protein markers (in thousands) are given.

could be shown that EHV-1 gM is present in the plasma membrane of both TCgM and RacL11-infected cells (data not shown). However, as in TCgM cells, no specific signal was obtained in intact infected cells reacted with the 13B2 antibody (Fig. 2D). Inasmuch as the 13B2 epitope is available for reaction with the antibody only when cells are permeabilized, it is concluded that the carboxy terminus of gM is located on the cytoplasmic side of the plasma membrane of the TCgM cell line and EHV-1-infected cells.

To further characterize the gM protein(s) specified by TCgM, lysates of TCgM cells, RacL11-infected cells (14 h p.i.), and purified RacL11 virions were prepared (28). The lysates were incubated in sample buffer (28) containing 2-mercaptoethanol at 56°C for 2 min, were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (15), transferred to a nitrocellulose membrane (14), and probed with MAb 13B2 or a gM-specific mouse polyclonal antiserum. The antiserum was prepared from mice that were immunized four times with pCgM DNA (100 μg in 50 μl of phosphate-buffered saline) at 15-day intervals. Bound antibodies were visualized with an anti-mouse immunoglobulin G–alkaline phosphatase conjugate and Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate (Sigma) as a substrate. Proteins with apparent M_rs of 46,000 to 48,000 and 50,000 to 55,000 were specifically detected by both MAb 13B2 (data not shown) and mouse polyclonal antiserum in TCgM cell lysates and in RacL11-infected cell lysates (Fig. 3). In purified RacL11 virions, only the higher-M_r species (50,000 to 55,000) was present. Additionally, a band with an apparent M_r of 110,000 to 115,000 was detected in lysates of purified RacL11 virions, suggesting that gM forms higher-order structures (Fig. 3). To ensure that the observed protein bands contained EHV-1 gM, we took advantage of the fact that gM aggregates at a high temperature (7, 23). Heating of the samples to 95°C led to the disappearance of the 46,000- to 48,000-, the 50,000- to 55,000-, and the 110,000- to 115,000-M_r proteins in lysates of TCgM cells, RacL11-infected cells, and RacL11 virions. Instead, a broad band in the stacking gel that did not enter the resolving gel reacted with gM-specific antibodies in all samples (Fig. 3). This indicated that the above-described proteins displayed the properties of gM. We therefore conclude that (i) the 46,000- to 48,000-, 50,000- to 55,000-, and 110,000- to 115,000-apparent-M_r proteins all contain gM protein and (ii) identical gM-containing protein species were present in the engineered TCgM cell line and EHV-1-infected cells.

Construction of a gM-negative RacL11 mutant. The *Sma*I-site located 400 bp downstream of the gM start codon contained in plasmid pSVgM (Fig. 1) was used for insertion of the *Escherichia coli lacZ* gene contained in pTT264A$^+$ (20) (kindly provided by W. Fuchs and T. C. Mettenleiter). Plasmid pSVgM was generated by releasing the EHV-1 gM gene from pPgM with *Eco*RI and *Pst*I and cloning into vector pSVK3 (Pharmacia). To insert the 4-kbp *lacZ* cassette into pSVgM, the cassette was released from pTT264A$^+$ by cleavage with *Sal*I and *Bam*HI, 5′ overhangs were filled in with the Klenow fragment (Boehringer Mannheim) (28), and the fragment was cloned into *Sma*I-linearized pSVgM, giving rise to plasmid pSVgMβ$^+$ (Fig. 1). Various amounts (0.5 to 2.0 μg) of RacL11 DNA prepared from purified virions were lipotransfected (DOTAP; Boehringer) with 1 to 10 μg of pSVgMβ$^+$ plasmid DNA into TCgM cells, and progeny virus was plated under an agarose overlay containing 300 μg of Bluogal (Gibco-BRL) per ml. Blue plaques were picked and subjected to five consecutive rounds of plaque purification, and the plaque-purified recombinant virus lacking a functional gM gene was designated L11ΔgM. To confirm the expected genotype of L11ΔgM, TCgM or Edmin337 cells were infected with L11ΔgM or the parental virus RacL11, respectively. Viral DNA was cleaved with *Bam*HI, *Pst*I, and *Eco*RI. After separation on 0.8% agarose gels, the DNA fragments were transferred to a nylon membrane (24) and were probed with labelled DNA fragments containing the gM gene or the 4-kbp insert of pTT264A$^+$ containing the *lacZ* gene. These analyses revealed the following. (i) In RacL11 DNA an 11.2-kbp *Bam*HI fragment, a 9.3-kbp *Pst*I fragment, and a 12.7-kbp *Eco*RI fragment were detected by the labelled gM probe. (ii) No reactivity was obtained with the *lacZ*-specific probe in RacL11 DNA, whereas in lanes containing L11ΔgM DNA, the *lacZ*-specific probe hybridized with a DNA fragment of 15.2 kbp after cleavage with *Bam*HI, a DNA fragment of 13.3 kbp after cleavage with *Pst*I, and two bands of 8.7 and 8.0 kbp after cleavage with *Eco*RI. In the last case, two *Eco*RI fragments hybridized with the *lacZ* probe because an additional *Eco*RI site was introduced by the insertion of the expression cassette. (iii) DNA fragments of electrophoretically identical sizes also hybridized with the gM DNA probe in L11ΔgM viral DNA. Since the fragments were increased in size by 4 kbp relative to the original wild-type DNA fragments and hybridized with the *lacZ* probe, we concluded that the β-galactosidase expression cassette was inserted in the gM gene locus of L11ΔgM DNA (Fig. 4A). To test for gM expression by the L11ΔgM virus, Edmin337 cells infected with L11ΔgM or RacL11 were prepared 36 h p.i. and identical samples were transferred to separate nitrocellulose membranes. One membrane was probed with the anti-gM MAb 13B2, and the other was probed with the anti-gB MAb 4B6 (22). Whereas gM was readily detectable in lysates of RacL11-infected cells, no reaction was obtained in cell lysates infected with L11ΔgM (Fig. 4B, panel gM). The absence of gM expression in L11ΔgM-infected cells was not due to the inability of L11ΔgM to infect cells, because probing with the gB-specific MAb 4B6 (22) showed that in both RacL11- and

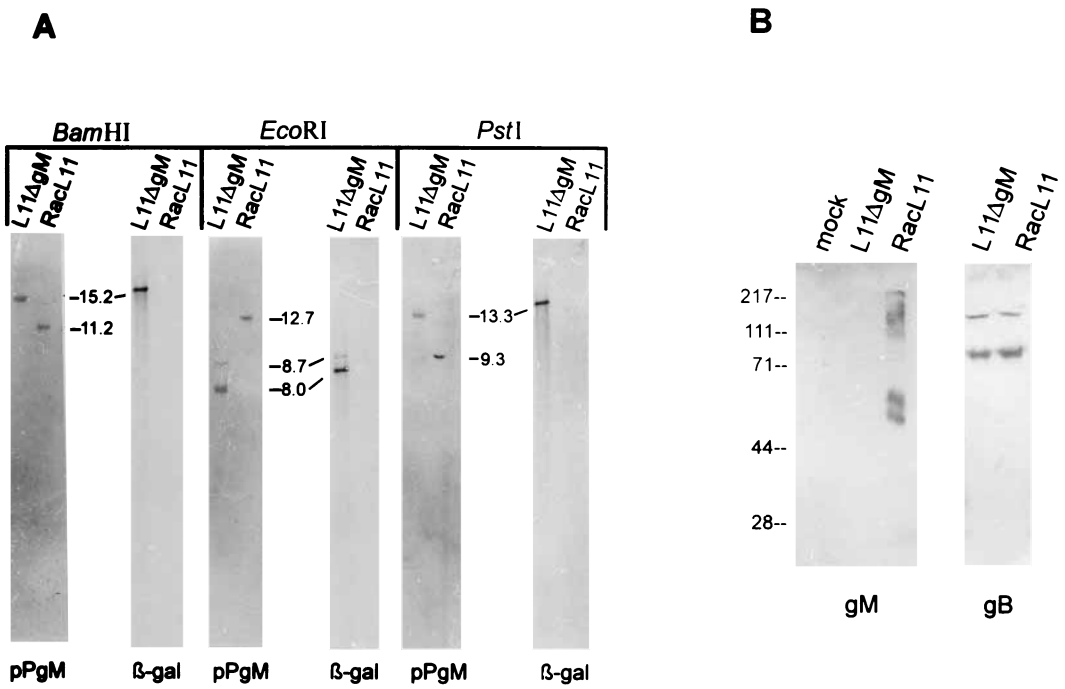

FIG. 4. (A) Photograph of RacL11 and L11ΔgM *Bam*HI, *Eco*RI, or *Pst*I DNA fragments hybridized with digoxigenin-labelled gM-specific (pPgM) or *lacZ*-specific (β-gal) DNA probes. The sizes of the reactive fragments are given in kilobase pairs. (B) Photographs of immunoblots containing mock-, RacL11-, or L11ΔgM-infected cell lysates and probed with the monospecific anti-gM antiserum (gM) or anti-gB MAb 4B6 (gB). The M_rs of the protein markers (in thousands) are indicated.

L11ΔgM-infected cell lysates, gB was detectable (Fig. 4B, panel gB). It is concluded that the gM protein cannot be detected in cells infected with L11ΔgM, although L11ΔgM is theoretically able to encode amino acids 1 to 128 of gM.

Growth characteristics of L11ΔgM on different cell lines. To characterize the growth of the gM-negative L11ΔgM virus, three separate experiments were conducted. (i) Edmin337 cells were infected (1 PFU per cell) with L11ΔgM or RacL11, and at different times p.i., virus yields from infected cell culture supernatants or cell lysates were determined. Viral titers determined for L11ΔgM were reduced at all time points compared with those for the parental RacL11, and an approximately 100-fold reduction was observed at 36 h p.i. There was virtually no difference between viral titers derived from supernatants of infected cells and those from infected cell lysates (data not shown). (ii) The relative abilities of the mutant and parental viruses to form plaques were tested by infection of various cell lines with wild-type and mutant virus; the cells were then overlaid with methylcellulose, which prevented the formation of secondary plaques. At day 4 after infection, the diameters of 50 plaques of each virus-cell combination were measured. It could be demonstrated that plaques produced by L11ΔgM were reduced in size by more than a factor of 2 on noncomplementing cells compared with those on the complementing cell line TCgM or the plaques produced by the parental RacL11 (Table 1). On Rk_{13} cells, the plaques produced by L11ΔgM were barely visible. (iii) To characterize the growth of the gM-deficient mutant at a low multiplicity of infection, the gM-complementing cell line TCgM, Rk_{13} cells, or Edmin337 cells were infected with the gM-negative L11ΔgM or wild-type RacL11 virus at 0.1 PFU per cell. Virus progeny was harvested at 48 h p.i. and titrated on TCgM, Rk_{13}, and Edmin337 cells. The results of these experiments are summarized in Table 2. The titers obtained upon infection with the parental RacL11 were very similar when grown and titrated on TCgM or Rk_{13} cells and were somewhat higher in Edmin337 cells (Table 2). In contrast, the titers obtained after infection with L11ΔgM were reduced about 100-fold compared with those obtained after infection with the parental RacL11 when grown and titrated on noncomplementing cells. When L11ΔgM was propagated on the complementing TCgM cells and titrated on noncomplementing cells, titers were reduced only about 10-fold compared with growth on complementing cells. The 10-fold reduction in L11ΔgM viral titers was also obtained when the mutant virus was grown on noncomplementing cells and titrated on TCgM (Table 2).

From these experiments we conclude the following: the EHV-1 gM is required for (i) efficient cell-to-cell spread and (ii) efficient production of infectious virus, in that both the number of PFU and the plaque size of L11ΔgM were markedly reduced when propagated and titrated on noncomplementing cells. The observation that virus produced on complementing cells was partially restored in its ability to replicate on noncomplementing cells was of particular interest since these results suggested that gM was incorporated into L11ΔgM virions during replication on complementing cells. This incorporation was addressed by immunoblot analysis of L11ΔgM virions pu-

TABLE 1. Plaque size of RacL11 and L11ΔgM on Edmin337 and Rk_{13} cells

Cell line	Plaque size (mean diam [μm] ± SD)[a] of:	
	RacL11	L11ΔgM
TCgM	360 ± 90	270 ± 80
Rk_{13}	310 ± 70	110 ± 50
Edmin337	500 ± 100	190 ± 70

[a] The plaque size was determined by using a magnifying glass with a metric scale. Values represent the mean of the diameters of 50 individual plaques.

TABLE 2. Comparison of RacL11 and L11ΔgM virus titers produced and titrated on different cell lines

Cell line for titration	Expt	Titer of virus prepared on cell line[a]:					
		TCgM		Rk_{13}		Edmin337	
		L11ΔgM	RacL11	L11ΔgM	RacL11	L11ΔgM	RacL11
TCgM	1	4.2×10^5	8.6×10^5	9.5×10^3	4.7×10^5	5.9×10^4	1.0×10^6
	2	5.8×10^5	5.3×10^5	1.6×10^4	5.3×10^5	4.3×10^4	7.3×10^5
Rk_{13}	1	7.2×10^4	1.1×10^6	5.1×10^3	3.9×10^5	7.4×10^3	6.9×10^5
	2	2.3×10^4	5.7×10^5	2.2×10^3	4.3×10^5	1.4×10^4	9.1×10^5
Edmin337	1	1.1×10^5	3.1×10^6	1.0×10^4	9.6×10^5	1.8×10^4	4.1×10^6
	2	7.6×10^4	1.0×10^6	9.3×10^3	1.2×10^6	8.3×10^3	1.3×10^6

[a] Cell line on which the RacL11 and L11ΔgM virus preparations were produced. The results of two independent experiments each are given. In the case of L11ΔgM, plaques were counted after staining with Bluogal.

rified by sucrose gradient centrifugation after production in TCgM cells. As in RacL11 virions, the monospecific anti-gM antiserum reacted with the 50,000- to 55,000- and 110,000- to 115,000-M_r proteins after incubation of the L11ΔgM lysate at 56°C and with a broad band in the stacking gel in an identical sample heated to 95°C (Fig. 3). These results demonstrated that gM provided by TCgM is present in L11ΔgM virions and improved the ability of the gM-negative virus to replicate in noncomplementing cells, supporting the possibility that EHV-1 gM plays a role early in the infectious cycle.

Kinetics of penetration of L11ΔgM. To test the hypothesis that gM plays a role in viral entry, both complementing and noncomplementing cells were infected with approximately 100 PFU of the L11ΔgM virus and parental RacL11 for 1 h at 4°C. At this temperature, attachment normally occurs but virus penetration does not (13). Indeed, less than 10% of input virus infectivity could be recovered from the medium after the 1-h adsorption period at 4°C. The incubation temperature of cells was shifted to 37°C; starting immediately after the temperature shift (time zero) and at various times thereafter, cells were washed briefly with citrate buffer (pH 3.0) to inactivate virus that had not entered cells (13). The low-pH buffer was then removed, the monolayers were washed, medium was added, and the percentage of plaques produced in citrate-treated cells relative to the number produced in nontreated cells was determined. The results indicated that whereas 60% of the infectious parental RacL11 was protected from extracellular acid treatment 30 min after shifting the temperature from 4 to 37°C (Fig. 5, closed rectangles), only 25% of L11ΔgM was protected from acid treatment at this time point on noncomplementing Edmin337 cells (Fig. 5, closed circles). After 60 min of incubation at 37°C, >85% of RacL11, but only 50% of L11ΔgM, was protected from low-pH treatment. The lower rate of penetration of L11ΔgM could be totally overcome by allowing the L11ΔgM virus produced on noncomplementing cells to interact with the complementing cell line TCgM (Fig. 5, open circles). L11ΔgM virus produced on complementing TCgM cells penetrated into both Edmin337 and TCgM cells (Fig. 5, triangles) with kinetics which were virtually indistinguishable from those of RacL11 or from those of L11ΔgM produced on noncomplementing cells and assayed on TCgM cells. From these experiments it is concluded that EHV-1 gM is required for optimal penetration of target cells by EHV-1 and that the TCgM cell line is able to complement virus penetration of a gM-negative EHV-1 mutant.

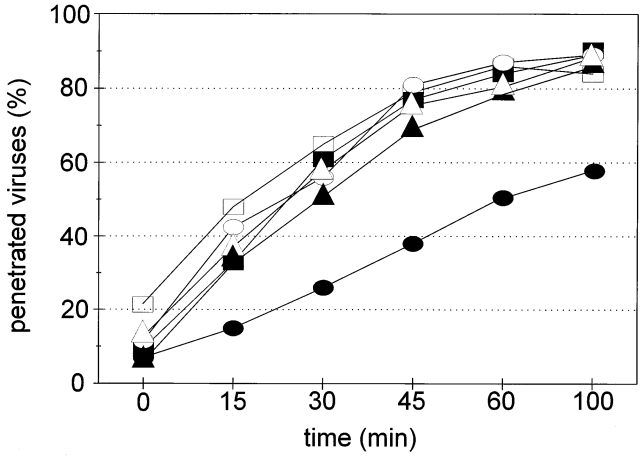

FIG. 5. Penetration of RacL11 (rectangles) and L11ΔgM produced on noncomplementing cells (circles) or complementing TCgM cells (triangles) into different cell lines. The rate of penetration on Edmin337 (closed symbols) or TCgM (open symbols) cells was determined as the percentage of plaques produced at the given time points after citrate treatment in relation to untreated cells. The values represent means of results of two experiments. The standard deviations ranged between 3.1 and 6.3%.

In sum, this study describes the analysis of an EHV-1 mutant lacking gM. The mutant was analyzed on equine and rodent cells as well as on a cell line which expresses EHV-1 gM and complements its function. Analysis of the mutant in noncomplementing cells presents the first evidence that a gM homolog is involved in viral entry and also indicates that gM is necessary for optimal viral spread in cultured cells. HSV-1 gM has similarly been implicated in cell-to-cell fusion, which may contribute to spread of virus in cultured cells (9). However, comparison of the functions of herpesvirus glycoproteins indicates that homologous glycoproteins may confer different functions in different herpesvirus systems. Thus, it is not surprising that the EHV-1 mutant lacking gM is significantly more debilitated than are gM-negative HSV-1 mutants (7, 17). Given the fact that herpesviruses must replicate in a number of different cell types for survival in nature and that, at least in the EHV-1 system, gM serves to facilitate both entry into target cells and cell-to-cell spread, it will be of interest to determine if gM contributes to viral spread within tissues of infected animals.

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REFERENCES

1. **Albrecht, J.-C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wittman, M. A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66:**5047–5058.
2. **Allen, G. P., and L. D. Coogle.** 1988. Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to herpes simplex virus glycoprotein C. J. Virol. **62:**2850–2858.
3. **Allen, G. P., and M. R. Yeargan.** 1987. Use of λgt11 and monoclonal antibodies to map the genes for the six major glycoproteins of equine herpesvirus 1. J. Virol. **61:**2454–2461.
4. **Audonnet, J. C., J. Winslow, G. Allen, and E. Paoletti.** 1990. Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI, and gE. J. Gen. Virol. **71:**2969–2978.
5. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310:**207–211.
6. **Baines, J. D., and B. Roizman.** 1991. The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J. Virol. **65:**938–944.
7. **Baines, J. D., and B. Roizman.** 1993. The UL10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. J. Virol. **67:**1441–1452.
8. **Colle, C. F., III, C. C. Flowers, and D. J. O'Callaghan.** 1992. Open reading frames encoding a protein kinase, homolog of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the unique short segment of equine herpesvirus type 1. Virology **188:**545–557.
9. **Davis-Poynter, N., S. Bell, T. Minson, and H. Browne.** 1994. Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J. Virol. **68:**7586–7590.
10. **Flowers, C. C., R. N. Harty, and D. J. O'Callaghan.** 1992. Equine herpesvirus 1 glycoprotein D: mapping of the transcript and a neutralization epitope. J. Virol. **66:**6451–6460.
11. **Flowers, C. C., and D. J. O'Callaghan.** 1992. The equine herpesvirus type 1 (EHV-1) homolog of herpes simplex virus type 1 US9 and the nature of a major deletion within the unique short segment of the EHV-1 KyA strain genome. Virology **190:**307–315.
12. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus. Virology **52:**456–467.
13. **Highlander, S. L., W. Cai, S. Person, M. Levine, and J. C. Glorioso.** 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. **62:**1881–1888.
14. **Kyhse-Andersen, J.** 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10:**203–214.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Lehner, R., H. Meyer, and M. Mach.** 1989. Identification and characterization of a human cytomegalovirus gene coding for a membrane protein that is conserved among human herpesviruses. J. Virol. **63:**3792–3800.
17. **MacLean, C. A., L. M. Robertson, and F. E. Jamieson.** 1993. Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo. J. Gen. Virol. **74:**975–983.
18. **Matsumura, T., R. H. Smith, and D. J. O'Callaghan.** 1993. DNA sequence and transcriptional analyses of the region of the equine herpesvirus type 1 Kentucky A strain genome encoding glycoprotein C. Virology **193:**910–923.
19. **Meredith, D. M., J.-M. Stocks, G. R. Whittaker, I. W. Halliburton, W. B. Snowden, and R. A. Killington.** 1989. Identification of the gB homologues of equine herpesvirus types 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. J. Gen. Virol. **70:**1161–1172.
20. **Mettenleiter, T. C., and I. Rauh.** 1990. A glycoprotein gX-β-galactosidase fusion gene as insertional marker for rapid identification of pseudorabies virus mutants. J. Virol. Methods **30:**55–66.
21. **Osterrieder, N., J. D. Baines, A. Neubauer, and C. Brandmüller.** Unpublished data.
22. **Osterrieder, N., R. Wagner, C. Brandmüller, P. Schmidt, H. Wolf, and O.-R. Kaaden.** 1995. Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). Virology **208:**500–510.
23. **Pilling, A., A. J. Davison, E. A. R. Telford, and D. M. Meredith.** 1994. The equine herpesvirus type 1 glycoprotein homologous to herpes simplex virus type 1 glycoprotein M is a major constituent of the virus particle. J. Gen. Virol. **75:**439–442.
24. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:**7207–7221.
25. **Robertson, G. R., N. A. Scott, J. M. Miller, M. Sabine, M. Zheng, C. W. Bell, and J. M. Whalley.** 1991. Sequence characteristics of a gene in equine herpesvirus 1 homologous to glycoprotein H of herpes simplex virus. DNA Seq. **1:**241–249.
26. **Roizman, B., and A. E. Sears.** 1993. Herpes simplex viruses and their replication, p. 11–68. *In* B. Roizman, R. J. Whitley, and C. Lopez (ed.), The human herpesviruses. Raven Press, Ltd., New York.
27. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Ehrlich.** 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Inc., Boca Raton, Fla.
30. **Sullivan, D. C., G. P. Allen, and D. J. O'Callaghan.** 1989. Synthesis and processing of equine herpesvirus type 1 glycoprotein 14. Virology **173:**638–646.
31. **Telford, E. A. R., M. S. Watson, H. C. Aird, J. Perry, and A. J. Davison.** 1995. The DNA sequence of equine herpesvirus 2. J. Mol. Biol. **249:**520–528.
32. **Telford, E. A. R., M. S. Watson, K. McBride, and A. J. Davison.** 1992. The DNA sequence of equine herpesvirus-1. Virology **189:**304–316.
33. **Whalley, J. M., G. R. Robertson, N. A. Scott, G. C. Hudson, C. W. Bell, and L. M. Woodworth.** 1989. Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein B. J. Gen. Virol. **70:**383–394.
34. **Zhao, Y., V. R. Holden, R. N. Harty, and D. J. O'Callaghan.** 1992. Identification and transcriptional analyses of the UL3 and UL4 genes of equine herpesvirus 1, homologs of the ICP27 and glycoprotein K genes of herpes simplex virus. J. Virol. **66:**5363–5372.