A glycoprotein M-deleted equid herpesvirus 4 is severely impaired in virus egress and cell-to-cell spread

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To analyse the function of the equid herpesvirus 4 (EHV-4) glycoprotein M homologue (gM), two different mutated viruses (E4DgM-GFP and E4DgM-w) were generated. Both gM-negative EHV-4-mutants were characterized on complementing and on non-complementing cells and compared with E4RgM, a virus where gM-expression had been repaired. It was demonstrated in virus growth kinetics that deleting gM had a more dramatic influence on EHV-4 replication than expected. Extracellular infectivity was detected 9–12 h later than in EHV-4-infected Vero cells and titres were reduced up to 2000-fold. In addition, mean maximal diameters of plaques were less than 20 % of diameters of wild-type plaques. These results are in contrast to most other alphaherpesviruses, including the closely related equid herpesvirus type 1, where deletion of gM only marginally influences the ability of viruses to replicate in cell culture. Nevertheless, analysis of infected cells by electron microscopy did not reveal a specific defect for deleting gM. It was concluded that EHV-4 gM is important for more than one step in virus replication in cell culture, influencing both efficient virus egress and cell-to-cell spread.

INTRODUCTION

Infections of horses with equid herpesvirus type 4 (EHV-4) are clinically hard to distinguish from those with equid herpesvirus 1 (EHV-1) as both affect the respiratory system. An EHV-1 infection, however, can spread further and cause abortions or neurological disorder, whereas it is broadly accepted that EHV-4 replication remains mostly restricted to the respiratory tract (Allen & Bryans, 1986). Both viruses belong in the genus Varicellovirus within the subfamily Alphaherpesvirinae (Roizman & Pellet, 2001), and comparison of the complete DNA sequence of EHV-4 strain NS80567 to EHV-1 strain Ab4p revealed a high degree of conservation ranging between 55 and 96 % in individual genes (Telford et al., 1998). Various studies have assessed the molecular biology of EHV-1 replication but as yet only a few data have been obtained on the EHV-4 life cycle. To date only the functions of EHV-4 gE and gl have been addressed by studying recombinant viruses (Damiani et al., 2000b).

Amidst a large number of herpesviral glycoproteins, gM is remarkable because it is conserved through all herpesvirus subfamilies but not essential for replication of most alphaherpesviruses in cell culture e.g. herpes simplex virus 1 (HSV-1), pseudorabies virus (PRV) or EHV-1 (Baines & Roizman, 1991; Dijkstra et al., 1996; Osterrieder et al., 1996). However, merely deleting the respective sequences of Marek’s disease virus (MDV), a strictly cell associated alphaherpesvirus, abolishes virus growth in vitro (Tischer et al., 2002).

EHV-1 gM has been studied in some detail. It is thus known that the product of gene 52 is translated at late times post-infection (p.i.) into a 44 kDa precursor protein that is co- and post-translationally processed into a 50–55 kDa protein (Osterrieder et al., 1997). The glycosylated type III transmembrane protein is predicted to cross membranes eight times with the 95 aa C-terminal tail of the polypeptide passing into the cytoplasm of infected cells (Telford et al., 1992; Pilling et al., 1994; Seyboldt et al., 2000). The most prominent epitope(s) are apparently located within this tail-region (Osterrieder et al., 1996; Day, 1999). gM-oligomers are incorporated into virions and the protein physically interacts with the EHV-1 UL49-5 homologue.
(gene 10). The meaning of this interaction is not yet fully understood but it is important for the transport of gM into the trans-Golgi-network, and only the mature gM is fully functional (Rudolph et al., 2002). Similar complex formations between gM and the respective UL49-5 products have been described for PRV, bovine herpesvirus 1 (BoHV-1), Epstein–Barr virus and human cytomegalovirus (Jongs et al., 1998; Lake et al., 1998; Wu et al., 1998; Mach et al., 2000). In most herpesviruses analysed to date, deleting gM does not result in a marked phenotype in vitro, causing its specific function still to be a matter of discussion. Studying a gM, gE and gI triple deletion mutant in PRV, however, revealed a clear defect in secondary envelopment of particles (Brack et al., 1999). The interruption of gM sequences in the EHV-1 low passage strain RacL11 leads to a reduction in plaque sizes of about 50% and to a 50–100-fold decrease in extracellular virus titres. In contrast, deletion of gM in its highly passaged cell culture derivative, the modified live vaccine strain RacH, has only a minor influence on plaque sizes (reduction of about 10%) or production of infectious virus progeny (about 10-fold decrease; Osterrieder et al., 1996; Neubauer et al., 1997b; Seyboldt, 2000; Rudolph & Osterrieder, 2002). Again, removing gM from a virus background lacking expression of gE and gI resulted in a major impact on virus release and plaque phenotype. A block in secondary envelopment of virions at Golgi vesicles was reported, suggesting that gM or rather the gM/UL49 complex and the gE/gI complex play additive or partially overlapping roles in EHV-1 virus egress (Seyboldt et al., 2000). Based on this structure, formation of an ion channel by gM was discussed. However, EHV-1 gM by itself does not constitute an ion channel as assessed by transient transfection into Xenopus laevis oocytes (Osterrieder et al., 1997). Interestingly, comparing the protective potential of a gM-negative RacH to that of RacH itself in a mouse model revealed an increased immunogenicity of the recombinant virus, indicating another role of EHV-1 gM. It is hypothesized that the multiple membrane spanning protein might also serve as a ligand for molecules modulating immune responses (Osterrieder et al., 2001).

The aim of this study was to investigate the function of EHV-4 gM in cell culture. Using an anti-EHV-1 gM serum, EHV-4 gM was identified and its role assessed after deleting a major part of gM-sequences in a wild-type EHV-4 strain. A major decrease in plaque sizes and a substantial impact on the ability to replicate in non-complementing cells was noted. These data together with an analysis of infected cells by electron microscopy allowed the conclusion that the deletion of gM strongly affects mechanisms influencing virus egress and also cell-to-cell spread. This study also represents the first step towards understanding the differences in the replication cycle of EHV-1 and EHV-4.

METHODS

Plasmids. The complete open reading frame (ORF) encoding EHV-4 gM (1352 bp; Telford et al., 1998) was amplified by PCR (5’-GCCTCTAGATTACGTAGTACCTCTGC-3’; 5’-AAGGATCCATGGGACAGCTGGG-3’) and inserted into vector pcDNA1/Amp (Invitrogen), resulting in plasmid pCMgM. Plasmid pgM4 was generated after PCR amplification of nt 91699–94808 (5’-AATCTGCGAGGTCTAGTACGGCTAGTCG-3’; 5’-AAGAATCTCCGGAATACGGCTATG-3’; Telford et al., 1998) and cloning of the product in vector pGEM-3Zf(+) (Promega) (see Fig. 2a). To manipulate the EHV-4-genome by homologous recombination several constructs were made. A fragment of EHV-4 gM (1110 bp) was replaced by either a lacZ- or a GFP-cassette or deleted without insertion. To this aim, flanking regions of 983 and 1017 bp necessary for DNA recombination were amplified separately by PCR (5’-CCGAGATCCCTAAGAGAACCCTATAA-3’; 5’-AAGAATCTCCGGAATACGGCTATG-3’; 5’TGAAGT- CGACATTGGAATAGAAACTCG-3’; see Fig. 2a). Then, the 3-kbp E. coli lacZ-cassette was released from plasmid pbt264A+ (Metteletter & Raub, 1990) by BamHI and SalI digest and combined step-wise with both flanking regions into plasmid pgM4β+ (vector; pTZ18R; Pharmacia), such that the marker gene cassette was inserted between EHV-4-specific sequences.

Plasmid pgM4β+ was converted into plasmid pgM4w by removing the lacZ-cassette (SalI and BamHI), filling in 5’-overhangs with the Klenow polymerase and religating (see Fig. 2c). To avoid synthesis of a truncated gM-polypeptide a frameshift between the N-terminal 208 nt and the remaining C-terminal 33 nt of the gM-sequence was designed. Similarly, in plasmid pgM4GFP+ (see Fig. 2b) the lacZ-cassette of pgM4β+ was blunt-end replaced by the GFP-cassette, which had been removed from the vector pGFP-C1 (Clontech) via Asd and MluI digest. Correct amplification of all PCR products was confirmed by cycle sequencing (MWG Biotech) and comparison to the published sequence of EHV-4 strain NS80567 (Telford et al., 1998).

Cells and viruses. Vero cell clone C1008, Edmin337 cells and RK13 cells were maintained as previously described (Neubauer et al., 1997a). Recombinant cell line Vero-gM was generated by Effectene-mediated co-transfection of Vero-gM cells with plasmid pgM4GFP (vector; pTZ18R; Pharmacia), such that the marker gene cassette was inserted between EHV-4-specific sequences.

The EHV-4 strain used had been isolated from a horse with rhinitis from the USA. It was initially characterized and then passaged in Vero cells. The eleventh to thirteenth cell culture passages were used in this study. EHV-1 strain RacH and its recombinant derivative ΔAgMgfp+ were propagated on RK13 cells (Hubert et al., 1996; Seyboldt, 2000).

Purification of virions. Cells were infected at a m.o.i. of 0.5, harvested when the cytopathic effect was complete, and subjected to two rounds of freeze-thawing. Cell detritus was then removed by low-speed centrifugation. The virus suspension was carefully layered two rounds of freeze-thawing. Cell detritus was then removed by low-speed centrifugation. The virus suspension was carefully layered over a 30% sucrose cushion and centrifuged for 3 h at 23000 r.p.m. in a Beckman SW 28 rotor. This step was repeated once.

Generation of recombinant viruses. Initial experiments had shown that the GFP-marker was easier to handle when selecting for EHV-4 recombinants than the lacZ-marker. Homologous recombination into EHV-4 was therefore achieved by calcium phosphate mediated co-transfection of Vero-gM cells with plasmid pgM4GFP+ (see Fig. 2b) and EHV-4 DNA. Recombining sequences of plasmid pgM4w with DNA of the generated gM-negative EHV-4, ΔAgM-GFP, resulted in another mutant virus, ΔAgMw (see Fig. 2c). Finally, the gM-repaired EHV-4, ΔAgM (see Fig. 2a), was isolated after co-transfection of plasmid pgM4R with DNA of ΔAgM-GFP into Vero-cells. Supernatants of transfected cells were plated on the
respective cells and GFP-positive plaques were detected under a methocellulose overlay using an inverted fluorescence microscope (Axiovert; Zeiss).

**Southern blot and control sequence analysis.** Viral DNA was prepared from Vero or Vero-gM cells infected with the respective viruses by using standard protocols. Agarose gel electrophoresis and Southern blotting were done as previously described (Osterrieder et al., 1996). GFP-sequences, released from the vector pEFGP-C1 (Clontech) and the viral fragment of plasmid pgM4R (see Fig. 2a) were used as probes. The region encompassing the manipulated sequences within recombinant virus E4AgM-w was PCR amplified and the nucleotide sequence of the PCR product determined using primers depicted in Fig. 2(c).

**Western blotting and antibodies.** Cells were infected with the indicated viruses (m.o.i. of 1) and lysates prepared at the stated time points p.i. To inhibit synthesis of viral DNA, cells were infected and incubated for 24 h in the presence of phosphonoacetic acid (PAA; 0.5 or 1.0 mg ml⁻¹). To digest N-linked carbohydrates, virions suspended in deglycosylation buffer (Klupp et al., 1998) were incubated for 16 h in the presence or absence of PNGase-F (2 U; Roche Molecular Biochemicals). Samples were mixed with buffer containing 5% 2-mercaptoethanol (Sambrook et al., 1989) and then either heated to 99°C for 5 min or kept on ice. Proteins were separated by using either SDS-10% Tris/glycine PAGE or SDS-16.5% Tris/tricine PAGE (UL11) and blotted onto nitrocellulose filters. The monoclonal anti-EHV-1 gB antibody (3F6; Allen & Yeargan, 1987), anti-EHV-1 UL11 (Schimmer & Neubauer, 2003) and gM polyclonal rabbit antibodies (Seyboldt et al., 2000), and polyclonal anti-EHV-4 gE and gI mouse sera (Damiani et al., 2000a) were used in this study. Antibody binding was visualized using anti-species immunoglobulin G peroxidase conjugates (Sigma) followed by ECL detection (Pharmacia-Amersham).

**Virus growth kinetics and plaque size measurements.** Viruses were allowed to adsorb to Vero, Vero-gM or Edmin337 cells in 24-or 6-well plates for 90 min at 4°C. After another 90 min of virus penetration at 37°C, extracellular infectivity was inactivated using a citrate buffer (pH 3-0). At the indicated time points parallel samples of supernatants and infected cells were harvested. Cell samples were treated with citrate buffer to exclude contamination with extracellular infectivity, while supernatants were cleared of cellular debris by low-speed centrifugation. Intracellular and extracellular virus titres were compared by plaque titration on Rk13, Vero or Vero-gM cells, respectively.

Plaque sizes were determined on cells in 6-well plates infected with 50 p.f.u. per well of the respective viruses and incubated for 4 days under a methocellulose overlay. Plaques were screened, immunofluorescently labelled as described elsewhere (Schimmer & Neubauer, 2003) and digitally documented in an Axiovert microscope (Zeiss) or stained with crystal violet and measured. For each virus, maximum diameters of 150 randomly selected plaques were determined using a magnifying glass with a metric scale and mean sizes calculated. Measurements were compared with those of parental plaques that were set to 100%.

**Penetration assays.** Penetration assays were performed as previously described (Neubauer et al., 1997a). Briefly, 100 p.f.u. of the respective viruses were allowed to adsorb for 90 min to Vero cells. The assay was started by shifting the incubation temperature to 37°C and replacing supernatants with fresh medium. At different times after this shift, extracellular virus was inactivated with citrate buffer (pH 3-0) and parallel control samples were washed with PBS. The penetration efficiency was taken as the percentage of plaques present after citrate treatment relative to the number of plaques present after control treatment.

**Electron microscopy.** Vero or Edmin337 cells were infected at an m.o.i. of 1 with the different viruses. Cells were fixed at the indicated times p.i. for 2 h in 5% glutaraldehyde, 4% formaldehyde, buffered with 0.1 M sodium phosphate buffer to pH 7.4 and then washed with 0.1 M sodium phosphate buffer. Cells were post-fixed with 1% OsO₄, 0.8% K₃Fe(CN)₆ in 0.1 M sodium phosphate buffer for 2 h. All samples were stained with 2% aqueous uranyl acetate for 90 min, dehydrated in graded ethanol and finally embedded in ERL 4206 (Spurr, 1969). The blocks were sectioned on an Ultracut microtome, stained with uranyl acetate and lead citrate (Plattner & Zingsheim, 1987) and examined with a Philips CM10 transmission electron microscope at 80 kV.

**RESULTS**

**Identification of EHV-4 gM**

Although the predicted amino acid sequence of EHV-4 gM is calculated to be 86-7% identical to that of EHV-1 gM (Telford et al., 1998), anti-EHV-1 gM mAb (13B2) reacts in Western blot with the type-specific protein only (Crabb et al., 1991). Nevertheless to try to identify the EHV-4 homologue, additional anti-EHV-1 gM antibodies (Day, 1999; Seyboldt et al., 2000) were tested, but the reactivity of all mAbs against EHV-4 gM was below the detection limit in Western blot (data not shown). Only the polyclonal antiserum, which had been generated in rabbits against a His-tagged EHV-1 gM-derived polypeptide (aa 376–450; Seyboldt et al, 2000), specifically recognized the heterologous gM as well, albeit at low sensitivity (Fig. 1). According to its predicted hydrophobic properties the protein identified aggregated upon boiling (data not shown) and samples were therefore kept on ice throughout. Using this antiserum, in purified EHV-4 virions, specific reactivities at about 50–55 and 100–130 kDa were observed that decreased to about 40 and 80 kDa after removing N-linked carbohydrates with PNGase-F. The purity and quantity of these virion preparations were controlled in parallel blots probing against gB (Meredith et al., 1989; Sullivan et al., 1989; Fig. 1a). In lysates of Vero cells infected with EHV-4 several bands between approximately 44 and 55 kDa occurred. Similar to gB, expression of gM was inhibited in the presence of high concentrations of PAA, indicating that expression of EHV-4 gM was dependent on the synthesis of viral DNA (Fig. 1a). Taken together, although most gM-epitopes are clearly different between EHV-1 and EHV-4 and although the low sensitivity of the antiserum to EHV-4 gM hampered a conclusive comparison, processing of EHV-4 gM seemed very similar to that of EHV-1 gM.

**Generation of a gM-negative EHV-4 virus mutant**

To assess the function of gM in EHV-4 replication, sequences of plasmid pgM4GFP + (Fig. 2b) were inserted into EHV-4 DNA, such that the GFP-marker cassette replaced 82% of the gM-ORF. A homogeneous virus population, E4AgM-GFP, was plaque purified on cell line Vero-gM and initial observations indicated a dramatic
growth defect. Consequently, a gM-repaired virus, E4RgM (Fig. 2a), and another gM-deleted EHV-4 without gM-sequences (Fig. 2c) were designed. The latter, E4ΔgM-w, allowed the exclusion of a putative influence of the inserted marker gene sequences on the viral phenotypes, and was the result of recombining plasmid pgM4w into E4ΔgM-GFP. The genotypes of generated viruses were assessed by sequencing of respective PCR amplicons, by agarose gel electrophoresis of DNA fragments and by Southern blot analysis. DNA of EHV-4, E4RgM, E4ΔgM-w and E4ΔgM-GFP were cleaved with PstI, EcoRV or HindIII and fragments blotted onto nylon membranes. Parallel membranes were either hybridized with GFP-specific sequences or with a probe, gM3-1, containing the EHV-4-specific sequences taken out of the plasmid pgM4R (Fig. 2d). The results were as follows: (i) on DNA of E4ΔgM-GFP the GFP-probe recognized fragments of 5531 bp when PstI cleaved, of 8383 bp after EcoRV digest and of 4528 bp after HindIII digest. Identical fragments plus fragments of 1792 bp (PstI), 1801 bp (EcoRV) and 826 plus 5487 bp (HindIII) reacted with probe gM3-1. (ii) As expected, neither parental EHV-4, the repaired virus E4RgM nor E4ΔgM-w carried any GFP-specific sequences. (iii) The gM3-1 reactive DNA fragments in EHV-4 and E4RgM were detected at 6806 bp (PstI), at 7874 bp plus 1801 bp (EcoRV) and at 4837 bp plus 5487 bp (HindIII), respectively. (iv) The gM- and GFP-cassette-negative virus, E4ΔgM-w, did not hybridize with GFP-sequences, but with the respective EHV-4-specific sequences (gM3-1). The latter probe detected fragments, lacking 1110 bp of gM-sequences, when compared with wild-type virus, i.e. at 5696 bp (PstI), at 6764 bp plus 1801 bp (EcoRV) and at 3727 bp plus 5487 bp (HindIII), respectively. Taken together it was demonstrated that the genotypes of the generated viruses E4ΔgM-GFP, E4ΔgM-w and E4RgM were as expected.

**EHV-4 gM is important for cell-to-cell spread**

To compare expression of selected proteins, Vero cells infected with EHV-4, E4RgM, E4ΔgM-w or E4ΔgM-GFP were analysed by Western blotting or indirect immunofluorescence. In Fig. 1(b), it was shown that expression of gM was found in EHV-4- and E4RgM-infected cells only. Moreover, the deletion of gM-sequences did not detectably influence the production of gB, gE, gI or of the UL11-protein (Figs 1b, 3b), indicating not only that expression of other early-late EHV-4-proteins was not altered, but also specifically that the expression of the adjacent UL11-ORF was unaffected.

To compare plaque sizes, maximal diameters of plaques were measured on Vero or Vero-gM cells. Mean maximal diameters of infectious foci are given relative to sizes of EHV-4-induced plaques (Fig. 3). Interestingly in the absence of gM-expression, plaque diameters were reduced by more than 80 %. Photographs of representative immunofluorescently labelled (gE and gI) plaques illustrate this marked difference in size. In contrast, infection with the rescuant virus or of complementing Vero-gM cells resulted in wild-type phenotypes, suggesting that the major reduction in EHV-4 plaque diameters observed was indeed caused by deleting gM (Fig. 3a, c). Plaques produced on Vero cells

**Fig. 1.** Western blot analysis of EHV-4 gM (a). (Blots i, iv) Lysates of Edmin377 cells infected either with EHV-1 strain RaciH (24 h) or with EHV-4 (24 h), or respective virions purified of Edmin377 cells (EHV-4-vir; EHV-1-vir) were separated by SDS-PAGE (10 % Tris/glycine). Aliquots of samples were probed for gM (i) or gB (iv). (Blots ii, iii, v) EHV-4-virions were purified from Vero cells (EHV-4-vir), suspended in deglycosylation buffer, and incubated for another 16 h at 37 °C in the absence or presence of either PNGase-F (+NGF) or endoglycosidase H (+EH). Corresponding preparations of EHV-1-virions were diluted by factor 20 relative to that of EHV-4-virions. Vero cells were infected with EHV-4 (24 h p.i.) in the presence or absence of PAA (+0.5, +1.0 mg ml⁻¹). All samples were subjected to SDS-PAGE (10 % Tris/glycine) and probed for gM (ii) or gB (v). Blot (iii) represents a prolonged exposure of the respective part of blot (ii). (b) Vero cells were lysed 16 h p.i. with EHV-4, E4RgM, E4ΔgM-w or E4ΔgM-GFP, subjected to SDS-PAGE (10 % Tris/glycine) (gB or gM) or SDS-PAGE (18-5 % Tris/tricine) (UL11), and probed with anti-EHV-1 gB mAb 3F6, anti-EHV-1 UL11 or anti-EHV-1 gM serum. Blots were scanned and digitally processed. Prestained protein marker sizes are given (Biolabs).
by EHV-1 strain RacH or its gM-deleted derivative HA\(\text{gM}F\)P + (Seyboldt, 2000), however, were small but alike, corroborating the conclusion that the drastic effect of gM on the plaque phenotype was EHV-4-specific (Fig. 3b).

Deletion of gM severely impairs EHV-4 egress

To compare virus growth kinetics of the various mutant viruses, Vero cells in 24-well plates were infected at an m.o.i. of 2 and extracellular, and intracellular virus titres were determined separately at different times p.i. (Fig. 4a). Growth properties of the repaired virus E4\(\text{RgM}\) resembled those of EHV-4, whereas E4\(\Delta gM\)-w and E4\(\Delta gM\)-GFP presented with a marked growth defect. Within these experiments extracellular infectivity could not be detected before 24 h p.i. Even at 30 h p.i. only extremely low extracellular titres were observed, although corresponding cells clearly showed cytopathic effect. The reduction in intracellular infectivity was substantial as well. However, it never reached 100-fold (maximal 84-fold between EHV-4 and E4\(\Delta gM\)-w at 24 h p.i.) and detection was only delayed by one time point. EHV-4 and E4\(\text{RgM}\) as well as E4\(\Delta gM\)-w and E4\(\Delta gM\)-GFP behaved virtually indistinguishably, respectively; therefore only the data for EHV-4 and E4\(\Delta gM\)-GFP are depicted in the following experiments. To prove the direct link between this growth defect and gM expression, a similar kinetic was generated on Vero-gM cells, showing that the gM-expressing cells were significantly able to rescue virus growth (Fig. 4a). More experiments were dedicated to the question of whether this effect on virus replication was cell type- or virus-specific. Therefore, growth was also addressed on equine dermal cell line Edmin337 in 6-well plates. However, as shown at 24 h p.i., intracellular titres of gM deletion viruses were again reduced by about 100-fold, and no extracellular infectious virus progeny was detectable (Fig. 4b). In addition, although EHV-1 replication was clearly ineffective on Vero...

**Fig. 2.** Map of viruses and constructs generated in this study. (a) BamHI-map of EHV-4 strain NS80567 (Telford et al., 1998). The enlarged Psf fragment encompasses the UL10 (gM) and neighboring ORFs. Plasmid pgM4R containing 3113 bp of EHV-4 sequences and the depicted priming sites are described in the text. (b) Plasmid pgM4GFP + was used for the generation of E4\(\Delta gM\)-GFP, the GFP-positive and gM-negative EHV-4. Recombination of DNA of E4\(\Delta gM\)-GFP with plasmid pgM4R resulted in E4\(\text{RgM}\), the gM-repaired EHV-4 (a), and with plasmid pgM4w in E4\(\Delta gM\)-w, the GFP- and gM-negative EHV-4 (c). Priming sites used for control sequence reactions in E4\(\Delta gM\)-w are given (c). Restriction sites: BamHI – B, EcoRI – E, EcoRV – EV, HindIII – H, Psf – P, Sau3A – S. b – blunt-end. MCS – Multiple cloning site. (d) DNA of EHV-4 (1), E4\(\text{RgM}\) (2), E4\(\Delta gM\)-w (3) and E4\(\Delta gM\)-GFP (4) was cleaved with Psf, EcoRV or HindIII as indicated, and probed with either a GFP-specific probe or the EHV-4 fragment removed from plasmid pgM4R (gM3-1). DNA hybrids were detected by chemiluminescence using CSPD. Molecular mass marker sizes (Biolabs) are given in kbp.
cells, the influence of deleting gM in EHV-1 (Seyboldt, 2000) was minor relative to the effect in EHV-4 (Fig. 4c, shown at 24 h p.i.; m.o.i. of 0-02). In the absence of gM the ability of EHV-4 to directly infect adjacent cells was markedly reduced, as shown in Fig. 3. Therefore to analyse the effect of cell-to-cell spread on EHV-4 growth kinetics, replication was assayed after inoculation with different m.o.i. values. It was assumed that the effect of cell-to-cell spread on growth kinetics should increase with lower m.o.i. and longer incubation. To this aim, Vero cells in 6-well plates were infected with m.o.i. values of 0-5, 0-02 or 0-001 and resulting virus titres were measured at 24 and 48 h p.i. Representative data are given in Fig. 4(d). As the observed relative reduction (given as the factor of difference between titres) remained similar, no matter what the input m.o.i. had been, it could be concluded that the replication defect of gM-negative EHV-4 detected in these kinetics (Fig. 4a) was not appreciably related to the efficiency of cell-to-cell spread. Taken together, the presented series of experiments allowed us to conclude that the marked defect in EHV-4 replication was a specific consequence of the deletion of EHV-4 gM and that it was not Vero-cell-specific.

**EHV-4 gM marginally influences virus penetration**

As EHV-1 gM is involved in virus penetration (Osterrieder et al., 1996; Seyboldt et al., 2000), the influence of the structural protein on entry kinetics of EHV-4 was addressed. In the first set of penetration assays, E4ΔgM-w and E4ΔgM-GFP inocula had been propagated on non-complementing Vero cells and were therefore phenotypically gM-negative. A mean of 52-4% (50-3%) of the parental EHV-4 (E4RgM) was protected from extracellular acid treatment after 40 min of penetration, whereas only 36-2% (E4ΔgM-w) and 38-2% (E4ΔgM-GFP) of gM-negative viruses were protected, respectively. At later time points of kinetics, the penetration rates started to overlap. A slight delay in entry kinetics of E4ΔgM-w and E4ΔgM-GFP was thus observed, whereas gM-deleted viruses derived from complementing Vero-gM cells penetrated as fast as wild-type viruses (Fig. 5). When compared to the known reductions in entry kinetics of gM-negative EHV-1 of about 20% (strain RacL11; Osterrieder et al., 1996) to up to 40% (strain KyA; Seyboldt et al., 2000), the effect exerted by deleting gM of EHV-4 seemed minor and could not account for the drastic defects in replication reported above.

**Electron microscopical analysis of infected cells**

To define finally the reported major defect in EHV-4 replication more precisely, Vero or Edmin337 cells were infected with EHV-4, E4ΔgM-GFP, E4ΔgM-w or E4RgM at an m.o.i. of 1 and fixed at 14, 16-5 or 24 h p.i. The cytopathic effect was prominent in either approach and several observations were made by electron microscopical analyses: virus particles were identified in about 70% of the sections screened (at 24 h p.i. in Edmin337 cells; E4ΔgM-GFP – 66%, EHV-4 – 73%). Regardless of what virus had been used for infection and at what time p.i. the cells had been fixed, all stages of particle formation could be identified, ranging from different types of capsids inside the nucleus to mature particles in vesicles within the cytoplasm (examples in Figs 6 and 7). Complete virions outside cells were rarely observed after wild-type infection, such that the complete absence of free particles at mutant-virus-infected cells seemed hard to interpret. Because no clear difference in any step of maturation was obvious, particles were counted in about 20 sections of Edmin337 cells infected with EHV-4 or E4ΔgM-GFP (14 and 24 h p.i.).
As demonstrated in Table 1, with the exception of extracellular particles (14 h p.i.), no statistically significant differences between the respective numbers of particles in various compartments could be discerned. Taken together, although gM is functionally important for EHV-4 egress and cell-to-cell spread – as has been demonstrated in this study – no clear morphological correlate could be identified in infected cells, thus suggesting that gM might not play just one simple role at some specific step but might rather facilitate the overall process.

**DISCUSSION**

None of the EHV-1 gM mAbs tested in this study reacted in Western blot analyses with the EHV-4-protein. Only a polyclonal serum (Seyboldt et al., 2000) detected the heterologous gM, albeit at clearly reduced sensitivity. It can thus be assumed that the gM proteins of EHV-1 and

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**Fig. 4.** Replication of EHV-4, E4RgM, E4ΔgM-w, E4ΔgM-GFP. (a) Vero, Vero-gM cells or (b) Edmin337 cells were infected with an m.o.i. of 2 of EHV-4 (■; 1), E4RgM (○; 2), E4ΔgM-w (×; 3), or E4ΔgM-GFP (▲; 4), as indicated. Kinetics of virus growth are depicted as virus titres (p.f.u. ml⁻¹) determined in supernatants of infected cells (extracellular) or within infected cells (intracellular) relative to the time point indicated. The values given in (b) represent a 24 h growth on Edmin337 cells. (c) Vero cells were infected (m.o.i. of 0-02; 24 h p.i.) with EHV-4 (1), E4ΔgM-GFP (4), EHV-1 strain RacH (5) or the gM-negative EHV-1, H3αMGFP+ (6), and resulting extracellular and intracellular infectivity are shown. (d) Vero cells were infected with EHV-4 or E4ΔgM-GFP at the indicated m.o.i. values (0-5, 0-02 or 0-001), and titres were compared after incubating for 24 or 48 h. Numbers above the bars give the factors of difference between E4ΔgM-GFP titres and EHV-4 titres. The respective means of two individual experiments are shown; standard deviations are given as error bars above symbols.

**Fig. 5.** Penetration kinetics of EHV-4 (■), E4RgM (○), E4ΔgM-w (×) or E4ΔgM-GFP (▲). Vero cells in 6-well plates were infected with 100 p.f.u. of the indicated viruses and penetration assays performed as described in the text. Penetration was stopped at the given times p.i. and resulting plaques counted 4 days p.i. Virus stocks were either prepared using Vero (a, non-complemented) or Vero-gM cells (b, complemented).
Fig. 6. Electron micrographs of sections of Edmin337 cells infected with EHV-4 (16·5 h p.i.). (a) An overview of a section of the infected cell is shown. (b) All classes of capsids were observed within the nucleus. (c, d) Particles budding in vicinity to the Golgi network and within vesicles are shown. Mature particles were found adjacent to sections of cells. Arrows indicate capsids, arrowheads point out enveloped particles. Bars, 500 nm (a) or 250 nm (b–d); nu, nucleus; *, elements of the Golgi network.

Fig. 7. Electron micrographs of sections of Edmin337 cells infected with E4ΔgM-w (16·5 h p.i.). (a) An overview over a section of the infected cell is depicted. (b) Normal sets of capsids were observed within the nucleus. (c, d) Enveloped and budding particles were found within sections of cells. Arrows indicate capsids, arrowheads point to enveloped particles. Bars, 500 nm (a) or 250 nm (b–d); nu, nucleus; *, elements of the Golgi network.

Table 1. Relative number of herpesvirus particles counted in sections of Edmin337 cells infected with EHV-4 or E4ΔgM-GFP

<table>
<thead>
<tr>
<th>Particle</th>
<th>14 h p.i.</th>
<th>24 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EHV-4 (%)</td>
<td>E4ΔgM-GFP (%)</td>
</tr>
<tr>
<td>Nuclear</td>
<td>57·2</td>
<td>55·4</td>
</tr>
<tr>
<td>In perinuclear space</td>
<td>1·0</td>
<td>2·3</td>
</tr>
<tr>
<td>Enveloped*</td>
<td>12·1</td>
<td>10·7</td>
</tr>
<tr>
<td>’Naked’†</td>
<td>28·4</td>
<td>31·6</td>
</tr>
<tr>
<td>Extracellular</td>
<td>1·3‡</td>
<td>0·0‡</td>
</tr>
</tbody>
</table>

*Virion particles within vesicles in the cytoplasm were counted as enveloped particles. No distinction was made between primarily enveloped and mature virions.
†Capsids within the cytoplasm were listed as ‘naked’ particles. It was not discerned whether particles were in proximity to membranes or not.
‡Differences were significant as determined by pairwise testing of counted numbers according to Student’s t-test (P<0·05).
EHV-4 carry more than one linear epitope between aa 376–450 and that major epitopes have to be distinct, although the amino acid sequences of EHV-1 and EHV-4 gM polypeptides are 86-7% identical (Telford et al., 1998). Using this antiserum, several forms of EHV-4 gM were identified, and the structural characteristics seemed similar to that of EHV-1 gM. Apparently, the second putative N-glycosylation motif within gM sequences of EHV-4 (one in EHV-1 gM) seemed of no particular influence. Initial observations, however, suggested differences in the efficiency of glycosylation between Edmin337 and Vero cells.

The primary focus of this study was to elucidate the function of EHV-4 gM. The gM deletion virus isolated was unexpectedly growth deficient, and to avoid an effect of the GFP-expression another recombinant EHV-4 without GFP-marker sequence was generated. In further experiments a set of four viruses was thus compared and it was readily observed that neither GFP-expression nor undetected mutations were of particular influence. These conclusions were sustained by demonstration of good complementation on Vero-gM cells, which constitutively express EHV-4 gM. Moreover, it was shown that expression of the UL11 product, which is encoded adjacent to gM (UL10), was unaffected as well as that of other structural proteins. If the expression of the second adjacent ORF, UL9, had been reduced, a general reduction in expression of late proteins would have been expected, as the HSV-1 UL9 homologue is known to be the essential DNA origin binding-protein (Roizman & Knipe, 2001).

Taking all data presented in this study into consideration, it could be concluded that EHV-4 gM is important for both virus egress and cell-to-cell spread. As the deletion only marginally influenced virus penetration and as expression of the protein depended on synthesis of viral DNA, newly synthesized gM could only function late in infection. Plaque sizes, which are generally taken as a surrogate marker for efficiency of cell-to-cell spread, were drastically diminished in the absence of gM, and irrespective of cell-to-cell spread an important function in assembly and egress was demonstrated. Assuming that similar to replication of other alphaherpesviruses in vitro (Johnson & Huber, 2002), the processes of EHV-4 cell-to-cell spread and virus egress are at least partially distinct in Vero cells, these data suggest that the replication defect in gM deletion mutants in cell culture involves a mechanism necessary for both pathways, or completely different mechanisms exerted by a multifunctional gM, or an early step in assembly influencing both processes. The latter appears to be less probable as one would then have expected to note a morphological correlate in ultra-thin sections of infected cells.

Deleting gM had a more pronounced effect on EHV-4 replication than reported for EHV-1. The question of whether this observation really was EHV-4-specific was addressed by using two different cell lines, Vero and Edmin337, in various experiments, thereby showing that the growth behaviour of gM-deleted EHV-4 was independent of the cell line used. Also, the growth disadvantage of the gM-deleted EHV-1, HAgMGFP+, was similar on Vero cells to what had been reported on Rk13 cells (Seyboldt, 2000), again corroborating the virus specificity of the observations in this study.

Apparently, EHV-4 gM is more important for a carefully balanced set of functional and structural interactions than the respective homologues in most other herpesviruses. Only the gM-homologue of the strictly cell associated MDV is essential for virus replication (Tischer et al., 2002). Therefore, it could be hypothesized that EHV-4 might be more cell-associated than EHV-1. Further experiments will be necessary to address such speculations. In some alphaherpesviruses, including EHV-1, the simultaneous deletion of gM, gE and gI increases the observed defects in plaque formation and virus growth, demonstrating that these three proteins fulfill somewhat overlapping functions in secondary envelopment (Brack et al., 1999; Seyboldt et al., 2000), but none is essential by itself. One of the reasons to initiate electron microscopic analysis was the idea that gM alone might be so important for secondary envelopment in EHV-4 that an accumulation of particles in the Golgi area might occur. This was not the case within the settings of our study. With the exception of a complete absence of extracellular particles, no distinct phenotype could be discerned in sections of cells infected with the gM-mutants. EHV-4 gM must therefore be important for more than just secondary envelopment. As gM probably spans membranes several times it might thereby influence or stabilize them, and it is tempting to assume a function in all steps of virus replication involving fusion of virus envelopes and cellular membranes. Although exact mechanisms are still unknown, fusion is certainly important in virus penetration, assembly of particles, egress and also cell-to-cell spread. EHV-1 and PRV gM have been shown to prevent syncytia formation efficiently in a PRV membrane fusion assay (Klupp et al., 2000) and thus directly to affect fusion of membranes, and both the EHV-1- and EHV-4-homologues have been shown to, at least slightly, facilitate virus penetration. As electron microscopic analyses can only give pictures of selected moments in replication, the stability, efficiency or even directionality of membrane fusion events might not be reflected in these analyses. In summary, a surprisingly marked replication defect was shown after deleting EHV-4 gM, strongly influencing the efficiency in cell-to-cell spread and virus egress. In combination with analyses of sections of infected cells by electron microscopy, it was demonstrated that the functions of EHV-4 gM are even more complex than had been assumed.

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