The equine herpesvirus 1 UL11 gene product localizes to the trans-Golgi network and is involved in cell-to-cell spread

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Abstract

Experiments were conducted to identify and characterize the equine herpesvirus type 1 (EHV-1) UL11 homologous protein. At early-late times after EHV-1 infection of Rk13 cells several proteins at an Mr of 8000 to 12,000 were detected using a UL11 protein-specific antiserum. Particularly, an Mr of 11,000 protein was found abundantly in purified virions and could be assigned to the tegument fraction. As demonstrated by confocal laser scanning microscopy, UL11 reactivity localized predominantly to the trans-Golgi network of infected cells, but was also noted at the plasma membrane, specifically of transfected cells. Deletion of UL11 sequences in EHV-1 vaccine strain RacH (H11) and in the virulent isolate RacL22 (L11) resulted in viruses that were able to replicate on noncomplementing cells. It was shown in one-step growth kinetics on Rk13 cells that the reduction of intracellular and of extracellular virus titers caused by the absence of UL11 expression in either virus was somewhat variable, but approximately 10- to 20-fold. In contrast, a marked influence on the plaque phenotype was noted, as mean maximal diameters of plaques were reduced to 23.2% (RacL22) or 34.7% (RacH) of parental virus plaques and as an effect on the ability of RacH to cause syncytia upon infection was noted. It was therefore concluded that the EHV-1 UL11 product is not essential for virus replication in Rk13 cells but is involved in cell-to-cell spread.

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INTRODUCTION

Equine herpesvirus type 1 (EHV-1), an important pathogen of horses, is a member of the subfamily Alphaherpesvirinae (Allen and Bryans, 1986). The pathway of Alphaherpesvirus assembly has been subject of discussion for some time and two main models were suggested (Roizman and Knipe, 2001; Mettenleiter, 2002). Recent findings favor that the maturation of most members of the subfamily involved at least two distinct steps of tegumentation and envelopment: Budding of newly formed capsids at the inner nuclear membrane results in the acquisition of a primary envelope that is then lost by fusion with the outer nuclear membrane. Capsids are thus released into the cytoplasm and become completed finally to mature virions by budding into vesicles of the trans-Golgi network (TGN) (Mettenleiter, 2002). To allow efficient assembly, numerous herpesviral structural proteins have to come together in an ordered fashion at defined cellular compartments. Various Alphaherpesvirus gene products have been proposed to play some role in egress, but as yet only few have been associated with a definite function (Sun et al., 1996; Osterrieder et al., 1998; Oettler et al., 2001; Roizman and Knipe, 2001; Mettenleiter, 2002). The understanding of assembly and egress is complicated additionally by the fact that mechanisms can differ not only between members of the subfamily but also between cell systems used (Baines et al., 1991; Baines and Roizman, 1992; Fuchs et al., 1997). Furthermore, functions of viral gene products can be connected to others or even redundant in vitro, such that only deleting simultaneously several genes might reveal a clear phenotype—a phenomenon that has for example been reported for glycoproteins M,
E, and I, that are all involved in secondary envelopment (Brack et al., 1999; Seyboldt et al., 2000).

The herpes simplex virus 1 (HSV-1) UL11 protein is a small, phosphorylated, palmitoylated, and myristoylated virion protein, forming up to five bands in polyacrylamide gels (MacLean et al., 1989; Baines et al., 1995; Loomis et al., 2001). The UL11 product is suggested to reside in the viral tegument and is associated with membranes of infected cells, although the structural prerequisites for a transmembrane anchor are not given (MacLean et al., 1992). By immune electron microscopic analyses UL11 reactivity was found in various undefined cytoplasmic ribbon-like structures or membranes but especially at the inner nuclear membrane and also in distinct sites within the nucleus (Baines et al., 1995). In contrast, when expressed in the absence of other HSV-1 proteins, the UL11 protein has been demonstrated to be targeted to the Golgi apparatus, exhibiting highly dynamic membrane-trafficking properties. The protein was shown to recycle from the plasma membrane to Golgi membranes mediated by a conserved acidic amino acid cluster. The relevance of myristylation and in particular palmitylation for a strong association with cellular membranes and for a specific localization to the Golgi apparatus has been emphasized. As the Golgi region represents the compartment of secondary envelopment, a role of the UL11 protein in virus assembly has been discussed (Bowzard et al., 2000; Loomis et al., 2001).

UL11-negative HSV-1 induce smaller plaques and show reduced growth in vitro, reaching a final yield 5- to 20-fold (MacLean et al., 1992) or 30- to 280-fold less than parental viruses relative to the cell systems used (Baines and Roizman, 1992). In Vero cells, as analyzed by Baines and Roizman (1992), a markedly delayed and reduced production (over 1000-fold) of extracellular virus titers was found, corroborating their electron microscopic data showing capsids accumulating at the inner nuclear membrane and increasing numbers of naked particles in the cytoplasm. The HSV-1 UL11 is therefore thought to influence budding at the inner nuclear membrane and to facilitate transport of virions into the extracellular space (Baines and Roizman, 1992). As UL11 reactivity was also found within distinct areas of the nucleus, again more as yet unidentified functions were suggested (Baines et al., 1995). Generally, the gene products of the UL11 homologs of the Alphaherpesvirinae exhibit low-sequence similarity, but the myristylation consensus sites and the presence of several cysteine residues in the amino-terminal regions of the polypeptides appear to be highly conserved and might indicate a common function of the UL11 homologs (Davison and Scott, 1986; Dijkstra et al., 1997; Telford et al., 1998; Osterrieder, 1999; Bowzard et al., 2000). So far, only the function of the HSV-1 UL11 protein has been investigated and discussed.

In this study, experiments were conducted to identify and analyze the EHV-1 UL11 homologous protein. Using a rabbit antiserum, several UL11 products were identified in infected as well as in transfected cells. The EHV-1 UL11 protein was abundantly detected within the tegument fraction of virions and shown to colocalize mainly with the trans-Golgi network of EHV-1-infected cells. The function of the protein was investigated after generating UL11 deletions in EHV-1 strains RacL22 and RacH. RacH does exhibit a more pronounced syncytial phenotype in cell culture than its low-passage derivative RacL22 (Neubauer et al., 1997) and therefore enabled us to assess a putative influence of the UL11 product on syncytia formation. Functional analysis revealed an impact, albeit minor, of the UL11 deletion on virus replication in cell culture and a prominent decrease in plaque size, suggesting a role in cell-to-cell spread of viruses.

RESULTS

Identification of the EHV-1 UL11 homologous protein

To identify the product of the UL11 homologous EHV-1 open reading frame (gene 51), an anti-UL11 serum was generated by immunizing rabbits against a purified UL11-mycHis fusion protein. The specificity of the anti-UL11 serum was ascertained (e.g., Fig. 1A, Fig. 3A) and the reagent then used in Western blot analyses of lysates of RacH or RacL22-infected Rk13 or Edmin337 cells. In the following series of experiments mostly the data obtained using EHV-1 wild-type strain RacL22 on Rk13 cells are shown. After separation of samples by 16.5% Tris–Tricine–polyacrylamide gel electrophoresis (PAGE), the UL11 antiserum bound specifically to several polypeptides in the range of Mr of 8000 to 12,000. In time-course experiments expression kinetics of the UL11 products were compared to that of the well-characterized EHV-1 glycoprotein B (gB). Similar to gB expression (Fig. 1B) UL11 specific reactivity was detected as early as 5 h postinfection (p.i.), and its intensity increased until late in infection (Fig. 1C). At least four different UL11 species were noted, the most prominent of which running at an Mr of 11,000 (Figs. 1A and 1C). When de novo viral DNA synthesis was inhibited by the presence of 0.5 to 1.0 mg of phosphono acetic acid (PAA) per milliliter of cell-culture medium, the expression of UL11 products was reduced but not completely precluded, again paralleling the expression kinetics of the early-late gB (data not shown). It was thus confirmed that the EHV-1 UL11 products belonged into the early-late class of protein expression.

The EHV-1 UL11 protein is tegument associated in extracellular virions

A strong UL11-specific signal was observed in Western blot analysis of EHV-1 virions that had been purified from supernatants of infected cells (Fig. 2A, 15% Tris–Glycine PAGE). The purity of all preparations and the integrity of virions had been controlled by electron microscopy (data...
not shown) and on parallel blots using anti-gB mAb 3F6 (Fig. 2A), taking advantage of the fact that only the fully processed form of gB is included into virus particles (Sullivan et al., 1989), or the anti-UL34 serum (Fig. 2B), using its reactivity with intracellular but not extracellular virions (Neubauer et al., 2002). To address the localization of the UL11 protein within virion structures, viral envelopes were separated from capsids after incubation with 1% Triton X-100 and both fractions were analyzed individually by Western blot. The major capsid protein (MCP) was detected only in the capsid-enriched pellet and the envelope protein gB in the envelope containing supernatant, whereas a similar intensity of UL11 reactivity was revealed in either sample (Fig. 2A). The strict separation of reactivity in control blots and the presence of UL11 in both virion fractions suggested a classification as a tegument protein of EHV-1. As obviously 15% Tris–Glycine PAGE does not resolve the different UL11 species (Fig. 2A), purified virus particles were probed in parallel after 16.5% Tris–Tricine PAGE and the exact banding pattern of UL11 reactivity in virions was determined. As shown in Fig. 2B mainly the Mr 11,000 form of UL11 was detected accompanied by a less intense protein at an Mr of 20,500 (Fig. 2B). As all samples have always been heat denatured, infected cells as well as purified virions, the Mr 20,500 protein species could reflect very stable higher order structures of either UL11 products alone or complex formation of UL11 with another viral protein. This interaction could theoretically also be mediated by putative fatty acid modifications. The Mr 20,500 band corresponded in size to formation of UL11 homodimers, but further experiments will be necessary to understand the composition of this species after all.

**Subcellular localization of the EHV-1 UL11 protein**

Although the overall homology of the known herpesviral UL11 polypeptides is low, encoding for myristylation consensus sequences and for cysteine residues that could serve for palmitylation appears to be conserved. The HSV-1 UL11 protein has been shown to indeed be myristylated and palmitoylated (MacLean et al., 1989; Loomis et al., 2001). Accordingly, the EHV-1 UL11 sequence carries corresponding motifs and no features are found within the 74 amino acid polypeptide, allowing the prediction of a putative transmembrane anchor. If the protein was membrane associated in infected or transfected cells, this should then be mediated by myristyl and palmitoyl anchoring or interaction. To determine the subcellular localization of the EHV-1 UL11 protein, RacL22- and un-infected Rk13 cells were separated into fractions, containing either nucleic, membranous, or soluble cytoplasmic components (Bogner et al., 1992). In Western blot analysis of these fractions UL11 reactivity was discovered principally with cellular membranes, just like the integral membrane protein gB, and both were found to a lesser extend in the nucleic fraction (data not shown). To analyze further the distribution of the UL11
product, Rk13 cells expressing UL11 at different times p.i. (RacL22) and cells infected with the UL11-negative virus LΔ11 (see below) were subjected to indirect immunofluorescence analysis, using the anti-UL11 serum and an Axioskope (Fig. 3A, green fluorescence). Since these initial tests indicated a UL11-specific cytoplasmic staining pattern in infected cells, other experiments were conducted to identify the compartment of enhanced UL11 presence. As shown in Fig. 3B, confocal laser scanning microscopic analysis of Rk13 cells (9 h p.i.), that were simultaneously stained for the trans-Golgi network marker protein γ-adaptin and for the UL11 protein, suggested a specific concentration of UL11 reactivity in the area of the TGN. In Rk13 or Cos7 cells transfected with either UL11-expression plasmid pC11 or pC11myc the localization to the TGN region was conserved, but a more diffuse pattern was noted. An example of pC11-transfected Rk13 cells is depicted (Fig. 3C), demonstrating that UL11 reactivity was observed throughout cells and somewhat concentrated at the plasma membrane. The fluorescence pattern was similar after transfection of either plasmid into either cell line, but appeared to be relative to the amount of UL11 protein expressed in the individual cell—the brighter the fluorescence, the less TGN specific.

As the current model of EHV-1 egress indicates a final assembly of virus particles by budding into vesicles of the Golgi area, the enhanced presence of the UL11 protein in this area could either simply reflect the necessity to pile up for incorporation into particles or point out a function of the UL11 protein at this process.

**Generation of UL11-negative RacL22 and RacH**

To investigate the function of the UL11 protein in the replication cycle of EHV-1 strains RacL22 and RacH, 67.4% of UL11 sequences each were replaced by the *Escherichia coli* LacZ cassette taken out of plasmid pTT264A+ (Mettenleiter and Rauh, 1990). As the UL12 homologous ORF partially overlaps with the UL11 ORF (Fig. 4B), sequences encoding for aa 1 to 24 of the UL11 polypeptide had to be left intact (Fig. 4C). To ensure additionally correct

![Fig. 2. Western blot analysis of RacL22 virions and of subviral fractions. (A) Virions purified from supernatants of RacL22-infected cells were incubated in 1% Triton X-100 and then separated into a pellet and a supernatant fraction. Parallel blots were reacted with the anti-UL11 serum (15% Tris–Glycine PAGE), with anti-gB mAb 3F6 or with mAb ZB4, directed against the EHV-1 major capsid protein (MCP; 12% Tris–Glycine PAGE). (B) UL11 reactivity of purified virions was compared to that of lysates of RacL22-infected cells (14h p.i.) after 16.5% Tris–Tricine PAGE. The same samples were assayed for UL34 reactivity after 12% Tris–Glycine PAGE. Sizes of a precision molecular weight marker (Bio-Rad) are indicated in thousands.](image-url)
termination of the UL14 to UL12 transcripts, that all do
coterminate with the UL11 transcript, extra polyadenylation
signal sequences were inserted between the stop codon of
UL12 and the marker gene cassette (Fig. 4C). The adjacent
transcripts should thus only be minimally changed, i.e.,
shortened by 48 nucleotides. Recombination was achieved
in Rk13 cells after cotransfection of plasmid pΔ11LacZ+
that was used for the generation of HΔ11 and LΔ11 is described under Materials and Methods. Restriction sites
are as follows: BamHI, B; HinIII, H; EcoRI, E; PstI, P; KpnI, K; SalI, S.

The EHV-1 UL11 protein is involved in cell-to-cell spread

Several experiments were conducted comparing proper-
ties of the different generated recombinant viruses to that of
parental viruses. First, Rk13 cells infected with RacL22,
LΔ11, LΔ11R, RacH, HΔ11, or HΔ11R were lysed 14 h p.i.
and expression of UL11 and of other early-late proteins was
judged by Western blot analysis. As shown in Fig. 5, no
UL11 specific reactivity at all was found in Rk13 cells
infected with the UL11-deleted EHV-1. These results al-
lowed the conclusion that the mutated viruses did not syn-
thesize any detectable aberrant or truncated UL11 product.
Moreover, other structural proteins, such as gB, gD, and
gM, were noted at similar levels, no matter whether UL11
was present or not. Apparently, a comparable amount of
cells had been infected initially and viral gene products
were produced efficiently. In a next step, kinetics of virus
growth were assessed. To this end, confluent monolayers of
Rk13 cells were infected at an m.o.i. of 3-5 and at different
times p.i. virus titers of supernatants (extracellular) and of
infected cells (intracellular) were determined independently
(Fig. 6). As expected, growth kinetics of the rescuant vi-
ruses LΔ11R or HΔ11R were virtually undistinguishable
from those of the parental viruses RacL22 and RacH. In
contrast, both UL11 negative viruses replicated constantly
to slightly reduced levels. The maximum reductions in virus
titers were detected in kinetics of extracellular RacH infec-
tivity (83-fold at 12 h p.i.). Titer decrease in RacL22 kinet-
ics never exceeded 25-fold (LΔ11 to LΔ11R at 9 h p.i.,

Fig. 3. Rk13 cells grown on glass coverslips were either infected with EHV-1 strain RacL22, the UL11 negative virus LΔ11 (A, B), or transfected with plasmid pC11 (C) and acetone-fixed. Times of fixation are given in A, whereas cells were analyzed at 9 h p.i. or at 48 h post transfection in B and C, respectively. The UL11 protein was visualized using the anti-UL11 serum followed by an anti-rabbit Alexa 488 secondary antibody (green). The EHV-1 glycoprotein 2, detected with mAb 3B12 (A), or the trans-Golgi marker protein γ-adaptin, detected with an anti-γ-adaptin mAb (Sigma) (B), were visualized by the Alexa 546 secondary antibody (red). Blue fluorescence (A) was caused by DAPI staining of nuclei of LΔ11-infected Rk13 cells. Cells were either analyzed by conventional microscopy (A) or by confocal laser scanning microscopy (B, C). Pictures were digitally processed.
extracellular) and mostly ranged between 5- and 20-fold. Likewise, the relation of virus yields did not change markedly when lower m.o.i. (0.1 or 0.01) was used for infection. The reduction in titers of UL11 negative viruses compared to wild-type viruses was then noted to be between 1.2- and 20-fold (tested at 15 h p.i., data not shown). It was deduced that neither virus suffered a severe growth defect after deleting UL11 expression. Then, diameters and phenotypes of RacL22-, LΔ11-, LΔ11R-, RacH-, HΔ11-, or HΔ11R-induced plaques were analyzed on Rk13 cells, and representative plaques are depicted in Fig. 7B. Maximal diameters of 150 plaques each were measured, means calculated, and results of parental viruses, i.e., RacH and RacL22, were set to 100%, respectively. As graphically demonstrated in Fig. 7A, maximal plaque diameters were drastically reduced for both UL11-deleted viruses. Without UL11 expression plaques reached only 23.2% (RacL22) or 34.7% (RacH) of maximal diameters of parental plaques, whereas the plaque size of the rescuant viruses corresponded well to wild-type. When plaques were analyzed on cell-line 51D3 that constitutively expresses a UL11-mycHis fusion protein, virtually no differences were observed between parental plaques and plaques of LΔ11 and HΔ11, underlining that the reported phenotype was solely due to the deletion of UL11 sequences and not to alterations of adjacent transcripts (Fig. 7A, 51D3). Taken together, both UL11-deletion mutants replicated quite efficiently in Rk13 cells, but when infection was directed from cell-to-cell, the deletion of UL11 resulted in a marked disadvantage of the recombinant viruses. In the next series of experiments, Rk13 cells were infected with RacH, HΔ11, or HΔ11R at an m.o.i. of 1. At 14 h p.i. cells were fixed, scraped off the support, pelleted, and embedded. Semithin sections of cell pellets were stained by Toluidine blue and the phenotypes of un-infected and infected cells were compared. To demonstrate specific and comparable EHV-1 infection of cells, parallel samples were infected accordingly but then fixed and processed by indirect immunofluorescence analysis (IIF), using anti-gp2 mAb 3B12.
In Fig. 8 pictures of IIF-processed cells are given next to examples of the respective semithin sections. In RacH- or HΔ11R-infected Rk13 cells numerous large multinucleated cells were evident when stained by IIF and especially in semithin sections. However, after infection with HΔ11—although cells clearly showed all typical signs of infection and expressed gp2 to high levels—rarely more than one nucleus accumulated per cell. Over 1000 nuclei each were counted in pictures of semithin sections, indicating that up to 10% of RacH-infected Rk13 cells carried three or more nuclei, whereas none such cells were found after HΔ11 infection. Because these results somewhat implicated an influence of the UL11 protein on RacH-induced membrane fusion events and as UL11 is a virion component, albeit of the tegument, the possibility of an effect on virus entry kinetics was also assessed. But no delay in virus penetration of HΔ11 compared to RacH could be observed (data not shown). Taken together, it was shown in this study that the deletion of the UL11 homologous gene 51 from EHV-1 strains RacL22 and RacH led to a minor defect in overall virus replication in cell culture and especially to a prominent decrease in cell-to-cell fusion and the spreading of infectivity to neighboring cells.

Discussion

In this article, data are presented indicating that the UL11 homolog of equine herpesvirus 1 is an early-late protein, which is abundantly found in extracellular virions. It was also shown that the EHV-1 UL11 protein localizes to cellular membranes, specifically to the TGN region of infected cells. Through functional analysis of UL11-negative viruses it was demonstrated that the EHV-1 UL11 homolog is not essential for virus replication in cell culture, but that its deletion has a remarkable effect on plaque sizes in Rk13 cells. Finally, an impact of deleting UL11 on the ability of the EHV-1 strain RacH to fuse Rk13 cells was observed. The EHV-1 UL11 ORF (gene 51) is predicted to encode an Mr 8400 polypeptide. After 16.5% Tris-Tricine PAGE several species between Mr 8000 and 12,000 were detected in RacL22 as well as in RacH-infected cells, using an anti-
serum that was produced in rabbits against the complete UL11 polypeptide. The addition of a mycHis-tag to the carboxyl-terminus of the UL11 sequence resulted in an impressive increase of the apparent \( M_r \) to an \( M_r \) between 13,000 and 18,000 (data not shown), indicating that generally changes in the UL11 polypeptide can have a remarkable effect on its running properties in PAGE. It is therefore difficult to assign the different species of UL11 reactivity yet, other than that the polypeptide has to be somehow modified. The minor species, that is running at an \( M_r \) of about 8000, could stand for an unprocessed backbone of UL11. Furthermore, the sequence of the EHV-1 UL11 homolog does carry a conserved myristylation consensus sequence (M-G-X-X-S/T) (Resh, 1999), as has been shown to be used in the HSV-1 homologous protein (MacLean et al., 1992). If myristylation occurred in EHV-1 also, this could explain for some cotranslational alteration in the running profile of the polypeptide. Similarly, palmitylation and phosphorylation that also affect the HSV-1 protein (MacLean et al., 1989; Loomis et al., 2001) could change the apparent \( M_r \) of the EHV-1 UL11 polypeptide. One species at an \( M_r \) of 11,000 was always particularly prominent and might therefore correspond to a mature and functionally important form of the UL11 products. By analysis of different cell fractions and by indirect immunofluorescence, an association could be demonstrated between the EHV-1 UL11 homolog and cellular membranes, and the UL11 protein was shown to accumulate in the region of the trans-Golgi network of infected cells. Surprisingly, when UL11 was expressed alone, the specific fluorescence was found to be more dispersed throughout the cell, no matter whether a mycHis-tag had been added or not. Moreover, the pattern of fluorescence appeared to be somewhat relative to the amount of protein expressed in the respective cell. Cells exhibiting a bright UL11 fluorescence also stained markedly in structures other than the TGN region, especially in the area of the cell-surface membrane. In these cells, an overflow of Golgi retention or retrieval mechanisms that have been demonstrated to be responsible for the localization of the HSV-1 homolog might occur. Of course other factors or products of viral infection might also be involved in concentrating the UL11 protein within the TGN region. It is
Fig. 8. Rk13 cells were infected with RacH, HΔ11, or HΔ11R at an m.o.i. of 1. Fourteen hours p.i. cells were fixed and embedded, and un-infected control cells were treated accordingly. Toluidine blue stained semithin sections of cells and parallel samples on glass coverslips that were stained by IIF with anti-gp2 mAb 3B12 were compared in an Axioscope (Zeiss, Germany). Pictures were taken digitally and processed. Bars represent 10 μm in the left and 100 μm in the right panels.
interesting to note that in contrast the HSV-1 UL11 protein strongly accumulates in the Golgi region when expressed alone, but is distributed differently during viral infection (Baines et al., 1995; Loomis et al., 2001).

Investigation of two independent UL11-deleted EHV-1 revealed that UL11 expression does not have a major impact on EHV-1 replication in Rk13 cells. Virus titers of UL11-deleted viruses were reduced slightly but constantly throughout all experiments. As the UL11 protein was shown to be a component of EHV-1 virions and to be synthesized at early-late times after infection, a role either in very early events of infection or in some step in virus maturation and egress has to be assumed. The HSV-1 homologous protein facilitates virus egress and has been discussed to play a role in assembly (Baines et al., 1995; Loomis et al., 2001). In this study, the possibility of a function in early events could not be excluded other than that the EHV-1 UL11 protein appears not to be involved in virus penetration and that infection and protein production were similarly effective without expression of UL11 as judged by Western blot analysis. If there then was an effect on early events of infection, it would be minor in the systems analyzed at present. Furthermore, deleting the UL11 protein did not obviously obliterate any very late transport step in virus egress, as one would then expect a clear reduction in extracellular titers against intracellular infectivity. Generally, virus egress into the supernatant appeared to be relatively effective, but a function in some transport processes, resulting in effective particle assembly and egress, is probable. However, the most prominent phenotype observed was a marked reduction in plaque size. Generally plaque size is taken as a marker for efficient cell-to-cell spread. Of course the cell-culture systems used often do not allow a clear distinction between the mechanisms of direct cell-to-cell spread and the mechanisms of infecting neighboring cells via extracellular virions. That the reported general reduction in virus yield of the UL11-deleted viruses might be exclusively responsible for the small plaque phenotype though can be excluded, as other mutant viruses that were tested on the very same Rk13 cell system, such as EHV-1 deleted in the early-late proteins UL45 or gM and gp2, do also replicate at reduced levels, but do not exhibit any plaque phenotype (Oettler et al., 2001; Rudolph and Osterrieder, 2002). It is thus concluded that the EHV-1 UL11 protein does somehow influence direct cell-to-cell spread. In this context the observation that the ability of RacH to fuse neighboring cells during infection was drastically reduced—if not lost—was very interesting. The phenomenon of an effect on cell-to-cell spread and on fusion of cells could be functionally connected. Mechanisms of direct cell-to-cell spread are still relatively unknown, but could involve directed and tightly regulated fusion of basolateral cellular membranes (Johnson and Huber, 2002). Taking the assumed localization of UL11 within infected cells into account, i.e., an orientation into the cytoplasm within the proximity of specific cellular membranes, especially the TGN, an indirect involvement of the UL11 protein in cell-to-cell spread could be speculated on. It is suggested that UL11 could direct or increase the efficiency of transport of major participants—cellular or viral—to their final localization, e.g., to specific areas of basolateral membranes.

Materials and methods

Plasmids

The complete UL11 ORF (gene 51) was amplified by PCR, including recognition sites for KpnI and XhoI into primer sequences, and the PCR product was then inserted into vectors pcDNA3.1/Myc-His (+) and pcDNAI/Amp (Invitrogen), resulting in plasmids pC11myc and pC11. All constructs were controlled by cycle sequencing (MWG Biotech). To express a UL11-mycHis fusion protein in E. coli, the sequences of the complete UL11-mycHis fusion gene were released of pC11myc by an NcoI and Pmel digest and cloned into vector pBADgIII (Invitrogen). Plasmid pBH3.1 carries the 2.9-kb EHV-1 BamHI-HindIII fragment, containing UL11 encoding and adjacent sequences (Fig. 4B) (Seyboldt et al., 2000) in vector pTZ18R (Stratagene). To construct plasmid pΔ11LacZ+, 956 and 962 bp on either side of the EHV-1 UL11 ORF were amplified by PCR, using primers containing appropriate restriction enzyme sites (recognition sites are in bold letters: 5‘-aagaattcgatgggaattaagtcccct-3’ and 5‘-aaggtaccagagctgctcagag-3’). Plasmid pH9004 carries the 2.9-kb EHV-1 BamHI-HindIII fragment, containing UL11 encoding and adjacent sequences (Fig. 4B) (Seyboldt et al., 2000) in vector pTZ18R (Stratagene). To construct plasmid pΔ11LacZ+, 956 and 962 bp on either side of the EHV-1 UL11 ORF were amplified by PCR, using primers containing appropriate restriction enzyme sites (recognition sites are in bold letters: 5‘-aagaattcgatgggaattaagtcccct-3’ and 5‘-aaggtaccagagctgctcagag-3’). Both these fragments and a 3.8-kb E. coli LacZ expression cassette that had been released of vector pTT264A+ (Mettenleiter and Rauh, 1990) were sequentially cloned into vector pGem3Zf(+) (Promega), such that the LacZ cassette was framed by viral sequences necessary for homologous recombination. Due to overlapping with the adjacent ORF UL12, UL11 nucleotides 1 to 73 had to be left unaffected. Therefore plasmid pΔ11LacZ+ aimed at the deletion of the remaining 151 bp (67.4%) of UL11 sequences. To ensure correct termination of the UL12 to UL14 transcripts, additional SV40 polyadenylation signal sequences, taken via KpnI and PvuII from plasmid pEGFP-C1 (Clontech; GenBank Accession No. U55763), were introduced directly upstream of the inserted cassette (into the respective KpnI and Smal site, such that the expected transcripts were reduced by 48 nucleotides only) (Fig. 4C).

Cells and viruses

Rk13 cells, equine dermal cell line Edmin337, and Cos7 cells were propagated in minimal essential medium supplemented with 5–10% fetal calf serum (Neubauer et al., 1997). The EHV-1 UL11 expressing cell-line 51D3 was isolated after transfection of Rk13 cells with plasmid pC11myc and subsequent selection for Genitocin-resistant cell clones.
(G418, 500 μg/ml, Calbiochem). Wild-type EHV-1 strain RacL22 and its attenuated cell-culture derivative RacH (Hüb- 
bert et al., 1996) as well as the recombinant viruses generated in this study were grown and analyzed on the respec-
tive cell lines. To analyze phenotypes of un-infected and infected cells, cells were embedded in ERL4206 as has been 
described before (Neubauer et al., 2002) and semithin sec-
tions were stained with Toluidine blue.

**Generation of recombinant viruses**

The UL11-negative EHV-1, HΔ11 and LΔ11, were iso-
lated after homologous recombination in Rk13 cells. Supernatants of cells, cotransfected with 5 μg of plasmid 
PΔ11LacZ+ (Fig. 4C) and 1-2 μg of DNA of either RacH or RacL22, were plated onto monolayers of Rk13 cells and 
single plaques of recombinant viruses were selected under an agarose overlay (0.5%) containing 0.05% X-gal substrate 
(Roche Molecular Biochemicals). Recombinant virus pop-
ulations were purified to homogeneity by four rounds of 
plaque isolation. The UL11 repaired viruses, HΔ11R and LΔ11R, were isolated accordingly after calcium phosphate 
mediated cotransfection of plasmid pBH3.1 (Fig. 4B) with DNA of HΔ11 or LΔ11 into Rk13 cells.

**Southern blot analysis**

Preparations of DNA from the respective viruses (Oster-
rieder et al., 1996) were subjected to restriction enzyme 
analysis using EcoRV or HindIII. DNA fragments were 
separated by 0.8% agarose gel electrophoresis and trans-
ferred onto positively charged nylon membranes, as previ-
ously described (Osterrieder et al., 1996; Neubauer et al., 
1997). UL11- and LacZ-specific sequences, as contained in 
plasmids pBH3.1 or pTT264A +, respectively, were labeled 
with digoxigenin 11-dUTP according to the manufacturer’s 
(Roche Molecular Biochemicals) instructions and used as 
specific probes. DNA hybrids were detected using an anti-
digoxigenin alkaline phosphatase conjugate followed by 
chemiluminescence detection (CSPD) (Roche Molecular 
Biochemicals).

**Purification of virions**

Supernatants of infected cells (m.o.i. of 2) were cleared 
by low-speed centrifugation (14 h p.i.). Extracellular virions 
were purified of supernatants by repeated centrifugation 
through a 30% sucrose cushion (Oettler et al., 2001).

Virion preparations were split into a fraction containing 
proteins that become solubilized after treatment of virions 
with 1% Triton X-100 in PBS (45°C for 20 min), i.e., 
proteins of the viral envelope and of the tegument, and 
another fraction, representing capsids and adhering tegu-
ment proteins. Fractions were separated by centrifugation at 
50,000 g for 30 min.

**Fractionation of cells and analysis of protein synthesis 
using phosphono acetic acid**

Fractionation of cells was performed exactly as de-
scribed by Bogner et al. (1992). Infected Rk13 cells 
(RacL22, m.o.i. of 3) were harvested 14 h p.i., washed with 
PBS, and suspended in fractionation buffer (5 mM Na 
phosphate, pH 7.5, 2 mM MgCl2, 0.5 mM CaCl2, 1 mM 
PMSF). After freeze-thawing and ultrasonic treatment, su-
crose was added (ad 0.3 M) and nuclei and cell detritus were 
pelleted by centrifugation at 800 g (10 min, pellet 1). The 
resulting supernatant was cleared at 10,000 g (10 min) and 
by another centrifugation step at 100,000 g (1 h, 4°C), a 
fraction containing cellular membranes and vesicles of the cell 
(pellet 2) and a fraction containing soluble components of the cytoplasm (supernatant) were collected. Pellet 1 was 
suspended in fractionation buffer followed by centrifugation 
over a sucrose cushion to purify nuclei (1.62 M) that were 
then lysed in fractionation buffer with 0.5% Triton X-100.

To inhibit synthesis of viral DNA, PAA was added to 
Rk13 cells that were infected at an m.o.i. of 5 with RacL22, 
at a concentration of 0.1, 0.5, or 1 mg per milliliter of 
cell-culture medium. At 14 h p.i. cells were lysed and 
analyzed by PAGE.

**Western blotting and indirect immunofluorescence**

Rk13 or Edmin337 cells, infected at an m.o.i. of 2-5 with 
the respective viruses, were harvested at the indicated time 
points p.i. The protein concentration of samples was ad-
tended to 1 mg/ml protein (BCA protein assay; Pierce) 
before they were mixed with sample buffer (5% 2-mercap-
toethanol) (Sambrook et al., 1989), heated to 95°C for 5 min 
or to 56°C for 2 min (for glycoprotein M detection only), 
and separated on SDS–Tris–Glycine polyacrylamide gels 
(PAGE; 12 or 15%). Alternatively, SDS–Tris–Tricine 
16.5% PAGE was used to improve separation of low M, 
proteins, such as UL11 (Schagger and von Jagow, 1987). In 
this case protein-adjusted lysates were mixed with sample 
buffer, containing Tris–HCl (pH 6.8; 200 mM), glycerol 
(40%), SDS (2%), and Coomassie blue G250 (0.04%) and 
heated to 95°C for 5 min. After electrophoresis, proteins 
were blotted onto nitrocellulose membranes and detected 
exactly as described before (Oettler et al., 2001).

For IIF, cells grown on glass coverslips were either 
infected with the indicated viruses or transfected with plas-
mids pC11 or pC11myc and fixed at the times given with 
80% acetone. Unspecific binding was blocked with PBS 
containing 10% FCS for 45 min. Cells were incubated with 
the primary antibody for 30 min, and for another 30 min 
with the anti-rabbit or anti-mouse IgG Alexa 546 or Alexa 
488 conjugates (Molecular Probes). Processed samples 
were analyzed and digitally documented using either an 
Axiovert, an Axioskope, or an LSM510 laser scanning mi-
croscope (Zeiss, Oberkochen, Germany).
Antibodies

The anti EHV-1-gB monoclonal antibody (mAb) 3F6 (Allen and Yeargan, 1987), anti EHV-1-gM mAb B8 (Day, 1999), the anti EHV-1-gD polyclonal anti-19mer rabbit antibody (Flowers and O’Callaghan, 1992), anti EHV-1-gp2 mAb 3B12, anti EHV-1 gB mAb 4B6 (both Meyer and Hübter, 1988), anti-major-capssid protein mAb ZB4 (kindly provided by G. B. Caughman), and the anti-UL34 antiserum (Neubauer et al., 2002) were used in this study. A UL11-specific antibody was generated by immunizing rabbits four times in 4-week intervals with 50-100 μg of a purified UL11 protein. To this aim the complete UL11 ORF was expressed in E. coli strain Top 10 as a mycHis fusion protein using the pBADgIII expression system (Invitrogen). The expressed protein was purified by affinity chromatography followed by elution of SDS-polyacrylamide gels using an Electro-Eluter (Millipore). The quantity and quality of the purified protein was confirmed by PAGE and by Western blot analysis using an anti-6-His monoclonal antibody (Invitrogen). The anti γ-adaptin mAb was obtained from Sigma Immunochemicals.

Virus growth kinetics and plaque size measurements

Rk13 cells in six-well plates were infected at 4°C with the indicated viruses at an m.o.i. as stated. After 90 min of incubation at 4°C, virus penetration was induced by shifting the temperature to 37°C. The penetration period was stopped after 90 min by inactivating remaining extracellular virus with a citrate buffer (pH 3.0). At the indicated time points supernatants and infected cells were collected separately. Supernatants were cleared by low-speed centrifugation, whereas cells were again treated with citrate buffer to inactivate adhering extracellular infectivity and subjected to freeze-thawing. Virus titers were determined independently on Rk13 cells. Plaque sizes of the different viruses on Rk13 cells or 51D3 cells (50 PFU/six-well) were measured after 3 days under a 0.25% methylcellulose overlay. Maximum cell or 51D3 cells (50 PFU/six-well) were measured after 3 days under a 0.25% methylcellulose overlay. Maximum

References

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Penetration assays

Penetration assays were performed as previously described (Neubauer et al., 1997). Briefly, 100 PFU of the respective viruses were allowed to adsorb for 90 min to Rk13 cells grown on six-well plates. The assay was started by shifting the incubation temperature to 37°C and at different times after the temperature shift, extracellular virus was inactivated using a citrate buffer (pH 3.0). The penetration effectiveness was determined by comparing the number of plaques present after citrate treatment with the number of plaques present after control treatment (PBS).

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