The role of the gene 71 product in the life cycle of equine herpesvirus 1

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Equine herpesvirus type 1 (EHV-1) gene 71 encodes a heavily O-glycosylated 192 kDa protein with no identified herpesvirus homologue. Isolation of a deletion mutant in gene 71 (ED71) demonstrated that its protein product is not essential in vitro. To investigate the role of the gene 71 protein in the virus life cycle, ED71 has been characterized in vitro in terms of cellular adsorption, penetration, egress and transmission compared to wild-type and revertant virus. ED71 virions adsorbed to cells less efficiently than wild-type and revertant virus with a consequential effect on virus penetration; virus egress was significantly impaired and the timing of release was also delayed. The percentage of both full and empty capsids accumulating in the nuclei of ED71-infected cells was significantly higher than in wild-type virus-infected cells but the most notable differences were the low number of particles and the low ratio of enveloped to unenveloped capsids in the cytoplasm. The primary mode of transmission of the mutant virus is by direct cell-to-cell spread and the fact that a neutralizing antiserum did not reduce ED71 plaque size, supported the conclusion that deletion of gene 71 impairs the ability of virus to spread via release and readsorption to uninfected cells. Thus, deletion of EHV-1 gene 71 results in a defect in virus maturation and capsid envelopment. Progeny virus is consequently impaired in adsorption/penetration presumably due to the particles lacking the glycoprotein spikes predicted to be encoded by this gene and hence spreads by direct cell-to-cell contact.

Introduction

Equine herpesvirus 1 (EHV-1), a member of the subfamily Alphaherpesvirinae, is a significant viral pathogen of horses and causes a variety of clinical problems including respiratory disease, abortion and neurological disorders (Bryans & Allen, 1989). EHV-1 strain Ab4 is a pathogenic UK isolate of EHV-1, the genome of which is 150 223 bp in size and contains 81 open reading frames (ORFs) predicted to encode polypeptides (Telford et al., 1992). At least 10 of these ORFs have been predicted to encode membrane glycoproteins. Herpesvirus glycoproteins play an important role in essential viral functions such as adsorption, penetration, egress and cell-to-cell transmission. Consequently they play a significant role in viral pathogenesis (Spear, 1993). Of the herpes simplex virus type 1 (HSV-1) glycoproteins identified, gB, gD, gH, gK and gL are essential for virus growth in vitro (Cai et al., 1988; Desai et al., 1988; Hutchinson et al., 1992; Ligas & Johnson, 1988). The glycoproteins gB, gD, gH and gL mediate virus attachment and penetration, as well as cell fusion and virus spread (Fuller et al., 1989; Fuller & Spear, 1987; Highlander et al., 1987, 1988; Johnson & Ciphers, 1988; Johnson et al., 1984; Desai et al., 1988; Noble et al., 1983). Glycoprotein C plays a principal role in the adsorption of virus to cellular membranes (Herold et al., 1991). Glycoproteins gC, gE and gI are believed to modulate the immune response to infection; gC acts as a C3b receptor (Eisenberg et al., 1987; Friedman et al., 1984; McNearney et al., 1987); gE and gI function together as an Fc receptor (Baucke & Spear, 1979; Frank & Friedman, 1989; Johnson et al., 1988).

EHV-1 gene 71, located in the unique short region of the genome encodes a 797 amino acid polypeptide and has no homologue in any sequenced herpesvirus. We have previously demonstrated by SDS-PAGE that EHV-1 gene 71 encodes a heavily O-linked 192 kDa glycoprotein (Sun et al., 1994). The gene 71 polypeptide is associated with cellular membranes and virion envelopes. The isolation of a deletion mutant, ED71 in which the majority of the gene 71 encoding sequence was removed and replaced with an Escherichia coli lacZ gene demonstrated that gene 71 is not essential for virus growth in vitro although mutant virus yield was 10-fold less than that of wild-type virus (Sun & Brown, 1994). In this paper, we demonstrate that the EHV-1 gene 71 product is involved in adsorption/penetration of virus, egress of virus from infected cell nuclei and lack of the gene product results in the mode of transmission of virus being predominantly by the direct cell-to-cell pathway.

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previously described (Sun & Brown, 1994; Sun reintanto Re71 in which the deletion in ED71 was restored have been
and adsorption was allowed to take place for various times at 4 °C.
multiplicity infection in equine dermal NBL-6 cells maintained in
essentially as described by Szili~gyi & Cunningham (1991) and Sun
(1994). BHK-21/C13 monolayers in roller bottles were infected with
experiment done in triplicate and the error bars indicate standard
deviations. Symbols : A, ED71-infected cells; II, EHV-1-infected cells;
O, Re71-infected cells.

Methods

Cells and virus. Baby hamster kidney clone 13 (BHK-21/C13; Macpherson & Stoker, 1962) were grown as previously described
(Brown et al., 1973). EHV-1 strain Ab4 was used as the wild-type strain
in this study. Stock preparation of virus at passage 13 was made by low
multiplicity infection in equine dermal NBL-6 cells maintained in MEM with 1% fetal calf serum. Mutant ED71 in which the gene 71
ORF was removed and replaced by the
E. coli lacZ gene and the
revertant Re71 in which the deletion in ED71 was restored have been
previously described (Sun & Brown, 1994; Sun et al., 1994).

Purification and quantification of virions. The procedure used was
essentially as described by Szili~gyi & Cunningham (1991) and Sun et al.
(1994). BHK-21/C13 monolayers in roller bottles were infected with
virus at an m.o.i. of 0.01 or 5 p.f.u. per cell. At either 72 h post-infection
(p.i.) or 20 h p.i. the supernatant was harvested and centrifuged at
2500 r.p.m. for 20 min to remove the cell debris. Supernatant virus
was pelleted for 2 h at 12000 r.p.m. and the pellet gently resuspended in 1 ml
Eagle’s medium without phenol red and laid onto a 5 to 15% Ficoll
gradient before centrifuging at 12000 r.p.m. for 2 h at 4 °C. The virion
band collected by side puncture was diluted and pelleted at 21 000 r.p.m.
for 2 h at 4 °C. The virion pellet was gently resuspended in 200 µl of
Eagle’s medium and stored at −70 °C. Infectivity was determined by
titraton on BHK-21/C13 cells. The number of particles was
determined by electron microscopy. The specific infectivities (particle:p.f.u. ratio) of the purified mutant, wild-type virus and Re71 are
presented in Table 1.

Assay for rate of virus adsorption. Monolayers of BHK-21/C13 cells
(4 × 10⁶) in 50 mm Petri dishes were precooled for 1 h at 4 °C and
inoculated with virus at an m.o.i. of 400 p.f.u. per plate and left at 4 °C
for various lengths of time up to 4 h. At various time points, the plates
were washed three times with PBS and overlaid with medium containing
1% carboxymethylcellulose. The plates were incubated at 37 °C for 2
days after which time they were fixed and stained. Plaques were
counted and the percentage of virus absorbed at each time-point
determined, relative to the final time point (4 h) which represented
100% adsorption.

Assay for rate of virus penetration. Virus penetration was assessed by
determining the rate at which adsorbed virus became resistant to
inactivation by a low-pH citrate buffer. The method used was as
described by Huang & Wagner, (1964) and Highlander et al. (1987)
with modifications. Confluent BHK-21/C13 cells (4 × 10⁶) in 50 mm
Petri dishes were separately infected with 400 p.f.u. per plate of either
purified wild-type virus, the mutant ED71 or Re71 and allowed to
adsorb for 2 h at 4 °C. The unbound virus was removed by washing
four times with PBS. The cells were then overlaid with Eagle’s medium
and shifted to 37 °C to allow viral penetration to proceed. At selected
time points after the temperature shift, plates were either treated with
1 ml of citrate buffer (40 mM-citric acid, 10 mM-KC1, 135 mM-NaCl
pH 3.0) or with 1 ml of PBS for 5 min as a control. The monolayers
then were washed three times with PBS and overlaid with Eagle’s
medium containing 1% carboxymethylcellulose and incubated at 37 °C
for 2 days. The plaques were visualized with Giemsa stain and counted.
Experiments were performed in triplicate.

Assay for virion release from infected cells. BHK-21/C13 monolayers
in 35 mm Petri dishes were separately infected with ED71, wild-type
virus or Re71 at an m.o.i. of 5 p.f.u. per cell. After 1-0 h adsorption at
37 °C, the unbound extracellular virus was removed by thoroughly
washing the monolayers four times with PBS containing 1% calf
serum. After each incubation period, the medium was removed,
clarified at 21000 r.p.m. for 20 min at 4 °C and stored at −70 °C. Cells
were separately harvested in the same volume, sonicated and stored at
−70 °C until the titres were assayed.

Electron microscopic analysis. BHK-21/C13 monolayers (4 × 10⁶
cells) infected with virus at 5 p.f.u. per cell, were scraped from Petri
dishes at 24 h p.i. and pelleted in Been Capsules (Taab Laboratories).
The pellet was fixed in 2.5% glutaraldehyde in PBS, then post-fixed in
1% (aqueous) OsO₄. The pellets were dehydrated through a series of
increasing ethanol concentrations to 100%, then infiltrated with Epon
812 epoxy resin (Taab Laboratories). After polymerization, 80 nm
sections were cut and stained with saturated uranyl acetate (in 50:50
ethanol/water) and subsequently stained with lead citrate. For
counting, 50 complete cell profiles (non-serial and including nuclei)
were examined.

Assay for virus spread from cell to cell. Confluent monolayers
(4 × 10⁶) of BHK-21/C13 cells were infected with virus at an m.o.i. of
0.01 p.f.u./cell. After adsorption at 37 °C for 1-5 h, the plates were
washed twice with PBS, overlaid with ETC10 plus and minus,
immunized horse serum (final concentration 1:25) and incubation
continued at 37 °C. The infected cells were harvested at various time
points after infection and the virus was titrated on BHK-21/C13 cells.
Results

ED71 entry into cells

We have previously demonstrated that ED71 grows 10-fold less well than wild-type virus or Re71. To analyse the reason for this, we have studied the various stages in the virus life cycle in which a glycoprotein may be expected to play a role. To determine the efficiency of adsorption onto cells, monolayers of BHK-21/C13 cells were infected with 400 p.f.u./plate of purified virus as described and the rate of adsorption was calculated as the percentage of plaques at the various times compared to the numbers at 4 h p.i. The results represented in Fig. 1 show that purified ED71 adsorbs more slowly onto cells than wild-type and revertant virus over the test period at 4 °C, its 50% adsorption time being 100 min compared to 60 min for both wild-type and revertant. These experiments were repeated several times with identical or very similar results. A comparable effect was also observed in an experiment performed at 37 °C. These results indicate that adsorption of ED71 virions onto cells is impaired. To determine whether there is a defect in penetration during entry of ED71 into cells, the amount of adsorbed virus which resisted an acid buffer wash was examined. The results presented in Fig. 2 show that the rate of penetration compared to the untreated controls was lower than for wild-type and revertant virus, suggesting that adsorbed ED71 penetrated less efficiently than wild-type virus and Re71 which had similar penetration rates. The 50% time for ED71 was 45 min compared to 20 min for wild-type virus and revertant and again this experiment was repeated several times with consistent results.

Egress of ED71 from cells

The specific infectivities of the virions purified from infected BHK-21/C13 cells, presented in Table 1 show that the particle/p.f.u. ratio of purified ED71 is significantly higher (10–20-fold) than that of wild-type virus and Re71 which could indicate an impairment in virus maturation. To test whether there was a defect in virus release, the amounts of infectious extracellular and intracellular virus at various times post-infection, were separately quantified. The results presented in Fig. 3 show that the final yield of released infectious virus from ED71-infected cells decreased about 250-fold compared to that from wild-type and Re71-infected cells. This compares to a decrease in total titre of 5-fold, leading to a relative decrease of 50-fold in the ratio of cell-released to cell-associated virus.

Furthermore the onset of release of infectious virus from ED71-infected cells was significantly delayed. The yield of both wild-type and Re71 virus released from

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Table 1. The specific infectivity (particle/p.f.u. ratio) of ED71, Re71 and wild-type virus EHV-1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Particle/p.f.u. ratio*</th>
<th>Particle/p.f.u. ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHV-1</td>
<td>1017/1</td>
<td>638/1</td>
</tr>
<tr>
<td>Re71</td>
<td>1035/1</td>
<td>1077/1</td>
</tr>
<tr>
<td>ED71</td>
<td>1440/1</td>
<td>2128/1</td>
</tr>
</tbody>
</table>

* Virus from 4 x 10⁸ BHK-21/C13 cells infected with virus at an m.o.i. of 0.01 p.f.u. per cell and harvested at 72 h p.i.
† Purified virions from 4 x 10⁸ BHK-21/C13 cells infected with virus at an m.o.i. of 5 p.f.u./cell and harvested at 20 h p.i.
infected cells increased by 1000-fold between 8 and 16 h p.i., whereas ED71 demonstrated no significant increase. Between 16 and 24 h p.i., the yield of infectious virus from ED71-infected cells increased by only 20-fold. This was a consistent finding in several experiments.

**ED71 virion formation**

The distribution of capsids and virions in infected cells was analysed by electron microscopy. The results presented in Table 2 show that in ED71-infected cells, a large number of capsids accumulate in the nuclei adjacent to the nuclear membranes and the percentage of capsids in the nuclei is much higher than that in the nuclei of wild-type- or Re71-infected cells. The most notable difference in capsid distribution between the ED71 and wild-type virus-infected cells was the extremely low number of particles (6.7% compared to 20–24%) and the low ratio of enveloped to unenveloped capsids in the cytoplasm of ED71-infected cells (1% of ED71 cytoplasm capsids were enveloped compared to 10% for both wild-type and Re71). In addition, the percentage of extracellular virions from ED71-infected cells was less than that from wild-type virus-infected cells. The revertant Re71 gave a similar pattern to wild-type virus.

**Cell-to-cell transmission of ED71**

Removal of the gene 71 product results in a defect in virus adsorption/penetration and a defect in virus egress from infected cells. This could mean that transmission of virus from cell-to-cell via the extracellular medium would proceed inefficiently. To determine whether this was the case, BHK-21/C13 monolayers were infected with ED71, Re71 or wild-type virus at a low m.o.i. (0.01 p.f.u./cell) and incubated in the presence or absence of a horse antiserum which equally neutralized the infectivities of ED71 and wild-type virus (data not shown). At various time points after infection, the cell-associated (CA) and cell-released (CR) infectious virus was harvested and titrated on BHK-21/C13 cells. The results showed that the yields of ED71 progeny virus were little reduced by

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Table 2. Distribution of capsids in different subcellular locations of infected BHK-21/C13 cells

The data are from 50 randomly selected sections of BHK-21/C13 cells infected with virus at 5 p.f.u./cell and harvested at 24 h p.i.

<table>
<thead>
<tr>
<th>Location and condition</th>
<th>EHV-1, Ab4</th>
<th>ED71</th>
<th>Re71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1649 (77.6)</td>
<td>1443 (92.5)</td>
<td>1200 (73.1)</td>
</tr>
<tr>
<td>Inside the inner lamellae</td>
<td>1649 (77.6)</td>
<td>1443 (92.5)</td>
<td>1200 (73.1)</td>
</tr>
<tr>
<td>Between the lamellae</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>425 (20.0)</td>
<td>104 (6.7)</td>
<td>389 (23.7)</td>
</tr>
<tr>
<td>Enveloped (%)*</td>
<td>8</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Unenveloped (%)*</td>
<td>92</td>
<td>99</td>
<td>83</td>
</tr>
<tr>
<td>Extracellular</td>
<td>51 (2.4)</td>
<td>13 (0.8)</td>
<td>52 (3.1)</td>
</tr>
<tr>
<td>Total (all locations)</td>
<td>2125 (100)</td>
<td>1560 (100)</td>
<td>1641 (100)</td>
</tr>
</tbody>
</table>

* Percentage of total cytoplasm capsids.
the presence of neutralizing antiserum. The yields of wild-type and RE71 progeny virus were reduced about 10-fold by the neutralizing antibodies, with the titres reaching a plateau approximately 24 h later in the presence of antibody (data not shown). This indicated that the yields of ED71 were mainly composed of progeny from direct cell-to-cell transmission. However, an unexpected difference between ED71 and wild-type virus was observed in that the yields of progeny virus from ED71-infected cells produced before 24 h p.i. were about 10-fold higher than that from both wild-type virus and revertant. Since the yields from revertant were
Table 3. Effect on the sizes of plaques formed by wild-type, revertant virus and ED71 by a horse anti-EHV-1 serum

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>EHV (gram)</th>
<th>Re71 (gram)</th>
<th>ED71 (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>2.74 (±0.18)</td>
<td>2.61 (±0.3)</td>
<td>0.572 (±0.041)</td>
</tr>
<tr>
<td>+</td>
<td>0.492 (±0.065)</td>
<td>0.480 (±0.05)</td>
<td>0.428 (±0.031)</td>
</tr>
</tbody>
</table>

Identical to that of wild-type virus the deletion of gene 71 appeared to enhance the efficiency of transmission by direct cell-to-cell contact.

To further analyze the transmission of ED71, the effect of the neutralizing antiserum on the size of plaques formed by virus was examined. The results, which are illustrated in Fig. 4 and summarized in Table 3, show that in the absence of antiserum the plaques formed by ED71 were smaller and tighter than those of wild-type and revertant. In the presence of antiserum, the plaques formed by wild-type virus and revertant were reduced and indistinguishable from those formed by ED71 which were not affected by the neutralizing antiserum. These findings show that the plaques formed by ED71 mainly result from direct cell-to-cell transmission, whereas plaques formed by wild-type and revertant virus are the result of both direct transmission plus release and readsoption, supporting the conclusion that the deletion of gene 71 impairs virus release from infected cells and readsoption onto uninfected cells.

**Discussion**

Mutations in a number of HSV-1 genes have been shown to affect the transmission of virus from nuclei to extracellular spaces (Baines et al., 1991; Campadelli-Fiume et al., 1991; Desai et al., 1988; Baines & Roizman, 1992). These genes encode gD, gH, gL and the UL11 and UL20 proteins, which are all membrane glycoproteins. Most of these proteins appear to have multiple functions and to be responsible for different steps in the process of virus egress. EHV-1 strain Ab4 gene 71 is an unique gene which has no known homologue in other herpesviruses (Telford et al., 1992). Gene 71 encodes a large (192 kDa) heavily O-linked glycoprotein which is associated with cellular membranes and the virion envelope (Sun et al., 1994). Sequence analysis suggested that the serine and threonine-rich region (amino acids 22-465) in the protein may form highly extended semi-flexible rods of 71 nm in length on the virion surface (McGeoch et al., 1993). In vivo ED71 is attenuated in a mouse model of EHV-1 pathogenesis (H. J. Field, personal communication).

Using a gene 71 deletion mutant, ED71, we have previously demonstrated that although the gene 71 product is not essential for virus growth in vitro, the yield of progeny virus is reduced 5-fold (Sun et al., 1994). In this paper we demonstrate that, compared to wild-type virus, ED71 is defective in adsorption/penetration; a conclusion consistent with the predicted features of the gene 71 protein as a long protruding membrane glycoprotein spike (McGeoch et al., 1993). However, we could not rule out the possibility that the demonstrated defect in adsorption resulted in a consequent decrease in the efficiency of virus penetration.

It appears that the gene 71 protein also plays a role in virus egress from cells. This conclusion was derived from the following evidence: (i) the yield of released infectious virus from ED71-infected cells is significantly lower than that from wild-type- and revertant-infected cells and the onset of virus release from ED71-infected cells is extremely delayed, compared to wild-type and revertant virus; (ii) the percentage of capsids in the nuclei of ED71-infected cells is markedly higher than that in wild-type virus-infected cells. The number of particles in the cytoplasm of ED71-infected cells is much lower than in wild-type virus-infected cells and the ratio of enveloped to unenveloped ED71 capsids in the cytoplasm is dramatically lower than that for wild-type virus. In addition, the proportion of extracellular virions in ED71-infected cells is less than in wild-type virus-infected cells. These observations indicate that the absence of the gene 71 protein impairs nucleocapsid envelopment and virus transmission from nucleus to cytoplasm. These defects must contribute to the high particle/p.f.u. ratio and the defective release of ED71 infectious virus.

As a result of the defect in egress and adsorption/penetration of ED71, it seemed likely that virus transmission via release and readsoption could be affected. The data on the effect of neutralizing antiserum on the plaque size confirmed this possibility. In the absence of neutralizing antiserum the plaques formed by ED71 are contained and smaller than those formed by wild-type and revertant, whereas in the presence of neutralizing antiserum, the plaques formed by ED71, wild-type virus and revertant are indistinguishable. This observation indicated that the smaller, tighter plaques formed by ED71 were mainly the result of direct cell-to-cell transmission of virus and demonstrated that the deletion of gene 71 impaired virus release and readsoption. Interestingly, it appears that the deletion of gene 71 affects the efficiency of virus transmission. The evidence for this is: (i) at low multiplicity of infection the yield of
progeny virus from ED71-infected cells before 24 h p.i. was about 10-fold higher than that of both wild-type and revertant and it was not significantly reduced in the presence of neutralizing antiserum (the same increase was not observed following a high multiplicity of infection); and (ii) the deletion of gene 71 did not affect capsid formation, since electron microscopy showed that very similar numbers of particles were produced from ED71-, wild-type virus- and revertant-infected cells.

Overall these studies show that the deletion of EHV-1 gene 71 protein mainly impairs virus egress from cells, which indicates that the EHV-1 gene 71 protein may play a role in virus maturation and egress. The deletion of the gene 71 protein also impairs virus entry into cells. At this stage it is fair to assume that this is the result of the predicted semi-flexible glycoprotein spikes being absent from the virus surface. These have a consequential role in the mode and efficiency of virus transmission. Finally, these defects could account for the mutant virus attenuation in vivo.

It should be noted that although only 80% of the gene 71 ORF is deleted in ED71, the gene 71 product is not detected by a specific antiserum (anti-71) and a monoclonal antibody (P19) (Sun et al., 1994). The lacZ insertion within the gene 71 ORF should not affect virus behaviour since a deletion mutant ED75 with a similar lacZ substitution in EHV-1 gene 75 ORF (Sun & Brown 1994) does not show any defect in virus penetration, egress or transmission (data not shown).

We are grateful to Christine MacLean for willingly giving advice. The neutralizing antiserum was kindly supplied by Dr Neil Edington, CAb W., Gu, B. & PERSON, S. (1988). Role of glycoprotein B of herpes simplex virus type 1: evidence that gH is essential for virus infectivity. Journal of General Virology 69, 1147–1156.


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