Identification and characterization of the protein product of gene 67 in equine herpesvirus type 1 strain Ab4

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Equine herpesvirus type 1 (EHV-1) strain Ab4 gene 67 has no counterpart in any herpesvirus sequenced to date. To identify and characterize the product of EHV-1 gene 67, we have expressed the putative amino acids 11 to 260 encoded by gene 67 as a β-galactosidase fusion protein in Escherichia coli. The expressed fusion protein has been used to generate an antiserum raised against the gene 67 product. Immunoblotting and immunoprecipitation experiments have revealed that the anti-67 serum specifically recognizes a polypeptide with an Mr of 36000 (the 36K polypeptide) in infected cell extracts. The gene 67 protein is regulated as an early polypeptide in EHV-1 strain Ab4 infected cells and post-translational modification experiments have revealed that the protein is phosphorylated, but not glycosylated. The gene 67 protein has been transiently expressed in BHK-21/C13 cells using plasmid pCMV67, which contains the putative gene 67 ORF under the control of the cytomegalovirus immediate early promoter. Immunoblotting experiments with anti-67 have shown that the 36K protein is expressed at high levels in transfected cells. From both immunofluorescence and cellular fractionation experiments it is concluded that the gene 67 protein is associated with intracellular membranes and produces novel ribbon or filament-like structures within the cytoplasm of infected cells. We have demonstrated that the gene 67 product is a component of the virion nucleocapsid/tegument.

Introduction

Equine herpesvirus type 1 (EHV-1), a member of the subfamily Alphaherpesvirinae, is a significant viral pathogen of horses causing a variety of clinical symptoms including respiratory disease, abortion and neurological disorders (Bryans & Allen, 1989). The EHV-1 genome is a linear dsDNA molecule of approximately 150 kb in size which can be divided into two covalently linked components, the long and short regions (Henry et al., 1981; Whalley et al., 1981). The complete DNA sequence of EHV-1 strain Ab4, a pathogenic UK isolate of EHV-1, has been determined (Telford et al., 1992). The genome consists of 150223 bp and contains 76 distinct ORFs. Among the predicted genes in EHV-1 strain Ab4 there are three genes, l, 67 and 75, which lack positional and sequence counterparts in varicella-zoster virus and herpes simplex virus type 1 (HSV-1) (Telford et al., 1992). Gene 67 in the short inverted repeat sequences (IRs/TRs) immediately adjacent to US is predicted to encode a 272 amino acid protein with an Mr of 30100. An equivalent gene, IR6, has been identified in the Ky A strain of EHV-1 (Breeden et al., 1992). In order to investigate the role of gene 67 in the virus life cycle, we have attempted to isolate a deletion mutant in the gene. It was not possible to purify the deletion mutant to homogeneity, suggesting that the gene 67 protein product may be essential for virus growth, at least in vitro (Sun & Brown, 1994). In the work presented in this paper we have identified and characterized the polypeptide product of gene 67 using an antiserum raised against a gene 67 fusion protein. The gene 67 protein is membrane-associated and forms novel filamentous structures in infected and transfected cells.

Methods

Cells and virus. Baby hamster kidney clone 13 cells (BHK-21/C13) (Macpherson & Stoker, 1962) were propagated in ETC10 medium as previously described (Brown et al., 1973). EHV-1 strain Ab4 (kindly supplied by Dr E. A. R. Telford) was the wild-type virus used in this study. Preparation of virus stock at passage 13 was made by passage at low multiplicity in NBL-6 cells maintained in MEM with 1% fetal calf serum.

Plasmids. pUEH67 was constructed by inserting a PstI–NaeI fragment (nucleotides 124411–125160) into the 3' end of the β-galactosidase gene of pUR278 in the correct orientation at the BamHI–HindIII sites (Rüther & Müller-Hill, 1983) using a BamHI–HindIII adaptor. To construct pCMV67, an 880 bp fragment (nucleotides 124319–125199) containing the gene 67 ORF was amplified by PCR with primers (5' TACTAGATCTTGAATACCGATTA 3'; 5' TATCAGATCTAGAGATACAGATTA 3') and cloned into the pCMV10 BamHI site (Stow et al., 1993). Restriction enzyme digestion
confirmed that the gene 67 ORF is in the correct orientation in pCMV67.

Lipotransfection of BHK-21/C13 cells with pCMV67 DNA. The method used was that described by Felgner et al. (1987). Briefly, 50% confluent BHK-21/C13 cells in 35 mm Petri dishes were transfected with 2 μg of pCMV67 DNA and 15 μl of liposomes in 1 ml Opti-MEM 1 (GIBCO BRL) and incubated at 37 °C for 4 h. The medium was then replaced with ETC5, and incubation continued at 37 °C for 26 h. The cells were then used for immunoblotting or immunofluorescence experiments.

Preparation of a bacterial fusion protein and rabbit antisera raised against the gene 67 product. The β-galactosidase fusion protein, pUEX67, was partially purified by the method of Harlow & Lane (1988). Two New Zealand White rabbits were each initially injected
subcutaneously with 0.8 mg total protein dissolved in 0.25 ml PBS and emulsified with an equal volume of Freund’s complete adjuvant. Each rabbit was boosted on days 14, 30, 60 and 90 with an emulsion of 0.2 mg fusion protein dissolved in 0.25 ml water plus an equal volume of Freund’s incomplete adjuvant. Final antisera were collected on day 100.

Preparation of radiolabelled cell extracts. Confluent BHK-21/C13 cell monolayers in 35 mm Petri dishes were infected with 20 p.f.u./cell in Eagle’s medium containing 2% calf serum. For immunoprecipitation, infected cells were labelled from 5 h to 20 h post-infection (p.i.) with 50 μCi/ml [35S]methionine (Amersham > 1000 mCi/mmol) in medium containing one-fifth the normal level of methionine, with 100 μCi/ml of [3H]glucosamine (30 mCi/mmol) (Amersham) in medium containing one-tenth the normal level of glucose. The cells were harvested at the end of the labelling period by washing the monolayers with PBS and scraping the cells into 300 μl of extraction buffer (100 mM-Tris-HCl pH 8.0, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate and 0.2 mM-PMSF) and analysed by immunoprecipitation. Lysates were frozen at −70 °C.

Immunoprecipitation. Infected cell polypeptides were harvested and immunoprecipitations were carried out essentially as described by MacLean et al. (1992), except that 200 μl of the extract was incubated with 20 μl of antiserum overnight at 4 °C and then transferred to ice for a further 2 h in the presence of 60 μl of a 50% (v/v) suspension of Protein A-Sepharose.

Immunoblotting. Confluent BHK-21/C13 cell monolayers in 35 mm Petri dishes were infected at an m.o.i. of 20 p.f.u./cell and maintained in medium containing 2% calf serum. To inhibit viral DNA synthesis, cells were maintained from 1 h before and then throughout infection in medium containing 300 μg/ml phosphonoacetic acid (PAA, Sigma). Infected cell polypeptides were harvested at various times up to 24 h p.i. in 0.15 ml of lysis buffer [2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol]/106 cells, and immunoblotting was carried out as described by Frame et al. (1987). The anti-67 serum was diluted 200-fold. The reactivities on Western blots of the were harvested and immunoprecipitations were carried out essentially as described by MacLean et al. (1992), except that 200 μl of the extract was incubated with 20 μl of antiserum overnight at 4 °C and then transferred to ice for a further 2 h in the presence of 60 μl of a 50% (v/v) suspension of Protein A-Sepharose.

Preparation of virus-induced immediate early polypeptides. Immediate early polypeptides were prepared under a cycloheximide/actinomycin D block essentially as described by Preston et al. (1978). BHK-21/C13 cells in 35 mm Petri dishes were infected with EHV-1 or HSV-1 and harvested into 0.75 ml extraction buffer.

Treatment with glycosylation inhibitors. Confluent monolayers of BHK-21/C13 cells were infected with virus at 20 p.f.u./cell or mock infected. Cells were treated with 2 μg/ml tunicamycin (Sigma) or 1 μM-monomennsin (Sigma) from the end of the absorption period (1 h) until 20 h p.i.

Subcellular fractionation. Subcellular fractionation was based on the method described by Yukio et al. (1982) as modified by MacLean et al. (1992). Infected cells were adjusted to 150 mM-NaCl and separated into supernatant (cytosol) and pellet (total membrane fraction). The total membrane fraction was then adjusted to 1 M-NaCl and further separated into a supernatant fraction containing those proteins which bound only at low ionic strength and a pellet fraction containing proteins which bound to membranes at high ionic strength. The pellet was resuspended in 20 ml 100 mM-sodium carbonate, pH 11.5 bringing the protein concentration to 1 mg/ml and kept at 4 °C for 30 min, before centrifuging at 4 °C for 1 h at 50000 r.p.m. in a Beckman AH650 rotor. The pellet was gently washed once with ice-cold distilled water and dissolved in 300 μl lysis buffer, before storing at −70 °C.

Purification of virus particles. The procedure used was essentially that described by Szilágyi & Cunningham (1991). Monolayers of BHK-21/C13 cells in 80 oz roller bottles were infected with 1000 p.f.u. of EHV-1 strain Ab4 and incubated until CPE was complete. Supernatant virus was separated into two fractions: a virion preparation and a L particle preparation which consists of nucleocapsid-free tegument-envelope structures.

NP40 extraction of virions. Purified virions were incubated in the presence of 5% NP40 and 600 mM-NaCl for 30 min at room temperature, and then diluted fivefold with 20 mM-Tris–HCl pH 7.5, and centrifuged in a Sorvall SS34 rotor at 12000 r.p.m. for 2 h at 4 °C (Frame et al., 1986). The non-soluble pellet was resuspended in lysis buffer in a volume equivalent to the supernatant fraction.

Immunofluorescence staining. BHK-21/C13 cells on glass coverslips in 24-well dishes were infected or transfected with EHV-1 (15 p.f.u./cell) or 2 μg pCMV67 DNA, respectively. At 16 h.p.i. and 30 h after transfection cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature, washed, exposed to 0.1% Triton-X 100 in PBS for 30 min at room temperature, incubated for 1 h with anti-67 at 37 °C and processed for indirect immunofluorescence by standard techniques.

Results

Synthesis of β-galactosidase fusion proteins for immunization of rabbits

Plasmid pUEH67 contains the sequence encoding the putative amino acids 11–260 of gene 67 fused to the C terminus of the pUR278 β-galactosidase gene. The junction between the β-galactosidase gene and the EHV-1 gene 67 sequence was sequenced by the double strand method of Masahira & Yoshiyuki (1988) to confirm that the EHV-1 insert was in the correct frame (data not shown). Plasmid pUEH67 was transformed into Escherichia coli XL1 and after induction by IPTG, the lysates were analysed by 6% SDS–PAGE. The fusion protein migrated at the expected position with an M, of 140000 (data not shown) and was subsequently partially purified as inclusion bodies and used to immunize two rabbits. Both antisera were checked by immunoblotting and immunoprecipitation against EHV-1 strain Ab4 infected BHK-21/C13 cell extracts. Only one of these antisera produced a strong reaction and was named anti-67.

Identification of the gene 67 product in EHV-1 infected cells

Anti-67 was used to identify the product of the putative gene 67 by immunoblotting (Fig. 1a) and immunoprecipitation (Fig. 1b). Anti-67 recognized a polypeptide (the 36K polypeptide) from EHV-1 strain Ab4 infected cell extracts which migrated at a position corresponding to an M, of approximately 36000 (Fig. 1a, lane 2 and Fig. 1b, lane 1) on 10% SDS–PAGE. This polypeptide was recognized in neither mock infected cell extracts
Regulation of expression of the gene 67 protein product

To study gene regulation, the time course of gene 67 expression was determined. Cells were harvested at various times p.i. and polypeptides were separated by 10% SDS-PAGE, transferred to a nitro-cellulose membrane and probed with anti-67 serum (Fig. 2a). The 36K protein was first detected at 1 h p.i., the amount increased until about 10 h and remained constant up to 24 h. In the presence of PAA, the expression of the 36K polypeptide was somewhat inhibited (Fig. 2a). At this concentration of PAA, EHV-1 DNA replication is reduced to < 5% of the level of the untreated control in BHK-21/C13 cells (data not shown). The 36K protein was not detected in an EHV-1 immediate early extract (Fig. 2b, lane 1). For comparison, the behaviour of two HSV-1 proteins, Vmw110, an immediate early protein (Stow et al., 1986; Sacks & Schaffer, 1987) (Fig. 2c) and 65KDBF, an early protein (Goodrich et al., 1989; data not shown) was examined. Under the conditions used to prepare the immediate early extracts Vmw110 was clearly synthesized whereas 65KDBF was not. These results suggest that gene 67 is regulated as an early gene in EHV-1-infected cells.

Four additional polypeptides with Mr's of 33000 and 31500 were also detected by anti-67 in EHV-1 infected cell extracts (Fig. 2a). These bands are not present in mock infected cell extracts, suggesting that they are specific. The time course experiment shows that the 26K species appears at 1 h p.i. and is absent at 6 h p.i.; the 29K peptide appears at 12 h p.i. and is absent by 24 h p.i.; the 33K and 31-5K peptides appear at 12 h p.i. and are maintained up to 24 h p.i. The precise relationship between these lower Mr products and the 36K product is unclear.
EHV-1 gene 67 protein

Fig. 2. (a) Results of a time course study of EHV-1 gene 67 expression. Proteins were extracted at various times up to 24 h p.i. from BHK-21/C13 cells infected with EHV-1 in the absence or presence of 300 μg/ml PAA and separated on 10% SDS-PAGE. Polypeptides were transferred to a nitrocellulose membrane and probed with anti-67. Mrs are indicated on the left. The gene 67 36K, 33K, 31.5K, 29K and 26K products are indicated by arrows on the right hand side. (b) EHV-1 and (c) HSV-1 induced polypeptides were analysed by immunoblotting with anti-67 and an antibody raised against Vmw110 respectively. Lane 1, infected cell polypeptides prepared under immediate early conditions; lane 2, mock infected extracts; lane 3, untreated infected cell extracts. Mrs are indicated on the left and virus specific bands on the right.

Location of the gene 67-encoded polypeptide within infected cells and virions

To determine the cellular distribution of the gene 67 polypeptide, infected cells were fractionated into a soluble cytosol fraction and a membrane-containing fraction. The product of gene 67 is detected in the membrane-associated fraction and is stably associated with membranes in the presence of 1 M-NaCl and 100 mM-sodium carbonate, pH 11.5. (Fig. 3, lane 5).

To study further the behaviour of the gene 67 product in mammalian cells, gene 67 was transiently expressed in BHK-21/C13 cells. Two polypeptides with Mrs of 36000 and 33000 were specifically detected by anti-67 in BHK-21/C13 cells transfected with the plasmid pCMV67 (Fig. 4, lane 3). Indirect immunofluorescence experiments with anti-67 revealed that the gene 67 protein is located in the cytoplasm as punctate, ribbon or filament-like structures in infected or transfected BHK-21/C13 cells (Fig. 5a, c). The pattern of filament staining showed a coalescence in

Fig. 3. BHK-21/C13 cells infected with EHV-1 (16 h p.i.) were separated into membrane and cytosolic fractions and the resultant extracts were analysed by 10% SDS-PAGE and immunoblotting using anti-67. Lane 1, infected cell medium; lane 2, cytosolic fraction; lane 3, membrane proteins stable in 1 M- NaCl and sodium carbonate, pH 11.5; lane 4, low affinity membrane proteins stable in only 150 mM- NaCl; lane 5, total cellular extract; lane 6, mock infected cell extracts.
the cytoplasm immediately adjacent to the nucleus such that a fairly solid band structure surrounded the nucleus. The staining pattern was reminiscent of the cytoskeleton but double labelling experiments showed no co-localization with either actin or tubulin. Staining with antibodies to intermediate filaments has not yet been carried out.

The 36K species was present in purified virions at low abundance (Fig. 6, lane 1). To determine whether the 36K protein is associated with the virion envelope, purified virions were treated with NP40 (Fig. 6, lane 4). The 36K species was detected in the non-soluble pellet, suggesting that it is a component of the virion capsid or tegument.

**Post-translational modification of the gene 67-encoded polypeptide**

The gene 67-encoded polypeptide contains two putative N-linked glycosylation sites and six predicted phosphorylation sites. To determine whether the gene 67 polypeptide is glycosylated and phosphorylated, extracts from cells infected with EHV-1 were either treated with the glycosylation inhibitors tunicamycin or monensin, or labelled with [14C]glucosamine or [32P]orthophosphate. Immunoprecipitation with anti-67 showed that the 36K polypeptide is phosphorylated (Fig. 7, lanes 2 and 4), but not labelled by [14C]glucosamine (data not shown). In addition the 36K protein was not altered in 

**Discussion**

Gene 67 appears to be unique to equine herpesvirus. It has been found in two strains of EHV-1 (Breeden et al., 1992; Telford et al., 1992) and in EHV-4 (Nagesha et al., 1993). Conservation of the gene may indicate that its protein product has a specific role in pathogenesis in horses. Our previous inability to isolate a viable mutant in EHV-1 strain Ab4 gene 67 indicates that it may be essential in the virus life cycle (Sun & Brown, 1994). Gene 67 in EHV-1 strain Ab4 was predicted to encode a 272 amino acid membrane-bound glycoprotein which contains two N-linked glycosylation sites and six phosphorylation sites.

Our results show that the EHV-1 strain Ab4 gene 67 encodes a protein with an \( M_r \) of 36000 which is first detectable by 1 h p.i., but is not synthesized under immediate early conditions. The transcriptional pattern of the genome of EHV-1 is similar to that of HSV-1 (Cohen et al., 1975; Gray et al., 1987; Honess & Roizman, 1974; Clements et al., 1977); therefore in comparison with two HSV-1 genes, an immediate early gene (IE1) and an early gene (UL42), we conclude that gene 67 polypeptide is in the early class. Labelling experiments have demonstrated that the protein is a phosphoprotein, but not a glycoprotein. As a non-glycoprotein, the strong association with membranes requires further analysis for post-translational modifications such as myristylation (Bryant & Ratner, 1990). The protein is part of the virion and removal of virion envelopes results in its remaining with the insoluble fraction, indicating that the gene 67 product is a component of the nucleocapsid or the tegument.

The relationship between the five polypeptides identi-
The intracellular localization of gene 67 was analysed by indirect immunofluorescence. Cells were fixed and stained with anti-67 (1:100), then analysed by indirect immunofluorescence. (a) EHV-1 infected BHK-21/C13 cells; (b) mock infected cells; (c) pCMV67 transfected cells; and (d) pCMV10 transfected cells.
Fig. 6. Localization of the gene 67 product. Purified EHV-1 virions were prepared, treated with NP40 and immunoblotted with anti-67. Lane 1, purified virions; lane 2, mock infected cell extracts; lane 3, supernatant of virions treated with NP40; lane 4, insoluble pellet from treated virions. $M_r$'s are indicated on the left and the 36K gene 67 product by an arrow on the right.

Fied by anti-67 is unclear. Pulse-chase experiments suggest that none of the four smaller peptides is likely to be the precursor of the 36K product (data not shown). Whether they are the post-translation modification products of the gene 67 polypeptide, due to overlapping control elements or to previously unrecognized expression of overlapping small ORFs/exons is under study.

One of the most interesting characteristics of the gene

Fig. 7. Phosphorylation of the gene 67 polypeptide. EHV-1-infected cells were labelled with $\text{[32P]}$orthophosphate, immunoprecipitated with anti-67 and analysed by 8% SDS-PAGE. Lane 1, infected cell extract; lanes 2-4, immunoprecipitated by anti-67 (lane 2 in the presence of 20 μg unrelated fusion protein; lane 3 in presence of 20 μg pUX67 fusion protein); lane 4, with anti-67 alone; lane 5, mock infected cell extract; lane 6, EHV-1 infected cell extracts precipitated with preimmune serum. $M_r$'s are indicated on the left and the 36K gene 67 product by an arrow on the right.

67 protein is the pattern it displays within the infected cell as detected by immunofluorescence. As far as we are aware this pattern has not been described previously for any herpesvirus polypeptide. The staining arrangement is striking and varies depending on the extent of the
infection. Specific filamentous, ribbon-like structures confined entirely to the cytoplasm are apparent in both virus-infected and transfected cells. Small rod-shaped structures appear to coalesce to form large filaments which become concentrated at a perinuclear location although strands continue to wind through the entire cytoplasm. This overt configuration suggests that the protein may be associating with some component of the cytoskeleton although the filaments are much thicker than those normally observed on routine staining of cytoskeletal elements. Double labelling fluorochrome staining with anti-67 and tubulin or actin demonstrated that the protein was not associated with either of these cellular proteins. We have not as yet looked for an association with intermediate filaments although the gross morphology of the staining pattern is not indicative of intermediate filaments. We are now carrying out immunoelectron microscopy to determine the localization of the gene 67 protein within specific cellular components or organelles. Whether these structures have any bearing on the pathogenesis of the virus remains to be determined. Isolation of a deletion mutant will be of major importance in determining the role of gene 67 in the virus life cycle.

Note added in proof. A deletion mutant ED67 has now been isolated and purified. In ED67 sequences encoding amino acids 88 to 272 of gene 67 were deleted and substituted with the lacZ gene. Recombination within the repeat region has resulted in the sequences being deleted in both copies of the gene. The isolation of ED67 demonstrates that gene 67 is non-essential. Subsequent to this manuscript being submitted for publication, similar findings have been reported for EHV-1 KyA (O’Callaghan et al., Journal of Virology 68, 5351–5364, 1994).

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References


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