Characterization of the protein encoded by gene UL49A of herpes simplex virus type 1

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Herpes simplex virus type 1 (HSV-1) gene UL49A potentially encodes a primary translation product of 91 residues with a signal sequence at the N terminus and a membrane anchor domain near the C terminus. Mutants were generated in this gene and utilized to characterize the encoded protein on SDS–PAGE as a 6.7 kDa species which fractionated with infected cell membranes, was a relatively abundant virion component, and was not detectably O-glycosylated. The protein was identified by microsequencing as a 68 residue polypeptide formed by removal of 23 residues from the N terminus of the primary translation product. Cleavage of the signal sequence was also demonstrated by in vitro transcription and translation in the presence of microsomal membranes. The UL49A protein was efficiently solubilized along with envelope proteins by treatment of virions with a non-ionic detergent but only in the presence of a reducing agent, suggesting that it may be an envelope protein that is disulphide-linked to the tegument. It is apparent from mutational analysis that the 10 amino acid residues at the C terminus are not essential for synthesis of the protein, signal sequence cleavage, targeting to membranes and virions, linkage to the tegument and growth of virus in cell culture.

Introduction

The virion of herpes simplex virus type 1 (HSV-1) has a complex structure. The DNA-containing icosahedral capsid is composed of five major proteins and is embedded in a layer (the tegument) comprising at least 20 proteins. The tegument is surrounded by a lipid envelope containing membrane proteins encoded by at least 12 viral genes. Most envelope proteins for which data are available exhibit the hydrophobic properties of membrane proteins and are processed by signal sequence cleavage, N- or O-linked glycosylation, sulphation, phosphorylation or the addition of fatty acid groups, or commonly by combinations of these types of modification. Envelope proteins, by virtue of their exterior location, are involved in adsorption to, penetration into and egress from cells, and have roles in transit of virus between different cell types in the infected host. They also play a primary role in the interplay between the virus and the host’s immune response.

An analysis of the complete DNA sequence of HSV-1 led to the identification of a total of 70 distinct genes (McGeoch et al., 1988). Evidence has since accrued for the existence of a few additional genes which are small, spliced or characterized by significant overlap with other genes. UL49A (or UL49.5) is in the first category, and was identified on the basis of the activity of its promoter and initiation codon in chimeric gene constructs inserted into the viral genome (Barker & Roizman, 1992) and the existence of counterparts in other herpesviruses (Barker & Roizman, 1992; Barnett et al., 1992). UL49A is expressed as a true late (or γ) transcript (Hall et al., 1982) which specifies a primary translation product of only 91 amino acid residues. Barnett et al. (1992) noted that the UL49A protein (henceforth called UL49A) has a putative signal sequence at the N terminus and a hydrophobic domain near the C terminus, and predicted it to be membrane-associated. They also noted that the protein has the potential to be O-glycosylated at serine or threonine residues but lacks consensus sequences for the addition of N-linked carbohydrates. Counterparts of UL49A in other herpesviruses share the sequence features of membrane proteins identified in HSV-1 UL49A, and hence were also predicted to be membrane-associated.

HSV-1 UL49A has not yet been characterized, but information is available on its counterparts in three other herpesviruses: pseudorabies virus (PRV), bovine herpesvirus 1 (BHV-1) and varicella-zoster virus (VZV). Jöns et al. (1996), using a specific antibody, showed that PRV UL49A is present...
in virions as an O-glycosylated 14 kDa envelope protein designated glycoprotein N. In contrast, the BHV-1 protein is an apparently non-glycosylated 9 kDa envelope protein which is disulphide-linked to a 39 kDa virion protein (Liang et al., 1996). VZV UL49A has been identified as a 7 kDa component of a membrane fraction from infected cells, but it is not known whether it is a virion component or whether it is glycosylated (Ross et al., 1997). Synthesis of UL49A is not essential for growth of HSV-1, BHV-1 or VZV in cell culture (Pyles et al., 1992; Liang et al., 1993; Ross et al., 1997). Barker & Roizman (1992) were unable to generate an HSV-1 mutant with an insertion in UL49A, but Pyles et al. (1992) constructed a viable mutant in which part of UL50 (encoding dUTPase) and the promoter and transcriptional initiation site (but not the coding region) of UL49A were deleted. This mutant did not express UL49A mRNA and replicated to wild-type (wt) levels in cell culture. A VZV mutant in which expression of UL49A was disrupted showed a modest (10-fold) reduction in yield (Ross et al., 1997).

HSV-1 UL49A was also found to encode a small virion protein which has properties similar to those of the BHV-1 counterpart, in that it is apparently not O-glycosylated and is disulphide linked to the tegument. In addition, data are presented regarding the abundance of UL49A in the virion, cleavage of the signal sequence and role of the C-terminal region.

**Methods**

**Cells and viruses.** BHK-21 C13 cells (MacPherson & Stoker, 1962) were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% (v/v) newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin B. MeWo cells (Bean et al., 1975) were grown in Dulbecco’s modification of MEM supplemented with 8% (v/v) foetal calf serum, 2 mM glutamine, non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin.

HSV-1 strain 17 syn+ was obtained from a master stock (Brown et al., 1973) or reconstructed by cosmid recombination (Cunningham & Davison, 1993).

Low multiplicity growth curves were obtained by infecting BHK cell monolayers in 50 mm Petri dishes at 0.001 p.f.u. per cell at 37 °C. Virus was harvested at various times after infection by scraping the cells into the medium, then sonicated and titrated in duplicate on BHK cells. Virus yields were calculated from the product of the titre and the volume.

**Plasmids and cosmids.** Plasmids and cosmids were constructed and propagated using standard procedures. The nucleotide numbers given below originate from the HSV-1 DNA sequence reported by McGeoch et al. (1988), and represent either the first base of a restriction site or the first and last bases of a protein-coding region (including the termination codon). Details of the encoded amino acid sequences of the wt and mutated UL49A proteins are shown in Fig. 1.

pFJ3 contains the Escherichia coli lacZ gene under the control of the simian virus 40 early promoter as a 4.1 kbp XbaI fragment (Rixon & McLauchlan, 1990). HSV-1 KmpI υ (104533–107355) contains parts of UL48 (104533–105079) and UL50 (107010–107355), and the entire coding regions of UL49 (105488–106391) and UL49A (106719–106933). UL50 is transcribed rightwards and the other three genes leftwards. A plasmid containing this fragment (pGX143; Davison & Rixon, 1985) possesses a unique BglII site in UL49A at 106750. The lacZ-containing XbaI fragment from pFJ3 was blunt-ended by treating with the Klenow fragment of E. coli DNA polymerase I in the presence of the four deoxynucleoside triphosphates, and ligated into the pGX143 BglII site which had also been blunt-ended. This plasmid (pR74) was used to generate an HSV-1 mutant containing lacZ inserted into UL49A (see below).

A 450 bp HindIII fragment (106562–107012) containing UL49A was isolated from pGX143, blunt-ended and cloned into the Smal site of the baculovirus expression vector pAcCL29-1 (Livingstone & Jones, 1989). By virtue of sites flanking the Smal site, a SacI–BamHI fragment containing UL49A was cloned into the corresponding sites of the T7/SP6 expression vector pGEM-1 (Melton et al., 1984) to form pR29, which contains UL49A in the appropriate orientation for expression from the T7 promoter. pR54.23 and pR54.44 were then generated by inserting a synthetic duplex DNA molecule into the UL49A BglII site in pR29, and the inserted sequences were confirmed by DNA sequencing. In the orientation present in pR54.44, the insertion recreates the 3' end of UL49A fused to an additional 11 C-terminal amino acid residues containing an epitope from human cytomegalovirus pp65. In the opposite orientation present in pR54.23, the 10 C-terminus residues are replaced by five others (Fig. 1). pR29, pR54.44 and pR54.23 were used to express wt and mutated UL49A proteins in coupled transcription and translation reactions.

A cosmid (cos56; 79442–115152; Cunningham & Davison, 1993) containing UL49A was linearized by partial digestion with BglII in the presence of 10 µg/ml ethidium bromide. The DNA was blunt-ended using T4 DNA polymerase, and linear molecules were isolated from agarose gels by electroelution, phenol extracted, ethanol precipitated and ligated. Cosmids in ampicillin-resistant colonies arising after transfection into E. coli were screened by restriction endonuclease digestion. This approach resulted in loss of the BglII site due to insertion of 4 bp at the site of mutation. One cosmid (cosUL49A) lacking the BglII site in UL49A was selected for use in constructing a mutant virus (see below).

**Construction of mutant viruses.** A mutant containing lacZ inserted into the UL49A BglII site (UL49A-lacZ) was constructed by co-transfecting BHK cells with pR74 and HSV-1 DNA, using the methods described by MacLean et al. (1991). One mutant was subjected to several rounds of plaque purification, and the genotype was confirmed by restriction endonuclease analysis.

A mutant with a 4 bp insertion at the UL49A BglII site (UL49A-cos) was regenerared from cosmids as described by Cunningham & Davison (1993). Five cosmids representing the entire genome (one, cosUL49A, containing the mutation in UL49A as described above), were digested with PstI to release the intact inserts and co-transfected into BHK cells. One plaque was subjected to a further round of plaque purification and the mutant genotype was confirmed by probing restriction endonuclease digests of infected cell DNA with an appropriate genomic fragment.

**Expression in vitro.** Coupled *in vitro* transcription and translation of plasmids was carried out in the presence or absence of pancreatic microsomal membranes (Promega) according to the manufacturer’s instructions. Translated proteins were separated from globin, which migrated in the same region of gels, by precipitation with ammonium sulphate at 35% (w/v) saturation.

**Purification and fractionation of virions.** Virions and L particles (virions lacking capsids) were purified from infected BHK or MeWo cells.
HSV-1 UL49A

(a) N-terminal region of wt UL49A and mutated derivatives

<table>
<thead>
<tr>
<th>Wt UL49A</th>
<th>cosUL49A (and derived virus UL49A-cos)</th>
<th>pR74 (and derived virus UL49A-lacZ)</th>
<th>pR54.44</th>
<th>pR54.23</th>
</tr>
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<tr>
<td>MGGPRPRVRVCRAGLIFVILVALAGDAGP</td>
<td>DLPETACGARLNPCCVG - 3ATCCAGAGGCTACGTCGCCCAGCCCTGAANCCGCCCTGTGGGGGTGA</td>
<td>LDPGRPRNSREICTAGACCCGGTCGACGCCGGAATCTCGAGAGATCTAA</td>
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(b) C-terminal regions

<table>
<thead>
<tr>
<th>wt UL49A</th>
<th>cosUL49A (and derived virus UL49A-cos)</th>
<th>pR74 (and derived virus UL49A-lacZ)</th>
<th>pR54.44</th>
<th>pR54.23</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQRLLAAGHA- 1GCCAGAGGCTACTGGCGGCCACGCCCTGA</td>
<td>DLPETACGARLNPCCVG - 3ATCCAGAGGCTACGTCGCCCAGCCCTGAANCCGCCCTGTGGGGGTGA</td>
<td>LDPGRPRNSREICTAGACCCGGTCGACGCCGGAATCTCGAGAGATCTAA</td>
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Fig. 1. DNA and aligned amino acid sequences of wt and mutated versions of UL49A. (a) N-terminal region of wt UL49A and mutated versions, from the ATG codon to the fifth base of the BglII site at which insertions were made. Hydrophobic residues predicted to constitute the signal and transmembrane sequences are underlined. (b) C-terminal sequences from the sixth base of the BglII site (or its replacement) to the relevant stop codon (wt UL49A, cosUL49A, pR74) or the end of the inserted oligonucleotide (pR54.44, pR54.23).

by centrifugation on 5–15% (w/v) Ficoll gradients as described by Szilagyi & Cunningham (1991). In some experiments, virions were labelled with [35S]methionine prior to purification as described by MacLean et al. (1992). Protein profiles were routinely checked by SDS–PAGE, and selected preparations were also assessed by electron microscopy for the absence of membranous or other non-viral particulate material.

Treatment of virions with a non-ionic detergent effectively fractionates soluble envelope proteins (and a proportion of certain tegument proteins) from insoluble capsid-tegument material (McLauchlan & Rixon, 1992). A solution of 10% (v/v) NP40 in PBS (140 mM NaCl, 2·7 mM KCl, 8 mM Na2HPO4, 1·4 mM KH2PO4, pH 7·2) was added to virion preparations resuspended in PBS to yield a final concentration of 1% NP40. The mixture was incubated on ice for 15 min, and microcentrifuged at 11000 g for 5 min at 4 °C. The supernatant (containing envelope proteins) was clarified by a second microcentrifugation step. The pellet (containing capsid-tegument proteins) was resuspended in PBS by bath sonication, microcentrifuged for 5 min, drained, and resuspended by bath sonication in a volume of PBS equal to that of the envelope fraction. L particles were treated similarly to yield envelope and tegument fractions.

■ Preparation of infected cell extracts. Extracts from infected cells labelled with 50 µCi/ml [35S]methionine from 4–24 h post-infection (p.i.) were prepared as described by MacLean et al. (1993). For
pulse–chase experiments, the radiolabel was added to a final concentration of 100 µCi/ml at 10 h p.i. for 15 or 120 min, and infected cell extracts were prepared immediately or after a 12 h chase in the presence of unlabelled methionine. To study O-glycosylation patterns, cells were infected and maintained continuously in 1 mM monensin.

Fractionation of infected cells into cytosolic and membrane components was based on the method described by Bryant & Ratner (1990) as modified by MacLean *et al.* (1992, 1993). Briefly, cells were disrupted by Dounce homogenization in a hypotonic buffer containing a reducing agent (7 mM 2-mercaptoethanol). Intact cells, nuclei and large membrane fragments were pelleted by low speed centrifugation (relative centrifugal force of 600 at 3 min) in the presence of 150 mM NaCl. The supernatant was adjusted to 1 M NaCl, and a membrane fraction obtained by high speed centrifugation (relative centrifugal force of 138 000 at 3 min) for 30 min. The final supernatant was retained as a cytosolic fraction.

**Gel electrophoresis.** Samples were reduced and denatured, and subjected to SDS–PAGE using 16.5% Tris–Tricine polyacrylamide gels which resolve small proteins (Schägger & von Jagow, 1987), or using standard 9.5% Tris–glycine polyacrylamide gels. Gels were either electro-blotted to PVDF membranes for mass spectrometric analysis and protein sequencing of non-radiolabelled proteins, or fixed and stained with Coomassie blue (non-radiolabelled proteins) or treated with EnHance, dried and autoradiographed (radiolabelled proteins).

**Mass spectrometry.** Proteins separated by SDS–PAGE were electro-blotted, stained with sulforhodamine and subjected to trypsin digestion as described previously (Coull & Pappin, 1990; Pappin *et al.*, 1995). The masses of resulting peptides were determined using a Finnigan Lasermat laser desorption mass spectrometer (Pappin *et al.*, 1993; Davison & Davison, 1995).

**Results**

**Construction of UL49A mutants**

The inability of Barker & Roizman (1992) to generate an HSV-1 mutant containing a small epitope-encoding insertion at the *BglII* site near the 3′ end of the UL49A gene suggested that the C terminus of UL49A is essential for virus viability. However, different approaches were used to produce two mutants with insertions at the same site (*UL49A-lacZ* and *UL49A-cos*). Neither could be distinguished from wt on the basis of plaque size and morphology, and *UL49A-lacZ* displayed only a very slight depression in yield (no more than twofold) in cell culture following infection at low multiplicity (Fig. 2). The experiments described below indicate that both mutants synthesized forms of UL49A with modified C termini (Fig. 1).

**Identification of UL49A in infected cell membranes**

A small methionine-containing protein was detected in the total cell extract and membrane fraction from wt-infected cells (Fig. 3, lanes 6 and 2), but not those from *UL49A-lacZ*. Instead, *UL49A-lacZ* specified a slightly larger protein most clearly seen in the membrane fraction (lanes 5 and 3). These were the only consistent differences observed between proteins induced by the two viruses. Migration of small proteins in Tris–Tricine gels appeared to be sensitive to the loading conditions, and thus the apparent sizes of these proteins estimated by comparison with molecular mass markers varied somewhat from gel to gel (6.3 and 8.2 kDa in Fig. 3). To avoid confusion, the most frequently observed sizes (6.7 and 9.6 kDa) are used in the subsequent discussion.
These results suggest that the 6–7 and 9–6 kDa proteins are the products of the wt and mutated UL49A-lacZ genes, respectively. The predicted sizes of the proteins differ by only 0–4 kDa (Fig. 1). The larger difference observed by SDS–PAGE may reflect aberrant mobility of one or both proteins, but the precise cause is unknown.

**Identification of UL49A in virions**

In order to assess whether UL49A is a virion component, [35S]methionine-labelled virions were examined by SDS–PAGE. A 6–7 kDa protein present in wt (Fig. 4a, lane 2) was absent from UL49A-cos (lane 7), and an 8–3 kDa protein in the mutant (lane 7) was absent from wt (lane 2). Detergent fractionation of virions was then carried out, and was shown effectively to solubilize recognized envelope proteins while leaving capsid proteins and the majority of tegument proteins as insoluble material (Fig. 4b). Fractionation of proteins was not in general affected by including a reducing agent during treatment of the virions (Fig. 4a, b). In the absence of reducing agent, however, the majority (60–80%) of the 6–7 or 8–3 kDa protein was present in the capsid-tegument fraction (in Fig. 4a compare lane 3 with 5, and lane 8 with 10), but in the presence of a reducing reagent in excess of 90% of the protein fractionated with the envelope (compare lane 4 with 6, and lane 9 with 11). The 6–7 and 8–3 kDa proteins were also present in L particles (which lack capsids), and fractionated similarly (data not shown). Differential fractionation of these proteins in the presence or absence of reducing agent was observed consistently in several experiments.

These results indicate that the 6–7 and 8–3 kDa virion proteins are encoded by the wt and UL49A-cos forms of UL49A, respectively. The requirement for reducing conditions for efficient solubilization suggests that UL49A, unlike other envelope proteins, may be disulphide-linked to a tegument component.

**Direct identification of UL49A**

In order to confirm directly the identity of the 6–7 and 8–3 kDa proteins, samples were subjected to mass spectrometric and protein sequencing analyses. The proteins were obtained from the capsid-tegument fraction of wt and UL49A-cos virions prepared in the absence of reducing agent. They were separated by SDS–PAGE and electro-blotted to a PVDF membrane, and digests of excised bands with sequencing grade trypsin were subjected to laser desorption mass spectrometry using α-cyano-4-hydroxycinnamic acid as a UV-absorbent matrix and insulin B-chain (protonated mass 3496±9 Da) as a calibration marker. As is usual in this type of analysis, not all peptides produced from the proteins were detected and the matrix itself produced several masses of less than 600 Da. Masses derived specifically from the excised proteins were identified by careful comparison with other samples, including several virion proteins as well as the matrix alone. The observed masses of tryptic peptides are listed in
Table 1. Masses of protonated tryptic peptides derived from UL49A proteins isolated from wt and UL49A-cos viruses

<table>
<thead>
<tr>
<th>Observed mass (Da)</th>
<th>Corresponding sequence</th>
<th>Residues in primary translation product</th>
<th>Predicted mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1609±5</td>
<td>GEPPGEEGGRDGIGGAR</td>
<td>29–45</td>
<td>1611±7</td>
</tr>
<tr>
<td>985±4</td>
<td>GEPPGEEGGR</td>
<td>29–38</td>
<td>985±0</td>
</tr>
<tr>
<td>646±2</td>
<td>DGIGGAR</td>
<td>39–45</td>
<td>645±7</td>
</tr>
<tr>
<td>Not detected</td>
<td>AR</td>
<td>91–92</td>
<td>246±3</td>
</tr>
</tbody>
</table>

Table 1, with their proposed locations in the UL49A primary translation products and the predicted masses of the putative peptides. Three peptides were detected in both samples and correspond to predicted UL49A tryptic products from residues 29–38 and 39–45, in addition to a partial digestion product consisting of a fusion of these two peptides. The smallest peptide was detected only in the mutant protein, and corresponds to residues 91–92 in the novel C-terminal region. The peptides detected in proteins derived from virions were also observed in an analysis of UL49A derived from L particles. These results strongly indicate that the 6±7 and 8±3 kDa proteins originated from the UL49A gene.

N-terminal sequencing of the 6±7 and 8±3 kDa proteins yielded the sequences DAGPRGGEPPGEGRDGIGGAR and DAGPRGGEPPGEGRDGIGGARIETQNY5g, respectively, where ? signifies an unidentified residue and g a probable G residue. This confirms the identification of the proteins as the products of UL49A and shows that the N terminus of the mature protein is located at residue 24 in the primary translation product. The mature N terminus is consistent with cleavage of a signal sequence for membrane translation from the primary translation product, as predicted by Barnett et al (1992). The predicted masses of the mature proteins (6±8 kDa in wt and 7±7 kDa in UL49A-cos) correspond reasonably well to those estimated from gel mobility (6±7 and 8±3 kDa).

Abundance of UL49A in virions

Despite its small size, UL49A was readily seen after staining gels with Coomassie blue or staining electro-blotted membranes with sulforhodamine (data not shown), thus indicating that the protein is a relatively abundant virion component. Moreover, densitometry of virion proteins shown in Fig. 4a, lane 2 indicated that UL49A contains one-third the amount of methionine label as VP26, a capsid protein which is also expressed with true late (or γ) kinetics. Taking into account the number of methionine residues in the two proteins (three in mature UL49A, four in VP26) and the fact that 900 copies of VP26 are present per virion (Trus et al., 1995), it is concluded that each virion contains approximately 400 copies of UL49A.

Post-translational modification of UL49A

Having shown that mature UL49A is generated in vivo by cleavage of the primary translation product, a system was utilized for transcribing and translating plasmids to investigate whether this modification occurs in vitro in the presence of pancreatic microsomes (Fig. 5; see Fig. 1 for sequences). UL49A translation products were identified by comparison with appropriate controls (data not shown). In this and other experiments, the total level of translation was variable but consistently less in the presence of microsomes, and cleavage was partial. The low level of cleavage in the experiment illustrated in Fig. 5 corresponded with that obtained using control RNAs (data not shown). The 11±3 kDa primary translation product from wt UL49A in pR29 (lane 4) was processed to a 6±7 kDa protein (lane 5) corresponding in mobility with UL49A from wt-infected cells (lane 3). The 110±0 kDa product from mutated UL49A in pR54.23 (lane 6)
was processed to a 6.5 kDa protein (lane 7), and the 13.1 kDa product of pR54.44 (lane 8) to a 9.4 kDa protein (lane 9). The somewhat larger sizes of the primary translation products of pR29, pR54.23 and pR54.44 estimated by SDS–PAGE compared with the predicted sizes (Fig. 1; 9.2, 8.8 and 10.4 kDa) presumably reflect aberrant mobility contributed by a region in the shared N-terminal sequences.

These results show that the primary translation product of UL49A can be cleaved in the presence of microsomal membranes in vitro to yield a protein that corresponds in mobility with the 6.7 kDa protein detected in wt-infected cells, and that forms of UL49A modified at the C terminus are also cleaved appropriately. They support the view that the N-terminal region of the translated protein constitutes a signal sequence (predicted size 2.4 kDa) that is cleaved after insertion into membranes.

The correspondence in size of mature UL49A with the protein produced in vitro in the presence of microsomal membranes indicates that it is not glycosylated like its BHV-1 counterpart. However, since the PRV counterpart is O-glycosylated, additional experiments were carried out to determine whether HSV-1 UL49A is similarly modified. Peptides detected by mass spectrometry and residues identified by protein sequencing are clearly not modified in mature UL49A. However, neither of these analyses yielded information about the bulk of the protein (residues 46–90).

The presence of monensin during infection did not affect synthesis of the 6.7 or 9.4 kDa proteins specified by wt or UL49A-lacZ, respectively (in Fig. 6a, compare lanes 4 and 5; lanes 7 and 6), although it efficiently inhibited synthesis of mature gD (Fig. 6b). The lack of effect of monensin on UL49A was observed in several experiments, and no other virus-
induced proteins were observed to differ consistently between wt and UL49A-lacZ in its presence. Attempts to radiolabel the wt or mutated forms of UL49A with [14C]glucosamine were uniformly unsuccessful, again suggesting that they are not O-glycosylated (data not shown).

In a further investigation of additional processing of the 6.7 kDa protein, pulse–chase experiments were carried out. Fig. 6c shows that the 6.7 kDa UL49A protein was synthesized in cells infected with wt virus that had been pulse-labelled at 10 h p.i. for a short period (15 min) with labelled methionine (lane 4). This protein was not reduced in relative abundance or altered in mobility during a 12 h chase with unlabelled methionine (lane 5; proteins in this lane are slightly retarded with respect to other lanes). The 6.7 kDa protein synthesized during labelling for 120 min was also not detectably processed before or after chasing (lanes 7 and 6). The 9.6 kDa protein synthesized by UL49A-lacZ was similarly not processed under these conditions (lanes 2 and 3; lanes 9 and 8). These results suggest that the 6.7 kDa protein is the first product of the UL49A gene detected during viral infection, and that the great majority is not processed into forms with different mobilities.

Discussion

Barnett et al. (1992) concluded from DNA sequence data that HSV-1 UL49A and its counterparts in other herpesviruses are potential components of the virion envelope which may be O-glycosylated. In accord with this, Jöns et al. (1996) identified PRV UL49A as a 14 kDa virion protein which, from virion fractionation experiments and immunoelectron microscopy, is associated with the virion envelope, and which, from glycosidase treatment experiments, is O-glycosylated. Liang et al. (1996) subsequently showed that BHV-1 UL49A is present in infected cells and virions as a 9 kDa protein which, from biontynilation and trypsinization studies, is exposed at the external surfaces of the virion envelope and infected cell membrane. Experiments involving glycosidase treatment, however, failed to demonstrate O-glycosylation of the BHV-1 protein. SDS–PAGE of virion proteins under reducing or non-reducing conditions implied that a proportion of BHV-1 UL49A is present in infected cells as a monomer and homodimer, and also as a heterodimer consisting of UL49A disulphide-linked to a 39 kDa virion protein. The gene encoding the 39 kDa protein was not identified.

Using a combination of genetic and physical techniques, HSV-1 UL49A is identified as a relatively abundant 6.7 kDa virion component which fractionates with membranes from infected cells. There was no evidence for O-glycosylation of the majority of UL49A, since the protein produced in vitro in the presence of microsomal membranes was indistinguishable in size from that in infected cells, and its mobility was not detectably altered by the presence of monensin in infected cells or in pulse–chase experiments. This conclusion is in line with that made for BHV-1 but not PRV.

Protein sequence data showed that mature HSV-1 UL49A is a 68 residue protein formed by cleavage of a 23 residue signal sequence from the 91 residue primary translation product. Cleavage of the signal sequence was also demonstrated in vitro. The majority of UL49A molecules fractionate with capsid-tegument proteins (or tegument proteins in L particles, which lack capsids) when virions were treated with a non-ionic detergent in the absence of a reducing agent, but most fractionated with envelope proteins in the presence of a reducing agent. These results are thus consistent with the situation proposed for BHV-1, in which UL49A molecules are located in the virion envelope and are disulphide-bonded to a component of the tegument.

The presence of potential signal sequence and transmembrane anchor domains in the primary translation product of HSV-1 gene UL49A led Barnett et al. (1992) to predict that HSV-1 UL49A and its counterparts in other herpesviruses are membrane-spanning proteins. UL49A is clearly associated with the virion envelope in PRV, BHV-1 and HSV-1, but formal proof that it is an integral membrane protein, such as would emerge from topological studies of regions of internal and external protein domains, is lacking in each case. Nonetheless, the presence of a potential transmembrane anchor and experimental demonstration of signal sequence cleavage in vitro constitutes strong evidence that HSV-1 UL49A is translated on membranes and is retained via its transmembrane anchor. The alternative, that it is secreted and retained on the exterior of the envelope via fatty acid modification or linkage to another membrane protein, appears less likely.

If UL49A is disulphide-linked to a tegument protein, the linkage is likely to be direct since no other virion protein was detected whose fractionation was dependent on a reducing environment. Residues in mature UL49A which may form disulphide linkages are located at C46, C73, C79 and C82 (Fig. 7), but the observation that the UL49A-cos protein was linked to the tegument implies that C82 is not specifically required. If linkage occurs via C46, the only completely conserved residue in HSV-1 UL49A and its counterparts (Fig. 7), the cognate domain of the tegument protein must protrude through the envelope. Alternatively, UL49A may be linked via C73 or C79, since its counterparts also possess at least one cysteine residue in the potential endodomain or in the adjacent region of the transmembrane domain (Fig. 7). These arguments are speculative at present, however, since PRV UL49A was reported to fractionate with envelope proteins in the absence of a reducing agent and thus is apparently not disulphide-linked, despite the presence of appropriate cysteine residues. If the linkage established by UL49A is functionally significant, it might be involved in an aspect of uncoating or envelopment of virions, or in promoting virion stability. Each of these possibilities is clearly open to experimental investigation.

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Fig. 7. Alignment of the amino acid sequences of HSV-1 UL49A and its counterparts in other herpesviruses. The sequences are shown in three groups corresponding to the α-, β- and γ-herpesviruses, and a consensus (con) of conserved residues is shown for each group. The four cysteine residues present in mature HSV-1 UL49A are marked by asterisks and numbered with respect to residues in the primary translation product, and the single cysteine residue that is conserved in all four sequences is shown by a single asterisk. The four consensus residues that are in bold type and shown by a single asterisk are also conserved in all four sequences. The sequences were derived from: HSV-1, McGeoch et al. (1988); HSV-2 and VZV, Barnett et al. (1992); equine herpesvirus 1 (EHV-1), Telford et al. (1992); EHV-4, E. Telford & A. Davison (unpublished data); PRV, Jons et al. (1993); Marek's disease virus (MDV), Yanagida et al. (1993); BHV-1, Liang et al. (1993); HCMV, Chee et al. (1990); mouse cytomegalovirus (MCMV), Rawlinson et al. (1996); human herpesvirus 6 (HHV-6), Gompels et al. (1995); HHV-7, Nicholas (1996); Epstein–Barr virus (EBV), Baer et al. (1984); EHV-2, Telford et al. (1995); herpesvirus saimiri (HVS); Albrecht et al. (1992); HHV-8, Russo et al. (1996).
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