The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold

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Herpes simplex virus type 1 (HSV-1) polypeptides specified by overlapping genes UL26 and UL26.5 form a scaffold around which the icosahedral capsid shell is assembled. In a series of cleavage events catalysed by the UL26-encoded protease, the full-length UL26 product is processed into capsid proteins VP24 and VP21 and the UL26.5 protein is converted into the capsid protein VP22a by the loss of 25 amino acids from its carboxy terminus. The roles of the UL26 and UL26.5 products were investigated using the baculovirus expression system, focusing on the function of the 25 residues cleaved from the UL26.5 protein. A key conclusion from electron microscopic analysis and protein expression studies is that the 25 amino acids at the carboxy terminus of the full-length UL26.5 protein are required for the interaction of the capsid shell proteins with the scaffold in the formation of intermediate capsids. When cells were multiply infected with baculoviruses expressing a truncated form of the UL26.5 product corresponding to VP22a and the essential components of the capsid shell, no capsids were detected, whereas large numbers of capsids were observed when the full-length UL26.5 product was used as a scaffold. The results are consistent with the proposal that cleavage of the UL26.5 product occurs after capsid assembly or when the UL26.5 protein is in a complex with one or more capsid shell proteins. Expression of VP22a in the absence or presence of capsid shell proteins resulted in the formation of large numbers of 60 nm scaffold-like particles. Since VP22a expressed from baculovirus was unable to participate in capsid assembly, these particles cannot be intermediates in the capsid assembly pathway but may be similar in structure to the protein cores present in HSV-1 immature (B) capsids.

Introduction

Herpes simplex virus type 1 (HSV-1) gene UL26 specifies a 635 amino acid protease that cleaves itself between alanine and serine residues at positions 247-248 and 610-611 to generate the capsid proteins VP24 and VP21 (McGeoch et al., 1988; Liu & Roizman, 1991 a; Davison et al., 1992; Preston et al., 1992; Dilanni et al., 1993a). Another gene, UL26.5, lies within the UL26 open reading frame (ORF) and encodes a protein identical in sequence to the 329 amino acids at the carboxy terminus of the UL26 product (Liu & Roizman, 1991 b, 1993). The protease cleaves the UL26.5 product at the site near the carboxy terminus to form the capsid protein VP22a (Fig. 1). The fate of the 25 amino acids cleaved from the carboxy terminus of UL26 and UL26.5 products is unknown.

The UL26.5 and UL26 products have a central role in the assembly of intermediate (B) capsids, which are the precursors of DNA-containing (C) capsids (Perdue et al., 1976; Ladin et al., 1980; Preston et al., 1983). The HSV intermediate capsid is composed of seven proteins encoded by six genes (Gibson & Roizman, 1972; Heilman et al., 1979; Cohen et al., 1980; Davison et al., 1992; Person et al., 1993). VP22a is the most abundant protein in these capsids, being present in approximately 1100 copies per capsid. VP21 and VP24, on the other hand, are minor components and are present in approximately 100 copies per capsid (Newcomb & Brown, 1989, 1991; Newcomb et al., 1993). VP22a and VP21 form the core around which the capsid shell proteins VP5, VP19C and VP23 are arranged (Rixon et al., 1988; Sherman & Bachenheimer, 1988; Newcomb & Brown, 1991). The capsid shell is organized into an icosahedral lattice consisting of 162 capsomers, of which 12 are pentons and the remainder are hexons (Wildy et al., 1960). The available evidence suggests that both the hexons and pentons are composed of VP5 (Trus et al., 1992; Newcomb et al., 1993; Zhou et al., 1994). The capsomers are connected by triplexes, formed by VP19C and VP23.
in a 1:2 ratio (Schrag et al., 1989; Booy et al., 1991; Newcomb et al., 1993). VP26 is present on the outer surface of the capsid on the tips of the hexons and is not required for the formation of the capsid shell (Tatman et al., 1994; Thomsen et al., 1994; Booy et al., 1994; Zhou et al., 1994). The precise location of VP24, which is proteolytically active, is unknown but it lies within the cavity of the capsid (Newcomb & Brown, 1989; Weinheimer et al., 1993).

The UL26 and UL26.5 products act as a template for capsid assembly and are analogous to the scaffolding proteins of dsDNA bacteriophages. VP22a and VP21, like their bacteriophage counterparts, are transiently associated with capsids, forming a central core which is thought to be released from the capsid as the viral DNA is packaged. VP22a self-assembles in vitro into distinct structures similar to those produced by the bacteriophage P22 scaffolding protein (Fuller & King, 1981; Newcomb & Brown, 1991). The construction of cell lines expressing the UL26 and UL26.5 products has enabled mutants lacking these proteins to be generated. A virus containing a deletion in the protease domain of the UL26 gene produced intermediate capsids but no DNA-containing capsids in non-complementing cells and in this respect resembled the temperature sensitive (ts) protease mutant ts1201, which fails to package viral DNA at the non-permissive temperature (Preston et al., 1983; Gao et al., 1994). In contrast to the UL26 deletion mutant, virus unable to express the UL26.5 product packaged DNA to a limited extent in non-complementing cells and produced low yields of viable virus (Matusick-Kumar et al., 1994). This unexpected result suggests that the UL26 product may be able to substitute partially for the UL26.5 product. A mutant containing a deletion in both the UL26 and UL26.5 genes failed to make capsids in non-complementing cells (Desai et al., 1994). Although the function of homologous genes in other herpesviruses has been less well characterized, studies on the human cytomegalovirus protease and assembly protein suggest that these proteins have very similar properties and roles to their HSV-1 counterparts (Gibson et al., 1990; Welch et al., 1991, 1993; Baum et al., 1993).

Recently, it has been shown that HSV-1 intermediate capsids are assembled in insect cells multiply infected with baculoviruses expressing HSV-1 capsid proteins (Tatman et al., 1994; Thomsen et al., 1994). Capsids were also formed in the absence of the UL26 protease, suggesting that UL26.5 product on its own could form a scaffold for production of the icosahedral shell. We have taken advantage of this finding together with the observation that cells co-infected with baculoviruses expressing the UL26 and UL26.5 products contained large numbers of scaffold-like particles (Preston et al., 1994) to investigate further the functions of the UL26 and UL26.5 products.

**Methods**

**Cells.** Spodoptera frugiperda S21-AE cells, subcloned from IPLB-S21 cells (Vaughn et al., 1977) were cultured in TC100 medium (Life Technologies) containing 5% fetal calf serum and 100 units/ml penicillin and streptomycin. BHK-21 clone 13 cells were grown in Eagle's MEM (Life Technologies) containing 10% tryptose phosphate and 10% newborn calf serum.

**Viruses.** The recombinant baculoviruses containing the HSV-1 strain 17 capsid genes UL18 (VP3), UL19 (VP5), UL26 (protease), UL26.5 (major scaffold protein) and UL38 (VP19C) have been described previously (Preston et al., 1994; Tatman et al., 1994). Mutant UL26 and UL26.5 genes in the baculovirus transfer vector were recombined into the baculovirus expression vector AcPAK6, which contains the β-galactosidase gene under the control of the polyhedrin promoter (Kitts & Possée, 1993; Fig. 1).

**Nomenclature of UL26 and UL26.5 products.** There are a variety of nomenclatures for the UL26 and UL26.5 proteins, none of which is completely satisfactory. Two of the most commonly used systems are given in Fig. 1. We have used the earliest version in which products are...
referred to as structural proteins (Gibson & Roizman, 1972). The form of VP21 containing the 25 amino acids at the carboxy terminus of the UL26 product has been referred to as pVP21 but this name does not imply that there is a product–precursor relationship. For consistency, in labelling proteins separated by SDS-PAGE, the full-length UL26.5 product has been termed pVP22a. The UL26.5 protein in the text refers to the uncleaved form of the protein.

Construction of mutant UL26 and UL26.5 genes. To analyse the functions of the UL26 and UL26.5 proteins, genes encoding VP24, VP21, pVP21, VP22a and a mutant UL26.5 gene with an inactivated cleavage site were constructed (Fig. 1).

1. VP24. The construct encoding VP24 was made by engineering a termination codon after the alanine codon 247 on the amino-terminal side of the scissile bond in the UL26 ORF, using primer extension PCR. A plasmid containing the UL26 ORF flanked by BgII sites was cleaved with HpaI and KpnI and the large fragment ligated to the HpaI- and KpnI-cleaved PCR product formed using the primers 5' ACCGGCTTTACAAATGATGTCACTGGCCGAGGACGGC 3' and 5' CTTGTCAACGCCGACCGGCACTGGCCGAGGACGGC 3'.

2. pVP21. The gene specifying pVP21 was constructed by introducing a methionine translational start codon after the alanine codon 247 in the UL26 ORF. A plasmid containing the UL26 ORF with a unique BgII site at the 5' end of the gene was digested with BgII and BstEII, and the large DNA fragment ligated to the BgII- and BstEII-cleaved PCR product generated using the primers 5' CCCGGAGAAGACTCTACACCATGAGGCCGAAAATTACATGCAGTTGGGGGCGG 3' and 5' CTTGTCAACGCCGACCGGCACTGGCCGAGGACGGC 3'. The pVP21 gene was flanked by BgII sites by inserting a BgII linker into the unique EcoRI site at the 3' end of the gene. This construct was referred to as pIM19.

3. VP22a. A termination codon was introduced after the codon for the alanine at the maturation cleavage site in the UL26.5 ORF, using the complementary oligonucleotides 5' CTGGTCACGGCAAGCCCTATGACCCAG 3' and 5' AGCTTTCTAGATCGTACCCCCGTGACCAACGCCG 3'. The plasmid pJK2 (Nicholson et al., 1994), containing the UL26.5 gene under the control of the human cytomegalovirus immediate early promoter, was cleaved with Apal and HindIII, the large DNA fragment purified and ligated to the annealed primers to generate the plasmid pJK33. The Xhol site in pJK33 was converted into a BgII site as described by Preston et al. (1994).

4. VP21. The encoding VP21 was constructed by ligating the Bsu36I–KpnI fragment from the pVP22A ORF into the plasmid pIM19 cleaved with Bsu36I and KpnI.

5. UL26.5M. The mutation of the alanine codon to valine (GTG) at the maturation cleavage site was accomplished by annealing complementary oligonucleotides 5' GATCCCAACGGAGGCATCACAAGCCGAGGCGGCGGCGGCCCTTGTCAACGTGACCAAGGCCGACGACAC3' and 5' GTGTGTCGCGCGCTACGTACCCACGTCGGCGAACGAGGCCGCCCTCGCGTTGATCTCCGGCGGTG 3' to form a BamHI–PmlI fragment which was ligated to the large BamHI–PmlI fragment from pGX262. The plasmid pGX262 has the UL26.5 gene flanked by BgII sites. The UL26.5 gene was reconstructed by inserting the cloned BamHI genomic fragment back into the mutated plasmid.

The regions in the pVP21 and VP24 constructs generated by PCR were sequenced to confirm that no adventitious mutations had been created. In addition, the presence of the correct mutations in the VP22a and maturation site mutant constructs was verified by sequencing. All the UL26 and UL26.5 mutant genes were cloned as BgII fragments into the BgII site of the baculovirus transfer vector pAcCL29.1B (Preston et al., 1994).

Antibodies. Mouse monoclonal antibodies LP12, generously provided by A.C. Minson (University of Cambridge, UK) and 5010 (Rixon et al., 1988), which recognize VP5 and the UL26.5 protein respectively, and a rabbit antiserum raised against glutathione S-transferase–UL26 fusion protein (Preston et al., 1994) were the primary antibodies used in indirect immunofluorescence experiments. These primary mouse and rabbit antibodies bound to antigen were detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC-GAM) and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (TRITC-GAR), respectively. In protein blot analysis the mouse monoclonal antibody 406 (Serotec), specific for sequences within VP22a, was used.

Plasmid transfections. BHK-21 cells (7.5 x 10^6) were transfected with 3 µg plasmid DNA using the DNA–calcium phosphate coprecipitation method of Shen et al. (1982). At 4 h after transfection the cells were treated with 25% DMSO in HEPES-buffered saline for 4 min at room temperature, washed, overlaid with tissue culture medium and incubated at 37°C for 20 h.

Immunofluorescence and immunoblotting. Immunofluorescence studies were performed as described by Nicholson et al. (1994). Cells on coverslips were examined under a Zeiss Axiosplan fluorescence microscope. The procedure for immunoblotting was carried out as outlined by Preston et al. (1994), using colloidal gold particles bound to Protein A followed by silver enhancement to detect antibodies bound to the UL26.5 protein.

Electron microscopy of cells. Electron microscopy was carried out essentially as described by Preston et al. (1983).

Radioactive labelling of polypeptides. At 30 h post-infection (p.i.) cell monolayers (2 x 10^6 cells per well of a 24-well plate), infected with 10 p.f.u. per cell of baculovirus or mock-infected, were labelled with 20µCi [35S]methionine per ml in TC100 containing 10% the normal concentration of methionine and 0.5% fetal calf serum. After further incubation for 18 h, the samples were harvested and analysed by SDS–PAGE. Radiolabelled suspension cultures of virus-infected SF21-SE cells (150 ml) were prepared in the same way as the cell monolayers except that the cells were labelled with 30 µCi [35S]methionine per ml in TC100 containing 20% the normal concentration of methionine and 0.5% fetal calf serum.

Sucrose gradient sedimentation of virus-infected cell extracts. Extracts of virus-infected suspension cultures, labelled with [35S]methionine, were prepared essentially as described by Tatman et al. (1994). The disrupted nuclei were centrifuged for 10 min at 5000 r.p.m. in a Sorvall TST41 rotor and the supernatant was layered on to a 1 ml cushion of 40% (w/v) sucrose in NTE (0.5 M-NaCl, 20mM-Tris-HC1 pH 7.4, 0.5 % fetal calf serum). The pellet, formed by centrifugation of the sample at 40000 r.p.m, for 1 h in a Sorvall TST41 rotor, was resuspended in a small volume of NTE by sonication and sedimented through a 12 ml 5 to 40% (w/v) sucrose gradient in NTE (0.5 M-NaCl, 20 mM-Tris–HCl pH 7.4, 1 mM-EDTA). The pellet, formed by centrifugation of the sample at 25000 r.p.m. for 1 h in a Sorvall TST41 rotor, was resuspended in a small volume of NTE by sonication and sedimented through a 12 ml 5 to 40% sucrose gradient in NTE at 25000 r.p.m. for 1 h in a Sorvall TST41 rotor. Eight-drop fractions were collected by dripping the gradient through an 18-gauge needle inserted near the bottom of the tube. Fractions of interest were pooled, diluted in NTE and centrifuged at 40000 r.p.m. for 1 h in a Sorvall TST41 rotor. The pellet was resuspended by sonication in a small volume of NTE, stained with 1% phosphotungstic acid and examined under the electron microscope.

Results

Construction of baculovirus recombinants expressing mutant UL26 and UL26.5 genes

Mutant UL26 and UL26.5 genes, under the control of the polyhedrin promoter, were recombined into the baculovirus expression vector AcPAK6, replacing lacZ.
Fig. 2. Analysis of polypeptides induced by recombinant baculoviruses carrying the HSV genes. Mock- and virus-infected cells were labelled with [35S]methionine and the polypeptides analysed on a SDS-single concentration (10%) polyacrylamide gel. The positions of the UL26 and UL26.5 gene products are indicated on the left and positions of molecular mass markers are indicated on the right.

Putative recombinants forming white plaques in the presence of X-gal were isolated and plaque-purified. To confirm that the viruses expressed proteins of the expected size for the mutant HSV gene product, the virus-infected cell polypeptides were labelled with [35S]methionine and analysed by SDS-PAGE (Fig. 2). The AcUL26-infected cell polypeptide profile contained two novel bands with molecular masses of about 46 kDa and 27 kDa, corresponding to VP21 and VP24 respectively (Fig. 2, lane 11). The recombinant AcVP21 induced a polypeptide which comigrated with the upper novel band in the AcUL26 polypeptide profile and the recombinant AcVP24 induced a species which comigrated with the lower band, suggesting that the correct HSV protein had been expressed by each of these recombinants (Fig. 2, lanes 10 and 12). VP24 produced in cells infected with AcVP24 was proteolytically active, cleaving the UL26.5 product to VP22a and pVP21 to VP21 (Fig. 2, lanes 5 and 9). The HSV protein induced in cells infected with AcVP22a comigrated with the cleaved form of the UL26.5 polypeptide (Fig. 2, lanes 3 and 4). No VP22a, however, was detected in cells dually infected with AcVP24 and the recombinant baculovirus AcUL26.5M expressing the mutant scaffolding protein (Fig. 2, lane 6). The mutation of Ala-610 to a valine residue in the UL26.5 ORF therefore resulted in the formation of a protein that was resistant to cleavage by VP24.

Proteolytic cleavage of UL26.5 is required for the formation of scaffold-like particles

Previous work by Preston et al. (1994) showed that insect cells infected with AcUL26.5, a recombinant baculovirus expressing UL26.5, contained large aggregates of HSV protein, varying in morphology from fibrous material to scaffold-like particles, with structures intermediate between these extremes predominating. By contrast, in cells infected with AcUL26, a recombinant expressing the UL26 protease, the HSV protein aggregates had a uniform morphology with a fibrous appearance. In cells co-infected with AcUL26 and AcUL26.5, semi-crystalline arrays of 60 nm scaffold-like particles and dispersed scaffolds were frequently observed whereas these structures were very rarely detected in UL26.5-infected cells (Preston et al., 1994).

To investigate which region of the UL26 gene ORF is required for the production of large numbers of scaffold-like particles in the mixed infections with AcUL26 and AcUL26.5, baculoviruses expressing VP21 (AcVP21) or VP24 (AcVP24) were assayed for the ability to stimulate the formation of scaffold-like particles in cells infected with AcUL26.5 (Fig. 3). Only AcVP24 had an effect on scaffold formation by the UL26.5 product. Cells co-infected with AcVP24 and AcUL26.5 (Fig. 3a) contained large numbers of scaffold-like particles, similar in appearance to those present in mixed infections of AcUL26 and AcUL26.5. In cells infected with AcVP24 alone, no distinctive aggregates of protein were observed whereas in cells infected with AcVP21 (Fig. 3b), fibrous material was detected, similar in appearance to the HSV-1 protein aggregates present in cells infected with AcUL26 (Fig. 3f).

Since VP24 is proteolytically active, it is likely that cleavage of the UL26.5 product to VP22a is responsible for the transition of the UL26.5 product into scaffold-like particles. To test this idea, a baculovirus recombinant AcUL26.5M, expressing a mutant UL26.5 product with an inactive cleavage site, was produced (Fig. 1). Electron microscopic examination of thin section preparations of cells co-infected with AcUL26.5M and AcVP24 revealed that the cells did not contain large numbers of scaffold-like particles and that aggregates of the mutant UL26.5 protein were similar in morphology to those observed in AcUL26.5-infected cells (Fig. 3c, e). The finding that cells infected with baculovirus expressing VP22a contained abundant scaffold-like particles confirmed that...
removal of the 25 carboxy-terminal amino acid residues from the UL26.5 product was required for the formation of large numbers of scaffold-like particles (Fig. 3d) and suggested that the expressed VP22a was adopting a similar conformation to VP22a generated from the UL26.5 product by proteolytic cleavage. Interestingly, the morphology of VP22a aggregates, like those of the uncleaved protein, was variable and, in addition to numerous scaffold-like particles, similar structures were observed to those present in AcUL26.5-infected cells.

VP22a is unable to function as a template for capsid assembly

To determine whether VP22a could participate in capsid assembly, cells were multiply infected with recombinant baculoviruses expressing the capsid shell proteins VP5, VP19C and VP23 together with a baculovirus expressing VP22a. No capsids were detected in thin section preparations of the infected cells examined under the electron microscope, suggesting that the scaffold-like particles produced by VP22a were abortive products rather than intermediates of capsid assembly (Fig. 4a, b). Limited assembly of the capsid shell proteins into spiral and sheet structures was, however, observed. These structures have also been detected in cells infected with baculoviruses expressing the capsid shell proteins in the absence of any scaffolding protein (Fig. 4d) and in cells infected with a HSV-1 mutant containing a deletion in both the UL26 and UL26.5 genes (Desai et al., 1994; Tatman et al., 1994; Thomsen et al., 1994). Large cored capsids, on the other hand, were readily observed in cells infected with viruses expressing the capsid shell proteins and the UL26.5 product prepared at the same time as those containing the capsid shell proteins and VP22a (Fig. 4c).

To verify that VP22a was unable to participate in capsid assembly, a suspension culture of virus-infected cells labelled with [35S]methionine was prepared and the clarified nuclear extract sedimented through a sucrose gradient. As a control, a nuclear extract from cells multiply infected with baculoviruses expressing the capsid shell proteins and the scaffold protein UL26.5 was also analysed. No HSV capsid band was observed in the gradient of the nuclear lysate containing VP22a whereas a capsid band was clearly visible in a sucrose
Fig. 4. Electron micrograph of a thin section of a cell multiply infected with (a, b) AcVP22a, AcUL18, AcUL19 and AcUL38, (c) AcUL26.5, AcUL18, AcUL19 and AcUL38 and (d) AcUL18, AcUL19 and AcUL38. In (a) the aggregate of VP22a adjacent to incomplete capsid shells has a fibrous appearance whereas in (b) VP22a forms scaffold-like particles. The arrow points to VP22a aggregates; (▲), large cored capsid; (▼), an incomplete capsid shell. The bar marker represents 0.5 μm.

gradient of the sample containing the full-length UL26.5 product. Fractions from each gradient were collected, the radioactivity incorporated into protein determined (Fig. 5a, b) and the polypeptides analysed by SDS-PAGE (Fig. 5c, d). In addition, proteins separated on a SDS-polyacrylamide gel were blotted on to nitrocellulose and probed with antibody 406 which recognizes amino acid sequences present within VP22a (Fig. 5e, f). A distinct peak of radioactivity was present in the fractions corresponding to the region where the capsid band was observed in the sucrose gradient of the lysate containing the capsid shell proteins VP5, VP19C, VP23 and the uncleaved UL26.5 product (Fig. 5a). As expected, the distribution of the four capsid proteins coincided with this peak (Fig. 5c). In addition, a second peak of UL26.5 protein was observed higher up in the gradient (Fig. 5c, e). A much broader peak of radioactivity was detected in the gradient of nuclear lysate
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Fig. 5. Sucrose gradient sedimentation of (a) extracts of cells multiply infected with AcUL26.5, AcUL18, AcUL19 and AcUL38 and (b) extracts of cells multiply infected with AcVP22a, AcUL18, AcUL19 and AcUL38. Radiolabelled extracts were sedimented through a 5 to 40% sucrose gradient. Successive fractions were collected from the bottom of each gradient and the radioactivity incorporated into protein determined by TCA precipitation and scintillation counting. Panels (c) and (d) show an autoradiograph of a SDS–single concentration (10%) polyacrylamide gel of samples 5 to 21 from gradient (a) and (b), respectively. The separated polypeptides in panels (c) and (d) were blotted on to nitrocellulose paper and UL26.5 (e) and VP22a (f) detected in the protein blot using monoclonal antibody 406. Purified HSV-1 intermediate capsids (C) and an extract (M) from cells infected with AcVP22a or AcUL26.5 were used as markers.

Although the capsid shell proteins were present in larger amounts on the right-hand side of the peak, supporting the electron microscopic data that capsids were not assembled (Fig. 5d, f). To examine capsid structures present in the gradient, fractions from the peak of radioactivity were pooled, diluted and centrifuged at high speed to concentrate any particles that might be present. No intact capsids were detected in negatively stained preparations of fractions from the gradient containing VP22a but large numbers of incomplete capsid shells were observed, confirming that VP22a was unable to interact with VP5, VP19C and VP23 to generate an intact capsid shell. Capsids, on the other hand, were present in large numbers in fractions from the peak of radioactivity of the gradient containing the UL26.5 product (Fig. 5b).
Fig. 6. The effect of VP22a on the intracellular distribution of VP5. Cells were cotransfected with the following plasmids: (a), pE19 (VP5); (b), pJK33 (VP22a); (c), pJK2 (UL26.5); (d) and (e), pJK2 and pE19; (f) and (g), pJK33 and pE19. Panels (d) and (e) and (f) and (g) represent duplicate exposures of single fields. The secondary antibody used in (a), (e) and (g) was FITC-GAM whereas in (b), (c), (d) and (f) TRITC-GAR was used.
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Interaction of VP22a with VP5

Previous work has shown that the full-length UL26.5 product interacts with VP5, redistributing it to the cell nucleus (Nicholson et al., 1994; Matusick-Kumar et al., 1994). Immunofluorescence experiments were carried out to determine whether VP22a could also affect the location of VP5. Cells were either singly transfected with a plasmid containing the UL19 gene (VP5) or the gene encoding VP22a, under the control of the human cytomegalovirus major immediate early promoter, or cotransfected with both plasmids. The distribution of the HSV proteins was examined by immunofluorescence using antibodies specific for these gene products. In addition, transfections were performed using a plasmid containing the full-length UL26.5 gene instead of the gene specifying VP22a. In cells transfected with the plasmid containing the UL19 gene, VP5 was present in large amounts in both the nucleus and cytoplasm (Fig. 6a) whereas in cells expressing the UL26.5 or VP22a proteins the HSV protein was largely confined to the nucleus (Fig. 6b, c). When VP5 was expressed with either the full-length UL26.5 gene product or VP22a, both proteins colocalized to the cell nucleus (Fig. 6d-g). Neither cross-reaction between antibodies nor any channel leakage in immunofluorescence was ever observed and in the particular experiment depicted all the positive cells in Fig. 6(d–g) contained both the scaffolding protein and VP5. These results show that the loss of 25 amino acids from the carboxy terminus of the UL26.5 polypeptide does not impair the ability of this protein to direct the transport of VP5 to the nucleus or retain VP5 in the nucleus.

The full-length UL26.5 product forms distinct structures with VP5

To investigate whether the uncleaved form of the UL26.5 product or VP22a could form distinct structures with VP5, electron microscopy was carried out on cells co-infected with AcUL19 and AcUL26.5 or AcVP22a. A striking feature of cells expressing VP5 and UL26.5 was the presence of numerous spherical structures, of variable size but usually slightly larger than scaffold-like particles, in the cell nuclei (Fig. 7a). These spherical particles, which had indistinct boundaries, were not detected in cells singly infected with AcUL19 or AcUL26.5, nor were they found in cells co-infected with AcUL19 and AcVP22a (Fig. 7b). Large numbers of scaffold-like particles and other aggregates of VP22a, however, were present in cells expressing VP5 and VP22a. The aggregates found in these cells were similar in appearance to those in cells infected with AcVP22a alone. In cells co-infected with AcUL19 and AcUL26.5, large aggregates of HSV protein were only occasionally observed, suggesting that VP5 was preventing aggregation of the UL26.5 product. This observation supports...
Discussion

We have exploited the finding that HSV-1 intermediate capsids are assembled in insect cells multiply infected with baculoviruses expressing HSV capsid proteins to examine the role of the UL26.5 and UL26 products. Proteolytic cleavage of the UL26.5 product by the UL26 protease resulted in the formation of large numbers of scaffold-like particles which may be similar in structure to the cores present in intermediate capsids of HSV. Since VP22a did not participate in capsid assembly, these scaffold-like particles are not intermediates in the capsid assembly pathway. The failure of VP22a to act as a template for capsid shell formation suggests that cleavage of the UL26.5 product occurs after capsid assembly or when the UL26.5 product is in a complex with one or more capsid shell proteins. This proposal is in keeping with evidence from early work on the ts protease mutant tsl201 which suggested that cleavage of UL26.5 product could take place inside the capsid (Preston et al., 1983). Inefficient cleavage of uncomplexed UL26.5 product by the protease is one strategy the virus could adopt to ensure that most proteolysis occurs after the initial stage of capsid assembly. The observation that cleavage of the UL26.5 product by the protease, both in vivo and in vitro, is slow, together with recent findings that the maturation cleavage sequence within the protease is suboptimum for proteolysis, further supports the idea that cleavage of the UL26.5 product occurs after the scaffolding protein has interacted with the capsid shell proteins (Preston et al., 1983; Dilanni et al., 1993b; Weinheimer et al., 1993; McCann et al., 1994).

Although the 25 amino acid residues at the carboxy terminus of the UL26.5 polypeptide are not required for the redistribution of VP5 to the nucleus, these sequences are essential for the formation of spherical particles and capsids. It is possible that there are multiple VP5 binding sites on the UL26.5 polypeptide, one of which lies within the 25 amino acids at the carboxy terminus. Alternatively, loss of the carboxy-terminal sequences from the UL26.5 product may lead to a conformation change which affects the interaction of VP5 with the scaffolding protein. The scaffold-like particles present in cells expressing VP5 and VP22a were similar in appearance to the structures detected in cells singly infected with AcVP22a, suggesting that VP5 failed to bind to these particles, bound in low numbers or bound in a different conformation. It is possible that VP5 only interacted with VP22a that had not been recruited into scaffold-like particles. Since proteolysis of the UL26.5 product probably occurs at a late stage in capsid assembly, the main function of cleavage of the UL26.5 product by the UL26 protease may be to weaken the interaction between the scaffold and capsid shell to facilitate removal of the protein core during encapsidation of the genome.

The poorly defined spherical particles, found in cells containing large amounts of VP5 and the UL26.5 product, may represent intermediates in the assembly pathway and consist of VP5 attached to the external surface of the UL26.5 scaffold. Further work, however, is required to determine their role in capsid formation.

VP21, in contrast to VP22a, did not form scaffold-like particles and aggregates had a similar appearance to those in cells infected by AcUL26. These results suggest that sequences within the 59 amino acids at the amino terminus of VP21 that are absent from VP22a prevent scaffold formation. This region is much less highly conserved between herpesviruses than the remaining sequences unique to the UL26 protease. It is clear from the results that the amino terminus of VP21, in addition to acting as a spacer between the protease and the scaffolding protein in the UL26 protein, has some other role, perhaps limiting the number of copies of the protease incorporated into capsids.

We thank Professor J. H. Subak-Sharpe and Pfizer Central Research, Sandwich, Kent, UK for their support. We are also grateful to Professor A. C. Minson for generously supplying us with LP12 monoclonal antibody.

References


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(Received 22 November 1994; Accepted 27 February 1995)