Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a

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The intracellular distributions of three herpes simplex virus type 1 (HSV-1) capsid proteins, VP23, VP5 and VP22a, were examined using vaccinia virus and plasmid expression systems. During infection of cells with HSV-1 wild-type virus, all three proteins were predominantly located in the nucleus, which is the site of capsid assembly. However, when expressed in the absence of any other HSV-1 proteins, although VP22a was found exclusively in the nucleus as expected, VP5 and VP23 were distributed throughout the cell. Thus nuclear localization is not an intrinsic property of these proteins but must be mediated by one or more HSV-1-induced proteins. Co-expression experiments demonstrated that VP5 was efficiently transported to the nucleus in the presence of VP22a, but the distribution of VP23 was unaffected by the presence of either or both of the other two proteins.

Introduction

Studies on the composition and structure of capsids of herpes simplex virus type 1 (HSV-1) have identified seven capsid proteins. Five of these (VP5, VP19C, VP23, VP24 and VP26) are present in all capsid forms, whereas two (VP21 and VP22a) form the internal scaffold of immature (B) capsids and are removed during DNA packaging (Gibson & Roizman, 1972; Cohen et al., 1980; Rixon et al., 1988; Sherman & Bachenheimer, 1988; Newcomb & Brown, 1989). The genes encoding these proteins have been identified using a variety of techniques. Thus, VP5 is specified by UL19 (Costa et al., 1984; Davison et al., 1992), VP19C by UL38 and VP23 by UL18 (Pertuiset et al., 1989; Rixon et al., 1990), and VP26 by UL35 (McNabb & Courtney, 1992; Davison et al., 1992). VP21, VP22a and VP24 have an interesting relationship, being encoded by the overlapping genes UL26 and UL26.5 (Liu & Roizman, 1991a). The abundant scaffolding protein VP22a belongs to a family of highly processed proteins encoded by the UL26.5 open reading frame (ORF). This is designated the ICP35 family but for convenience VP22a will be used as a general term throughout this paper regardless of the precise form of the protein concerned. The protein encoded by UL26 is a protease which is responsible for the maturational cleavage of the UL26.5-encoded protein and for cleaving itself into C-terminal and N-terminal fragments (Liu & Roizman, 1991b, 1992; Preston et al., 1992) which are present in the capsid as VP21 and VP24 respectively (Davison et al., 1992; Person et al., 1993; Weinheimer et al., 1993).

The roles of the individual proteins in capsid assembly are not fully understood. The major capsid protein VP5 encoded by UL19 is known to form the hexons and pentons (Trus et al., 1992; Newcomb et al., 1993). Temperature-sensitive (ts) mutants in the UL19 gene have shown that VP5 is required for capsid assembly. Mutations in UL38 (Pertuiset et al., 1989) and UL18 (Desai et al., 1993) have shown that these genes also are essential for capsid assembly. No mutations in UL35 or UL26.5 have been described. However, there is a ts mutation in UL26 (Preston et al., 1983) that results in reduced processing of the protease and the UL26.5 gene product (Preston et al., 1992). This mutant makes large numbers of immature capsids at non-permissive temperatures but is unable to package viral DNA.

As in many other complex systems a better understanding of the mechanism of capsid assembly is likely to require analysis of the behaviour of the individual proteins involved. We report here the cloning and expression of three of the capsid protein genes (UL18, UL19 and UL26.5) and the examination of their properties when introduced into cells, alone or in combination.

Methods

Cells and virus. All experiments were performed in BHK-21 C13 cells cultured in Glasgow-modified MEM supplemented with 10% tryptose phosphate and 10% newborn calf serum (ETC10). HSV-1 strain 17 (Brown et al., 1973) and tsG8 (Weller et al., 1987) were grown in BHK-21 cells at 37 °C and 31 °C respectively. Recombinant and parental
vaccinia viruses (strain WR) were grown at 37 °C on CV1 cells cultured in Dulbecco’s modified MEM supplemented with 5% fetal calf serum.

**Gel electrophoresis.** To prepare capsids, fully confluent BHK-21 cells in 21 plastic roller bottles were infected with 0.001 p.f.u./cell of HSV-1 and incubated at 31 °C in ETC10. After 2 days the culture medium was replaced with MEM containing 20% of the standard concentration of L-methionine, supplemented with 2% newborn calf serum (EC2) and containing 10 μCi/ml of [35S]methionine. When complete c.p.e. had developed the cells were harvested by shaking into the medium and HSV-1 capsids were purified essentially according to the method of Gibson & Roizman (1972).

For vaccinia virus proteins, BHK-21 cells in 35 mm plates were infected at a multiplicity of 10 p.f.u./cell. After 1 h at 37 °C the cells were washed and overlaid with ETC10. At 3 h after infection the culture medium was replaced with EC2 containing 20 μCi/ml [35S]methionine and incubated for a further 21 h.

Protein samples were analysed by SDS-PAGE on 9% (w/v) polyacrylamide gels containing 2.5% N,N’-methylenebisacrylamide.

**Plasmid transfection.** The transfection procedure used a synthetic cationic lipid reagent prepared according to the method of Rose et al. (1991). Thirty μl of lipid reagent was mixed with 70 μl of HBS (20 mM-HEPES pH 7.4 and 150 mM-NaCl) in a polystyrene tube and added to 100 μl of HBS containing 1 μg of DNA in a separate polystyrene tube. The solution was left at room temperature for 10 min prior to the addition of 2 ml of Opti-MEM 1 medium (Gibco BRL). The culture fluid was removed from a 70% confluent monolayer of BHK-21 cells and replaced with the transfection mixture. After a 5 h incubation at 37 °C, ETC10 (2 ml) was added and incubation was continued for a further 19 h at 37 °C.

**Antibodies.** Mouse monoclonal antibodies LP12, 1060 and 5010 recognize VP5, VP23 and VP22a respectively. The rabbit antiserum 20999 was raised against a synthetic peptide comprising the 14 amino acids.
acids immediately upstream from the C-terminal residue of the UL26 ORF product (Preston et al., 1992). Antibodies were used at dilutions of 1:200 (LP12, 1060), 1:100 (5010) or 1:50 (20999) in PBS containing 0.05% Tween 20 and 5% newborn calf serum (solution A).

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (GAM) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (GAR; Sigma) were each used at a dilution of 1:200. Immunofluorescence. All experiments were performed on BHK-21 C13 cells grown on 13 mm glass coverslips placed in 24-well tissue culture dishes. One set of cells was infected with 5 p.f.u./cell of HSV-1 strain 17 or tsG8, and incubated for 9 h at 31 °C or 38.5 °C. A second set of cells, which had been infected with vaccinia viruses or transfected with plasmids, was incubated at 37 °C for 18 to 24 h. At the appropriate times cells were washed in PBS then fixed with 100% methanol at −20 °C for a minimum of 30 min. Subsequent steps were carried out at room temperature in a humidified chamber. Antibodies were diluted in solution A. Samples were incubated for 10 min in solution A, drained and incubated in 50 μl of primary antibody for 45 min. After three 10 s washes in solution A, they were incubated for 45 min in 50 μl of the appropriate secondary antibody. After five 10 s washes in solution A and two rinses in deionized water, the coverslips were mounted in Citifluor, examined using a Zeiss Axioplan fluorescence microscope and photographed on Fujichrome 400 film. For double labelling experiments the primary mouse and rabbit antisera were mixed prior to addition to the coverslips, as were the secondary FITC- and TRITC-GAR antibodies.

Construction of recombinant vaccinia viruses. To express the HSV-1 capsid genes UL18, UL19 and UL26.5 in vaccinia virus the coding sequences first had to be placed under the control of the virus transcriptional promoter and terminator signals. To do this the vaccinia virus expression plasmid pMJ601 (Davison & Moss, 1990) was used, which contains a synthetic vaccinia virus late promoter, designed to give maximized expression.

(i) UL18-vaccinia virus. All nucleotide positions given refer to the sequence of HSV-1 strain 17 (McGeoch et al., 1988). NarI cleaves two residues upstream from the UL18 ATG initiation codon and 381 residues downstream from the stop codon at positions 36053 and 34713 respectively. The 1340 bp NarI fragment, containing the UL18 ORF, was purified from the KpnI fragment present in the plasmid pGX128 and ligated into the NarI site in the multiple cloning sequence of pMJ601. One clone, designated pMJ521, in which the UL18 ORF was in the correct orientation, was selected.

(ii) UL19-vaccinia virus. Construction of the plasmid pJN6 containing the UL19 ORF is shown in Fig. 1. Digestion of pJN6 with BglII released UL19 in a 4189 bp fragment which was cloned into the BamHI site of pCMV10 to give pCMV10Bgl. The KpnI fragment containing UL18 was inserted into pCMV10 to give pE18. To clone UL26.5, a BglII linker was first inserted into the SmaI site of pCMV10 to give pCMV10Bgl. The BglII fragment from pGX263 was then subcloned into pCMV10Bgl to give pJK2.

Results

Expression of HSV-1 capsid proteins by recombinant vaccinia viruses

To determine whether the recombinant vaccinia viruses expressed the HSV-1 proteins, BHK-21 cells infected with each of the recombinant vaccinia viruses were labelled with [35S]methionine and harvested at 18 h post-infection. The recombinant vaccinia viruses were grown to high titre. One of each was selected for further study and these were designated vMJ521 (UL18), vMJ535 (UL19) and vMJ542 (UL26.5). Several plaque isolates of each recombinant were selected and grown to high titre. One of each was selected for further study and these were designated vMJ521 (UL18), vMJ535 (UL19) and vMJ542 (UL26.5).

Construction of expression plasmids. Expression plasmids were constructed by cloning the capsid protein genes under the control of the human cytomegalovirus immediate early promoter present in the plasmid pCMV10 (Stow et al., 1993). UL19 was subcloned from pJN6 to give VP5, VP19C, VP21, VP22a and VP23. Novel polypeptides synthesized by the recombinant viruses are marked with arrowheads.
Fig. 3. Intracellular localization of VP22a, VP5 or VP23. BHK-21 cells were infected with wild-type vaccinia virus (a, b and c), with recombinant vaccinia viruses expressing VP22a (vMJ542; d), VP5 (vMJ535; e) or VP23 (vMJ521; f), or were transfected with plasmids expressing VP22a (pJK2; g), VP5 (pE19; h) or VP23 (pE18; i). The distributions of VP22a (a, d and g), VP5 (b, e and h) and VP23 (c, f and i) were examined using the primary mouse monoclonal antibodies 5010, LP12 and 1060 respectively.

with 10 p.f.u./cell of vMJ521, vMJ535 or vMJ542 were labelled from 3 to 24 h after infection and the protein profiles were analysed by SDS-PAGE (Fig. 2). Each of the recombinant viruses expressed abundant proteins which were not present in cells infected with the parental vaccinia virus. The vMJ535- (lane 3) and vMJ521- (lane 5) specific proteins comigrated with VP5 and VP23 from a preparation of purified HSV-1 capsids (lane 6) and had apparent \( M_r \)s of 155000 (vMJ535) and 33000 (vMJ521). For vMJ542 (lane 4), two bands with approximate \( M_r \)s of 45000 were observed which were considerably larger than the VP22a bands in the capsid sample. These bands
Localization of HS V capsid proteins

Fig. 4. Localization of VP22a, VP5 and VP23 in HSV-1-infected cells. BHK-21 cells were infected with 5 p.f.u./cell of HSV-1 wild-type virus (a, b and c) or tsG8 (d, e and f) and incubated at 38.5 °C (non-permissive for tsG8) for 9 h. The distributions of VP22a (a and d), VP5 (b and e) and VP23 (c and f) were examined using the primary mouse monoclonal antibodies 5010, LP12 and 1060 respectively.

correspond to the unprocessed UL26.5 gene products designated ICP35 c and d. Their identity was confirmed by Western blot analysis using the monoclonal antibody 5010 (data not shown).

Subcellular localization of VP5, VP23 and VP22a

To examine the intracellular distributions of the individual proteins, immunofluorescence was performed on recombinant vaccinia virus-infected cells using the monoclonal antibodies LP12 (for VP5), 1060 (for VP23) and 5010 (for VP22a). No fluorescent labelling was apparent with any of these antibodies on cells infected with the parental strain WR vaccinia virus (Fig. 3a to c). In vMJ542-infected cells, VP22a was confined to the nucleus (Fig. 3d). The pattern of labelling was distinctive, with fluorescence in many cells localized in discrete spots. By contrast, in vMJ535-infected cells VP5 was widely distributed throughout the cell (Fig. 3e) and a similar pattern was apparent for VP23 in vMJ521-infected cells (Fig. 3f). Thus neither VP5 nor VP23 was sequestered efficiently in the nucleus when expressed from a vaccinia virus vector.

It appeared unlikely that the failure of VP5 and VP23 to localize to the nucleus was a consequence of the vector interfering with the normal transport mechanisms, since under similar conditions VP22a did accumulate in the nucleus. However, to eliminate this possibility, the distribution of each of the proteins when expressed from a plasmid vector was examined. BHK-21 cells were transfected with pJK2, pE19 or pE18 (see Methods) and incubated at 37 °C for 24 h. When their distributions were examined, VP22a was again found to be confined to the nucleus (Fig. 3g) while VP5 (Fig. 3h) and VP23 (Fig. 3i) were distributed throughout the cells. This confirmed the patterns observed in cells infected with the vaccinia virus recombinants. Thus it appears that the differing localization patterns of the individual capsid proteins reflect their inherent properties and are not consequences of the vector used to express them.
Fig. 5. For legend see opposite.
Dependence of nuclear localization on capsid assembly

The failure of VP5 and VP23 to locate specifically to the nucleus when expressed from vaccinia virus- or plasmid-based vectors could reflect the absence of capsid formation under these conditions, since assembly into capsids might serve to anchor these proteins in the nucleus. To assess this possibility, immunofluorescence was performed on cells infected with wild-type HSV-1 or with a ts mutant, tsG8, which fails to assemble capsids under non-permissive conditions (Weller et al., 1987). The mutation in tsG8 maps to UL19 (Weller et al., 1987; Nicholson, 1992) and only low levels of VP5 are made at non-permissive temperatures. BHK-21 cells infected at 31 °C or 38.5 °C were processed for immunofluorescence. Duplicate samples grown on 35 mm Petri dishes were incubated at the appropriate temperatures until 24 h after infection, when the cells were fixed and processed for electron microscopy. This confirmed the capsid-negative phenotype of the mutant (data not shown).

As expected, examination of wild-type HSV-1-infected cells revealed that VP22a (Fig. 4a), VP5 (Fig. 4b) and VP23 (Fig. 4c) were all confined to the nucleus. In cells infected with tsG8 at 38.5 °C, VP5 could not be detected (Fig. 4e) but both VP22a (Fig. 4d) and VP23 (Fig. 4f) were predominantly nuclear and showed the characteristic distribution observed with wild-type virus (Fig. 4a and c), or with tsG8 grown at the permissive temperature (data not shown). Thus VP23 can be localized in the nucleus in the absence of capsid assembly.

Co-expression of capsid proteins

The inability of VP5 and VP23 to locate to the nucleus in the absence of other HSV-1 proteins implies that some additional factor is required to ensure correct targeting of these proteins. One possibility is that another virus-encoded protein acts as a chaperonin to transport them to the nucleus. An obvious candidate is VP22a which localizes with them in HSV-1-infected cells. Cells were therefore transfected with pJK2 (VP22a) together with either pE18 or pE19. In all combinations, VP22a retained its ability to localize in the nucleus (Fig. 5a, iv to vi). The simultaneous expression of VP5 and VP22a had a marked effect on the distribution of VP5, causing it to relocate into the nucleus (Fig. 5a, i). However, the localization of VP23 appeared unaffected by the presence of VP22a, and it remained widely distributed throughout the cell (Fig. 5a, ii). No additional effects were observed in cells transfected with all three plasmids together, and VP23 did not localize in the nucleus under these conditions (Fig. 5a, iii).

Similar results were obtained with proteins expressed from the recombinant vaccinia viruses. Parental strain WR vaccinia virus controls are shown in Fig. 3(a) to (c). Thus, the distribution of VP23 was unaffected by the presence of VP22a, either alone (Fig. 5b, ii) or in conjunction with VP5 (Fig. 5b, iii), whereas VP5 became predominantly nuclear in cells expressing VP22a (Fig. 5b, i).

From these results, it is clear that VP22a is responsible for efficiently sequestering VP5 in the nucleus but that it does not perform a similar role with respect to VP23.

Discussion

Capsid assembly takes place in the nucleus and therefore it is not surprising that studies of HSV-1-infected cells have found the capsid proteins to be predominantly nuclear (Fig. 4; Powell & Watson, 1975; Cohen et al., 1980). However, the results presented here reveal that two of the capsid proteins, VP5 and VP23, have little intrinsic capacity for nuclear localization when expressed in the absence of the other HSV-1 proteins. VP22a was nuclear under all conditions examined but when expressed by itself tended to have a punctate distribution. This was particularly obvious in cells infected with the recombinant vaccinia virus. In some cases very well defined spots were observed, suggesting local accumulation of VP22a. Newcomb & Brown (1991) have shown that VP22a prepared from purified capsids can self-assemble into 60 nm diameter spheres which appear to resemble the B capsid cores from which the protein is derived. This ability of VP22a to self-assemble probably accounts for the punctate distribution observed here, although the apparent size of the spots suggests that the aggregates being formed are much larger than those found with the purified protein.

![Fig. 5. Influence of VP22a on the distributions of VP5 and VP23. (a) Cells were co-transfected with plasmids pJK2 (VP22a) and pE19 (VP5; i and iv), pJK2 and pE18 (VP23; ii and v) or pJK2, pE19 and pE18 (iii and vi). (b) Cells co-infected with 5 p.f.u./cell of vMJ542 (VP22a) and 0.5 p.f.u./cell of vMJ535 (VP5; i and iv); cells co-infected with 5 p.f.u./cell of vMJ542 and 0.5 p.f.u./cell of vMJ521 (VP23; ii and v); cells co-infected with 5 p.f.u./cell of vMJ535 and vMJ521 and with 0.5 p.f.u./cell of vMJ542 (iii and vi). In both (a) and (b), panels (i) and (iv), (ii) and (v), (iii) and (vi) represent duplicate exposures of single fields visualized using FITC-conjugated GAM (i to iii) and TRITC-conjugated GAR (iv to vi) antisera respectively. The distribution of VP22a was examined using the rabbit antiserum 20999 (iv to vi), VP5 was examined using the mouse antibody LP12 (i) and VP23 using the mouse antibody 1060 (ii and iii). Double labelling was performed as described in Methods.](http://www.microbiologyresearch.org/)
Localization of VP5 to the nucleus during HSV-1 infection clearly depends, at least in part, on its interaction with VP22a. The alteration in the behaviour of VP5 in the presence of VP22a, and their co-localization, strongly suggest that the two proteins form a complex. The nature and composition of this complex is not known and it is not possible at present to determine whether the complex initially forms in the cytoplasm or in the nucleus. In the former case the transport of VP5 to the nucleus would be a direct consequence of binding to VP22a. In the latter case VP22a might act to anchor and concentrate VP5 in the nucleus. Knipe & Spang (1982) suggested that the delay they observed in the translocation of VP5 to the nucleus could be explained by the time required for it to assemble into a multicomponent complex in the cytoplasm and we feel that a direct role for VP22a in transport is inherently more likely. Newly synthesized VP5 has been reported to bind rapidly to the cytoskeleton and to pass from the cytoplasmic to the nuclear framework without appearing in the soluble phase (Quinlan & Knipe, 1983; Bibor-Hardy et al., 1985). This being the case, the binding of VP5 to the cytoskeleton may be mediated through VP22a.

A pathway of assembly can be envisaged in which newly synthesized VP5 binds to VP22a and is transported to the nucleus. In the nucleus the VP5–VP22a units come together, by virtue of the ability of VP22a to interact with itself, thereby forming a basic framework to which the other capsid proteins would attach to form the archetypal B capsid. A subjective impression, gained from the fluorescence patterns, was that the distribution of VP22a was more uniform when VP5 was present. This might indicate that the presence of the second protein modulates the self-association of VP22a, thereby preventing the formation of extensive aggregates.

The existence of mutants in UL18 and UL38 which fail to form capsids suggests that VP5 and VP22a together are insufficient to form recognizable capsids. The role of the other proteins or the stage at which they become incorporated into the nascent capsid is not known. The inability of VP22a to alter the distribution of VP23 suggests that there is no direct interaction between these proteins. This accords with the location proposed for VP23 on the surface of the capsid, probably as a component of the trivalent connections between the capsomers (Schrag et al., 1989; Baker et al., 1990; Newcomb et al., 1993). In this position it could be inaccessible to the internal proteins. However, the fact that it also remained in the cytoplasm in the presence of both VP22a and VP5 is more surprising. This suggests that either additional components are needed to link VP23 to the VP22a–VP5 complex, or transport of VP23 to the nucleus involves a separate pathway. Studies are currently underway with the remaining capsid protein genes to determine their mutual effects on localization and transport of capsid proteins.

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