Differences in the intracellular localization and fate of herpes simplex virus tegument proteins early in the infection of Vero cells

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The fate of herpes simplex virus 1 (HSV-1) tegument proteins during infection in Vero cells was investigated immunochemically. Input virion-associated VP13/14 and VP16 localized to the nucleus early in infection, while VP1/2 localized to the nuclear envelope of the cell and VP22 could not be detected using monoclonal antibody P43. Western blotting suggested that virion-associated VP13/14, VP16 and VP22 were stable in infected cells whereas VP1/2 appeared to be processed or modified. Further studies showed that P43 recognized a phosphorylation-sensitive epitope in VP22 and suggested that virion-associated VP22 was phosphorylated upon entry to the cell. VP13/14 and VP16 were easily extracted from cells early in infection whereas VP22 was largely insoluble. Phosphatase treatment of soluble extracts caused a shift in the molecular mass of VP16 showing it was phosphorylated. As infection progressed VP16 was observed in discrete nuclear compartments where it co-localized with ICP8 and the capsid-associated protein VP22a. VP13/14 was also observed in the nucleus. P43 immunostaining appeared around 6 h post-infection as punctate nuclear foci which often localized to the edge of VP16-immunoreactive areas. Punctate P43 cytoplasmic staining appeared around 12 h post-infection. By 18 h the nuclear pattern had disappeared and an extensive cytoplasmic stain was observed which closely overlapped that of other tegument proteins. On the basis of these data we suggest that virion-associated VP22 is phosphorylated upon entry of the virus into the cell and that unphosphorylated VP22, which is preferentially recognized by P43, becomes available later in infection, initially in the nucleus, for packaging into virions.

Introduction

The herpes simplex virus 1 (HSV-1) tegument is an electron-opaque region of the virion located between the capsid and the virus envelope (Spear & Roizman, 1972). Some of the proteins constituting the tegument provide critical virus functions in the time between virus penetration of the cell and the beginning of de novo synthesis of virally encoded proteins. Some tegument proteins may also play an important structural role within the HSV-1 virion (Heine et al., 1974). The function of many others is still obscure. The tegument includes four major structural proteins, VP1/2, VP13/14, VP16 and VP22 (Spear & Roizman, 1972; Honess & Roizman, 1973), the products of the UL36, UL47, UL48 and UL49 genes, respectively (Campbell et al., 1984; McLean et al., 1990; Whittaker et al., 1991; McNabb & Courtney, 1992a; Elliott & Meredith, 1992). Although HSV-1 tegument proteins provide important virus functions both early and late in infection, little is known about their fate in infected cells, particularly at early times after virus entry. For example, it is not known which, if any, of the tegument proteins remain associated with the capsid after virus penetration of a cell. Some, such as VP16, would be expected to dissociate from the virion at this time, whereas others could play a role in transporting the virion along microtubules to the nuclear envelope (Sodeik et al., 1997) or in liberating the HSV-1 genome from the nucleocapsid. Furthermore, the mechanisms underlying tegument assembly late in infection and the site at which it occurs are unknown. We have therefore used antibodies specific for HSV-1 tegument proteins for the immunochemical analysis of Vero cells infected with HSV-1, concentrating initially on early
times post-infection. Exploiting the unusual properties of a monoclonal antibody to VP22 we then examined the localization of the major tegument proteins later in infection.

**Methods**

**Cells.** Vero cells were grown in Dulbecco’s modification of Eagle’s medium (DMEM) containing Glutamax (Life Technologies), supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. For immunocytochemical experiments the cells were grown on sterile glass coverslips.

**Virus infections.** Cells were washed in PBS before incubation with virus in pre-warmed DMEM containing 2% heat-inactivated FCS. An m.o.i. of 100 was used for the immunocytochemical study of input virion proteins, in which case the cells were incubated with virus for 30 min in the presence of cycloheximide (CHX), whereas an m.o.i. of 10 in the absence of CHX was used in the study of de novo-synthesized viral proteins. In this situation the cells were incubated with virus for 1 h at 37 °C, after which the virus was removed and the cells washed twice in PBS, before incubation for various periods of time in DMEM containing 2% FCS. For Western blotting analysis of input viral proteins the cells were incubated with virus at an m.o.i. of 50 for 30 min followed by extensive washes in PBS and further incubation for up to 4 h, all in the presence or absence of CHX. The wild-type virus used in these experiments was HSV-1 strain 171. Purified virion preparations were obtained as follows: confluent cell monolayers in roller bottles were infected at an m.o.i. of 0.02 p.f.u. per cell in medium containing 2% heat-inactivated serum. Cells were incubated at 37 °C until complete CPE was achieved. Cells were detached and the cell suspension was spun at 1600 g for 30 min. The supernatant was removed and spun at 16000 g for 90 min. The resulting pellet was resuspended in PBS and layered onto a gradient of 10–40% (w/v) sucrose in PBS. The gradient was centrifuged at 30000 g in a swing-out rotor for 45 min and the opaque virus band in the centre of the gradient was removed, taking care to avoid disturbance of the diffuse layer of light particles above it. The virus fraction was then diluted in PBS and centrifuged at 80000 g again in a swing-out rotor. The resulting pellet was resuspended in PBS and a virus titre obtained by standard plaque-assay procedures using BHK cells. Purified virion preparations were stored at –80 °C.

**Antibodies.** Antibodies used to detect virus proteins were as follows: R218, specific for VP1/2; R220, specific for VP13/14; R230, specific for VP16. R218, R220 and R230 were prepared by inoculation of rabbits with VP1/2 and VP16 purified by preparative SDS–PAGE in an identical manner to R220 (Whittaker et al., 1991). R205 is a rabbit polyclonal antiserum raised against purified capsid/tegument samples and recognizes VP13/14, VP16 and VP22 among others (Morrison et al., 1998). All polyclonal antisera were used at a dilution of 1/100. VP22 was also detected using the mouse monoclonal IgM P43 (Elliott & Meredith, 1992). ICp8 was detected using monoclonal antibody 39(S), used as neat culture supernatant. VP22a was detected using the pooled monoclonal antibodies CY45 and CY46 (Stevenson et al., 1997).

**Immunofluorescence.** Cells on coverslips were washed in PBS before fixing in acetone at –20 °C for 5 min. After fixing the cells were washed in PBS before incubation in 10% normal swine serum in PBS (NSwS, Vector Laboratories) for 1 h at room temperature to block non-specific binding. The cells were washed briefly in 1% NSwS before incubation with primary antibody at a 1/100 dilution in 10% NSwS for 2 h at room temperature. After this the cells were washed in 1% NSwS before incubation with the appropriate fluorophore-conjugated secondary antibodies (goat anti-rabbit IgG (Vector Laboratories) and/or goat anti-mouse immunoglobulins (Dako)) at a 1/100 dilution in 10% NSwS for 1 h. In double-labelling experiments both secondary antisera were diluted in the same aliquot of NSwS before adding to cells. No cross-reaction between the two antisera was observed. The coverslips were then washed in PBS before mounting in Mowiol (Sigma). Controls included normal rabbit serum, normal mouse immunoglobulins or pre-immune serum in place of primary antibody, and incubation with secondary antibodies alone. The slides were viewed using a Zeiss Axiowert 135TV inverted fluorescence microscope. Images were captured as TIF files using a Hamamatsu cooled CCD camera (model C4742) and the ImproVision Fluovision image analysis package running on an Apple Macintosh computer. The images were exported to Adobe Photoshop and reproduced using a dye-sublimation printer.

**SDS–PAGE and Western Blotting.** SDS–PAGE and Western blotting of infected-cell extracts and purified virus were carried out essentially as described previously (Whittaker et al., 1991). Samples were solubilized under reducing conditions in the presence of DTT and a cocktail of protease inhibitors. Gels contained acrylamide cross-linked with N,N’-diallyltartardiamide (DATD) (Heine et al., 1974). Proteins were electrophoretically transferred to nitrocellulose, after which non-specific binding was blocked using 1% (w/v) skimmed milk powder in PBS. Primary antibody incubations were carried out in the same solution for 2 h at 37 °C. After washing in PBS, blots were developed using HRP-conjugated secondary antiserum and the Pierce SuperSignal ECL system (Pierce and Warriner) or chloronaphthol.

**Infected cell extractions and phosphatase treatments.** Cells infected in the presence of CHX as described above were washed thoroughly in warm PBS, detached by scraping, pelleted by centrifugation at 1600 g for 10 min and resuspended in 5 vols of one of three buffers consisting of 50 mM Tris–HCl, pH 8 with a cocktail of protease inhibitors [leupeptin, aprotinin, PMSF, antipain and chymostatin (Sigma), used at concentrations recommended by supplier] and one, the first two or all three of the following detergents: 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS. After mixing for 10 min at room temperature the samples were spun at full speed in a microcentrifuge for 10 min. Soluble material and the insoluble pellet were separated and frozen until required. Material solubilized in 1% Triton X-100 was incubated in the presence or absence of 150 U calf intestinal phosphatase (CIP, Sigma) for 30 min at 37 °C before SDS–PAGE.

**Phosphorylation of purified virion preparations.** Equal volumes of purified virion samples were resuspended on ice in final volumes of 100 µl 50 mM Tris–HCl, pH 8, 1% NP40. To one sample MgCl2 and ATP were added to final concentrations of 5 mM each. To a second sample the same reagents were added along with 150 U CIP. The third sample was left untouched. All samples were incubated at 37 °C for 1 h following which 100 µl of the three-detergent-containing buffer described above was added. A further 200 µl SDS–PAGE sample loading buffer was added and the samples were boiled for 5 min before SDS–PAGE and Western blotting.

**Results**

The distribution of input HSV-1 virion-associated tegument proteins after 30 min incubation of Vero cells at an m.o.i. of 100 in the presence of CHX was as follows. VP1/2 (Fig. 1B) primarily gave a weak speckled cytoplasmic and strong perinuclear staining pattern (arrows), with no nuclear staining observed. In marked contrast to this, VP13/14 and VP16 (Fig.
Fig. 1. Immunofluorescence staining patterns of HSV-1 virion-associated tegument proteins in Vero cells 30 min after infection at high m.o.i. in the presence of CHX. (A) An infected cell immunostained using normal rabbit serum. No specific immunofluorescence is seen. (B) An infected cell immunostained with the VP1/2-specific antiserum R218. Note the strong immunolocalization of this protein to structures resembling the nuclear envelope of the cell (arrows). (C) An infected cell immunostained with the VP13/14-specific antiserum R220. Note the strong nuclear immunofluorescence (large arrow) and the punctate cytoplasmic staining (small arrows). (D) An infected cell immunostained using the VP16-specific antiserum R230. Note again the strong nuclear immunofluorescence (large arrow) and punctate cytoplasmic staining (small arrows). Note also the nuclear compartment not immunostained by this antibody (arrowhead). Bar, 20 μm.

1C, D respectively) displayed very similar patterns of weak speckled cytoplasmic staining (small arrows) accompanied by strong nuclear immunofluorescence (large arrows). This pattern was not homogeneous, with distinct areas of the nucleus free of any staining (arrowhead, Fig. 1D). These areas may represent nucleoli. Interestingly, no staining was apparent for VP22 using monoclonal antibody P43 at this point in the infection (results not shown).

The fate of VP1/2, VP13/14, VP16 and VP22 early in infection was investigated by Western blotting (Fig. 2) using the antibodies R230 (panel A), R205 (panel B), P43 (panel C), R220 (panel D) and R218 (panel E). Panels (A)–(D) show gels loaded with equal samples from the same purified virus preparation or infected-cell extracts, and the positions of the proteins of interest are indicated. Cells were incubated (with the same amount of virus loaded in lane 1) for 30 min in the presence or absence of CHX to prevent viral protein synthesis, before thorough washing in PBS and subsequent incubation for up to 4 h, again in the presence or absence of CHX. Cells were then scraped from the flask into PBS and pelleted by low-speed centrifugation before extraction in SDS–PAGE sample loading buffer containing a cocktail of protease inhibitors and 5 mM DTT. R230 produced a single band of around 65 kDa in blots of purified virus (Fig. 2A, lane 1). Lanes 2 and 3 in Fig. 2(A)
Fig. 2. Western blotting analysis of tegument proteins early in infection of Vero cells. Five blots are shown: (A) was developed using R230, (B) using R205, (C) using P43, (D) using R220 and (E) using R218. The lanes in each blot represent the following samples, equal quantities of which were loaded on blots (A)–(D): lane 1, purified HSV-1; lane 2, Vero cells incubated with purified HSV-1 at an m.o.i. of 50 in the presence of CHX for 2 h; lane 3, as lane 2, incubated for 4 h; lane 4, Vero cells incubated with HSV-1 at an m.o.i. of 50 for 4 h; lane 5, mock-infected Vero cells incubated with CHX for 4 h; lane 6, mock-infected Vero cells. The positions of VP13/14, VP16 and VP22 are indicated by arrows. The quantity of purified virus loaded in lane 1 on each blot represents the same quantity used in the incubations with cells. Note that whereas R205 recognizes VP22 in both purified virus and all cells incubated with HSV-1 (Fig. 2B, lanes 1–4), P43 only recognizes the protein in purified virus (Fig. 2C, lane 1). Note also that VP13/14 is detected more efficiently in infected-cell extracts by R220 (Fig. 2D, lanes 2–4) than R205 (Fig. 2B). Fig. 2(E) includes samples from a different experiment to blots (A)–(D), but carried out under the same conditions. Purified virions were extracted in 5 mM DTT, cell extracts in 20 mM DTT. Note the multiple immunoreactive bands in infected-cell extracts under all conditions. The arrow labelled VP1/2 represents the major form observed in purified virions. The first arrow above that labelled VP1/2 indicates the position of a 250 kDa molecular-mass marker in this gel.

demonstrate that in the presence of CHX VP16 persisted in the infected cell for up to 4 h. Lane 4 shows cells incubated with virus in the absence of CHX for 4 h. The increase in band intensity and the appearance of VP16-immunoreactive bands of different molecular mass is characteristic of productive infection. R205 (Fig. 2B) reveals multiple bands in purified viral preparations run on a 9% acrylamide gel (lane 1), prominent among them VP16, VP13/14 and VP22 (indicated). This panel confirms the finding that VP16 persisted for up to 4 h in the presence of CHX in this system (Fig. 2B, lanes 2 and 3). However, although VP13/14 was readily detectable in purified virions (lane 1) and could also be detected after 4 h infection in the absence of CHX (lane 4), it was not detected in the CHX-treated samples. Fig. 2(D) demonstrates that this result was an artefact probably resulting from a lower affinity for VP13/14 of R205 compared to R220. We note however that prolonged exposure of the immunoblot to film was required for the detection of these bands; note the overexposure of lanes 1 and 4 in Fig. 2(D). Repeating this experiment and probing with R205 followed by a prolonged developing time which overexposed the VP16 and VP22 signals also resulted in the detection of VP13/14-specific bands (not shown). Like VP16, VP22 was detected in purified virions and under all conditions in infected cells using R205, and it also showed an increase in intensity after 4 h incubation in the absence of CHX (Fig. 2B, lane 4). The blot developed using P43 to detect VP22 (Fig. 2C) gave a contradictory but reproducible result. In this case the antibody only recognized VP22 in preparations of purified virus (lane 1), although a very faint band was also observed after 4 h incubation in the presence of CHX (lane 3). No immunoreactive bands were seen even after 4 h in the absence of CHX, at a time when from the other blots viral protein synthesis is suggested to have occurred. Furthermore, increasing the primary antibody concentration followed by prolonged exposure of the blot to film (up to 2 h, not shown) resulted in no signal, suggesting the problem was not one of sensitivity, as was the case with R205 and VP13/14. This phenomenon corroborates the lack of immunostaining ob-
Fate of HSV-1 tegument during uncoating

Fig. 3. Solubilities of tegument proteins early in infection of Vero cells. (A) Cells infected as described above were extracted in 1% Triton X-100, incubated in the presence (lane 2) or absence (lane 1) of CIP and analysed by SDS–PAGE and Western blotting with R205. Note that both VP13/14 and VP16 were readily soluble. Note also that CIP treatment caused a decrease in the apparent molecular mass of VP16, showing that this protein was phosphorylated. (B) Infected cells were extracted in buffers containing 1% Triton X-100 and 0.5% sodium deoxycholate (lanes 1 and 3), or with these detergents plus 0.1% SDS (lanes 2 and 4) before separation into soluble (lanes 1 and 2) or insoluble (lanes 3 and 4) material by centrifugation. Note that VP22 is found solely in the insoluble fraction.

erved with P43 at early times post-infection, and was investigated further (see below). Immunoblotting for VP1/2 under these conditions (Fig. 2E) provided unexpected difficulties related to the insolubility of the protein in infected-cell extracts. The protein was easily solubilized from purified virions in buffer containing 5 mM DTT (Fig. 2E, lane 1). Under similar conditions little or no signal was observed in cell extracts (not shown). Increasing the DTT concentration gave progressively more signal in subsequent blots. In the blot shown extracts were solubilized in the presence of 20 mM DTT. The bands observed in infected-cell extracts are marked to the right of the blot by arrows, and those which appear as faint bands on this exposure were confirmed by prolonged exposures which also increased background signals significantly and are therefore not shown. The observed uneven transfer is not unusual for large insoluble proteins. A band corresponding to that seen in purified virions was observed in infected-cell extracts under all conditions, and this is labelled as VP1/2. Furthermore, multiple bands of higher and lower molecular mass were seen. Those of lower molecular mass may be explained as possible products of proteolysis. We believe that those of higher molecular mass correspond to protein aggregates containing VP1/2, as the difference in molecular mass between ‘authentic’ VP1/2 and these higher molecular mass forms would appear to be too large to be accounted for by modifications such as phosphorylation (McNabb & Courtney, 1992b). This conjecture is open to further testing through the use of harsher reducing agents in the solubilization of infected-cell extracts. Note that in all panels no immunoreactive bands were observed in uninfected cells in either the presence or absence of CHX (Fig. 2A–E, lanes 5 and 6).

The above immunochemical data showed that although VP22 was readily detectable in CHX-treated cells by Western blotting using a polyclonal antibody it was not recognized by P43 (Fig. 2B, C). We hypothesized that the epitope recognized by P43 was masked in some way after virus penetration of the infected cell. An obvious candidate for this masking was modification of VP22 by phosphorylation (Knopf & Kaerner, 1980; Meredith et al., 1991; Elliott et al., 1996; Morrison et al., 1998). We therefore attempted to solubilize VP22 from Vero cells infected as described above in a buffer containing 1% Triton X-100. The extract was divided into two and half was incubated with CIP for 30 min at 37 °C. The other half was incubated without phosphatase. The samples were analysed by SDS–PAGE and Western blotting using both R205 and P43, the rationale being that P43 should preferentially detect dephosphorylated VP22 in the CIP-treated samples. The phosphatase treatment appeared to be successful, since a decrease in the molecular mass of VP16 was observed in the CIP-treated extract, suggesting that input virion-associated VP16 is phosphorylated in infected cells (Fig. 3 A). VP13/14 was also detected in the soluble fraction. However, no VP22 was detected by either R205 or P43, suggesting that VP22 was insoluble in the extraction buffer used. Since CIP is inactive in strong detergents we attempted to solubilize VP22 under harsher conditions as a prelude to immunoprecipitation using R205, as the immunoprecipitate could later be washed in milder buffers before CIP treatment. Two extraction buffers were tested. The first contained 1% Triton X-100 in combination with 0.5% sodium deoxycholate, a standard ‘two-detergent’ buffer. No VP22 was detected in the soluble extract (Fig. 3B, lane 1) but it was readily detected in the insoluble pellet (Fig. 3B, lane 3). A similar result was obtained using a standard three-detergent buffer containing Triton X-100, sodium deoxycholate and 0.1% SDS. VP22 could however be solubilized by boiling in SDS–PAGE sample buffer (see e.g. Fig. 2B). This approach to characterizing P43 was therefore discarded in favour of another.

Incubation of purified, detergent-permeabilized HSV-1 virions with ATP in the presence of Mg2+ ions results in the rapid and efficient phosphorylation of a number of tegument structural proteins including VP22 (Morrison et al., 1998).

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Fig. 4. Monoclonal antibody P43 recognizes a phosphorylation-sensitive epitope in VP22. (A) Purified virions were solubilized in 50 mM Tris–HCl, pH 8 containing 1% NP40. To one half of the sample was added 5 mM MgCl$_2$ and 5 mM ATP. This sample (lane 1) was then incubated at 37 °C for 30 min, while the other (lane 2) was stored on ice before SDS–PAGE analysis and Western blotting with either R205 or P43. Note that in the treated sample R205 recognizes a doublet corresponding to phosphorylated and dephosphorylated VP22, whereas P43 recognizes only the dephosphorylated form (arrow). (B) Purified virions were solubilized as above and divided into three equal samples. The first was left untreated. MgCl$_2$ and ATP were added to the remaining two, and 150 U CIP was added to one of these. All three samples were incubated at 37 °C for 1 h before SDS–PAGE and Western blotting with P43. In the untreated sample (lane 1) a single band is seen (arrow). In the sample incubated with MgCl$_2$ and ATP (lane 2) P43 immunostaining is abolished. In the sample incubated with MgCl$_2$, ATP and CIP (lane 3) a single band is seen (arrow).

4(A) shows Western blots of virions incubated in the presence or absence of 5 mM ATP and 5 mM MgCl$_2$, probed with R205 and P43. In untreated, unincubated virions R205 recognizes a single VP22 band. In treated virions incubated at 37 °C it recognizes a doublet of bands representing phosphorylated and unphosphorylated VP22 (Elliott et al., 1996; Morrison et al., 1998). Note the decrease in intensity of the unphosphorylated band as VP22 in the virions is converted to a phosphorylated form. In untreated virions P43 also recognizes a single band representing unphosphorylated VP22. In treated virions, in contrast to R205, P43 only recognizes the lower molecular mass unphosphorylated VP22 band. Note again the decrease in intensity of this band. Overexposure of the P43 blot resulted in no appearance of further bands. It has previously been demonstrated that phosphatase treatment can resolve the characteristic doublet seen with a mixture of phosphorylated and dephosphorylated VP22 (Elliott et al., 1996; Morrison et al., 1998). To prove conclusively, therefore, that phosphorylation was responsible for the masking of VP22 from P43, a similar incubation to the one above was performed. However, CIP was added to one of the samples to inhibit the phosphorylation of VP22. Immuno blotting showed that in untreated virions incubated at 37 °C P43 revealed a single band (Fig. 4B). In treated virions P43 immunoreactivity was abolished. In the presence of CIP a single band was again seen. In a similar experiment, R205 also recognizes a single band after CIP treatment of phosphorylated virion-associated VP22 (Morrison et al., 1998). We therefore conclude that P43 recognizes a phosphorylation-sensitive epitope in VP22. Note that the P43 band in Fig. 4(B) lane 1 is less intense than that seen in lane 3 with CIP treatment. This suggests that even in the absence of added MgCl$_2$ and ATP limited phosphorylation occurs in purified virion preparations incubated at 37 °C (Morrison et al., 1998). In the light of previous work the P43 epitope is likely to lie in the N-terminal portion of VP22 (Elliott et al., 1996).

Three hours after infection of Vero cells at an m.o.i. of 10, in the absence of CHX to visualize early de novo-synthesized proteins, the staining patterns observed differed radically to those seen early after virus entry. VP1/2 was observed primarily in the perinuclear region of the cell, and was also seen in association with the nuclear membrane (Fig. 5B). The staining pattern for VP13/14 (Fig. 5C) now localized the protein to the perinuclear region of the cell and small, distinct structures at the edge of the nucleus. VP16 (Fig. 5D) displayed a diffuse cytoplasmic localization, with no obvious perinuclear concentration. Strikingly, VP16 was also observed in specific structures in the nucleus, very different to those seen early in the infection (Fig. 5D). These structures were globular spots, predominantly around the edge of the nucleus (large arrows). The spots were often linked by narrow channels of immunoreactivity, a feature which became more common as the infection progressed. Once again, VP22 was not seen in the infected cell at this time using P43.

To characterize further the distinctive nuclear compartment containing VP16, double staining with antibody R230 and the ICP8-specific monoclonal antibody 39(S) was carried out in Vero cells 4 h after infection. The results of this experiment are shown in Fig. 6(A, B), and demonstrate that VP16 and ICP8 extensively co-localized in the nucleus of infected cells. This co-localization was stable up to 8 h post-infection (not shown), and the intranuclear structures which were immunolabelled by both antibodies tended to increase in size over time. No co-localization was observed outside nuclei since ICP8 labelling was exclusively nuclear. Furthermore, nuclear VP16 immunoreactivity also extensively overlapped with that seen for VP22a, a scaffolding protein which is associated with maturing nucleocapsids, although it is not a structural component of mature virions. This suggests that the VP16-immunoreactive compartment is also the site of capsid biosynthesis at this time in infection.

As P43 preferentially recognizes unphosphorylated VP22 we would expect to see P43 immunostaining associated with maturing virions in infected cells, since virion-associated VP22 is not phosphorylated. P43 immunoreactivity initially appeared in some cells at distinct intranuclear sites around 6 h post-infection (not shown). We therefore investigated the distribution of P43 immunoreactivity at 8 h post-infection for comparison with that of VP16 by double immunostaining with R230 (Fig. 6E, F). P43 immunoreactive foci were not specifically associated with the VP16-immunoreactive nuclear com-
Fate of HSV-1 tegument during uncoating

Fig. 5. Intracellular localization of HSV-1 tegument proteins in Vero cells 3 h after infection. (A) Infected cells immunostained with normal rabbit serum. No specific immunofluorescence is seen. (B) Infected cells immunostained for VP1/2. Note the perinuclear staining (small arrow), and the staining of the nuclear envelope (large arrows). (C) Infected cells immunostained for VP13/14. Note the strong perinuclear staining (small arrow) and the staining of areas at the edge of the nucleus (large arrows). (D) Infected cells immunostained for VP16. Note the diffuse cytoplasmic staining throughout the cell and the strong staining of defined intranuclear compartments (large arrows). Bar, 20 µm.

dpartment, as some were seen to lie outside this area. Many of the foci appeared to localize to the edge of the VP16 immunoreactivity (arrows). A typical P43 staining pattern 8 h post-infection is also shown in Fig. 7(A). Note again that the immunoreactive foci are exclusively nuclear. By 12 h post-infection some cells had begun to display immunoreactive foci in the cytoplasm as well (Fig. 7B, arrows), although nuclear foci were still apparent. The nuclear foci did not appear to change significantly in size as infection progressed.

The majority of HSV-1 virion production and egress from the infected cell are thought to occur from around 18 h after initial infection. We therefore compared P43 immunoreactivity at 18 h post-infection with that of the other tegument proteins by double immunostaining. Fig. 8 shows the results obtained with P43 and R218, which were identical to those seen with P43 in conjunction with R220 and R230 (not shown). The previously observed intranuclear staining is essentially abolished for all of the proteins studied, although sites on the nuclear membrane are still heavily labelled. P43 immunostaining is almost entirely cytoplasmic and often overlaps with
Fig. 6. VP16 co-localizes with ICP8 and VP22a in infected-cell nuclei. (A), (C) and (E) show infected cells 4 h (A) or 8 h (C, E) post-infection immunostained for VP16. (B) shows the same cells as (A) immunostained with a monoclonal antiserum to ICP8. Note the specific co-localization of the two nuclear staining patterns (arrows). (D) shows the same cells as (C) immunostained with a monoclonal antiserum to VP22a. Note again the specific co-localization of the two nuclear staining patterns (arrows). (F) shows the same cell as (E) immunostained with monoclonal antibody P43 to visualize unphosphorylated VP22. Note the immunoreactive nuclear foci primarily located outside or on the edge of the VP16-immunoreactive area (arrows).
large immunoreactive masses (Fig. 8B, arrows). These masses are also immunolabelled for VP1/2 (Fig. 8A, arrows). Given (a) the late time in infection, (b) the co-immunostaining of all the tegument proteins studied in cytoplasmic masses (this was the only time in the study that overlapping immunostaining for all of the tegument proteins studied was observed) and (c) the finding that P43 preferentially recognized unphosphorylated VP22, we believe that a reasonable interpretation of the cytoplasmic masses is that they represent maturing virions exiting the infected cell. We therefore suggest that monoclonal antibody P43 will be a useful tool for identifying the presence and subcellular localization of mature virions, and investigating the site and mechanism of tegument acquisition by these virions in infected cells.

Discussion

VP1/2 has been hypothesized to play several roles during the HSV-1 replicative cycle based upon studies using a temperature-sensitive mutant (Knipe et al., 1981). It is thought to be important in transferring the viral genome from the capsid into the nucleoplasm of an infected cell (Batterson et al., 1983). The results presented here do not disagree with this hypothesis. We saw no evidence for a specific intranuclear localization of VP1/2 early in infection, but structures resembling the nuclear envelope were stained (Fig. 1B), consistent with the putative role of the protein in liberating the...
viral genome from the capsid at the nuclear pore. Recent studies have demonstrated the accumulation of HSV-1 capsid proteins at the nuclear envelope in a similar experimental set-up, also using immunofluorescence (Sodeik et al., 1997). This may suggest that VP1/2 remains associated with the capsid early in infection. It seems likely that virion-associated VP1/2 has no further role to play after the viral genome has been liberated; the detection of apparent VP1/2-containing aggregates and processed forms of the protein by Western blotting (Fig. 2E) suggests that it may then be degraded. Although no nuclear VP1/2 was visible at 3 h post-infection it was observed later in infection (results not shown), in agreement with previous work (McNabb & Courtney, 1992b).

VP13/14 is thought to be a modulator of VP16-mediated induction of immediate-early genes (McKnight et al., 1987; Zhang et al., 1991). This implies a nuclear localization for these proteins early in infection. Our results demonstrate that both VP13/14 and VP16 localized to the nucleus of newly infected cells in a similar manner (Fig. 1C, D). Both of these proteins are therefore liberated from the virion during infection.

VP16 immunoreactivity was present in the nucleus throughout infection, in a manner which suggested a role for VP16 aside from its trans-inducing function. The likely role for VP16 early in infection is to ‘kick-start’ viral gene transcription in cells infected at a low m.o.i. (as might be seen in vivo). This function would become redundant as the transcriptional cascade regulating virus replication begins. Virion-associated VP16 however appears to be a persistent protein in the infected cell as determined by Western blot (Fig. 5A). The demonstration that input virion-associated VP16 is phosphorylated early in infection is perhaps unsurprising since it has recently been demonstrated that phosphorylation of VP16 is required for its trans-activating function (O’Reilly et al., 1997).

Although VP16 was shown by immunocytochemistry to remain in the nucleus throughout infection, its distribution radically changed. This change coincided with the arrival of de novo-synthesized VP16, since cytoplasmic VP16 staining also appeared at this time (Fig. 5). The nuclear immunoreactivity was seen in discrete areas which grew and merged. The resemblance of the VP16-immunoreactive sites to those previously designated as ‘replication compartments’ (Quinlan et al., 1984) was noted. These are postulated to be intranuclear areas within which HSV-1 DNA replication occurs. A number of HSV-1 proteins (see e.g. Liptak et al., 1996) and cellular proteins (Wilcock & Lane, 1991) have been localized to these structures, including the early major DNA-binding protein ICP8 (Randall & Dinwoodie, 1986; Knipe et al., 1987; Yao & Courtney, 1989). The nuclear co-localization demonstrated in this paper between VP16 and ICP8 (Fig. 6) shows that VP16 also specifically localizes to replication compartments in infected cells, while the co-localization of VP16 and VP22A also demonstrates the presence of proteins involved in capsid biosynthesis in these compartments. The characterization of nuclear compartments in cells infected with HSV-1 is a rapidly expanding field with sometimes contradictory findings (Phelan & Clements, 1997). The VP16-immunoreactive compartment identified in this study most closely resembles that seen by previous workers at a similar stage of infection with antibodies to immature capsids (Ward et al., 1996). This compartment resolves into multiple, partially overlapping functional domains later in infection.

VP22 is an abundant protein in both the HSV-1 virion and the infected cell (Gibson & Roizman, 1974), present in quantities at least equivalent to that of VP16. Despite this, using monoclonal antibody P43 no VP22 immunoreactivity was apparent at very early times of infection (Figs 1 and 2), and VP22 immunoreactivity was only present in a minority of infected-cell nuclei by 6 h post-infection (not shown) whereas other tegument proteins could be seen by 3 h post-infection (Fig. 5). VP22 is subjected to a range of post-translational modifications in infected cells, including phosphorylation (Knopf & Kaerner, 1980; Meredith et al., 1991; Elliott et al., 1996). P43 was raised against VP22 protein purified from HSV-1 virions, which is not phosphorylated (Elliott & Meredith, 1992; Elliott et al., 1996). It is therefore possible that P43 recognizes an epitope on VP22 which is masked by a modification such as phosphorylation upon virus penetration of a cell (Morrison et al., 1998). This hypothesis is supported by the observation that P43 does not recognize input virion-associated VP22 on Western blots when the protein is readily detectable in the same extract by the polyclonal antiserum R205 (Fig. 2). We therefore used ATP-treated virions as a rich source of phosphorylated VP22 to investigate this hypothesis further. The logical conclusion to be drawn from these experiments is that P43 recognizes a phosphorylation-sensitive epitope in VP22. When we apply this knowledge to our immunofluorescence results we can therefore hypothesize that the staining pattern produced by P43 primarily represents unphosphorylated VP22 becoming available prior to incorporation into the virion. Whether this represents specific dephosphorylation of VP22 by phosphatases or simply an accumulation of unphosphorylated de novo-synthesized protein is unclear. The punctate nuclear immunostaining shown in Figs 6 and 7 may therefore represent sites of VP22 acquisition by assembling virions. How these foci relate to nuclear compartments previously identified in infected cells late in infection is unclear; their characteristics appear similar to structures recently termed ‘assemblions’ (Ward et al., 1996). Finally, the observation that antibodies to all of the tegument proteins studied, including P43, immunostain cytoplasmic masses very late in infection suggests that these masses represent mature virions during egress from the infected cell.

VP22 contains multiple phosphorylation sites in an N-terminal portion of the protein (Elliott et al., 1996). Preliminary data suggest that P43 can immunoprecipitate 32P-labelled VP22 from infected cells (Morrison et al., 1998). This is an unsurprising observation since the likelihood that the P43
epitope is sensitive to all phosphorylation sites in VP22 is very small. Strictly speaking therefore, the form of VP22 recognized by P43 need not be completely phosphate-free, but may simply lack phosphorylation at a specific site. Definition of this site should be possible at a future point, as it is already clear that both protein kinase C and casein kinase II can phosphorylate VP22 in vitro (Elliott et al., 1996). It is worth noting however that in virion phosphorylation experiments the majority of VP22 phosphorylation appears to be dependent upon the UL13 virion kinase (Coulter et al., 1993; Morrison et al., 1998).

The relative insolubility of VP22 early in infection when compared to VP13/14 and VP16 could have many explanations. Like VP1/2, VP22 may remain associated with the capsid. Alternatively, VP22 may tightly associate with cellular components as part of its function, still currently unknown. For example, an association of VP22 with the nuclear matrix has been suggested (Pinard et al., 1987), which would account for this increased resistance to detergent extraction.

In summary, we have elucidated the intracellular localizations of three of the major tegument proteins of HSV-1 early in infection and have presented data which demonstrate the compartmentalization of VP16 in infected-cell nuclei later in infection. We have shown that monoclonal antibody P43 preferentially recognizes unphosphorylated VP22 such as that in infection. We have shown that monoclonal antibody P43 compartmentalization of VP16 in infected-cell nuclei later in infection and have presented data which demonstrate the localizations of three of the major tegument proteins of HSV-1 early stages of infection the P43 epitope is masked by the phosphorylation of VP22 phosphorylation appears to be dependent upon the UL13 virion kinase (Coulter et al., 1993; Morrison et al., 1998).

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