The Products of Herpes Simplex Virus Type 1 Gene UL26 which Are Involved in DNA Packaging Are Strongly Associated with Empty but Not with Full Capsids

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SUMMARY

We report on the properties of a family of related herpes simplex virus type 1 polypeptides (designated p40) of Mr around 40000. The intracellular localization of these polypeptides has been examined using monoclonal antibodies and their association with viral capsids within the nuclei of infected cells has been demonstrated directly by immuno-electron microscopy. Specific DNA staining and the use of mutants defective for DNA packaging has revealed, in contrast to earlier findings, that p40 is present in empty capsids. Protein p40 is not present as a major component of full capsids or of mature virions indicating that it is transiently associated with capsids and that its removal from capsids is linked with the process of DNA packaging.

INTRODUCTION

Members of a family of herpes simplex virus type 1 (HSV-1) late polypeptides of approx. Mr 40000 (p40, VMw40, ICP35, VP22a, henceforth referred to as p40) which are products of gene UL26 (Preston et al., 1983; McGeoch et al., 1988) have been found to have a differential association with different forms of the HSV-1 capsid. The p40 family was identified as a major component of full, DNA-containing viral capsids, but not of empty capsids (Gibson & Roizman, 1972; Braun et al., 1984). Studies on other herpesviruses have also found that major structural proteins in the size range of 30K to 50K Mr show differential association with different (full and empty) capsid forms. This has been shown for equine herpesvirus type 1 (EHV-1; Perdue et al., 1975), for human cytomegalovirus (HCMV; Gibson, 1981) and for pseudorabies virus (Ladin et al., 1982). From the results obtained with these herpesviruses it is clear that these proteins, if related, are characteristic components of the capsid. There is uncertainty however, concerning the relationship between the occurrence of these proteins on capsids and packaging of viral DNA. All the studies mentioned above have used capsid purification procedures to analyse the different capsid forms. We have examined the behaviour of p40 in HSV-1-infected cells using a different approach, namely immuno-electron microscopy.

Previously we have described a temperature-sensitive (ts) mutant of HSV-1, ts1201, which has a defect in processing of p40 at the non-permissive temperature (NPT) (Preston et al., 1983). Mutant ts1201 makes but fails to package viral DNA at the NPT and accumulates large numbers of partially cored (DNA-) capsids in the nuclei of infected cells. When cells infected with ts1201 were shifted from the NPT to the permissive temperature (PT) in the presence of cycloheximide, both processing of p40 and packaging of viral DNA took place, suggesting that p40 is closely involved in the packaging process. A second mutant, ts1203 (Matz et al., 1983), which makes but fails to package viral DNA at the NPT, also assembles partially cored capsids. In contrast to ts1201, ts1203 processes p40 normally at the NPT. We have used immuno-electron microscopy to examine the distribution of p40 within cells infected with HSV-1 wild-type (wt)
METHODS

Cells. BHK-21 clone 13 cells were used throughout for virus titrations and virus propagation. These cells were also used for all electron microscopy and for the Western blotting experiments.

Viruses. The wt HSV-1 virus was strain 17 (Brown et al., 1973). Isolation and characterization of mutants tsI201 and tsI203 has been described elsewhere (Preston et al., 1983; Matz et al., 1983).

Antibodies. Monoclonal antibodies 5010, 1028 and 5028 were produced and characterized by A.M.C. as described in Cross et al. (1987) except that the spleen cell donors were immunized with HSV-1-infected BHK cells.

Purification of HSV-1 virions. BHK cells were grown in 80 oz plastic roller bottles, infected at low multiplicity (10^-4 p.f.u./cell) with wt HSV-1 and left for three days at 37 °C. Cells were shaken into the culture medium and pelleted by low speed centrifugation (1000 g, 10 min, 4 °C). The supernatant medium was then combined with that from [35S]methionine-labelled cells prepared at the same time (see below) and further clarified by a second low speed centrifugation. Virions were pelleted by centrifugation at 9000 g for 2 h. The virus pellet was resuspended and layered onto a 5 to 15% Ficol 400 gradient and centrifuged at 4 °C for 2 h at 20000 g. The band was collected in a syringe by side puncture, diluted and pelleted by centrifugation at 20000 g for 1 h.

Radioactive labelling. Eighty oz plastic roller bottles of BHK cells were infected with 5 p.f.u./cell of wt HSV-1 and incubated with 100 μCi/ml [35S]methionine from 5 to 16 h post-infection (p.i.) in Eagle's medium containing 2% calf serum and one-fifth the normal concentration of methionine. Cells were collected from the medium by low speed centrifugation. The cell pellet was used as an infected cell sample in immunoblotting experiments. Where required the supernatant medium was combined with that from unlabelled roller bottles and virion purification was carried out as described above.

Immunoprecipitation. Labelling of cells with [35S]methionine for immunoprecipitation, and the immunoprecipitation procedures were as described in Preston et al. (1983). For the immunoprecipitations shown in Fig. 5 [35S]methionine-labelled cell extracts were prepared as described by Zweig et al. (1980) except that labelling was from 1 to 9 h p.i. Purified virion preparations for Fig. 5 were labelled with [35S]methionine for 2 days at 31 °C in Eagle's medium with 2% calf serum. Virions were then purified as above and solubilized with 1% NP40.

Immunoblotting. Immunoblotting was carried out as described by Towbin et al. (1979) with slight modifications. Briefly, proteins were separated electrophoretically on 7.5% SDS-polyacrylamide gels (Marsden et al., 1978) and transferred to nitrocellulose sheets. Duplicate sets of samples were run on each gel. Following transfer to nitrocellulose, one set of samples was probed with the appropriate monoclonal antibody and the other set with control serum. The nitrocellulose sheets were incubated at 65 °C for 1 h in the presence of 3% gelatin, then reacted with antisera for 20 h at room temperature. After washing, bound antibody was detected using 125I-labelled Protein A. Radioactive labelling was detected by exposing the nitrocellulose to three sheets of X-Omat XS-1 film (Harrr et al., 1985). Sheet 1 was placed in direct contact with the nitrocellulose and detected both 35S-labelled and 125I-labelled polypeptides. Sheets 2 and 3 were separated from sheet 1 by a piece of opaque black paper and an intensifying screen was placed outside them. These two sheets detected only 125I-labelled polypeptides.

Preparation of samples for electron microscopy. BHK cells, infected with 10 p.f.u./cell of wt HSV-1, tsI201 or tsI203, were harvested at 10 h p.i. by scraping into phosphate-buffered saline (PBS). Duplicate samples were pelleted into BEEM capsules (Agar Aids) and fixed for 1 h with 2.5% glutaraldehyde in PBS. Samples were then submerged briefly in PBS containing 0.05% Tween 20. This solution was used for all antibody dilutions and for washing the sections after treatment with antibody. The sections were transferred into the primary antibody for 45 min, then jet-washed and placed into a 1:50 dilution of either Protein A tagged with 10 nm gold particles (pA10) or similarly tagged goat anti-mouse antibody (GAM10) (Janssen Life Sciences). After a further 45 min they were jet-washed, rinsed in distilled water and dried. Contrast was enhanced by exposing sections to OsO4 vapour for 1 h and in certain cases this was followed by staining in uranyl acetate and lead acetate (Roth, 1986). Sections were examined using a JEOL 100S electron microscope.
HSV-1 40K protein localization

Fig. 1. Immunoprecipitation of p40 induced by ts1201, ts1203 and wt HSV-1. Autoradiogram of
immuno precipitation of p40 from cells infected at 38.5 °C, with 20 p.f.u./cell of wt HSV-1 (lanes 4 and
10), ts1201 (lanes 5 and 11) or ts1203 (lanes 6 and 12) and from mock-infected cells (lanes 3 and 9).
Whole cell extracts of mock-infected (lanes 1 and 7) and wt HSV-1-infected cells (lanes 2 and 8) are also
shown. Virus-infected cells were pulse-labelled with [35S]methionine at 5 h p.i. After 30 min, the
medium was removed, and the cells were harvested either immediately (lanes 1 to 6) or after a chase of
5 h (lanes 7 to 12). Immunoprecipitation was carried out as described by Preston et al. (1983) using 5010
antibody. Samples were analysed on a 9% SDS-polyacrylamide gel. Arrowheads indicate p40 bands.

RESULTS

Immunoprecipitation of p40 induced by ts1201, ts1203 and wt HSV-1

Cells were infected at 31 °C and 38.5 °C (NPT) with wt HSV-1, ts1201 or ts1203. Immunoprecipitation with a monoclonal antibody (5010) against p40 revealed (Fig. 1) that p40 was processed to the lower Mr forms in wt HSV-l-infected cells at 38.5 °C. This was also the case in cells infected with ts1203. However, as found previously (Preston et al., 1983), in ts1201-infected cells processing proceeded normally at 31 °C (data not shown) but very little processing took place at the NPT.

Subcellular localization of p40

The distribution of p40 in cells infected with wt HSV-1 or ts1201 at 31 °C and 38.5 °C was examined by immunofluorescence using anti-p40 monoclonal antibody 5010, visualized with fluorescein isothiocyanate-conjugated goat anti-mouse antibody. In each of the infected samples fluorescence was predominantly nuclear (data not shown). The nuclear localization of p40 in cells infected with ts1201 at the NPT was interesting since Braun et al. (1984) stated that the immature forms of p40, which are the only ones present at this temperature (Fig. 1), were found only in the cytoplasm. To investigate this apparent contradiction in more detail the subcellular localization of p40 was examined using immunoelectron microscopy.

Capsid structure in ts1201, ts1203 and wt HSV-1-infected cells

Monolayers of cells infected with 5 p.f.u./cell of wt HSV-1, or ts1201 or ts1203 for 7 h at
38.5 °C were fixed with glutaraldehyde. Half of each sample was prepared for immunoelectron microscopy (Fig. 2 and 3) and the remainder was embedded in Epon 812 resin for morphological examination (Fig. 2).

Wild-type virus produced the normal spectrum of herpesvirus capsid forms (Fig. 2a), namely full or dense cored (DNA+) and partially cored and empty (DNA-) capsids. As described previously (Preston et al., 1983), the nuclei of cells infected with ts1201 at the NPT contained no dense cored or empty capsids but had large aggregations of partially cored capsids (Fig. 2c). The nuclei of cells infected with ts1203 also contained only partially cored capsids; however, in this case the capsids do not form large aggregations but are randomly distributed throughout the nucleoplasm (Fig. 2e).

Intranuclear localization of p40

The distribution of p40 within infected cell nuclei was examined using immunogold localization procedures. The duplicate samples of those prepared for morphological
examination were embedded in Lowicryl K4M resin. Sections were incubated with a mouse monoclonal antibody against p40 and this was visualized using either pAl0 or GAM10 antibodies. Two monoclonal antibodies (5010 and 1028) that were directed against different epitopes on p40 were used in these experiments. As the results obtained with the two antibodies were indistinguishable only the data obtained with 5010 are illustrated here. Normal rabbit serum or normal mouse serum was used as a control in all experiments. In these control samples apparent localization of antibody-bound gold was sometimes observed on cellular membranes. We believe that this is due to interaction between the antibodies used and a virus-induced membrane protein (or proteins) since no cross-reaction was seen with uninfected cells. However, no non-specific localization was ever seen within the nucleus and at the antibody dilutions used (1/200) the localization on membranes was weak and could not be confused with that observed with the anti-p40 antiserum.

In sections from cells infected at the PT (not shown) or at the NPT with wt HSV-1 (Fig. 2b), tsl201 (Fig. 2d) or ts1203 (Fig. 2f), p40 was predominantly nuclear. Furthermore, the distribution of the gold particles indicated that much of the p40 was closely associated with virus capsids. This association was very marked, with approximately 65% of total nuclear gold particles present on capsids in HSV wt-infected cells. This result suggests that after synthesis p40 is rapidly transported to the nucleus and assembled into capsids leaving little free in either nucleus or cytoplasm. The gold label was not uniformly distributed over the capsid but tended to lie around the perimeter. This finding appears to support the suggestion of Braun et al. (1984) that p40 is not a component of the core but is present on the outside of capsids. However, in certain particularly striking examples the gold particles appeared to be aligned along the inner margin of the capsid (Fig. 2b, f). This agrees with the suggestion of Vernon et al. (1981) that p40 might be located slightly interior to the major capsid protein. The clearest localization was found using pA10 as the secondary marker since in this case only one gold particle is present per anti-p40 antibody molecule rather than with GAM10 where several secondary markers can bind each primary antibody leading to clustering of gold particles.

**Association of p40 with full and empty capsids**

Lowicryl sections from the same wt virus sample as that described above were reacted with anti-p40 antibody. In this case GAM10 was used as a secondary marker. The sections were stained with uranyl acetate and lead acetate to distinguish between DNA+ and DNA− capsids. Full capsids represented the same proportion (9%) of total capsids in the Lowicryl sections as in the Epon sections indicating that embedding in Lowicryl does not cause loss of DNA from capsids. Representative electron micrographs are shown in Fig. 3 and the overall distribution of gold particles from a number of micrographs is presented in Fig. 4. The majority of dense cored (DNA+) capsids and dense cored, enveloped virions present in the cytoplasm and around the cell surface have no gold associated with them and in no case were more than four gold particles present. By contrast gold particles were present on the majority of capsids which lacked DNA, and many of these had considerable numbers of associated gold particles. This was a consistent observation and large numbers of gold particles were not seen on dense cored capsids when either monoclonal antibody 5010 or 1028 was used.

**Presence of p40 in HSV-1 virions**

The immunoelectron microscopy results indicated that p40 was only a minor component of, or absent from DNA-containing capsids and virions. To examine this possibility further,
Fig. 4. Distribution of p40 on full capsids, empty capsids and enveloped virions. All capsids and virions on 20 micrographs were analysed for their association with gold particles. The micrographs were taken at a low magnification which allowed a large area of each cell to be examined. The results are plotted as a histogram showing the percentage of the total capsids in each class which were associated with different numbers of gold particles. Shaded columns represent enveloped virions; hatched columns represent dense-cored (DNA+) capsids and unshaded columns represent partially cored and coreless (DNA-) capsids.

immunoprecipitation was performed on purified virions using 5010 antibody (Fig. 5). Although p40 was readily precipitated from infected cells it was not observed in immunoprecipitations of extracts of purified virions. In addition no obvious bands comigrating with p40 were present in the protein profile of purified virions, suggesting that the inability to precipitate p40 reflected its low concentration in the virion preparation rather than inaccessibility or lack of recognition by the antibody.

To investigate further whether p40 was a virion component, purified virions were analysed by immunoblotting. The blots were incubated with a different monoclonal antibody against p40 (5028) from those used for the earlier experiments. Antibody 5028 reacted well on blots but did not work in immunoelectron microscopy. In both infected cells and in the purified virion sample p40 was detected (Fig. 6). However, considerably less p40 was present in the virion sample than in the infected cell sample. Since equal amounts of protein were run in each lane it is clear that p40 forms a smaller proportion of the total amount of protein in the virion preparation than in the infected cells. This in turn implies that p40 is at most a very minor component of the HSV-1 virion. It is also notable that once again p40 bands were not visible in the purified virion sample, but that bands similar in size to those of p40 were present in the infected cell sample.

**DISCUSSION**

Our data show unambiguously that p40 is present on empty (DNA-) capsids both in wt HSV-1 and in two ts mutants which fail to package viral DNA at the NPT. Our results also suggest strongly that p40 is only a minor species or a contaminant of DNA+ capsids and enveloped virions. These observations directly contradict those of Gibson & Roizman (1972) and Braun et al. (1984) who identified p40 as a major component of B (full) but not of A (empty) capsids and of Gibson & Roizman (1974) who speculated that the abundant virion protein VP22 might be a
Fig. 6. Immunoblotting with anti-p40 (5028) antiserum. Equal amounts of protein (40 μg) were run in each lane. Lanes 1 and 5, wt HSV-1-infected cells; lanes 2, 4 and 6, purified HSV-1 virions; lanes 3 and 7, mock-infected cells. Lanes 1 to 3 show [35S]methionine-labelled proteins transferred to nitrocellulose. These lanes were incubated in control serum which did not cross-react with any polypeptides. Lanes 5 to 7 show the polypeptides on the nitrocellulose that were detected by 5028. This autoradiogram is from the third sheet of X-ray film (see Methods) and therefore did not show [35S]-labelled bands. Lane 4 shows the autoradiogram obtained from lane 6 with the first sheet of X-ray film. This detects both [35S]-labelled and [125I]-labelled bands and allows the different samples to be aligned. Arrowheads indicate p40 bands recognized by 5028.

processed form of capsid protein 22a (p40). We think it very unlikely that the association of gold particles with empty capsids of both wt and mutant viruses is artefactual. The association was observed with two different monoclonal antibodies and was not seen with any of the control antisera. We have never obtained a similar localization with any monoclonal antibody against other HSV proteins. In addition, the association we see is very strong and very specific, being restricted to the perimeter of DNA capsids. Since the cells used in the immunolocalization
experiments underwent no disruptive treatment before fixing and embedding we believe that the localization of p40 we have observed reflects the true distribution of this polypeptide within the infected cells. Recently, Schenk et al. (1988) described a monoclonal antibody against p40 which also localizes on capsids within the nucleus of HSV-1-infected cells but they did not distinguish different capsid forms.

It is difficult to reconcile the seemingly opposite conclusions about the localization of p40 which have been reached using the different approaches. It is possible that the absence of p40 from purified empty capsids in the experiments of Gibson & Roizman (1972) and Braun et al. (1984) might be due to extraction of loosely bound p40 from these capsids during purification [as has been shown to be the case with HCMV (Gibson, 1981)]. This would imply that p40 is more tightly bound to certain capsid forms than to others since p40 was found by these authors on full capsids prepared by the same procedure. This suggestion is supported by the immunolocalization data for ts1201 at the NPT which show that immature forms of p40 are present on capsids within the nucleus. This conflicts with the conclusion of Braun et al. (1984) that the immature forms of p40 are restricted to the cytoplasm. Thus the immature forms of p40 may have been preferentially extracted from capsids and eluted from the nucleus under the isolation conditions used in their experiments. However, it is also possible that this apparent contradiction is due to incomplete separation of the different capsid forms as discussed below.

The differences between the observations concerning DNA+ capsids cannot be due to extraction of p40 during capsid purification. Our finding that p40 is present on DNA+ capsids in much lower amounts than on DNA− capsids contrasts with the earlier conclusion that p40 is a major component of full capsids (Gibson & Roizman, 1972). It might be postulated that changes in the conformation of p40 result in loss of, or masking of, the epitopes recognized by our antibodies. We consider this unlikely, since two distinct monoclonal antibodies gave identical results. In addition the immunoprecipitation and Western blotting data suggest that p40 is not a major component of the virus particle. We believe that a more likely explanation may be that the purification procedures used in the earlier studies did not completely separate the various capsid forms. From the micrographs presented by Gibson & Roizman (1972), it seems clear that the A capsids which lack any core structure at all correspond to the empty capsids seen in thin sections of infected cells. The B capsids are described as having 'a centrally located, densely staining structure' and therefore resemble the dense cored capsids observed in thin sections of infected cells. However the most abundant intranuclear capsid form normally comprises partially cored capsids which do not appear to be present in either of the purified capsid bands. It is possible that these partially cored capsids have in fact copurified with dense cored capsids and have been identified as B capsids. If this was the case it would account for the presence of p40 on these 'full' capsids and would partially resolve the apparent contradiction between these two sets of data.

After this manuscript was submitted for publication Sherman & Bachenheimer (1988) published a study on ts mutants of HSV-1 which fail to package DNA. These mutants make partially cored capsids that contain p40 and cosediment with B capsids from wt HSV-1-infected cells. This provides strong support for our suggestion that the p40 that is found on B capsids is due to the presence of partially cored capsids in these preparations. The detection by Western blotting of a small amount of p40 in the purified virion preparations might indicate that not all the p40 is removed from the capsids when the DNA is packaged. Alternatively, it might reflect the presence in these preparations of low numbers of enveloped empty capsids which have been observed in electron micrographs of HSV-infected cells.

Due to the relatively poor staining obtained with the hydrophilic resin used in our studies, it was not possible to distinguish with certainty between partially cored and empty capsids. Thus, since not all DNA− capsids had associated gold particles, it is possible that p40 was not present on either the dense cored or the coreless (A) capsids but only on the partially cored forms. This would be consistent with the alternative interpretation of the data of Gibson & Roizman (1972) suggested here.

Work on other herpesviruses has not established a clear cut picture of the relationships between the various capsid forms and the presence of putative equivalents to p40. Ladin et al. (1982) showed that empty capsids contained a polypeptide of Mr 35K which was
underrepresented in mature virions. However, they did not distinguish between coreless and partially cored empty capsids. Friedrichs & Grose (1986) using varicella-zoster virus found that a group of proteins (the p32/p36 complex) which they thought were equivalent to HSV-1 p40, was a component of both empty and full capsids. However, it is not certain that these proteins are equivalents of p40. Perdue et al. (1975) were successful in separating three capsid forms from the nuclei of cells infected with EHV-1. These were designated H, I and L and it seems clear that these correspond to the dense cored, partially cored and empty capsids respectively which are seen in thin sections of HSV-1-infected nuclei. Analysis of the protein composition of the different forms identified two species of M, 46K and 30K which are found in H and I capsids but are not present in L capsids. It is not clear whether these proteins are related to one another or if either is equivalent to the HSV-1 p40. However, their abundance in H capsids does not agree with our findings for p40. Examination of HCMV has shown that a 36K protein is a component of B capsids but not of A capsids and that it is present in non-infectious enveloped particles (NIEP) but not in virions (Irmiere & Gibson, 1985). The B and A capsids are thought to correspond to the similarly named HSV capsids. The B capsid preparations in this case are known to include capsids with little or no DNA and the NIEPs, which also lack DNA, are thought to be enveloped B capsids. This therefore would agree with our suggestion that the HSV B capsid preparations are a mixture of DNA⁺ and DNA⁻ capsids.

The results we have obtained show that p40 is a major component of partially cored HSV capsids but is absent from or a minor component of dense-cored capsids and mature virions. Previous studies on ts1201 suggested that partially cored capsids are precursors of dense cored capsids and virions and possibly also of coreless capsids (Preston et al., 1983). Thus it appears that p40 becomes transiently associated with capsids at an early stage in their assembly and is lost from the capsids during the process of viral DNA packaging which is itself linked to processing of p40. This reinforces our earlier suggestion (Preston et al., 1983) that p40 is closely involved in DNA packaging and poses interesting questions as to the functions of the different processed forms of p40 in this process. However, direct examination of the role of the different forms of p40 will require the use of antibodies which recognize specific forms of p40.

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HSV-1 40K protein localization


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