On the Intracellular Transport and the Nuclear Association of Human Cytomegalovirus Structural Proteins

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(Accepted 3 December 1984)

SUMMARY

In cells productively infected with human cytomegalovirus (HCMV) AD169, large amounts of two viral proteins, the 150K major capsid and the 68K major matrix proteins, are continuously produced during the late phase of infection. In the present study, the mechanism for the intracellular transport of the 150K and 68K proteins was investigated. Infected cells were labelled for 30 min at 72 h post-infection with [35S]methionine, chased for various periods of time at 37 °C and fractionated into cytoplasmic and nuclear fractions. Immediately after 30 min of labelling, the 68K protein was already associated with the nuclear fraction. In contrast, the major proportion of the 150K protein remained in the cytoplasm for more than 1 h; the migration of the 150K protein was much slower than that of the 68K protein. Both the 150K and the 68K proteins were associated with the perinuclear cytoskeletal fraction in the process of migration. After migration into the nucleus, these proteins were resistant to extraction with DNase and high salt, indicating that they were associated with the nuclear skeleton (nuclear matrix). Effects of various inhibitors on the migration of the 150K protein showed that cycloheximide inhibited the transport of the 150K protein, but other inhibitors such as arabinosyl cytosine, cytochalasin D, colchicine or sodium azide did not. The results suggest that the cytoskeletal structure may play a role in the intracellular transport of HCMV structural proteins from the cytoplasm into the nucleus.

INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the herpesvirus group, the viruses of which are morphologically characterized by an enveloped icosahedral capsid (Smith & Rasmussen, 1963). Like other herpesviruses, HCMV replicates its DNA and forms its capsid within the nucleus of the host cell, and becomes enveloped in the process of budding (Kanich & Craighead, 1972; Fong et al., 1979). In contrast to herpes simplex virus (HSV), HCMV is less cytocidal and has a much longer growth cycle (Smith & De Harven, 1973). In cells productively infected with HCMV, viral DNA synthesis begins 12 to 24 h after infection and persists over 5 days (Stinski, 1977; Nishiyama et al., 1982). During the late phase of infection, large amounts of viral proteins are continuously produced without a shut-off of cellular protein synthesis; in particular, two structural viral proteins, the 68K and 150K proteins, are preferentially synthesized (Stinski, 1977). Most of these structural proteins are transported to the nucleus after synthesis in the cytoplasm and accumulate in the nucleus where they are assembled into virions. However, the process of viral morphogenesis and the mechanism for intracellular transport to the nucleus have not been elucidated. Recent studies have shown that the cytoskeleton (cytoplasmic network structure) interacts with virions and subviral components, which suggests that it may play a significant role in virus replication (Luftig, 1982; Rowe et al., 1983). Ben-Ze'ev et al.
The final LM pellet was extracted for 15 rain in 1 ml HS buffer (2 M-NaCl, 0.2 mM-MgCl₂, 10 mM-Tris-HCl pH 7.4) and examined with a Hitachi H-12 electron microscope.

Infection and isotope labelling. Confluent monolayers of HEF in plastic culture dishes were infected with HCMV at a multiplicity of approximately 3 p.f.u./cell. After a 1 h adsorption period at 37 °C, cells were washed with phosphate-buffered saline (PBS) and incubated at 37 °C with MEM containing 2% FCS. At appropriate times after infection, culture medium was replaced with L-[³⁵S]methionine in MEM lacking unlabelled methionine or replaced with [³²P]orthophosphate in MEM lacking unlabelled phosphate.

Cell fractionation. Monolayers of infected cells were treated on ice for 3 min with PBS containing 0.02% EDTA and 0.25% trypsin, and scraped from culture dishes into PBS containing 20% calf serum. The cells were collected by low-speed centrifugation, washed twice with PBS, and then suspended in reticulocyte standard buffer (RSB: 10 mM-Tris-HCl pH 7.4, 10 mM-NaCl, 1.5 mM-MgCl₂) at a concentration of 1.5 × 10⁶ cells per ml, and left for 5 min on ice. After addition of NP40 to a final concentration of 1% (w/v), the suspension was allowed to stand on ice for another 5 min, with occasional vortex mixing, and then separated into the crude nuclear and cytoplasmic fractions by low-speed centrifugation (1500 r.p.m., 5 min). The crude nuclear fraction was resuspended in 1 ml RSB by gentle vortex mixing, and NP40 and deoxycholate (DOC) were added to final concentrations of 0.5% (w/v). After gentle mixing, the nuclear fraction was separated by low-speed centrifugation (1500 r.p.m., 10 min), and the supernatant, subsequently referred to as the perinuclear cytoskeletal fraction, was collected. The cytoplasmic fraction was further separated into the NP40-soluble and the insoluble pellet fractions by centrifugation at 10000 x g for 15 min (Naso et al., 1975).

Nuclear matrix was prepared according to the method of Buckler-White et al. (1980). Nuclei from 5 × 10⁶ cells were suspended in 1 ml TM sucrose buffer (0.25 M-sucrose, 5 mM-MgCl₂, 50 mM-Tris-HCl pH 7.4) and Triton X-100 was added to a final concentration of 1%. After centrifugation at 15000 x r.p.m. for 10 min, the nuclei were resuspended in 1 ml TM sucrose buffer and incubated with 20 µg/ml DNase I for 20 min at room temperature with continuous shaking, followed by three washings with 1 ml LM buffer (0.2 mM-MgCl₂, 10 mM-Tris-HCl pH 7.4). The final LM pellet was extracted for 15 min in 1 ml HS buffer (2 mM-NaCl, 0.2 mM-MgCl₂, 10 mM-Tris-HCl pH 7.4) on ice and then pelleted at 3000 x r.p.m. for 15 min. The high-salt extraction was repeated once more, and the nuclear matrix fraction was finally washed with LM buffer.

Electron microscopy. Pelleted cells and nuclei were fixed on ice for 1 h with 2% glutaraldehyde in PBS, washed with PBS overnight, and post-fixed with 1% osmium tetroxide for 1 h. The specimens were then dehydrated in ethanol and embedded in Epon 812. Sections were contrasted with 2% uranyl acetate and lead citrate and examined with a Hitachi H-12 electron microscope.

Polycrylamide slab gel electrophoresis (PAGE). PAGE was carried out by the method of Laemmli (1970). Samples were dissociated in 0.0625 M-Tris-HCl pH 6.8 containing 5% SDS, 2% 2-mercaptoethanol (2-ME), 10% glycerol and 0.001% bromophenol blue, followed by heating at 100 °C for 1 min. The acrylamide concentrations were 8.5 or 10% for the separating gel and 3% for the stacking gel. RNA polymerase (165K, 155K, 39K) and bovine serum albumin (68K) were used as reference protein markers. After electrophoresis, the gels were fixed, dried and then exposed to Kodak Royal X-Omat film at −80 °C.

Chemicals. L-[³⁵S]Methionine (600 Ci/mmol) and inorganic [³²P]phosphate (carrier-free) were purchased from Amersham and Japan Radioisotope Association, respectively. Actinomycin D, cycloheximide, cytosine-1-β-D-arabinofuranoside (Ara-C), cytochalasin D and colchicine were obtained from Sigma. Ouabain and vinblastine were purchased from Merck and Shionogi Pharmaceutical Company, respectively. N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) was purchased from the biochemical Industry Company.

RESULTS

Kinetics of the intracellular transport of 150K and 68K viral proteins from cytoplasm to nucleus

Fig. 1 shows an autoradiograph of PAGE of HCMV-infected HEF proteins labelled with [³⁵S]methionine every 24 h after infection. As reported previously (Stinski, 1977), the 150K (1983) have also suggested that the cytoskeletal structure may be involved in the transport of HSV proteins into the nucleus. In the present study, we examined the role of the cytoskeletal and nuclear frameworks in the intracellular transport and nuclear accumulation of HCMV proteins.
Intracellular transport of HCMV proteins

Fig. 1. Protein synthesis in HCMV-infected cells. Confluent monolayers of HEF were mock-infected or infected with HCMV at a multiplicity of approximately 3 p.f.u./ml and labelled for 1 h with [35S]methionine (10 µCi/ml) at the appropriate time (1, 2, 3 or 4 days) after infection. Whole cells were subjected to SDS gel electrophoresis on 10% acrylamide gels, followed by autoradiography.

Major capsid and the 68K major matrix proteins were clearly detectable by 48 h after infection. Thereafter, the production of these late proteins continued at a high rate, and the formation of viral particles became detectable 72 h after infection (Fig. 2). Although infected cells appeared enlarged in size 72 h after infection, no extensive cell damage was observed. Therefore, we examined the kinetics of the intracellular transport of the HCMV proteins in cells at 72 h after infection.

HCMV-infected cells were labelled with 10 µCi/ml [35S]methionine for 30 min and chased in unlabelled medium for various periods of time at 37°C. The cells were then separated into the crude nuclear and cytoplasmic fractions as described in Methods, and both fractions were processed for PAGE analysis. The autoradiograms were scanned with a densitometer and the relative amounts of the 68K and 150K proteins in the crude nuclear and cytoplasmic fractions were quantified (Fig. 3). After 30 min of labelling, more than 50% of the total amount of the 68K matrix protein was already associated with the crude nuclear fraction. In contrast, only a trace amount of the 150K capsid protein was present in the crude nuclear fraction. The migration of the 150K protein into the nucleus was much slower than that of 68K protein.
Fig. 2. Electron microscopy of intact HCMV-infected HEF (a), crude nuclear (b) and purified nuclear fractions (c). HCMV-infected cells at 72 h post-infection were fractionated as described in Methods. Samples were fixed with 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in ethanol, and processed for electron microscopy. Bar markers represent 2 μm.

Association of 150K and 68K proteins with cytoskeletal fraction

As shown in Fig. 2, the crude nuclear fractions contained the perinuclear filamentous framework associated with electron-dense structures. These perinuclear structures, however, were separated from the nucleus by washing with a buffer containing DOC and NP40. By this
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Fig. 3. Migration of 68K (a) and 150K (b) proteins into the crude nuclear fractions. HCMV-infected cells at 72 h post-infection were labelled with [³⁵S]methionine (10 µCi/ml) for 30 min, chased for 1 h, 2 h and 3 h at 37°C after the addition of excess unlabelled methionine, and then fractionated into the cytoplasmic (○) and crude nuclear (●) fractions. The proteins from each fraction were subjected to SDS-PAGE and autoradiography. The amount of 150K and 68K proteins in each fraction was quantified by a densitometer scanning of the autoradiogram and expressed as a percentage of the total amount at the time.

Fig. 4. Association of 150K and 68K proteins with various subcellular fractions. HCMV-infected cells were labelled with [³⁵S]methionine (10 µCi/ml) for 30 min at 72 h post-infection and chased for (a) 1 h and (b) 3 h at 37°C. The cells were then fractionated into four fractions: C, NP40-soluble cytoplasmic fraction; CS1, NP40-insoluble cytoplasmic fraction; CS2, DOC-soluble perinuclear fraction; N, nuclear fraction. The proteins in each fraction were subjected to SDS-PAGE and autoradiography.

fractionation procedure, cells were separated into three fractions. In addition, the cytoplasmic fraction was separated into the NP40-soluble supernatant and the NP40-insoluble pellet by centrifugation for 15 min at 10000 g. Experiments were then performed to examine the association of the 150K and 68K proteins with each of the four fractions during the process of migration in the cell.

HCMV-infected cells were labelled with [³⁵S]methionine for 30 min at 72 h post-infection, chased in MEM containing unlabelled methionine, and then fractionated after various periods of chase (Fig. 4). After 1 h of chase, a considerable amount of newly synthesized 150K protein was still present in the NP40 soluble cytoplasmic fraction (C) and a minor amount was found in the NP40-insoluble (CS1), DOC-soluble perinuclear (CS2) and nuclear (N) fractions. After 3 h of chase, the majority of the 150K protein was found in the nuclear fraction. In contrast, after a 1 h chase no 68K protein was detected in the NP40-soluble fraction and after a 3 h chase, more than half was already present in the nuclear fraction. These experiments showed that both the 150K and the 68K proteins were associated with the NP40-insoluble and DOC-soluble perinuclear fraction which contained most of the cytoskeletal framework.

Modification of the proteins was also examined in pulse–chase experiments. As shown in Fig. 5, the 68K matrix protein in the nuclear fraction was found to be phosphorylated and the extent of phosphorylation in the 68K protein increased with time of chase. The 150K capsid protein
was not phosphorylated. Neither the 150K nor the 68K protein was labelled by [3H]glucosamine in the process of migration (data not shown).

Effects of various inhibitors on the intracellular transport of the 150K capsid protein

The association of the 150K protein with the nucleus occurred at a much slower rate than that of the 68K protein, and both proteins were associated with the cytoskeletal framework in the process of migration. Furthermore, newly synthesized 150K protein did not migrate into the nucleus when pulse-labelled infected cells were chased at 4°C (data not shown). From these results, it seemed reasonable to assume that a cellular function(s) might be positively involved in the transport of the viral proteins into the nucleus. To examine this, various compounds which are known specifically to inhibit different cell functions were tested for their effect on the migration of the 150K protein.

Infected cells were labelled with 10 μCi/ml [35S]methionine for 30 min from 72 h post-infection in the absence of inhibitors. After a chase period of 4 h in the presence or absence of an inhibitor, the purified nuclear and cytoplasmic fractions were prepared and the labelled proteins were analysed by PAGE and autoradiography. Only cycloheximide exhibited a significant inhibitory effect on the transport of the 150K protein (Fig. 6), indicating that continuous protein synthesis was necessary for the migration of the capsid protein into the nucleus. However, other inhibitors that inhibit the synthesis of DNA, RNA and ATP, or the function of microfilaments, microtubules, calmodulin and the Golgi apparatus did not inhibit the migration of the 150K capsid protein into the nucleus, as summarized in Table 1.

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Fig. 5. Phosphorylation of proteins in HCMV-infected cells. HCMV-infected cells were labelled with [32P]orthophosphate (100 μCi/ml) or [35S]methionine (10 μCi/ml) for 30 min at 72 h post-infection, chased for 1 to 3 h, and then fractionated into the cytoplasmic (C), DOC-soluble perinuclear (CS) and nuclear (N) fractions. The proteins in each fraction were analysed by SDS-PAGE and autoradiography.
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Fig. 6. Effect of protein synthesis and DNA synthesis inhibitors on the distribution between purified nuclear and cytoplasmic fractions of HCMV-infected cell proteins. Infected cells were labelled with [35S]methionine for 30 min from 72 h post-infection in the absence (None) of inhibitors, and chased for 4 h in the presence of cycloheximide (Cyclo., 500 μg/ml) or Ara-C (50 μg/ml). The purified nuclear (N) and cytoplasmic (C) fractions were prepared as described in Methods, and the labelled proteins were analysed by PAGE and autoradiography.

Table 1. Effect of various inhibitors on the intracellular transport of 150K capsid protein

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dose</th>
<th>Targets or action of inhibitors</th>
<th>Effect</th>
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<tr>
<td>Ara-C</td>
<td>50 μg/ml</td>
<td>DNA synthesis</td>
<td>−</td>
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<tr>
<td>Actinomycin D</td>
<td>2 μg/ml</td>
<td>RNA synthesis</td>
<td>−</td>
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<tr>
<td>Cycloheximide</td>
<td>500 μg/ml</td>
<td>Protein synthesis</td>
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<td>Monensin</td>
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<td>Ionophore</td>
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<td>Cytochalasin D</td>
<td>10 μg/ml</td>
<td>Microfilaments</td>
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<td>Colchicine</td>
<td>1 mM</td>
<td>Microtubules</td>
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<td>Vinblastine</td>
<td>100 μM</td>
<td>Microtubules</td>
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<tr>
<td>Sodium azide</td>
<td>10 mM</td>
<td>ATP synthesis</td>
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<td>Ouabain</td>
<td>100 μM</td>
<td>Na+K+ ATPase</td>
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<td>W-7</td>
<td>100 μM</td>
<td>Calmodulin</td>
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Association of 150K and 68K proteins with the nuclear matrix fraction

The nucleoskeletal framework, termed the nuclear matrix, has been reported to be involved in DNA replication, transcription and RNA processing (Berezney & Buchholtz, 1981; Nakayasu et al., 1982; Robinson et al., 1982), and recent studies from several laboratories have shown an
Fig. 7. Association of 150K and 68K proteins with the nuclear matrix fractions. HCMV-infected cells were labelled with [35S]methionine for 1 h at 72 h post-infection, and chased for (a) 4 h or (b) 24 h. The crude nuclear fractions were treated as described in Methods to isolate the nuclear matrix fraction (NM). The supernatants resulting from treatments with Triton X-100 (S1), DNase (S2), hypotonic (S3) and hypertonic (S4) buffers were concentrated by ethanol precipitation. Proteins in each fraction were dissolved in the same volume of dissociation buffer and subjected to SDS-PAGE and autoradiography.

**DISCUSSION**

When HEF were infected with HCMV, two structural viral proteins were continuously produced in abundance during the late phase of infection. These are the 150K and 68K proteins, which have been identified as major capsid and major matrix proteins, respectively (Gibson, 1981). Since all HCMV proteins are produced in the cytoplasm, both proteins must be associated with viral capsids with nuclear matrices prepared from HSV-infected cells (Bibor-Hardy et al., 1982; Tsutsui et al., 1983). These observations prompted us to examine whether the major capsid and matrix proteins of HCMV transported into the nuclei were associated with the nuclear matrix.

Infected cells were labelled with [35S]methionine for 1 h from 72 h post-infection, chased for 4 h or 24 h at 37°C, and then fractionated. The crude nuclear fraction was sequentially treated with Triton X-100, DNase, hypotonic and hypertonic buffer as described in Methods. The resultant supernatant and nuclear matrix fractions were concentrated by ethanol precipitation and the proteins analysed by PAGE. As shown in Fig. 7, both the 150K and the 68K proteins were resistant to treatment with DNase and high-salt buffer, and remained with the nuclear matrix. Even after 24 h of chase, the majority of the 150K capsid protein was still associated with the nuclear matrix but a considerable amount of the 68K matrix protein was washed off by high-salt buffer.
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transported from the cytoplasm to the nucleus to be assembled into virions. The pulse–chase experiments showed that more than 50% of newly synthesized 68K matrix protein was associated with the nuclear fraction within 30 min, but the majority of newly synthesized 150K capsid protein remained in the soluble cytoplasmic fraction for more than 30 min, suggesting that a selective process may be involved in the intracellular transport of viral proteins into the nucleus.

The relatively slow transport of the capsid protein has been observed also in HSV-infected cells, where the HSV capsid protein (150K to 155K) is transported to the nucleus much more slowly than the major DNA-binding protein (128K to 130K) (Knipe & Spang, 1982; Ben-Ze’ev et al., 1983). Although the replication cycle of HCMV is more than five times as long as that of HSV, the kinetics of the association of the HCMV capsid protein with the nucleus was comparable with that of the HSV capsid protein. The lag time might be explained by a modification or an assembly of the capsid protein in the process of transport, although the rate of transport would be dependent on the shape, size and charge of the proteins. Neither phosphorylation nor glycosylation of the capsid protein was detected, whereas the 68K matrix protein was phosphorylated. Therefore, it is unlikely that modification of the capsid protein was involved in the process of transport. Since the transport of the capsid protein required the continuous synthesis of protein, the lag might be related to a limiting amount of a protein which is necessary for transport.

It has been shown that there are three major cytoskeletal filament classes inside culture cells: microtubules, microfilaments and intermediate filaments (Luftig, 1982) and that these network structures play significant roles in the replication of animal viruses such as adenoviruses (Luftig & Weihing, 1975), reoviruses (Sharpe et al., 1982) and poliovirus (Lenk & Penman, 1979). Recently, Ben-Ze’ev et al. (1983) have shown an association of the HSV capsid protein with the cytoskeleton in the process of migration into the nucleus. In HCMV-infected cells, newly synthesized capsid protein was initially found in the NP40-soluble cytoplasmic fraction and next in the NP40-insoluble cytoplasmic and DOC-soluble perinuclear fractions which contained most of the cytoskeletal network. These results suggest an involvement of the cytoskeletal network in the transport of the capsid protein, while the inhibitors of microfilament and microtubule formation did not inhibit the migration of the capsid protein. Taken together, it is tempting to infer that intermediate filaments may play a role in the intracellular transport of the HCMV major capsid protein into the nucleus.

After entering the nucleus, the majority of both the 150K and the 68K proteins were resistant to DNase and high-salt treatments, indicating that these proteins were associated with nuclear skeletal structures. After 4 h of chase, both proteins were already associated with the nuclear matrix fractions. Since it took about 3 h for the 150K protein to be transported into the nucleus, the observations suggest that the capsid protein was associated with the nuclear matrix immediately after entering the nucleus. Although recent studies have revealed that the nuclear skeletal filaments are morphologically and biochemically different from the perinuclear cytoskeletal filaments (Capco et al., 1982), our observations suggest an interaction between them in the intracellular transport of the viral proteins. There might be a functional and structural continuity between perinuclear cytoskeletal filaments and nuclear skeletal filaments through the nuclear membrane and pores.

We would like to thank H. Aoki and T. Tsurumi for helpful discussion and advice. We also thank T. Tsuruguchi and E. Iwata for their technical assistance. This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

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(Received 3 September 1984)