Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells

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Cells infected with a number of alphaherpesviruses produce non-infectious virion-related particles, termed L particles, in addition to infectious virions. L particles consist of the tegument and envelope components, but lack the virus capsid and DNA. Using a herpes simplex virus type 1 (HSV-1) temperature-sensitive mutant, ts1201, which fails to produce mature virions, we show that L particle production is independent of virion formation. Moreover, the quantity and protein composition of L particles generated by this mutant at the non-permissive temperature are indistinguishable from those produced in wild-type HSV-1 infections. Electron microscopy studies suggest that the processes governing the assembly of tegument and envelope components into L particles are similar to those involved in virion maturation.

Introduction

A number of alphaherpesviruses have recently been shown to produce a second type of virion-like particle (termed the L particle) in addition to infectious virions (Szlágyi & Cunningham, 1991; McLauchlan & Rixon, 1992). In infectious virions, the virus DNA is contained in an icosahedral nucleocapsid that is in turn enclosed by an amorphous proteinaceous tegument and a glycoprotein-containing envelope (Dargan, 1986). L particles consist of tegument surrounded by an envelope, but lack the virus capsid and DNA, and consequently are non-infectious.

The appearance and composition of L particles suggest that their genesis is related to that of virions, although differences in the compositions of the two types of particle indicate that their pathways of assembly are not identical (Szlágyi & Cunningham, 1991). The conditions under which L particles were initially characterized did not reveal whether their formation was directly coupled to and dependent upon that of virions.

To address this question, we have examined the production of virion-like particles using ts1201, a temperature-sensitive mutant of herpes simplex virus type 1 (HSV-1) with a defect in gene UL26 (Preston et al., 1983, 1991). At non-permissive temperatures, ts1201 makes viral DNA and a full spectrum of viral proteins, including the late structural species. Although capsid assembly takes place, viral DNA is not packaged. The immature capsids are retained in the nucleus and do not acquire a tegument or envelope, thus mature virions are not assembled.

Methods

Cells and viruses. All experiments were performed in BHK C13 cells cultured in Glasgow modified Eagle's medium supplemented with tryptose phosphate and 10% calf serum (ETC10). For virus titration, cells were overlaid with ETC10 containing 1.25% methylcellulose. Stocks of HSV-1 strain 17 and ts1201 were obtained by infecting 10 roller bottles of cells at a multiplicity of 1/300 p.f.u./cell. Following infection at 31 °C for 4 days, virus was harvested and purified on 5 to 15% Ficoll gradients as described previously (Szlágyi & Cunningham, 1991). Bands were collected by side puncture, diluted with Eagle's medium lacking phenol red and pelleted by centrifugation at 21000 r.p.m. in a Sorvall T41 rotor for 2 h at 4 °C. Pellets were resuspended in Eagle's medium lacking phenol red and stored at −70 °C.

Electron microscopy (EM). Cells to be used for EM were grown in 30 mm Petri dishes each containing a 13 mm diameter glass coverslip. At appropriate times after infection the cells were washed with PBS and fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C. Cells were then washed in PBS and osmium tetroxide (1% in PBS) was added for 1 h.

Replica formation. The coverslips were removed to a 24-well tissue culture tray, dehydrated through a graded alcohol series and dried in a critical point drier. The coverslips were shadowed with platinum/palladium for 7 s at an angle of 75° in a Balzers BAE121 shadowing unit, then rotary shadowed with carbon to provide additional support. The shadowed surface was overlaid with 50 µl of 0.25% paraloid in amyl acetate and allowed to dry. Each coverslip was broken in half and the shadowed surface was scored into approximately 2 mm squares. To release replicas, the coverslips were floated on hydrofluoric acid until they dissolved. The replica squares were picked up on a platinum wire loop and washed on distilled water. Cell material was removed by
Table 1. Virus titres and particle :p.f.u. ratios of inoculating virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>31 °C</th>
<th>38.5 °C</th>
<th>Particles/ml</th>
<th>Particle :p.f.u. ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>$3.8 \times 10^{10}$</td>
<td>$3.6 \times 10^{10}$</td>
<td>$1.02 \times 10^{12}$</td>
<td>27:1</td>
</tr>
<tr>
<td>ts1201</td>
<td>$1 \times 10^{10}$</td>
<td>$&lt;10^4$</td>
<td>$4.89 \times 10^{11}$</td>
<td>49:1</td>
</tr>
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</table>

* Ratios determined using virus titres at 31 °C.

Floating replicas on 66% chromic acid for 1 h. Clean replicas were washed three times on water, then dried onto 400-mesh copper microscope grids. When dry, the parlodion support film was removed by immersing the grids in amyl acetate and the replicas were examined in a JEOL 100S electron microscope.

Thin sectioning. The remaining cells on each 30 mm Petri dish were harvested, pelleted in BEEM capsules, dehydrated and embedded in Epon 812 resin as described previously (Preston et al., 1983). Sections of 120 nm thickness were cut and stained with uranyl acetate and lead citrate.

PAGE. Proteins were separated on 5 to 12% gradient polyacrylamide gels cross-linked with 5% N,N'-methylene-bis-acrylamide using the buffer system described by Laemmli (1970). Proteins were visualized by silver staining as described by McLean et al. (1990).

Results

Appearance of L particles in mutant-infected cells

To examine the production of virion-like particles under conditions of productive and non-productive infection, their appearance within and on the surfaces of cells was monitored by EM. Purified virions were used in the following experiments to reduce possible artefacts resulting from impurities in the virus inocula. Both wild-type (wt) and ts1201 virus, grown at 31 °C, were purified on gradients by the method of Szilágyi & Cunningham (1991). Purified virion preparations were titrated at 31 °C (permissive) and 38.5 °C (non-permissive temperature) and their particle/p.f.u. ratios determined (Table 1). Titration of the ts1201 virion stock confirmed that it was temperature-sensitive under the conditions used, with a reduction in titre of greater than 10^6-fold.

In the following experiments the production of virion-like particles was analysed by titration and EM. In order that the results obtained from these different approaches should be directly comparable, cells were grown on 30 mm Petri dishes which each contained a 13 mm glass coverslip. Following infection with 5 p.f.u./cell of either wt virus or ts1201, cells were incubated at 38.5 °C. A portion of each inoculum was retained for subsequent titration (these constituted the 0 h samples). At 1 h after infection, the input inocula were removed and were also retained (these constituted the 1 h samples and were used to measure unadsorbed virus). The cells were washed with ETC10 and incubation was continued at 38.5 °C. At 6, 10 and 24 h, supernatant medium was collected from each plate and retained for titration. The cells from each time were used for formation of surface replicas, and the cells remaining on the dish were prepared for thin sectioning.

(i) Virus growth

The titres of viruses present at each time are shown in Fig. 1. As expected, the wt virus, after an initial decline in titre during the eclipse phase, showed a rapid increase in virus production to a level about 10-fold higher than that of the input virus. By contrast, ts1201 titres declined throughout the period of infection to about 100-fold lower than that of the input virus, thereby confirming that the ts1201 used in this experiment was behaving in a fashion typical of this mutant (V. Preston, personal communication). This ensured that the results obtained from the ts1201-infected cells were not a result of leakiness or reversion of the mutant.

(ii) Appearance of particles on the cell surface

Examination of replicas prepared from wt virus-infected cells revealed a characteristic series of changes to the cell surface (Fig. 2). At 1 h, the cell surface was smooth and showed no evidence of infection compared with mock-
infected samples (Fig. 2a). By 6 h, many cells had small numbers of particles of approximately 250 nm diameter on their surfaces (Fig. 2c), and by 10 h large numbers of these particles had accumulated on most cells (Fig. 2e). The size of these particles was consistent with that of virions. At 24 h the cells were decidedly rounded and had dense coverings of particles (Fig. 2g). The distribution of these particles on the cell surface was frequently asymmetric, with marked concentrations over the area of the nucleus (identified as a swelling in the cell surface) and around regions at the periphery of the cell being typical.

Examination of ts1201-infected cells revealed a very similar pattern of changes. Thus, at 1 h after infection their surfaces were indistinguishable from those of mock-infected cells apart from the presence of occasional particles which presumably were derived from the inoculum (Fig. 2b). By 6 h, small numbers of particles were present on the cell surface, and their number had increased at 10 h and 24 h (Fig. 2d, f and h). The distribution and abundance of particles were comparable to those found with wt virus, but they appeared slightly less uniform in size.

(iii) **Nature of the particles on the cell surface**

To determine the nature of the particles present on the surfaces of infected cells, thin sections prepared from the remainder of each sample were examined. Both virions and particles lacking obvious capsids, which we consider to be L particles, were present on wt virus-infected cells (Fig. 3a). Similar particles were also found with virions in intracellular vacuoles (Fig. 3c).

Thin sections of ts1201-infected cells demonstrated the phenotype typical of this mutant (Preston et al., 1983). Thus, the nuclei contained aggregations of characteristically large cored capsids which lacked DNA (data not shown; Preston et al., 1983). Full, DNA-containing capsids were not apparent and there was no evidence of mature virions either in the cytoplasm or on the cell surface. The particles that were present on the surface lacked capsids and closely resembled L particles in appearance (Fig. 3b); particles of a similar type were also seen frequently in cytoplasmic vacuoles (Fig. 3d). A number of features were also seen which could be considered as L particles in the process of formation by budding into vacuoles (data not shown). However, due to the lack of diagnostic capsid, it is impossible to be dogmatic as to the nature of these features.

**Protein composition of particles released from ts1201-infected cells**

To establish whether the particles produced by ts1201 at the non-permissive temperature were typical L particles, five roller bottles of cells were infected with either gradient-purified ts1201 or wt virus. Following incubation at 38.5 °C for 24 h, particulate matter present in the supernatant medium was pelleted, resuspended and banded on 5 to 15% Ficoll gradients as described by Szilágyi & Cunningham (1991). The gradient containing the wt virus preparation (Fig. 4a) contained two bands which corresponded to those described by Szilágyi & Cunningham (1991); these were a sharp lower band of virions and a diffuse upper band of L particles. By contrast, the virion band was not observed in the gradient containing ts1201 (Fig. 4b). However, a diffuse band was present which comigrated with, and was of a similar intensity to, the L particle band present in the wt virus preparation. When collected and examined by negative staining, this band was shown to contain material which appeared identical to typical L particles (data not shown). Analysis of their protein composition by PAGE confirmed this assignment; the compositions of the wt virions and wt L particles conformed to the patterns described previously (Fig. 5, lanes 1 and 2).

Thus, the Mr 155000 and 35000 capsid proteins which were abundant in virions were greatly reduced in amount in L particles, whereas Vmw175 was found predominantly in L particles.

The protein profile of the ts1201 band was indistinguishable from that of the wt L particles, and the diagnostic Vmw175 band was clearly visible (Fig. 5, lane 3). Interestingly, despite the very low levels of infectious virions in this band, the Mr 155000 capsid protein was present in trace quantities similar to those found in wt L particles. This suggests that the presence of this protein may not be due solely to contaminating virions.

**Discussion**

The identification of a class of non-infectious virion-related particles (L particles) posed questions concerning their origin and pathway of assembly. We have shown that the mutant ts1201 produces L particles at the non-permissive temperature in quantities similar to those generated by wt virus. Under these conditions, ts1201 does not form virions and its capsids do not leave the nucleus (Preston et al., 1983). However, the ability of this mutant to produce L particles shows that their formation can take place without the involvement of capsids. Thus, for ts1201, and we propose for wt virus also, assembly of L particles can be independent of virion assembly. This implies that the signals controlling condensation of tegument and acquisition of an envelope are intrinsic to components of L particles.

The route by which capsids within the nucleus acquire tegument and envelope is not fully understood and
Fig. 2. Surface replicas of cells infected with wt virus (a, c, e and g) or ts1201 (b, c, f and h) at 38.5 °C. Replicas were prepared from cells infected for 1 h (a and b), 6 h (c and d), 10 h (e and f) and 24 h (g and h). All photographs are shown at the same final magnification. Bar marker represents 2 μm.
Fig. 3. Thin sections of cells infected with wt virus (a and c) or ts1201 (b and d) at 38.5 °C for 24 h. Particles are shown at the cell surface (a and b) and within cytoplasmic vacuoles (c and d). Bar marker represents 1 μm.
similarly the pathway of L particle formation is unclear. Original reports of envelopment at the inner nuclear membrane were based on observations of capsids in the process of budding through this membrane and of enveloped, apparently mature virions between the lamellae of the nuclear envelope (Nii et al., 1968; Schwartz & Roizman, 1969). However, the presence of non-enveloped capsids in the cytoplasm is a consistent feature of infected cells and apparent budding into cytoplasmic vacuoles is frequently observed (Nii et al., 1968; personal observation). These observations suggest a possible cytoplasmic site for envelopment, although an alternative explanation that cytoplasmic capsids represent terminally de-enveloped virions has been proposed (Campadelli-Fiume et al., 1991). Studies on the betaherpesvirus, human cytomegalovirus (HCMV), support the belief that envelopment can take place in the nucleus. In their model, capsids pass through a maturation pathway in which an envelope gained at the inner nuclear membrane is removed by fusion with the outer nuclear membrane, releasing the capsid into the tegusome. Assembly of tegument around the capsid takes place in this compartment and final envelopment occurs by budding into cytoplasmic vacuoles.

Evidence from EM is not incompatible with a similar pathway for maturation of HSV-1 virions, although a tegusome-like compartment has not been implicated. This being the case, L particles would represent the products of later stages of this pathway which have been uncoupled from the early stages. In a parallel study, we have shown that the structural integrity of tegument is not dependent on either the capsid or envelope (McLauchlan & Rixon, 1992). Thus, we envisage that tegument proteins assemble either around a capsid or as independent condensations, probably within the cyto-
plasm, with envelopment occurring upon entry into cytoplasmic vacuoles (Fig. 6). One implication of the model shown in Fig. 6 is that at least some of the tegument and envelope components need not enter the nucleus. Although we cannot exclude the possibility that initial assembly of tegument takes place in the nucleus, no evidence was obtained from either wt virus- or ts1201-infected cells of L particles between the lamellae of the nuclear envelope. The presence of L particles alongside virions in intracytoplasmic vacuoles (Fig. 3c) suggests that the final stages of transport out of the cell are common to both types of particle.

L particles offer obvious potential for a non-infectious subunit vaccine. However, the inability to produce L particles free from contaminating wt virus has limited this potential. Although virions are still produced at low levels by ts1201-infected cells, our demonstration that L particles can be produced under conditions in which infectious virions are not made suggests one route by which material suitable for vaccine purposes could be prepared.

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References


