Herpes simplex virus L particles contain spherical membrane-enclosed inclusion vesicles

J. F. Szilágyi1* and J. Berriman2

1MRC Virology Unit, Institute of Virology, University of Glasgow, Glasgow G11 5JR and 2MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, U.K.

The fine structure of light (L) particles of herpes simplex virus type 1 was examined by cryo-electron microscopy and compared to that of virions. The L particles appeared to be spherical entities with significant variation in size, on average smaller in diameter than virions (140 nm compared to 180 nm). The technique confirmed that L particles are composed of an outer envelope, i.e. a bilaminar membrane with protruding glycoprotein spikes, and a uniformly granular tegument, but lack any nucleocapsid. In addition it revealed the presence of one or occasionally more spherical objects, termed inclusion vesicles (IVs), embedded in the tegument of a large proportion of L particles but not observed in virions, suggesting that presence of IVs is unique to the L particles. The IVs vary in size and appear to be composed of a bilaminar membrane without surface projections and filled with material of relatively low electron density, suggesting that the composition of IVs is distinct from that of the envelope and tegument of the L particles.

The herpes simplex virus (HSV) virion consists of four distinct morphological components: the DNA-containing core, the icosahedral capsid that encloses this core, the envelope, a bilaminar membrane with glycoprotein projections on its surface, and the tegument, a dense granular material located between the capsid and the envelope (Wildy et al., 1960; Wildy, 1986; Roizman & Furlong, 1974; Schrag et al., 1989; Roizman & Sears, 1990). Recently, Szilágyi & Cunningham (1991) demonstrated that tissue culture cells infected with HSV type 1 (HSV-1) release into the culture medium not only virions but also similar amounts of non-infectious particles, termed L particles. These were shown by electron microscopy of negatively stained preparations to consist of an outer envelope, with surface projections, enclosing tegument-like material, but lacking the nucleocapsid of the virions. Biochemical analysis confirmed that L particles share most, if not all, membrane and tegument proteins with the virions, but lack the capsid polypeptides and the viral DNA. However, L particles also contain five phosphoproteins (175K, 134K, 92K, 60K and 55K) not observed in the virions. One of these, the 175K polypeptide, has been identified as the immediate early regulatory protein IE3 (McLauchlan & Rixon, 1992). The presence of the 175K polypeptide (IE3) in the tegument of the virion had been reported previously by Bibor-Hardy & Sakr (1989) and Yao & Courtney (1989). However, we suggest that as yet unidentified L particles contaminating their virion preparations were responsible for these observations. Morphogenesis of L particles appears to be independent of virion formation, since cells infected with the temperature-sensitive mutant ts1201 (Preston et al., 1983, 1991) produce L particles at the restrictive temperature even though assembly of the virion is inhibited (Rixon et al., 1992). The production of L particles appears to be a characteristic feature of all alphaherpesviruses so far examined (A. MacLean & S. M. Brown, personal communication; McLauchlan & Rixon, 1992).

In the present investigation we used cryo-electron microscopy to study the morphology and the fine structure of HSV-1 L particles in comparison with the virions. The advantage of this technique is that rapid vitrification prevents dehydration and allows the original shape and size of the particles to be retained. Furthermore, since the particles are examined without the use of a negative stain their internal structures remain visible.

HSV-1 strain 17 (Brown et al., 1973) was grown in BHK-21 C13 cell monolayers and virions and L particles were purified, with minor modifications, as described by Szilágyi & Cunningham (1991). To reduce the stresses that might distort the particles the purification procedure was modified by omitting the pelleting stage between the first and second gradient separations (the suspensions of particles after the first gradient centrifugation were diluted with equal volumes of medium before placing them on the second gradients) and by reducing the
Fig. 1. Low magnification cryo-electron micrographs of HSV-1 virions and L particles. (a) Suspension of purified virions (→) and three contaminating L particles (←). The observation that these L particles were significantly larger (respectively 300, 270 and 170 nm) than the average L particle (140 nm) may explain their presence in the virus band. (b) Suspension of L particles, many with clearly visible IVs (●). A single contaminating virion is indicated (→). Instrument magnification 13200 ×; scale bar represents 500 nm.

For cryo-electron microscopy the suspensions of purified virions and L particles were adsorbed onto holey carbon films (made hydrophilic with air glow-discharge) and, after the excess liquid had been blotted in a high humidity chamber, were rapidly frozen by plunging into melting ethane (Adrian et al., 1984). Micrographs were obtained at 120 kV using a Philips 420T electron microscope. The specimens were maintained at −175 °C with a Gatan 626 cold stage. The images were taken from regions across holes in the carbon where the thickness of the frozen sample was 150 to 200 nm (40 to 50% scattering beyond the 10 milliradian cut-off of a 50 μm objective aperture). The exposures were determined in order to produce an optical density of about 1.0 on Kodak SO-163 film following development in full-strength D19 for 12 min. Magnification values of 13200 × and 34000 × were used, maintaining 1% accuracy by using a constant objective lens setting and varying the goniometer height to set the focus mechanically to within 1 μm. The underfocus values used were 1.2 μm to emphasize the phase contrast of the unit membrane and DNA, and 3.6 μm to emphasize the capsid and membrane spikes.

The low magnification images were used to compare large numbers of virions and L particles (Fig. 1). In good agreement with previous observations (Wildy et al., 1960; Wildy, 1986; Roizman & Furlong, 1974; Schrag et al., 1989; Roizman & Sears, 1990) the intact virions (a) appeared as reasonably homogeneous spherical particles, consisting of the limiting envelope, the underlying tegument and the icosahedral capsid with the strongly electron-dense viral DNA. The L particles (b) also appeared to be spherical, although they exhibited less uniformity of shape and size than the virions. As was shown previously with negative staining (Szilágyi & Cunningham, 1991), the L particles consisted of the tegument and the surrounding envelope, but lacked a nucleocapsid. Furthermore, cryo-electron microscopy revealed that a large proportion of them also contained within their tegument one or more spherical membrane-enclosed vesicles, which had never previously been visualized. We designated these inclusion vesicles (IVs).

High resolution cryo-electron microscopy was used to compare the fine structure of virions and L particles (Fig. 2). Images of virions (a) show the viral envelope to consist of a bilaminar membrane with a thickness of 5 nm and a fringe of glycoprotein spikes extending approximately 15 nm from its surface. The underlying tegument appears as granular material without any discernible structure. The icosahedral capsid is only faintly visible, and the electron-dense paracrystalline DNA exhibits the characteristic fingerprint motif reported earlier (Booy et al., 1991). The envelope and the tegument of the L particles (b) are indistinguishable from those of the virions. Under the focusing condition used, the high contrast of their bilaminar membranes clearly demonstrates the presence of the spherical IVs in a large proportion of L particles, although the relatively high electron density of the tegument hindered critical study of their fine structure. However, some of the L particles had been ruptured, especially when stored at −70 °C prior to cryo-electron microscopy, releasing their IVs, which could be now studied in isolation (b and c; large arrows). Such liberated IVs consist of a limiting membrane and internal content. The membrane is bilaminar and similar in thickness to the membrane of...
Fig. 2. High magnification cryo-electron micrograph of HSV-1 virions and L particles. Images were taken at high instrument magnification (34000 x) with either 1-2 μm underfocus (a and b) to emphasize the contrast in the bilayer separation of the membranes and the paracrystalline order of the DNA within the viral capsid, or 3.4 μm underfocus (c) to highlight the presence of glycoprotein spikes emanating from the surface of the membranes. Virions (a) and L particles (b) were purified and examined by the modified method described in the text; another preparation of L particles purified by the original method (c) was stored at -70 °C and subsequently melted at room temperature prior to cryo-electron microscopy. Inclusion vesicles liberated from ruptured L particles (†) and arrays of glycoprotein spikes on the surface of L particles (♦) are indicated. On the surface of L particles prepared by the original method we often observed tufts (*) which may have been either a conglomeration of glycoprotein spikes or tegument extruded through broken envelope. Scale bar represents 200 nm.

the L particle envelope (approximately 5 nm) but, unlike the envelope, is devoid of any detectable surface projections. The internal content of IVs appears as homogeneous relatively low electron density material, very different from the granular tegument and occasionally showing organization as weak concentric rings or striations.

Size distributions of virions, L particles, viral capsids and IVs are shown in Fig. 3. Virions (a) varied in size within a relatively narrow range (between 160 and 230 nm, average 180 nm), forming a sharp peak at 175 nm. A considerably wider range of sizes (between 80 and 200 nm, average 140 nm) was observed in the case of L particles (b). A small number of L particles (about 2%) had very much larger diameters, varying between 200 and 400 nm. The viral capsids (c) fall within the narrow range of 110 and 120 nm (average 115 nm), whereas the IVs (d) varied in size over a fairly wide range (from 30 to 160 nm, average 75 nm) with a relatively sharp peak at around 75 nm. No correlation was observed between the sizes of IVs and L particles carrying them.

Frequency of IVs in L particles are shown in Table 1. The majority of L particles (on average 64%) contained identifiable IVs in their tegument. Most (59%) contained a single IV, but a small proportion (about 5%) contained multiple IVs (mainly two, but up to six could be detected on rare occasions). In the remaining 36% of the L particles we were unable to detect any IVs. We detected no IVs in the tegument of any of the 1026 separate virion images we examined.

Thus cryo-electron microscopy showed that L particles are, on average, somewhat smaller than the virions, but with a wider range of sizes, suggesting that their morphogenesis is under less stringent control than that
of the virions. Since the novel IVs could be seen in the L particles only by virtue of the contrast provided by the bilaminar nature of their membrane it is possible that all L particles contain IVs, but in some instances the higher electron density of the tegument, in which the IVs are embedded, masks their presence. No structure other than the capsid was ever detected within the virions, suggesting that the presence of IVs may be unique to the L particles. The appearance of their membrane and internal content suggests that the chemical composition of IVs is distinct from that of the rest of the L particles (i.e. envelope and tegument), and we postulate that at least some of the five phosphopolypeptides unique to the L particles (Szilágyi & Cunningham, 1991) may be components of the IVs. Direct analysis of the chemical composition of IVs will require their isolation from purified L particles (which has so far been prevented by aggregation following L particle lysis).

In conclusion, the chemical composition, structural organization and relative abundance indicate that the L particles may have a significant role to play in the infectious cycle, and thus their study may yield important information relevant to the growth and morphogenesis of the virions.

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Table 1. Frequency of IVs in HSV-1 L particles

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<thead>
<tr>
<th>L particle preparation</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without detectable IVs</td>
<td>270</td>
<td>181</td>
<td>15</td>
<td>466</td>
</tr>
<tr>
<td>With one IV</td>
<td>477</td>
<td>230</td>
<td>62</td>
<td>769</td>
</tr>
<tr>
<td>With two or more IVs</td>
<td>47</td>
<td>15</td>
<td>4</td>
<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>794</td>
<td>426</td>
<td>81</td>
<td>1301</td>
</tr>
</tbody>
</table>

* Three independently grown and purified L particle preparations (A, B and C) were examined.
† Of the 66 L particles with multiple IVs, 43 (65%) contained two, 17 (25%) contained three and six (9%) contained four to six IVs in their tegument.

References


integrity of the tegument does not depend on the presence of capsid or envelope. *Journal of General Virology* 73, 269–276.


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