Electron microscopic evidence for budding process-independent assembly of double-shelled rotavirus particles during passage through endoplasmic reticulum membranes

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Slowing down of the maturation process of human rotavirus particles on ice allowed the clear demonstration of two different assembly pathways through the endoplasmic reticulum (ER) membrane. One was the ‘enveloped’ and single-shelled (ss) particle assembly pathway, in which a transient envelope is acquired through the budding of subviral particles from the cytoplasm to the ER lumen, and later these ‘enveloped’ particles are released as ss particles in the ER lumen. The other was a double-shelled particle assembly pathway by which subviral particles acquire the outer capsid proteins during their transport across the ER membrane.

It is considered from electron microscopic evidence that rotaviruses undergo a unique mode of morphogenesis (Altenburg et al., 1980; Estes et al., 1982; Holmes, 1983; Bellamy & Both, 1990). Single-shelled (ss) particles become transiently enveloped by a membrane portion derived from the endoplasmic reticulum (ER) and thereby acquire viral proteins associated with this membrane. During the maturation process within the ER lumen, elements of the transient membrane envelope are subsequently lost and ultimately, in place of the membrane, a thin layer of protein is present as the outer capsid of mature virions. However, there are still many questions concerning morphogenesis which remain to be answered, particularly with regard to the morphogenesis of the outer capsid.

In our previous work, we described an alternative double-shelled (ds) particle assembly pathway (Suzuki et al., 1984b) in which subviral particles selectively acquired the outer capsid proteins during their transport across the ER membrane. However, this process appeared quite rare compared with the standard budding process. Kinetic studies have revealed that the VP4 and VP7 components of the outer rotavirus shell appear in ds particles with a lag time of 10 to 15 min, possibly the time required for outer capsid assembly (Kabcenell et al., 1988). In the light of this finding, we calculated that this lag time might be too short to allow morphological features of the above process to be frequently observed under the electron microscope. Therefore, in the present study, we slowed down the maturation of the virus by exposure to a low temperature of 4 °C, to facilitate demonstration of rotavirus entry mechanisms (Suzuki et al., 1986), and confirm our previous observations.

Rhesus monkey cells (MA104 cell line) were infected with KUN strain rotavirus and incubated at 37 °C for 24 h. Bars represent 100 nm. V, Viroplasm.
Fig. 2. (a to f) Ds particle assembly. As a first step, electron-dense granular substances become studded on the ER membrane (arrows). Subviral particles then appear to acquire the outer capsid proteins during their transport across the ER membrane, so that when ds particles are released from the ER membrane to the ER lumen, fine reticular material is present on the luminal side of the projecting particles. As a last step, the material-encased particles are pinched off (arrowheads). The cells illustrated were infected with KUN strain rotavirus and incubated at 37 °C for 7 h and at 4 °C for a further 2 h. Bars represent 100 nm.

with the human rotavirus KUN strain (Kutsuzawa et al., 1982), at a multiplicity of 10 p.f.u./ml, in serum-free maintenance medium with 0-5 μg/ml trypsin (Sigma, type IX). To slow down the rotavirus maturation, cells were kept in an ice bath for 2 h after inoculation and incubation for 7 or 8 h at 37 °C, and then fixed for electron microscopy (EM) as previously described (Suzuki et al., 1984a, b). As a control, infected cells were incubated for 7, 8 or 24 h after inoculation at 37 °C, and then similarly fixed for EM. After block staining with uranyl acetate during dehydration, thin sections were prepared, double-stained with 1% uranyl acetate and lead citrate, and then examined with an electron microscope (JEM 100 C). For ultrastructural immuno-
cytochemistry, cells were kept in an ice bath for 2 h after inoculation and incubated for 7 h at 37 °C in the absence of trypsin as previously described (Kitaoka et al., 1986). They were washed once in PBS, and fixed for 7 min in 0-2% glutaraldehyde as described by Petrie et al. (1984). After primary fixation, cells were made permeable to the immunological reagents by incubation for 30 min at room temperature in PBS containing 0-02% saponin and 1% BSA. Permeabilized cells were incubated for 3 h with 0-5 ml (10 μg/ml) of the anti-VP4 (K-1532) monoclonal antibody (MAb; murine IgG2b) diluted 10-fold, which reacted with the viral protein VP8 in a radio-immunoassay, and demonstrated neutralizing and haem-
agglutination inhibition activities (Kitaoka et al., 1986).
The cells were then washed for 30 min to remove unabsorbed antiserum and re-incubated overnight with gold-conjugated goat anti-mouse IgG (Zymed Laboratories). After a 30 min wash to remove excess gold conjugates, the cells were fixed for EM as described above.

Rotavirus-infected cells demonstrated several distinct morphological features as previously described (Suzuki et al., 1984b). The ss particle was 60 to 65 nm in diameter (Fig. 1), whereas the ds particle was 70 to 75 nm in diameter (Fig. 2). 'Enveloped' particles were approximately 100 nm in diameter, consisting of a core, an inner shell, a halo and an 'envelope' (Fig. 1 to 4).

By slowing down the maturation process on ice, two different assembly pathways of rotaviruses through the ER membrane were evident (Fig. 1, 2 and 3). One was the 'enveloped' particle assembly pathway in which subviral particles (possibly ss particles) acquire a transient envelope by budding from the cytoplasm into the ER lumen (Fig. 1a). The 'envelope' subsequently swelled and ruptured, and ss particles were released later during cytolysis as previously described (Fig. 1b, c). Even at the low temperature there was no indication that ds particles were formed from 'enveloped' particles. Previous studies from our laboratories, and others, have demonstrated that 'enveloped' particles accumulate in tunicamycin-treated cells and that ss particles are released into the culture medium (Petrie et al., 1983; Suzuki et al., 1984a). It is thus suggested that the 'enveloped' particles are the result of inefficient virus maturation, and the process involved serves simply as a vehicle to transport ss particles from the cytoplasm to the ER lumen.

The alternative ds particle assembly pathway, in which subviral particles (possibly ss particles) selectively acquire the outer capsid proteins during their transport across the ER membrane, was observed much more frequently in the low temperature treatment of cells (Fig. 2) than in the controls (Fig. 3). A number of steps could be clearly distinguished in infected cells.

As a first step in the ds particle assembly, electron-dense granular materials (probably virus precursor) became studded on the ER membrane as previously described (Suzuki et al., 1984b) (Fig. 1e, i). These appeared to acquire outer capsid proteins during their transport across the ER membrane: when ds particles were observed at their release from the ER membrane into the ER lumen, fine reticular material was observed on the luminal side of projecting particles (Fig. 2 and 3). A trilaminar boundary line between this material and the ER lumen connecting with the ER membrane was not observed. Therefore it is likely to be a protein layer. This material was also observed on the cytoplasmic side of projecting particles (Fig. 2a to e). It is envisaged that pinching off of the above material-encased particles constitutes the last step in this process (Fig. 2f to i), although many ds particles remain membrane-associated despite cell lysis, as reported by Musalem & Espejo (1985). Thus, it is suggested that the above process may be a novel morphological pathway for production of ds particles (Fig. 4).

The viral haemagglutinin, VP4, which is a component of the outer capsid layer was localized using an immunogold-labelled MAb to VP4 (K-1532). The outer capsid layer of ds particles within the cisternae of the ER was labelled, but ss and 'enveloped' particles were not (Fig. 4). The present results confirmed the negative staining observed in the previous study, with the MAb binding only to ds particles of KUN (Suzuki et al., 1991). Furthermore, the luminal side of membrane-associated...
ds particles on the ER membrane was labelled, whereas their cytoplasmic side was not, probably due to the nature of the MAb used (Fig. 5). Thus, it is suggested that the ds particles formed during the above process may contain mature VP4 and be mature in character.

A number of basic studies in cellular and molecular biology have shown the localization of VP4, VP7 and non-structural glycoprotein NS28 in infected cells is a major factor for ds particle assembly (Kabcenell et al., 1988; Estes & Cohen, 1989; Bellamy & Both, 1990; Poruchynsky & Atkinson, 1991). Our observations suggest that these proteins are present in the fine reticular material and play a role at the site of assembly of ds particles, particularly around the junction area between virus particles and the ER membrane. Further studies will be required to determine the nature of the material.

Studies of the maturation of a variant of SA11 (clone 28), which produces a non-glycosylated VP7, have shown that whereas glycosylation of VP7 is not necessary for producing ds particles, glycosylation of NS28 is essential for mature particle forms (Estes et al., 1982). NS28 acts as a receptor to bind ss particles prior to their transport across the ER membrane into the ER lumen and is a transmembrane glycoprotein in the ER membrane (Au et al., 1989; Bellamy & Both, 1990). Based on these findings and our electron microscopic evidence, we propose that NS28 might play an active role in the regulation of the two different assembly pathways involving the ER membrane. Furthermore, it is suggested that the ds particle assembly may have similarities to the transport of proteins across the ER membrane (Rapoport, 1992) and particularly that NS28 acts in the actual transfer of virus particles across the ER membrane. However, no information is available concerning the possible multimeric state of NS28 in the membrane. It is of interest in this context that calcium interactions with NS28 have also been proposed to play a role in virus morphogenesis (Holmes, 1983; Au et al., 1989). Further studies of the function of NS28 in the translocation sites of the ER membrane are warranted.

References


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