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Review

Rotavirus Replication: Gaps of Knowledge on Virus Entry and Morphogenesis

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In 1973, rotaviruses A (RVAs) were discovered as major causative agents of acute gastroenteritis in infants and young children worldwide. The infectious RV virion is an icosahedral particle composed of three concentric protein layers surrounding the 11 double-stranded (dsRNA) segments. An *in vitro* replication system for RVs in permanent cell lines was developed in 1982 and expanded to replication in intestinal organoids in 2015. However, the details of rotavirus (RV) entry into cells and particle maturation mechanisms at the molecular level remain incompletely understood. Slowing down human RVA replication in cell culture on ice allowed morphological visualization of virus particle entry and the assembly of triple-layered particles (virion). Although RVAs are non-enveloped viruses, after virus attachment to the cell membrane, the virus enters the cell by perforating the plasma membrane by a fusion mechanism involving VP5* of the cleaved VP4 protein, as the alternative virus entry route besides the receptor-mediated endocytosis which is generally accepted. After assembling double-layered particles (DLPs) in viroplasm or cytoplasm, they appear to be connected with the endoplasmic reticulum (ER) membrane and become coated with outer capsid proteins (VP4 and VP7) in a coating process. The perforation of the ER membrane is caused by an unknown mechanism following interaction between non-structural protein 4 (NSP4) and the inner capsid protein VP6 of the DLPs. The coating process is closely related to the formation of a hetero-oligomeric complex (NSP4, VP4 and VP7). These lines of evidence suggest the existence of novel mechanisms of RV morphogenesis.

Keywords: membrane penetration; morphogenesis; recoating; rotavirus; virus cell entry
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Introduction

Rotaviruses A (RVAs) were discovered in 1973 as major causative agents of acute gastroenteritis (AGE) in infants and young children (Bishop et al. 1973; Flewett et al. 1973). Rotaviruses (RVs) are excreted in stools and frequently associated with diarrheal disease. RV infects enterocytes and induces diarrheal illness through the destruction of absorptive enterocytes, intestinal secretion stimulated by RV non-structural protein 4 and activation of the enteric nervous system (Estes and Greenberg 2013). The management of RV infection focuses on the prevention and treatment of dehydration. RV vaccine is recommended for routine use in all countries globally. Despite the global introduction of vaccinations for RV over a decade ago, RV infections still result in > 200,000 deaths annually, most in low-income countries (Tate et al. 2016).

RVs are nonenveloped viruses, members of the family *Reoviridae*. RV has a genome consisting of 11 segments of double-stranded (ds)RNA surrounded by a triple-layered protein capsid. The RNA segments encode six structural viral proteins (VP1 to VP4, VP6 and VP7) and six non-structural proteins (NSP1 to NSP6) (Table 1). RVs are classified serologically by a scheme that allows for the presence of multiple groups (based on VP6) and of multiple serotypes within each group (based on VP4 and VP7 neutralizing epitopes).

The successful establishment of a cell culture model for human rotavirus (Sato et al. 1981; Kutsuzawa et al. 1982) and the achievement of RVA replication in intestinal organoids (Saxena et al. 2016) vastly improved our understanding of rotavirus replication. However, virus cell entry and virion morphogenesis are complex processes that are not yet fully understood. In the case of RVs, findings have suggested two methods for virus entry and virion assembly. Triple-layered particles (TLPs) can enter the cell by either endocytosis or direct cell membrane penetration (Suzuki et al. 1984b, 1985, 1986; Kaljot et al. 1988; Estes and Greenberg 2013; Desselberger 2014), whereas virion assembly occurs by either a budding process (Estes and

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Greenberg 2013; Desselberger 2014) or membrane perforation (Suzuki et al. 1984b, 1993; Suzuki 1996). We previously reported a series of morphological investigations of RV using transmission electron microscopy (TEM) (Suzuki and Konno 1975; Suzuki et al. 1981, 1984a, b, 1985, 1986, 1993; Suzuki 1996) and virological investigations (Kitaoka et al. 1984, 1986a, b). Our knowledge of RV cell entry and virion assembly as explored by these methods has been complemented by other similar studies (Altenburg et al. 1980; Petrie et al. 1982, 1983, 1984; Poruchynsky et al. 1991) and been expanded by numerous more recent investigations (Kaljot et al. 1988; Fukudome et al. 1989; Jourdan et al. 1997; Lawton et al. 1997; Dormitzer et al. 2004; Jayaram et al. 2004; Silvestri et al. 2004; Lopez et al. 2005; Patton et al. 2006; Trask and Dormitzer 2006; Lu et al. 2008; Maruri-Avidal et al. 2008; Chen et al. 2009; Baker and Prasad 2010; Kim et al. 2010; Aoki et al. 2011; Trask et al. 2012; Abdelhakim et al. 2014; Rodriguez et al. 2014; Arias et al. 2015; Salgado et al. 2017). This paper discusses gaps in current knowledge of RV morphogenesis, presenting findings obtained by TEM and relevant molecular biological studies.

Rotavirus Structure and Classification

RV particles have a distinctive morphological appearance and three types of particle have been observed by TEM using negative staining (Fig. 1) (Altenburg et al. 1980; Suzuki et al. 1981, 1984a, b, 1985; Kabcenell et al. 1988; López et al. 2005). The RV virions are non-enveloped and TLPs of 100 nm in diameter, including the VP4 that spans the VP6 and VP7 layers, and extends out from the particle (Figs. 1a and 2). Double-layered particles (DLPs) lack the outer layer (VP7 and VP4) and have VP6 of the middle layer exposed on their surface (Figs. 1a and 2). Single-layered particles, also termed cores, lack the middle layer, resulting in the exposure of VP2. As a unique morphogenic pathway of RV, transient enveloped particles (TEPs) are formed by the budding of DLPs into the endoplasmic reticulum (ER) lumen (Estes and Greenberg 2013), and can be observed by TEM using a thin section method (Figs. 1b and 2).

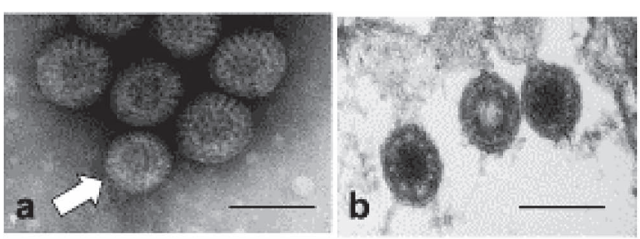

Fig. 1. Rotavirus structures.
Panel a: Four TLPs and one DLP (white arrow) in a negative contrast preparation. Panel b: Three TEPs in the cisternae of the rough endoplasmic reticulum in thin section. TEPs revealed radial bars between the envelope and DLPs, like the spokes of a wheel. Bars represent 100 nm.

The infectious RV virion is an icosahedral particle composed of three concentric protein layers surrounding the 11 dsRNA segments (Table 1) (Settembre et al. 2011). Each segment encodes one protein, with the exception of segment 11, which encodes two NSPs, NSP5 and NSP6, in the case of RVA (Estes and Greenberg 2013). Depending on the particular RV species, certain RNA segments encode two or more viral proteins. In addition, the core contains the RNA-dependent RNA polymerase, VP1 and the capping enzyme, VP3. For several RV strains, complete gene-protein assignment has been achieved (Estes and Greenberg 2013).

According to the serological reactivity and genetic variability of the middle layer protein VP6, at least 10 different groups, also termed species, have been differentiated (termed RVA-RVJ) (Matthijnssens et al. 2012; Mihálov-Kovács et al. 2015; Bányai et al. 2017). The outer capsid proteins, VP4 and VP7, are used in a dual classification system (Table 1), where VP4 determines the P type and VP7 determines the G type. Although 32 G types and 47 P types are currently known (Rotavirus Classification Working Group 2017), only a few G and P genotype combinations (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8]) substantially contribute to the burden of disease in humans (Matthijnssens et al. 2009). An extension of the dual classification system into genotyping of all 11 segments was described in 2008 (Matthijnssens et al. 2008) and is increasingly used for studying the molecular epidemiology, evolution of RVs, and the impact of rotavirus vaccination. In humans, the existence of two major genotype constellations, referred to as Wa-like and DS-1-like strains, has been reported (Matthijnssens et al. 2009).

Rotavirus Replication

Attachment

RV attachment is a complex process (López and Arias 2004; López et al. 2005); however, progress is being made owing to new molecular and structural information on the outer capsid proteins and an understanding of differences in virus strains (Estes and Greenberg 2013). The TLP interacts *via* its VP4 spikes with cellular receptors (attachment receptors), which contain sialic acid (SA) at terminal or sub-terminal positions. The initial cell attachment step is followed by the interactions of viral proteins [VP8* (produced by cleavage of VP4 into VP5* and VP8*) and VP7] with multiple co-receptors, including integrins ($\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha v\beta$, and $\alpha x\beta 2$) and hsc70, which have been found to be associated with cell membrane lipid microdomains (Fukudome et al. 1989; López and Arias 2004; Baker and Prasad 2010; Estes and Greenberg 2013; Desselberger 2014). RV attachment to host cells is also mediated by binding partners on the host cell surface, including sialoglycans, such as gangliosides GM1 and GM1a and host-blood group antigens (HBGAs) (Arias et al. 2015). RV-HBGA interactions are dependent on the RV P genotype, and studying these interactions has provided new insight into

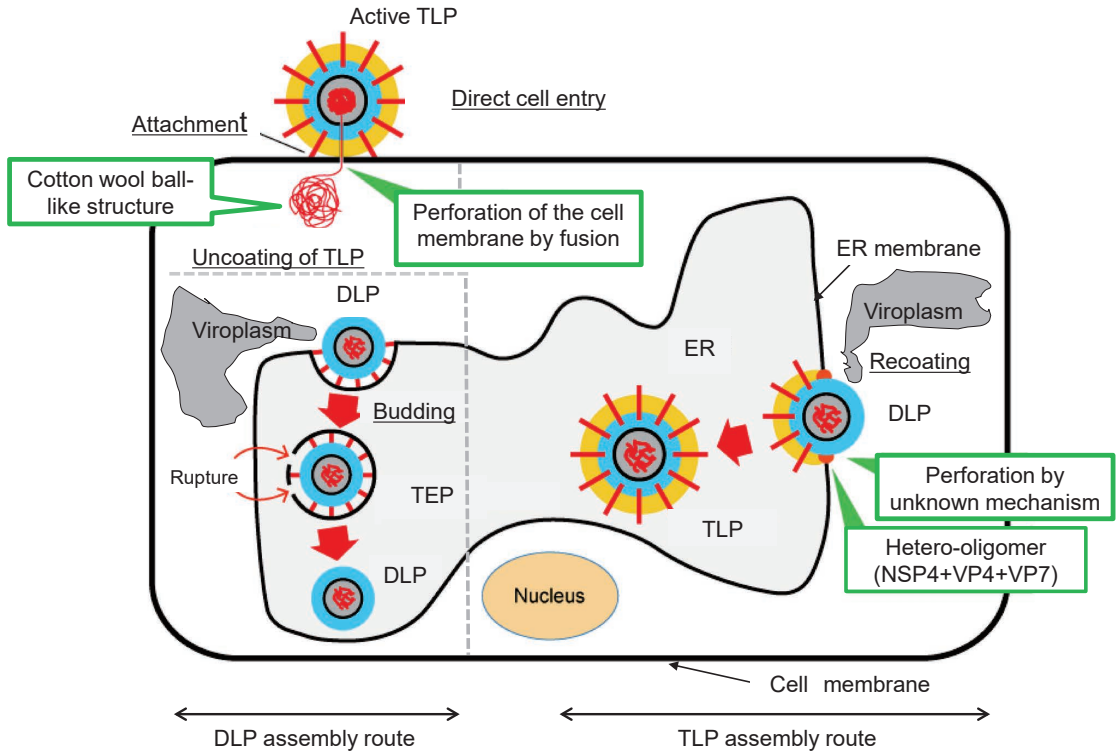

Fig. 2. The proposed schematic of the rotavirus replication cycle.
The triple-layered particle (TLP) is schematically shown at top. The outer layers of TLP consist of VP4 (magenta bars) and VP7 (yellow). Inner layer of TLP and the outer layer of double-layered particle (DLP) consist of VP6 (blue), VP2 (beneath VP6, in black), VP1 and VP3 (greenish brown), and double-stranded RNAs (red). The schematic also delineates virus entry (top left half), DLP assembly (bottom left half), and TLP assembly (right half), marked with dotted lines. For details, see each section on the replication cycle (*direct cell membrane penetration*, *TEP formation*, and *recoating of outer proteins on DLP*).
Top left half indicates direct cell entry and uncoating of transcriptionally active TLP by perforation of the cell membrane. In the TLP, red fine line from inner capsid of TLP (probably (+)RNAs?) results in cotton wool ball-like structure in cytoplasm.
Bottom left half indicates the DLP assembly route through the formation of transient enveloped particle (TEP) that consists of DLP, VP4 (short magenta bars) and envelope (black). VP4 of TEP weakly attaches to VP6 via NSP4, and the attached VP4 is repressed by envelope (represented as shortened bars). Thus, the DLP assembly is achieved *via* viroplasm-TEP-DLP system. In viroplasm, replication and packaging of the viral genome into the viral capsids take place. DLPs assemble at the periphery of viroplasm and bud across the endoplasmic reticulum (ER) membrane with NSP4 acting as an intracellular receptor interacting with VP6, forming TEP. TEPs swell and rupture, resulting in DLPs in the ER lumen, and DLPs are released from a cell through cell lysis. The budding process serves to create a vehicle to transport DLPs from the cytoplasm to the ER lumen, but does not participate in the TLP assembly.
Right half indicates the TLP assembly route *via* recoating of outer proteins on DLP in collaboration with hetero-oligomer (orange half balls) at the perforation area of the ER membrane. DLPs assemble at the periphery of viroplasm and attach to the ER membrane. The formed TLP is released through cell lysis.

host range restrictions and interspecies transmission of different RV strains (Saxena et al. 2015).

Virus Cell Entry

For non-enveloped viruses, the mechanism appears to rely on viral capsid proteins disrupting or forming pores in cellular plasma membranes (Sieczkarski and Whittaker 2005). An important issue is how the viral genome is detached from the core. RV contains 11 segments of dsRNA as its genome and RNA polymerase to synthesize its own RNAs endogenously (Table 1) (Estes and Greenberg 2013). After RV attachment, TLPs can enter the cell by either endocytosis (Lawton et al. 1997; Chen et al. 2009; Trask et al. 2012; Estes and Greenberg 2013; Periz et al. 2013; Abdelhakim et al. 2014; Arias et al. 2015; Salgado et al. 2017) or direct cell membrane penetration (Kaljot et al. 1988; Estes and Greenberg 2013; Desselberger 2014), as also evidenced by TEM studies (Suzuki et al. 1984b, 1985, 1986).

Receptor-mediated endocytosis

Following RV attachment to cellular receptors, it is generally accepted that the TLPs are internalized into the cytoplasm by receptor-mediated endocytosis (Estes and Greenberg 2013; Abdelhakim et al. 2014; Desselberger 2014). RVs are internalized into cells by clathrin-dependent

Table 1. Rotavirus proteins.

Genome segment	Encoded protein	Location in virus particles	Functions
1	VP1	Core	ssRNA binding, RNA dependent RNA polymerase, complex with VP3
2	VP2	Core	RNA binding, required for replicase activity of VP1
3	VP3	Core	ssRNA binding complex with VP1, guanylyltransferase, methyltransferase
4	VP4	Outer capsid	P-type neutralization angigen, attachment protein, protease-enhanced infectivity, virulence, fusion with cell membrane <Mature protein modified; cleaved VP5*+VP8* (cleaved by trypsin or cellular protease) >
5	NSP1	Nonstructural inner capsid	RNA binding, interferon antagonist, E3 ligase
6	VP6	Inner capsid	Subgroup antigen, species deteminant, protection (intracellular neutralization), required for transcription
7	NSP3	Nonstructural	Inhibits host protein translation
8	NSP2	Nonstructural	Essential for viroplasm formation
9	VP7	Outer capsid	G-type neutarlization antigen, glycoprotein calsium-dependent trimer <Mature protein modified; cleaved signal peptide glycosylation>
10	NSP4	Nonstructural	RER transmembrane glycoprotein, viroporin, intracellular receptor for DLPs, role in morphogenesis of TLPs, interacts with viroplasms and autophagy pathway, modulates intracellular calsium and RNA replicatioon, enterotoxin (secreted), virulence
11	NSP5	Nonstructural	Essential for viroplasms formation, RNA binding, protein kinase, interaction with VP2 and NSP6
	NSP6	Nonstructural	Interaction with NSP5, localization in viroplasms

ssRNA, single stranded RNA; mRNA, messenger RNA; RER, rough endoplasmic reticulum; TLPs, triple-layered particles; DLPs, double-layered particles.

or -independent endocytic pathways, depending on the virus strain (Arias et al. 2015). Following entry, RVs travel to different endosomal compartments, such as early endosomes, maturing endosomes and late endosomes, and lose their outer layer before existing in the cytosolic space (Arias et al. 2015). Promptly (typically within 10 min), the outer-layer proteins are lost and transcriptionally active DLPs enter the cytosol (Salgado et al. 2017).

Uncoating of TLPs via endocytosis

It has been proposed that, at the exit of TLP from the endosomal compartment, the RV spike protein VP4 undergoes an initial conformational change, triggered by unknown factors (Arias et al. 2015). The decrease of calcium in endosomes promotes the release of VP7, and VP4 is believed to undergo a rearrangement of VP5* to a fold-back conformation, which leads to the interaction of a hydrophobic domain of this VP4 subunit with the endosomal membrane to disrupt it (Abdelhakim et al. 2014; Salgado et al. 2018).

In DLPs released into the cytoplasm, the polymerase complex (VP1 and VP3) is activated to transcribe capped positive-sense RNA [(+)RNAs] from each of the 11 double-stranded RNA (dsRNA) genome segments (Table 1) (Lawton et al. 1997; Chen et al. 2009; Trask et al. 2012; Periz et al. 2013; Salgado et al. 2017). In transcriptionally active DLPs, the class I channels at the five-fold-vertices increase in diameter, permitting the release of ss(+)RNA transcripts into the cytoplasm (Trask et al. 2012). Monoclonal antibodies binding to epitopes near the class I channels narrow their lumen and block transcription (Kohli et al. 1994; Aiyegbo et al. 2013). It is still unclear how the 5′ ends of the (+)RNAs are capped by VP3 before exit through the class I channels (Lu et al. 2008). This is the widely accepted view that the uncoating of TLPs occurs after endocytosis and results in DLPs in the cytoplasm. A recent report indicated that after endocytosis the uptake of TLP leads promptly to loss of the outer-layer proteins and escape of the transcriptionally active DLP into the cytoplasm (Salgado et al. 2017).

Direct cell entry through membrane penetration

RVs usually grow in vitro in the presence of trypsin, which cleaves the viral VP4 into its subunits VP5* and VP8* to gain maximal infectivity (Table 1) (Estes et al. 1981; Kitaoka et al. 1986b; Estes and Greenberg 2013). To obtain a non-infectious RV with uncleaved VP4, monolayer infected with infectious RV is incubated at 4°C, and 60 min later is washed with cold MEM. Thereafter, the cultures are kept in trypsin-free MEM for 24 h at 37°C. Non-infectious RVs with uncleaved VP4 are capable of being adsorbed on cells at 4°C (Kitaoka et al. 1986b). VP4 spikes of RV grown in the absence of trypsin are indistinguishable from those of particles grown in the presence of trypsin

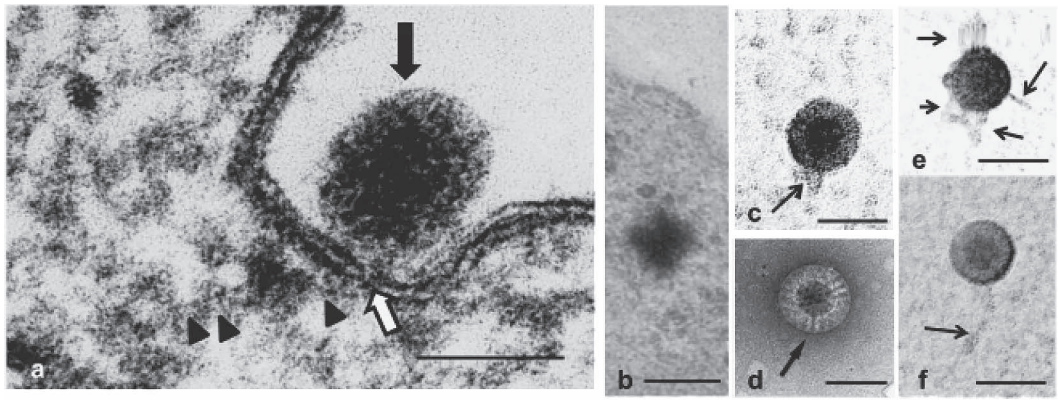

Fig. 3. Overview of the process of rotavirus cell entry.
For details, see the section on the direct cell membrane penetration. The black arrow indicates the changes of the total and internal shapes of TLP from round to oval (a). The white arrow indicates the point of perforation of the ER membrane, and the arrowheads indicate the long electron-dense thin line ejected from the core of TLP into the cytoplasm through the space of radial capsomere and the pore of the cell membrane (a). The series of processes in rotavirus cell entry result in cotton-wool-like structures in the cytoplasm (b). The panels (c, e, f) are TLPs visualized by a combination of staining and shadowing: mild disruption of TLP by EDTA (c, f) and by heat shock (e). Projections (arrows) are observed on the periphery of TLPs (c, e, f), suggesting the release of nucleic acids from active TLP. Some components of the nucleocapsid inside the projection of inner capsid form a funnel-shaped structure (arrow), which is observed clearly in trypsin-treated culture fluid (d). These figures are modified from a previous paper (Suzuki et al. 1986). Bars represent 100 nm.

(Rodriguez et al. 2014), suggesting that proteolytic cleavage of VP4 mainly achieves conformational changes enabling viral entry into cells.

To determine the effect of trypsin treatment of non-infectious human RVs on hemagglutinin (HA) activities and infectivity, RV suspensions are treated with various concentrations of trypsin for several minutes at 37°C. Trypsin treatment decreases the HA titer of non-infectious human RV and conversely increases their infectivity (Kitaoka et al. 1984, 1986b). Most strains isolated from humans and many animal rotaviruses do not have HA activities, bind to and infect NA-treated cells (Ciarlet and Estes 1999).

Kinetic studies have revealed that RVs enter cells rapidly (Estes and Greenberg 2013). To visualize the cell entry step by TEM, we slowed down the virus entry process at a low temperature (4°C) and successfully showed direct cell membrane penetration by infectious human RV (Figs. 2 and 3) (Suzuki et al. 1985, 1986). The TEM observations of virus cell entry indicated that, at the attachment site of a TLP to the cell membrane, an electron-dense thin line is expelled from the virus core into the cytoplasm through radial space between the capsomere and the cell membrane pore forming its attachment site, suggesting perforation of the cell membrane (Figs. 2, 3a and b) (Suzuki et al. 1985, 1986). This is the first such observation among animal viruses. These results are supported by a paper describing that infectious RVs increase cell membrane permeability, as measured by ^{51}Cr, [^{14}C]choline and [^{3}H]inositol release from prelabelled cells (Kaljot et al. 1988). In contrast, when human RV grown in a trypsin-free medium is used to infect cells, its mode of penetration into the cytoplasm is mediated by endocytosis (Fig. 4) (Suzuki et al. 1985).

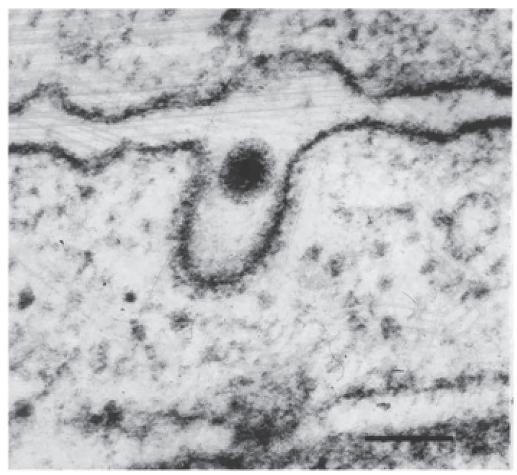

Fig. 4. The endocytosis of non-infectious rotavirus.
This figure indicates endocytosis. The cell is inoculated with non-infectious rotavirus grown in trypsin-free medium and cultivated in trypsin-free medium at 37°C for 24 h. This figure is modified from a previous paper (Suzuki et al. 1985). Bar represents 100 nm.

Nevertheless, we could not detect any evidence of viral replication.

These early results were supported by subsequent molecular studies. It is conceivable that trypsin plays an important role in dissolution of the outer layer of TLP at the cell membrane to facilitate the entry of viral nucleoids into the cell. VP4 contains discrete functional domains: *in vitro* treatment of virions with trypsin results in specific cleavage of VP4, and yields subunits consisting of polypeptides VP8* and VP5* with concomitant enhancement of viral

infectivity (Table 1) (Estes et al. 1981; Gilbert and Greenberg 1998; Kim et al. 2010; Rodriguez et al. 2014). The likely membrane-binding surfaces are the set of three hydrophobic loops at the apex of each VP5* β barrel, which is distal to the foot in the spike conformation (Dormitzer et al. 2004). Their conformational similarity to the fusion loops of class II and class III viral fusion proteins has been described (Dormitzer et al. 2004), and one of them has an amino-acid sequence related to the sequence of the E1 fusion loop of the Semliki Forest virus (Mackow et al. 1988; Levy-Mintz and Kielian 1991). Like the fusion loops and peptides of enveloped virus entry proteins, these hydrophobic surfaces are hidden on the mature virion and exposed only during a conformational change triggered by events that accompany entry (Settembre et al. 2011; Trask et al. 2012; Abdelhakim et al. 2014).

Uncoating of TLPs via perforation of the cell membrane

Upon higher-magnification TEM observation using a thin section method, the shapes of TLP and its core change from round to oval (Fig. 3a), suggesting TLP activation (Suzuki et al. 1985, 1986). An electron-dense thin line, probably consisting of nucleic acids of TLPs, is extruded from TLPs into the cytoplasm through perforation of the cell membrane, and finally results in a cotton wool ball-like structure in the cytoplasm, which is visible at a depth of less than 200 nm from the adhering cell surface and soon disappears (Figs. 2, 3a, and b), in the same way as in poliovirus cell entry (Brandenburg et al. 2007). Transcriptase activity is masked in TLPs, but heat shock and EDTA treatments release it (Cohen 1977). Our early studies under a TEM using a combination of staining and shadowing methods (Bartlett et al. 1974; Yazaki and Miura 1980) indicated that EDTA and heat shock treatments cause mild damage to virus particles and viral RNA of TLPs is extruded (Fig. 3c, e, and f) (Suzuki et al. 1986). After discharge of the nucleoprotein under EDTA treatment, empty TLPs with funnel-shaped structures which allow the passage of nucleic acids are shown (Fig. 3c), and the same structures are found in culture fluids (Fig. 3d). Upon higher-magnification TEM observation using negative staining, some parts of the nucleocapsid aligned within the funnel-shaped structure are apparently derived from the inner capsid, which consist of a space of radial capsomere sheathed inside by components of the nucleocapsid (Fig. 3d), probably the class I channel. It appears that the nucleocapsid plays an important role in the process of viral RNA ejection, probably *via* the activation of RNA polymerase. However, these early studies had a limitation of not confirming the specific character of the above materials expelled from TLPs. Viral mRNA is used for translation or as a template for RNA synthesis during genome replication. The RNA is then packaged into new DLPs within viroplasm (Fig. 2).

The loss of outer capsid VP7 activates the internal polymerase complex to transcribe capped positive-sense RNA [(+) RNA] from each of the 11 dsRNA genome segments for release into the cytosol (Lawton et al. 1997; Chen et al. 2009; Trask et al. 2012; Salgado et al. 2017). The capped transcripts are released through aqueous channels at the five-fold axes of these intact particles (Hu et al. 2012). Once these initial transcripts have been translated, RV NSPs then coordinate various stages of genome replication and viral assembly by adapting and modifying the cellular machinery during this movement of genome dsRNA. VP7 completely covers the tip of VP6, and inhibits transcription by preventing the conformational change of VP6 (Thouvenin et al. 2001). This is supported by the findings of transcriptional inhibition by VP6-specific antibodies (Kohli et al. 1994; Aiyegbo et al. 2013). These molecular studies and TEM observations provide grounds for the suggestion that even partial disruption of VP7 protein at the fusion point results in transcriptionally active TLP. However, these key lines of evidence require further detailed investigation.

These results led us to conclude that RV uncoating proceeds via two steps. At the first step, a change of the outer layer VP4 of TLP occurs by proteolytic digestion by trypsin. The endogenous transcriptase in such particles is in a switched-off state. At the second step, TLP results in fusion of the cell membrane, probably with partial disruption of the VP7 outer layer at the fusion point, followed by conversion into particles with activated transcriptase. It is assumed that this two-step process through the perforation of the cell membrane induces RV uncoating.

Triple-Layered Particle (TLP) Assembly

The review of rotaviruses indicated important points: (a) precise mechanisms of how the envelope on particles was removed, (b) the hetero-oligomeric complexes function in particle budding through the ER, and (c) how the outer capsid was assembled onto the newly made DLPs (Estes and Greenberg 2013). There are two TLP assembly processes in the relationship between DLP and ER membrane, namely, a budding process (Estes and Greenberg 2013; Desselberger 2014) and a perforation process (Suzuki et al. 1993; Suzuki 1996). Notably, NSP4 plays an important role in TLP assembly, especially in terms of selecting one of the above two processes.

DLP assembly

Replication and packaging of the viral genome into the viral capsids take place in specialized, cytoplasmic compartments, called viroplasm, which form at an early stage of infection. These are large, non-membrane-bound, electron-dense structures rich in viral RNA and viral proteins (VP1, VP2 and VP3) and VP6 and non-structural proteins (NSP2, NSP5 and NSP6) (Table 1) (Petrie et al. 1982, 1984; Fabbretti et al. 1999; Silvestri et al. 2004; Patton et al. 2006; Hu et al. 2012; Trask et al. 2012; Estes and Greenberg 2013; Desselberger 2014; Viskovska et al. 2014). NSP2 and NSP5 are critically important for the nucleation of viroplasm and virus replication (Table 1)

(Silvestri et al. 2004; Vascotto et al. 2004; Campagna et al. 2005). The ss+RNAs released from DLPs either remain in the cytoplasm, where they serve as templates for the translation of RV proteins, or are trapped in viroplasm, where they will be used for assortment, packaging and replication to become genomic dsRNA (McDonald and Patton 2011; Trask et al. 2012). The function of any given (+)RNA during infection is thought to be determined by its intracellular localization (Silvestri et al. 2004). NSP2 and NSP5 are assumed to co-localize around transcribing DLP. NSP5 has been shown to self-associate and to interact with RNA and NSP2 (Table 1) (Martin et al. 2011). The numerous interactions of NSP2 and NSP5 suggest that viroplasm forms as large, semiregular networks designed to sequester viral RNAs and capsid proteins for assembly into nascent virions (Trask et al. 2012). Consistent with this model, the RNA-dense bodies are the sites of early virion assembly and further (secondary) (+)RNA transcription by nascent progeny DLPs. Viroplasm-associated RV (+)RNAs are selectively packaged into assembling VP2 cores and replicated by VP1 into the dsRNA genome (Silvestri et al. 2004).

TEP formation

It is generally accepted that DLPs assembled at the periphery of viroplasm bud across the ER membrane with NSP4 acting as an intracellular receptor interacting with VP6 (Au et al. 1989; O'Brian et al. 2000; Xu et al. 2000; López et al. 2005), although no specific mechanism for the exit of DLPs from viroplasm is currently known (Trask et al. 2012). NSP4 has VP6 and VP4 binding domains, and topographically VP4 is localized on the cytoplasmic side of the ER membrane (Au et al. 1989; Maass and Atkinson 1990; Hu et al. 2012; Estes and Greenberg 2013; Viskovska et al. 2014). During the budding process, VP4 (that interacts with the cytosolic domain of NSP4), NSP4 and VP7 are incorporated into TEPs (López et al. 2005). Once TEPs are inside the ER, the high-calcium environment of the lumen triggers the lateral interaction of VP7 molecules (located on the outside of the lipid envelope) promoting the surface protein layer to tighten and to interact with the VP6 layer, excluding the lipids and NSP4 during this process (López et al. 2005). There is some evidence that disturbance of the lipid homeostasis of RV-infected cells decreases TLP formation more profoundly than DLP formation (Cheung et al. 2016).

The observations from TEM suggest that DLPs assemble at the periphery of viroplasm and bud across the ER membrane (Fig. 5a), as described in other report (Estes and Greenberg 2013). Furthermore, interestingly, DLPs, which appear not to be associated with viroplasm, also show budding (Fig. 5d) (Suzuki et al. 1993; Suzuki 1996) (Table 1). This finding contradicts the viroplasm-associated DLP assembly theory as the current model for the assembly of TLP. We generally use the human RV for TEM study, in which viroplasm is relatively rare. The critical concentration of NSP2 outside of viroplasm is far too low for DLPs assembly (Borodavka et al. 2017, 2018). It has been shown that primary human RV clinical isolates grow very well in secondary monkey kidney cells, far better than in MA104 African green monkey kidney cells (Ward et al. 1984). Furthermore, the simian rotavirus SA11 can be grown at a high titer (Estes et al. 1979) and has a high frequency of viroplasm in infected cells (Altenburg et al. 1980; Petrie et al. 1982, 1984). Therefore, SA11 is frequently used for the molecular study of viroplasms in relation to DLP assembly (Petrie et al. 1982, 1984; Fabbretti et al. 1999; Silvestri et al. 2004; McDonald and Patton 2011; Patton et al. 2006; Hu et al. 2012; Trask et al. 2012; Estes and Greenberg 2013; Desselberger 2014; Viskovska et al. 2014). These results suggest that the number of viroplasm is related to the growth of RV.

TEM study revealed the important finding that TEPs

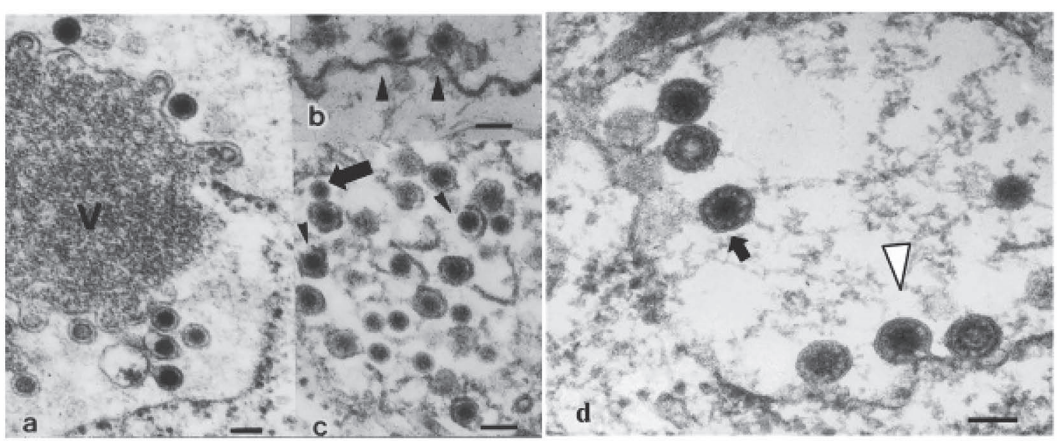

Fig. 5. Budding process.
The panel (a) indicates the budding process at the periphery of viroplasm (V) and TEP formation. The panels (b, c) show the disruption of TEPs (arrow heads) and DLP formation (arrow). The panel (d) suggests that TEP assembly *via* budding process (white arrow) is not associated with viroplasm, and the TEP interior exhibits radial bars between the envelope and core (DLP) particle (arrow). These figures are modified from our previous paper (Suzuki et al. 1984b, 1993). Bars represent 100 nm.

swell and rupture, resulting in DLPs in the ER lumen (Figs. 2, 5b and c) (Suzuki et al. 1984a, 1993; Suzuki 1996). Higher-magnification analysis of TEPs revealed radial bars between the envelope and DLPs, like the spokes of a wheel (Fig. 5d) (Suzuki et al. 1993). It is assumed that the spoke structures correspond to VP4 because TEPs contain VP4 (López, et al. 2005). VP4 weakly attaches to VP6, and only the additional assembly of VP7 to the particles lock VP4 proteins in place (Trask and Dormitzer 2006; Aoki et al. 2009). Disruption of TEPs is related to viroporin-mediated calcium-activated autophagy (Crawford et al. 2012; Crawford and Estes 2013). By using an immunogold-labelled mAb to VP4 (K-1532) (Kitaoka et al. 1986b), the outer capsid layer of TLP is labelled, but neither DLP nor TEP is (Fig. 6b and c) (Suzuki 1996). Serial TEM figures indicated TEP assembly and its disruption to associated with the reappearance of DLP (Fig. 5b and c). These observations led us to conclude that the budding process serves to create a vehicle to transport DLPs from the cytoplasm to the ER lumen, but does not participate in TLP assembly (Fig. 2).

Since EM studies with viruses are performed with a large number of particles and since virus preparations usually contain a vast excess of noninfectious particles, it is not possible to determine by this method alone whether individual events are part of a pathway leading to productive infection in the case of rotaviruses. It is known that the ratio of physical to infectious viral particles may vary between 100 and 10,000 (Méndez et al. 1999). The uncoating efficiency is 20 to 50%; of the uncoated particles, about 10 to 15% synthesized detectable RNA (Salgado et al. 2017). These results suggest that the high rates of detection of viroplasm and TEPs in infected cells have misled researchers to believe that they play key roles in TLP assembly.

Several factors mediate the increase of DLPs. RV-infected cells treated with tunicamycin (TM, N-linked glycosylation inhibitor) are known to accumulate TEPs in the ER lumen and DLPs in the culture medium (Petrie et al. 1983; Suzuki et al. 1984a; Trask et al. 2012). Although TM inhibits the glycosylation of both VP7 and NSP4, studies of the maturation of a variant of SA11 (clone 28), which produces a non-glycosylated VP7, have shown that the glycosylation of NSP4, but not necessarily VP7, is essential for removal of the envelope (Estes et al. 1982; Kabcenell and Atkinson 1985; Estes and Cohen 1989). Viruses produced in calcium free medium, and in the presence of the calcium ionophore A23187, have been found to be exclusively TEPs and DLPs (Poruchynsky et al. 1991). The structural plasticity of NSP4 is regulated by pH and Ca^{2+} (Sastri et al. 2014). Based on these facts, it is conceivable that both the glycosylation of NSP4 and the presence of calcium are indispensable for virion assembly.

Recoating of outer proteins on DLP

Arguably, one of the least understood aspects of RV biology is the process by which the assembling particle penetrates the ER membrane to acquire its outer capsid (Trask et al. 2012). We successfully and comprehensively characterized the process of recoating of DLPs during their transport across the ER membrane (Fig. 6a) (Suzuki et al. 1993; Suzuki 1996). At the junction of DLPs and the ER membrane, the lower half of DLPs in the cytoplasm appears to present a smooth surface, which is an original DLP characteristic, whereas the upper half of DLP is an irregular surface with high-density materials in the ER lumen, as in the recoating of VP4 and VP7 (Fig. 6a). The outer capsid layer of TLP is labelled by an immunogold-labelled mAb to VP4 (K-1532) (Fig. 6b and c) (Kitaoka et al. 1986b; Suzuki 1996). These results provide evidence that the transport of DLP from the cytoplasm to the ER lumen through the ER membrane results in TLP assembly (Fig. 2). However, the perforation of the ER membrane is caused by an unknown mechanism following interaction between NSP4 and the inner capsid protein VP6 of DLP.

A number of basic studies in cellular and molecular biology have indicated that the formation of a hetero-oligomeric complex (NSP4, VP4 and VP7) is an important factor for TLP assembly (Kabcenell et al. 1988; Maass and Atkinson 1990; Poruchynsky et al. 1991). Thus, the TEM observations support the view that, during the recoating process, the VP4 and VP7 outer surface proteins fold onto the DLPs as a hetero-oligomeric complex and result in TLPs, although the correct order of attachment (first VP4 and then VP7) to DLPs is essential for optimal TLP assembly (Trask and Dormitzer 2006). This is the first direct evidence of outer capsid recoating and TLP assembly as a novel recoating process.

The novel mechanism involved in the recoating of outer shell proteins on DLPs remains an important issue in terms of how both DLP and the ER membrane participate in the perforation of the ER membrane. However, no study has specifically focused on this, so further studies are required. In conjunction with these two processes, namely, the budding and perforation processes, TEM observations suggested that the single attachment of VP4 to the ER membrane through NSP4 leads to the budding process, whereas the attachment of a hetero-oligomeric complex to the ER membrane leads to the perforation process (Fig. 2). These results give rise to the view that NSP4 is critical for the selection of the above two processes, and is regulated by NSP4, probably as an ER chaperone. Among the ER chaperones, grp78 (also known as BiP), protein disulphide isomerase, calnexin and calreticulin are important for the formation of infectious virus, but grp94 (also known as endoplasmin) and ERp57 are not (Maruri-Avidal et al. 2008). Against this background, there is a need for further study to clarify NSP4's role as the key ER chaperone for virion assembly.

Final step of intact virion assembly

In the ER of SA11-infected cells, there are two pools

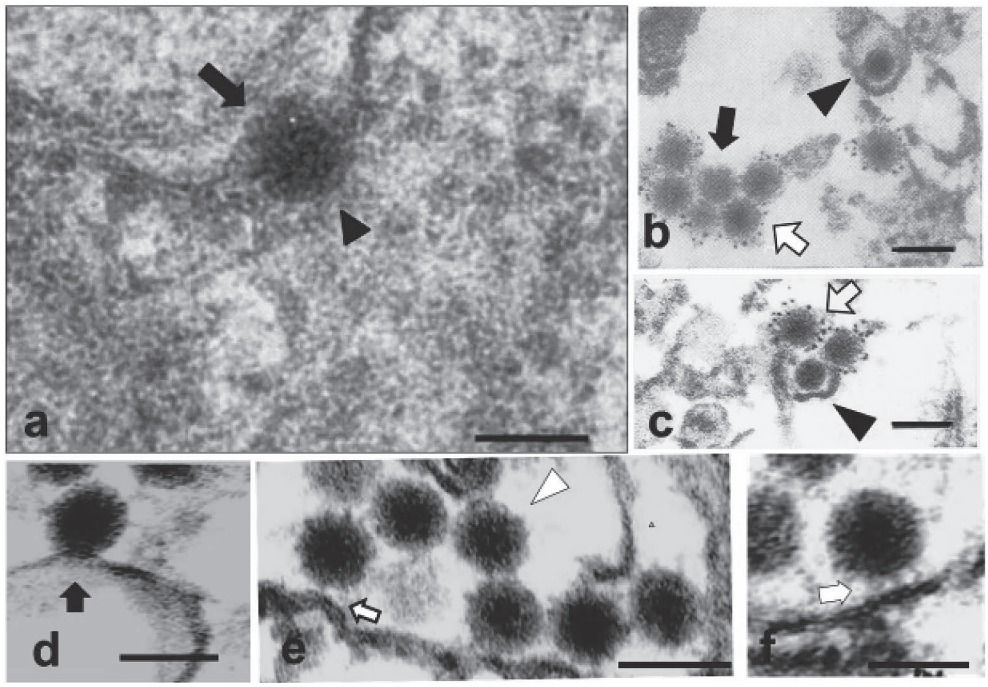

Fig. 6. Overview of virion assembly.
For details, see the section on the recoating of outer proteins on DLP and final step of intact virion assembly. The panel a indicates that DLPs appear to acquire outer capsid proteins during their transport across the ER membrane as a recoating process. The arrowhead indicates the DLP without an outer surface in cytoplasm, whereas the arrow indicates the DLP recoated with outer proteins in the ER lumen (a). For details, see the section on the process of recoating of outer proteins. By using immunogold labelling of KUN-infected cells with mAb to VP4, the outer capsid layers of TLPs are labelled (white arrows) but neither DLP (arrow) no TEPs (arrow heads) are (b, c). The panels (d, e, f) indicate final steps of intact virion assembly. The reticular materials of encased TLPs are expanded from the ER membrane (arrow in the panel d), disrupted (white arrows in the panels e, f), and TLPs are released to the ER lumen as an intact virion (white arrows head in the panels e, f). These figures are modified from our previous paper (Suzuki et al. 1984b, 1993). Bar represents 100 nm.

of VP7, namely, virus-associated VP7 of intact particles and membrane-associated VP7 (Kabcenell et al. 1988). VP7 has a peptidase cleavage site between Ala50 and Gln51, resulting in the removal of a signal peptide (Table 1) (Stirzaker et al. 1987; Estes and Greenberg 2013). ER retention of VP7, a prerequisite for these steps, requires both the VP7 signal peptide and the first ~31 residues of the mature protein (Stirzaker and Both 1989; Maass and Atkinson 1994). However, the precise mechanism by which RV particles disconnect from the ER membrane is not well understood.

TEM study has indicated the existence of a final step of virion assembly (Fig. 6d, e and f) (Suzuki et al. 1993; Suzuki 1996). The outer surface of the TLP stretches from the ER membrane forming bridge, which is eventually disrupted. It is assumed that this process, which corresponds to the scission of stretching VP7 tail from the ER membrane, results in the emergence of virus-associated VP7 of intact particles, as membrane-associated VP7. These TEM observations led us to conclude that the last step of intact virion assembly is key evidence supporting the existence of the perforation pathway for TLP assembly (Fig. 2). The infectious cycle ends when progeny virus is released by host cell lysis in nonpolarized cells (Estes and Greenberg 2013), but is released from the apical pole of human intestinal cells before cell lysis (Jourdan et al. 1997).

NSP4 releases Ca^{2+} from intracellular stores (Tian et al. 1994) by acting as a viroporin (Hyser et al. 2010). The NSP4-triggered increase of intracellular (Ca^{2+}) activates a kinase-dependent pathway, which leads to autophagy (Crawford et al. 2012; Crawford and Estes 2013). However, at present, the involvement of unknown factors in the final step of intact virion assembly cannot be ruled out.

Perspective

Although RV is a non-enveloped virus, interestingly, RV utilizes cell membrane perforation twice during its life cycle: cell entry and virion assembly. Further research is needed to fully elucidate the details of these processes. A major challenge for future RV research is to elucidate how viral proteins cooperate during membrane penetration.

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Conflict of Interest

The author declares no conflict of interest.

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