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Electron microscopic analysis of rotavirus assembly-replication intermediates

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ABSTRACT

Rotaviruses (RVs) replicate their segmented, double-stranded RNA genomes in tandem with early virion assembly. In this study, we sought to gain insight into the ultrastructure of RV assembly-replication intermediates (RIs) using transmission electron microscopy (EM). Specifically, we examined a replicase-competent, subcellular fraction that contains all known RV RIs. Three never-before-seen complexes were visualized in this fraction. Using in vitro reconstitution, we showed that ~15-nm doughnut-shaped proteins in strings were nonstructural protein 2 (NSP2) bound to viral RNA transcripts. Moreover, using immunoaffinity-capture EM, we revealed that ~20-nm pebble-shaped complexes contain the viral RNA polymerase (VP1) and RNA capping enzyme (VP3). Finally, using a gel purification method, we demonstrated that ~30–70-nm electron-dense, particle-shaped complexes represent replicase-competent core RIs, containing VP1, VP3, and NSP2 as well as capsid proteins VP2 and VP6. The results of this study raise new questions about the interactions among viral proteins and RNA during the concerted assembly–replicase process.

Introduction

Rotaviruses (RVs) are eleven-segmented, double-stranded RNA (dsRNA) viruses and important causes of acute gastroenteritis in humans and other animal species (Estes and Kapikian, 2007). Despite their medical significance, critical gaps in knowledge exist about how RVs replicate within infected host cells. In particular, the mechanism by which RVs synthesize their dsRNA genome segments during the early stages of virion particle assembly is poorly understood (Guglielmi et al., 2010; Patton et al., 2007; Trask et al., 2012a). The coupling of genome replication with assembly may have evolved to ensure that the viral dsRNA remains sequestered away from cellular antiviral sentries during infection (Zinzula and Tramontano, 2013). Yet, this concerted assembly–replicase process has been difficult to study in the context of infected cells because it occurs within electron-dense cytosolic inclusions called viroplasms (Eichwald et al., 2004; Fabbretti et al., 1999; Patton et al., 2006). When viewed by transmission electron microscopy (EM) in negatively-stained, resin-embedded cell sections, complexes located within the boundaries of viroplasms cannot be resolved (Altenburg et al., 1980; Eichwald et al., 2012; Saif et al., 1978; Suzuki et al., 1981).

High-resolution structures have been determined for several of the viral proteins involved in RV genome replication and assembly, as well as for intact RV triple-layered particles (TLPs) and double-layered particles (DLPs) (Aoki et al., 2009; Chen et al., 2009; Dormitzer et al., 2002; Jayaram et al., 2002; Li et al., 2009; Lu et al., 2008; McClain et al., 2010; Rodriguez et al., 2014; Settembre et al., 2011; Zhang et al., 2008). The mature RV TLP is non-enveloped, ~80–100-nm in diameter, and comprised of three capsid layers with icosahedral symmetry. The outermost layer ($T=13$) of the TLP is formed mostly by the VP7 glycoprotein and is embedded with 60 trimers of the protease-sensitive VP4 spike attachment protein. This outer VP7–VP4 layer is shed during viral entry into the host cell, revealing a DLP, which is deposited into the cytosol. The intermediate layer ($T=13$) of the TLP and outer layer of the DLP is made up of VP6. Beneath VP6 resides a thin, smooth VP2 core shell ($T=1$), which directly encases eleven dsRNA genome segments as well as several copies each of the viral RNA polymerase (VP1) and RNA capping enzyme (VP3). Although the structural details of the RV particle interior are not fully resolved, it is thought that VP1 and VP3 form a heterodimer situated beneath most if not all of the fivefold icosahedral vertices (Estrozi et al., 2013; Prasad et al., 1996). During RV transcription in the context of a DLP, VP1 proteins simultaneously synthesize eleven non-polyadenylated plus-strand RNAs (+RNAs) using the minus-strands of dsRNAs as templates

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(Jayaram et al., 2004; Trask et al., 2012a). Nascent +RNAs acquire a 5′ me^{7}G cap via the activities of VP3 prior to their extrusion from the DLP through aqueous channels at or near the fivefold vertices (Lawton et al., 1997). In addition to serving as templates for protein synthesis by cellular ribosomes, +RNAs are also templates for genome replication. Specifically, viral +RNAs are selectively assorted and packaged into assembly-replication intermediates (RIs), where they are converted into dsRNAs by a single round of VP1-mediated minus-strand RNA synthesis. However, very little is known about the macromolecular architectures of RV RIs, including that of the viral replicase complex.

The limited insight that we have into the organization and composition of RV RIs is mainly based upon early biochemical studies. Helmberger-Jones and Patton (1986) showed that a subcellular fraction of infected monkey kidney cells, called a subviral particle (SVP) preparation, can be prepared using differential centrifugation and sucrose gradient fractionation. Upon incubation of this SVP preparation with nucleotides (NTPs) and divalent cations (Mg^{2+} and Mn^{2+}) at $\geq$30 °C, minus-strand RNA synthesis occurs on eleven endogenous +RNAs to produce dsRNA genome segments in vitro. This result indicates that the SVP preparation is enriched for functional viral replicase complexes. In addition to the viral RNA polymerase and RNA capping enzyme (i.e., VP1 and VP3), the preparation was also suggested to include the VP2 core shell protein, the VP6 middle virion layer protein, two multifunctional nonstructural proteins (NSP2 and NSP5), as well as numerous unspecified cellular proteins. The RV RIs in the SVP preparation are heterogeneous, as they are captured at different stages of assembly and/or minus-strand RNA synthesis. However, Gallegos and Patton (1989) showed that an RI population with maximal replicase activity, called core RIs, can be resolved using non-denaturing Tris-glycine agarose gel electrophoresis. The replicase-competent core RI population was suggested to contain the same viral proteins as the SVP preparation (i.e., VP1, VP2, VP3, VP6, NSP2, and NSP5). However, in these early studies, the protein composition of SVPs and gel-purified core RIs was not confirmed by immunoblot analyses, and the particles within the preparations were not directly visualized using EM.

Based solely upon its biochemical attributes before and after genome replication, Patton and Gallegos (1990) made some predictions about the ultrastructure of the RV replicase complex. Specifically, prior to being subjected to in vitro minus-strand RNA synthesis, core RIs migrated more slowly in the agarose gels than did TLPs and DLPs, and they were exquisitely sensitive to inactivation by RNases. In contrast, after in vitro genome replication, core RIs migrated more quickly in the gels, and they were more resistant to RNase inactivation. These results led Patton and Gallegos (1990) to hypothesize that the RV replicase complex began as a >100-nm particle with +RNA replication templates extending away from its VP2–VP6 capsid surface. During minus-strand RNA synthesis, the +RNAs were predicted to be "pulled into" the particle interior, thereby condensing the complex to <50-nm in diameter and protecting the +RNA templates. In the current study, we sought to employ EM to visualize, for the first time, complexes found in both the replicase-competent SVP preparation and in the gel-purified, replicase-competent core RI population. Our EM imaging data suggest a new model for the ultrastructure of the viral replicase complex, and they raise important questions about the interactions among viral proteins and RNA during the early stages of RV particle assembly.

Results

EM imaging of a replicase-competent, subcellular fraction derived from RV-infected cells

To isolate the subcellular fraction of infected cells containing all RV RIs (i.e., the SVP preparation) we used an approach described by Helmberger-Jones and Patton (1986). Mock-infected or strain SA11 simian RV-infected monkey kidney (MA104) cells were lysed at 10 h post-infection (p.i.) using a Dounce homogenizer, and the lysates were clarified by low-speed centrifugation. Large particulate in the cell supernatant were then pelleted thru a 15–30% sucrose gradient by ultracentrifugation. The pellet was resuspended, and a small amount was analyzed for protein content using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). The results showed that the subcellular fractions derived from mock-infected and RV-infected cells contained numerous cellular proteins of unknown identity. However, prominent protein bands consistent with the molecular masses of several viral proteins were also detected in the SVP preparation. Immunoblot analyses confirmed the identities of viral proteins VP1 (125 kDa), VP2 (102 kDa), VP6 (45 kDa), NSP2 (35 kDa), NSP3 (35 kDa), and NSP5 (30–34 kDa) (Fig. S1A). Unfortunately, we lacked antisera to detect VP3 (98 kDa), VP4 (86 kDa), VP7 (37 kDa), and NSP4 (20–28 kDa) by immunoblot.

To determine whether the SVP preparation contained active replicase complexes, an aliquot was incubated at 30 °C along with NTPs, divalent cations, and [^{32}P]-UTP. No exogenous +RNA templates were added to the reaction, requiring VP1 to utilize associated +RNA templates for in vitro minus-strand RNA synthesis. The [^{32}P]-labeled dsRNA products of the reaction were recovered using phenol–chloroform extraction, and then they were separated by SDS-PAGE (Fig. 1B). The results showed that all 11 [^{32}P]-labeled dsRNA gene segments were detected in the gel for the reaction containing the SVP preparation, suggesting that VP1 elongated associated +RNA templates. These results indicate that we isolated an impure subcellular fraction of RV-infected cells that contains viral replicase complexes.

To visualize the ultrastructures of complexes in the subcellular fractions, diluted aliquots were added onto glow-discharged, carbon-coated EM grids. The complexes on the grids were negatively stained, and then they were imaged at 40,000× magnification using a transmission EM (Fig. 1C-H). As controls, grids containing purified TLPs, DLPs, and cores (i.e., DLPs with VP6 removed) were also prepared (Fig. 1I-L).I-J). For the mock subcellular fraction, only ~50–200-nm cloud-shaped complexes were visualized (Fig. 1C). These same cloud-shaped complexes were also seen on the grids prepared with the SVP preparation. However, many unique complexes were also visualized in the SVP preparation, which were not found in the mock-infected control fraction (Fig. 1D-H). As expected, ~80–100-nm particles resembling TLPs and DLPs were detected (Fig. 1D-G and J-K). Also, ~15-nm doughnut-shaped proteins resembling NSP2 octamers were seen either as free complexes (Fig. 1D) or associated in strings (Fig. 1F), possibly bound to single-stranded RNA or bound to each other (Hu et al., 2012). Even more, in the SVP preparation, we also detected dispersed pebble-shaped complexes, which were ~20 nm in diameter (Fig. 1E). Interestingly, these pebble-shaped complexes were visually indistinguishable from those that appeared to be released from disrupted virion-derived cores (Fig. 1L). Finally, in the SVP preparation, we also detected ~30–70 nm particle-shaped complexes with electron-dense centers (Fig. 1G and H). These particles were distinct in both in their sizes and morphologies from TLPs, DLPs, and cores (Fig. 1J-L). They also appeared to vary in relative size compared with each other (i.e., small vs. large particle-shaped complexes). Together, the EM images provided a first glimpse of complexes in the SVP preparation.

EM imaging of NSP2 bound to +RNA transcripts

Having observed ~15-nm doughnut-shaped proteins arranged as strings in the SVP preparation (Fig. 2A), we next sought to determine whether they were NSP2 octamers bound to single-