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Interactions among Capsid Proteins Orchestrate Rotavirus Particle Functions

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Abstract

Rotaviruses are members of the *Reoviridae* family of non-enveloped viruses and important etiologic agents of acute gastroenteritis in infants and young children. In recent years, high-resolution structures of triple-layered rotavirus virions and the constituent proteins have provided valuable insights into functions. Of note, structural studies have revealed the position of the viral RNA-dependent RNA polymerase, VP1, within the inner capsid, which in turn provides clues about the location of the viral capping machinery and the route of viral transcript egress. Mechanisms by which the viral spike protein, VP4, mediates receptor binding and membrane penetration have also been aided by high-resolution structural studies. Future work may serve to fill the remaining gaps in understanding of rotavirus particle structure and function.

Introduction

Virus capsids protect the viral genome and mediate entry into target cells. The segmented, double-stranded (ds) RNA genomes of *Reoviridae* viruses are packaged into multi-layered, icosahedral capsids that never fully disassemble; instead, RNA transcripts that direct viral protein expression and genome replication are synthesized within the confines of a subviral particle and extruded into the cytosol. Recent technological advances have enabled determination of high-resolution structures for the virion of rotavirus, the medically most relevant of the *Reoviridae* viruses. In combination with biochemical studies, virion structures have clarified many aspects of rotavirus biology, including the interaction of the viral polymerase with the inner capsid protein during transcription and replication and the conformational changes in the outer capsid that accompany trypsin proteolysis (maturation) and membrane penetration during entry.

Overview of the rotavirus virion

The inner capsid of the rotavirus virion is formed by VP2 (102 kDa), a comma-shaped, plate-like protein (Fig. 1A–B) [1,2]. In two alternate conformations (A and B), VP2 forms a *pseudo* T=1 capsid from 60 asymmetric dimers: VP2-A converge around the icosahedral five-fold axes, while VP2-B interdigitate between the VP2-A and sit farther away from the five-fold axes (Fig. 1B) [1]. It is convenient to think of the VP2 capsid as twelve decameric arrangements of VP2, each centered on an icosahedral five-fold axis of rotation (Fig. 1B).

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Both the multimeric configuration and the monomeric fold of VP2 are highly conserved among the inner capsid proteins of the *Reoviridae* [1,3,4]. Packaged within the VP2 capsid are the eleven dsRNA segments (ranging from 0.7–3.3 kbp) of the rotavirus genome, roughly arranged as concentric spheres [5]. One copy each of the viral RNA-dependent RNA polymerase (RdRp), VP1 (125 kDa), and the RNA capping enzyme, VP3 (88 kDa), is thought to be associated with each genome segment. The VP1-VP3 pairs are anchored to the interior side of the VP2 capsid through contacts made near the five-fold axes. Because there are eleven genome segments, but twelve vertices within the VP2 capsid, one of the five-fold positions may be “empty”, lacking an associated VP1 and/or VP3 component.

Surrounding the VP2 capsid and its contents is a T=13 layer formed by 260 trimers of the VP6 protein (45 kDa; Fig. 1A) [2]. VP6 subunits intertwine in a right-handed twist, forming a roughly pear-shaped trimer [6]. VP6 shares structural similarity with the orthoreovirus (reovirus) and aquareovirus membrane penetration proteins [6–8], but instead functions as an adapter for the rotavirus outer capsid proteins (VP4 and VP7) that mediate entry. Trimeric spikes of VP4 (87 kDa) are anchored into depressions in the VP6 layer that surround the five-fold icosahedral axes (Fig. 1A, C) [9]. Up to 60 spikes can potentially decorate the virion, although the precise occupancy of VP4 may vary among rotavirus strains. VP4 has receptor binding activities and functions as the membrane penetration protein during entry. The trimeric glycoprotein VP7 (~ 34 kDa) covers the virion surface and locks the VP4 spikes into place (Fig. 1A, C) [9]. VP7 trimerization is entirely dependent on coordination of calcium ions that mediate the interactions between each subunit [10]. A VP7 trimer sits atop each VP6 trimer, arranged in the same T=13 configuration, completing the outer capsid [9,11]. The rotavirus triple-layered particle (TLP) is the infectious form of the virion and measures approximately 100 nm in diameter, spike-to-spike (Fig. 1A). Outer capsid proteins VP4 and VP7 are shed during entry, delivering a double-layered particle (DLP) to the cytosol to initiate infection (Fig. 1A).

The inner capsid orchestrates transcription

Transcription of rotavirus positive-sense RNAs ([+]RNAs) by VP1 occurs within the DLP [12]. Since little structural information about VP1 could be gleaned from virion reconstructions alone, structures of purified VP1 were solved [13]. VP1 is a roughly globular protein with a large central cavity that houses the active site for RNA polymerization. The VP1 polymerase domain has a “cupped right-hand” architecture common to polymerases, but large N- and C-terminal domains (common to the *Reoviridae*) form a “cage” that encloses the VP1 catalytic center [13]. Four tunnels, each with a discrete function, permit access to the active site (Fig. 2A): (1) entry of the template RNA, (2) exchange of nucleotides and pyrophosphate during catalysis, (3) egress of the template RNA (during transcription) or dsRNA (during genome replication), and (4) release of [+]RNA during transcription.

At the 12 five-fold axes of icosahedral symmetry (each corresponding to the ‘center’ of a VP2 decamer) are small channels in the virion that permit extrusion of [+]RNAs (Fig. 1B). The diameter of these channels is larger in the DLP than in the TLP, suggesting that removal of VP7 permits conformational changes that trigger transcription [11,14]. It has been inferred that both VP1 and VP3 must occupy positions near the five-fold channels to direct nascent transcripts out of the DLP. Consistent with this model, the RdRp (λ 3) of reovirus is located proximal to, but off-center from, the five-fold channels of the virion [15]. Fitting of the VP1 crystal structure into rotavirus reconstructions has confirmed that VP1 adopts a similar orientation and association with the VP2 capsid (Fig. 1B, 2A) [16]. This position and orientation enable VP1 to direct transcripts towards the five-fold channel (Fig. 2A). Mutagenesis of VP2 mapped the site required for activation of dsRNA synthesis by VP1 to

the “apical” and “central” domains of VP2 [17], precisely the region of VP1 binding identified in particle reconstructions, which suggests that VP1 interacts with the same site on the VP2 capsid during both transcription and genome replication. The striking similarity between the reovirus core- λ 3 and rotavirus VP2-VP1 interactions suggest that the *Reoviridae* may have a highly conserved capsid-RdRP interaction mechanism that functions to promote virion assembly and to direct [+]RNAs during transcription. The N-termini of the VP2 proteins are not fully resolved in virion reconstructions, but the resolved regions are directed towards the five-fold axis of each decamer (Fig. 2B) [1]. It appears as though the VP2 N-termini directly contact the bound VP1 on one side, suggesting that these appendages may function to support or orient the RdRp (Fig. 2B; S. Harrison, personal communication). Accordingly, biochemical analyses suggest that the VP2 N-termini are required for VP1 encapsidation [18]. VP2 N-termini vary in sequence and length between rotavirus strains but generally contain numerous positively charged amino acids [19]. Thus, a primary function may be to interact with [+]RNAs during virion assembly, dsRNAs within the assembled core, or both.

The least well understood aspect of the rotavirus polymerase complex is the RNA capping enzyme, VP3. The structure of VP3 is not known, nor has it been located within virion reconstructions. However, multiple enzymatic RNA capping functions, including guanylation, N7-methylation, and 2'-O-methylation, have been assigned to VP3 [20,21]. The structure of bluetongue virus (BTV) VP4, the functional homolog of VP3, has been solved [22]. Based on sequence alignments and structure predictions, VP3 is anticipated to adopt a structure similar to BTV VP4, particularly with regard to the predicted methyltransferase domains (K. Ogden, unpublished data). VP3 is larger than BTV VP4; yet, VP4 has been shown to encode an enzymatic activity (RNA 5'-triphosphatase) that has not been observed for VP3 *in vitro* [20,23,24]. The localization of VP1 near the five-fold channels of the virion necessitates that VP3 must occupy an adjacent position (Fig. 2A). The [+]RNA exit tunnel of VP1 is oriented near the five-fold channel (S. Harrison, personal communication). Unlike the capping enzyme turrets of reovirus [4,15], which sit on the exterior of the inner capsid, VP3 must intercept and modify the 5' end of rotavirus [+]RNAs shortly after transcription is initiated. Consistent with this idea, biochemical data indicate that rotavirus [+]RNAs are capped as they reach only five to seven nucleotides in length [25]. Conformational flexibility or positional variability are likely reasons for the inability to detect VP3 in rotavirus particle reconstructions, which rely on icosahedral symmetry and averaging functions to observe proteins at near-atomic resolution. It is likely that VP3 has a transient interaction with VP1 and [+]RNAs, as it must quickly bind, cap, and release RNAs as they are directed out of the core and into the cytosol. A prolonged or highly stable interaction with RNA (or VP1) would presumably slow the rate at which rotavirus could generate and release [+]RNAs. These observations, as a whole, suggest several challenges that must be addressed to better understand VP3, an essential component of the rotavirus replication machinery.

Interactions among the outer capsid proteins prime the spike for entry

Outer capsid assembly appears to be a step-wise process in which VP4 is added to an assembled DLP, and subsequent budding through the endoplasmic reticulum membrane provides access to VP7, which locks VP4 in place [9,26,27]. The trimeric VP7 structure has been solved, both as a soluble protein bound by a neutralizing antibody and assembled onto the virus particle [9–11]. The VP7 trimer is a triangular plate with a slight depression in the center (Fig. 3Aii). Two calcium ions are bound between subunits near each of the corners of the triangle [10]. VP7 assembles onto the DLP using flexible N-terminal arms that latch onto VP6 [6,11]. VP7 functions as a molecular switch during entry. As trimers uncouple (likely

due to calcium depletion within an endosomal vesicle) and dissociate from the virion surface, they cue membrane penetration by the mature form of the VP4 spike [28–30].

To become fully infectious, the virion must be released from the cell, and VP4 must be proteolytically cleaved by trypsin-like proteases in the intestinal lumen. Prior to trypsin cleavage, the VP4 spikes are flexible and cannot be observed in particle reconstructions. Proteolysis triggers a reorganization in which the spike adopts a distinct, rigid conformation [31]. VP4 is cleaved within a defined region to generate two fragments (VP8* and VP5*) that remain non-covalently associated. VP8* (~ 27 kDa) is the smaller fragment derived from the VP4 N-terminus. The structures of the globular domain of VP8* from several rotavirus strains have been solved; all have a galectin-like fold, and many have been shown to bind cellular glycans [32–34]. VP8* has been shown to bind terminal sialic acid residues in many rotavirus strains (Fig. 3Ai) [33]. Recently, though, it has been reported that certain strains, notably those that infect humans, bind a broader range of glycans [35–38]. Further categorization of VP8* binding specificities, and possible correlations with host-restriction and pathogenicity, are strongly needed. Consistent with a role in receptor binding and attachment to target cells, VP8* forms the two distal lobes of the rotavirus spike (Fig. 3Ai).

The remainder of the spike is formed by three copies of the VP5* proteolysis product (~ 60 kDa) and the extended N-terminal regions of VP8* [9,39]. The VP5* spike adopts a highly unusual conformation that has elements of both trimeric and dimeric symmetry (Fig. 3A). The trimeric, globular base of VP5* docks into a quasi-six-fold symmetric depression in the VP6 layer, such that each VP5* subunit approaches two VP6 trimers [9]. However, aside from general shape-complementarity, few interactions exist to stably anchor VP4 to the DLP. This observation is consistent with many aspects of VP4 biology, including a requirement for viral chaperone (NSP4)-mediated assembly and rapid release from the DLP upon VP7 dissociation [28,30,40].

Assembly of VP7 onto VP6 restricts dissociation of VP4 by constricting the diameter above the VP5* base (Fig. 3A) [9,26]. As the spike extends out of the virus particle, trimeric symmetry is broken. One of the VP5* subunits lies nearly flat to the particle surface (VP5*C), and the other two form dimeric interactions and project away from the virion [9,39]. The upright VP5* subunits bind the two VP8* lobes described above. The globular VP8* partner of VP5*C is presumed (but not definitively known) to dissociate from the virion after proteolysis, as it has not been observed in any rotavirus particle reconstruction. However, given the nature of the interactions known to exist between VP8* and VP5* at the tip of the spike (Fig. 3B), it is unlikely that a third VP8* could be present as its globular shape would sterically preclude VP5*C from adopting the conformation that is seen for it at the base of the spike.

Specific interactions with VP7 appear to be the key regulator of VP4 conformational changes during maturation and entry. Subtle asymmetry of the VP6 and VP7 layers suggests a mechanism driving VP5*C to adopt its unusual conformation at every identical position on the virion. A gap between two VP7 trimers enables specific interactions with the base of the VP5*C β -barrel domain (Fig. 3B) [9]. In contrast, the other VP7 trimers more closely surround the spike and are unavailable for interaction. Thus, the VP7 capsid, in part, dictates VP5* conformation after trypsin proteolysis. The function of VP4 maturation is thought to be analogous to that of enveloped virus fusion proteins, namely, to prime VP5* for refolding during membrane penetration [9,39,41]. During entry, VP7 uncoating releases constraints on the spike and permits VP5* to snap-back into a collapsed trimer structure [29,39]. This conformation of VP5* is considered a post-penetration state, analogous to the post-fusion state of enveloped virus fusion proteins [39,40].

Conclusions

High-resolution reconstruction of icosahedral viruses has become a rapid and reasonably facile approach to gain significant insights into virion functions, including assembly and entry [2,8,9]. The limitations of this technology, however, are illustrated in the paucity of information obtained for rotavirus VP3 and the pre-cleavage conformation of the VP4 spike. Cryo-EM tomography, which can image individual (non-averaged) virions [42], and novel methods of orienting particles [43], may be helpful in deriving additional data from “known” structures, and developing new hypotheses for mechanisms of virus biology.

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Highlights

Structures of rotavirus have greatly informed studies of replication and entry

The *Reoviridae* may have a conserved capsid-polymerase interaction mechanism

The rotavirus polymerase orientation suggests the location of the capping enzyme

The conformation of the rotavirus spike is regulated by the outer capsid protein

New methods are needed to study the rotavirus spike precursor and capping enzyme

Figure 1. Overview of the rotavirus virion

(A) Hybrid view of the rotavirus virion (PDB IDs 3IYU, 3N09, and 2R7U) [9,13]. The outer capsid proteins of the triple-layered particle (TLP), VP4 (red) and VP7 (yellow), mediate attachment and entry of the virus into target cells. Entry releases a double-layered particle, whose outer surface is composed of VP6 (green), into the cytosol. The inner capsid protein, VP2 (blue), encloses the dsRNA genome and multiple copies of the viral polymerase, VP1 (pink), and capping enzyme, VP3 (not shown). (B) View of an icosahedral five-fold axis from inside the particle. Each five-fold axis is immediately surrounded by 5 copies of VP2 (VP2-A; light blue), while the alternate conformation of VP2 (VP2-B; dark blue) sit farther away and interdigitate between VP2-A. Current evidence indicates that VP1 (pink silhouette) can occupy one of five rotationally symmetric positions near the five-fold axis. Note the channel formed at the five-fold axis (obscured by VP1). The channel is thought to be the exit for [+]RNAs during transcription. (C) Enlarged side view of the capsid. Note that several trimers of VP6 contact each VP2 molecule. The pear-like shape of VP6 forms large depressions in the DLP surface; the pseudo-six-fold symmetric depressions that flank each five-fold axis allow docking of VP4 trimers deep into the VP6 capsid. Trimers of VP7 latch onto each VP6 trimer and partially obscure the depressions in the VP6 layer, thereby preventing VP4 dissociation.

Figure 2. Interactions among the inner capsid proteins during [+]RNA transcription

(A) Model for transcription of capped (red circle) rotavirus [+]RNAs (yellow), based on empirical evidence and modeling on the capsid and polymerase proteins of reovirus (PDB IDs 1EJ6 and 1UON) [4,14–16]. VP1 (pink) binds the VP2 (blue) capsid, simultaneously interacting with multiple VP2 molecules (see panel B). Structural information, and comparison with the RdRP of reovirus indicate the functions and orientations of the four tunnels of VP1 that direct substrates in and out of the central active site: 1. Entry of the template negative-sense RNA (“–”, blue); 2. Exchange of nucleotides and pyrophosphate; 3. Egress of the template RNA; 4. Release of [+]RNA. It is thought that a “cap-binding site” near tunnel 1 allows VP1 to retain the cognate [+]RNA to the template during transcription. The presumed location of VP3 is indicated. The proximity of VP1 tunnel 4 to the five-fold channel indicates that VP3 must quickly bind and cap (white arrow) the nascent [+]RNA before it is extruded out of the virion. (B) Detailed view of the VP1-VP2 interaction. The inset panel represents an approximately 90° vertical rotation of the view in (A). VP2-B conformers are shown in ribbon to highlight the N-terminal arms (yellow) that project towards the five-fold axis and VP1 (the arms of VP2-A are disordered and not observed in particle reconstructions). VP1 is shown in ribbon and colored according to the three primary domains described in the text: the *Reoviridae*-specific N-terminal (green) and C-terminal (magenta) domains that flank the central polymerase domain (pink). Rotation (~45°) and magnification of the inset view provides greater detail of the VP2-B N-terminal arm that approaches and likely interacts with VP1 (white arrowhead in inset).

Figure 3. VP7 interacts with VP5* to regulate structural transitions of the spike

(A) Three-quarters profile of the rotavirus capsid. A single VP4 spike trimer (red/brick/orange) is embedded in the VP6 (green) capsid. The A and B subunits of the spike project away from the virion, while the C subunit lies nearly flat to the particle surface. The distal tips of the spike are formed by two globular domains of VP8* (pink; inset i), which bind sialic acid (SA) moieties (or other glycans) to mediate attachment of the virion to target cells (PDB IDs 1KQR and 3IYU). The VP7 (yellow) layer binds VP6 and encloses the VP4 base to restrict its dissociation from the virion. Note the N-terminal arms of VP7 that extend down and latch onto VP6; this is the primary mode of VP7-VP6 interaction [11]. VP7 trimers are stabilized by two bound calcium (Ca^{2+}) ions at each subunit interface (inset ii).

(B) Top view of the VP7 capsid surrounding the VP4 spike, highlighting the asymmetry that influences the spike conformation. Relative to the view in (A), the structure has been tipped towards the viewer. The two VP7 trimers immediately counter-clockwise of the black-and-white arrowhead are not shown in (A). The arrowhead indicates a gap in between two adjacent VP7 trimers that imparts asymmetry on the spike binding site and permits interaction of VP5*C with the two VP7 trimers on either side of the gap (see black-and-white arrowhead in (A)) [9]. In this view, the outer features of the spike complex have been truncated to highlight both the trimeric configuration of the spike base, and to illustrate how VP7 occludes the base, preventing dissociation of the spike.